EMERGING MECHANISMS IN DYNAMIC GABAergic INHIBITION

EDITED BY: Shiva K. Tyagarajan, Arianna Maffei and Andrea Barberis
PUBLISHED IN: Frontiers in Molecular Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-766-8
DOI 10.3389/978-2-88971-766-8

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

EMERGING MECHANISMS IN DYNAMIC GABAergic INHIBITION

Topic Editors:
Shiva K. Tyagarajan, University of Zurich, Switzerland
Arianna Maffei, Stony Brook University, United States
Andrea Barberis, Italian Institute of Technology (IIT), Italy

Citation: Tyagarajan, S. K., Maffei, A., Barberis, A., eds. (2021). Emerging Mechanisms in Dynamic GABAergic Inhibition. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-766-8

Table of Contents

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 05 February 2019
doi: 10.3389/fnmol.2019.00012

Bumetanide Prevents Brain Trauma-Induced Depressive-Like Behavior

Emmanuelle Goubert[1], Marc Altvater[2], Marie-Noelle Rovira[1], Ilgam Khalilov[1,3], Morgane Mazzarino[1], Anne Sebastiani[2], Michael K. E. Schaefer[2], Claudio Rivera[1,4†] and Christophe Pellegrino[1*†]*

[1] INSERM, Institute of Mediterranean Neurobiology, Aix-Marseille University, Marseille, France, [2] Department of Anesthesiology and Research Center Translational Neurosciences, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany, [3] Laboratory of Neurobiology, Kazan Federal University, Kazan, Russia, [4] Neuroscience Center, University of Helsinki, Helsinki, Finland

Brain trauma triggers a cascade of deleterious events leading to enhanced incidence of drug resistant epilepsies, depression, and cognitive dysfunctions. The underlying mechanisms leading to these alterations are poorly understood and treatment that attenuates those sequels are not available. Using controlled-cortical impact as an experimental model of brain trauma in adult mice, we found a strong suppressive effect of the sodium-potassium-chloride importer (NKCC1) specific antagonist bumetanide on the appearance of depressive-like behavior. We demonstrate that this alteration in behavior is associated with an impairment of post-traumatic secondary neurogenesis within the dentate gyrus of the hippocampus. The mechanism mediating the effect of bumetanide involves early transient changes in the expression of chloride regulatory proteins and qualitative changes in GABA(A) mediated transmission from hyperpolarizing to depolarizing after brain trauma. This work opens new perspectives in the early treatment of human post-traumatic induced depression. Our results strongly suggest that bumetanide might constitute an efficient prophylactic treatment to reduce neurological and psychiatric consequences of brain trauma.

Keywords: psychiatric disease, depression, potassium chloride cotransporter 2 (KCC2), bumetanide, neurogenesis, interneuron cell death

OPEN ACCESS

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

Reviewed by:
Alexander A. Mongin, Albany Medical College, United States
Jamie Maguire, Tufts University School of Medicine, United States

***Correspondence:**
Claudio Rivera
claudio.rivera@helsinki.fi;
claudio.rivera@inserm.fr
Christophe Pellegrino
christophe.pellegrino@inserm.fr

† These authors have contributed equally to the work

Received: *06 November 2018*
Accepted: *14 January 2019*
Published: *05 February 2019*

Citation:
Goubert E, Altvater M, Rovira M-N, Khalilov I, Mazzarino M, Sebastiani A, Schaefer MKE, Rivera C and Pellegrino C (2019) Bumetanide Prevents Brain Trauma-Induced Depressive-Like Behavior. Front. Mol. Neurosci. 12:12. doi: 10.3389/fnmol.2019.00012

INTRODUCTION

Brain trauma is the main cause of disability all over the world with a very high prevalence in developed countries (Meyer et al., 2008; Bondi et al., 2015). According to the World Health Organization and the Centers for Disease Control and Prevention (Meyer et al., 2008), brain trauma classification is based on multiple factors such as altered neurological functions, brain area of interest and genetic variations. Altogether, these factors lead to highly individualized injuries. Sequels of trauma include low prevalence post-traumatic epilepsies (PTEs), with a severity and occurrence dependent on trauma severity (Kelly et al., 2015; Bragin et al., 2016), and cognitive dysfunctions and depression-like phenotypes are also commonly associated (Peeters et al., 2015; Perry et al., 2015; Stein et al., 2015). Following brain trauma, neuronal cell death occurs and more particularly within the neurons of

the dentate gyrus of the hippocampus (Ren et al., 2015; Samuels et al., 2015), leading to hippocampal volume reduction (Samuels et al., 2015; Anacker and Hen, 2017). These observations could be related to changes in post-traumatic neurogenesis in the hippocampus. This has been proposed to be a useful marker of therapeutic treatment efficacy (Brandon and McKay, 2015; Alvarez et al., 2016).

In a wide range of neurological and psychiatric disorders, GABAergic signaling is affected through chloride homeostasis impairment triggered by a down regulation of the main neuronal-specific chloride and potassium extruder, KCC2, and up regulation of the chloride importer NKCC1, respectively (Medina et al., 2014). Similar changes in GABAergic transmission have been reported in a different model of brain trauma (Ben-Ari, 2017). This leads to depolarization and also an excitatory action of GABA that could perturb the generation of behaviorally relevant oscillations and integrative properties of brain networks (Rivera et al., 1999; Luscher et al., 2011; Kahle et al., 2013; Medina et al., 2014; Ben-Ari, 2017). These shifts have been observed notably in developmental disorders including autism spectrum disorders (ASDs) (Tyzio et al., 2014), stroke (Jaenisch et al., 2010; Xu et al., 2016) and epilepsy (Pallud et al., 2014; Tyzio et al., 2014; Kelley et al., 2016). The interaction between major depressive disorders (MDDs) and GABAergic neurotransmission has been suggested in a genetic mice model of GABA(B)-R *knock-out* (Mombereau et al., 2005) and in studies showing an antidepressant effect of potent and selective blockage of GABA(A) transmission (Rudolph and Knoflach, 2011) at both the hippocampus (Boldrini et al., 2013) and mesolimbic system (Kandratavicius et al., 2014). In addition, several observations link chloride homeostasis to secondary neurogenesis through GABA(A) neurotransmission (Luscher et al., 2011; Ostroumov et al., 2016). The generation of new neurons within the DG requires different steps: first, the transition from quiescent to proliferative progenitors, then their differentiation to immature neurons in a GABAergic-dependent manner (Chell and Frisén, 2012; Moss and Toni, 2013). In that context, it's well-accepted that brain trauma alters neurogenesis (Perry et al., 2015; Stein et al., 2015). In the past decade, the relationship between GABA neurotransmission and neurogenesis has been well-established. Ge and collaborators have shown that GABA receptors are expressed in the progenitor cells and that GABA itself, either ambient or synaptically-released GABA, could act at different steps during neurogenesis from proliferation to cell differentiation and finally synaptic integration (Ge et al., 2006; Anacker and Hen, 2017). In addition, the GABAergic polarity acts on the cell integration (Ge et al., 2006) but also in cell proliferation (Sun et al., 2012), thus establishing a causal link between cell cycling and cell cycle exit on depolarizing GABA condition (Scharfman and Bernstein, 2015; Hu J.J. et al., 2017). Apart from the monoamine hypothesis, a new theory based on the GABA release itself has been proposed to contribute to depression. GABA release has been demonstrated to be impaired in psychiatric disorders and particularly in depression (Luscher et al., 2011; Gabbay et al., 2012). More particularly, the GABAergic receptors have been shown to be decreased in expression and function in the dentate gyrus of depressed patients (Luscher et al., 2011; Lüscher and Fuchs, 2015) and brain tissues collected from suicide patients with a history of depression and anxiety (Merali et al., 2004). One of the first phenomenon linking depression and the hippocampus is the change in hippocampal volume observed both in rodent and in human (Savitz et al., 2010; Schuhmacher et al., 2013; Roddy et al., 2018). This is a common trait observed when the hypothalamic–pituitary–adrenal (HPA) axis is impaired. Other brain regions such as cingulate cortex, prefrontal cortex or even amygdala are also associated with depression (Drevets et al., 2008). In addition to volume changes other functions are changed in the hippocampus of animal displaying DLB, e.g., modified volume (Roddy et al., 2018), impaired GABAergic function (Merali et al., 2004), increase in excitability and monoamine dysfunction (Samuels et al., 2015) as well as impaired secondary neurogenesis and cognitive deficit (Ferguson et al., 2016; Anacker and Hen, 2017). Taken together, this makes the hippocampal formation a important and valuable structure to study depression in TBI models.

Parvalbumin-containing interneurons are the principal source of GABA release within the dentate gyrus and thus potential candidates to explain controlled-cortical impact (CCI)-induced dysregulations through their role in the synchronicity of hippocampal networks (Curia et al., 2008; Drexel et al., 2011; Shiri et al., 2014). Moreover, it is accepted that the activity of this class of interneurons could act on secondary neurogenesis by providing a source of ambient GABA (Song et al., 2012; Butler et al., 2016; Hu D. et al., 2017; Pérez-Domínguez et al., 2017), but little is known about the relationship that exists between parvalbumin-containing interneurons and the establishment of post-traumatic depression (Earnheart et al., 2007; Luscher et al., 2011; Fenton, 2015). Moreover, in human depression, their action is far from being established (Khundakar et al., 2011; Pehrson and Sanchez, 2015; Smiley et al., 2016).

Interestingly, the NKCC1 chloride importer antagonist bumetanide has been shown to attenuate many disorders like ASD, Parkinson's disease, and schizophrenia as well as some CCI-induced consequences. This stresses the therapeutic potential of restoring low $(Cl^-)_i$ levels and an efficient GABAergic inhibition (Lemonnier et al., 2013, 2016; Damier et al., 2016; Xu et al., 2016; Ben-Ari, 2017). Although, it has been previously shown that bumetanide could have various positive effects on TBI models (Hui et al., 2016; Zhang et al., 2017) and could also act on secondary neurogenesis in stroke condition (Xu et al., 2016), yet nothing is known about the early action of this compound prior to the establishment of depressive-like behaviors (DLB). Our results showed that brain trauma disrupts chloride homeostasis, leading to hippocampal network disturbances and impaired neurogenesis associated with DLB. Early restoration of chloride homeostasis, using the NKCC1 inhibitor bumetanide rapidly after trauma, attenuates the severity of post-traumatic alterations notably by reducing interneuron loss. This, taken together, suggests a therapeutic potential of this FDA-approved compound after trauma.

MATERIALS AND METHODS

The French ethical approved all experimental procedures (No. APAFIS#2797-2015112016427629v8). All experiments were performed in blind.

Controlled-Cortical Impact Model (CCI)

Ten-weeks old C57bl6-J males are housed individually in an enriched environment, consisting in thick rolled paper (Diamon twist, Envigo) and Dome Home (Envigo) allowing correct nesting of the animals as requested by our French ethical committee. They are maintained in a 12 h light/12 h dark cycle environment with controled temperature (23 ± 2°C), food and water are given *ad libitum*. The CCI procedure is performed using aseptic technique. Buprenorphine (0.03 mg/kg) is given intra-peritonealy (i.p.) 30 min before surgery. Anesthesia induction is done using 4% isoflurane mixed with air and enriched with oxygen (0.3%), for the procedure isoflurane is set to 2–2.5%, before animals are positioned in a stereotaxic frame (David Kopf Instruments). Body temperature is maintained at 37 ± 2°C with a heating pad (Harvard Apparatus). The impact is done on the right cortex within the boundaries of the bregma and lambda after a craniotomy is done, using a leica impactor (tip diameter 3 mm, 6 m/s speed, 1.5 mm depth and 200 ms duration). Sham animals receive complete surgery without the impact. Before experiment, animals were randomly assigned to each group, e.g., sham-vehicle, sham-bumetanide (as there were no differences in all considered tests in between sham-vehicle and sham-bumetanide, and to clarify the message of the manuscript, this group will not be presented in the figures), CCI-vehicle and CCI-bumetanide.

Drug Delivery

Bumetanide stock solution 20 mM (Sigma-aldrich, B3023) is prepared by dissolving 36.4 mg of powder in 1 ml absolute ethanol. The injected solution consists of 40 µl of stock solution diluted in 4 ml PBS 1X. A volume of 26.7 µl par gram of Bumetanide is injected intra-peritonealy, twice daily (9 am and 5 pm), thus corresponding to 2 mg/kg. The vehicle solution consists in the same preparation but lacks the bumetanide powder to respect volume and diluent. Imipramine (30 mg/kg) is i.p injected 30 min before testing the animals for depression.

Western Blot Analysis

Animals were killed by decapitation after deep isoflurane anesthesia. Hippocampi are quickly dissected out, flash-frozen in liquid nitrogen and stored at −80°C until processed. Brain tissue are homogenized in RIPA buffer (50 mM Tris-HCl pH 8; 150 mM NaCl; SDS 0.1%; Deoxycholic Acid 0.5%; 1% Triton X-100), containing complete Protease/Phosphatase Inhibitor Tablet (Thermo Fisher) and loaded with Laemmli 3X loading buffer. The samples are separated in 4–15% SDS-PAGE gel (Criterion gel, Bio-Rad) and transferred to a nitrocellulose membrane (Whatman). After blocking in Tris-buffered saline/0.1% tween/5% bovine serum albumin (BSA), membranes are exposed overnight at 4°C to primary antibody diluted in blocking solution (Tris-buffered saline/0.1% tween/2.5% BSA), anti-NKCC1 (DHSB, 1:2000), KCC2 [non-commercial (Ludwig et al., 2003); 1:5000] and Phospho-Serine 940 (Rockland, 612-415-E15, 1:1000). Secondary antibodies (anti-mouse HRP, #31430, and anti-rabbit HRP, #31460, Thermo Fisher Scientific) are applied for 2 h at room temperature, before a chemiluminescence assay is performed using horseradish peroxidase-conjugated detection. Signals are revealed using ECL-plus Western blotting reagents (ECL-plus kit, Pierce Biotech) on the image analysis software G box (Syngene). Membranes are then stripped using 50 mM DTT/2% SDS in 50 mM Tris-HCl, pH 7.0 for 30 min at 65°C, and blocked again in Tris-buffered saline/0.1% tween/5% BSA and finally probed with anti-α-tubulin (#62204, Life Technologies) or anti-β-tubulin (TUBB3 18020, Biolegend) for normalization. Signal detection and revelation are done following the same procedure as the one for primary antibodies. Quantifications are performed using Gel Plot Analyzer plugin (ImageJ).

Immunohistochemistry

Mice are transcardially perfused with 4% paraformaldehyde then 60 µm coronal sections are made and stained overnight at 4°C using KCC2 (1:3000; Ludwig et al., 2003), Bromo-deoxy-Uridin (BrdU) (Dako M0744, 1:100), DCX (Abcam, AB18723, 1:1000) and parvalbumin (Sigma P3088, 1:500), The Alexa Fluor-conjugated secondary antibodies (1/500, Invitrogen) used 2 h at room temperature and slices are finally counterstained with Hoechst 33258 (10 µg/mL, Sigma-Aldrich 861405). Images are acquired using a confocal microscope with 10, 20, 40, or 63X objectives. The KCC2 antibody used in this study is a custom-made antibody recognizing both a and b isoforms of KCC2 (Uvarov et al., 2007), the epitope is localized on the N-terminus part of the protein.

KCC2 Subcellular Localization Analysis

The measure of the distribution of KCC2 fluorescence associated with cytosolic regions, in sham and CCI condition, is performed at high magnification (x63 objective) using the Image J software. Plot Profiles are done using a line scan analysis through ImageJ software. Briefly, the same straight-line is applied from the extracellular compartment to the nucleus. The intensity profile of each point of the line, separated by 0.1 µm, is analyzed and compared between sham and CCI groups using *t*-test.

BrdU Injections and Neurogenesis Staining

Intra-peritoneal injections of a 1 mg Bromodeoxyuridine solution (BrdU, Sigma, 10 mg/ml) are performed at 6 days and 1-month post brain trauma to label dividing cells in the S-phase. BrdU is dissolved in PBS 1X. Mice received two BrdU injections (9 am and 5 pm), the day before brain collection. Immunohistochemistry is done using a mouse-BrdU antibody (M0744, 1:100, Dako) to monitor dividing cells and using double cortin (DCX) antibody to label immature newly born granular cells. The total number of either BrdU- or DCX-immunopositive cells are assessed within the granular layer of the dentate gyrus (DG) after images acquisition using an apotome module at 20X

objective (BX 40 Olympus). Pictures consist in 1 μm stack images, the total number of stacks gives the total volume of the DG. The total number of positive cells are expressed within the reconstructed volume and reported to the volume, in order to avoid any biases due to thickness differences.

Behavioral Studies

Animals are habituated to the testing room 1 h before testing. For the open field test (OFT), mice are allowed to freely explore the arena for 10 min (Noldus apparatus, 38.5 cm × 38.5 cm). Parameters are detected and analyzed using the Ethovision software (Noldus).

The forced swim test (FST) paradigm is performed in a 25°C water with first a 2-min habituation period followed by a 4 min recording. The time of immobility is quantified to discriminate between swimming and non-swimming movements. Stabilization movements are not counted as swimming movements.

The tail suspension test (TST) is performed on a 6-min trial and the time of immobility is again measured by the experimenter to discriminate between movements and swinging movements. The splash test consists of spraying a 10%-sucrose solution to the fur of the animal, and then animals are video-monitored for a 5-min period, during which latency to first complete sequence of grooming and total grooming time is measured.

For the novel object recognition test, animals are exposed to an empty open field arena (38.5 cm × 38.5 cm × 38.5 cm) for a 3-min habituation time. In a second time, animals are exposed, in the same arena, to two identical objects for a 3-min period. Finally, after a 1-h delay, animals return to the arena, for a third 3-min period, where one of the objects has been replaced by a new one. The time spent close to the objects is measured and plotted as a new versus familiar object ratio.

Acute Slices Preparation

Animals are collected on the first post-traumatic week. After cervical dislocation, brains are rapidly removed, the hippocampi dissected, and transverse 350 to 450 μm thickness slices are produced using a Leica VT1000S tissue slicer (Leica VTS1200S, Germany) in oxygenated (95% O_2 and 5% CO_2) modified artificial cerebrospinal fluid (mACSF) containing, in mM: 132 choline, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 7 $MgCl_2$, 0.5 $CaCl_2$, and 8 D-glucose. Slices are then transferred at room temperature for 1–2 h before chloride and electrophysiological recordings in oxygenated (95% O_2 and 5% CO_2) normal artificial CSF (ACSF) containing, in mM: 126 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 2.0 $CaCl_2$, and 10 D-glucose, pH 7.4.

In vitro Electrophysiological Recordings

Hippocampal slices are individually transferred to a recording chamber maintained at 30–32°C and continuously perfused (2 mL/min) with oxygenated normal or adapted ACSF. Extracellular field recordings are made using tungsten wire electrodes (diameter: 50 μm, California Fine Wire, Grover Beach, CA, United States). Recording electrodes are positioned in a pyramidal cell layer of CA3 subfield, and signals are amplified using custom- DAM-8A amplifiers (WPI, GB; low-pass filter: 0.1 Hz; high-pass filter: 3 kHz; gain: x1000) and then acquired using an A/D converter (Digidata 1440A, Axon Instruments). Clampfit 10.1 (Axon Instruments) software is used for the acquisition and analysis of the network activity. Isoguvacine and bumetanide are purchased from Sigma.

Volumetry Analysis

Coronal 10 μm thick cryostat sections were stained by cresyl violet, digitized and analyzed using an 1.25X objective and computer image analysis system (Optimas 6.51, Optimas Corporation, Bothell, WA, United States). Lesion volume measurement was performed essentially as previously described (Schaible et al., 2014).

Parvalbumin-Containing Interneurons Quantification

Forty μm sections were stained using a mouse-Parvalbumin antibody (Sigma-Aldrich, 1:500) and counterstained with Hoechst 33258 (Sigma-Aldrich, 10 μg/mL in PBS). The quantification was done in granular layer of the DG at 40X objective. All experiments were manually performed in blind. As done with the BrdU images, pictures consist of 1 μm stack images, the total number of stacks gives the total volume of the DG. The number of positive cells is expressed in percentage within the reconstructed volume to avoid any biases.

Statistical Analysis

All mean values are given with the standard error mean (SEM). Normality was tested for each distribution and was set to 5%. Two-tailed Student's, Mann–Whitney test or one-way ANOVA were used accordingly using Prism software (GraphPad Software, Inc., La Jolla, CA, United States). Box plot report the median, the interquartile range and the total range data and represent as following: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

Behavioral Analysis of Depressive-Like Behavior

The CCI protocol triggers the appearance of comorbidity factors at later stages, e.g., depression-like behavior. We first performed behavioral tests to ensure that the mice model of brain trauma used in this study exhibited DLB. We performed a FST (Poleszak et al., 2006; Tao et al., 2016), TST (Castagné et al., 2011; Fan et al., 2016), OFT (Tao et al., 2016), splash test (Marrocco et al., 2014; Petit et al., 2014) and finally novel object recognition (García-Pardo et al., 2016; Egeland et al., 2017). All those experiments were carried out 1 month after the CCI (1mpCCI). In the OFT, we observed significant changes in the time spent by the animal in the center of the arena (sham 50.83 ± 13 vs. CCI 60.86 ± 22 s, sham $n = 30$, CCI $n = 20$, $p = 0.04$, **Figure 1A**) whereas there was no significant difference in the total distance (sham 3308 ± 160 vs. CCI 3571 ± 155 cm, $p = 0.2$, **Figure 1A**), nor in the average speed of the animals (sham 5.8 ± 0.2 vs. CCI 6.2 ± 0.3 m/s, $p = 0.3$, **Figure 1A**). Then, we moved to more specific tests

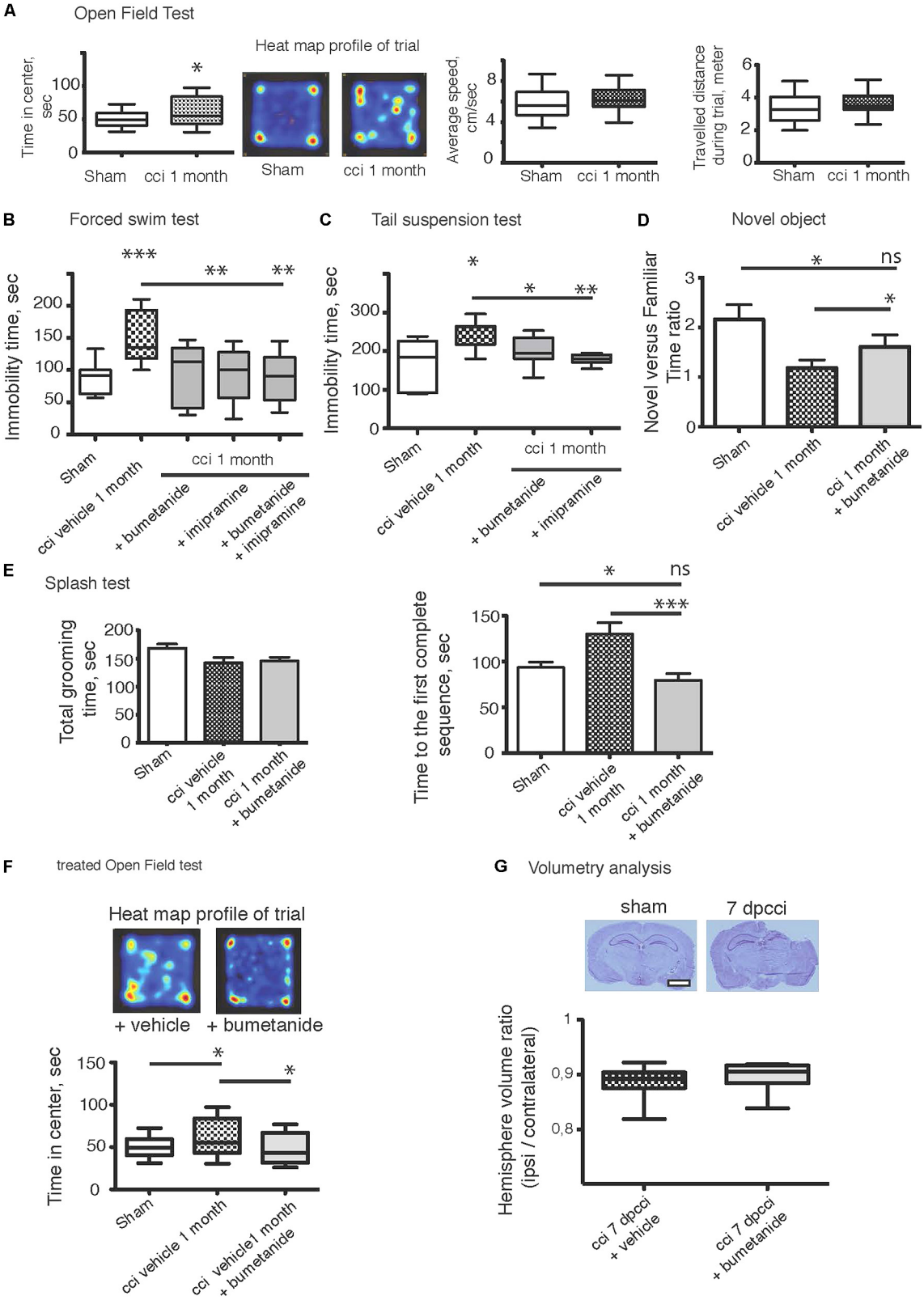

FIGURE 1 | Continued

FIGURE 1 | Bumetanide ameliorates CCI induced behavioral changes. **(A)** Open field test (OFT): plots represent both the time spent by the animal in the arena center, the total distance traveled and the average speed of the animal during the 10 min test, sham $n = 30$ and CCI $n = 20$, we used unpaired t-test for the comparison on the two population. **(B)** Forced swim test (FST): immobility time in a 25°C water for 4 min, sham $n = 15$, CCI $n = 12$, CCI + bum = 6, CCI + imipramine = 6 and CCI + bum + imipramine = 6. **(C)** Tail suspension test (TST): immobility time for 6 min (sham $n = 10$ and CCI $n = 10$. **(D)** The Novel Objet Recognition shows changes in the exploration time, the results are presented on a ratio of time of new versus familiar, $n = 16$, 15, and 17, respectively. **(E)** Splash test analyzes the total grooming time and the latency to the first complete sequence, $n = 26$, 27, 28, respectively. **(F)** After 1 week of i.p. bumetanide twice daily injection (20 μM). Treated animals showed improvement in the OFT compared to non-treated animals, $n = 20$. Statistic analysis is done using one-way ANOVA test with either Kruskal–Wallis, for non-parametric data or Tukey's comparison of multiple test for parametric data as *post hoc* treatment to compare between conditions. **(G)** Volumetry analysis: volume are calculated by summation of areas multiplied by distance between sections (500 μm) $n = 10$ brains per condition. The graph shows the ipsi over contralateral volume ratio, 7 days post cci from bumetanide- and vehicle-treated mice. Statistical significance is tested using Mann–Whitney test, $p = 0.21$. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

for depression using the FST and TST paradigm. We found a significant increase in the immobility time of CCI animals versus sham (sham FST 88.8 ± 6 vs. CCI FST 152.8 ± 12 s, $p < 0.0001$, $n = 12$ and 15 animals respectively; **Figure 1B**) (sham TST 165 ± 54 vs. CCI TST 246.7 ± 18 s, $p = 0.007$; **Figure 1C**). To confirm this was indeed a DLB, we injected imipramine (30 mg/kg, $n = 20$ animals), a classical anti-depressant compound given before the test. Interestingly, this compound could be used during behavioral tests, both in an acute and chronic manner. In the acute way, as we used in the study, it is given 30 min before testing animals (Cryan et al., 2005; Castagné et al., 2011; Zhao et al., 2015). In agreement with the literature, we observed a strong effect on the phenotype (CCI FST 152.8 ± 12 vs. CCI imipramine 89.6 ± 30 s for imipramine treated animals, six animals). The same effect on immobility time was observed on the TST (CCI 246.7 ± 18 versus CCI imipramine 168.6 ± 31 s, $n = 10$ per condition) (**Figure 1C**). Performing a novel object recognition test, a well-known test for MDD (Egeland et al., 2017), we observed a significant change in the time spent by the animal around the new object after CCI, as shown in the new versus familiar time ratio depicted in **Figure 1D** (sham 2.15 ± 0.2 vs. CCI 1.18 ± 0.15). Finally, after a 10%-sucrose solution was sprayed to the fur, the grooming behavior was assessed (Amini-Khoei et al., 2017). We observed an increase in the time to perform the first entire grooming sequence (sham 93.5 ± 5 vs. CCI 129.9 ± 12 s, $n = 23$ and 24 per condition) without any significant change in the total grooming time (sham 168 ± 7 vs. CCI 142 ± 9 s) (**Figure 1E**).

Early Application of Bumetanide Rescues CCI-Induced Depressive-Like Behavior

We then subjected CCI mice to twice-daily i.p. injections of bumetanide (2 mg/kg) during the first week after CCI. The sham and the CCI-vehicle animals received the same procedure with the vehicle solution as described in "Materials and Methods." This time window was chosen as the blood–brain barrier (BBB) is considered to remain open (Dachir et al., 2014). Behavioral analyses of the cohorts were performed again after 1 month. Analysis of these results revealed a potent action of bumetanide on all the behavioral tests. This indicates a major role of CCI-induced changes in chloride homeostasis in the induction of DLB. The effect of bumetanide was significant in both on the immobility time using FST ($p = 0.0002$, **Figure 1B**) and the TST ($p = 0.03$, **Figure 1C**), but also on the exploratory paradigm of the OFT ($p = 0.04$, **Figure 1F**). The other sets of experiments also showed a beneficial role of bumetanide on the grooming behavior when using the splash test ($p = 0.0005$, **Figure 1E**) and finally we observed a very potent effect on the novel object recognition paradigm ($p = 0.0005$, **Figure 1D**). Interestingly, we did not observe any significant difference in the FST paradigm between imipramine-treated animals and bumetanide-imipramine double treated animals (**Figure 1B**), indicating no additional effect of bumetanide over imipramine.

The accumulation of intracranial pressure is a comorbidity of closed-head TBI and this is produced by the formation of edema. Previous work suggested that changes in chloride homeostasis can have an ameliorating effect on trauma-induced edema (Lu et al., 2007) that could mediate the positive effects of inhibiting changes in chloride transport. Significant formation of intracranial pressure is not expected in this study, as it is an open-head CCI model. To investigate the effect of bumetanide on lesion size, we performed volumetric analysis on both bumetanide- and vehicle-treated CCI brains. By performing cresyl-violet staining, we defined the brain lesion size (**Figure 1G**). The volume ratio between contralateral and ipsilateral hemispheres showed that, at 7 dpCCI, there was no significant modification in the lesion size after bumetanide application (CCI 0.88 ± 0.01 vs. CCI bumetanide 0.89 ± 0.01, $n = 10$) (**Figure 1G**).

CCI-Induced Changes in Hippocampal Network Activity and Inhibitory Strength of GABAergic Signaling

Studying changes in neuronal activity is important to understand the appearance of MDD. As the hippocampus is one of the main regions known to be involved in the occurrence of DLB, the behavioral tests led us to focus on the hippocampal network activity. The action of bumetanide on the prevention of post-traumatic DLB suggests that changes in chloride homeostasis and in GABAergic neurotransmission in the hippocampus may be involved in the process. Indeed, impairment in chloride homeostasis after TBI has already been shown in the hippocampus in *ex vivo* paradigm (Rivera et al., 2004; Shulga et al., 2012). In order to assess whether GABA(A) transmission was affected in our model, we monitored the effect of GABA(A) receptor activation on extracellular field potentials in the hippocampus. As spontaneous activity of the DG is known to be quite low (Spruston and Johnston,

1992; Kvajo et al., 2011) (**Supplementary Figure 1**), we decided to record multi-unit activity (MUA) from the CA3 hippocampal region. Acute hippocampal slices, both from ipsi- and contra-lesional hemispheres at 3 days after CCI were recorded in the presence of 10 μM isoguvacine, a potent and selective GABA(A) agonist. Such treatment exerts an excitatory action on the action potential spiking frequency on ipsi- but not on the contralateral hippocampus at 3 dpCCI (**Figure 2B**), as compared to the sham condition (**Figure 2A**). This set of results suggests that GABAergic transmission is modified, rendering the network more excitable. The strong block of the depolarizing effect of isoguvacine by bath application of 10 μM bumetanide (n = 2 animals, 4 to 5 slices per animal) (**Figure 2C**) indicates the involvement of chloride imbalance in the CCI-induced changes in GABA(A) responses.

Changes in Chloride Regulatory Proteins After CCI

We then investigated whether changes in network excitability could be explained by changes in dynamics of chloride extrusion efficacy. To estimate to what extent chloride-regulatory proteins are affected during the early post-traumatic time window, CCI, NKCC1, and KCC2 protein expression levels were followed during the first post-traumatic week in the hippocampus.

We observed a significant decrease in KCC2 protein expression rapidly after the trauma with a recovery on the 7th day at the ipsi and contralateral hippocampus (**Figures 3A,B**). For NKCC1 analysis, we did not observe any significant changes in protein levels in the hippocampi (**Figures 3A,B**). Similar results were obtained for KCC2 and conversely for NKCC1 at mRNA levels in the injured hemisphere (**Supplementary Figure 3**).

CCI-Induced Internalization of KCC2 Plasma Membrane

To study a possible link between protein expression and network activity changes, we then investigated whether KCC2 expression and more specifically its subcellular distribution were affected. We used a specific KCC2 antibody to examine the cellular distribution of KCC2. We decided to focus on DG, a hippocampal region where changes in network activity is already reported after TBI (Bonislawski et al., 2007) and as the DG region is involved in depression. In sham granular cells, KCC2 was mainly located near the membrane of cell bodies (**Figure 3C**). In contrast, the labeling of KCC2 in granular cells was largely cytoplasmic 3 dpCCI (**Figure 3D**). The cellular distribution of KCC2 in sham and 3 dpCCI was significantly different with a peak around the membrane for sham granular cells (sham 64.09 ± 4.550 N = 2 n = 60 vs. 3 dpCCI 44.34 ± 1.83 N = 3 n = 88), together with staining dispersion over the cytoplasmic compartment in granular cells at 3 dpCCI (sham 13.56 ± 1.011 N = 2 n = 60 vs. 3 dpCCI 31.92 ± 1.54 N = 3 n = 88) (**Figure 3E**). This suggests an internalization of KCC2 after TBI and is in agreement with robust changes in chloride homeostasis and GABAergic transmission in the DG.

To examine if GABAergic transmission is altered all over the hippocampus, we decided to also study the functionality of chloride transport using Clomeleon mice in the CA1 region (Berglund et al., 2006) (**Supplementary Figure 2**). We found that chloride extrusion was significantly reduced at 3 and 5 but not at 7 dpCCI compared to sham condition, thus confirming the phenotype observed using the MUA approach and the biochemical techniques. Our results suggest a general effect of CCI on chloride transport into the hippocampal formation through the reduction of KCC2 expression and function.

Taken together, these results show an imbalance in the NKCC1/KCC2 ratio in favor of NKCC1 and a loss of function of KCC2.

Bumetanide Rescues Post-traumatic Impairment in Secondary Neurogenesis

Previous results suggested that the effect of antidepressants on proliferation of adult born neurons of the DG might be involved in the mechanism of action of these compounds. Considering the prophylactic anti-depressant effect of bumetanide found in this study, it is plausible that part of the antidepressive effect is mediated by changes in proliferation. Thus, we monitored both the proliferative cells and newly born neurons at the end of the first post-traumatic week. Neurons were labeled with double-cortin (DCX), a marker of immature neurons (Ren et al., 2015), and proliferative cells were stained with bromo-deoxy-Uridin (BrdU) to assess the relative number of dividing cells within the granular layer of the DG (Samuels et al., 2015). The number of positive cells was calculated on a defined slice volume (see section "Materials and Methods") and expressed as the total number of positive cells per volume. We observed a significant CCI-induced reduction in the number of the DCX positive neurons within the DG both in the ipsi- and contra-lesional hippocampi at 7 dpCCI (sham ipsi 168.3 ± 26.8 vs. CCI ipsi 45.9 ± 11.09 and sham contra 158.8 ± 15.3 vs. CCI contra 89 ± 21.8; respectively 6 and 4 animals, 4 slices per animal) (**Figures 4A,C**), together with an increase in the number of BrdU positive cells within the DG (sham ipsi 45 ± 5.8 vs. CCI ipsi 53.5 ± 4.3 and sham contra 43.5 ± 4.2 vs. CCI contra 81.3 ± 6.7, n = 4 and 6 animals, 6 slices per animal) (**Figures 4A,C**). Bumetanide treatment reduced first the number of BrdU positive cells (CCI bumetanide ipsi 27.9 ± 3.8 and contra 54 ± 3, n = 4 animals, 6 slices per animal) (**Figures 4A–C**) and triggered an increase in the number of newly generated neurons (CCI bumetanide ipsi 69.2 ± 11 and contra 138.4 ± 11, n = 4 animals, 4 slices per animal) (**Figures 4A–C**).

Interestingly, 1 month after trauma, it was not possible to find any significant differences in the number of BrdU+ (sham contra 29 ± 1.3 vs. CCI contra 26 ± 1.6) and in the number of DCX+ neurons (sham contra 124 ± 6.1 vs. CCI contra 91.3 ± 8.5) within the contralateral hippocampus of CCI animals compared to sham animals (**Figures 4B–D**). On the contrary, a significant and persistent loss of newly generated neurons (sham ipsi 145.3 ± 7.3 vs. CCI ipsi 85.3 ± 11.3) with no significant change in BrdU positive cells (sham ipsi 28.3 ± 1.9 vs. CCI ipsi 34.9 ± 3.9) (**Figures 4B,D**) was still present at the ipsilateral side, indicating a permanent change at the DCX level compared to

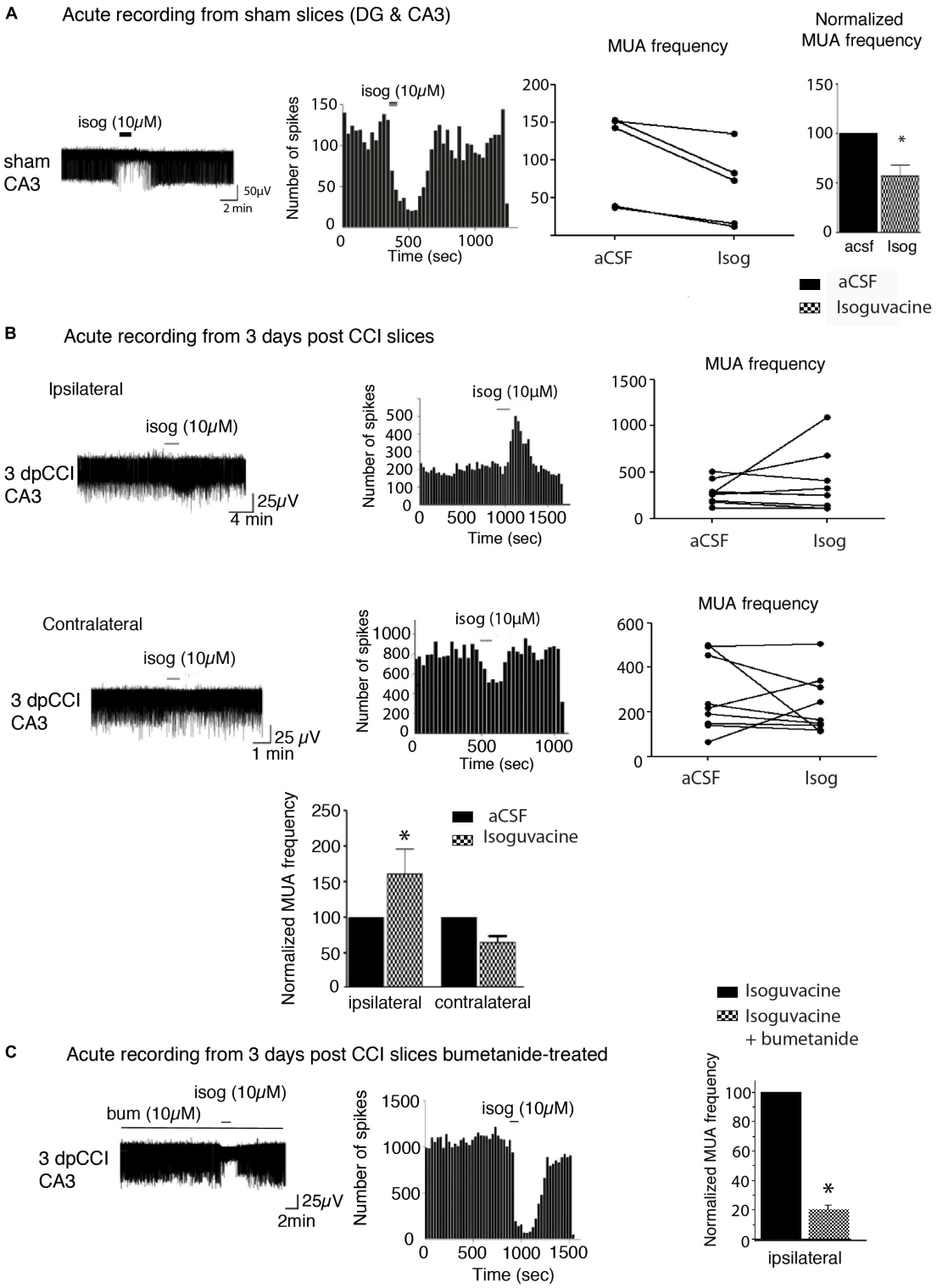

FIGURE 2 | Network activity recording and chloride extrusion efficacy. **(A)** Effect of isoguvacine (10 μM) on hippocampal networks from ipsi and contralateral hippocampus from sham animals. **(B)** Effect of isoguvacine (10 μM) on hippocampal networks at 3 days post-CCI, Top left: example trace of spontaneous extracellular field potentials recorded in ipsilateral hippocampus. Middle: corresponding time course of spike frequency changes. Top right: graph of non-normalized spike frequencies. Middle left: example trace of spontaneous extracellular field potentials recorded in contralateral hippocampus. Middle: corresponding time course of spike frequency changes. Middle right: graph of non-normalized spike frequencies. Bottom: average histograms of normalized spike frequencies. **(C)** The same as in **(B)** with acute pre-treatment of bumetanide (10 μM). 3 days post-CCI (n = 2 animals, 4–5 slices per animal). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

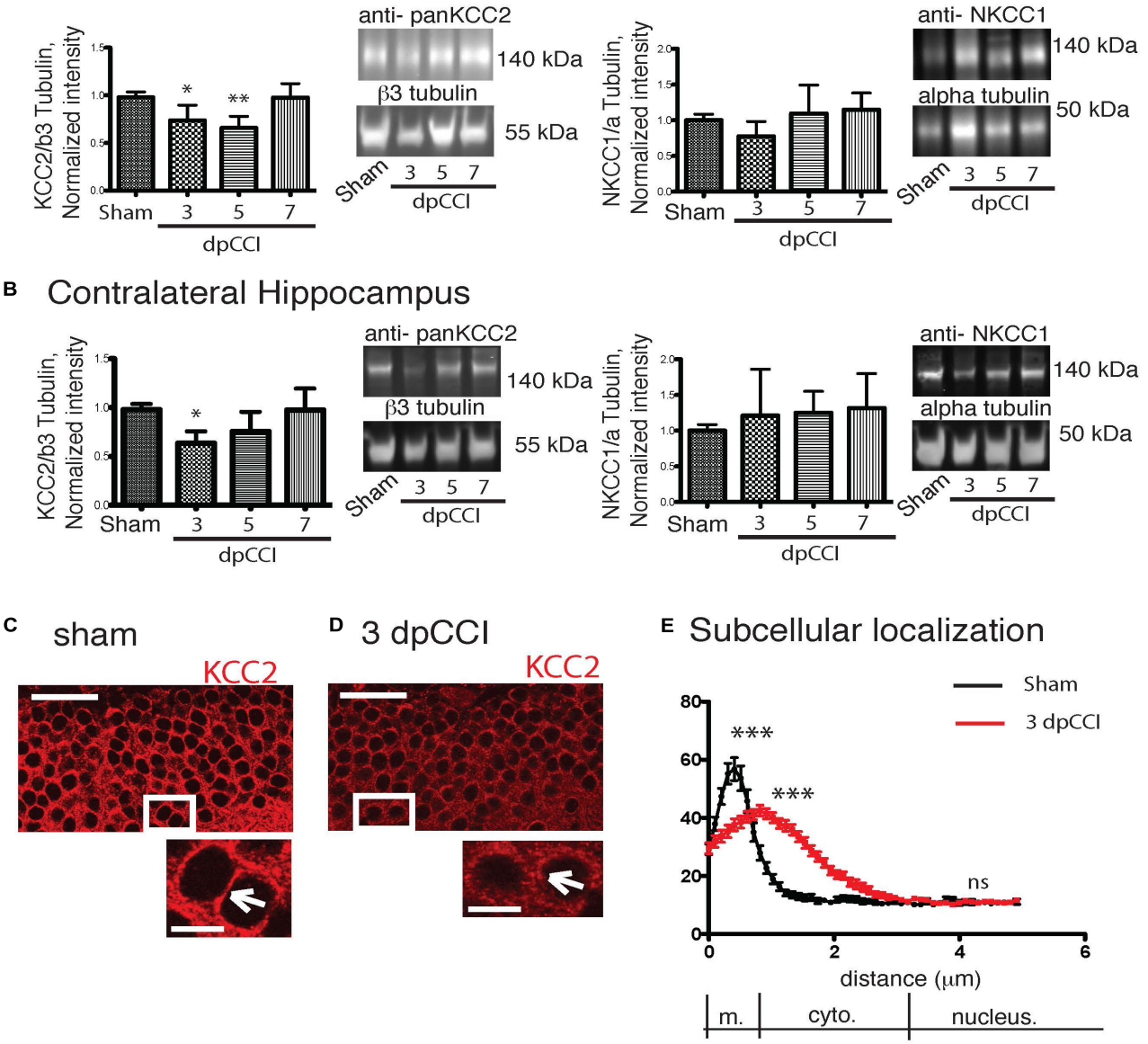

FIGURE 3 | CCI-induced changes in chloride co-transporters expression. **(A)** The left panel represents the KCC2 protein expression normalized to the neuronal marker β3-tubulin on the ipsilateral hippocampus. Protein expression over the time is expressed in comparison to the sham conditions. On the right panel, NKCC1 protein expression is shown normalized to the ubiquitous marker α-tubulin. Protein expression over the time is expressed in comparison to sham conditions, $n = 8$ per condition. One-way ANOVA test is performed and expressed as following $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ together with Kruskal–Wallis *post hoc* test. **(B)** Same as **(A)** but in the contralateral hippocampus. **(C–E)** KCC2 staining in granule cells. **(C)** Sham at 3 dpCCI. The labeling is at the cellular membrane (arrowhead) and the cytoplasm is almost devoid of KCC2 labeling. **(D)** 3 dpCCI. KCC2 is found in the cytoplasmic cell compartments (arrowheads). **(E)** Histograms representing the distribution and quantification of the intensity of fluorescence in 3 dpCCI cells (red curve) in sham (black curve). Statistical analysis represents the difference in each sub region of the cell, namely membrane, cytoplasm, and nuclear staining. Scale bars: 50 and 10 μm.

the transient one observed in the contralateral side. This suggests that both hemispheres are involved in the early settling up of post-traumatic depression.

Ambient GABA, that is provided by the activity of DG interneurons, may play a role in the proliferation and migration of granular cell progenitors (Duan et al., 2008). We then wonder if GABAergic signaling known as a neurogenesis modulator (Moss and Toni, 2013; Samuels et al., 2015; Alvarez et al., 2016) could contribute to the etiology of post-traumatic depression (Cryan et al., 2005). Therefore, it appeared interesting to quantify parvalbumin interneurons, which are known to play a critical role in post-traumatic consequences (Drexel et al., 2014; Hsieh et al., 2014; Khodaie et al., 2014). We quantified the parvalbumin-containing interneurons survival in the granular

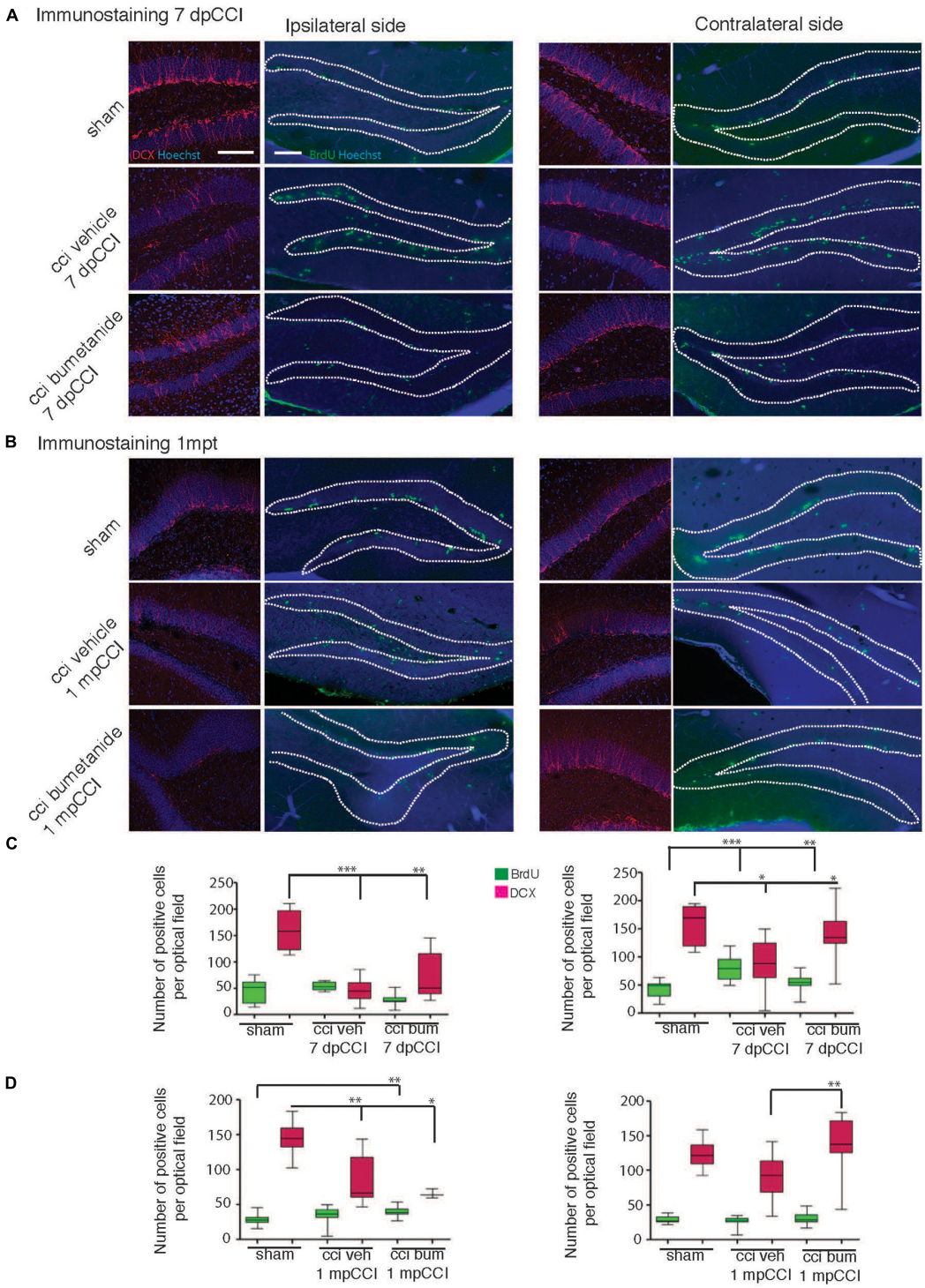

FIGURE 4 | Effect of bumetanide on CCI-induced changes in secondary neurogenesis. Secondary neurogenesis in the dentate gyrus. **(A)** Double-cortin (DCX) and BrdU labeling at 7 days post-CCI in the ipsilateral (left) and contralateral (right) dentate gyrus of sham, CCI vehicle and bumetanide-treated animals. Dotted lines delimit granular layer of dentate gyrus (scale bar = 100 μm). **(B)** Same as in **(A)** at 1 month post-CCI. **(C)** Quantification of BrdU and DCX positive cells 7 dpCCI in the ipsilateral (left) and contralateral (right) dentate gyrus of sham, CCI vehicle and bumetanide-treated animals. **(D)** Same as in **(C)** at 1 month post-CCI. DCX 7 days post-CCI: $n = 6$ animals per condition, 3 slices per animal; 1 month post-CCI; $n = 4$ animals per condition, 2–4 slices per animal. BrdU 7 days post-CCI: $n = 5$ sham $n = 6$ CCI vehicle and 6 CCI bumetanide, 2–6 slices per animal, 1 month post-CCI: $n = 3$ sham $n = 4$ CCI vehicle and 4 CCI bumetanide, 3–4 slices per animal. All sets of data were analyzed using one-way ANOVA test with Tukey's *post hoc* test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

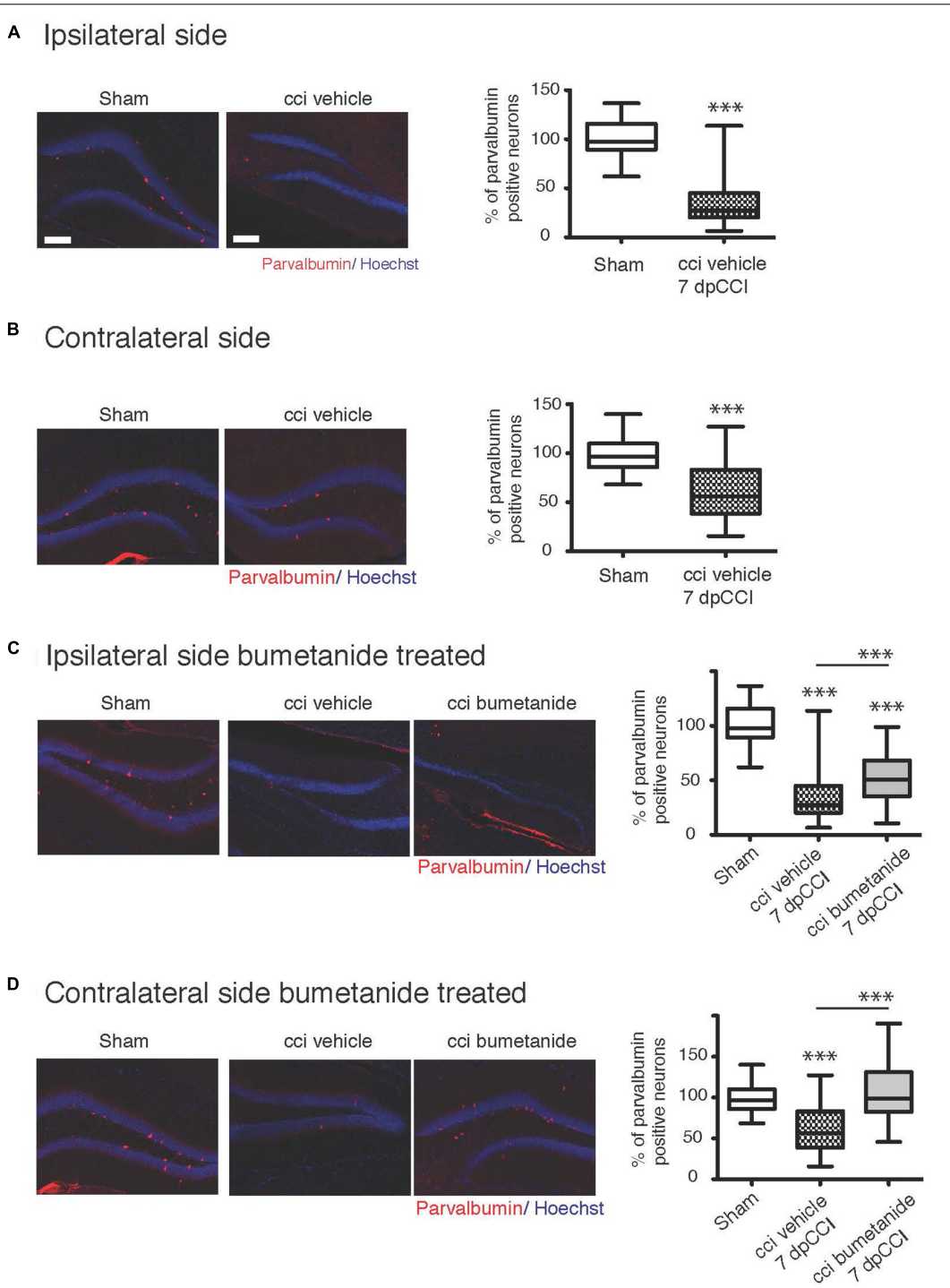

FIGURE 5 | Effect of bumetanide on CCI-induced parvalbumin positive interneuron death. **(A)** Ipsilateral hippocampus: left panel example of parvalbumin and Hoechst immunostaining, from sham and CCI mice. On the right panel, quantification of parvalbumin positive interneurons in the dentate gyrus normalized to sham values. $n = 5$ animals per condition. **(B)** Same as **(A)** but in contralateral hippocampus, the histogram shows reduction in the number of parvalbumin-containing cells in the DG, $n = 5$ animals per condition. **(C)** Effect of bumetanide in parvalbumin interneuron survival in the ipsilateral hippocampus. The histogram shows a significant reduction in the cell loss in the presence of bumetanide but this is though significantly less as compared to sham, $n = 5$ animals per condition. **(D)** Contralateral hippocampus: bumetanide injection reduces interneurons loss, $n = 5$ animals per condition. All sets of data were analyzed using one-way ANOVA test with Tukey's *post hoc* test. One-way ANOVA test is expressed as following $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

layer of the DG, both from ipsi and contralesional hippocampi at 7 dpCCI. Both sides showed a significant reduction of the number of parvalbumin-positive interneurons, compared to sham condition (ipsi 67 ± 4.6%, ***, n = 5 animals, 25 slices; contra 40 ± 6%, ***, n = 5 animals, 26 slices, **Figures 5A,B**). This loss was significantly reduced by bumetanide application at the contralateral (101 ± 5.3%, ***, n = 5 animals, 3 to 4 slices per animal, **Figure 5D**) and ipsilateral side (52 ± 3.9%, **, n = 5 animals, 3 to 4 slices per animal, **Figure 5C**), compared to the CCI condition itself. Thus, the effect of bumetanide on DG secondary neurogenesis could be partly caused by changes in ambient GABA that is provided by the activity of DG interneurons.

DISCUSSION

Our fundamental issue was to understand the consequences of the depolarizing GABA at both early and late stages after brain trauma. Using our experimental model, we found that CCI-induced DLB is strongly sensitive to trauma-induced changes in GABA(A)-mediated responses. The depolarizing GABA(A) responses, at very early stages after CCI, lead to DLB phenotypes at chronic stages. The question raised by these results is how changes in GABA(A) transmission are involved in the CCI-induced rearrangements in the hippocampal network, leading to abnormal behavior (**Figure 6**). Our analyses after CCI highlight long-term impairment of mood-associated behavior. CCI mice exhibited a phenotype that mimicks a decreased defensive behavior in an unfamiliar environment, as it has been described in other anxiety-like behavioral tests (Pandey et al., 2009; Stemper et al., 2015). Surprisingly, blocking GABA(A)-mediated depolarization, with the specific inhibitor bumetanide at early stages after CCI, resulted in a significant long-term reduction in DLB, long after the end of the treatment with bumetanide. These results pinpoint an important role of the qualitative changes in GABA(A) responses and suggest that bumetanide itself could act in a prophylactic manner as an anti-depressant compound; this was proven to be independent of its effect on lesion volume in a model of cerebral ischemia (Xu et al., 2016) and in our hands. Consistent changes in KCC2 and NKCC1 expression have been found in a number of trauma models, as well as in resections form temporal lobe epilepsy (Pallud et al., 2014). Qualitative changes in GABA(A) transmission and levels of chloride regulatory proteins have not however been well-characterized in CCI models (Robel et al., 2015; Hui et al., 2016). In the present study, we show that KCC2 expression is significantly changed in the hippocampus during the first week following trauma. Interestingly, although these changes occur shortly after CCI in both the ipsi and contralesional hippocampi, they remain transient as KCC2 expression levels return to normal values in both hippocampi. Changes in chloride extrusion efficacy are consistent with this biochemical conclusion, thus resulting in a switch in GABA(A)-mediated network excitability as opposed to the control condition. As expected, the effects observed in the contralateral hippocampus are milder but present. This put the question to how these transient changes could have a long-term effect on brain circuitry. Previous results showed that almost all antidepressants have significant effects on proliferation in the DG and production of newborn neurons. This has led to the hypothesis that proliferation in the DG is associated with DLB. While CCI induced a significant increase in proliferation in the contralateral DG alone, the number of DCX positive immature neurons was significantly diminished on both sides. The acute effect of bumetanide that we observe, leads to increased neuron production and reduced proliferation. The results we show here propose that the neurogenesis itself is modified leading to a reduction in neuron production. The remaining question is how this transient KCCs effect could last longer. Our results provide evidences that transition from BrdU positive cells to double-cortin positive immature neurons after CCI is significantly affected by blocking GABA(A)-mediated responses at early stages. This, together with the interneurons cell death, leads us to propose that GABAergic neurotransmission, either qualitative and quantitative, impacts the secondary neurogenesis (Scharfman and McCloskey, 2009; Toda and Gage, 2017). Further studies are now needed to discriminate the exact role of chloride transporters in both cell cycle and cell differentiation.

Other TBI features must be taken into account to explain the action of bumetanide seen in this study. Among them, inflammation is the most common phenomenon happening after brain insults (Solmaz et al., 2009; Anderson, 2013; Anderson et al., 2014). This inflammation could have several effects, among which, cell-death is one of the most prominent effects. It has been shown in other models that inflammation is associated with a massive loss of hippocampal formation write in epilepsy (Aldenkamp and Bodde, 2005; Swartz et al., 2006; Jefferys, 2010; Peng et al., 2013) or TBI models (Swartz et al., 2006; Morrison et al., 2011; Belousov et al., 2012; Acosta et al., 2013) and more particularly in the DG region (Lowenstein et al., 1992; Kourdougli et al., 2015; Sun et al., 2015). In that context, neuronal cell death and more specifically Parvalbumin positive cell death is one particular phenomenon both in epilepsy (Huusko et al., 2015) and in TBI models (Santhakumar et al., 2001; Cantu et al., 2014; Huusko and Pitkänen, 2014). In those studies, even with a milder model, they were able to show a really strong effect in Parvalbumin cell survival. Although it's known that principal cells required KCC2 for their survival (Lee et al., 2007; Pellegrino et al., 2011), nothing is known about interneurons survival in that particular context. One explanation could be that interneurons are not dying but lose their biochemical identity after trauma since this has been demonstrated in other trauma models (Unal-Cevik et al., 2004; Todkar et al., 2012; Kelley et al., 2016). In our model, interneurons also lose their GAD67 immunoreactivity but this loss is not transient as shown in other models (Kelley et al., 2016) since it persists after 1 month, thus, confirming a significant and permanent reduction in the number of parvalbumin-positive interneurons. Disruption of the BBB is also an important in leading to cell death and brain invasion. In our model the bbb remains open during the first post-traumatic week, thus the crossing of the BBB is possible during that specific time period (Dachir et al., 2014). In other studies, its disruption was observed (Cernak, 2005; Davidsson and Risling, 2011; Zhu et al., 2018) and proposed to be linked to glial and microglial invasion. Again, further studies

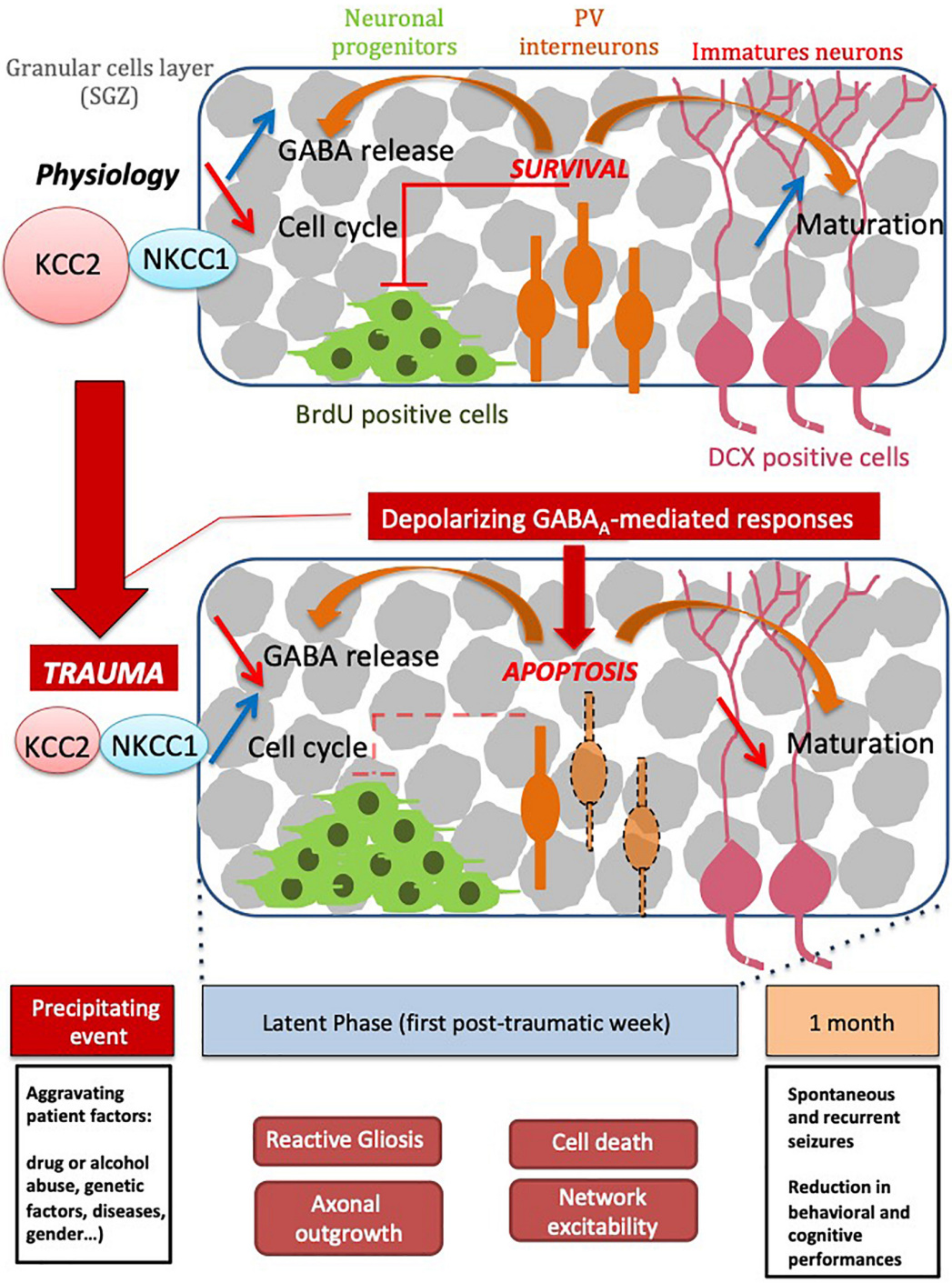

FIGURE 6 | Schematic scheme of TBI time course events. Please note that series of events taking place right and shortly after trauma are sequentially arranged leading to both short and long term consequences leading to decreased cognitive performance.

are necessary to determine the exact contribution of chloride homeostasis in inflammation. One other brain trauma feature we need to add to this discussion is the cell volume regulation and more particularly, the neuronal volume regulation. It has been proposed that, after brain insults, neurons could have volume variation in an aquaporin-independent process (Hoffmann et al., 2009; Zeuthen, 2010; Ullah et al., 2015). This aspect needs to be linked with the structural plasticity observed in the principal cells after trauma in which both the dendritic tree (Winston et al., 2013; Wang et al., 2016) and dendritic spines are affected

(Swann et al., 2000). One possibility could be that bumetanide, through its action on hyperexcitability, could prevent protein remodeling observed after TBI, thus preventing volume changes. One of the questions of interest in our case regards bumetanide action and the so-called therapeutic window we used. We decided to work on the first post-traumatic week due to the poor BBB permeability for bumetanide (Töllner et al., 2014), and as BBB is known to be opened at that stage (Dachir et al., 2014). Even if the peripheral action of bumetanide could have effect in the CNS, we consider our main effect to be central as shown by the effect on parvalbumin survival and on neurogenesis.

Although the CCI-induced changes in chloride regulatory proteins and GABA(A) transmission are consistent with short-term effects on excitability, it is less obvious how this is involved in a long-term effect after trauma. A possible contributing explanation could be related to cell death of interneurons. We have previously shown that the qualitative changes in GABA(A) responses is tightly linked with the survival mechanism of injured neurons (Pellegrino et al., 2011; Shulga et al., 2012). Changes in the interneuron population leading to changes in GABA release could significantly change the excitability of the network (Hsieh et al., 2014; Shiri et al., 2014). In some pathological contexts, such as temporal lobe epilepsy and TBI as well as in another different model of acquired epilepsy, parvalbumin interneurons are known to be very sensitive to death (Drexel et al., 2011; Hsieh et al., 2014) and their loss is involved in the dentate gyrus hyperexcitability triggered by aberrant sprouting (Zhang and Buckmaster, 2009). Altogether this highlights that both can lead to changes GABA polarity at early stages and impaired GABAergic signaling. We have proposed that bumetanide could prevent trauma induced cell death (Shulga and Rivera, 2013; Hui et al., 2016). This mechanism involved the block of the post-traumatic depolarizing effect of GABA(A) receptor that is produced by KCC2 functional downregulation (Lee et al., 2011; Pellegrino et al., 2011; Winkelmann et al., 2015; Hui et al., 2016). The results presented here clearly show that bumetanide prevents CCI-induced interneuron death at least in the DG. Thus, the previously shown mechanism for trauma-triggered apoptosis of principal cells could also be relevant for interneurons. The activity of parvalbumin interneurons has been linked to changes in secondary neurogenesis in the DG. Released ambient GABA from hilar interneurons, and this is of primary importance for regulating the proliferation state of cells within the DG (Boldrini et al., 2013; Moss and Toni, 2013; Samuels et al., 2015; Alvarez et al., 2016). Thus, the effect of bumetanide on interneuron survival could contribute to the short and long-term effects on DG proliferation (Sun et al., 2012).

In the present study we have mainly focused on DLB but bumetanide amelioration of other pathological behaviors may be also present. It will be highly interesting to supplement these studies with other cognitive tests for, e.g., learning and memory together with social interaction paradigms. We show that each hemisphere reacts to the brain trauma to different extents and with different kinetics. This highlights that the consequences in the contralateral hemisphere are as important as in the ipsilateral side (Khalilov et al., 2003), and thus cannot be considered as an independent structure in the etiology of DLB. This result also prompts testing of the specific compound to block NKCC1 able to penetrate the BBB or promote KCC2 function after CCI to restore GABAergic activity (Gagnon et al., 2013; Medina et al., 2014; Puskarjov et al., 2014).

CONCLUSION

Our study pinpoints the contribution of the depolarizing GABA in the establishment of the TBI-induced DLB. This work opens new perspectives to treat TBI-associated psychiatric disorders and suggests the use of bumetanide as a potential prophylactic agent.

AUTHOR CONTRIBUTIONS

EG was responsible for biochemistry, IHC, TBI model, electrophysiology, and surgery. MA performed the volumetric analysis and IHC. MNR performed the behavioral analysis. MM performed the behavioral analysis and IHC. IK performed the multi-unit recording. AS performed the neuroscoring and surgery. MS and CR designed the experiment and wrote the manuscript. CP performed the behavioral analysis, designed the experiment, wrote the manuscript.

FUNDING

This work was supported by the French National Agency for Research, ANR through CR and EG ANR-Traumep-13-BSV4-0012-01, the Eranet Neuron III program through Acrobat grant to CR, the Akademy of Finland (AK1308265) to CR and by the "fondation des gueules cassées" through EG, part of the behavioral unit has been funded by an FRC grant "espoir en tête 2015" to Y. Ben-Ari. The Ministry of Education and Research of Russian Federation (6.2313.2017/4.6 and Top-100 to IK). This research article was also available at BioRxiv website, please visit http://biorxiv.org/cgi/content/short/443739v1. DAAD research mobility grant to MA and MS in the funding sources.

ACKNOWLEDGMENTS

We are grateful to Pr. Y. Ben-Ari and Drs. G. Chazal and F. Molinari for critical reading of the manuscript. Some data in this article are part of the doctoral thesis of MA presented to the Medical Faculty of the Johannes Gutenberg University Mainz, Germany.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2019.00012/full#supplementary-material

REFERENCES

Acosta, S. A., Diamond, D. M., Wolfe, S., Tajiri, N., Shinozuka, K., Ishikawa, H., et al. (2013). Influence of post-traumatic stress disorder on neuroinflammation and cell proliferation in a rat model of traumatic brain injury. *PLoS One* 8:e81585. doi: 10.1371/journal.pone.0081585

Aldenkamp, A. P., and Bodde, N. (2005). Behaviour, cognition and epilepsy. *Acta Neurol. Scand. Suppl.* 182, 19–25. doi: 10.1111/j.1600-0404.2005.00523.x

Alvarez, D. D., Giacomini, D., Yang, S. M., Trinchero, M. F., Temprana, S. G., Büttner, K. A., et al. (2016). A disynaptic feedback network activated by experience promotes the integration of new granule cells. *Science* 354, 459–465. doi: 10.1126/science.aaf2156

Amini-Khoei, H., Mohammadi-Asl, A., Amiri, S., Hosseini, M.-J., Momeny, M., Hassanipour, M., et al. (2017). Oxytocin mitigated the depressive-like behaviors of maternal separation stress through modulating mitochondrial function and neuroinflammation. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 76, 169–178. doi: 10.1016/j.pnpbp.2017.02.022

Anacker, C., and Hen, R. (2017). Adult hippocampal neurogenesis and cognitive flexibility — linking memory and mood. *Nat. Rev. Neurosci.* 18, 335–346. doi: 10.1038/nrn.2017.45

Anderson, G. D. (2013). Comparison of the effects of erythropoietin and anakinra on functional recovery and gene expression in a traumatic brain injury model. *Front. Pharmacol.* 4:129. doi: 10.3389/fphar.2013.00129

Anderson, M. A., Ao, Y., and Sofroniew, M. V. (2014). Heterogeneity of reactive astrocytes. *Neurosci. Lett.* 565, 23–29. doi: 10.1016/j.neulet.2013.12.030

Belousov, A. B., Wang, Y., Song, J.-H., Denisova, J. V., Berman, N. E., and Fontes, J. D. (2012). Neuronal gap junctions play a role in the secondary neuronal death following controlled cortical impact. *Neurosci. Lett.* 524, 16–19. doi: 10.1016/j.neulet.2012.06.065

Ben-Ari, Y. (2017). NKCC1 chloride importer antagonists attenuate many neurological and psychiatric disorders. *Trends Neurosci.* 40, 536–554. doi: 10.1016/j.tins.2017.07.001

Berglund, K., Schleich, W., Krieger, P., Loo, L. S., Wang, D., Cant, N. B., et al. (2006). Imaging synaptic inhibition in transgenic mice expressing the chloride indicator, Clomeleon. *Brain Cell Biol.* 35, 207–228. doi: 10.1007/s11068-008-9019-6

Boldrini, M., Santiago, A. N., Hen, R., Dwork, A. J., Rosoklija, G. B., Tamir, H., et al. (2013). Hippocampal granule neuron number and dentate gyrus volume in antidepressant-treated and untreated major depression. *Neuropsychopharmacology* 38, 1068–1077. doi: 10.1038/npp.2013.5

Bondi, C. O., Semple, B. D., Noble-Haeusslein, L. J., Osier, N. D., Carlson, S. W., Dixon, C. E., et al. (2015). Found in translation: understanding the biology and behavior of experimental traumatic brain injury. *Neurosci. Biobehav. Rev.* 58, 123–146. doi: 10.1016/j.neubiorev.2014.12.004

Bonislawski, D. P., Schwarzbach, E. P., and Cohen, A. S. (2007). Brain injury impairs dentate gyrus inhibitory efficacy. *Neurobiol. Dis.* 25, 163–169. doi: 10.1016/j.nbd.2006.09.002

Bragin, A., Li, L., Almajano, J., Alvarado-Rojas, C., Reid, A. Y., Staba, R. J., et al. (2016). Pathologic electrographic changes after experimental traumatic brain injury. *Epilepsia* 57, 735–745. doi: 10.1111/epi.13359

Brandon, N. J., and McKay, R. (2015). The cellular target of antidepressants. *Nat. Neurosci.* 18, 1537–1538. doi: 10.1038/nn.4144

Butler, C. R., Boychuk, J. A., and Smith, B. N. (2016). Differential effects of rapamycin treatment on tonic and phasic GABAergic inhibition in dentate granule cells after focal brain injury in mice. *Exp. Neurol.* 280, 30–40. doi: 10.1016/j.expneurol.2016.03.022

Cantu, D., Walker, K., Andresen, L., Taylor-Weiner, A., Hampton, D., Tesco, G., et al. (2014). Traumatic brain injury increases cortical glutamate network activity by compromising GABAergic control. *Cereb. Cortex* 25, 2306–2320. doi: 10.1093/cercor/bhu041

Castagné, V., Moser, P., Roux, S., and Porsolt, R. D. (2011). Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. *Curr. Protoc. Neurosci.* 55, 8.10A.1–8.10A.14. doi: 10.1002/0471142301.ns0810as55

Cernak, I. (2005). Animal models of head trauma. *NeuroRx* 2, 410–422. doi: 10.1602/neurorx.2.3.410

Chell, J. M., and Frisén, J. (2012). Noisy neurons keep neural stem cells quiet. *Cell Stem Cell* 11, 282–284. doi: 10.1016/j.stem.2012.08.009

Cryan, J. F., Mombereau, C., and Vassout, A. (2005). The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci. Biobehav. Rev.* 29, 571–625. doi: 10.1016/j.neubiorev.2005.03.009

Curia, G., Longo, D., Biagini, G., Jones, R. S. G., and Avoli, M. (2008). The pilocarpine model of temporal lobe epilepsy. *J. Neurosci. Methods* 172, 143–157. doi: 10.1016/j.jneumeth.2008.04.019

Dachir, S., Shabashov, D., Trembovler, V., Alexandrovich, A. G., Benowitz, L. I., and Shohami, E. (2014). Inosine improves functional recovery after experimental traumatic brain injury. *Brain Res.* 1555, 78–88. doi: 10.1016/j.brainres.2014.01.044

Damier, P., Hammond, C., and Ben-Ari, Y. (2016). Bumetanide to treat Parkinson disease: a report of 4 cases. *Clin. Neuropharmacol.* 39, 57–59. doi: 10.1097/WNF.0000000000000114

Davidsson, J., and Risling, M. (2011). A new model to produce sagittal plane rotational induced diffuse axonal injuries. *Front. Neurol.* 2:41. doi: 10.3389/fneur.2011.00041

Drevets, W. C., Price, J. L., and Furey, M. L. (2008). Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Struct. Funct.* 213, 93–118. doi: 10.1007/s00429-008-0189-x

Drexel, M., Preidt, A. P., Kirchmair, E., and Sperk, G. (2011). Parvalbumin interneurons and calretinin fibers arising from the thalamic nucleus reuniens degenerate in the subiculum after kainic acid-induced seizures. *Neuroscience* 189, 316–329. doi: 10.1016/j.neuroscience.2011.05.021

Drexel, M., Puhakka, N., Kirchmair, E., Hörtnagl, H., Pitkänen, A., and Sperk, G. (2014). Expression of GABA receptor subunits in the hippocampus and thalamus after experimental traumatic brain injury. *Neuropharmacology* 88, 122–133. doi: 10.1016/j.neuropharm.2014.08.023

Duan, X., Kang, E., Liu, C. Y., Ming, G.-L., and Song, H. (2008). Development of neural stem cell in the adult brain. *Curr. Opin. Neurobiol.* 18, 108–115. doi: 10.1016/j.conb.2008.04.001

Earnheart, J. C., Schweizer, C., Crestani, F., Iwasato, T., Itohara, S., Mohler, H., et al. (2007). GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. *J. Neurosci.* 27, 3845–3854. doi: 10.1523/JNEUROSCI.3609-06.2007

Egeland, M., Guinaudie, C., Du Preez, A., Musaelyan, K., Zunszain, P. A., Fernandes, C., et al. (2017). Depletion of adult neurogenesis using the chemotherapy drug temozolomide in mice induces behavioural and biological changes relevant to depression. *Transl. Psychiatry* 7:e1101. doi: 10.1038/tp.2017.68

Fan, Q.-Y., Xue, R., Li, Y., Zhang, T.-T., He, X.-H., Fan, S.-Y., et al. (2016). Antidepressant-like effects of ZBH2012001, a novel potent serotonin and norepinephrine reuptake inhibitor. *CNS Neurosci. Ther.* 22, 700–706. doi: 10.1111/cns.12567

Fenton, A. A. (2015). Excitation-inhibition discoordination in rodent models of mental disorders. *Biol. Psychiatry* 77, 1079–1088. doi: 10.1016/j.biopsych.2015.03.013

Ferguson, S., Mouzon, B. C., Paris, D., Aponte, D., Abdullah, L., Stewart, W., et al. (2016). Acute or delayed treatment with anatabine improves spatial memory and reduces pathological sequelae at chronic timepoints after repetitive mild TBI. *J. Neurotrauma* 34, 1676–1691. doi: 10.1089/neu.2016.4636

Gabbay, V., Mao, X., Klein, R. G., Ely, B. A., Babb, J. S., Panzer, A. M., et al. (2012). Anterior cingulate cortex γ-aminobutyric acid in depressed adolescents: relationship to anhedonia. *Arch. Gen. Psychiatry* 69, 139–149. doi: 10.1001/archgenpsychiatry.2011.131

Gagnon, M., Bergeron, M. J., Lavertu, G., Castonguay, A., Tripathy, S., Bonin, R. P., et al. (2013). Chloride extrusion enhancers as novel therapeutics for neurological diseases. *Nat. Med.* 19, 1524–1528. doi: 10.1038/nm.3356

García-Pardo, M. P., Roger-Sánchez, C., Rodríguez-Arias, M., Miñarro, J., and Aguilar, M. A. (2016). Cognitive and behavioural effects induced by social stress plus MDMA administration in mice. *Behav. Brain Res.* 319, 63–72. doi: 10.1016/j.bbr.2016.11.012

Ge, S., Goh, E. L. K., Sailor, K. A., Kitabatake, Y., Ming, G.-L., and Song, H. (2006). GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature* 439, 589–593. doi: 10.1038/nature04404

Hoffmann, E. K., Lambert, I. H., and Pedersen, S. F. (2009). Physiology of cell volume regulation in vertebrates. *Physiol. Rev.* 89, 193–277. doi: 10.1152/physrev.00037.2007

Hsieh, L.-T., Gruber, M. J., Jenkins, L. J., and Ranganath, C. (2014). Hippocampal activity patterns carry information about objects in temporal context. *Neuron* 81, 1165–1178. doi: 10.1016/j.neuron.2014.01.015

Hu, D., Yu, Z.-L., Zhang, Y., Han, Y., Zhang, W., Lu, L., et al. (2017). Bumetanide treatment during early development rescues maternal separation-induced susceptibility to stress. *Sci. Rep.* 7:11878. doi: 10.1038/s41598-017-12183-z

Hu, J.-J., Yang, X.-L., Luo, W.-D., Han, S., Yin, J., Liu, W.-H., et al. (2017). Bumetanide reduce the seizure susceptibility induced by pentylenetetrazol via inhibition of aberrant hippocampal neurogenesis in neonatal rats after hypoxia-ischemia. *Brain Res. Bull.* 130, 188–199. doi: 10.1016/j.brainresbull.2017.01.022

Hui, H., Rao, W., Zhang, L., Xie, Z., Peng, C., Su, N., et al. (2016). Inhibition of Na^+-K^+-2Cl- cotransporter-1 attenuates traumatic brain injury-induced neuronal apoptosis via regulation of Erk signaling. *Neurochem. Int.* 94, 23–31. doi: 10.1016/j.neuint.2016.02.002

Huusko, N., and Pitkänen, A. (2014). Parvalbumin immunoreactivity and expression of GABAA receptor subunits in the thalamus after experimental TBI. *Neuroscience* 267, 30–45. doi: 10.1016/j.neuroscience.2014.02.026

Huusko, N., Römer, C., Ndode-Ekane, X. E., Lukasiuk, K., and Pitkänen, A. (2015). Loss of hippocampal interneurons and epileptogenesis: a comparison of two animal models of acquired epilepsy. *Brain Struct. Funct.* 220, 153–191. doi: 10.1007/s00429-013-0644-1

Jaenisch, N., Witte, O. W., and Frahm, C. (2010). Downregulation of potassium chloride cotransporter KCC2 after transient focal cerebral ischemia. *Stroke* 41, e151–e159. doi: 10.1161/STROKEAHA.109.570424

Jefferys, J. G. R. (2010). Advances in understanding basic mechanisms of epilepsy and seizures. *Seizure* 19, 638–646. doi: 10.1016/j.seizure.2010.10.026

Kahle, K. T., Deeb, T. Z., Puskarjov, M., Silayeva, L., Liang, B., Kaila, K., et al. (2013). Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter KCC2. *Trends Neurosci.* 36, 726–737. doi: 10.1016/j.tins.2013.08.006

Kandratavicius, L., Hallak, J. E., Carlotti, C. G., Assirati, J. A., and Leite, J. P. (2014). Neurotrophin receptors expression in mesial temporal lobe epilepsy with and without psychiatric comorbidities and their relation with seizure type and surgical outcome. *Acta Neuropathol. Commun.* 2:81. doi: 10.1186/s40478-014-0081-2

Kelley, M. R., Deeb, T. Z., Brandon, N. J., Dunlop, J., Davies, P. A., and Moss, S. J. (2016). Compromising KCC2 transporter activity enhances the development of continuous seizure activity. *Neuropharmacology* 108, 103–110. doi: 10.1016/j.neuropharm.2016.04.029

Kelly, K. M., Miller, E. R., Lepsveridze, E., Kharlamov, E. A., and Mchedlishvili, Z. (2015). Posttraumatic seizures and epilepsy in adult rats after controlled cortical impact. *Epilepsy Res.* 117, 104–116. doi: 10.1016/j.eplepsyres.2015.09.009

Khalilov, I., Holmes, G. L., and Ben-Ari, Y. (2003). In vitro formation of a secondary epileptogenic mirror focus by interhippocampal propagation of seizures. *Nat. Neurosci.* 6, 1079–1085. doi: 10.1038/nn1125

Khodaie, B., Lotfinia, A. A., Ahmadi, M., Lotfinia, M., Jafarian, M., Karimzadeh, F., et al. (2014). Structural and functional effects of social isolation on the hippocampus of rats with traumatic brain injury. *Behav. Brain Res.* 278, 55–65. doi: 10.1016/j.bbr.2014.09.034

Khundakar, A., Morris, C., and Thomas, A. J. (2011). The immunohistochemical examination of GABAergic interneuron markers in the dorsolateral prefrontal cortex of patients with late-life depression. *Int. Psychogeriatr.* 23, 644–653. doi: 10.1017/S1041610210001444

Kourdougli, N., Varpula, S., Chazal, G., and Rivera, C. (2015). Detrimental effect of post status epilepticus treatment with ROCK inhibitor Y-27632 in a pilocarpine model of temporal lobe epilepsy. *Front. Cell. Neurosci.* 9:413. doi: 10.3389/fncel.2015.00413

Kvajo, M., McKellar, H., Drew, L. J., Lepagnol-Bestel, A.-M., Xiao, L., Levy, R. J., et al. (2011). Altered axonal targeting and short-term plasticity in the hippocampus of Disc1 mutant mice. *Proc. Natl. Acad. Sci. U.S.A.* 108, E1349–E1358. doi: 10.1073/pnas.1114113108

Lee, H. H. C., Deeb, T. Z., Walker, J. A., Davies, P. A., and Moss, S. J. (2011). NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor–mediated currents. *Nat. Neurosci.* 14, 736–743. doi: 10.1038/nn.2806

Lee, H. H. C., Walker, J. A., Williams, J. R., Goodier, R. J., Payne, J. A., and Moss, S. J. (2007). Direct protein kinase C-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter KCC2. *J. Biol. Chem.* 282, 29777–29784. doi: 10.1074/jbc.M705053200

Lemonnier, E., Lazartigues, A., and Ben-Ari, Y. (2016). Treating schizophrenia with the diuretic bumetanide: a case report. *Clin. Neuropharmacol.* 39, 115–117. doi: 10.1097/WNF.0000000000000136

Lemonnier, E., Robin, G., Degrez, C., Tyzio, R., Grandgeorge, M., and Ben-Ari, Y. (2013). Treating Fragile X syndrome with the diuretic bumetanide: a case report. *Acta Paediatr.* 102, e288–e290. doi: 10.1111/apa.12235

Lowenstein, D. H., Thomas, M. J., Smith, D. H., and McIntosh, T. K. (1992). Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. *J. Neurosci.* 12, 4846–4853. doi: 10.1523/JNEUROSCI.12-12-04846.1992

Lu, K.-T., Wu, C.-Y., Yen, H.-H., Peng, J.-H. F., Wang, C.-L., and Yang, Y.-L. (2007). Bumetanide administration attenuated traumatic brain injury through IL-1 overexpression. *Neurol. Res.* 29, 404–409. doi: 10.1179/016164107X204738

Ludwig, A., Li, H., Saarma, M., Kaila, K., and Rivera, C. (2003). Developmental up-regulation of KCC2 in the absence of GABAergic and glutamatergic transmission. *Eur. J. Neurosci.* 18, 3199–3206. doi: 10.1111/j.1460-9568.2003.03069.x

Lüscher, B., and Fuchs, T. (2015). GABAergic control of depression-related brain states. *Adv. Pharmacol.* 73, 97–144. doi: 10.1016/bs.apha.2014.11.003

Luscher, B., Shen, Q., and Sahir, N. (2011). The GABAergic deficit hypothesis of major depressive disorder. *Mol. Psychiatry* 16, 383–406. doi: 10.1038/mp.2010.120

Marrocco, J., Reynaert, M.-L., Gatta, E., Gabriel, C., Mocaër, E., Di Prisco, S., et al. (2014). The effects of antidepressant treatment in prenatally stressed rats support the glutamatergic hypothesis of stress-related disorders. *J. Neurosci.* 34, 2015–2024. doi: 10.1523/JNEUROSCI.4131-13.2014

Medina, I., Friedel, P., Rivera, C., Kahle, K. T., Kourdougli, N., Uvarov, P., et al. (2014). Current view on the functional regulation of the neuronal K(+)-Cl(-) cotransporter KCC2. *Front. Cell. Neurosci.* 8:27. doi: 10.3389/fncel.2014.00027

Merali, Z., Du, L., Hrdina, P., Palkovits, M., Faludi, G., Poulter, M. O., et al. (2004). Dysregulation in the suicide brain: mRNA expression of corticotropin-releasing hormone receptors and GABA(A) receptor subunits in frontal cortical brain region. *J. Neurosci.* 24, 1478–1485. doi: 10.1523/JNEUROSCI.4734-03.2004

Meyer, K., Helmick, K., Doncevic, S., and Park, R. (2008). Severe and penetrating traumatic brain injury in the context of war. *J. Trauma Nurs.* 15, 185–189; quiz 190–191. doi: 10.1097/01.JTN.0000343324.55087.de

Mombereau, C., Kaupmann, K., Gassmann, M., Bettler, B., van der Putten, H., and Cryan, J. F. (2005). Altered anxiety and depression-related behaviour in mice lacking GABAB(2) receptor subunits. *Neuroreport* 16, 307–310. doi: 10.1097/00001756-200502280-00021

Morrison, B., Elkin, B. S., Dollé, J.-P., and Yarmush, M. L. (2011). In vitro models of traumatic brain injury. *Annu. Rev. Biomed. Eng.* 13, 91–126. doi: 10.1146/annurev-bioeng-071910-124706

Moss, J., and Toni, N. (2013). A circuit-based gatekeeper for adult neural stem cell proliferation: parvalbumin-expressing interneurons of the dentate gyrus control the activation and proliferation of quiescent adult neural stem cells. *Bioessays* 35, 28–33. doi: 10.1002/bies.201200136

Ostroumov, A., Thomas, A. M., Kimmey, B. A., Karsch, J. S., Doyon, W. M., and Dani, J. A. (2016). Stress increases ethanol self-administration via a shift toward excitatory GABA signaling in the ventral tegmental area. *Neuron* 92, 493–504. doi: 10.1016/j.neuron.2016.09.029

Pallud, J., Le Van Quyen, M., Bielle, F., Pellegrino, C., Varlet, P., Labussiere, M., et al. (2014). Cortical GABAergic excitation contributes to epileptic activities around human glioma. *Sci. Transl. Med.* 6:244ra89. doi: 10.1126/scitranslmed.3008065

Pandey, D. K., Yadav, S. K., Mahesh, R., and Rajkumar, R. (2009). Depression-like and anxiety-like behavioural aftermaths of impact accelerated traumatic brain injury in rats: a model of comorbid depression and anxiety? *Behav. Brain Res.* 205, 436–442. doi: 10.1016/j.bbr.2009.07.027

Peeters, W., van den Brande, R., Polinder, S., Brazinova, A., Steyerberg, E. W., Lingsma, H. F., et al. (2015). Epidemiology of traumatic brain injury in Europe. *Acta Neurochir.* 157, 1683–1696. doi: 10.1007/s00701-015-2512-7

Pehrson, A. L., and Sanchez, C. (2015). Altered γ-aminobutyric acid neurotransmission in major depressive disorder: a critical review of the supporting evidence and the influence of serotonergic antidepressants. *Drug Des. Devel. Ther.* 9, 603–624. doi: 10.2147/DDDT.S62912

Pellegrino, C., Gubkina, O., Schaefer, M., Becq, H., Ludwig, A., Mukhtarov, M., et al. (2011). Knocking down of the KCC2 in rat hippocampal neurons increases intracellular chloride concentration and compromises neuronal survival. *J. Physiol.* 589, 2475–2496. doi: 10.1113/jphysiol.2010.203703

Peng, Z., Zhang, N., Wei, W., Huang, C. S., Cetina, Y., Otis, T. S., et al. (2013). A reorganized GABAergic circuit in a model of epilepsy: evidence from optogenetic labeling and stimulation of somatostatin interneurons. *J. Neurosci.* 33, 14392–14405. doi: 10.1523/JNEUROSCI.2045-13.2013

Pérez-Domínguez, M., Tovar-y-Romo, L. B., and Zepeda, A. (2017). Neuroinflammation and physical exercise as modulators of adult hippocampal neural precursor cell behavior. *Rev. Neurosci.* 29, 1–20. doi: 10.1515/revneuro-2017-0024

Perry, D. C., Sturm, V. E., Peterson, M. J., Pieper, C. F., Bullock, T., Boeve, B. F., et al. (2015). Association of traumatic brain injury with subsequent neurological and psychiatric disease: a meta-analysis. *J. Neurosurg.* 124, 511–526. doi: 10.3171/2015.2.JNS14503

Petit, A.-C., Quesseveur, G., Gressier, F., Colle, R., David, D. J., Gardier, A. M., et al. (2014). Converging translational evidence for the involvement of the serotonin 2A receptor gene in major depressive disorder. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 54, 76–82. doi: 10.1016/j.pnpbp.2014.04.013

Poleszak, E., Wlaź, P., Kedzierska, E., Nieoczym, D., Wyska, E., Szymura-Oleksiak, J., et al. (2006). Immobility stress induces depression-like behavior in the forced swim test in mice: effect of magnesium and imipramine. *Pharmacol. Rep.* 58, 746–752.

Puskarjov, M., Seja, P., Heron, S. E., Williams, T. C., Ahmad, F., Iona, X., et al. (2014). A variant of KCC2 from patients with febrile seizures impairs neuronal Cl- extrusion and dendritic spine formation. *EMBO Rep.* 15, 723–729. doi: 10.1002/embr.201438749

Ren, Z., Sahir, N., Murakami, S., Luellen, B. A., Earnheart, J. C., Lal, R., et al. (2015). Defects in dendrite and spine maturation and synaptogenesis associated with an anxious-depressive-like phenotype of GABAA receptor-deficient mice. *Neuropharmacology* 88, 171–179. doi: 10.1016/j.neuropharm.2014.07.019

Rivera, C., Voipio, J., Payne, J. A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., et al. (1999). The K^+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397, 251–255. doi: 10.1038/16697

Rivera, C., Voipio, J., Thomas-Crusells, J., Li, H., Emri, Z., Sipilä, S., et al. (2004). Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2. *J. Neurosci.* 24, 4683–4691. doi: 10.1523/JNEUROSCI.5265-03.2004

Robel, S., Buckingham, S. C., Boni, J. L., Campbell, S. L., Danbolt, N. C., Riedemann, T., et al. (2015). Reactive astrogliosis causes the development of spontaneous seizures. *J. Neurosci.* 35, 3330–3345. doi: 10.1523/JNEUROSCI.1574-14.2015

Roddy, D. W., Farrell, C., Doolin, K., Roman, E., Tozzi, L., Frod, T., et al. (2018). The Hippocampus in Depression: more than the sum of its parts? Advanced hippocampal substructure segmentation in depression. *Biol. Psychiatry* doi: 10.1016/j.biopsych.2018.08.021 [Epub ahead of print].

Rudolph, U., and Knoflach, F. (2011). Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. *Nat. Rev. Drug Discov.* 10, 685–697. doi: 10.1038/nrd3502

Samuels, B. A., Anacker, C., Hu, A., Levinstein, M. R., Pickenhagen, A., Tsetsenis, T., et al. (2015). 5-HT1A receptors on mature dentate gyrus granule cells are critical for the antidepressant response. *Nat. Neurosci.* 18, 1606–1616. doi: 10.1038/nn.4116

Santhakumar, V., Ratzliff, A. D., Jeng, J., Toth, Z., and Soltesz, I. (2001). Long-term hyperexcitability in the hippocampus after experimental head trauma. *Ann. Neurol.* 50, 708–717. doi: 10.1002/ana.1230

Savitz, J., Nugent, A. C., Bogers, W., Liu, A., Sills, R., Luckenbaugh, D. A., et al. (2010). Amygdala volume in depressed patients with bipolar disorder assessed using high resolution 3T MRI: the impact of medication. *Neuroimage* 49, 2966–2976. doi: 10.1016/j.neuroimage.2009.11.025

Schaible, E. V., Windschügl, J., Bobkiewicz, W., Kaburov, Y., Dangel, L., Krämer, T., et al. (2014). 2-Methoxyestradiol confers neuroprotection and inhibits a maladaptive HIF-1α response after traumatic brain injury in mice. *J. Neurochem.* 129, 940–954. doi: 10.1111/jnc.12708

Scharfman, H. E., and Bernstein, H. L. (2015). Potential implications of a monosynaptic pathway from mossy cells to adult-born granule cells of the dentate gyrus. *Front. Syst. Neurosci.* 9:112. doi: 10.3389/fnsys.2015.00112

Scharfman, H. E., and McCloskey, D. P. (2009). Postnatal neurogenesis as a therapeutic target in temporal lobe epilepsy. *Epilepsy Res.* 85, 150–161. doi: 10.1016/j.eplepsyres.2009.03.006

Schuhmacher, A., Lennertz, L., Wagner, M., Höfels, S., Pfeiffer, U., Guttenthaler, V., et al. (2013). A variant of the neuronal amino acid transporter SLC6A15 is associated with ACTH and cortisol responses and cognitive performance in unipolar depression. *Int. J. Neuropsychopharmacol.* 16, 83–90. doi: 10.1017/S1461145712000223

Shiri, Z., Manseau, F., Lévesque, M., Williams, S., and Avoli, M. (2014). Interneuron activity leads to initiation of low-voltage fast-onset seizures. *Ann. Neurol.* 77, 541–546. doi: 10.1002/ana.24342

Shulga, A., Magalhães, A. C., Autio, H., Plantman, S., di Lieto, A., Nykjær, A., et al. (2012). The loop diuretic bumetanide blocks posttraumatic p75NTR upregulation and rescues injured neurons. *J. Neurosci.* 32, 1757–1770. doi: 10.1523/JNEUROSCI.3282-11.2012

Shulga, A., and Rivera, C. (2013). Interplay between thyroxin, BDNF and GABA in injured neurons. *Neuroscience* 239, 241–252. doi: 10.1016/j.neuroscience.2012.12.007

Smiley, J. F., Hackett, T. A., Bleiwas, C., Petkova, E., Stankov, A., Mann, J. J., et al. (2016). Reduced GABA neuron density in auditory cerebral cortex of subjects with major depressive disorder. *J. Chem. Neuroanat.* 76, 108–121. doi: 10.1016/j.jchemneu.2015.10.008

Solmaz, I., Kural, C., Temiz, C., Seçer, H. I., Düz, B., Gönül, E., et al. (2009). Traumatic brain injury due to gunshot wounds: a single institution's experience with 442 consecutive patients. *Turk. Neurosurg.* 19, 216–223.

Song, J., Zhong, C., Bonaguidi, M. A., Sun, G. J., Hsu, D., Gu, Y., et al. (2012). Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. *Nature* 489, 150–154. doi: 10.1038/nature11306

Spruston, N., and Johnston, D. (1992). Perforated patch-clamp analysis of the passive membrane properties of three classes of hippocampal neurons. *J. Neurophysiol.* 67, 508–529. doi: 10.1152/jn.1992.67.3.508

Stein, M. B., Kessler, R. C., Heeringa, S. G., Jain, S., Campbell-Sills, L., Colpe, L. J., et al. (2015). Prospective longitudinal evaluation of the effect of deployment-acquired traumatic brain injury on posttraumatic stress and related disorders: results from the army study to assess risk and resilience in servicemembers (Army STARRS). *Am. J. Psychiatry* 172, 1101–1111. doi: 10.1176/appi.ajp.2015.14121572

Stemper, B. D., Shah, A. S., Pintar, F. A., McCrea, M., Kurpad, S. N., Glavaski-Joksimovic, A., et al. (2015). Head rotational acceleration characteristics influence behavioral and diffusion tensor imaging outcomes following concussion. *Ann. Biomed. Eng.* 43, 1071–1088. doi: 10.1007/s10439-014-1171-9

Sun, D., Daniels, T. E., Rolfe, A., Waters, M., and Hamm, R. (2015). Inhibition of injury-induced cell proliferation in the dentate gyrus of the hippocampus impairs spontaneous cognitive recovery after traumatic brain injury. *J. Neurotrauma* 32, 495–505. doi: 10.1089/neu.2014.3545

Sun, L., Yu, Z., Wang, W., and Liu, X. (2012). Both NKCC1 and anion exchangers contribute to Cl^- accumulation in postnatal forebrain neuronal progenitors. *Eur. J. Neurosci.* 35, 661–672. doi: 10.1111/j.1460-9568.2012.08007.x

Swann, J. W., Al-Noori, S., Jiang, M., and Lee, C. L. (2000). Spine loss and other dendritic abnormalities in epilepsy. *Hippocampus* 10, 617–625. doi: 10.1002/1098-1063(2000)10:5<617::AID-HIPO13>3.0.CO;2-R

Swartz, B. E., Houser, C. R., Tomiyasu, U., Walsh, G. O., DeSalles, A., Rich, J. R., et al. (2006). Hippocampal cell loss in posttraumatic human epilepsy. *Epilepsia* 47, 1373–1382. doi: 10.1111/j.1528-1167.2006.00602.x

Tao, W., Dong, Y., Su, Q., Wang, H., Chen, Y., Xue, W., et al. (2016). Liquiritigenin reverses depression-like behavior in unpredictable chronic mild stress-induced mice by regulating PI3K/Akt/mTOR mediated BDNF/TrkB pathway. *Behav. Brain Res.* 308, 177–186. doi: 10.1016/j.bbr.2016.04.039

Toda, T., and Gage, F. H. (2017). Review: adult neurogenesis contributes to hippocampal plasticity. *Cell Tissue Res.* 373, 693–709. doi: 10.1007/s00441-017-2735-4

Todkar, K., Scotti, A. L., and Schwaller, B. (2012). Absence of the calcium-binding protein calretinin, not of calbindin D-28k, causes a permanent impairment of murine adult hippocampal neurogenesis. *Front. Mol. Neurosci.* 5:56. doi: 10.3389/fnmol.2012.00056

Töllner, K., Brandt, C., Töpfer, M., Brunhofer, G., Erker, T., Gabriel, M., et al. (2014). A novel prodrug-based strategy to increase effects of bumetanide in epilepsy. *Ann. Neurol.* 75, 550–562. doi: 10.1002/ana.24124

Tyzio, R., Nardou, R., Ferrari, D. C., Tsintsadze, T., Shahrokhi, A., Eftekhari, S., et al. (2014). Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring. *Science* 343, 675–679. doi: 10.1126/science.1247190

Ullah, G., Wei, Y., Dahlem, M. A., Wechselberger, M., and Schiff, S. J. (2015). The role of cell volume in the dynamics of seizure, spreading depression, and anoxic depolarization. *PLoS Comput. Biol.* 11:e1004414. doi: 10.1371/journal.pcbi.1004414

Unal-Cevik, I., Kilinç, M., Gürsoy-Ozdemir, Y., Gurer, G., and Dalkara, T. (2004). Loss of NeuN immunoreactivity after cerebral ischemia does not indicate neuronal cell loss: a cautionary note. *Brain Res.* 1015, 169–174. doi: 10.1016/j.brainres.2004.04.032

Uvarov, P., Ludwig, A., Markkanen, M., Pruunsild, P., Kaila, K., Delpire, E., et al. (2007). A novel N-terminal isoform of the neuron-specific K-Cl cotransporter KCC2. *J. Biol. Chem.* 282, 30570–30576. doi: 10.1074/jbc.M705095200

Wang, C.-F., Zhao, C.-C., Jiang, G., Gu, X., Feng, J.-F., and Jiang, J.-Y. (2016). The role of posttraumatic hypothermia in preventing dendrite degeneration and spine loss after severe traumatic brain injury. *Sci. Rep.* 6:37063. doi: 10.1038/srep37063

Winkelmann, A., Semtner, M., and Meier, J. C. (2015). Chloride transporter KCC2-dependent neuroprotection depends on the N-terminal protein domain. *Cell Death Dis.* 6:e1776. doi: 10.1038/cddis.2015.127

Winston, C. N., Chellappa, D., Wilkins, T., Barton, D. J., Washington, P. M., Loane, D. J., et al. (2013). Controlled cortical impact results in an extensive loss of dendritic spines that is not mediated by injury-induced amyloid-beta accumulation. *J. Neurotrauma* 30, 1966–1972. doi: 10.1089/neu.2013.2960

Xu, W.-S., Sun, X., Song, C.-G., Mu, X.-P., Ma, W.-P., Zhang, X.-H., et al. (2016). Bumetanide promotes neural precursor cell regeneration and dendritic development in the hippocampal dentate gyrus in the chronic stage of cerebral ischemia. *Neural Regen. Res.* 11, 745–751. doi: 10.4103/1673-5374.182700

Zeuthen, T. (2010). Water-transporting proteins. *J. Membr. Biol.* 234, 57–73. doi: 10.1007/s00232-009-9216-y

Zhang, J., Pu, H., Zhang, H., Wei, Z., Jiang, X., Xu, M., et al. (2017). Inhibition of Na(+)-K(+)-2Cl(-) cotransporter attenuates blood-brain-barrier disruption in a mouse model of traumatic brain injury. *Neurochem. Int.* 111, 23–31. doi: 10.1016/j.neuint.2017.05.020

Zhang, W., and Buckmaster, P. S. (2009). Dysfunction of the dentate basket cell circuit in a rat model of temporal lobe epilepsy. *J. Neurosci.* 29, 7846–7856. doi: 10.1523/JNEUROSCI.6199-08.2009

Zhao, J., Jung, Y.-H., Jang, C.-G., Chun, K.-H., Kwon, S. W., and Lee, J. (2015). Metabolomic identification of biochemical changes induced by fluoxetine and imipramine in a chronic mild stress mouse model of depression. *Sci. Rep.* 5:8890. doi: 10.1038/srep08890

Zhu, C. S., Grandhi, R., Patterson, T. T., and Nicholson, S. E. (2018). A review of traumatic brain injury and the gut microbiome: insights into novel mechanisms of secondary brain injury and promising targets for neuroprotection. *Brain Sci.* 8:E113. doi: 10.3390/brainsci8060113

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 Molecular Neuroscience

MINI REVIEW
published: 28 March 2019
doi: 10.3389/fnmol.2019.00072

The Functional Role of Spontaneously Opening $GABA_A$ Receptors in Neural Transmission

*Nathanael O'Neill and Sergiy Sylantyev**

Center for Clinical Brain Sciences, University of Edinburgh, Edinburgh, United Kingdom

Ionotropic type of γ-aminobutyric acid receptors ($GABA_A$Rs) produce two forms of inhibitory signaling: phasic inhibition generated by rapid efflux of neurotransmitter GABA into the synaptic cleft with subsequent binding to $GABA_A$Rs, and tonic inhibition generated by persistent activation of extrasynaptic and/or perisynaptic $GABA_A$Rs by GABA continuously present in the extracellular space. It is widely accepted that phasic and tonic GABAergic inhibition is mediated by receptor groups of distinct subunit composition and modulated by different cytoplasmic mechanisms. Recently, however, it has been demonstrated that spontaneously opening $GABA_A$Rs (s-$GABA_A$Rs), which do not need GABA binding to enter an active state, make a significant input into tonic inhibitory signaling. Due to GABA-independent action mode, s-$GABA_A$Rs promise new safer options for therapy of neural disorders (such as epilepsy) devoid of side effects connected to abnormal fluctuations of GABA concentration in the brain. However, despite the potentially important role of s-$GABA_A$Rs in neural signaling, they still remain out of focus of neuroscience studies, to a large extent due to technical difficulties in their experimental research. Here, we summarize present data on s-$GABA_A$Rs functional properties and experimental approaches that allow isolation of s-$GABA_A$Rs effects from those of conventional (GABA-dependent) $GABA_A$Rs.

Keywords: GABA-A receptor, GABA-independent inhibition, phasic conductance, tonic conductance, G-proteins

OPEN ACCESS

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

Reviewed by:
Tija Jacob,
University of Pittsburgh, United States
Paul Andrew Davies,
Tufts University School of Medicine, United States

****Correspondence:***
Sergiy Sylantyev
s.sylantyev@ed.ac.uk
orcid.org/0000-0002-1358-0601

Received: *22 January 2019*
Accepted: *08 March 2019*
Published: *28 March 2019*

Citation:
O'Neill N and Sylantyev S (2019) The Functional Role of Spontaneously Opening $GABA_A$ Receptors in Neural Transmission. Front. Mol. Neurosci. 12:72. doi: 10.3389/fnmol.2019.00072

INTRODUCTION

Ionotropic receptors of γ-aminobutyric acid (GABA receptors of type A, $GABA_A$Rs) are the main receptor type that generates inhibitory interneuronal signaling in the brain. The classical form of $GABA_A$R-induced inhibitory signal is phasic inhibition: a short synchronized opening of $GABA_A$Rs in a synapse, generated by the binding of GABA released from a presynaptic terminal. However, there is an alternative form of inhibition: charge transfer through continuously active $GABA_A$Rs, or tonic inhibition, detected in peripheral nervous system in the 1970s (Brown, 1979) but documented for the central nervous system only in the 1990s (Otis et al., 1991; Brickley et al., 1996). The classical view is that tonic inhibition is generated in response to GABA, which is continuously present in the extracellular space of neural tissue due to spillover from synapses or release from astroglia and/or neurogliaform cells (Farrant and Nusser, 2005; Kozlov et al., 2006; Oláh et al., 2009). This implies the generation of a continuous inhibitory tone mainly by perisynaptic and extrasynaptic $GABA_A$Rs, since the vast majority of transporters which perform reverse uptake of GABA are localized in synapses or in their immediate vicinity (Minelli et al., 1996; Chiu et al., 2002; Conti et al., 2004). Hence, the magnitude of tonic $GABA_A$Rs-delivered current is considered to be regulated by the

availability of extracellular GABA, and by the quantity of $GABA_A$Rs at an extrasynaptic surface of a given neuron (Glykys and Mody, 2007). Later research, however, revealed that a significant part of tonic inhibition mediated by $GABA_A$Rs is independent of GABA binding, i.e., it is delivered by spontaneously opening $GABA_A$Rs (s-$GABA_A$Rs). s-$GABA_A$Rs in that study were shown to be insensitive to the competitive GABA antagonist SR-95531 (SR), but could be suppressed by the $GABA_A$R open channel blocker picrotoxin (PTX), and, to the less extent, by competitive GABA antagonist bicuculline (BIC; McCartney et al., 2007).

In the last few decades, studies of $GABA_A$Rs-mediated tonic currents have attracted a considerable interest, and have described a functional role of this form of inhibition in a number of brain areas; in particular, its important input into neural excitability, synaptic plasticity, neurogenesis and network oscillations (Mody and Pearce, 2004; Farrant and Nusser, 2005; Glykys and Mody, 2007). Since our understanding of underlying mechanisms is still far from excellent, the newly discovered type of tonic conductance delivered *via* s-$GABA_A$Rs promises a conceptual breakthrough in the field. Nevertheless, despite the phenomenon of GABA-independent gating of $GABA_A$Rs being reported in numerous publications (Neelands et al., 1999; Birnir et al., 2000; Maksay et al., 2003; Miko et al., 2004), until recently the functional role of s-$GABA_A$Rs in living neural tissue has remained beyond the focus of neuroscience research.

In this article, we try to summarize the data available to date on s-$GABA_A$Rs function in neural transmission and to discuss perspective directions for further studies which should clarify the role of s-$GABA_A$Rs under normal conditions and in pathology.

FUNCTIONAL PROPERTIES OF s-GABARs

s-GABARs: Problem of the Isolation of GABA-Independent Effects

One of the main factors which prevent a detailed study of s-$GABA_A$Rs functioning is a lack of specific pharmacological tools: the independence of s-$GABA_A$Rs gating from GABA binding makes impossible the use of competitive GABA antagonists for selective s-$GABA_A$Rs silencing, whereas allosteric modulators such as benzodiazepines display a lack of specificity, tuning both GABA-dependent and GABA-independent effects (Bianchi and Macdonald, 2001; McCartney et al., 2007; Gerak, 2009).

Hence, to clarify the input of s-$GABA_A$Rs into a given effect, differences in molecular mechanisms of SR- and PTX-induced $GABA_A$Rs silencing have been used. SR is a competitive antagonist and thus negates $GABA_A$R activity induced by GABA binding (i.e., it acts on conventional $GABA_A$Rs); in contrast, PTX binds inside the $GABA_A$R ion channel, and thus blocks all open channels, independently of the presence of GABA binding (i.e., it acts on both conventional $GABA_A$Rs and s-$GABA_A$Rs). Therefore, conventional $GABA_A$R activity can be assessed as the change in the given effect obtained in the control vs. after application of SR, whereas s-$GABA_A$R activity can be measured as the change in the effect obtained after SR application vs. after subsequent application of SR+PTX (Wlodarczyk et al., 2013)—see **Figure 1**. SR is a "silent" competitor for the GABA-binding site, i.e., it does not display inverse agonist properties. Obviously, competitive antagonists such as BIC, which display inverse agonism, cannot be used for the quantitative assessment of s-$GABA_A$Rs effects: BIC was shown not only to suppress synaptic events as SR does but also to induce an outward shift of holding current (Wlodarczyk et al., 2013).

s-GABARs Single-Channel Properties

The obvious step in the biophysical characterization of different subgroups of ionotropic receptors is a dissection of single-channel properties, such as electrical conductance, opening frequency and average open time. Single-channel recordings have repeatedly demonstrated similar or very close conductance values for s-$GABA_A$Rs and conventional $GABA_A$Rs (Mathers, 1985; Neelands et al., 1999; Birnir et al., 2000;

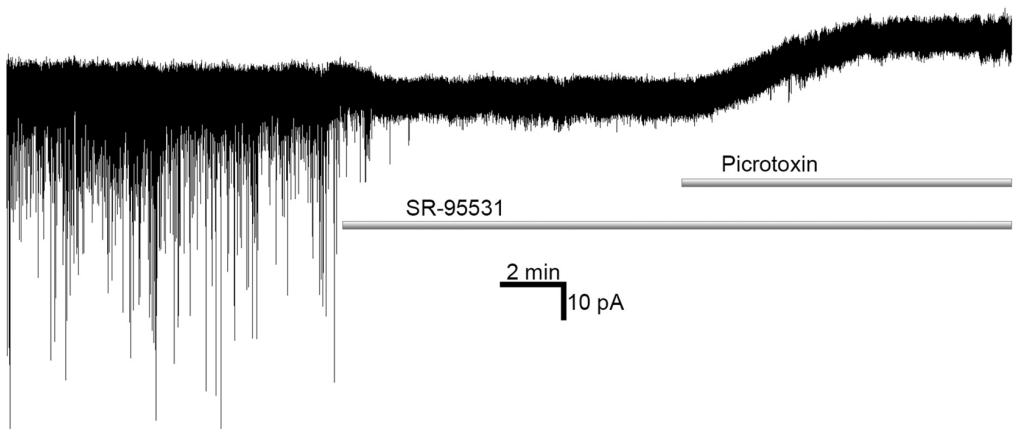

FIGURE 1 | Competitive γ-aminobutyric acid (GABA) antagonist SR-95531 suppresses spontaneous GABA-ergic synaptic signaling, but does not affect tonic conductance; on the contrary, open-channel blocker picrotoxin applied after SR-95531 shuts spontaneously opening GABA-receptors (s-$GABA_A$Rs), revealing the amount of inhibitory current passing through s-$GABA_A$Rs independently of GABA binding.

O'Neill and Sylantyev, 2018a,b) thus making this parameter hardly applicable for distinguishing between two receptor subtypes. Similarly, the dependence of $GABA_A$Rs opening frequency on the concentration of GABA, makes this parameter inapplicable for discrimination of effects of s-$GABA_A$Rs and conventional $GABA_A$Rs in single-channel recordings. In contrast, the average open time was found to be significantly lower for s-$GABA_A$Rs than for conventional $GABA_A$Rs. This generates a two-peak distribution of opening time values under physiological conditions when free GABA is present in extracellular space (O'Neill and Sylantyev, 2018a). Earlier observations demonstrated that the two-peak Gaussian distribution of average open times is a characteristic feature of $GABA_A$Rs of at least three different subunit compositions (Mortensen et al., 2010). It is important to note that the mode values for shorter durations in that work were found to be similar, irrespective of the agonist's type and concentration, thus representing an agonist-independent input. This suggests that: (i) s-$GABA_A$Rs activity is a common element of integral $GABA_A$R response; and (ii) that s-$GABA_A$Rs represent a functionally similar receptor subgroup composed of receptors of various subunit compositions.

Another method of distinguishing between s-$GABA_A$Rs and conventional $GABA_A$Rs at a level of single-channel effects may potentially develop from the recent observation about the ability of benzodiazepine flurazepam to modulate GABA-dependent and GABA-independent $GABA_A$R gating *via* different molecular mechanisms (Jatczak-Śliwa et al., 2018).

s-GABARs Input Into Tonic Conductance

Overall, charge transfer with phasic events mediated by $GABA_A$Rs (and induced by GABA binding) compared to that delivered by tonic conductance through $GABA_A$Rs, displays a ratio of more than 9/1 (Cope et al., 2005; O'Neill and Sylantyev, 2018a). Taking into account that GABA-induced tonic current was found to be negligible under physiological concentrations of extracellular GABA, whereas under these conditions s-$GABA_A$Rs generated a significant amount of tonic current (Wlodarczyk et al., 2013), s-$GABA_A$Rs should be considered as a potential key element in the generation of lasting inhibitory tone and, in a wider context, in inter-neuronal crosstalk.

Tonic inhibition has been widely accepted to be a strong modulator of action potential (AP) generation (Hamann et al., 2002; Bonin et al., 2007), AP firing patterns (Häusser and Clark, 1997) and the coincidence detection time window for synaptic inputs (Tang et al., 2011). Experiments on s-$GABA_A$Rs have readily confirmed their significant input into the regulation of the following phenomena: the modulation of AP generation (O'Neill and Sylantyev, 2018b), firing patterns (Botta et al., 2015; O'Neill and Sylantyev, 2018a), neurons' rheobase, and the time window of coincidence detection of excitatory inputs (O'Neill and Sylantyev, 2018a).

s-$GABA_A$Rs Input Into Phasic Conductance

Several classical studies have demonstrated that $GABA_A$Rs of specific subunit compositions (e.g., δ-$GABA_A$Rs) which may be responsible for a lion's share of tonic current (Nusser and Mody, 2002; Stell et al., 2003; Mortensen et al., 2010) are localized exclusively at the extrasynaptic membrane (Nusser et al., 1998; Wei et al., 2003). However, if s-$GABA_A$Rs are a functionally similar group of receptors of different subunit composition (see "s-GABARs Single-Channel Properties" section), their absence in synapses would be highly doubtful. This, in turn, raises a question as to how (and whether) s-$GABA_A$Rs modify synaptic (phasic) GABA-ergic inhibitory responses (inhibitory post-synaptic currents, IPSCs). In truth, recent studies have demonstrated their significant input into IPSC decay kinetics: s-$GABA_A$Rs introduced a slow element of decay profile (O'Neill and Sylantyev, 2018a), probably due to their higher potency to GABA (Yeung et al., 2003) and/or modified receptor efficacy.

It was shown earlier that $GABA_A$R-generated IPSC may contain fast and slow components with different sensitivities to GABA competitive antagonists, which resembles the functional profile of s-$GABA_A$Rs (Kapur et al., 1997). In this research, the generation of fast and slow components of whole-cell IPSC was attributed to different cell regions: dendritic and somatic, respectively. On the other hand, later direct recordings of s-$GABA_A$Rs activity confirmed a significant input of this receptor subtype into both whole-cell IPSCs (which are generated in synapses), and into IPSCs evoked in nucleated membrane patches, i.e., generated by $GABA_A$Rs localized at a neural cell soma (O'Neill and Sylantyev, 2018a). On top of that, a significant input of δ-$GABA_A$Rs into IPSCs was recently demonstrated (Sun et al., 2018), which confirms once again both the synaptic and extrasynaptic localization of $GABA_A$Rs which display high tonic activity.

Intracellular Regulatory Mechanisms of s-$GABA_A$Rs Activity

The particular intracellular mechanisms which are used by neural cells to modulate the activity of $GABA_A$Rs are still far from being completely understood; however, it has long been established that direct phosphorylation is of major importance (Brandon et al., 2002). It was shown that $GABA_A$Rs functions can be modulated differentially (potentiated or suppressed) depending on the receptor subunit composition, the type of neuron, et cetera by cAMP-dependent protein kinase A (PKA), tyrosine kinase Src and PKC: refer to Brandon et al. (2002) for review. In particular, $GABA_A$R-mediated tonic inhibitory currents were shown to be downregulated by PKC Bright and Smart, 2013, whereas PKA was found to enhance this type of inhibition (Carlson et al., 2016). In addition, $GABA_A$Rs effects were repeatedly shown to be modulated by G-protein-coupled receptors *via* G-proteins of different types (Cai et al., 2002; Wang et al., 2002) which are, in turn, tightly connected to the regulation of PKC and PKA activity (Neves et al., 2002). Hence, the clarification of impact on s-$GABA_A$Rs function delivered by intracellular regulatory factors (specifically, by various kinases and G-proteins), is one of the key steps needed for understanding and predicting s-$GABA_A$Rs functional input into a neural transmission.

To date, there is little data on this. It has been demonstrated that in dentate gyrus granule cells of hippocampus PKC regulates tonic GABA-dependent inhibitory conductance but has no significant impact on the GABA-independent effects of s-$GABA_A$Rs (O'Neill and Sylantyev, 2018b). However, at a longer time scale it was repeatedly shown that PKC and Ca^{2+}/calmodulin-dependent protein kinase II increase tonic inhibition in hippocampus and amygdala due to enhanced phosphorylation and membrane insertion of β3-containing $GABA_A$Rs (Saliba et al., 2012; Modgil et al., 2017) and α4-containing $GABA_A$Rs; this PKC action can be potentiated by neurosteroids such as THDOC (Abramian et al., 2010, 2014; Romo-Parra et al., 2015). In turn, s-$GABA_A$Rs-mediated tonic inhibition in dentate gyrus granule cells is controlled by G-proteins: non-specific block of G-proteins by pertussis toxin decreases the tonic current *via* the reduction of the s-$GABA_A$Rs opening frequency (O'Neill and Sylantyev, 2018b).

In contrast to PKC, activation of PKA was found to increase the tonic current through α4β3δ and, to a lesser extent, α4β3γ2L-$GABA_A$Rs in absence of GABA due to upregulation of single-channel opening frequency. Addition of GABA to an ambient solution, however, gradually decreased the sensitivity of $GABA_A$Rs of both subunit compositions to modulation by PKA; such a modulation became insignificant when GABA concentration reached micromolar values (Tang et al., 2010).

It is important to note, however, that a significant part of GABA-independent s-$GABA_A$Rs activity was found to be out of the control of any soluble cytoplasmic factors. GABA-independent openings of $GABA_A$Rs were recorded from outside-out patches excised from dentate gyrus granule cells somata: in this preparation, all cytoplasmic signaling chains are surely destroyed (O'Neill and Sylantyev, 2018b). However, anchored kinases that modulate ionotropic receptors (Brandon et al., 2003; Carnegie and Scott, 2003) may still be responsible for at least a part of the s-$GABA_A$Rs activity observed in outside-out patches.

CONCLUSIONS AND FURTHER RESEARCH DIRECTIONS

To date, there have been only a few publications highlighting the functional properties of s-$GABA_A$Rs in living neurons. This imposes obvious limitations on conclusions in terms of the applicability for different brain regions and types of neurons. Nevertheless, the significant input of s-$GABA_A$Rs into the modulation of output signal generation and into the integration of input signaling in a given neuron, suggests that s-$GABA_A$R activity is one of the key actors that regulate neural inhibition.

Indeed, the relative importance of GABA-independent s-$GABA_A$Rs signaling in a given region of the brain depends critically on the native concentration of GABA in the extracellular space. Different groups report *in vivo* concentrations varying by more than an order of magnitude: from less than 100 (Wlodarczyk et al., 2013) or 200 (Glaeser and Hare, 1975) nM to units of micromoles (Tossman et al., 1986; Takagi et al., 1993). Moreover, there may be local inhomogeneities of GABA concentrations due to cell-specific differences in the distribution and/or activity of GABA transporters and the elements of the GABA synthesis system. This was indirectly confirmed by the observation that the silencing of GAD-65 activity reduces tonic inhibitory currents in interneurons, but not in the pyramidal neurons of the hippocampal CA1 area (Song et al., 2011). A recent study on the hippocampus has demonstrated that at a GABA concentration of ∼100 nM, the amount of GABA-induced tonic current (which can be suppressed by SR) is close to statistical noise (see example at **Figure 1**), and negligible when compared to that through GABA-independent openings of s-$GABA_A$Rs (Wlodarczyk et al., 2013); on the contrary, SR has been shown to reveal a huge amount of tonic GABA-dependent current in thalamus (Cope et al., 2005). These data suggest that the relative impact of s-$GABA_A$Rs into neural signaling varies widely, depending on the particular brain region and cell type. To the best of our knowledge, previous articles that discuss lower EC_{50} values (i.e., higher potency) of extrasynaptic $GABA_A$Rs *in vivo* do not consider spontaneous channels and how they influence such measurements. This fact enforces the importance of the work on s-$GABA_A$Rs pharmacology for an understanding of biophysical phenomena in living neurons.

The important question regarding s-$GABA_A$Rs is whether or not these receptors represent a convergent group with similar functional properties, or if they share common receptor subunit(s). Numerous studies have attributed the majority (up to 75%) of $GABA_A$R-delivered tonic inhibition to δ-containing $GABA_A$Rs (Stell et al., 2003), which are abundant at extrasynaptic membranes (Nusser et al., 1998) but have been also found in synapses where they make a significant input into phasic inhibition (Sun et al., 2018), and in perisynaptic loci (Wei et al., 2003). The remaining portion of tonic inhibition is, to a large extent but not fully, produced by receptors containing the α5-subunit (Farrant and Nusser, 2005). Furthermore, the agonist-independent $GABA_A$R openings were observed under similar conditions for receptors of three different subunit compositions (Mortensen et al., 2010). In addition, the observation that mutations in α1 and β2 subunits modulate spontaneous $GABA_A$Rs gating (Baptista-Hon et al., 2017) prevents us from ruling out these subunits as potential alternative candidates to be involved in the formation of s-$GABA_A$Rs. Combined with the facts of the GABA-independent tonic activity of α4-$GABA_A$Rs (Tang et al., 2010) and spontaneous openings of α2β1ε-$GABA_A$Rs which contribute to the baseline currents in whole-cell recordings (Wagner et al., 2005), the abovementioned data on GABA-independent activity suggest that GABA-independent inhibition is of poly-subtype origin, with a substantial part inherent in the non-δ- and non-α5-containing receptors.

In view of numerous subunits and subunit compositions of $GABA_A$R which demonstrate spontaneous gating, the obvious question is: are there $GABA_A$Rs subtype(s) which do not demonstrate GABA-independent activity? The existence of such $GABA_A$Rs was suggested by the study showing that, in contrast to the α2α1ε receptor, responses of α2β1 and α2β1γ2-$GABA_A$Rs do not produce a "baseline overshoot" associated with spontaneous openings (Wagner et al., 2005).

Therefore, data collected to date suggest revision of two traditional views, now common in fundamental neuroscience: (i) that tonic inhibitory conductance is generated by ambient GABA (due to proven significance of s-$GABA_A$Rs input); and (ii) that tonic and phasic inhibition are mediated by different $GABA_A$Rs subtypes (due to growing evidence that typical extrasynaptic $GABA_A$Rs can make a significant contribution into IPSCs *via* a synaptic and/or perisynaptic presence).

It has been demonstrated that a scarcity of α1 subunit is correlated with resistance to anti-epileptic drugs (Bethmann et al., 2008), whereas increased α1-$GABA_A$R expression in the hippocampus suppresses the development of temporal lobe epilepsy (TLE; Raol et al., 2006). Apart from that, it was shown that phasic GABA-ergic inhibition is lowered in TLE, whereas tonic GABA-ergic conductance remains intact (Palma et al., 2007; Pavlov et al., 2011), making tonic GABA-ergic current a perspective target for TLE treatment. The classical paradigm, where extracellular GABA triggers tonic GABA-ergic current, implies that the most effective therapeutic approach is to increase the concentration of GABA in the cerebrospinal fluid, and thus augment inhibitory conductance. However, this approach was repeatedly found to be ineffective (Cohen et al., 2002; Glykys et al., 2009) or even one that leads to epileptogenesis (Palma et al., 2006; Cope et al., 2009) due to various side effects. These side effects impose limitations on the clinical use of specific antiepileptic drugs that increase the concentration of GABA in cerebrospinal fluid (Sander and Hart, 1990; Leppik, 1995). In contrast, the modulation of s-$GABA_A$Rs in GABA-independent manner promises an alternative for TLE treatment through the regulation of tonic conductance without the need to interfere with extracellular GABA concentration, thus avoiding the afore mentioned side effects.

Apart from the potential of α1-$GABA_A$Rs for TLE treatment, α5-$GABA_A$Rs (which also display GABA-independent activity) were found to be a perspective target for schizophrenia treatment (Lodge and Grace, 2011). Taking into account similar concentration of GABA found *in vivo* in the brains of schizophrenic patients and of a control group (Tayoshi et al., 2010), and the well-established fact that changes in tonic GABA-ergic inhibition are involved in the generation of schizophrenia symptoms (Damgaard et al., 2011), these data suggest a potentially important role of drugs targeting s-$GABA_A$Rs in the suppression of schizophrenia development, since action through s-$GABA_A$Rs in GABA-independent manner eliminates the need to modify GABA concentration in cerebrospinal fluid.

Another clinical implication of s-$GABA_A$Rs rises from the fact that sedative and analgesic effects of gaboxadol (THIP) are mediated exclusively by α4-containing $GABA_A$Rs (Chandra et al., 2006), that demonstrate GABA-independent activity.

AUTHOR CONTRIBUTIONS

NO and SS contributed to the conception and design of the article. NO received data displayed at the figure and analyzed literature connected to the topic, contributed to manuscript revision. SS wrote the manuscript.

FUNDING

This work was supported by the Rosetrees Research Grant A-1066.

REFERENCES

Abramian, A. M., Comenencia-Ortiz, E., Vithlani, M., Tretter, E. V., Sieghart, W., Davies, P. A., et al. (2010). Protein kinase C phosphorylation regulates membrane insertion of $GABA_A$ receptor subtypes that mediate tonic inhibition. *J. Biol. Chem.* 285, 41795–41805. doi: 10.1074/jbc.m110.149229

Abramian, A. M., Comenencia-Ortiz, E., Modgil, A., Vien, T. N., Nakamura, Y., Moore, Y. E., et al. (2014). Neurosteroids promote phosphorylation and membrane insertion of extrasynaptic $GABA_A$ receptors. *Proc. Natl. Acad. Sci. U S A* 111, 7132–7137. doi: 10.1073/pnas.1403285111

Baptista-Hon, D. T., Gulbinaite, S., and Hales, T. G. (2017). Loop G in the $GABA_A$ receptor α1 subunit influences gating efficacy. *J. Physiol.* 595, 1725–1741. doi: 10.1113/jp273752

Bethmann, K., Fritschy, J. M., Brandt, C., and Löscher, W. (2008). Antiepileptic drug resistant rats differ from drug responsive rats in $GABA_A$ receptor subunit expression in a model of temporal lobe epilepsy. *Neurobiol. Dis.* 31, 169–187. doi: 10.1016/j.nbd.2008.01.005

Bianchi, M. T., and Macdonald, R. L. (2001). Agonist trapping by $GABA_A$ receptor channels. *J. Neurosci.* 21, 9083–9091. doi: 10.1523/jneurosci.21-23-09083.2001

Birnir, B., Everitt, A. B., Lim, M. S., and Gage, P. W. (2000). Spontaneously opening $GABA_A$ channels in CA1 pyramidal neurones of rat hippocampus. *J. Membr. Biol.* 174, 21–29. doi: 10.1007/s002320001028

Bonin, R. P., Martin, L. J., MacDonald, J. F., and Orser, B. A. (2007). α5$GABA_A$ receptors regulate the intrinsic excitability of mouse hippocampal pyramidal neurons. *J. Neurophysiol.* 98, 2244–2254. doi: 10.1152/jn.00482.2007

Botta, P., Demmou, L., Kasugai, Y., Markovic, M., Xu, C., Fadok, J. P., et al. (2015). Regulating anxiety with extrasynaptic inhibition. *Nat. Neurosci.* 18, 1493–1500. doi: 10.1038/nn.4102

Brandon, N., Jovanovic, J., and Moss, S. (2002). Multiple roles of protein kinases in the modulation of gamma-aminobutyric $acid_{(A)}$ receptor function and cell surface expression. *Pharmacol. Ther.* 94, 113–122. doi: 10.1016/s0163-7258(02)00175-4

Brandon, N. J., Jovanovic, J. N., Colledge, M., Kittler, J. T., Brandon, J. M., Scott, J. D., et al. (2003). A-kinase anchoring protein 79/150 facilitates the phosphorylation of $GABA_A$ receptors by cAMP-dependent protein kinase via selective interaction with receptor β subunits. *Mole. Cell. Neurosci.* 22, 87–97. doi: 10.1016/s1044-7431(02)00017-9

Brickley, S. G., Cull-Candy, S. G., and Farrant, M. (1996). Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. *J Physiol.* 497, 753–759. doi: 10.1113/jphysiol.1996.sp021806

Bright, D. P., and Smart, T. G. (2013). Protein kinase C regulates tonic $GABA_A$ receptor-mediated inhibition in the hippocampus and thalamus. *Eur. J. Neurosci.* 38, 3408–3423. doi: 10.1111/ejn.12352

Brown, D. A. (1979). Extrasynaptic GABA systems. *Trends Neurosci.* 2, 271–273. doi: 10.1016/0166-2236(79)90107-3

Cai, X., Flores-Hernandez, J., Feng, J., and Yan, Z. (2002). Activity-dependent bidirectional regulation of $GABA_A$ receptor channels by the $5\text{-}HT_{(4)}$ receptor-mediated signalling in rat prefrontal cortical pyramidal neurons. *J. Physiol.* 540, 743–759. doi: 10.1113/jphysiol.2001.013391

Carlson, S. L., Bohnsack, J. P., Patel, V., and Morrow, A. L. (2016). Regulation of extrasynaptic $GABA_A$ α4 receptors by ethanol-induced protein kinase, A, but not protein kinase C activation in cultured rat cerebral cortical neurons. *J. Pharmacol. Exp. Ther.* 356, 148–156. doi: 10.1124/jpet.115.228056

Carnegie, G. K., and Scott, J. D. (2003). A-kinase anchoring proteins and neuronal signaling mechanisms. *Genes Dev.* 17, 1557–1568. doi: 10.1101/gad.1095803

Chandra, D., Jia, F., Liang, J., Peng, Z., Suryanarayanan, A., Werner, D. F., et al. (2006). $GABA_A$ receptor α4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. *Proc. Natl. Acad. Sci. U S A* 103, 15230–15235. doi: 10.1073/pnas.0604304103

Chiu, C. S., Jensen, K., Sokolova, I., Wang, D., Li, M., Deshpande, P., et al. (2002). Number, density and surface/cytoplasmic distribution of GABA transporters at presynaptic structures of knock-in mice carrying GABA transporter subtype 1-Green fluorescent protein fusions. *J. Neurosci.* 22, 10251–10266. doi: 10.1523/jneurosci.22-23-10251.2002

Cohen, I., Navarro, V., Clemenceau, S., Baulac, M., and Miles, R. (2002). On the origin of interictal activity in human temporal lobe epilepsy *in vitro*. *Science* 298, 1418–1421. doi: 10.1126/science.1076510

Conti, F., Minelli, A., and Melone, M. (2004). GABA transporters in the mammalian cerebral cortex: localization, development and pathological implications. *Brain Res. Rev.* 45, 196–212. doi: 10.1016/j.brainresrev.2004.03.003

Cope, D. W., Hughes, S. W., and Crunelli, V. (2005). $GABA_A$ receptor-mediated tonic inhibition in thalamic neurons. *J Neurosci.* 25, 11553–11563. doi: 10.1523/jneurosci.3362-05.2005

Cope, D. W., Di Giovanni, G., Fyson, S. J., Orbán, G., Errington, A. C., Lorincz, M. L., et al. (2009). Enhanced tonic $GABA_A$ inhibition in typical absence epilepsy. *Nat. Med.* 15, 1392–1398. doi: 10.1038/nm.2058

Damgaard, T., Plath, N., Neill, J. C., and Hansen, S. L. (2011). Extrasynaptic $GABA_A$ receptor activation reverses recognition memory deficits in an animal model of schizophrenia. *Psychopharmacology* 214, 403–413. doi: 10.1007/s00213-010-2039-9

Farrant, M., and Nusser, Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat. Rev. Neurosci.* 6, 215–229. doi: 10.1038/nrn1625

Gerak, L. R. (2009). Selective changes in sensitivity to benzodiazepines, and not other positive $GABA_A$ modulators, in rats receiving flunitrazepam chronically. *Psychopharmacology* 204, 667–677. doi: 10.1007/s00213-009-1497-4

Glaeser, B. S., and Hare, T. A. (1975). Measurement of GABA in human cerebrospinal fluid. *Biochem. Med.* 12, 274–282. doi: 10.1016/0006-2944(75)90129-5

Glykys, J., and Mody, I. (2007). Activation of $GABA_A$ receptors: views from outside the synaptic cleft. *Neuron* 56, 763–770. doi: 10.1016/j.neuron.2007.11.002

Glykys, J., Dzhala, V. I., Kuchibhotla, K. V., Feng, G., Kuner, T., Augustine, G., et al. (2009). Differences in cortical versus subcortical GABAergic signaling: a candidate mechanism of electroclinical uncoupling of neonatal seizures. *Neuron* 63, 657–672. doi: 10.1016/j.neuron.2009.08.022

Hamann, M., Rossi, D. J., and Attwell, D. (2002). Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron* 33, 625–633. doi: 10.1016/s0896-6273(02)00593-7

Häusser, M., and Clark, B. A. (1997). Tonic synaptic inhibition modulates neuronal output pattern and spatiotemporal synaptic integration. *Neuron* 19, 665–678. doi: 10.1016/s0896-6273(00)80379-7

Jatczak-Śliwa, M., Terejko, K., Brodzki, M., Michaowski, M. A., Czyzewska, M. M., Nowicka, J. M., et al. (2018). Distinct modulation of spontaneous and GABA-evoked gating by flurazepam shapes cross-talk between agonist-free and liganded $GABA_A$ receptor activity. *Front. Cell. Neurosci.* 12:237. doi: 10.3389/fncel.2018.00237

Kapur, A., Pearce, R. A., Lytton, W. W., and Haberly, L. B. (1997). $GABA_A$-mediated IPSCs in piriform cortex have fast and slow components with different properties and locations on pyramidal cells. *J. Neurophysiol.* 78, 2531–2545. doi: 10.1152/jn.1997.78.5.2531

Kozlov, A. S., Angulo, M. C., Audinat, E., and Charpak, S. (2006). Target cell-specific modulation of neuronal activity by astrocytes. *Proc. Natl. Acad. Sci. U S A* 103, 10058–10063. doi: 10.1073/pnas.0603741103

Leppik, I. E. (1995). Tiagabine: the safety landscape. *Epilepsia* 36, S10–S13. doi: 10.1111/j.1528-1157.1995.tb06009.x

Lodge, D. J., and Grace, A. A. (2011). Hippocampal dysregulation of dopamine system function and the pathophysiology of schizophrenia. *Trends Pharmacol. Sci.* 32, 507–513. doi: 10.1016/j.tips.2011.05.001

Maksay, G., Thompson, S. A., and Wafford, K. A. (2003). The pharmacology of spontaneously open α1 β3 ε $GABA_A$ receptor-ionophores. *Neuropharmacology* 44, 994–1002. doi: 10.1016/s0028-3908(03)00116-3

Mathers, D. A. (1985). Spontaneous and GABA-induced single channel currents in cultured murine spinal cord neurons. *Can. J. Physiol. Pharmacol.* 63, 1228–1233. doi: 10.1139/y85-203

McCartney, M. R., Deeb, T. Z., Henderson, T. N., and Hales, T. G. (2007). Tonically active $GABA_A$ receptors in hippocampal pyramidal neurons exhibit constitutive GABA-independent gating. *Mol. Pharmacol.* 71, 539–548. doi: 10.1124/mol.106.028597

Miko, A., Werby, E., Sun, H., Healey, J., and Zhang, L. (2004). A TM2 residue in the β1 subunit determines spontaneous opening of homomeric and heteromeric γ-aminobutyric acid-gated ion channels. *J. Biol. Chem.* 279, 22833–22840. doi: 10.1074/jbc.m402577200

Minelli, A., DeBiasi, S., Brecha, N. C., Zuccarello, L. V., and Conti, F. (1996). GAT-3, a high-affinity GABA plasma membrane transporter, is localized to astrocytic processes, and it is not confined to the vicinity of GABAergic synapses in the cerebral cortex. *J. Neurosci.* 16, 6255–6264. doi: 10.1523/jneurosci.16-19-06255.1996

Modgil, A., Parakala, M. L., Ackley, M. A., Doherty, J. J., Moss, S. J., and Davies, P. A. (2017). Endogenous and synthetic neuroactive steroids evoke sustained increases in the efficacy of GABAergic inhibition via a protein kinase C-dependent mechanism. *Neuropharmacology* 113, 314–322. doi: 10.1016/j.neuropharm.2016.10.010

Mody, I., and Pearce, R. A. (2004). Diversity of inhibitory neurotransmission through $GABA_A$ receptors. *Trends Neurosci.* 27, 569–575. doi: 10.1016/j.tins.2004.07.002

Mortensen, M., Ebert, B., Wafford, K., and Smart, T. G. (2010). Distinct activities of GABA agonists at synaptic- and extrasynaptic-type $GABA_A$ receptors. *J. Physiol.* 588, 1251–1268. doi: 10.1113/jphysiol.2009.182444

Neelands, T. R., Fisher, J. L., Bianchi, M., and Macdonald, R. L. (1999). Spontaneous and γ-aminobutyric acid (GABA)-activated $GABA_A$ receptor channels formed by ε subunit-containing isoforms. *Mol. Pharmacol.* 55, 168–178. doi: 10.1124/mol.55.1.168

Neves, S. R., Ram, P. T., and Iyengar, R. (2002). G protein pathways. *Science* 296, 1636–1639. doi: 10.1126/science.1071550

Nusser, Z., and Mody, I. (2002). Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J. Neurophysiol.* 87, 2624–2628. doi: 10.1152/jn.2002.87.5.2624

Nusser, Z., Sieghart, W., and Somogyi, P. (1998). Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J. Neurosci.* 18, 1693–1703. doi: 10.1523/jneurosci.18-05-01693.1998

O'Neill, N., and Sylantyev, S. (2018a). Spontaneously opening $GABA_A$ receptors play a significant role in neuronal signal filtering and integration. *Cell Death Dis.* 9:813. doi: 10.1038/s41419-018-0856-7

O'Neill, N., and Sylantyev, S. (2018b). Feature Article: selective modulation of tonically active $GABA_A$ receptor functional subgroups by G-proteins and protein kinase C. *Exp. Biol. Med.* 243, 1046–1055. doi: 10.1177/1535370218800980

Oláh, S., Füle, M., Komlósi, G., Varga, C., Báldi, R., Barzó, P., et al. (2009). Regulation of cortical microcircuits by unitary GABA-mediated volume transmission. *Nature* 461, 1278–1281. doi: 10.1038/nature08503

Otis, T. S., Staley, K. J., and Mody, I. (1991). Perpetual inhibitory activity in mammalian brain slices generated by spontaneous GABA release. *Brain Res.* 545, 142–150. doi: 10.1016/0006-8993(91)91280-e

Palma, E., Amici, M., Sobrero, F., Spinelli, G., Di Angelantonio, S., Ragozzino, D., et al. (2006). Anomalous levels of Cl- transporters in the hippocampal subiculum from temporal lobe epilepsy patients make GABA excitatory. *Proc. Natl. Acad. Sci. U S A* 103, 8465–8468. doi: 10.1073/pnas.0602979103

Palma, E., Roseti, C., Maiolino, F., Fucile, S., Martinello, K., Mazzuferi, M., et al. (2007). $GABA_A$-current rundown of temporal lobe epilepsy is associated with repetitive activation of $GABA_A$ "phasic" receptors. *Proc. Natl. Acad. Sci. U S A* 104, 20944–20948. doi: 10.1073/pnas.0710522105

Pavlov, I., Huusko, N., Drexel, M., Kirchmair, E., Sperk, G., Pitkänen, A., et al. (2011). Progressive loss of phasic, but not tonic, $GABA_A$ receptor-mediated inhibition in dentate granule cells in a model of post-traumatic epilepsy in rats. *Neuroscience* 194, 208–219. doi: 10.1016/j.neuroscience.2011.07.074

Raol, Y. H., Lund, I. V., Bandyopadhyay, S., Zhang, G., Roberts, D. S., Wolfe, J. H., et al. (2006). Enhancing $GABA_A$ receptor alpha 1 subunit levels in hippocampal dentate gyrus inhibits epilepsy development in an animal model of temporal

lobe epilepsy. *J. Neurosci.* 26, 11342–11346. doi: 10.1523/jneurosci.3329-06.2006

Romo-Parra, H., Blaesse, P., Sosulina, L., and Pape, H. C. (2015). Neurosteroids increase tonic GABAergic inhibition in the lateral section of the central amygdala in mice. *J. Neurophysiol.* 113, 3421–3431. doi: 10.1152/jn.00045.2015

Saliba, R. S., Kretschmannova, K., and Moss, S. J. (2012). Activity-dependent phosphorylation of $GABA_A$ receptors regulates receptor insertion and tonic current. *EMBO J.* 31, 2937–2951. doi: 10.1038/emboj.2012.109

Sander, J. W., and Hart, Y. M. (1990). Vigabatrin and behaviour disturbances. *Lancet* 335:57. doi: 10.1016/0140-6736(90)90190-g

Song, I., Savtchenko, L., and Semyanov, A. (2011). Tonic excitation or inhibition is set by $GABA_A$ conductance in hippocampal interneurons. *Nat. Commun.* 2:376. doi: 10.1038/ncomms1377

Stell, B. M., Brickley, S. G., Tang, C. Y., Farrant, M., and Mody, I. (2003). Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by delta subunit-containing $GABA_A$ receptors. *Proc. Natl. Acad. Sci. U S A* 100, 14439–14444. doi: 10.1073/pnas.2435457100

Sun, M. Y., Shu, H. J., Benz, A., Bracamontes, J., Akk, G., Zorumski, C. F., et al. (2018). Chemogenetic isolation reveals synaptic contribution of δ $GABA_A$ receptors in mouse dentate granule neurons. *J. Neurosci.* 38, 8128–8145. doi: 10.1523/jneurosci.0799-18.2018

Takagi, K., Ginsberg, M. D., Globus, M. Y., Dietrich, W. D., Martinez, E., Kraydieh, S., et al. (1993). Changes in amino acid neurotransmitters and cerebral blood flow in the ischemic penumbral region following middle cerebral artery occlusion in the rat: correlation with histopathology. *J. Cereb. Blood Flow Metab.* 13, 575–585. doi: 10.1038/jcbfm.1993.75

Tang, X., Hernandez, C. C., and Macdonald, R. L. (2010). Modulation of spontaneous and GABA-evoked tonic α4β3δ and α4β3γ2L $GABA_A$ receptor currents by protein kinase A. *J. Neurophysiol.* 103, 1007–1019. doi: 10.1152/jn.00801.2009

Tang, Z. Q., Dinh, E. H., Shi, W., and Lu, Y. (2011). Ambient GABA-activated tonic inhibition sharpens auditory coincidence detection via a depolarizing shunting mechanism. *J. Neurosci.* 31, 6121–6131. doi: 10.1523/jneurosci.4733-10.2011

Tayoshi, S., Nakataki, M., Sumitani, S., Taniguchi, K., Shibuya-Tayoshi, S., Numata, S., et al. (2010). GABA concentration in schizophrenia patients and the effects of antipsychotic medication: A proton magnetic resonance spectroscopy study. *Schizophr. Res.* 117, 83–91. doi: 10.1016/j.schres.2009.11.011

Tossman, U., Jonsson, G., and Ungerstedt, U. (1986). Regional distribution and extracellular levels of amino acids in rat central nervous system. *Acta Physiol. Scand.* 127, 533–545. doi: 10.1111/j.1748-1716.1986.tb07938.x

Wagner, D. A., Goldschen-Ohm, M. P., Hales, T. G., and Jones, M. V. (2005). Kinetics and spontaneous open probability conferred by the ε subunit of the $GABA_A$ receptor. *J. Neurosci.* 25, 10462–10468. doi: 10.1523/jneurosci.1658-05.2005

Wang, X., Zhong, P., and Yan, Z. (2002). Dopamine D4 receptors modulate GABAergic signaling in pyramidal neurons of prefrontal cortex. *J. Neurosci.* 22, 9185–9193. doi: 10.1523/jneurosci.22-21-09185.2002

Wei, W., Zhang, N., Peng, Z., Houser, C. R., and Mody, I. (2003). Perisynaptic localization of delta subunit-containing $GABA_A$ receptors and their activation by GABA spillover in the mouse dentate gyrus. *J. Neurosci.* 23, 10650–10661. doi: 10.1523/jneurosci.23-33-10650.2003

Wlodarczyk, A. I., Sylantyev, S., Herd, M. B., Kersanté, F., Lambert, J. J., Rusakov, D. A., et al. (2013). GABA-independent $GABA_A$ receptor openings maintain tonic currents. *J. Neurosci.* 33, 3905–3914. doi: 10.1523/jneurosci.4193-12.2013

Yeung, J. Y., Canning, K. J., Zhu, G., Pennefather, P., MacDonald, J. F., and Orser, B. A. (2003). Tonically activated $GABA_A$ receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. *Mol. Pharmacol.* 63, 2–8. doi: 10.1124/mol.63.1.2

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 Molecular Neuroscience

MINI REVIEW
published: 07 May 2019
doi: 10.3389/fnmol.2019.00115

Deep Survey of GABAergic Interneurons: Emerging Insights From Gene-Isoform Transcriptomics

*Lin Que, Jochen Winterer and Csaba Földy**

Laboratory of Neural Connectivity, Faculties of Medicine and Natural Sciences, Brain Research Institute, University of Zurich, Zurich, Switzerland

GABAergic interneuron diversity is a key feature in the brain that helps to create different brain activity patterns and behavioral states. Cell type classification schemes—based on anatomical, physiological and molecular features—have provided us with a detailed understanding of the distinct types that constitute this diversity and their contribution to brain function. Over recent years, the utility of single-cell RNAseq has majorly complemented this existing framework, vastly expanding our knowledge base, particularly regarding molecular features. Single-cell gene-expression profiles of tens of thousands of GABAergic cells from many different types are now available. The analysis of these data has shed new lights onto previous classification principles and illuminates a path towards a deeper understanding of molecular hallmarks behind interneuron diversity. A large part of such molecular features is synapse-related. These include ion channels and receptors, as well as key synaptic organizers and trans-synaptic signaling molecules. Increasing evidence suggests that transcriptional and post-transcriptional modifications further diversify these molecules and generate cell type-specific features. Thus, unraveling the cell type-specific nature of gene-isoform expression will be a key in cell type classification. This review article discusses progress in the transcriptomic survey of interneurons and insights that have begun to manifest from isoform-level analyses.

Keywords: GABAergic interneuron, single-cell RNA seq, cell type classification, trans-synaptic signaling, gene isoforms

OPEN ACCESS

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

Reviewed by:
Lisa Topolnik,
Laval University, Canada
Yuri Bozzi,
University of Trento, Italy

***Correspondence:**
Csaba Földy
foldy@hifo.uzh.ch

Received: *08 March 2019*
Accepted: *23 April 2019*
Published: *07 May 2019*

Citation:
Que L, Winterer J and Földy C (2019) Deep Survey of GABAergic Interneurons: Emerging Insights From Gene-Isoform Transcriptomics. Front. Mol. Neurosci. 12:115. doi: 10.3389/fnmol.2019.00115

INTRODUCTION

The activity of excitatory neurons is shaped by a highly diverse population of GABAergic interneurons (Klausberger and Somogyi, 2008; Rudy et al., 2011; Kepecs and Fishell, 2014). A clear comprehension of these GABAergic cells in the brain is therefore crucial for understanding the brain circuits in which they participate. To address the complexity of this diverse population, grouping of neurons into distinct cell types is a key to delineate and ultimately understand their function.

Classification of interneurons depends on an unambiguous identification of features that are unique for one cell type compared to others. These features typically encompass morphological and physiological characteristics, as well as molecular markers (Freund and Buzsáki, 1996; Klausberger and Somogyi, 2008; Ascoli et al., 2008; Booker and Vida, 2018). In addition, developmental origin has emerged as another distinctive feature for describing GABAergic cells (Pelkey et al., 2017; Wamsley and Fishell, 2017; Lim et al., 2018). However, whether

unique features exist for every potential distinct cellular identity or if, additionally, continuous modes of variables are needed to cover the diversity of interneurons, is an ongoing matter of debate.

Ever since next generation sequencing methods have touched upon the molecular features of cell type classification, an explosion of new information has set the field in motion and thereby increased the expectation to track down the problem of neuronal cell identity (Poulin et al., 2016). The immense power of transcriptomic surveys has not only been highlighted by their capacity to closely match previous cell type classifications, but also in facilitating the discovery of potential new cell types. However, these surveys disclose additional layers of complexity. First, the analysis of large-scale transcriptomic data required introducing the concept of "transcriptomic cell types," which are defined as clusters of cells with differential gene expression patterns. Differences between clusters may be discrete or continuous (Tasic et al., 2016, 2018; Harris et al., 2018; Muñoz-Manchado et al., 2018). Continuous differences, also referred to as continuous variability, between clusters could be the result of similarities in the molecular heterogeneity of cells from neighboring and even overlapping transcriptomic cell types. Second, transcriptomic surveys revealed that cell states, which may be defined as the cells' progress through their developmental trajectory (La Manno et al., 2018; Mayer et al., 2018; Mi et al., 2018) or as a consequence of neuronal activity (Tasic et al., 2018), need to be accounted for when classifying single cells. As a result, there is a high level of granularity in identifying single cells, which makes their classification still challenging.

In addition to their ability to detect differential gene expression levels, transcriptomic surveys began to reveal insights into cell type-specific transcriptional and post-transcriptional modifications, such as differential promoter usage and alternative splicing. Consequently, it has been shown that different gene-isoforms can correlate with cell type identity (Fuccillo et al., 2015; Tasic et al., 2016; Karlsson and Linnarsson, 2017; Nguyen et al., 2016; Wamsley et al., 2018).

In this review article, we discuss insights gained from single-cell transcriptomics and how they augment the existing classification schemes of GABAergic cell types. More specifically, we will elaborate on the importance of introducing the analysis of gene-isoform expression levels in cell type taxonomies and how this could facilitate the classification of cell type identities.

TRANSCRIPTOMIC CELL TYPES AND GABAergic INTERNEURON DIVERSITY

On-going progress in single-cell transcriptomics provides a major technological driving force for understanding cell-type identity. One of the first single-cell transcriptomic census of neural cell types was done in somatosensory cortex and hippocampus (Zeisel et al., 2015). Since then, molecular profiles of distinct cell types have been characterized in the developing brain to understand the developmental trajectory that gives rise to the high diversity of cell types (Mayer et al., 2018; Rosenberg et al., 2018; Zhong et al., 2018). In the adolescent and adult brain, molecular profiling studies in the hippocampus, dorsal striatum and different cortical areas have started to establish an indispensable framework of static and dynamic transcriptomic states behind cell taxonomies (Tasic et al., 2016, 2018; Harris et al., 2018; Muñoz-Manchado et al., 2018).

The hippocampus is one of the most investigated circuitry in the brain and therefore provides a well-established reference of morphological, immunohistochemical and electrophysiological characterizations. Transcriptional profiling of single-cell samples from hippocampal CA1 interneurons using the pan-GABAergic Slc32a1-Cre line revealed 10 major GABAergic "transcriptomic continents" (Harris et al., 2018), which further differentiated in 49 transcriptomic interneuron types. Referencing single-cell gene expression patterns to the extensive knowledge base of the hippocampus revealed that the 49 transcriptomic clusters were organized according to the previously described 23 interneuron classes. However, due to an overlap of gene expression levels of cells that initially fall into different clusters, transitions between populations remained and a continuous variability persisted throughout the dataset.

In another categorizing study in the dorsal striatum, transcriptomic analysis of GABAergic cells could not rely on back referencing to an existing knowledge-base because an elaborated description of resident interneurons was not available (Muñoz-Manchado et al., 2018). Using *Htr3a, Lhx6* and *Pvalb* specific transgenic lines, this study revealed discrete cell types, albeit fewer than within the hippocampus. In contrast to what was found in the hippocampus (Harris et al., 2018), *Pvalb* expressing interneurons did not cluster into one or more discrete groups, but continuously rendered into a larger population of *Pthlh* expressing cells that displayed a spatial gradient of *Pvalb* expression levels along the dorsoventral and mediolateral axis of the dorsal striatum. In addition, this gradual *Pvalb* expression was likewise reflected in electrophysiological characteristics of these cells, where higher expression levels of *Pvalb* correlated with fast-spiking signatures, such as shorter action potential half-width.

To study cell classification in the primary visual cortex, Tasic et al. (2016) chose for more detailed, pre-determined specificity by using 25 different *Cre*-driver lines. This allowed for a selection of specific subsets of cortical cells and access to both abundant as well as rare cell types. Differential gene expression analysis revealed robust separation of cells into 49 clusters, incidentally the same number as in hippocampus study, of presumed GABAergic, glutamatergic and non-neuronal cell types. These clusters were based on both cells that were consistently classified into the same cluster and cells of which their identity could not be determined using clustering algorithms, respectively referred to as core and intermediate cells. Discreteness of clusters largely depended on core cells, whilst intermediate cells caused a continuous gene expression variation. Nonetheless, identities of both core and intermediate cells could be inferred from referencing to previous studies and knowledge about the cre-driver line from which the cell was collected. Subsequent to identifying transcriptomic cell types, examination of transcriptional and post-transcriptional modifications revealed differential exon usage for 567 exons in

320 genes in a cell type-specific manner, indicating that isoform-level analyses could further facilitate the classification of cell types (see also Karlsson and Linnarsson, 2017).

In a more recent article, Tasic et al. (2018) revisited the primary visual cortex, and in addition studied the anterior lateral motor cortex in mice. An extensive dataset of 23,822 single-cell transcriptomes was created, based on 47 cre-driver lines. This allowed definition of 133 transcriptomic neuronal cell types (61 GABAergic) in both areas. Similar to their previous article, both discrete and continuous gene expression in the dataset was observed. Interestingly, while the heterogeneity in layer 4 cells was previously accounted for by both core and intermediate types (Tasic et al., 2016), this new survey rendered them as one continuous type without separable features, possibly owing to higher cell numbers and improved gene detection.

In contrast to large-scale approaches, patch pipette-based transcriptomics has been introduced as a method to combine electrophysiological with transcriptomic analyses. First, probe-based single-cell RT-PCR, qRT-PCR and whole-genome microarrays were performed after manual cell picking with a patch-pipette, either with or without electrophysiological recordings (Geiger et al., 1995; Okaty et al., 2009; Tricoire et al., 2011; Fuccillo et al., 2015). More recently, single-cell RNAseq approaches were introduced, allowing an unbiased access to all RNA species (Cadwell et al., 2016; Fuzik et al., 2016; Földy et al., 2016). Although manual cell picking results in a lower throughput, it grants *ad hoc* electrophysiological and morphological confirmation of the cell. Consequently, patch pipette-based transcriptomics enables the correlation of electrophysiological properties (e.g., spike patterns) to the molecular profile (e.g., ion channel composition) of the cell of interest, as has already been shown in several studies (Fuzik et al., 2016; Földy et al., 2016; Muñoz-Manchado et al., 2018). Furthermore, it enables a transcriptomic analysis of connected neurons, as evidenced by electrophysiological signatures of synaptic transmission, and molecules that define circuit connectivity motifs (Földy et al., 2016). In addition, recent studies introduced the use of driver lines that more precisely label specific morphological cell types, which grants a distinction that allows direct comparisons between these types (Paul et al., 2017; Favuzzi et al., 2019). These studies revealed cell type-specific expression of synaptic molecules, highlighting their close relation to cell type identity and connectivity (Földy et al., 2016; Paul et al., 2017; Favuzzi et al., 2019). Because these methods do not require clustering-based inferences and rely less on back referencing to an existing knowledge base, such approaches offer a straightforward access to the transcriptomic signature of cell types.

CHALLENGES ASSOCIATED WITH INTERPRETING TRANSCRIPTOMIC CELL TYPES

There are several challenges that need to be addressed when interpreting single-cell RNAseq data. First, RNAseq captures only a static snapshot at the time when the cell is collected. With the aim to address this snapshot nature of single-cell transcriptomes, based on the fraction of spliced vs. un-spliced RNA, RNA velocity analysis can predict the future state of developing cells at a timescale of hours, and describe fate decisions of major neural lineages in the hippocampus (La Manno et al., 2018). Furthermore, distinct cell states may influence transcriptomic profiles by forming "temporal" clusters. For example, the comparison of single-cell transcriptomes collected from dark-reared vs. light-exposed animals revealed that several glutamatergic and GABAergic types displayed statistically significant enrichment or depletion of early- and/or late-response genes (Tasic et al., 2018).

Second, the detected discreteness or continuity between clusters largely depends on technical parameters, such as gene detection, cell sampling, and the stringency of clustering criteria (Harris et al., 2018; Tasic et al., 2018). In addition, different clustering algorithms revealed different grouping of cells, as is highlighted by the comparison between algorithms of two studies (Tasic et al., 2016; Harris et al., 2018). Therefore, the identification of intrinsic biological variations remains challenging. Considering the pace at which transcriptomic methods continue to develop, and the number of sequenced cells continues to increase, it is likely that these problems will have to be revisited upfront in the light of new data.

Current classification efforts using single-cell transcriptomic data rely on neuronal clusters that are generated based on differential gene-expression but not on gene-isoform analysis. However, even in single genes, cell-intrinsic gene editing during transcription, the use of different promoters and alternative splicing sites can generate a level of diversity that affects gene-expression level readouts, and thereby cell classification. When cell types differentially express isoforms of a single gene of interest, all cells will be recognized as expressing that gene and will not be separated into different clusters. However, using single-cell isoform RNA-seq, a recent study identified more than 10,000 RNA isoforms in cerebellar cell types, and cell type-specific combination patterns of distant splice sites, indicating that many isoforms exist in a cell type-specific fashion (Gupta et al., 2018). By looking at differential gene expression, isoforms would reveal at most apparent gene expression differences, when differences in length between isoforms are large. Cell type-specific RNA modifications may be read out as a minor difference in the gene's expression while exerting a major impact in determining or correlating extremely well with cell identities. Depending on the method that is implemented, processing of single-cell samples can result in partial or full-length recovery of RNA molecules (Stegle et al., 2015). Although partial recovery is sufficient for the detection of genes, application of full length-cDNA would allow going beyond gene-expression analyses and examine the expression of different gene isoforms in detail. Partial and full-length recovery of RNA can be applied in both large-scale as well as pipette-based approaches (demonstrated in Fuccillo et al., 2015; Földy et al., 2016; Tasic et al., 2018). If all isoforms would be included in clustering analyses, would cell types clearly subdivide (**Figure 1**)?

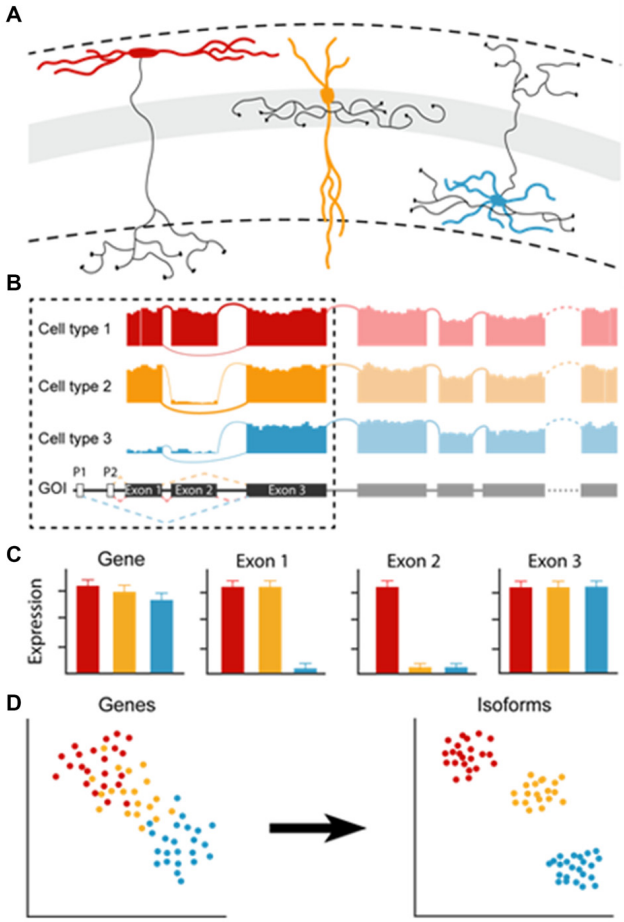

FIGURE 1 | The effect of cell type-specific isoform expression on gene expression pattern analysis. **(A)** Examples of different cell types based on their morphological appearance. **(B)** Modifications such as alternative splicing (cell type 2) or differential promotor usage (cell type 3) generate different isoforms. **(C)** Large differences in isoform length may result in apparent differential gene expression, small differences go unnoticed ("Gene"). However, looking at cell type-specific differential exon usage would reveal distinctions between cell types (in "Exon1" and "Exon2"). **(D)** Clustering by gene expression may result in cell type clusters with continuous variability ("Genes"). Clustering of the same cells based on isoform usage could possibly result in discrete clustering ("Isoforms").

ISOFORMS THAT MAKE THE DIFFERENCE

Transcriptional and post-transcriptional RNA modifications greatly extend on the molecular repertoire (Barash et al., 2010; Gabut et al., 2011; Zhang et al., 2016; Mauger and Scheiffele, 2017; Gandal et al., 2018; Wamsley et al., 2018) that may also generate cell type-specific features. For example, clustered protocadherins, which are important for determining cell identity and circuit assembly of olfactory sensory neurons, display cell-specific promoter usage (**Figure 2A**; Lefebvre et al., 2012; Chen W. V. et al., 2017; Mountoufaris et al., 2017). In addition, isoforms of cell adhesion molecule neurexins have been demonstrated to show cell type specificity between hippocampal neurons (**Figure 2B**; Fuccillo et al., 2015; Nguyen et al., 2016), and between nucleus accumbens projection and nucleus accumbens targeting neurons (Fuccillo et al., 2015). Furthermore, as is the case for alternative splicing of neurexin-3 in CA1 neurons, inclusion/exclusion of a single-cassette exon renders a lack or presence of long-term plasticity in output synapses depending on the identity of postsynaptic subicular cell type (**Figure 2C**; Aoto et al., 2013). Finally, due to gene editing, two consecutive exons ("flip" and "flop") in *Gria1* and *Gria2* displayed cell type specificity in primary visual cortex between layer 2/3 (flip) vs. layer 4 pyramidal cell types (flop; **Figure 2D**; Tasic et al., 2016). This difference renders a high Ca^{2+} permeability and mediates significant Ca^{2+} influx (flip) or lack thereof (flop; Sommer et al., 1990; Lomeli et al., 1994; Geiger et al., 1995). These examples illuminate that the lack of inclusion of different isoforms, which could have the capacity to disambiguate cell types, potentially underpowers current transcriptomic surveys.

Below, we describe emerging evidence on GABAergic signaling, which suggests that a large isoform diversity exists both pre- and post-synaptically. As synaptic molecules exist at the intersection of connectivity and cell identity, isoform diversity could possibly reflect distinct cell types. If so, isoform specificity could serve as a platform for cell type classification.

GENE ISOFORMS IN GABAergic SIGNALING

Within the synapse, cell surface molecules mediate transsynaptic signaling and are emerging as key organizers of synapse function (de Wit and Ghosh, 2016; Südhof, 2017). There is an increasing interest in understanding how key synaptic organizers drive circuit assembly for GABAergic interneurons and correlate with cell type identity (Földy et al., 2016; Li H. et al., 2017; Paul et al., 2017; Favuzzi et al., 2019). Such molecules may ubiquitously dictate the establishment and maturation of GABAergic synapses (e.g., neuroligin-2 and Slitrk-3; Li J. et al., 2017), or have been proposed to display cell type-specific expression.

Presynaptically, neurexins are key organizers that are expressed in thousands of alternatively spliced isoforms (Schreiner et al., 2014, 2015; Treutlein et al., 2014) and have been shown to restrict and rank trans-synaptic binding preference to their postsynaptic partners (Takahashi and Craig, 2013; de Wit and Ghosh, 2016; Südhof, 2017). Transcriptomic analysis of a few isoforms and cell types has begun to reveal that cell type-specific expression of neurexins may be important for brain function (Fuccillo et al., 2015; Traunmüller et al., 2016; Chen L. Y. et al., 2017; Nguyen et al., 2016). Intriguing differences in alternative splicing have been shown for hippocampal *Pvalb* and *Cck* interneurons. Especially, the single exon cassette at alternative splicing site 3 was uniformly retained in *Pvalb* interneurons and spliced out in *Cck* interneurons. Although the impact of neurexins on the build-up of the presynaptic active zone remains elusive, this striking difference in synapse organizing molecules is reflected in the diametrically different use of presynaptic Ca^{2+}-channels and release-modulating

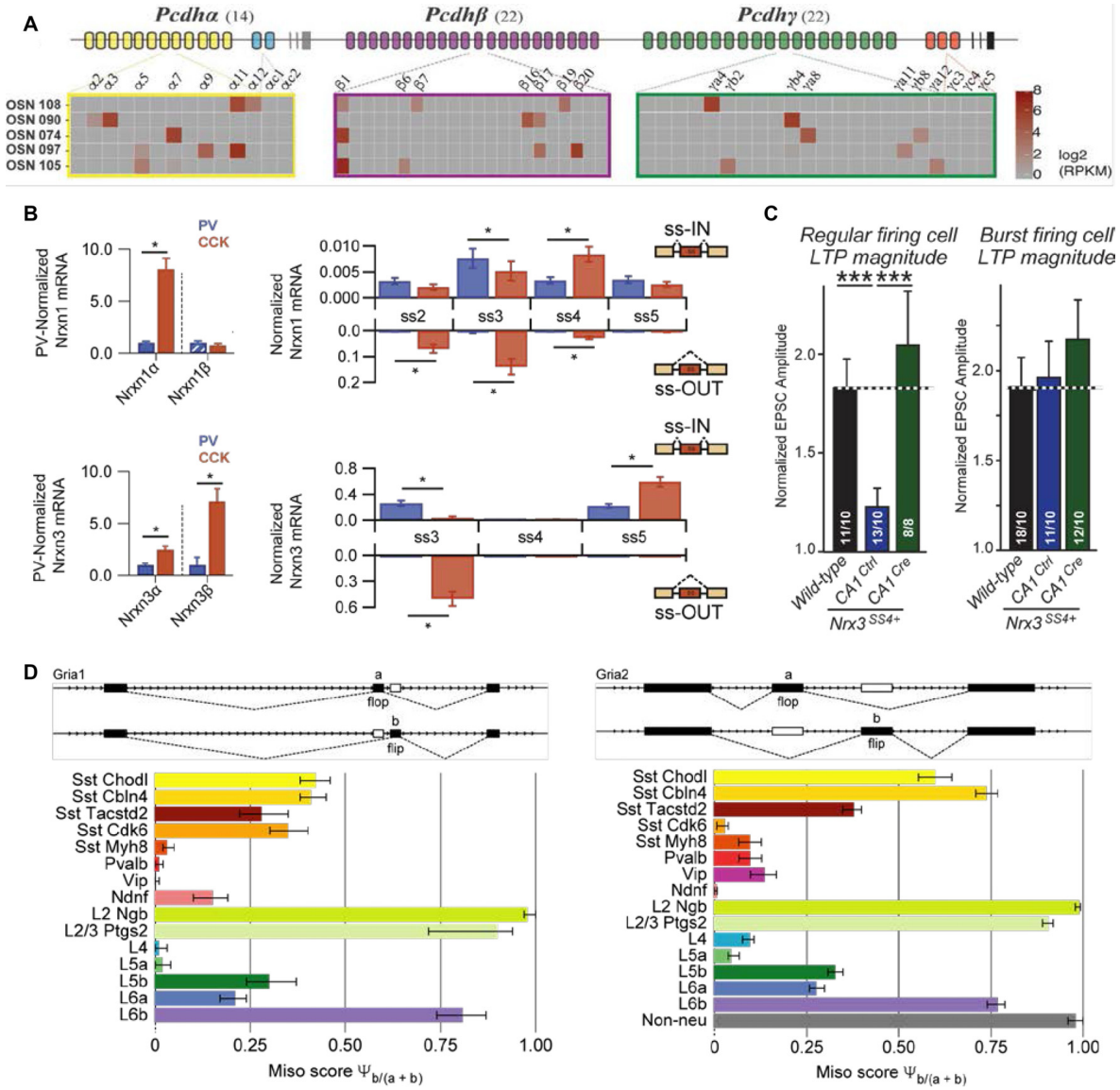

FIGURE 2 | Examples of cell- and cell type-specific isoform expression. **(A)** Protocadherin isoforms may be divided in two categories, the alternate (yellow, green and purple), and the C type (blue and red). Shown here are single-cell expression examples of the protocadherin alpha/beta/gamma in five cells, where the presence of individual protocadherin isoform mRNA is represented by a gradient of gray (no expression) to red (high expression; adapted from Mountoufaris et al., 2017 with permission). **(B)** Left: neurexin alpha/beta isoform expression in hippocampal *Cck (CCK)* and *Pvalb (PV)* GABAergic cells, normalized to the average level in *Pvalb (PV)* cells. Right: splice-site graph that displays averaged single-cell splice isoform expression values for either being "spliced in" (up-ward bars) or "spliced out" (down; adapted from Fuccillo et al., 2015 with permission). **(C)** Averaged EPSC amplitudes, recorded from subiculum neurons and elicited by electrical stimulation of CA1 pyramidal cells, over the last 10 min of LTP recordings and normalized to the baseline for cells of inactive ("Ctrl") or active excision of SS4 ("Cre"). The magnitude of LTP ("Ctrl") depended on the identity of postsynaptic cells; left graph represents postsynaptic regular firing cells, right graph postsynaptic burst firing cells (adapted from Aoto et al., 2013 with permission). **(D)** Both *Gria1* and *Gria2* display similar, but cell type-specific alternative exon usage of "flip" and "flop," which suggests a shared mechanism for alternative splicing (adapted from Tasic et al., 2016 with permission). ***$P < 0.001$, *indicates significant difference between groups ($P < 0.05$; Mann-Whitney U test).

receptors by these interneurons (Freund and Katona, 2007). In addition, neurexin alternative splicing might also affect transsynaptic binding and thereby synapse function: while loss of neurexin-ligand neuroligin-3 function affects inhibitory postsynaptic potentials from *Cck* interneurons by disinhibition of tonic endocannabinoid signaling, synaptic transmission from *Pvalb* interneurons remains unaltered (Földy et al., 2013). In the prefrontal cortex, genetic deletion of all neurexins in *Pvalb* interneurons decreased synapse numbers without affecting GABA release in surviving synapses while in *Sst* interneurons, the same manipulation impaired presynaptic Ca^{2+} influx without changing synapse numbers (Chen L. Y. et al., 2017).

However, neurexin isoform expression has not been identified in these cells.

Postsynaptically, gephyrin and collybistin are key organizers for the GABAergic density. Their roles were first highlighted by experiments in which forced expression of synaptogenic cell-adhesion molecule neuroligin-2 lead to gephyrin and collybistin aggregation and recruitment of GABA receptors (Poulopoulos et al., 2009). Gephyrin is subject to extensive alternatively splicing in at least 10 canonical sites (Prior et al., 1992; Paarmann et al., 2006), which can potentially generate a large number of possible isoforms. Alternative splicing of gephyrin occurs predominantly in the C-domain, where most phosphorylation sites have been reported. Because phosphorylation ultimately determines gephyrin folding and clustering (Zacchi et al., 2014), it is conceivable that different isoforms generate different gephyrin function. For collybistin, three major C-terminal isoforms have been described (CB1-3, Harvey et al., 2004). While distinct CB2 isoforms regulate translocation of gephyrin to the cell surface and formation and maintenance of gephyrin clusters at GABAergic sites (Tyagarajan et al., 2011), the CB1 isoform has been shown to selectively facilitate gephyrin clustering at distal portions of immature dendrites (de Groot et al., 2017). Alternative splicing in both the CB1 and CB2 isoforms have been observed (Harvey et al., 2004; de Groot et al., 2017). However, cell type-specific expression of these gephyrin/collybistin isoforms has not been investigated.

Parallel to alternative splicing, expression of neurexin splicing regulators *SLM1 and SLM2* appear in a cell type-specific manner (Iijima et al., 2014). Similarly, reports on gephyrin splicing factors *Nova1* and *Nova2* also indicate cell type specificity (Yuan et al., 2018). Intriguingly, alternative splicing of neurexins, gephyrin and collybistin are commonly regulated by *Sam68*, which, in contrast to the above factors displays ubiquitous expression (Witte et al., 2018).

Isoform diversity of synaptic molecules plays an important role in synapse connectivity. These synaptic connections are at the base of neuronal connectivity, which in turn defines the anatomical identity of a cell. Therefore, the transcriptomic identity, including cell type-specific isoform diversity, may correspond well with the anatomical identity of a cell (Li H. et al., 2017). To this end, single-cell transcriptomics would allow a precise, cell type-specific isoform dissection of synaptic molecules associated with GABAergic synapses, and give insight into the transcriptomic and anatomical identities of distinct cell types.

NEUROANATOMY-BASED INTERPRETATION OF TRANSCRIPTOMIC CELL TYPES

Cajal's work has become the foundation for the classification of neurons based on morphological characteristics and although behavior-specific activity of cell types has become an almost unambiguous marker for identifying GABAergic cell types (Klausberger and Somogyi, 2008; Kepecs and Fishell, 2014), specific axonal and dendritic features still provide fundamental reference points in cell type classification. As highlighted above, transcriptomic surveys are closing the gap between genetic content and cell identities. In this effort, inferences made between transcriptomic cell types and an existing knowledge base have played crucial roles. In specific cases, however, it appears to be inevitable to generate transcriptomic data directly from anatomically-defined cells. Development of transgenic lines that specifically label anatomically-defined cell types will be important for direct comparisons between the transcriptomic signatures of these cells. Patch pipette-based transcriptomics could largely facilitate the development of such lines, as it potentially allows identification of driver genes behind anatomical features.

Thus far, transcriptomic cell type definitions based on differential gene expression alone have not been consistently able to relate all transcriptomic cell types to specific anatomical features. For example, hippocampal *Pvalb* interneurons, basket and bistratified cells display non-overlapping axonal projections (to pyramidal layer and to oriens/radiatum layers, respectively; Klausberger and Somogyi, 2008), but this fundamental difference could not be resolved as distinct clusters (Harris et al., 2018). In this specific case, the existence of two types of *Pvalb* basket cells adds an extra layer of complexity. In one, soma are located within or in close proximity of the pyramidal layer and their dendrites extend radially along the oriens-radiatum axis (Booker and Vida, 2018). By contrast, soma of the other type are located in the oriens layer and their dendrites run horizontally within the oriens (Maccaferri, 2005), implicating different engagement in CA1 microcircuitry and therefore different function. It is important to note, that the same survey distinctly separated axo-axonic *Pvalb* cells, which establish synapses on the axon initial segment of pyramidal cells. If and how this molecular difference relates to different function remains a question. These transcriptomic insights appear thus far to support an alternative view on the classification of *Pvalb* cells, which suggests that non axo-axonic *Pvalb* neurons comprise a morphologically continuous cell class in which the above defined types represent only prototypes with higher preponderance, but to which not all *Pvalb* cells can be unambiguously assigned based on morphology. Therefore, a combined comprehension of their molecular and morphological identity would be important. Such combined comprehension is equally important for characterizing potential new cell types (Boldog et al., 2018). Overall, combined electrophysiology, anatomical tracing, and molecular profiling that is now equipped with transcriptomics, is defining new standards for cell type discovery.

Finally, a question that still remains is when and how distinct interneuron subtypes, such as the above defined *Pvalb* types, gain their final identity and how this is reflected in their transcriptome. One hypothesis suggests that that interneuron identity is determined at the cell's birth ("progenitor hypothesis"). Another suggests that progenitors first establish a cardinal identity, that is later refined into a definitive identity through post-mitotic

determinants ("progressive maturation hypothesis"; Wamsley and Fishell, 2017). Single-cell RNAseq surveys on anatomically-defined cells over the course of maturation would provide insights into the anatomical as well as the transcriptomic maturation process over time. It would allow the examination of transcriptome dynamics, whether transcriptomic cell types are consistently distinct, or only display a temporal distinction, and how this relates to the anatomical maturation of the cell.

To fully understand the intricacy behind interneuron diversity, single-cell transcriptomics provides a platform to identify the deep molecular architecture determining ontogenesis, morphology and synaptic connectivity of distinct cell types. It has hereby a tremendous influence on our current stand on cell type classification and identity. It not only reflects, but additionally complements previous knowledge on classification based on electrophysiology and morphology, and facilitates cell type discovery. Capitalizing on the capacity of transcriptomic surveys to study transcriptional and post-transcriptional RNA modifications will highlight its potential in understanding cell type-specific molecular mechanisms underlying connectivity, synapse function and cell identity.

AUTHOR CONTRIBUTIONS

LQ, JW and CF wrote the manuscript.

FUNDING

This work was supported by grants from the Swiss National Science Foundation (Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung; CRETP3_166815 and 31003A_170085 to CF) and from the Slack-Gyr Foundation.

ACKNOWLEDGMENTS

We thank Dr János Szabadics and David Lukacsovich for discussions and suggestions on the manuscript.

REFERENCES

Aoto, J., Martinelli, D. C., Malenka, R. C., Tabuchi, K., and Südhof, T. C. (2013). Presynaptic neurexin-3 alternative splicing trans-synaptically controls postsynaptic AMPA-receptor trafficking. *Cell* 154, 75–88. doi: 10.1016/j.cell.2013.05.060

Barash, Y., Calarco, J. A., Gao, W., Pan, Q., Wang, X., Shai, O., et al. (2010). Deciphering the splicing code. *Nature* 465, 53–59. doi: 10.1038/nature09000

Boldog, E., Bakken, T. E., Hodge, R. D., Novotny, M., Aevermann, B. D., Baka, J., et al. (2018). Transcriptomic and morphophysiological evidence for a specialized human cortical GABAergic cell type. *Nat. Neurosci.* 21, 1185–1195. doi: 10.1038/s41593-018-0205-2

Booker, S. A., and Vida, I. (2018). Morphological diversity and connectivity of hippocampal interneurons. *Cell Tissue Res.* 373, 619–641. doi: 10.1007/s00441-018-2882-2

Cadwell, C. R., Palasantza, A., Jiang, X., Berens, P., Deng, Q., Yilmaz, M., et al. (2016). Electrophysiological, transcriptomic and morphologic profiling of single neurons using Patch-seq. *Nat. Biotechnol.* 34, 199–203. doi: 10.1038/nbt.3445

Chen, L. Y., Jiang, M., Zhang, B., Gokce, O., and Südhof, T. C. (2017). Conditional deletion of all neurexins defines diversity of essential synaptic organizer functions for neurexins. *Neuron* 94, 611.e4–625.e4. doi: 10.1016/j.neuron.2017.04.011

Chen, W. V., Nwakeze, C. L., Denny, C. A., O'Keeffe, S., Rieger, M. A., Mountoufaris, G., et al. (2017). Pcdhαc2 is required for axonal tiling and assembly of serotonergic circuitries in mice. *Science* 356, 406–411. doi: 10.1126/science.aal3231

de Groot, C., Floriou-Servou, A., Tsai, Y. C., Früh, S., Kohler, M., Parkin, G., et al. (2017). RhoGEF9 splice isoforms influence neuronal maturation and synapse formation downstream of α2 $GABA_A$ receptors. *PLoS Genet.* 13:e1007073. doi: 10.1371/journal.pgen.1007073

de Wit, J., and Ghosh, A. (2016). Specification of synaptic connectivity by cell surface interactions. *Nat. Rev. Neurosci.* 17, 22–35. doi: 10.1038/nrn.2015.3

Favuzzi, E., Deogracias, R., Marques-Smith, A., Maeso, P., Jezequel, J., Exposito-Alonso, D., et al. (2019). Distinct molecular programs regulate synapse specificity in cortical inhibitory circuits. *Science* 363, 413–417. doi: 10.1126/science.aau8977

Földy, C., Darmanis, S., Aoto, J., Malenka, R. C., Quake, S. R., and Südhof, T. C. (2016). Single-cell RNAseq reveals cell-adhesion profiles in electrophysiologically defined neurons. *Proc. Natl. Acad. Sci. U S A* 113, E5222–E5231. doi: 10.1073/pnas.1610155113

Földy, C., Malenka, R. B., and Südhof, T. C. (2013). Autism-associated Neuroligin-3 mutation commonly disrupts endocannabinoid signalling. *Neuron* 78, 498–509. doi: 10.1016/j.neuron.2013.02.036

Freund, T., and Buzsáki, G. (1996). Interneurons of the hippocampus. *Hippocampus* 6, 347–370. doi: 10.1002/(sici)1098-1063(1996)6:4<347::aid-hipo1>3.0.co;2-i

Freund, T. F., and Katona, I. (2007). Perisomatic inhibition. *Neuron* 56, 33–42. doi: 10.1016/j.neuron.2007.09.012

Fuccillo, M., Földy, C., Gökce, M., Rothwell, P., Sun, G., Malenka, R. C., et al. (2015). Single-cell mRNA profiling reveals cell-type specific expression of neurexin isoforms. *Neuron* 87, 326–340. doi: 10.1016/j.neuron.2015.06.028

Fuzik, J., Zeisel, A., Máté, Z., Calvigioni, D., Yanagawa, Y., Szabó, G., et al. (2016). Integration of electrophysiological recordings with single-cell RNA-seq data identifies neuronal subtypes. *Nat. Biotechnol.* 34, 175–183. doi: 10.1038/nbt.3443

Gabut, M., Samavarchi-Tehrani, P., Wang, X., Slobodeniuc, V., O'Hanlon, D., Sung, H. K., et al. (2011). An alternative splicing switch regulates embryonic stem cell pluripotency and reprogramming. *Cell* 147, 132–146. doi: 10.1016/j.cell.2011.08.023

Gandal, M. J., Zhang, P., Hadjimichael, E., Walker, R. L., Chen, C., Liu, S., et al. (2018). Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science* 362:eaat8127. doi: 10.1126/science.aat8127

Geiger, J. R., Melcher, T., Koh, D. S., Sakmann, B., Seeburg, P. H., Jonas, P., et al. (1995). Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron* 15, 193–204. doi: 10.1016/0896-6273(95)90076-4

Gupta, I., Collier, P. G., Haase, B., Mahfouz, A., Joglekar, A., Floyd, T., et al. (2018). Single-cell isoform RNA sequencing characterises isoforms in thousands of cerebellar cells. *Nat. Biotechnol.* 36, 1197–1202. doi: 10.1038/nbt.4259

Harris, K. D., Hochgerner, H., Skene, N. G., Magno, L., Katona, L., Bengtsson Gonzales, C., et al. (2018). Classes and continua of hippocampal CA1 inhibitory neurons revealed by single-cell transcriptomics. *PLoS Biol.* 16:e2006387. doi: 10.1371/journal.pbio.2006387

Harvey, K., Duguid, I. C., Alldred, M. J., Beatty, S. E., Ward, H., Keep, N. H., et al. (2004). The GDP-GTP exchange factor collybistin: an essential determinant of neuronal gephyrin clustering. *J. Neurosci.* 24, 5816–5826. doi: 10.1523/JNEUROSCI.1184-04.2004

Iijima, T., Iijima, Y., Witte, H., and Scheiffele, P. (2014). Neuronal cell type-specific alternative splicing is regulated by the KH domain protein SLM1. *J. Cell Biol.* 204, 331–342. doi: 10.1083/jcb.201310136

Karlsson, K., and Linnarsson, S. (2017). Single-cell mRNA isoform diversity in the mouse brain. *BMC Genomics* 18:126. doi: 10.1186/s12864-017-3528-6

Kepecs, A., and Fishell, G. (2014). Interneuron cell types are fit to function. *Nature* 505, 318–326. doi: 10.1038/nature12983

Klausberger, T., and Somogyi, P. (2008). Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science* 321, 53–57. doi: 10.1126/science.1149381

La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., et al. (2018). RNA velocity of single cells. *Nature* 560, 494–498. doi: 10.1038/s41586-018-0414-6

Lefebvre, J. L., Kostadinov, D., Chen, W. V., Maniatis, T., and Sanes, J. R. (2012). Protocadherins mediate dendritic self-avoidance in the mammalian nervous system. *Nature* 488, 517–521. doi: 10.1038/nature11305

Li, J., Han, W., Pelkey, K. A., Duan, J., Mao, X., Wang, Y. X., et al. (2017). Molecular dissection of neuroligin 2 and slitrk3 reveals an essential framework for GABAergic synapse development. *Neuron* 96, 808.e8–882.e8. doi: 10.1016/j.neuron.2017.10.003

Li, H., Horns, F., Wu, B., Xie, Q., Li, J., Li, T., et al. (2017). Classifying *Drosophila* olfactory projection neuron subtypes by single-cell RNA sequencing. *Cell* 171, 1206.e22–1220.e22. doi: 10.1016/j.cell.2017.10.019

Lim, L., Mi, D., Llorca, A., and Marín, O. (2018). Development and functional diversification of cortical interneurons. *Neuron* 100, 294–313. doi: 10.1016/j.neuron.2018.10.009

Lomeli, H., Mosbacher, J., Melcher, T., Höger, T., Geiger, J. R., Kuner, T., et al. (1994). Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* 266, 1709–1713. doi: 10.1126/science.7992055

Maccaferri, G. (2005). Stratum orient horizontal interneurone diversity and hippocampal network dynamics. *J. Physiol.* 562, 73–80. doi: 10.1113/jphysiol.2004.077081

Mauger, O., and Scheiffele, P. (2017). Beyond proteome diversity: alternative splicing as a regulator of neuronal transcript dynamics. *Curr. Opin. Neurobiol.* 45, 162–168. doi: 10.1016/j.conb.2017.05.012

Mayer, C., Hafemeister, C., Bandler, R. C., Machold, R., Batista Brito, R., Jaglin, X., et al. (2018). Developmental diversification of cortical inhibitory interneurons. *Nature* 555, 457–462. doi: 10.1038/nature25999

Mi, D., Li, Z., Lim, L., Li, M., Moissidis, M., Yang, Y., et al. (2018). Early emergence of cortical interneuron diversity in the mouse embryo. *Science* 360, 81–85. doi: 10.1126/science.aar6821

Mountoufaris, G., Chen, W. V., Hirabayashi, Y., O'Keeffe, S., Chevee, M., Nwakeze, C. L., et al. (2017). Multicluster Pcdh diversity is required for mouse olfactory neural circuit assembly. *Science* 356, 411–414. doi: 10.1126/science.aai8801

Muñoz-Manchado, A. B., Bengtsson Gonzales, C., Zeisel, A., Munguba, H., Bekkouche, B., Skene, N. G., et al. (2018). Diversity of interneurons in the dorsal striatum revealed by single-cell RNA sequencing and PatchSeq. *Cell Rep.* 24, 2179.e7–2190.e7. doi: 10.1016/j.celrep.2018.07.053

Nguyen, T. M., Schreiner, D., Xiao, L., Traunmüller, L., Bornmann, C., and Scheiffele, P. (2016). Alternative splicing switch shapes neurexin repertoires in principal neurons versus interneurons in the mouse hippocampus. *Elife* 5:e22757. doi: 10.7554/elife.28013

Okaty, B. W., Miller, M. N., Sugino, K., Hempel, C. M., and Nelson, S. B. (2009). Transcriptional and electrophysiological maturation of neocortical fast-spiking GABAergic interneurons. *J. Neurosci.* 29, 7040–7052. doi: 10.1523/JNEUROSCI.0105-09.2009

Paarmann, I., Schmitt, B., Meyer, B., Karas, M., and Betz, H. (2006). Mass specrometric analysis of glycine receptor-associated gephyrin splice variants. *J. Biol. Chem.* 281, 34918–34925. doi: 10.1074/jbc.m607764200

Paul, A., Crow, M., Raudales, R., He, M., Gillis, J., and Huang, Z. J. (2017). Transcriptional architecture of synaptic communication delineates GABAergic neuron identity. *Cell* 171, 522.e20–539.e20. doi: 10.1016/j.cell.2017.08.032

Pelkey, K. A., Chittajallu, R., Craig, M. T., Tricoire, L., Wester, J. C., and McBain, C. J. (2017). Hippocampal GABAergic inhibitory interneurons. *Physiol. Rev.* 97, 1619–1747. doi: 10.1152/physrev.00007.2017

Petilla Interneuron Nomenclature Group, Ascoli, G. A., Alonso-Nanclares, L., Anderson, S. A., Barrionuevo, G., Benavides-Piccione, R., et al. (2008). Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat. Rev. Neurosci.* 9, 557–568. doi: 10.1038/nrn2402

Poulin, J. F., Tasic, B., Hjerling-Leffler, J., Trimarchi, J. M., and Awatramani, R. (2016). Disentangling neural cell diversity using single-cell transcriptomics. *Nat. Neurosci.* 19, 1131–1141. doi: 10.1038/nn.4366

Poulopoulos, A., Arumuni, G., Meyer, G., Soykan, T., Hoon, M., Papadopoulos, T., et al. (2009). Neuroligin-2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* 63, 628–642. doi: 10.1016/j.neuron.2009.08.023

Prior, P., Schmitt, B., Grenningloh, G., Pribilla, I., Multhaup, G., Beyreuther, K., et al. (1992). Primary structure and alternative splice variants of gephyrin, a putative glycine receptor-tubulin linker protein. *Neuron* 8, 1161–1170. doi: 10.1016/0896-6273(92)90136-2

Rosenberg, A. B., Roco, C. M., Muscat, R. A., Kuchina, A., Sample, P., Yao, Z., et al. (2018). Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. *Science* 360, 176–182. doi: 10.1126/science.aam8999

Rudy, B., Fishell, G., Lee, S. H., and Hjerling-Leffler, J. (2011). Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. *Dev. Neurobiol.* 71, 45–61. doi: 10.1002/dneu.20853

Schreiner, D., Nguyen, T. M., Russo, G., Heber, S., Patrignani, A., Ahrné, E., et al. (2014). Targeted combinatorial alternative splicing generates brain region-specific repertoires of neurexins. *Neuron* 84, 386–398. doi: 10.1016/j.neuron.2014.09.011

Schreiner, D., Simicevic, J., Ahrné, E., Schmidt, A., and Scheiffele, P. (2015). Quantitative isoform-profiling of highly diversified recognition molecules. *Elife* 4:e07794. doi: 10.7554/elife.07794

Sommer, B., Keinanen, K., Verdoorn, T. A., Wisden, W., Burnashev, N., Herb, A., et al. (1990). Flip and flop: a cell-specific functional switch in glutamateoperated channels of the CNS. *Science* 249, 1580–1585. doi: 10.1126/science.1699275

Stegle, O., Teichmann, S. A., and Marioni, J. C. (2015). Computational and analytical challenges in single-cell transcriptomics. *Nat. Rev. Genet.* 16, 133–145. doi: 10.1038/nrg3833

Südhof, T. C. (2017). Synaptic neurexin complexes: a molecular code for the logic of neural circuits. *Cell* 171, 745–769. doi: 10.1016/j.cell.2017.10.024

Takahashi, H., and Craig, A. M. (2013). Protein tyrosine phosphates PTPδ, PTPσ, and LAR: presynaptic hubs for synapse organization. *Trends Neurosci.* 36, 522–534. doi: 10.1016/j.tins.2013.06.002

Tasic, B., Menon, V., Nguyen, T. N., Kim, T. K., Jarsky, T., Yao, Z., et al. (2016). Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nat. Neurosci.* 19, 335–346. doi: 10.1038/nn.4216

Tasic, B., Yao, Z., Graybuck, L. T., Smith, K. A., Nguyen, T. N., Bertagnolli, D., et al. (2018). Shared and distinct transcriptomic cell types across neocortical areas. *Nature* 563, 72–78. doi: 10.1038/s41586-018-0654-5

Traunmüller, L., Gomez, A. M., Nguyen, T. M., and Scheiffele, P. (2016). Control of neuronal synapse specification by a highly dedicated alternative splicing program. *Science* 352, 982–986. doi: 10.1126/science.aaf2397

Treutlein, B., Gokce, O., Quake, S. R., and Südhof, T. C. (2014). Cartography of neurexin alternative splicing mapped by single-molecule long-read mRNA sequencing. *Proc. Natl. Acad. Sci. U S A* 111, E1291–E1299. doi: 10.1073/pnas.1403244111

Tricoire, L., Pelkey, K. A., Erkkila, B. E., Jeffries, B. W., Yuan, X., and McBain, C. J. (2011). Blueprint for the spatiotemporal origins of mouse hippocampal interneuron diversity. *J. Neurosci.* 31, 10948–10970. doi: 10.1523/JNEUROSCI.0323-11.2011

Tyagarajan, S. K., Ghosh, H., Harvey, K., and Fritschy, J. M. (2011). Collybistin splice variants differentially interact with gephyrin and Cdc42 to regulate gephyrin clustering at GABAergic synapses. *J. Cell Sci.* 124, 2786–2796. doi: 10.1242/jcs.086199

Wamsley, B., and Fishell, G. (2017). Genetic and activity-dependent mechanisms underlying interneuron diversity. *Nat. Rev. Neurosci.* 18, 299–309. doi: 10.1038/nrn.2017.30

Wamsley, B., Jaglin, X. H., Favuzzi, E., Quattrocolo, G., Nigro, M. J., Yusuf, N., et al. (2018). Rbfox1 mediates cell-type-specific splicing in cortical interneurons. *Neuron* 100, 846.e7–859.e7. doi: 10.1016/j.neuron.2018.09.026

Witte, H., Schreiner, D., and Scheiffele, P. (2018). A Sam68-dependent alternative splicing program shapes postsynaptic protein complexes. *Eur. J. Neurosci.* doi: 10.1111/ejn.14332 [Epub ahead of print].

Yuan, Y., Xie, S., Darnell, J. C., Darnell, A. J., Saito, Y., Phatnani, H., et al. (2018). Cell type-specific CLIP reveals that NOVA regulates cytoskeleton interactions in motorneurons. *Genome Biol.* 19:117. doi: 10.1186/s13059-018-1493-2

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

Zeisel, A., Muñoz-Manchado, A. B., Codeluppi, S., Lönnerberg, P., La Manno, G., Juréus, A., et al. (2015). Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science* 347, 1138–1142. doi: 10.1126/science.aaa1934

Zhang, X., Chen, M. H., Wu, X., Kodani, A., Fan, J., Doan, R., et al. (2016). Cell-type-specific alternative splicing governs cell fate in the developing cerebral cortex. *Cell* 166, 1147.e15–1162.e15. doi: 10.1016/j.cell.2016.07.025

Zhong, S., Zhang, S., Fan, X., Wu, Q., Yan, L., Dong, J., et al. (2018). A single-cell RNA-seq survey of the developmental landscape of the human prefrontal cortex. *Nature* 555, 524–528. doi: 10.1038/nature25980

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 Molecular Neuroscience

PERSPECTIVE
published: 25 June 2019
doi: 10.3389/fnmol.2019.00152

Posttranscriptional Gene Regulation of the GABA Receptor to Control Neuronal Inhibition

Rico Schieweck and Michael A. Kiebler**

Department of Cell Biology and Anatomy, Medical Faculty, Biomedical Center (BMC), Ludwig-Maximilians-University of Munich, Munich, Germany

Behavior and higher cognition rely on the transfer of information between neurons through specialized contact sites termed synapses. Plasticity of neuronal circuits, a prerequisite to respond to environmental changes, is intrinsically coupled with the nerve cell's ability to form, structurally modulate or remove synapses. Consequently, the synaptic proteome undergoes dynamic alteration on demand in a spatiotemporally restricted manner. Therefore, proper protein localization at synapses is essential for synaptic function. This process is regulated by: (i) protein transport and recruitment; (ii) local protein synthesis; and (iii) synaptic protein degradation. These processes shape the transmission efficiency of excitatory synapses. Whether and how these processes influence synaptic inhibition is, however, widely unknown. Here, we summarize findings on fundamental regulatory processes that can be extrapolated to inhibitory synapses. In particular, we focus on known aspects of posttranscriptional regulation and protein dynamics of the GABA receptor (GABAR). Finally, we propose that local (co)-translational control mechanism might control transmission of inhibitory synapses.

Keywords: posttranscriptional gene regulation, GABA receptors, inhibitory synapse, co-translational folding/assembly, RNA binding, RNA transport, local translation, RNA-binding proteins

OPEN ACCESS

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

Reviewed by:
Carlos B. Duarte,
University of Coimbra, Portugal
Katharine R. Smith,
University of Colorado Denver, United States

****Correspondence:***
Rico Schieweck
rico.schieweck@med.uni-muenchen.de
Michael A. Kiebler
mkiebler@lmu.de

Received: *25 March 2019*
Accepted: *29 May 2019*
Published: *25 June 2019*

Citation:
Schieweck R and Kiebler MA (2019) Posttranscriptional Gene Regulation of the GABA Receptor to Control Neuronal Inhibition. Front. Mol. Neurosci. 12:152. doi: 10.3389/fnmol.2019.00152

INTRODUCTION

The enormous capacity of the brain to store information and respond to different environmental conditions and challenges crucially rely on underlying mechanisms like synaptic plasticity. This depends on the ability to modulate the strength of transmission between two nerve cells as well as the growth and removal of synapses. Synapses consist of (at least) hundreds of proteins that need to be organized and correctly assembled to ensure proper synaptic function. Changes in synaptic transmission and structure are accompanied and conveyed by local alterations in protein levels. Understanding the regulation of synaptic protein composition is, therefore, crucial to gain insight into complex neurological processes such as learning and memory and, eventually, into neuropsychiatric diseases such as autism spectrum disorders, schizophrenia and bipolar disorders.

In order to remodel the synaptic proteome, neurons exploit different mechanisms that allow spatial and temporal control of protein levels. Protein synthesis was one of the first molecular mechanisms that were discovered to be indispensable for memory formation (Hershkowitz et al., 1975; Shashoua, 1976). Pioneer experiments showed that inhibiting translation blocked the ability of an animal to remember after training (Flexner et al., 1963). In line with this observation, several experiments have shown that strengthening and weakening of synaptic transmission, so called long-term potentiation (LTP) and depression (LTD), respectively, need active translation in a time-dependent manner (Krug et al., 1984; Linden, 1996).

The spatial selectivity of synapses to undergo changes upon stimulation raised the question of how a cell knows, which synapse is destined for functional and structural remodeling. This inspired Frey and Morris (1997) to the idea of "synaptic tagging." Repetitive activation of synapses, therefore, equips such a synapse with a labile molecular "tag." Eventually, the synaptic tag allows the synapse to recruit newly synthesized proteins. The concept of "synaptic tagging" is a very elegant model to explain processes such as LTP and LTD at excitatory synapses (Frey and Morris, 1997). The precise identity of the tag(s) is still lacking. Furthermore, synaptic plasticity depends on additional processes such as mRNA localization, which is mainly independent of translation activity (Steward et al., 1998). mRNA transport and localization are important determinants of synaptic function (Jung et al., 2014). To date, it is generally believed that mRNAs are assembled into ribonucleoprotein particles (RNPs) consisting of mRNAs and RNA-binding proteins (RBPs). The protein and mRNA composition of these particles differ substantially (Kanai et al., 2004; Fritzsche et al., 2013) giving raise to the idea that different subtypes of particles or granules co-exist in a nerve cell. The function of these RNA granules is: (i) to transport mRNA—in a translationally dormant stage—along cytoskeletal elements such as microtubules to their destination at the synapse; and (ii) to regulate the translation of their target mRNAs. Activity-dependent disassembly of these RNA granules then allows the release of mRNAs and subsequent induction of translation. How neuronal stimulation, recruitment of mRNAs and unpacking of RNPs are synchronized is largely unknown. A pioneer study identified the kinase mechanistic target of rapamycin (mTOR) as a central hub to recruit RNAs. The authors suggest that mTOR might be the tag that controls mRNA recruitment at the synapse (Sosanya et al., 2015). mTOR is essential for proper neuronal function (Costa-Mattioli and Monteggia, 2013; Pernice et al., 2016). It needs to be experimentally verified though whether it might represent an universal synaptic tag or whether it might be specific for a subset of mRNAs.

Local protein expression control comprising mRNA transport, local protein synthesis and recruitment of newly synthesized protein remodel the synaptic proteome. Consequently, protein degradation is compulsive to complete synaptic remodeling. Synaptic protein degradation is induced in an activity-dependent manner (Bingol and Schuman, 2006). Moreover, it is tightly linked to translation to balance the protein need (Klein et al., 2015). In line with this finding, the translation repressor poly(A)-binding protein interacting protein 2A (PAIP2A) is degraded by calpain in neurons upon stimulation (Khoutorsky et al., 2013). Interestingly, calpain also degrades gephyrin (Gphn), a major scaffold protein at inhibitory synapses (Tyagarajan and Fritschy, 2014). This finding indicates that translational activation at excitatory synapses may modulate inhibitory synapses to alter transmission.

In this review article, we provide insight into posttranscriptional regulatory mechanisms that control synaptic protein expression. Since most of these studies investigated these processes at excitatory synapses, we aim to expand these fundamental aspects to inhibitory synapses. We speculate that local expression control also regulates inhibitory transmission to balance neuronal excitation.

TO LOCALIZE OR NOT TO LOCALIZE—IT'S A MATTER OF RBP BINDING TO THE 3′-UTR

With the emergence of the individual-nucleotide resolution UV crosslinking and immunoprecipitation (iCLIP) technology (Huppertz et al., 2014), transcriptome-wide identification of RBP mRNA targets and binding site became experimentally addressable. iCLIP has now been performed for a series of RBPs (**Tables 1**, **2**). Interestingly, most of the RBP binding occurs within the 3′-untranslated region (3′-UTR) of transcripts (Andreassi and Riccio, 2009). In addition, it was shown that the median of the 3′-UTR length of mRNAs bound to the RBP Staufen2 that is necessary for RNA transport (Heraud-Farlow and Kiebler, 2014) is longer than the median of the transcriptome (Heraud-Farlow et al., 2013). This finding indicates that a certain 3′-UTR length is needed to allow association with RBPs and, consequently, mRNA transport and/or expression control (Heraud-Farlow and Kiebler, 2014). To test whether mouse GABA receptor (GABAR) subunits show a similar tendency towards longer 3′-UTR length, we analyzed the nucleotide length of their 3′-ends of all $GABA_A$ and $GABA_B$ receptor subunit isoforms (see "Methods" section). Strikingly, GABAR subunits reveal a significant increase in their 3′-UTR compared to the total mouse 3′-UTRome (**Figure 1A**). Moreover, the 3′-UTR length was significantly extended when comparing the GABAR subunits with the 3′-UTRome of the somatic and neuropil layer of the hippocampal CA1 region (Cajigas et al., 2012; **Figure 1A**). An increase in 3′-UTR length is linked with decreased translational activity in HEK cells and human neurons (Floor and Doudna, 2016; Blair et al., 2017) probably due to a higher number of miRNA and RBP binding sites. In addition, 3′-UTR length is extended during neuronal development indicating increased translation regulation in mature neurons compared to developing nerve cells (Blair et al., 2017). Of note, GABAR subunits exhibited a trend towards longer 3′-ends when compared with ionotropic glutamate receptor subunits (**Figure 1B**). Together, these results suggest that GABAR subunit 3′-UTRs have a high(er) potential to be bound by RBPs. Supportive for this hypothesis is the fact that GABAR subunit mRNAs are enriched in the dendrite containing neuropil layer of CA1 neurons in the hippocampus (Cajigas et al., 2012) suggesting that these mRNAs are localized there. The recognition of mRNA targets by RBPs relies on binding sites within their 3′-UTRs and that each mRNA might have its own specific RNA signature. In detail, these binding sequences consist of both sequence and structural elements (Kiebler and Bassell, 2006; Doyle and Kiebler, 2011; Jung et al., 2014; Sugimoto et al., 2015). Interestingly, GABAR subunits exhibited a lower GC content compared to the total, somatic CA1 and neuropil 3′-UTRome (**Figure 1C**). Concomitantly, we observed a higher AT content (**Figure 1C**). Moreover, the same statistically significant effects were detected when comparing

TABLE 1 | Hand-selected list of RBPs with RNAs related to GABAR as targets.

Rbp	Method	Tissue	RNA targets related to GABAR	Reference
Nova	iCLIP	Brain	Gabbr2, Gabrg2	Ule et al. (2003)
FMRP	iCLIP	Brain	Gabbr1, Gabbr2	Darnell et al. (2011)
Staufen1	iCLIP	Brain	Gabbr2	Sugimoto et al. (2015)
Staufen2	RIP, iCLIP	Embryonic brain	Gabra2, Gabra3, Gabbr1, Gabbr2, Gabrb1, Gabrb2, Gabrb3, Gabrg3	Heraud-Farlow et al. (2013) and Sharangdhar et al. (2017)
Unkempt	iCLIP	Embryonic brain	Gabra3, Gabrb2	Murn et al. (2015)
Celf4	iCLIP	Brain	Gabra1, Gabra2, Gabra3, Gabra4, Gabra5, Gabrb1, Gabrb2, Gabrb3, Gabbr1, Gabbr2, Gabrg1, Gabrg2, Gabrg3, Gabrd	Wagnon et al. (2012)
Rbfox1, 2, 3	iCLIP	Brain	Gabra1, Gabra3, Gabra6, Gabbr1, Gabrb2, Gabrb3, Gabrg1, Gabrg2	Lee et al. (2016)
Pumilio1	iCLIP	Brain	Gabra1, Gabra5, Gabbr1, Gabrb2, Gabrg2	Zhang et al. (2017)
Pumilio2	iCLIP	Brain	Gabra4, Gabrb2, Gabrg2, Gabrq	Zhang et al. (2017) and Zahr et al. (2018)
4E-T	RIP	Embryonic brain	Gabrg2	Yang et al. (2014)
hnRNP R	iCLIP	Embryonic primary mouse motorneurons	Gabra4, Gabbr1, Gabrb1, Gabrb3, Gabrg2, Gabrg3	Briese et al. (2018)
CPEB1	RIP	Striatum	Gabrb1, Gabrb2	Parras et al. (2018)
CPEB4	RIP	Striatum	Gabra1, Gabra2, Gabra4, Gabrb1, Gabrb2, Gabrb3, Gabrg3	Parras et al. (2018)
nELAV	iCLIP	Human dorsolateral prefrontal cortex	Gabra4, Gabrb2, Gabrb3, Gabrg1, Gabrg3	Scheckel et al. (2016)

TABLE 2 | Hand-selected list of RBPs with RNAs related to scaffold protein, GABAR auxiliary and transport proteins as targets.

Rbp	Method	Tissue	RNA targets related to GABAR	Reference
Nova	iCLIP	Brain	Gphn	Ule et al. (2003)
FMRP	iCLIP	Brain	NSF, Trak2, Ubqln1	Darnell et al. (2011)
Staufen1	iCLIP	Brain	KCTD12, GABARAPL3, NSF, Arfgef2, Ubqln1	Sugimoto et al. (2015)
Staufen2	RIP, iCLIP	Embryonic brain	Gphn, Arhgef9, KCTD16, NSF, Arfgef2, GABARAPL1, Zdhhc3, Plcl1, Ubqln1	Heraud-Farlow et al. (2013) and Sharangdhar et al. (2017)
Rbfox1, 2, 3	iCLIP	Brain	Gphn, NSF, Arfgef2, Ubqln1	Lee et al. (2016)
Pumilio1	iCLIP	Brain	KCTD12, Trak2	Zhang et al. (2017)
Pumilio2	iCLIP	Brain	Gphn, KCTD12, Arfgef2, Trak2, Plcl1	Zhang et al. (2017) and Zahr et al. (2018)
4E-T	RIP	Embryonic brain	Gphn, Trak2	Yang et al. (2014)
hnRNP R	iCLIP	Embryonic primary mouse motorneurons	Gphn, Arhgef9, KCTD16, NSF, Arfgef2, Zdhhc3, Trak2, Plcl1	Briese et al. (2018)
CPEB1	RIP	Striatum	Arfgef2, Zdhhc3	Parras et al. (2018)
CPEB4	RIP	Striatum	Gphn, Arfgef2, Zdhhc3, Trak2, Ubqln1	Parras et al. (2018)
nELAV	iCLIP	Human dorsolateral prefrontal cortex	KCTD16, Plcl1	Scheckel et al. (2016)

ionotropic GluR and GABAR subunit mRNAs (**Figure 1D**). A lower GC content accounts for less stable secondary structures in the 3′-UTRs of GABAR compared to the total, somatic CA1 and neuropil 3′-UTRome as well as to GluR 3′-ends. Interestingly, the cytoplasmic polyadenylation binding element binding protein (CPEB) binds a short, AT-rich sequence within the 3′-UTR of target mRNAs to control translation and to induce the elongation of polyA tails (Mendez and Richter, 2001). By using RNA immunoprecipitation (RIP), it was shown that CPEB1 and 4 bind different GABAR subunits as well as mRNAs coding for scaffold protein such as Gphn (Parras et al., 2018; see also **Tables 1**, **2**). Moreover, ELAV proteins, among others, bind so-called AU-rich elements (ARE) to stabilize its target mRNAs (Fan and Steitz, 1998; Peng et al., 1998). Therefore, it is tempting to speculate that ELAV proteins also bind mRNAs coding for GABAR subunits to regulate their abundance. Supportive for this idea is an iCLIP-based ELAV target screen from human brain, which detected selective mRNAs encoding GABAR subunits, $GABA_B$ receptor auxiliary proteins and GABAR transport proteins (Scheckel et al., 2016; see also **Tables 1**, **2**).

To date, several GABAR subunits, scaffold, auxiliary and GABAR transport proteins have been detected as targets for RBPs by iCLIP or RIP (**Tables 1**, **2**). Among those, known translation regulators such as fragile X mental retardation protein (FMRP), Pumilio1, 2, 4E-T as well as CPEB1 and 4 all bind GABAR subunit mRNAs. However, how these RBPs act together to locally control the expression of GABAR subunits in dendrites is still unknown. Future studies are clearly needed to unravel the role of RBP mediated protein expression control.

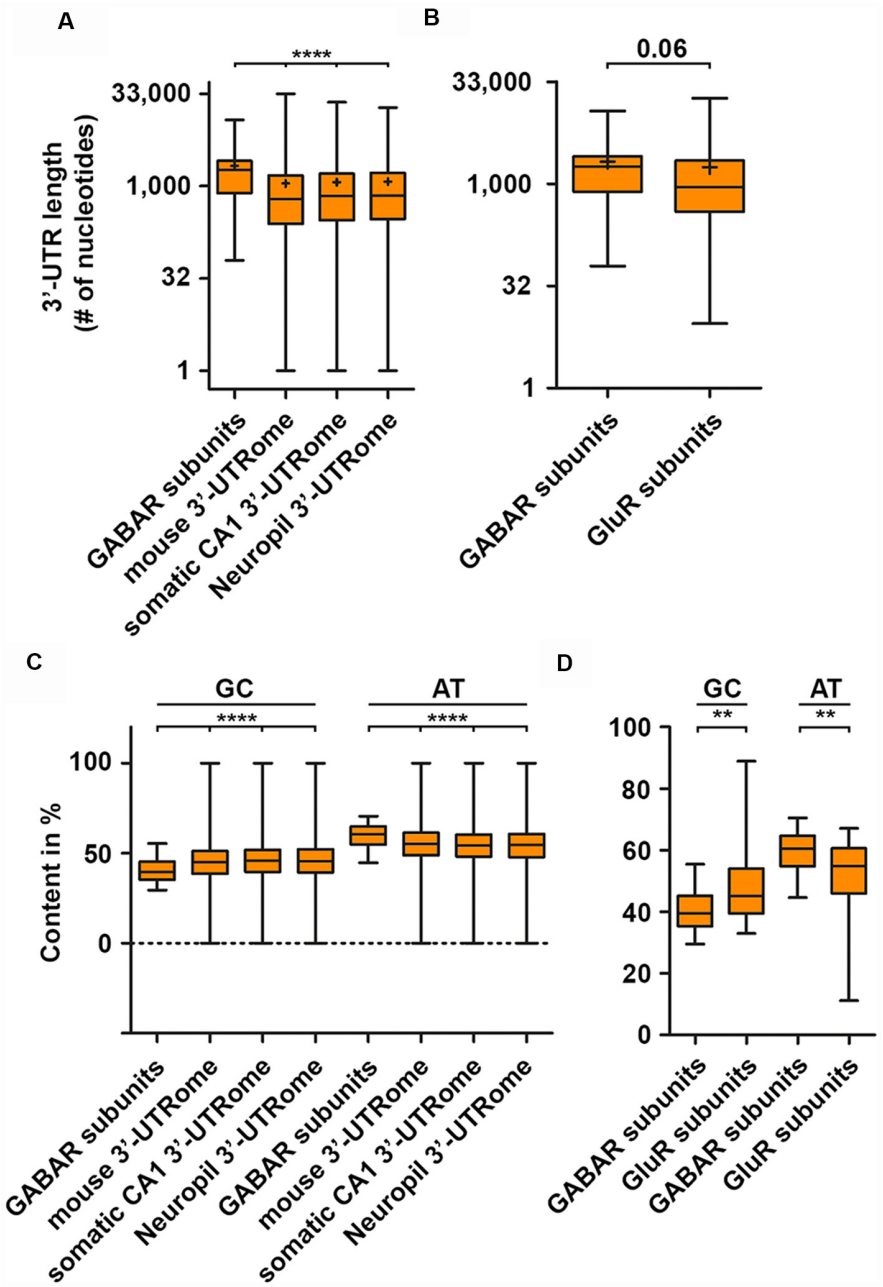

FIGURE 1 | GABA receptor (GABAR) subunits exhibit extended 3′-untranslated region (3′-UTR) length. 3′-UTR lengths of GABAR ($GABA_A$ and $GABA_B$ receptor) subunits compared to the global mouse, hippocampal CA1, neuropil 3′-UTRome **(A)** and the 3′-UTR lengths of ionotropic GluR subunits **(B)**. GC and AT content of GABAR subunits 3′-UTRs compared to the global mouse, hippocampal CA1 and neuropil 3′-UTRome **(C)** as well as ionotropic GluR subunits **(D)**. Abbreviation: $^+$represents the mean. *P*-values were calculated using the Mann-Whitney *U*-test, $^{**}p < 0.01$, $^{****}p < 0.0001$.

TRANSLATION CONTROL: A POSSIBLE REGULATION OF GABA RECEPTOR PROTEIN ABUNDANCE AND COMPLEX ASSEMBLY

Translation is a multistep process that is regulated by versatile proteins (Jackson et al., 2010). Different sequence features of the mRNA that influence translation activity and association with ribosomal polysomes have been characterized in human cell lines (Floor and Doudna, 2016). In detail, the length and structural stability of the 3′-UTR, the number of miRNA binding sites as well as AU elements in the 3′-UTR are main drivers of translation activity located at the 3′-end of the untranslated region. An increase in these features is associated with decreased translation activity in non-neuronal cells (Floor and Doudna, 2016) as well

as nerve cells (Blair et al., 2017). For GABAR subunit 3′-UTRs, we observed an increase in 3′-UTR length and AT content (**Figures 1A,C,D**). These results suggest that translation of these subunits is strongly regulated. Supportive for this idea is the finding that GABAR subunit mRNAs are recognized and subsequently bound by different RBPs (**Table 1**). In the last decade, several studies revealed that RBPs control translation of their target mRNAs (Hentze et al., 2018). One extensively studied example is the FMRP. FMRP mediated translational control is crucial for neuronal homeostasis and function since loss-of-function leads to severe neurological impairments in synaptic plasticity which cause intellectual disability and social deficits hallmarked for autism spectrum disorders (Bassell and Warren, 2008; Darnell and Klann, 2013). Furthermore, recent studies showed that FMRP is needed for proper differentiation of neuronal stem cells (Castrén et al., 2005; Gao et al., 2018). FMRP has been shown to co-migrate with translationally active ribosomal polysomes (Stefani et al., 2004). However, this finding was challenged by the same study showing that polysomal co-migration is detergent sensitive (Stefani et al., 2004). A mechanistic study combining *in vitro* assays and cryoelectron microscopy reported that FMRP inhibits translation through binding to the ribosomal intersubunit space thereby precluding binding of tRNAs and translation elongation factors (Chen et al., 2014). A transcriptome-wide screen for FMRP targets associated with polysomes identified mRNAs coding for subunits of the $GABA_B$ receptor complex (Darnell et al., 2011; see **Table 1**). Moreover, a recent study showed that the $GABA_A$ receptor subunit δ was downregulated in an FMRP knock-out mouse model (Gantois et al., 2006). These findings suggest that FMRP may regulate selected subunits of the $GABA_B$ and/or $GABA_A$ receptor, most likely at the translational level. Another known translation regulator is Pumilio2 (Pum2). For Pum2, it was shown that it represses translation by competing with the eukaryotic initiation factor (eIF4E) for mRNA 5′-cap binding (Cao et al., 2010), an essential step to start translation initiation (Jackson et al., 2010). Moreover, Pum2 is able to form a complex with the miRNA binding protein Argonaute (Ago) and the eukaryotic translation elongation factor 1A to repress translation elongation (Friend et al., 2014). Next to its role as translation regulator, Pum2 regulates transcript stability through recruitment of the polyA deadenylase complex CCR4-NOT (Van Etten et al., 2012), which is the major protein complex to induce RNA degradation (Collart, 2016). Based on a published iCLIP dataset, Pum2 is able to bind subunits of the $GABA_A$ and $GABA_B$ receptor (**Table 1**). Interestingly, double knockdown of Pumilio1 and 2 lead to a decrease in the mRNA levels of certain GABAR subunits (Zhang et al., 2017) indicating that they may be regulated posttranscriptionally by Pumilio proteins. Another RBP that impacts the expression of $GABA_A$ receptor subunits, is the non-octamer, POU-domain DNA-binding protein (NONO, also known as p54NRB). NONO belongs to the family of polypyrimidine tract-binding protein-associated splicing factors that are known to regulate various aspects of the RNA lifecycle including transcription regulation, splicing, RNA processing and RNA transport (Yarosh et al., 2015). Interestingly, mutations in the NONO locus causes intellectual disability in humans (Mircsof et al., 2015). Moreover, the authors found that the $GABA_A$ receptor-mediated inhibition is mainly affected when NONO is depleted (Mircsof et al., 2015) suggesting that this RBP regulates directly or indirectly the expression of the $GABA_A$ receptor. Nonetheless, it is widely unknown which GABAR subunits are translationally regulated. However, the binding of RBPs that are known to control RNA metabolism and translation, clearly suggests the existence of posttranscriptional gene regulation mechanisms for GABARs.

It is commonly accepted that the 3′-UTR allows for translational regulation of mRNAs. Research in the last years, however, has shown that the coding sequence (CDS) can also regulate protein synthesis rate, protein folding and protein complex assembly (Hanson and Coller, 2018). Dynamic translation regulation mediated by the CDS became experimentally accessible with the emergence of deep sequencing technologies and ribosome profiling protocols (Ingolia et al., 2009). Studies in cell lines and cultured neurons revealed that longer CDS are associated with translationally active "heavy" polyribosomes; most likely because a longer CDS can accumulate more ribosomes (Floor and Doudna, 2016; Blair et al., 2017). Interestingly, subunits of the $GABA_AR$ receptor complex display a shorter CDS compared to ionotropic GluR subunits (**Figure 2A**) suggestive for differences in translation activity. Another exciting possibility to regulate protein synthesis rate and output is the usage of synonymous codons. Twenty-one amino acids are encoded by 64 codons including three stop codons in the eukaryotic genome (Alberts et al., 2014). This degeneration of the genetic code leads to a codon bias, the preferred usage of certain codons over others to encode the same amino acid. Research in the last decades has shown that the usage bias is not random, but in contrast is driven and influenced by certain features such as translation activity, mRNA stability, protein folding, protein assembly and transcription factor binding (Grantham et al., 1980; Stergachis et al., 2013; Hanson and Coller, 2018). Codons can influence translation speed (Sørensen and Pedersen, 1991) most likely through the levels of cognate and near-cognate tRNAs (Anderson, 1969; Zhang and Ignatova, 2011; Fedyunin et al., 2012; Yu et al., 2015; Hanson and Coller, 2018). Since the nascent chain initiates folding already in the ribosomal exit tunnel (Lu and Deutsch, 2005), the elongation rate can also influence protein folding and, thereby, the protein conformation as it has been shown for the Cystic Fibrosis Transmembrane Regulator (CFTR) in mammalian cells (Kirchner et al., 2017). In line with this finding, Yu et al. (2015) showed using an *in vitro* translation system that codon usage determines co-translational folding through variation in the elongation rate. In particular for a multi-domain protein, it has been suggested that cluster of rare codons flank the parts of the mRNA that code for protein domains. Thus, ribosomes attenuate at these sites allowing the nascent domains to fold first to prevent misfolding (Schieweck et al., 2016; Hanson and Coller, 2018). Protein domains, that are encoded by the downstream mRNA, can then interact with already folded protein substructures to form a functional complex. Moreover, codon usage dependent protein folding can also influence protein specificity, which

was reported for the Multi-Drug Resistance 1 protein (MDR1). A silent mutation in a rare codon changes the specificity of MDR1 (Kimchi-sarfaty et al., 2007). Together, these results strongly indicate that dynamics in the translation elongation rate determine trajectories of (co-)translational folding. Based on these results, an intriguing question raises: can codon usage influence protein folding of transmembrane proteins such as subunits of the $GABA_A$ receptor? Interestingly, $GABA_AR$ subunits contain more transmembrane helices compared to ionotropic GluR subunits (**Figure 2B**). This suggests that $GABA_AR$ subunits may need more variation in translation speed to allow co-translational folding than ionotropic GluR subunits. Furthermore, $GABA_AR$ subunits differ in their codon usage compared to GluR subunits (**Figure 2C**). Overall, the codon usage profiles between the two receptor groups are similar. For some codons, however, we detected significant differences in their frequency (**Figures 2D,E**). Interestingly, impaired translation of AGA codons leads to neurodegeneration in a mouse model (Ishimura et al., 2014). Moreover, $GABA_AR$ and GluR subunits exploit different stop codons. While $GABA_AR$ subunit mRNAs display an almost 1:1:1 ratio, GluR subunits prefer the TGA stop codon that yields the highest readthrough potential in mammalian cell lines (Howard et al., 2000; Bidou et al., 2004; Loughran et al., 2014; Manuvakhova et al., 2014). In addition to co-translational folding, the assembly of large protein complexes can also occur co-translationally (Balchin et al., 2016). It has been shown that this process is crucial for the complex formation in eukaryotic cells (Shiber et al., 2018). It is tempting to speculate that for large neuronal protein complexes such as $GABA_A$ receptors, a similar mechanism exists to ensure proper protein-protein interaction. Of note, codon usage and optimality differ dramatically in their impact on RNA stability comparing neurons and non-neuronal cells (Burow et al., 2018). Therefore, a thorough analysis of the neuronal translatome and tRNAome is needed to understand the impact of codon usage on $GABA_A$ receptor functioning.

To sum up, findings from different model organisms and cells demonstrate that translation is a highly dynamic process necessary for many aspects of the protein life cycle. For $GABA_A$ receptors, it is widely unknown: (i) whether and how they are translationally regulated; and (ii) whether co-translational folding/assembly is necessary for proper GABAR function. However, our bioinformatic predictions suggest that for some aspects, GABAR are prone to be subject to posttranscriptional regulation. Future studies will be clearly needed to unravel the dynamics and regulatory factors of their translation.

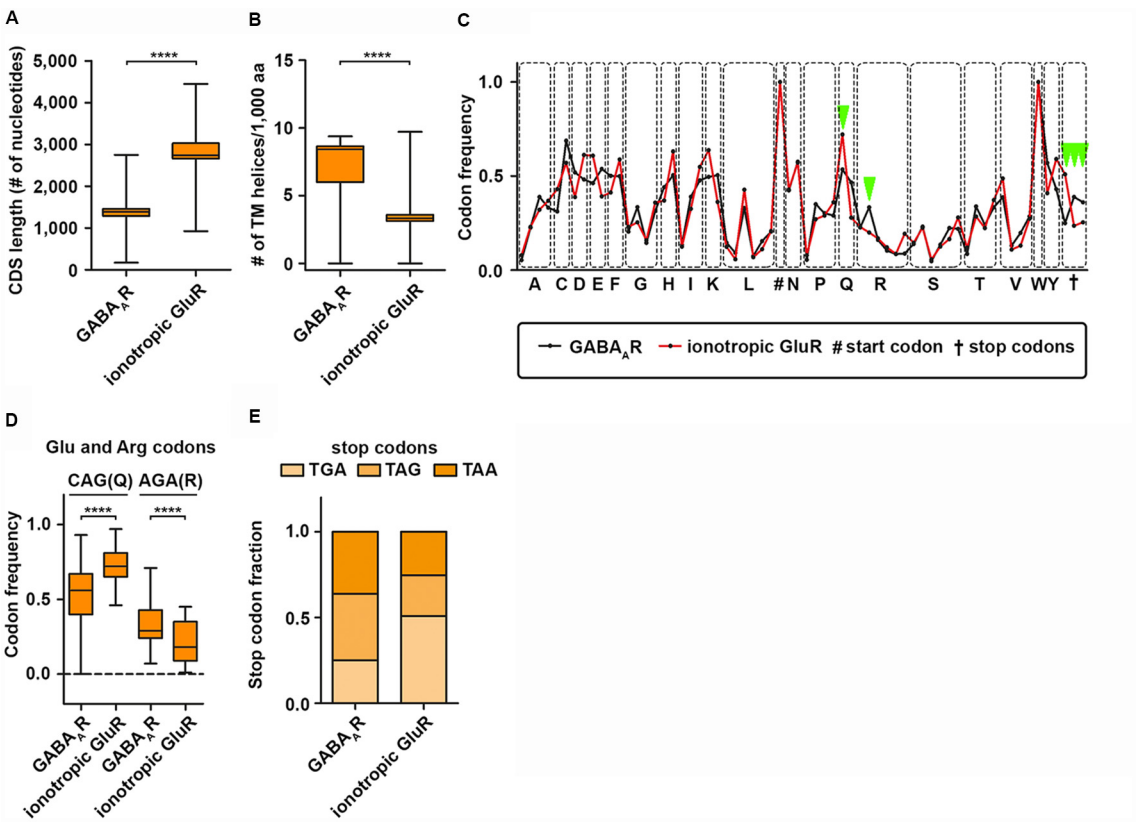

FIGURE 2 | $GABA_A$ receptor codon usage differ from ionotropic glutamate receptors. CDS length **(A)** and the number of transmembrane (TM) helices **(B)** in $GABA_AR$ and ionotropic GluR subunits. **(C)** Codon usage frequency of $GABA_AR$ and GluR for 20 amino acids and stop codons. Dots represent synonymous codons. **(D)** Codon frequency for CAG (Q) and AGA (R). **(E)** Relative fraction of stop codon usage between $GABA_AR$ and GluR subunits. Abbreviations: CDS, coding sequence; aa, amino acid. *P*-values were calculated using the Mann-Whitney *U*-test, ****$p < 0.0001$.

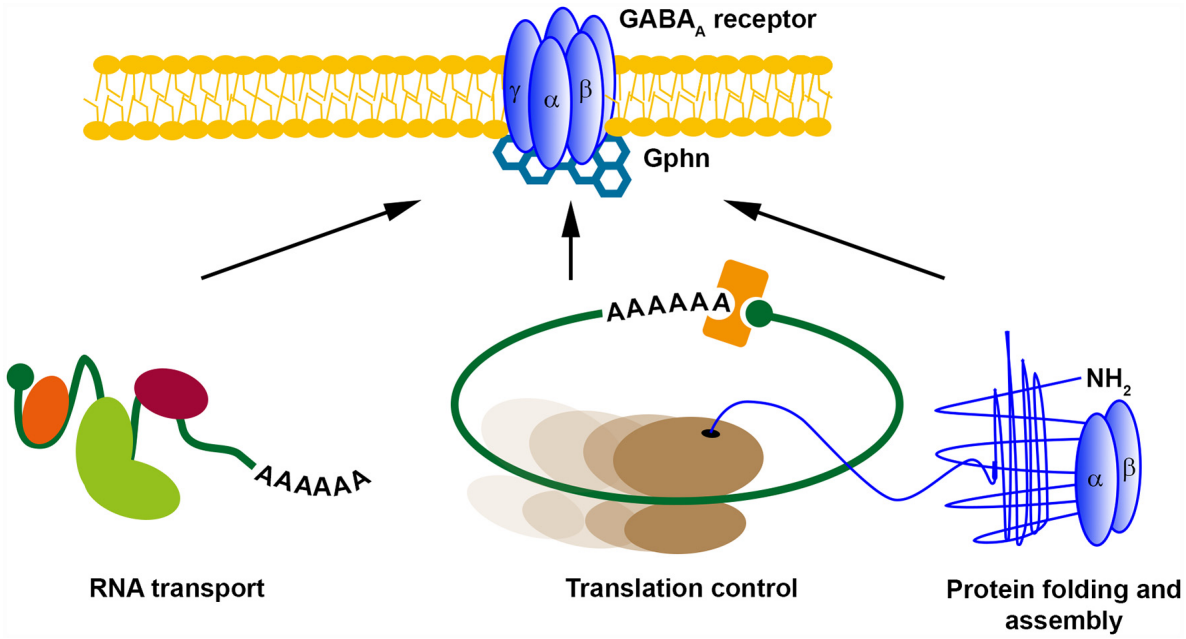

FIGURE 3 | Possible posttranscriptional regulation mechanisms for GABA$_A$ receptors. Different posttranscriptional regulatory mechanisms exist. RNA transport, translational control and (co-translational) protein folding and assembly control local protein expression. We propose that GABARs might be regulated at inhibitory synapses in a similar manner. Abbreviation: Gphn, Gephyrin.

IS LOCAL PROTEIN SYNTHESIS A PREREQUSITE FOR PLASTICITY OF INHIBITORY SYNAPSES: A PERSPECTIVE

Since the discovery of LTP by Bliss and Lomo (1973), numerous studies have unraveled the plasticity of excitatory synapses in the brain aiming to explain the mechanism of learning and memory formation (Kandel et al., 2014). However, how inhibitory synapses undergo structural and molecular plasticity has been widely overlooked for some time (Gaiarsa and Ben-Ari, 2006). One of the first examples that inhibitory synapses show long-term plasticity was a study on Purkinje cells in the cerebellum published in 1998 (Aizenman et al., 1998). Since that time, various studies have addressed the mechanisms of how inhibitory LTP is conveyed (Castillo et al., 2011). Interestingly, in some aspects, inhibitory and excitatory LTP share similar mechanisms including the exchange of synaptic receptors (de Luca et al., 2017) as well as the importance of scaffold proteins for LTP (Petrini et al., 2014). In this context, it was shown that clustering of Gephyrin (Gphn), the major scaffold protein for inhibitory synapses (Tyagarajan and Fritschy, 2014), is essential for GABA$_A$ receptor surface dynamics and iLTP (Petrini et al., 2014). In line with its importance for iLTP, Gphn is posttranslationally modified in response to neuronal activity (Flores et al., 2015; Ghosh et al., 2016), which may represent a molecular hub to control inhibitory transmission. Arguably, one of the most impressive examples showing the dynamics of inhibitory synapse formation is the study by Oh et al. (2016). Upon GABA stimulation, newly formed Gphn cluster appear that are the structural basis for inhibitory synapse formation (Tyagarajan and Fritschy, 2014). Based on our bioinformatic predictions (**Figures 1**, **2**) and RBP target screens (**Tables 1**, **2**), it is tempting to speculate that the appearance of Gphn clusters upon GABA stimulation requires mRNA transport and, subsequently, translation. We propose that these mechanisms are necessary for inhibitory synapse formation (**Figure 3**). In general, future studies are clearly necessary to address the importance of posttranscriptional gene regulation for GABAergic synaptic transmission. Therefore, it needs to be investigated: (i) which GABAR component is regulated by RBPs; (ii) whether their expression is regulated at the translation, splicing and/or stability level; and (iii) whether their posttranscriptional regulation occurs locally at the synapse. Unraveling the role of RBPs in neuronal inhibition will clearly improve our understanding how neuronal networks are coordinated to find the balance between excitation and inhibition.

METHODS

For analysis, 3′-UTR sequences and length of transmembrane domains were extracted from the EMSEMBL database (genome assembly GRCm38.p6) using the Gene Ontology ID "GO:0016917" for GABARs, "GO:0008066" for glutamate receptors and "GO:0004970" for ionotropic glutamate receptors. Only annotated mRNA isoforms were analyzed. Statistics were calculated using GraphPad Prism (version 5; GraphPad, San Diego, CA, USA).

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

RS and MK conceived, executed and discussed the research that is presented in this article. RS generated the figures, the table and wrote the manuscript. RS and MK edited together.

FUNDING

This work was supported by the DFG (FOR2333, SPP1738 to MK) and the Boehringer Ingelheim Fonds (to RS).

ACKNOWLEDGMENTS

We apologize to all colleagues whose work could not be cited or discussed owing to space constraints. We thank members of the Kiebler lab for critical comments.

REFERENCES

Aizenman, C. D., Manis, P. B., and Linden, D. J. (1998). Polarity of long-term synaptic gain change is related to postsynaptic spike firing at a cerebellar inhibitory synapse. *Neuron* 21, 827–835. doi: 10.1016/s0896-6273(00)80598-x

Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K., et al. (2014). *Molecular Biology of the Cell, 6th Edn*. New York and Abingdon: Garland Science.

Anderson, W. F. (1969). The effect of tRNA concentration on the rate of protein synthesis. *Proc. Natl. Acad. Sci. U S A* 62, 566–573. doi: 10.1073/pnas.62.2.566

Andreassi, C., and Riccio, A. (2009). To localize or not to localize: mRNA fate is in 3′UTR ends. *Trends Cell Biol.* 19, 465–474. doi: 10.1016/j.tcb.2009.06.001

Balchin, D., Hayer-Hartl, M., and Hartl, F. U. (2016). *In vivo* aspects of protein folding and quality control. *Science* 353:aac4354. doi: 10.1126/science.aac4354

Bassell, G. J., and Warren, S. T. (2008). Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. *Neuron* 60, 201–214. doi: 10.1016/j.neuron.2008.10.004

Bidou, L., Hatin, I., Perez, N., Allamand, V., Panthier, J. J., and Rousset, J. P. (2004). Premature stop codons involved in muscular dystrophies show a broad spectrum of readthrough efficiencies in response to gentamicin treatment. *Gene Ther.* 11, 619–627. doi: 10.1038/sj.gt.3302211

Bingol, B., and Schuman, E. M. (2006). Activity-dependent dynamics and sequestration of proteasomes in dendritic spines. *Nature* 441, 1144–1148. doi: 10.1038/nature04769

Blair, J. D., Hockemeyer, D., Doudna, J. A., Bateup, H. S., and Floor, S. N. (2017). Widespread translational remodeling during human neuronal differentiation. *Cell Rep.* 21, 2005–2016. doi: 10.1016/j.celrep.2017.10.095

Bliss, T. V. P., and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate are of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* 232, 331–356. doi: 10.1113/jphysiol.1973.sp010273

Briese, M., Saal-Bauernschubert, L., Ji, C., Moradi, M., Ghanawi, H., Uhl, M., et al. (2018). hnRNP R and its main interactor, the noncoding RNA 7SK, coregulate the axonal transcriptome of motoneurons. *Proc. Natl. Acad. Sci. U S A* 115, E2859–E2868. doi: 10.1073/pnas.1721670115

Burow, D. A., Martin, S., Quail, J. F., Alhusaini, N., Coller, J., and Cleary, M. D. (2018). Attenuated codon optimality contributes to neural-specific mRNA decay in *Drosophila*. *Cell Rep.* 24, 1704–1712. doi: 10.1016/j.celrep.2018.07.039

Cajigas, I. J., Tushev, G., Will, T. J., tom Dieck, S., Fuerst, N., and Schuman, E. M. (2012). The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. *Neuron* 74, 453–466. doi: 10.1016/j.neuron.2012.02.036

Cao, Q., Padmanabhan, K., and Richter, J. D. (2010). Pumilio 2 controls translation by competing with eIF4E for 7-methyl guanosine cap recognition. *RNA* 16, 221–227. doi: 10.1261/rna.1884610

Castillo, P., C.Q, C., and Carroll, R. C. (2011). Long-term synaptic plasticity at inhibitory synapses. *Curr. Opin. Neurobiol.* 21, 328–338. doi: 10.1016/j.conb.2011.01.006

Castrén, M., Tervonen, T., Kärkkäinen, V., Heinonen, S., Castrén, E., Larsson, K., et al. (2005). Altered differentiation of neural stem cells in fragile X syndrome. *Proc. Natl. Acad. Sci. U S A* 102, 17834–17839. doi: 10.1073/pnas.0508995102

Chen, E., Sharma, M. R., Shi, X., Agrawal, R. K., and Joseph, S. (2014). Fragile X mental retardation protein regulates translation by binding directly to the ribosome. *Mol. Cell* 54, 407–417. doi: 10.1016/j.molcel.2014.03.023

Collart, M. A. (2016). The Ccr4-not complex is a key regulator of eukaryotic gene expression. *Wiley Interdiscip. Rev. RNA* 7, 438–454. doi: 10.1002/wrna.1332

Costa-Mattioli, M., and Monteggia, L. M. (2013). mTOR complexes in neurodevelopmental and neuropsychiatric disorders. *Nat. Neurosci.* 16, 1537–1543. doi: 10.1038/nn.3546

Darnell, J. C., and Klann, E. (2013). The translation of translational control by FMRP: therapeutic targets for FXS. *Nat. Neurosci.* 16, 1530–1536. doi: 10.1038/nn.3379

Darnell, J. C., Van Driesche, S. J., Zhang, C., Hung, K. Y. S., Mele, A., Fraser, C. E., et al. (2011). FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* 146, 247–261. doi: 10.1016/j.cell.2011.06.013

de Luca, E., Ravasenga, T., Petrini, E. M., Polenghi, A., Nieus, T., Guazzi, S., et al. (2017). Inter-synaptic lateral diffusion of GABAA receptors shapes inhibitory synaptic currents report inter-synaptic lateral diffusion of GABAA receptors shapes inhibitory synaptic currents. *Neuron* 95, 63–69.e5. doi: 10.1016/j.neuron.2017.06.022

Doyle, M., and Kiebler, M. A. (2011). Mechanisms of dendritic mRNA transport and its role in synaptic tagging. *EMBO J.* 30, 3540–3552. doi: 10.1038/emboj.2011.278

Fan, X. C., and Steitz, J. A. (1998). Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the *in vivo* stability of ARE-containing mRNAs. *EMBO* 17, 3448–3460. doi: 10.1093/emboj/17.12.3448

Fedyunin, I., Lehnhardt, L., Böhmer, N., Kaufmann, P., Zhang, G., and Ignatova, Z. (2012). TRNA concentration fine tunes protein solubility. *FEBS Lett.* 586, 3336–3340. doi: 10.1016/j.febslet.2012.07.012

Flexner, J., Flexner, L., and Stellar, E. (1963). Memory in mice as affected by intracerebral puromycin. *Science* 141, 57–59. doi: 10.1126/science.141.3575.57

Floor, S. N., and Doudna, J. A. (2016). Tunable protein synthesis by transcript isoforms in human cells. *Elife* 5:e10921. doi: 10.7554/eLife.10921

Flores, C. E., Nikonenko, I., Mendez, P., Fritschy, J., and Tyagarajan, S. K. (2015). Activity-dependent inhibitory synapse remodeling through gephyrin phosphorylation. *Proc. Natl. Acad. Sci. U S A* 112, 65–72. doi: 10.1073/pnas.1411170112

Frey, U., and Morris, R. G. M. (1997). Synaptic tagging and long-term potentiation. *Nature* 385, 533–536. doi: 10.1038/385533a0

Friend, K., Campbell, Z. T., Cooke, A., Kroll-Conner, P., Wickens, M. P., and Kimble, J. (2014). A conserved PUF-Ago-eEF1A complex attenuates translation elongation. *Nat. Struct. Mol. Biol.* 19, 176–183. doi: 10.1038/nsmb.2214

Fritzsche, R., Karra, D., Bennett, K. L., Ang, F., Heraud-Farlow, J. E., Tolino, M., et al. (2013). Interactome of two diverse RNA granules links mRNA localization to translational repression in neurons. *Cell Rep.* 5, 1749–1762. doi: 10.1016/j.celrep.2013.11.023

Gaiarsa, J., and Ben-Ari, Y. (2006). "Long-term plasticity at inhibitory synapses: a phenomenon that has been overlooked," in *The Dynamic Synapse: Molecular Methods in Ionotropic Receptor Biology*, eds J. T. Kittler and S. J. Moss (Boca Raton, FL: CRC Press/Taylor & Francis).

Gantois, I., Vandesompele, J., Speleman, F., Reyniers, E., D'Hooge, R., Severijnen, L. A., et al. (2006). Expression profiling suggests underexpression

of the GABAA receptor subunit δ in the fragile X knockout mouse model. *Neurobiol. Dis.* 21, 346–357. doi: 10.1016/j.nbd.2005.07.017

Gao, Y., Li, Y., Stackpole, E. E., Zhao, X., Richter, J. D., Liu, B., et al. (2018). Regulatory discrimination of mRNAs by FMRP controls mouse adult neural stem cell differentiation. *Proc. Natl. Acad. Sci. U S A* 115, E11397–E11405. doi: 10.1073/pnas.1809588115

Ghosh, H., Auguadri, L., Battaglia, S., Thirouin, Z. S., Zemoura, K., Dieter, A., et al. (2016). Several posttranslational modifications act in concert to regulate gephyrin scaffolding and GABAergic transmission. *Nat. Commun.* 7:13365. doi: 10.1038/ncomms13365

Grantham, R., Gautier, C., Gouy, M., Mercier, R., and Pavé, A. (1980). Codon catalog usage and the genome hypothesis. *Nucleic Acids Res.* 8, r49–r62. doi: 10.1093/nar/8.1.197-c

Hanson, G., and Coller, J. (2018). Codon optimality, bias and usage in translation and mRNA decay. *Nat. Rev. Mol. Cell Biol.* 19, 20–30. doi: 10.1038/nrm.2017.91

Hentze, M. W., Castello, A., Schwarzl, T., and Preiss, T. (2018). A brave new world of RNA-binding proteins. *Nat. Rev. Mol. Cell Biol.* 19, 327–341. doi: 10.1038/nrm.2017.130

Heraud-Farlow, J. E., and Kiebler, M. A. (2014). The multifunctional Staufen proteins: conserved roles from neurogenesis to synaptic plasticity. *Trends Neurosci.* 37, 470–479. doi: 10.1016/j.tins.2014.05.009

Heraud-Farlow, J. E., Sharangdhar, T., Li, X., Pfeifer, P., Tauber, S., Orozco, D., et al. (2013). Staufen2 regulates neuronal target RNAs. *Cell Rep.* 5, 1511–1518. doi: 10.1016/j.celrep.2013.11.039

Hershkowitz, M., Wilson, J. E., and Glassman, E. (1975). The incorporation of radioactive amino acids into brain subcellular proteins during training. *J. Neurochem.* 25, 687–694. doi: 10.1111/j.1471-4159.1975.tb04389.x

Howard, M. T., Shirts, B. H., Petros, L. M., Flanigan, K. M., Gesteland, R. F., and Atkins, J. F. (2000). Sequence specificity of aminoglycoside-induced stop codon readthrough: potential implications for treatment of Duchenne muscular dystrophy. *Ann. Neurol.* 48, 164–169. doi: 10.1002/1531-8249(200008)48:2%3C164::AID-ANA5%3E3.0.CO;2-B

Huppertz, I., Attig, J., D'Ambrogio, A., Easton, L. E., Sibley, C. R., Sugimoto, Y., et al. (2014). iCLIP: protein-RNA interactions at nucleotide resolution. *Methods* 65, 274–287. doi: 10.1016/j.ymeth.2013.10.011

Ingolia, N. T., Ghaemmaghami, S., Newman, J. R. S., and Weissman, J. S. (2009). Genome-wide analysis *in vivo* of translation with nucleotide resolution using ribosome profiling. *Science* 324, 218–323. doi: 10.1126/science.1168978

Ishimura, R., Nagy, G., Dotu, I., Zhou, H., Yang, X.-L., Schimmel, P., et al. (2014). RNA function. Ribosome stalling induced by mutation of a CNS-specific tRNA causes neurodegeneration. *Science* 345, 455–459. doi: 10.1126/science.1249749

Jackson, R. J., Hellen, C. U. T., and Pestova, T. V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat. Rev. Mol. Cell Biol.* 11, 113–127. doi: 10.1038/nrm2838

Jung, H., Gkogkas, C. G., Sonenberg, N., and Holt, C. E. (2014). Remote control of gene function by local translation. *Cell* 157, 26–40. doi: 10.1016/j.cell.2014.03.005

Kanai, Y., Dohmae, N., and Hirokawa, N. (2004). Kinesin transports RNA. *Neuron* 43, 513–525. doi: 10.1016/j.neuron.2004.07.022

Kandel, E. R., Dudai, Y., and Mayford, M. R. (2014). The molecular and systems biology of memory. *Cell* 157, 163–186. doi: 10.1016/j.cell.2014.03.001

Khoutorsky, A., Yanagiya, A., Gkogkas, C. G., Fabian, M., Prager-Khoutorsky, M., Cao, R., et al. (2013). Control of synaptic plasticity and memory via suppression of poly(A)-Binding protein. *Neuron* 78, 298–311. doi: 10.1016/j.neuron.2013.02.025

Kiebler, M. A., and Bassell, G. J. (2006). Neuronal RNA granules: movers and makers. *Neuron* 51, 685–690. doi: 10.1016/j.neuron.2006.08.021

Kimchi-sarfaty, C., Oh, J. M., Kim, I., Sauna, Z. E., Calcagno, A. M., Ambudkar, S. V., et al. (2007). A silent polymorphism in the MDR1 gene changes substrate specificity. *Science* 315, 525–528. doi: 10.1126/science.1135308

Kirchner, S., Cai, Z., Rauscher, R., Kastelic, N., Anding, M., Czech, A., et al. (2017). Alteration of protein function by a silent polymorphism linked to tRNA abundance. *PLoS Biol.* 15:e2000779. doi: 10.1371/journal.pbio.2000779

Klein, M. E., Castillo, P. E., and Jordan, B. A. (2015). Coordination between translation and degradation regulates inducibility of mGluR-LTD. *Cell Rep.* 10, 1459–1466. doi: 10.1016/j.celrep.2015.02.020

Krug, M., Lössner, B., and Ott, T. (1984). Anisomycin blocks the late phase of long-term potentiation in the dentate gyrus of freely moving rats. *Brain Res. Bull.* 13, 39–42. doi: 10.1016/0361-9230(84)90005-4

Lee, J. A., Damianov, A., Lin, C. H., Fontes, M., Parikshak, N. N., Anderson, E. S., et al. (2016). Cytoplasmic Rbfox1 regulates the expression of synaptic and autism-related genes. *Neuron* 89, 113–128. doi: 10.1016/j.neuron.2015.11.025

Linden, D. J. (1996). A protein synthesis-dependent late phase of cerebellar long-term depression. *Neuron* 17, 483–490. doi: 10.1016/s0896-6273(00)80180-4

Loughran, G., Chou, M. Y., Ivanov, I. P., Jungreis, I., Kellis, M., Kiran, A. M., et al. (2014). Evidence of efficient stop codon readthrough in four mammalian genes. *Nucleic Acids Res.* 42, 8928–8938. doi: 10.1093/nar/gku608

Lu, J., and Deutsch, C. (2005). Folding zones inside the ribosomal exit tunnel. *Nat. Struct. Mol. Biol.* 12, 1123–1129. doi: 10.1038/nsmb1021

Manuvakhova, M., Keeling, K., Bedwell, D. M., Manuvakhova, M., Keeling, K., and Bedwell, D. M. (2014). Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. *Cold Spring Harb. Lab. Press Oct.* 1, 1044–1055. Available at: https://rnajournal.cshlp.org/content/6/7/1044.long.

Mendez, R., and Richter, J. D. (2001). Translational control by CPEB: a means to the end. *Nat. Rev. Mol. Cell Biol.* 2, 521–529. doi: 10.1038/35080081

Mircsof, D., Langouët, M., Rio, M., Moutton, S., Siquier-pernet, K., Bole-Feysot, C., et al. (2015). Mutations in NONO lead to syndromic intellectual disability and inhibitory synaptic defects. *Nat. Neurosci.* 18, 1731–1736. doi: 10.1038/nn.4169

Murn, J., Zarnack, K., Yang, Y. J., Durak, O., Murphy, E. A., Cheloufi, S., et al. (2015). Control of a neuronal morphology program by an RNA-binding zinc finger protein, Unkempt. *Genes Dev.* 29, 501–512. doi: 10.1101/gad.258483.115

Oh, W. C., Lutzu, S., Castillo, P. E., and Kwon, H. B. (2016). De novo synaptogenesis induced by GABA in the developing mouse cortex. *Science* 353, 1037–1040. doi: 10.1126/science.aaf5206

Parras, A., Anta, H., Santos-Galindo, M., Swarup, V., Elorza, A., Nieto-González, J. L., et al. (2018). Autism-like phenotype and risk gene mRNA deadenylation by CPEB4 mis-splicing. *Nature* 560, 441–446. doi: 10.1038/s41586-018-0423-5

Peng, S. S.-Y., Chen, C. A., Xu, N., and Shyu, A. (1998). RNA stabilization by the AU-rich element binding, HuR, an ELAV protein. *EMBO J.* 17, 3461–3470. doi: 10.1093/emboj/17.12.3461

Pernice, H. F., Schieweck, R., Kiebler, M. A., and Popper, B. (2016). mTOR and MAPK: from localized translation control to epilepsy. *BMC Neurosci.* 17:73. doi: 10.1186/s12868-016-0308-1

Petrini, E. M., Ravasenga, T., Hausrat, T. J., Iurilli, G., Olcese, U., Racine, V., et al. (2014). Synaptic recruitment of gephyrin regulates surface GABAA receptor dynamics for the expression of inhibitory LTP. *Nat. Commun.* 5:3921. doi: 10.1038/ncomms4921

Scheckel, C., Drapeau, E., Frias, M. A., Park, C. Y., Fak, J., Zucker-Scharff, I., et al. (2016). Regulatory consequences of neuronal ELAV-like protein binding to coding and non-coding RNAs in human brain. *Elife* 5:e10421. doi: 10.7554/eLife.10421

Schieweck, R., Popper, B., and Kiebler, M. A. (2016). Co-translational folding: a novel modulator of local protein expression in mammalian neurons? *Trends Genet.* 32, 788–800. doi: 10.1016/j.tig.2016.10.004

Sharangdhar, T., Sugimoto, Y., Heraud-Farlow, J., Fernández-Moya, S. M., Ehses, J., Ruiz de los Mozos, I., et al. (2017). A retained intron in the 3′-UTR of *Calm3* mRNA mediates its Staufen- and activity-dependent localization to neuronal dendrites. *EMBO Rep.* 18, 1762–1774. doi: 10.15252/embr.201744334

Shashoua, V. E. (1976). Identification of specific changes in the pattern of brain protein synthesis after training. *Science* 193, 1264–1266. doi: 10.1126/science.959837

Shiber, A., Döring, K., Friedrich, U., Klann, K., Merker, D., Zedan, M., et al. (2018). Cotranslational assembly of protein complexes in eukaryotes revealed by ribosome profiling. *Nature* 561, 268–272. doi: 10.1038/s41586-018-0462-y

Sørensen, M. A., and Pedersen, S. (1991). Absolute *in vivo* translation rates of individual codons in Escherichia coli. The two glutamic acid codons GAA and GAG are translated with a threefold difference in rate. *J. Mol. Biol.* 222, 265–280. doi: 10.1016/0022-2836(91)90211-n

Sosanya, N. M., Cacheaux, L. P., Workman, E. R., Niere, F., Perrone-Bizzozero, N. I., and Raab-Graham, K. F. (2015). Mammalian Target of

Rapamycin (mTOR) tagging promotes dendritic branch variability through the capture of Ca^{2+}/Calmodulin-dependent protein kinase II α (CaMKIIα) mRNAs by the RNA-binding Protein HuD. *J. Biol. Chem.* 290, 16357–16371. doi: 10.1074/jbc.M114.599399

Stefani, G., Fraser, C. E., Darnell, J. C., and Darnell, R. B. (2004). Fragile X mental retardation protein is associated with translating polyribosomes in neuronal cells. *J. Neurosci.* 24, 7272–7276. doi: 10.1523/JNEUROSCI.2306-04.2004

Stergachis, A. B., Haugen, E., Shafer, A., Fu, W., Vernot, B., Reynolds, A., et al. (2013). Exonic transcription factor binding directs codon choice and affects protein evolution. *Science* 342, 1367–1372. doi: 10.1126/science.1243490

Steward, O., Wallace, C. S., Lyford, G. L., and Worley, P. F. (1998). Synaptic activation causes the mRNA for the leg Arc to localize selectively near activated postsynaptic sites on dendrites. *Neuron* 21, 741–751. doi: 10.1016/s0896-6273(00)80591-7

Sugimoto, Y., Vigilante, A., Darbo, E., Zirra, A., Militti, C., D'Ambrogio, A., et al. (2015). hiCLIP reveals the *in vivo* atlas of mRNA secondary structures recognized by Staufen 1. *Nature* 519, 491–494. doi: 10.1038/nature14280

Tyagarajan, S. K., and Fritschy, J. M. (2014). Gephyrin: a master regulator of neuronal function? *Nat. Rev. Neurosci.* 15, 141–156. doi: 10.1038/nrn3670

Ule, J., Jensen, K. B., Ruggiu, M., Mele, A., Ule, A., and Darnell, R. B. (2003). Identifies nova-regutated RNA networks in the brain. *Science* 302, 1212–1215. doi: 10.1126/science.1090095

Van Etten, J., Schagat, T. L., Hrit, J., Weidmann, C. A., Brumbaugh, J., Coon, J. J., et al. (2012). Human Pumilio proteins recruit multiple deadenylases to efficiently repress messenger RNAs. *J. Biol. Chem.* 287, 36370–36383. doi: 10.1074/jbc.M112.373522

Wagnon, J. L., Briese, M., Sun, W., Mahaffey, C. L., Curk, T., Rot, G., et al. (2012). CELF4 regulates translation and local abundance of a vast set of mRNAs, including genes associated with regulation of synaptic function. *PLoS Genet.* 8:e1003067. doi: 10.1371/journal.pgen.1003067

Yang, G., Smibert, C. A., Kaplan, D. R., and Miller, F. D. (2014). An eIF4E1/4E-T complex determines the genesis of neurons from precursors by translationally repressing a proneurogenic transcription program. *Neuron* 84, 723–739. doi: 10.1016/j.neuron.2014.10.022

Yarosh, C. A., Iacona, J. R., Lutz, C. S., and Lynch, K. W. (2015). PSF: nuclear busy-body or nuclear facilitator? *Wiley Interdiscip. Rev. RNA* 6, 351–367. doi: 10.1002/wrna.1280

Yu, C. H., Dang, Y., Zhou, Z., Wu, C., Zhao, F., Sachs, M. S., et al. (2015). Codon usage influences the local rate of translation elongation to regulate co-translational protein folding. *Mol. Cell* 59, 744–754. doi: 10.1016/j.molcel.2015.07.018

Zahr, S. K., Yang, G., Kazan, H., Borrett, M. J., Yuzwa, S. A., Voronova, A., et al. (2018). A translational repression complex in developing mammalian neural stem cells that regulates neuronal specification. *Neuron* 97, 520.e6–537.e6. doi: 10.1016/j.neuron.2017.12.045

Zhang, M., Chen, D., Xia, J., Han, W., Cui, X., Neuenkirchen, N., et al. (2017). Post-transcriptional regulation of mouse neurogenesis by Pumilio proteins. *Genes Dev.* 31, 1354–1369. doi: 10.1101/gad.298752.117

Zhang, G., and Ignatova, Z. (2011). Folding at the birth of the nascent chain: coordinating translation with co-translational folding. *Curr. Opin. Struct. Biol.* 21, 25–31. doi: 10.1016/j.sbi.2010.10.008

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 Molecular Neuroscience

MINI REVIEW
published: 26 June 2019
doi: 10.3389/fnmol.2019.00154

Molecular Specialization of GABAergic Synapses on the Soma and Axon in Cortical and Hippocampal Circuit Function and Dysfunction

*April Contreras, Dustin J. Hines and Rochelle M. Hines**

Department of Psychology, University of Nevada, Las Vegas, Las Vegas, NV, United States

The diversity of inhibitory interneurons allows for the coordination and modulation of excitatory principal cell firing. Interneurons that release GABA (γ-aminobutyric acid) onto the soma and axon exert powerful control by virtue of proximity to the site of action potential generation at the axon initial segment (AIS). Here, we review and examine the cellular and molecular regulation of soma and axon targeting GABAergic synapses in the cortex and hippocampus. We also describe their role in controlling network activity in normal and pathological states. Recent studies have demonstrated a specific role for postsynaptic dystroglycan in the formation and maintenance of cholecystokinin positive basket cell terminals contacting the soma, and postsynaptic collybistin in parvalbumin positive chandelier cell contacts onto the AIS. Unique presynaptic molecular contributors, *LGI2* and *FGF13*, expressed in parvalbumin positive basket cells and chandelier cells, respectively, have also recently been identified. Mutations in the genes encoding proteins critical for somatic and AIS inhibitory synapses have been associated with human disorders of the nervous system. Dystroglycan dysfunction in some congenital muscular dystrophies is associated with developmental brain malformations, intellectual disability, and rare epilepsy. Collybistin dysfunction has been linked to hyperekplexia, epilepsy, intellectual disability, and developmental disorders. Both *LGI2* and *FGF13* mutations are implicated in syndromes with epilepsy as a component. Advancing our understanding of the powerful roles of somatic and axonic GABAergic contacts in controlling activity patterns in the cortex and hippocampus will provide insight into the pathogenesis of epilepsy and other nervous system disorders.

Keywords: GABAergic synapse development, epilepsies and epileptic syndromes, cholecystokinin, parvalbumin, interneuron, $GABA_A$ receptor subunits, somatic inhibitory synapse, axon initial segment inhibitory synapse

OPEN ACCESS

Edited by:
Shiva Tyagarajan,
University of Zurich, Switzerland

Reviewed by:
Marco Sassoè-pognetto,
University of Turin, Italy
Yuchio Yanagawa,
Gunma University, Japan

****Correspondence:***
Rochelle M. Hines
rochelle.hines@unlv.edu

Received: *29 March 2019*
Accepted: *31 May 2019*
Published: *26 June 2019*

Citation:
Contreras A, Hines DJ and Hines RM (2019) Molecular Specialization of GABAergic Synapses on the Soma and Axon in Cortical and Hippocampal Circuit Function and Dysfunction. Front. Mol. Neurosci. 12:154. doi: 10.3389/fnmol.2019.00154

INTRODUCTION

The functional output of the nervous system relies upon coordinated patterns of activity within neuronal circuitry. Neuronal circuits in the cortex and hippocampus are composed of not only excitatory pyramidal cells, but a multitude of diverse interneuron types that express unique complements of proteins and play distinct functional

roles (Petilla Interneuron Nomenclature Group et al., 2008). The diversity of inhibitory interneuron signaling allows for multiple levels of modulation of excitatory principal cell firing (Kubota et al., 2016). Interneurons release the neurotransmitter γ-aminobutyric acid (GABA) onto postsynaptic targets, which then binds to $GABA_A$ receptors ($GABA_A$Rs). Diversity is also present in the postsynaptic targets of interneuron types (**Figure 1**). In particular, interneurons releasing GABA onto the principal cell soma and axon exert powerful control by virtue of proximity to the site of action potential generation at the axon initial segment (AIS; Miles et al., 1996; Klausberger and Somogyi, 2008). As with specialization of the presynaptic interneuron partner, the postsynapse is also specialized by enrichment of $GABA_A$R subtypes. $GABA_A$Rs are heteropentamers, and those enriched at the postsynapse are most commonly composed of 2α, 2β, and a γ subunit. Some of the postsynaptic specialization of $GABA_A$Rs is conferred by the α subunit, with α1 containing receptors enriched on the dendrites and soma, and α2 containing receptors enriched on the soma and AIS (Jacob et al., 2008). Further complexity is added due to brain circuits likely relying on unique mechanisms to control synapse targeting, specificity, and molecular specialization. In this review, we examine the cellular and molecular regulation of soma and axon targeting GABAergic synapses in the cortex and hippocampus, as well as clarify their role in controlling network activity in these respective circuits. Because unique mechanisms likely exist in each circuit, we will compare and contrast soma and axon targeting GABAergic synapses in the cortex and hippocampus based on current research. We also examine the role of soma and axon targeting GABAergic synapse dysfunction in pathological states, linking animal phenotypes and human syndromes to key molecular contributors at soma and axon targeting synapses of cortical and hippocampal circuits.

SOMA AND AXON TARGETING INTERNEURONS

The function of a neuronal circuit relies upon inhibitory interneuron modulation of principal cell activity, with interneurons that contact the perisomatic region exerting powerful control over axonal output of principal cells (Miles et al., 1996). The cortex and hippocampus feature complex circuitry exemplified by interneuron diversity. Interneurons can be classified by their morphology, connectivity, firing pattern, and gene expression pattern. Based on morphology, 16 or more types of interneuron have been distinguished in the hippocampus (Cajal, 1893; Lorente and De Nó, 1934; Parra et al., 1998) while transcriptomic cell typing has identified 23 interneuron types in the cortex (Tasic et al., 2016). Interneurons targeting the perisomatic region generally have a small number of large terminals in comparison to those contacting dendrites. While dendrite-targeting inhibitory synapses can suppress Ca^{2+} dependent spiking, those contacting the perisomatic region can suppress repetitive discharge of Na^{+} dependent action potentials (Miles et al., 1996). Despite the morphological and molecular complexity of cortical and hippocampal circuits, the majority of interneurons in these regions express either the neuropeptide cholecystokinin or the calcium binding protein parvalbumin (CCK; PV; Whissell et al., 2015).

CCK positive basket cells target the soma and proximal dendrites of cortical and hippocampal pyramidal cells (**Figure 1**), and do not appear to innervate the AIS (Panzanelli et al., 2011). Proportions of CCK basket cells express the ionotropic serotonin receptor (5-HT_3; Morales and Bloom, 1997) and the metabotropic Cannabinoid receptor type 1 (CB1), which modulate GABA release from the presynaptic terminal (Katona et al., 1999; Lee and Soltesz, 2011). Functionally, CCK positive basket cells provide long-lasting inhibition, modulating cortical and hippocampal cell activity based upon motivation, emotion, and autonomic information from subcortical regions (Buzsáki, 1996; Freund and Katona, 2007).

PV positive interneurons include basket cells which target the soma and proximal dendrites of excitatory pyramidal cells, and chandelier cells whose terminals synapse onto the AIS (**Figure 1**; Defelipe et al., 1985). PV positive basket cell terminals express the metabotropic serotonin receptor 5-HT_{2A}, with electrophysiological data showing that activation of 5-HT_{2A} depolarizes PV positive GABAergic interneurons (Weber and Andrade, 2010). PV positive basket and chandelier cells are fast-spiking, with the potential to robustly influence the activity of hundreds of pyramidal cells (Hu et al., 2014), and are responsible for the generation of network oscillations in both the cortex and hippocampus (Freund and Buzsáki, 1996). While both CCK positive and PV positive interneurons inhibit the perisomatic region of pyramidal cells, they feature molecular specialization and have unique functional contributions to network activity.

MOLECULAR SPECIALIZATION OF POSTSYNAPTIC SITES ON THE SOMA AND AXON

Since the identification of distinct interneuron types, research has focused on the unique molecular and functional characteristics of their synaptic specializations. CCK positive somatic terminals are enriched with $GABA_A$Rs containing the α2/α3 subunits (Nyíri et al., 2001), and the formation and maintenance of CCK terminals is linked to dystroglycan (DG) of the dystrophin glycoprotein complex (DGC; **Figure 1**; Früh et al., 2016). The DGC is composed of a number of interacting proteins dependent upon tissue type, with brain DGC including dystrophin (or utrophin), syntrophin, dystrobrevin, and DG. Pyramidal cells express DGC in perisomatic clusters postsynaptic to GABAergic terminals (Knuesel et al., 1999; Brünig et al., 2002; Lévi et al., 2002). DG interacts with neurexins to form GABAergic synapses (Sugita et al., 2001), and disruption of the DGC alters synaptic clustering of $GABA_A$Rs (Knuesel et al., 1999; Vaillend et al., 2010).

Conditional deletion of DG (DG cKO) leads to a loss of the DGC and a modification in $GABA_A$R subunit clustering, but does not prevent the formation of GABAergic terminals (**Table 1**; Früh et al., 2016). DG cKO mice exhibit a decrease in cluster size

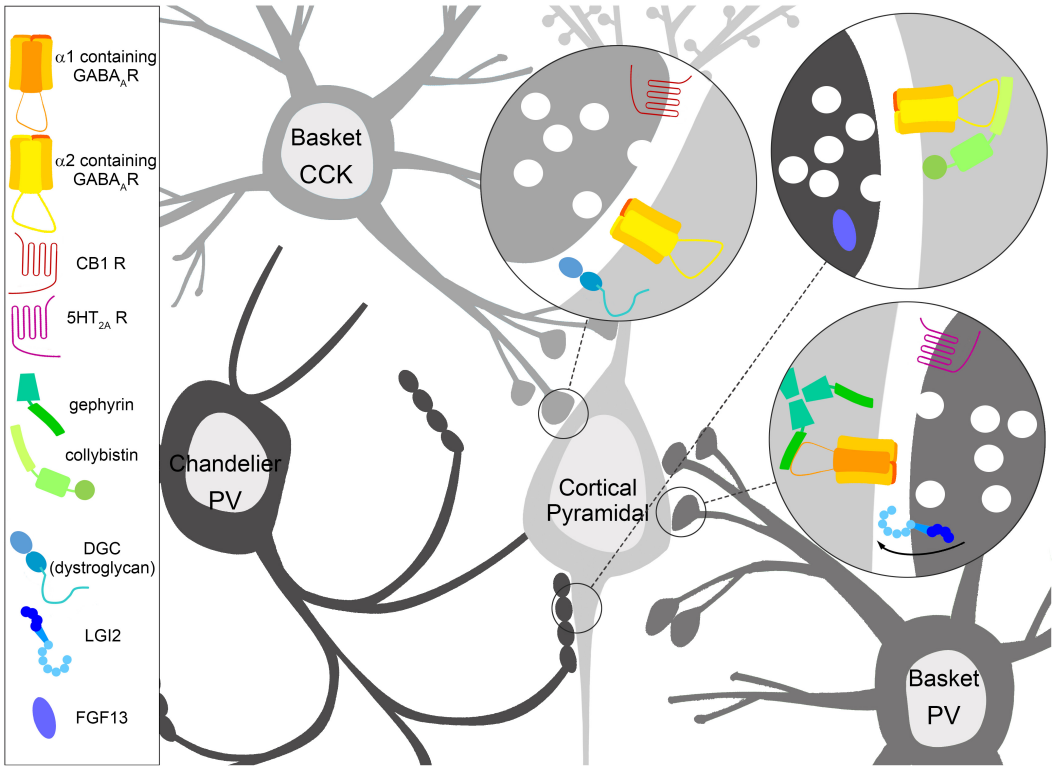

FIGURE 1 | Specialization of inhibitory GABAergic synapse subtypes. Interneurons have molecular specifications which help guide, form, and maintain GABAergic synapses onto distinct areas of the cortical pyramidal cell, which in turn feature molecular specialization in terms of enriched $GABA_A$ receptor ($GABA_AR$) subtypes and interacting proteins. Cholecystokinin (CCK) positive basket cells target the soma of pyramidal cells, where the dystrophin glycoprotein complex (DGC) containing dystroglycan and $GABA_ARs$ containing the α2 subunit are robustly expressed. The CCK positive presynaptic terminal is enriched with Cannabinoid receptor type 1 (CB1). Parvalbumin (PV) positive basket cells target the soma of pyramidal cells enriched with $GABA_ARs$ containing the α1 subunit anchored by gephyrin. The PV positive presynaptic terminal contains the serotonin 5-HT_{2A} receptor, which is thought to depolarize PV positive basket cells. LGI2 protein is enriched in PV positive basket cells during synaptogenesis, and regulates the formation of these synapses. PV positive chandelier cell cartridges target the axon initial segment (AIS) of pyramidal cells. $GABA_ARs$ containing the α2 subunit are enriched here, and collybistin interaction plays a key role in AIS localization, although both collybistin and α2 are found at other inhibitory contact sites. The non-secreted protein FGF13 is enriched in PV positive chandelier cells during synaptogenesis, and regulates the formation and maintenance of these synapses.

of $GABA_ARs$ containing the α1 subunit, along with an increase in cluster density of $GABA_ARs$ containing the α2 subunit. Examination of DG cKO mice reveals a specific loss of CCK positive basket cell terminals onto pyramidal cells of the cortex and hippocampal CA1. Induction of DG cKO in mature mice reveals that DG is necessary for the maintenance of CCK positive basket terminals, with the absence of DG leading to a reduction in already formed connections with CCK positive terminals (Früh et al., 2016). The role of DG in the maintenance of CCK positive terminals is independent of neurexin, demonstrated using mice that express the T190M variant of DG, which lacks neurexin binding. Disruption of DG alters functional connectivity of CCK positive terminals, with administration of carbachol to DG cKO slices showing a loss of carbachol-induced increases in inhibitory currents (Früh et al., 2016). Carbachol increases perisomatic inhibitory transmission in pyramidal cells in control slices (Früh et al., 2016), which is mediated by direct excitation of CB1 receptor expressing CCK positive interneurons (Nagode et al., 2014). DG cKO mice also appear to have a reduction in body and brain weight compared to controls (Früh et al., 2016).

PV positive cells terminate onto the soma and AIS, with synapses on the AIS enriched with $GABA_ARs$ containing the α2 subunit (**Figure 1**; Nusser et al., 1996; Nyíri et al., 2001). Recently, the α2 subunit was shown to have a strong interaction with the collybistin-SH3 domain, but a relatively weak interaction with the gephyrin-E domain (Hines et al., 2018). Conversely, the α1 subunit interaction with the collybistin-SH3 domain is relatively weak, with the gephyrin-E domain interaction being strong. These studies also showed that *in vitro*, collybistin (CB) and gephyrin compete for interaction with the α2 subunit (Hines et al., 2018). This sets interaction with CB as a possible means of regulating postsynaptic enrichment of α2 subunit containing receptors. To examine this possibility, a substitution mutation was made to introduce the gephyrin-preferring portion of the α1 subunit large intracellular loop into α2 (*Gabra2*-1). The *Gabra2*-1 mutation reduces interaction with CB, and results in an increase in total α2, but a decrease in CB expression in both the cortex and hippocampal CA1 (**Table 1**; Hines et al., 2018). The *Gabra2*-1 mutation reduces clustering of α2-containing receptors, but does not

appear to reduce the overall size or density of inhibitory presynaptic terminals stained by VGAT or GAD65 (Hines et al., 2018). Specific examination of AIS synapses showed a loss of α2-containing receptors, and a loss of VGAT positive terminals opposed to the AIS (Hines et al., 2018). A subset of heterozygous and homozygous *Gabra2*-1 pups die during postnatal (PN) development, with a peak in mortality at postnatal day 20, and during this time spontaneous seizures are observed. *Gabra2*-1 mice also show abnormalities in electroencephalogram (EEG) recordings, with elevations in δ-power (Hines et al., 2018).

MOLECULAR SPECIALIZATION OF SOMA AND AXON TARGETING INTERNEURONS

During synaptogenesis, contact between opposite yet complementary pre- and post-synaptic terminals is essential for proper circuit formation. In addition to postsynaptic specializations on pyramidal cells, recent papers have identified molecular specialization of presynaptic interneuron subtypes critical for their postsynaptic targeting. Cell sorting of interneurons during peak synaptogenesis, followed by RNA-sequencing and whole-transcriptome analyses, has recently identified molecular programs for synaptogenesis specific to soma, axon, and dendrite targeting interneurons (Favuzzi et al., 2019). Gene ontology analysis showed that the most enriched genes are those belonging to synaptic membrane compartments and processes that contribute to synaptogenesis, which were not enriched in mature cortex (Favuzzi et al., 2019). *Lgi2*, a member of the leucine-rich glioma inactivated protein gene family, was identified as a chief regulator for the establishment of perisomatic inhibitory synapses by a population of PV positive basket cells (**Figure 1**; Favuzzi et al., 2019). *Lgi2* encodes a secreted protein (LGI2) that consists of leucine-rich repeat and epilepsy-associated/epitempin (EPTP) domains. Prior studies have implicated the related family member LGI1 in maturation of excitatory synapses (Senechal et al., 2005; Kegel et al., 2013). LGI proteins have been shown to interact with a disintegrin and metalloprotease (ADAM) proteins (Seppälä et al., 2011).

Through cell sorting *Fgf13* was identified as a candidate for AIS-targeting chandelier synapses (**Figure 1**; Favuzzi et al., 2019). *Fgf13* is a member of the fibroblast growth factor gene family, which encodes proteins (FGFs) critical for development (Wu et al., 2012; Pablo et al., 2016). Unlike many FGF family members,

TABLE 1 | Overview of key proteins involved in specification and maintenance of soma and axon targeting inhibitory synapses, and implications for disorders of the nervous system.

Mouse model	Molecular/cellular phenotype	Network/behavioral phenotype	Associated disorders
Gabra2-1 (Hines et al., 2018)	↓ α2 subunit interaction with collybistin *in vitro* ↑ total α2 in hippocampus, cortex ↓ total collybistin in hippocampus, cortex ↓ α2 containing receptors at cortical cell AIS ↓VGAT positive terminals onto cortical cell AIS	Gabra2-1 mouse model (Hines et al., 2018): Reduced amplitude and decay of sIPSC in hippocampal CA1; Spontaneous seizures during development; Developmental mortality (peaks ∼ PND 20); Elevations in δ-power in surviving adults; Increased anxiety in light-dark boxes and elevated plus maze	α2 subunit - Generalized epilepsy associated with *GABRA2* (The International League Against Epilepsy Consortium on Complex Epilepsies, 2018) Collybistin - Hyperekplexia (Striano and Zara, 2017) - Epilepsy (Wang et al., 2018) - Anxiety and aggression (Kalscheuer et al., 2009) - Mental retardation (Shimojima et al., 2011)
Nex-Cre/*Dag1* conditional KO (DG cKO) (Früh et al., 2016)	↓ perisomatic dystrophin-glycoprotein complex (DGC) in hippocampus ↓ α1 subunit cluster size ↑ α2 subunit density ↓ CCK basket cell terminals onto hippocampal, cortical cell soma	DG cKO (Früh et al., 2016): Carbachol induced increase in inhibitory currents in slice; No change in hippocampal CA1 sIPSC frequency or amplitude; Reduced body and brain weight; Peak mortality at 10 weeks	Dystrophin - Duchenne muscular dystrophy (DMD; McNally and Pytel, 2007) Dystroglycan - DMD associated with mental retardation (Knuesel et al., 1999; Daoud et al., 2009; Desguerre et al., 2009)
Lhx6$^{Cre/+}$*shLgi2* (Favuzzi et al., 2019)	↓ density of presynaptic inputs onto cortical cell soma	Canine Benign Familial Juvenile Epilepsy (Seppälä et al., 2011): Unilateral epileptic discharges in central-parietal and occipital lobes; Epilepsy onset at 5–9 weeks with remission by 4 months; Seizures and whole body tremors	- Epilepsy Canine Benign Familial Juvenile Epilepsy; (Seppälä et al., 2011); Partial Epilepsy with Pericentral Spikes (Kinton et al., 2002; Limviphuvadh et al., 2010)
Nkx2-1$^{CreER/+}$*shFgf13* (Favuzzi et al., 2019)	↓ density of presynaptic inputs onto cortical cell AIS Axonal disorganization in *Fgf13* deficient PV positive chandelier cells when downregulated at P2	Fgf13 +/- (Puranam et al., 2015): Frequency of IPSCs amplitude of mIPSCs reduced in whole cell recordings in hippocampal slice; Age-dependent susceptibility to hyperthermia-induced seizures ≤ PND15 (Puranam et al., 2015); *Fgf13* mutation lethal in male offspring Spontaneous recurrent seizures	- Epilepsy (Guillemot and Zimmer, 2011); Febrile Seizures Plus (Puranam et al., 2015); Borjeson-Forssman-Lehmann syndrome (Malmgren et al., 1993; Gecz et al., 1999)

FGF13 is non-secretory and functions independent of FGF receptors. FGF13 has been shown to be a microtubule stabilizing protein enriched in the growth cones of cortical cells (Wu et al., 2012). FGF13 is also known to limit localization of voltage-gated sodium channels to the somatodendritic compartment of principal neurons, while FGF14 promotes localization to the proximal axon (Pablo et al., 2016).

To investigate the role of *Lgi2* and *Fgf13*, interneuron cell type-specific Cre-driver lines were combined with adeno-associated virus (AAV) vectors carrying miR-based short-hairpin RNAs (Favuzzi et al., 2019). Cell-specific down-regulation of *Lgi2* and *Fgf13* led to a decrease in density of presynaptic inputs from interneurons expressing the short-hairpin RNAs. A decrease in somatic inhibitory synapses made by PV positive basket cells was observed upon down-regulation of *Lgi2* at P2 (**Table 1**; Favuzzi et al., 2019). ADAM22, the proposed postsynaptic partner of expressed LGI2 was also shown to be colocalized with gephyrin clusters on the soma, opposite GAD-65+ terminals (Favuzzi et al., 2019). Interestingly, *Fgf13* deficient PV positive chandelier cells showed axonal disorganization in addition to a loss of AIS innervation when down regulation was induced at P2 (**Table 1**; Favuzzi et al., 2019). Axonal disorganization itself may contribute to the decrease in AIS innervation observed, although this may also represent a dual role for FGF13. Interestingly, chandelier cell synaptic boutons were decreased in the absence of axonal disorganization if *Fgf13* was down-regulated after P14, confirming that expressed FGF13 also plays a role in maintenance of chandelier cell contacts onto the AIS, after the axon has reached its target (Favuzzi et al., 2019).

IMPLICATIONS OF GABAERGIC SYNAPSES ON THE SOMA AND AXON IN DISORDERS OF THE NERVOUS SYSTEM

The coordination of excitatory principal cell firing relies on interneuron function, and dysregulation of soma and axon targeting interneurons has been identified in disorders of the nervous system (DeFelipe et al., 1993; Rubenstein and Merzenich, 2003; Ali Rodriguez et al., 2018). Disruption of neuronal DG directly impacts the maintenance of CCK positive basket terminals onto pyramidal cell somas, leading to impaired CCK positive interneuron mediated neurotransmission and functional connectivity. Mutations in DGC components such as dystrophin are the most common cause of muscular dystrophies, which are movement disorders characterized by a robust degeneration of muscle tissue (**Table 1**; McNally and Pytel, 2007). Muscular dystrophies with neurological aberrations can be caused by varying genetic mutations, and are associated with a lack of available glycosylated DG (**Table 1**; Brancaccio, 2005; Barresi and Campbell, 2006). Varying ranges of intellectual disability have been identified in individuals with muscular dystrophies, and cognitive deficits are associated with neuronal DG alterations (Knuesel et al., 1999; Moore et al., 2002; Daoud et al., 2009; Desguerre et al., 2009; Vaillend et al., 2010).

Disruption in inhibitory signaling mediated by PV positive cells on a global level has been associated with neurodevelopmental disorders (Ali Rodriguez et al., 2018). Schizophrenia, through post-mortem studies and in animal models, has been associated with soma and axon targeting inhibitory synapses (Lewis et al., 2008, 2012; Hines et al., 2013). Autism spectrum and related disorders such as Angelman syndrome and Rett syndrome have also been linked to PV cell dysfunction, and notably these disorders have a high incidence of epilepsy (**Table 1**; Kalscheuer et al., 2009; Shimojima et al., 2011; Ali Rodriguez et al., 2018). Mutations in the $GABA_AR$ subunit genes have been implicated in genetic epilepsies (Baulac et al., 2001; Wallace et al., 2001; Hines et al., 2018). The gene encoding the α2 subunit (*GABRA2*) was identified as one of the most likely biological epilepsy genes in a recent genome-wide mega-analysis (The International League Against Epilepsy Consortium on Complex Epilepsies, 2018). In the *Gabra2*-1 animal model, altered clustering of $GABA_ARs$ containing the α2 subunit led to developmental seizure and mortality, as well as anxiety-like phenotypes (Hines et al., 2018). In humans, mutations in the gene encoding collybistin (*ARHGEF9*), lead to hyperekplexia syndromes that include intellectual disability and mental retardation (**Table 1**; Shimojima et al., 2011; Striano and Zara, 2017). *ARHGEF9* mutations are also associated with epilepsies and anxiety in humans (Kalscheuer et al., 2009; Wang et al., 2018).

LGI2 and *FGF13* dysfunction have also been linked to epilepsy. *LGI1* mutations account for about half of Autosomal Dominant Lateral Temporal lobe Epilepsy (ADLTE; Kalachikov et al., 2002). Mutations in *LGI2* have also been associated with an epileptic phenotype, especially that of canine Benign Familial Juvenile Epilepsy (**Table 1**; Fukata et al., 2010; Seppälä et al., 2011). LGI2 is also a leading candidate for mutations in the 4p15 region thought to be responsible for Partial Epilepsy with Pericentral Spikes (PEPS; Kinton et al., 2002; Limviphuvadh et al., 2010). Mutations in *FGF13* are linked to Genetic Epilepsy and Febrile Seizures Plus (GEFS+; Guillemot and Zimmer, 2011; Puranam et al., 2015), as well as Börjeson-Forssman-Lehmann syndrome, which is a rare X-linked disorder characterized by intellectual disability, obesity, seizures, hypogonadism, and distinctive facial features (**Table 1**; Malmgren et al., 1993; Gecz et al., 1999).

DISCUSSION

The modulation of excitatory pyramidal cells by GABAergic interneurons is determined by interneuron diversity, allowing for the complex computations performed by these neuronal circuits (Tremblay et al., 2016). Of the many interneuron subtypes, those that release GABA onto the soma and axon can powerfully influence and fine-tune neuronal activity (Miles et al., 1996). CCK positive cells target the soma and proximal dendrites of pyramidal cells in cortex and hippocampus, and rely on DG for targeting of α2/α3 containing $GABA_ARs$ to postsynaptic sites on the hippocampal pyramidal cell soma (Früh et al., 2016). Interestingly, clustering of $GABA_ARs$ at sites opposing CCK positive terminals appears to be independent of DG, as

α2 containing $GABA_A$Rs still cluster in DG cKO mice in the absence of CCK positive terminals (Früh et al., 2016). Consistent with this, gephyrin clustering was also unaffected in DG cKO, while CB was not assessed (Früh et al., 2016). It remains unclear how gephyrin might selectively stabilize specific subtypes of $GABA_A$Rs at postsynaptic sites despite its ubiquitous presence, but leading hypotheses point to posttranslational modification (Ghosh et al., 2016), or subtleties in multi-protein complex arrangements (Saiepour et al., 2010). Somatic contacts from CCK positive basket cells were unaffected by a DG mutation that interferes with neurexin binding (Früh et al., 2016), encouraging the exploration of possible novel presynaptic partners in CCK positive terminals that are needed for transsynaptic signaling during synapse formation and maintenance at sites contacting the soma.

PV positive chandelier cells terminate onto the AIS which is enriched with $GABA_A$Rs containing the α2 subunit, and the high affinity interaction between α2 and CB appears essential in this enrichment and in the maintenance of these synapses on cortical pyramidal cells (Hines et al., 2018). Despite evidence of a preferential interaction between α2 and CB, as well as a preferential role of this complex at AIS synapses onto cortical pyramidal cells, several points remain to be clarified. Although the interaction strength between α2 and gephyrin was comparatively weak, a prominent effect of α2 KO is a loss of gephyrin clustering at perisomatic synapses onto CA1 pyramidal cells (Panzanelli et al., 2011). The molecular mechanism regulating this loss of gephyrin remains unclear but may relate to an indirect interaction between α2 and gephyrin. Although the interaction between α2 and CB appears critical for AIS synapses in the cortex, α2 and CB are well known to be present at other synapse types. Conversely, α1 and α3 containing receptors can also be detected at AIS synapses, particularly in the hippocampus and amygdala (Gao and Heldt, 2016), thus analysis of the impact of the *Gabra2*-1 mutation on AIS synapses in other brain regions is needed.

Developmental RNA-seq focusing on the period of peak inhibitory synapse formation demonstrated that distinct types of interneurons rely on a largely unique complement of molecular programs for the specific subcellular contact sites that they establish (Favuzzi et al., 2019). During synaptogenesis, genes involved in targeting and matching PV positive interneuron axons to their postsynaptic targets include expression of *Lgi2* for basket cells, and *Fgf13* for chandelier cells (Favuzzi et al., 2019). Details of how the expressed proteins function at presynaptic terminals during synaptogenesis remain to be uncovered, including further illumination of specific interacting partners and effectors. In addition to the genes that were characterized in more detail, a number of others were identified to have relatively specific regulated expression patterns related to synapse formation (Favuzzi et al., 2019). Many of these were genes encoding adhesion proteins, as well as extracellular components such as proteins that make up the peri-neuronal net (Favuzzi et al., 2019). Investigation into some of the other candidates will allow for more detailed illumination of the steps involved in building each unique type of inhibitory contact on the soma and axon.

In general, studies have yet to replicate or contrast these mechanisms in regulating somatic and axon targeting inhibitory synapse formation, maintenance, and function in distinct brain circuits. As a point of comparison, the cerebellum has a more limited repertoire of cell types, and the cellular and molecular specialization of soma and axon targeting interneurons in the cerebellum is relatively well established (Somogyi and Hámori, 1976; Somogyi et al., 1983; Li et al., 1992; Ango et al., 2004). In the cerebellum, principal Purkinje cells receive GABAergic innervation from stellate and basket cells. Stellate cells target dendritic domains, while basket cells innervate the perisomatic region and ensheath the AIS (pinceau formation) of Purkinje cells. Guidance of the basket cell axon to the Purkinje AIS is mediated by Semaphorin 3A and its receptor neuropilin-1, which interacts directly with the adhesion molecule NF186 at the AIS target (Cioni et al., 2013; Telley et al., 2016). Maturation of the Purkinje AIS and pinceau formation relies upon neurofascin interaction with Ankyrin-G (Ango et al., 2004; Zonta et al., 2011; Buttermore et al., 2012). Somatic synapses on Purkinje cells are enriched with both α1 and α3 containing $GABA_A$Rs (Fritschy et al., 2006). The maintenance of these synapses does not depend on α1 expression (Fritschy et al., 2006), and α1 expression on the Purkinje soma is maintained in CB knockout in the absence of gephyrin (Papadopoulos et al., 2007), leaving the mechanisms required to build the postsynaptic compartment of somatic synapses of Purkinje cells unclear. Also of interest, another intracellular FGF family member, FGF14, is localized to the AIS, and has been implicated in Purkinje neuron excitability by impacting voltage gated Na+ channel kinetics (Goldfarb et al., 2007; Xiao et al., 2013); thus distinct FGFs may play unique but complementary roles at the AIS. Additional studies should compare and contrast the contributions of this subclass of FGFs in formation and maintenance of axon targeting synapses across multiple circuits.

The function of inhibitory synapses on the soma and axon is perhaps best illustrated by the effects observed upon mutation (**Table 1**). Epilepsy is an interesting common thread among soma and axon targeting inhibitory synapse gene syndromes. Given the role of soma and axon targeting interneurons in coordinating principal cell activity, discoordination of neuronal activity patterns is a logical extension. Yet further studies are needed to understand the distinction between disrupting specific synapse subtypes and functional implications for circuit activity. Examination of animal models for these disorders focusing on abnormalities in the development and maintenance of specific inhibitory synapse subtypes will be helpful in confirming a selective contribution. Identification of specific synapse subtypes, along with key molecular players at these sites may allow the development of molecular and pharmacological interventions that more precisely modulate the development and maintenance of specific inhibitory synapse subtypes. Further knowledge of specific synapse subtypes in these disorders will ultimately aid with the refinement or development of novel therapeutic strategies.

AUTHOR CONTRIBUTIONS

AC, DH, and RH wrote the manuscript. RH conceived the work and prepared the figure.

FUNDING

This work was supported by the UNLV University Libraries Open Article Fund.

REFERENCES

Ali Rodriguez, R., Joya, C., and Hines, R. M. (2018). Common ribs of inhibitory synaptic dysfunction in the umbrella of neurodevelopmental disorders. *Front. Mol. Neurosci.* 11:132. doi: 10.3389/fnmol.2018.00132

Ango, F., di Cristo, G., Higashiyama, H., Bennett, V., Wu, P., and Huang, Z. J. (2004). Ankyrin-based subcellular gradient of neurofascin, an immunoglobulin family protein, directs GABAergic innervation at purkinje axon initial segment. *Cell* 119, 257–272. doi: 10.1016/j.cell.2004.10.004

Barresi, R., and Campbell, K. P. (2006). Dystroglycan: from biosynthesis to pathogenesis of human disease. *J. Cell Sci.* 119, 199–207. doi: 10.1242/jcs.02814

Baulac, S., Huberfeld, G., Gourfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J. F., et al. (2001). First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. *Nat. Genet.* 28, 46–48. doi: 10.1038/88254

Brancaccio, A. (2005). α-Dystroglycan, the usual suspect? *Neuromuscul. Disord.* 15, 825–828. doi: 10.1016/j.nmd.2005.08.003

Brünig, I., Suter, A., Knuesel, I., Lüscher, B., and Fritschy, J.-M. (2002). GABAergic terminals are required for postsynaptic clustering of dystrophin but not of GABAA receptors and gephyrin. *J. Neurosci.* 22, 4805–4813. doi: 10.1523/JNEUROSCI.22-12-04805.2002

Buttermore, E. D., Piochon, C., Wallace, M. L., Philpot, B. D., Hansel, C., and Bhat, M. A. (2012). Pinceau organization in the cerebellum requires distinct functions of neurofascin in Purkinje and basket neurons during postnatal development. *J. Neurosci. Off. J. Soc. Neurosci.* 32, 4724–4742. doi: 10.1523/JNEUROSCI.5602-11.2012

Buzsáki, G. (1996). The hippocampo-neocortical dialogue. *Cereb. Cortex* 6, 81–92. doi: 10.1093/cercor/6.2.81

Cajal, S. (1893). Estructura del asta de Ammon. *Ann. Soc Esp Hist Nat. Madr.* 22, 53–114.

Cioni, J.-M., Telley, L., Saywell, V., Cadilhac, C., Jourdan, C., Huber, A. B., et al. (2013). SEMA3A signaling controls layer-specific interneuron branching in the cerebellum. *Curr. Biol.* 23, 850–861. doi: 10.1016/j.cub.2013.04.007

Daoud, F., Angeard, N., Demerre, B., Martie, I., Benyaou, R., Leturcq, F., et al. (2009). Analysis of Dp71 contribution in the severity of mental retardation through comparison of Duchenne and Becker patients differing by mutation consequences on Dp71 expression. *Hum. Mol. Genet.* 18, 3779–3794. doi: 10.1093/hmg/ddp320

DeFelipe, J., Garcia Sola, R., Marco, P., del Río, M. R., Pulido, P., and Ramón y Cajal, S. (1993). Selective changes in the microorganization of the human epileptogenic neocortex revealed by parvalbumin immunoreactivity. *Cereb. Cortex* 3, 39–48. doi: 10.1093/cercor/3.1.39

Defelipe, J., Hendry, S. H. C., Jones, E. G., and Schmechel, D. (1985). Variability in the terminations of GABAergic chandelier cell axons on initial segments of pyramidal cell axons in the monkey sensory-motor cortex. *J. Comp. Neurol.* 231, 364–384. doi: 10.1002/cne.902310307

Desguerre, I., Christov, C., Mayer, M., Zeller, R., Becane, H.-M., Bastuji-Garin, S., et al. (2009). Clinical heterogeneity of duchenne muscular dystrophy (DMD): definition of sub-phenotypes and predictive criteria by long-term follow-up. *PLoS One* 4:e4347. doi: 10.1371/journal.pone.0004347

Favuzzi, E., Deogracias, R., Marques-Smith, A., Maeso, P., Jezequel, J., Exposito-Alonso, D., et al. (2019). Distinct molecular programs regulate synapse specificity in cortical inhibitory circuits. *Science* 363, 413–417. doi: 10.1126/science.aau8977

Freund, T. F., and Buzsáki, G. (1996). Interneurons of the hippocampus. *Hippocampus* 6, 347–470. doi: 10.1002/(sici)1098-1063(1996)6:4<347::aid-hipo1>3.0.co;2-i

Freund, T. F., and Katona, I. (2007). Perisomatic inhibition. *Neuron* 56, 33–42. doi: 10.1016/j.neuron.2007.09.012

Fritschy, J.-M., Panzanelli, P., Kralic, J. E., Vogt, K. E., and Sassoè-Pognetto, M. (2006). Differential dependence of axo-dendritic and axo-somatic GABAergic synapses on GABAA receptors containing the alpha1 subunit in purkinje cells. *J. Neurosci. Off. J. Soc. Neurosci.* 26, 3245–3255. doi: 10.1523/JNEUROSCI.5118-05.2006

Früh, S., Romanos, J., Panzanelli, P., Bürgisser, D., Tyagarajan, S. K., Campbell, K. P., et al. (2016). Neuronal dystroglycan is necessary for formation and maintenance of functional CCK-positive basket cell terminals on pyramidal cells. *J. Neurosci. Off. J. Soc. Neurosci.* 36, 10296–10313. doi: 10.1523/JNEUROSCI.1823-16.2016

Fukata, Y., Lovero, K. L., Iwanaga, T., Watanabe, A., Yokoi, N., Tabuchi, K., et al. (2010). Disruption of LGI1–linked synaptic complex causes abnormal synaptic transmission and epilepsy. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3799–3804. doi: 10.1073/pnas.0914537107

Gao, Y., and Heldt, S. A. (2016). Enrichment of GABAA receptor α-subunits on the axonal initial segment shows regional differences. *Front. Cell. Neurosci.* 10:39. doi: 10.3389/fncel.2016.00039

Gecz, J., Baker, E., Donnelly, A., Ming, J. E., McDonald-McGinn, D. M., Spinner, N. B., et al. (1999). Fibroblast growth factor homologous factor 2 (FHF2): gene structure, expression and mapping to the Börjeson-Forssman-Lehmann syndrome region in Xq26 delineated by a duplication breakpoint in a BFLS-like patient. *Hum. Genet.* 104, 56–63. doi: 10.1007/s004390050910

Ghosh, H., Auguadri, L., Battaglia, S., Simone Thirouin, Z., Zemoura, K., Messner, S., et al. (2016). Several posttranslational modifications act in concert to regulate gephyrin scaffolding and GABAergic transmission. *Nat. Commun.* 7:13365. doi: 10.1038/ncomms13365

Goldfarb, M., Schoorlemmer, J., Williams, A., Diwakar, S., Wang, Q., Huang, X., et al. (2007). Fibroblast growth factor homologous factors control neuronal excitability through modulation of voltage-gated sodium channels. *Neuron* 55, 449–463. doi: 10.1016/j.neuron.2007.07.006

Guillemot, F., and Zimmer, C. (2011). From cradle to grave: the multiple roles of fibroblast growth factors in neural development. *Neuron* 71, 574–588. doi: 10.1016/j.neuron.2011.08.002

Hines, R. M., Hines, D. J., Houston, C. M., Mukherjee, J., Haydon, P. G., Tretter, V., et al. (2013). Disrupting the clustering of GABAA receptor α2 subunits in the frontal cortex leads to reduced γ-power and cognitive deficits. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16628–16633. doi: 10.1073/pnas.1308706110

Hines, R. M., Maric, H. M., Hines, D. J., Modgil, A., Panzanelli, P., Nakamura, Y., et al. (2018). Developmental seizures and mortality result from reducing GABAA receptor α2-subunit interaction with collybistin. *Nat. Commun.* 9:3130. doi: 10.1038/s41467-018-05481-1

Hu, H., Gan, J., and Jonas, P. (2014). Fast-spiking, parvalbumin+ GABAergic interneurons: from cellular design to microcircuit function. *Science* 345:1255263. doi: 10.1126/science.1255263

Jacob, T. C., Moss, S. J., and Jurd, R. (2008). GABAA receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat. Rev. Neurosci.* 9, 331–343. doi: 10.1038/nrn2370

Kalachikov, S., Evgrafov, O., Ross, B., Winawer, M., Barker-Cummings, C., Boneschi, F. M., et al. (2002). Mutations in *LGI1* cause autosomal-dominant partial epilepsy with auditory features. *Nat. Genet.* 30, 335–341. doi: 10.1038/ng832

Kalscheuer, V. M., Musante, L., Fang, C., Hoffmann, K., Fuchs, C., Carta, E., et al. (2009). A balanced chromosomal translocation disrupting ARHGEF9 is associated with epilepsy, anxiety, aggression, and mental retardation. *Hum. Mutat.* 30, 61–68. doi: 10.1002/humu.20814

Katona, I., Sperlágh, B., Sík, A., Käfalvi, A., Vizi, E. S., Mackie, K., et al. (1999). Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J. Neurosci. Off. J. Soc. Neurosci.* 19, 4544–4558. doi: 10.1523/jneurosci.19-11-04544.1999

Kegel, L., Aunin, E., Meijer, D., and Bermingham, J. R. (2013). LGI proteins in the nervous system. *ASN Neuro* 5:AN20120095. doi: 10.1042/AN20120095

Kinton, L., Johnson, M. R., Smith, S. J. M., Farrell, F., Stevens, J., Rance, J. B., et al. (2002). Partial epilepsy with pericentral spikes: a new familial epilepsy

syndrome with evidence for linkage to chromosome 4p15. *Ann. Neurol.* 51, 740–749. doi: 10.1002/ana.10221

Klausberger, T., and Somogyi, P. (2008). Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science* 321, 53–57. doi: 10.1126/science.1149381

Knuesel, I., Mastrocola, M., Zuellig, R. A., Bornhauser, B., Schaub, M. C., and Fritschy, J. M. (1999). Short communication: altered synaptic clustering of GABAA receptors in mice lacking dystrophin (mdx mice). *Eur. J. Neurosci.* 11, 4457–4462. doi: 10.1046/j.1460-9568.1999.00887.x

Kubota, Y., Karube, F., Nomura, M., and Kawaguchi, Y. (2016). The diversity of cortical inhibitory synapses. *Front. Neural Circ.* 10:27. doi: 10.3389/fncir.2016.00027

Lee, S.-H., and Soltesz, I. (2011). Requirement for CB1 but not GABAB receptors in the cholecystokinin mediated inhibition of GABA release from cholecystokinin expressing basket cells. *J. Physiol.* 589, 891–902. doi: 10.1113/jphysiol.2010.198499

Lévi, S., Grady, R. M., Henry, M. D., Campbell, K. P., Sanes, J. R., and Craig, A. M. (2002). Dystroglycan is selectively associated with inhibitory GABAergic synapses but is dispensable for their differentiation. *J. Neurosci.* 22, 4274–4285. doi: 10.1523/JNEUROSCI.22-11-04274.2002

Lewis, D. A., Cho, R. Y., Carter, C. S., Eklund, K., Forster, S., Kelly, M. A., et al. (2008). Subunit-selective modulation of GABA type a receptor neurotransmission and cognition in schizophrenia. *Am. J. Psychiatry* 165, 1585–1593. doi: 10.1176/appi.ajp.2008.08030395

Lewis, D. A., Curley, A. A., Glausier, J. R., and Volk, D. W. (2012). Cortical parvalbumin interneurons and cognitive dysfunction in schizophrenia. *Trends Neurosci.* 35, 57–67. doi: 10.1016/j.tins.2011.10.004

Li, X. G., Somogyi, P., Tepper, J. M., and Buzsáki, G. (1992). Axonal and dendritic arborization of an intracellularly labeled chandelier cell in the CA1 region of rat hippocampus. *Exp. Brain Res.* 90, 519–525.

Limviphuvadh, V., Chua, L. L., Rahim, R. A. A. B., Eisenhaber, F., Maurer-Stroh, S., and Adhikari, S. (2010). Similarity of molecular phenotype between known epilepsy gene LGI1 and disease candidate gene LGI2. *BMC Biochem.* 11:39. doi: 10.1186/1471-2091-11-39

Lorente, and De Nó, R. (1934). Studies on the structure of the cerebral cortex. II. Continuation of the study of the ammonic system. *J. Für Psychol. Neurol.* 46, 113–177.

Malmgren, H., Sundvall, M., Dahl, N., Gustavson, K. H., Annerén, G., Wadelius, C., et al. (1993). Linkage mapping of a severe X-linked mental retardation syndrome. *Am. J. Hum. Genet.* 52, 1046–1052.

McNally, E. M., and Pytel, P. (2007). Muscle diseases: the muscular dystrophies. *Annu. Rev. Pathol. Mech. Dis.* 2, 87–109. doi: 10.1146/annurev.pathol.2.010506.091936

Miles, R., Tóth, K., Gulyás, A. I., Hájos, N., and Freund, T. F. (1996). Differences between somatic and dendritic inhibition in the hippocampus. *Neuron* 16, 815–823. doi: 10.1016/s0896-6273(00)80101-4

Moore, S. A., Saito, F., Chen, J., Michele, D. E., Henry, M. D., Messing, A., et al. (2002). Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. *Nature* 418, 422–425. doi: 10.1038/nature00838

Morales, M., and Bloom, F. E. (1997). The 5-HT3 receptor is present in different subpopulations of GABAergic neurons in the rat telencephalon. *J. Neurosci. Off. J. Soc. Neurosci.* 17, 3157–3167. doi: 10.1523/jneurosci.17-09-03157.1997

Nagode, D. A., Tang, A.-H., Yang, K., and Alger, B. E. (2014). Optogenetic identification of an intrinsic cholinergically driven inhibitory oscillator sensitive to cannabinoids and opioids in hippocampal CA1. *J. Physiol.* 592, 103–123. doi: 10.1113/jphysiol.2013.257428

Nusser, Z., Sieghart, W., Benke, D., Fritschy, J. M., and Somogyi, P. (1996). Differential synaptic localization of two major gamma-aminobutyric acid type A receptor alpha subunits on hippocampal pyramidal cells. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11939–11944. doi: 10.1073/pnas.93.21.11939

Nyíri, G., Freund, T. F., and Somogyi, P. (2001). Input-dependent synaptic targeting of alpha(2)-subunit-containing GABA(A) receptors in synapses of hippocampal pyramidal cells of the rat. *Eur. J. Neurosci.* 13, 428–442. doi: 10.1046/j.1460-9568.2001.01407.x

Pablo, J. L., Wang, C., Presby, M. M., and Pitt, G. S. (2016). Polarized localization of voltage-gated Na+ channels is regulated by concerted FGF13 and FGF14 action. *Proc. Natl. Acad. Sci. U.S.A.* 113, E2665–E2674. doi: 10.1073/pnas.1521194113

Panzanelli, P., Gunn, B. G., Schlatter, M. C., Benke, D., Tyagarajan, S. K., Scheiffele, P., et al. (2011). Distinct mechanisms regulate GABAA receptor and gephyrin clustering at perisomatic and axo-axonic synapses on CA1 pyramidal cells. *J. Physiol.* 589, 4959–4980. doi: 10.1113/jphysiol.2011.216028

Papadopoulos, T., Korte, M., Eulenburg, V., Kubota, H., Retiounskaia, M., Harvey, R. J., et al. (2007). Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. *EMBO J.* 26, 3888–3899. doi: 10.1038/sj.emboj.7601819

Parra, P., Gulyás, A. I., and Miles, R. (1998). How many subtypes of inhibitory cells in the hippocampus? *Neuron* 20, 983–993. doi: 10.1016/s0896-6273(00)80479-1

Petilla Interneuron Nomenclature Group, Ascoli, G. A., Alonso-Nanclares, L., Anderson, S. A., Barrionuevo, G., Benavides-Piccione, R., et al. (2008). Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat. Rev. Neurosci.* 9, 557–568. doi: 10.1038/nrn2402

Puranam, R. S., He, X. P., Yao, L., Le, T., Jang, W., Rehder, C. W., et al. (2015). Disruption of Fgf13 causes synaptic excitatory-inhibitory imbalance and genetic epilepsy and febrile seizures plus. *J. Neurosci.* 35, 8866–8881. doi: 10.1523/JNEUROSCI.3470-14.2015

Rubenstein, J. L. R., and Merzenich, M. M. (2003). Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav.* 2, 255–267. doi: 10.1034/j.1601-183X.2003.00037.x

Saiepour, L., Fuchs, C., Patrizi, A., Sassoè-Pognetto, M., Harvey, R. J., and Harvey, K. (2010). Complex role of collybistin and gephyrin in GABAA receptor clustering. *J. Biol. Chem.* 285, 29623–29631. doi: 10.1074/jbc.M110.121368

Senechal, K. R., Thaller, C., and Noebels, J. L. (2005). ADPEAF mutations reduce levels of secreted LGI1, a putative tumor suppressor protein linked to epilepsy. *Hum. Mol. Genet.* 14, 1613–1620. doi: 10.1093/hmg/ddi169

Seppälä, E. H., Jokinen, T. S., Fukata, M., Fukata, Y., Webster, M. T., Karlsson, E. K., et al. (2011). LGI2 truncation causes a remitting focal epilepsy in dogs. *PLoS Genet.* 7:e1002194. doi: 10.1371/journal.pgen.1002194

Shimojima, K., Sugawara, M., Shichiji, M., Mukaida, S., Takayama, R., Imai, K., et al. (2011). Loss-of-function mutation of collybistin is responsible for X-linked mental retardation associated with epilepsy. *J. Hum. Genet.* 56, 561–565. doi: 10.1038/jhg.2011.58

Somogyi, P., and Hámori, J. (1976). A quantitative electron microscopic study of the purkinje cell axon initial segment. *Neuroscience* 1, 361–365.

Somogyi, P., Nunzi, M. G., Gorio, A., and Smith, A. D. (1983). A new type of specific interneuron in the monkey hippocampus forming synapses exclusively with the axon initial segments of pyramidal cells. *Brain Res.* 259, 137–142. doi: 10.1016/0006-8993(83)91076-4

Striano, P., and Zara, F. (2017). ARHGEF9 mutations cause a specific recognizable X-linked intellectual disability syndrome. *Neurol. Genet.* 3:e159. doi: 10.1212/NXG.0000000000000159

Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K., and Südhof, T. C. (2001). A stoichiometric complex of neurexins and dystroglycan in brain. *J. Cell Biol.* 154, 435–446. doi: 10.1083/jcb.200105003

Tasic, B., Menon, V., Nguyen, T. N., Kim, T. K., Jarsky, T., Yao, Z., et al. (2016). Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nat. Neurosci.* 19, 335–346. doi: 10.1038/nn.4216

Telley, L., Cadilhac, C., Cioni, J.-M., Saywell, V., Jahannault-Talignani, C., Huettl, R. E., et al. (2016). Dual function of NRP1 in axon guidance and subcellular target recognition in cerebellum. *Neuron* 91, 1276–1291. doi: 10.1016/j.neuron.2016.08.015

The International League Against Epilepsy Consortium on Complex Epilepsies (2018). Genome-wide mega-analysis identifies 16 loci and highlights diverse biological mechanisms in the common epilepsies. *Nat. Commun.* 9:5269. doi: 10.1038/s41467-018-07524-z

Tremblay, R., Lee, S., and Rudy, B. (2016). GABAergic interneurons in the neocortex: from cellular properties to circuits. *Neuron* 91, 260–292. doi: 10.1016/j.neuron.2016.06.033

Vaillend, C., Perronnet, C., Ros, C., Gruszczynski, C., Goyenvalle, A., Laroche, S., et al. (2010). Rescue of a dystrophin-like protein by exon skipping in vivo restores GABAA-receptor clustering in the hippocampus of the mdx mouse. *Mol. Ther. J. Am. Soc. Gene Ther.* 18, 1683–1688. doi: 10.1038/mt.2010.134

Wallace, R. H., Marini, C., Petrou, S., Harkin, L. A., Bowser, D. N., Panchal, R. G., et al. (2001). Mutant GABA(A) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. *Nat. Genet.* 28, 49–52. doi: 10.1038/88259

Wang, J.-Y., Zhou, P., Wang, J., Tang, B., Su, T., Liu, X.-R., et al. (2018). ARHGEF9 mutations in epileptic encephalopathy/intellectual disability: toward understanding the mechanism underlying phenotypic variation. *Neurogenetics* 19, 9–16. doi: 10.1007/s10048-017-0528-2

Weber, E. T., and Andrade, R. (2010). Htr2a gene and 5-HT2A receptor expression in the cerebral cortex studied using genetically modified mice. *Front. Neurosci.* 4:36. doi: 10.3389/fnins.2010.00036

Whissell, P. D., Cajanding, J. D., Fogel, N., and Kim, J. C. (2015). Comparative density of CCK- and PV-GABA cells within the cortex and hippocampus. *Front. Neuroanat.* 9:124. doi: 10.3389/fnana.2015.00124

Wu, Q.-F., Yang, L., Li, S., Wang, Q., Yuan, X.-B., Gao, X., et al. (2012). Fibroblast growth factor 13 is a microtubule-stabilizing protein regulating neuronal polarization and migration. *Cell* 149, 1549–1564. doi: 10.1016/j.cell.2012.04.046

Xiao, M., Bosch, M. K., Nerbonne, J. M., and Ornitz, D. M. (2013). FGF14 localization and organization of the axon initial segment. *Mol. Cell. Neurosci.* 56, 393–403. doi: 10.1016/j.mcn.2013.07.008

Zonta, B., Desmazieres, A., Rinaldi, A., Tait, S., Sherman, D. L., Nolan, M. F., et al. (2011). A critical role for neurofascin in regulating action potential initiation through maintenance of the axon initial segment. *Neuron* 69, 945–956. doi: 10.1016/j.neuron.2011.02.021

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 Molecular Neuroscience

MINI REVIEW
published: 26 June 2019
doi: 10.3389/fnmol.2019.00162

Targeting $GABA_AR$-Associated Proteins: New Modulators, Labels and Concepts

Vladimir Khayenko [1,2] *and Hans Michael Maric* [1,2]*

[1]*Institute of Structural Biology, Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg, Würzburg, Germany,* [2]*Department of Biotechnology and Biophysics, Biocenter, University of Würzburg, Würzburg, Germany*

γ-aminobutyric acid type A receptors ($GABA_ARs$) are the major mediators of synaptic inhibition in the brain. Aberrant $GABA_AR$ activity or regulation is observed in various neurodevelopmental disorders, neurodegenerative diseases and mental illnesses, including epilepsy, Alzheimer's and schizophrenia. Benzodiazepines, anesthetics and other pharmaceutics targeting these receptors find broad clinical use, but their inherent lack of receptor subtype specificity causes unavoidable side effects, raising a need for new or adjuvant medications. In this review article, we introduce a new strategy to modulate GABAeric signaling: targeting the intracellular protein interactors of $GABA_ARs$. Of special interest are scaffolding, anchoring and supporting proteins that display high $GABA_AR$ subtype specificity. Recent efforts to target gephyrin, the major intracellular integrator of GABAergic signaling, confirm that $GABA_AR$-associated proteins can be successfully targeted through diverse molecules, including recombinant proteins, intrabodies, peptide-based probes and small molecules. Small-molecule artemisinins and peptides derived from endogenous interactors, that specifically target the universal receptor binding site of gephyrin, acutely affect synaptic $GABA_AR$ numbers and clustering, modifying neuronal transmission. Interference with $GABA_AR$ trafficking provides another way to modulate inhibitory signaling. Peptides blocking the binding site of $GABA_AR$ to AP2 increase the surface concentration of $GABA_AR$ clusters and enhance GABAergic signaling. Engineering of gephyrin binding peptides delivered superior means to interrogate neuronal structure and function. Fluorescent peptides, designed from gephyrin binders, enable live neuronal staining and visualization of gephyrin in the post synaptic sites with submicron resolution. We anticipate that in the future, novel fluorescent probes, with improved size and binding efficiency, may find wide application in super resolution microscopy studies, enlightening the nanoscale architecture of the inhibitory synapse. Broader studies on $GABA_AR$ accessory proteins and the identification of the exact molecular binding interfaces and affinities will advance the development of novel $GABA_AR$ modulators and following *in vivo* studies will reveal their clinical potential as adjuvant or stand-alone drugs.

Keywords: $GABA_A$ receptors, gephyrin, collybistin, protein-protein interaction (PPI), super resolution microscopy, fluorescent probes, dimeric peptide, peptide inhibitor design

OPEN ACCESS

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

Reviewed by:
Christian G. Specht,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Josef Kittler,
University College London, United Kingdom

***Correspondence:**
Hans Michael Maric
hans.maric@uni-wuerzburg.de

Received: *31 March 2019*
Accepted: *12 June 2019*
Published: *26 June 2019*

Citation:
Khayenko V and Maric HM (2019) Targeting $GABA_AR$-Associated Proteins: New Modulators, Labels and Concepts. Front. Mol. Neurosci. 12:162. doi: 10.3389/fnmol.2019.00162

INTRODUCTION

γ-aminobutyric acid type A receptors ($GABA_ARs$) are the principal mediators of phasic and tonic inhibition in the human brain, being a vital part of the molecular machinery that creates cognition, behavior, and consciousness (Fritschy and Panzanelli, 2014). Dysfunctional $GABA_ARs$ are involved in the pathogenesis of neurodevelopmental disorders (Ali Rodriguez et al., 2018), schizophrenia (de Jonge et al., 2017), postpartum depression (Mody, 2019), epilepsy (Palma et al., 2017; Hines et al., 2018), Alzheimer's disease (Govindpani et al., 2017), autism (Vien et al., 2015) and stroke (Darmani et al., 2016; Wang et al., 2018). Structurally, these receptors belong to the pentameric ligand-gated ion channels harboring an extracellular domain (ECD), followed by four helical transmembrane domains (TMDs) and loops connecting these helices. $GABA_ARs$ display a highly subtype-specific cellular and sub-cellular distribution and exhibit distinct physiological properties, making them very attractive pharmaceutical targets.

First $GABA_AR$ targeting compounds have been discovered more than a century ago. In 1904, Bayer marketed barbital, the first barbiturate and positive allosteric modulator of $GABA_ARs$ (Löscher and Rogawski, 2012). In the 1960s, benzodiazepines, a new class of $GABA_AR$ allosteric modulators (Sancar and Czajkowski, 2011), became commercially available. Today, modulators of $GABA_AR$ activity find broad clinical use as anesthetics (Propofol; Olsen, 2018), anticonvulsants (Gabapentin) or as hypnotics, muscle-relaxants and anxiolytics (Clonazepam, Diazepam), and new experimental medicines are developed. Nonetheless, wider application of these classical $GABA_AR$ modulators is limited by their lack of receptor subtype specificity, due to the fundamental structural and functional constraints: pharmacologically exploited sites are small hydrophobic pockets with high subunit sequence homology located at the folded ECDs and TMDs of the ion channels (**Figure 1**; Miller et al., 2017; Kasaragod and Schindelin, 2018; Masiulis et al., 2019). Additionally, binding sites on the interface between two subunits, such as the benzodiazepine binding site, are shared among different synaptic receptor subtypes. Consequently, the action of classical clinically relevant $GABA_AR$ ligands can be unspecific and provoke unavoidable side effects.

Molecules modulating receptor signaling through accessory proteins in the central nervous system (CNS; **Figure 1**) emerged as a new class of pharmaceuticals with superior receptor specificity and potential to treat epilepsy, neuropathic pain, fibromyalgia, migraines, and other diseases (Maher et al., 2017). Therefore, targeting $GABA_AR$-associated proteins might be a superior pharmacological strategy compared to the classical approaches. This rational approach, however, requires detailed knowledge and advanced understanding of the intracellular signaling of distinct $GABA_AR$ subtypes. The large number of post-synaptic candidate proteins that directly or indirectly associate with $GABA_ARs$ is still increasing (Krueger-Burg et al., 2017), with functional studies exploring some of their physiological roles and organization (Uezu et al., 2016; Lu et al., 2017), yet, the specific molecular details of these interactions remain largely unknown. We hypothesize that the identification of the exact molecular binding interfaces and binding affinities of known and newly identified $GABA_AR$ associated proteins will not only greatly expand our basic understanding of CNS function, but also provide new pharmaceutical opportunities.

ADJUSTING GABAERGIC SIGNALING THROUGH INTRACELLULAR MODULATION

The majority of $GABA_ARs$ assemble as heteropentamers to form GABA-gated chloride channels. Different subunit combinations possess unique pharmacology (Olsen and Sieghart, 2009), divergent brain region distribution (Wisden and Seeburg, 1992), cell-type specific expression (Lee and Maguire, 2014), and varying subcellular localization between synaptic and extra-synaptic sites (Mody and Pearce, 2004). Thus, subtype-specific modulators of $GABA_AR$ signaling should affect distinct circuits, brain regions or subcellular populations with improved accuracy and more selective pharmacology. Combined structural and functional studies have revealed the molecular details of the interplay of the ECD and TMDs in channel gating (Miller and Aricescu, 2014; Lu et al., 2017; Kasaragod and Schindelin, 2018; Zhu et al., 2018; Laverty et al., 2019). Structural studies of the receptors could, so far, not resolve most of the presumably intrinsically disordered intracellular regions of $GABA_ARs$. Short intracellular receptor regions, however, do adopt defined conformations when engaged with structured intracellular interactors, such as gephyrin (Maric et al., 2014) and the AP2 complex (Kittler et al., 2008; **Table 1**). Functional studies validated that distinct motifs within these unstructured regions exert tight control over channel biosynthesis, recycling, diffusion and synaptic recruitment (Tretter et al., 2012; Nakamura et al., 2015; Groeneweg et al., 2018; Lorenz-Guertin and Jacob, 2018). Remarkably, these intracellular regions display the highest level of sequence heterogeneity among receptor subunits, thereby enabling subtype-specific modulation of GABAergic signaling. Agents targeting these discrete regions will probably be highly selective and could affect $GABA_AR$ subtypes with distinct functional and pharmacological properties. It is noteworthy that, so far, all intracellular $GABA_AR$ interactions that displayed sufficient affinity and specificity ended up being exploited to modulate neuronal communication (**Table 1**).

AFFECTING POSTSYNAPTIC $GABA_AR$ ACCUMULATION BY TARGETING INTRACELLULAR SCAFFOLDS

The concept of neurotransmission modulation through targeting receptor-scaffolding protein interactions originated from studies investigating PSD-95/Discs-large/ZO-1 (PDZ) domain carrying proteins. These showed that through modulation of receptor-scaffolding protein interactions a variety of responses could be achieved, ranging from disruption of glutamate signaling to

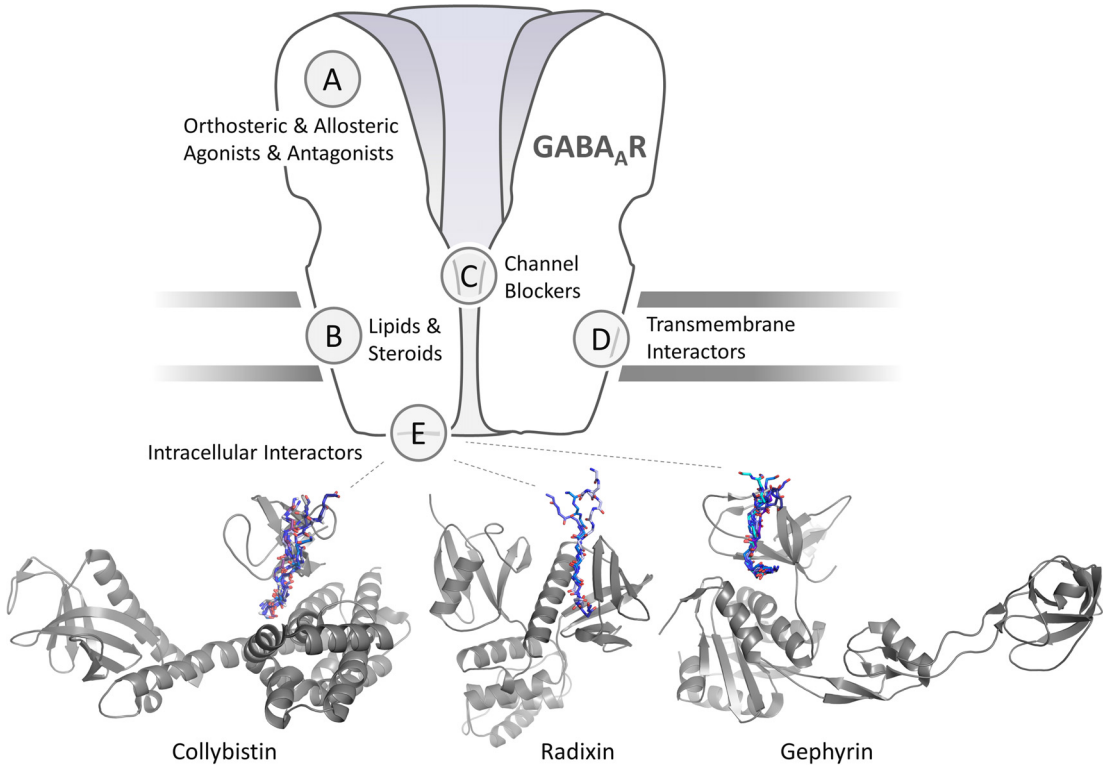

FIGURE 1 | Schematic representation of a γ-aminobutyric acid type A receptors ($GABA_ARs$) and sites for pharmaceutical intervention. Orthosteric and allosteric agonists and antagonist are classical activity modulators that bind directly to the extracellular domain (ECD) or, in case of lipids and neurosteroids to the transmembrane domain (TMD) of $GABA_ARs$. Each of the sites could occur in five subunits or at five interfaces, or only in distinct subunits and specific interfaces. Channel blockers bind within the ion pore formed by the $GABA_AR$ pentamer. Intracellular interactors such as Collybistin (CB; Hines et al., 2018) and gephyrin (Maric et al., 2014) interact with distinct intracellular regions of a subset of $GABA_AR$ subunits. Transmembrane interactors such as LHFPL4 interact with the TMDs of γ2 subunit containing $GABA_ARs$ (Davenport et al., 2017; Yamasaki et al., 2017). Cartoon representation of structurally characterized and predicted scaffold-$GABA_AR$ complexes. CB is shown in gray in its extended conformation (PDB-ID 4mt7) with its SH3 domain (PDB-ID 4mt6; Soykan et al., 2014) binding to a fragment of the $GABA_AR$ α2 subunit (Hines et al., 2018). Peptide backbones of resolved SH3 domain ligands (PDB-IDs 2df6, 4hvu, 4hvv, 4hvw, 4j9f, 4ln2 and 4rtz) are superimposed to indicate the putative $GABA_AR$ α2 binding site. The radixin FERM domain is shown in gray. Peptide backbones of resolved radixin FERM domain ligands (PDB-IDs 1j19, 2ems, 2d2q) are superimposed to indicate the putative $GABA_AR$ α5 binding site. Cartoon representation of the gephyrin E domain in complex with short linear $GABA_AR$ (PDB-IDs 4tk1, 4tk2, 4tk3, 4tk4) and GlyR derived peptides (PDB-IDs 2fts, 4u90, 4u91).

neuroprotective effects in ischemic brain damage (Hammond et al., 2006; Sainlos et al., 2011; Bach et al., 2012; **Figure 2A**). These results suggested that modulation of the inhibitory neurotransmission could be accomplished in a similar way, a concept recently proved with the inhibitory scaffold protein gephyrin (Maric et al., 2017).

Scaffolding proteins, such as gephyrin (Specht et al., 2013), radixin (Loebrich et al., 2006; Hausrat et al., 2015) and (collybistin, CB; Mayer et al., 2013; Hines et al., 2018), dynamically regulate the cell membrane distribution of postsynaptic and extrasynaptic $GABA_ARs$. Interestingly, their scaffolding functions are highly receptor specific, potentially allowing a fine tuning of neurotransmission.

Radixin

Radixin is involved in the anchoring of numerous membrane proteins to the actin cytoskeleton (Kawaguchi et al., 2017). Its C-terminal domain mediates actin binding, while the N-terminal FERM domain functions as a universal protein-binding module that directly interacts with receptors, extracellular matrix components, transmembrane and adhesion proteins (Kitano et al., 2006; Takai et al., 2007; Terawaki et al., 2007, 2008; Yogesha et al., 2011; **Figure 1**). Radixin also harbors a central α-helical domain, which either adopts a closed or elongated conformation to allow its auto-inhibitory module to mask the FERM domain. In neurons, radixin is activated through phosphorylation, which enables its simultaneous binding to cytoskeletal elements and transmembrane proteins, including α5 subunit containing $GABA_ARs$ (Loebrich et al., 2006; Hausrat et al., 2015). In primary hippocampal neuronal cultures, the association of radixin with α5-containing $GABA_ARs$ at extrasynaptic sites decreases upon maturation, in contrast, the number of α5-containing $GABA_ARs$, that associate with gephyrin at post-synaptic sites remains constant (Brady and Jacob, 2015). Structural and thermodynamic details of the radixin-$GABA_AR$ α5 complex will reveal whether modulation can be achieved without simultaneously affecting the binding of other ligands.

TABLE 1 | List of all known intracellular $GABA_AR$-associated proteins that display micromolar or better binding affinity.

$GABA_AR$ Interactor	$GABA_AR$ Subunit	Mapped Binding Site	Affinity [mM]	PDB ID	Physiological function	Inhibition or Interference
Gephyrin	α1	LIKKNNTYAPTATSYT[1]	17[7]	-	Clustering of distinct subsets of $GABA_ARs$ at post-synaptic sites.[1,2,3,7]	Redistribution of post-synaptic GABAARs towards extra-synaptic sites. Decreased amplitude and frequency of phasic inhibitory currents.
	α3	FNIVGTTYPIN[2]	5[3,7]	4TK1[8] 4U90[9]		
	β2	AGLPRHSFGRNALERHVAQKKSRL[3]	17[3]	-		
AP-2	β3	KTHLRRRSS[4]	1[4]	-	Surface stabilization; increased receptor numbers, enhanced inhibitory post-synaptic currents[5]	Surface stabilization; increased receptor numbers, enhanced inhibitory post-synaptic currents[5]
	γ2	YECL[5]	0.4[5]	2PR9[5]		
Collybistin	α2	VMIQNNAYAVAVANYAPNL[6]	1[6]	-	Clustering of α2 subunit containing $GABA_ARs$ at post-synaptic sites. Pronounced importance for receptors at the axon initial segment.[6]	Reduced $GABA_AR$ α2 cluster size and loss of $GABA_AR$ α2 subunit containing receptors. Reduced inhibitory post synaptic current amplitudes and decay times. Anxiety and seizure susceptibility.[6]

Interacting $GABA_AR$ subunits, mapped binding sites, determined affinities and PDB-IDs of structures are listed as well as the reported function and the consequences of an acute inhibition or genetic interference. [1](Mukherjee et al., 2011); [2](Tretter et al., 2011); [3](Kowalczyk et al., 2013); [4](Kittler et al., 2005); [5](Kittler et al., 2008); [6](Hines et al., 2018); [7](Maric et al., 2011); [8](Maric et al., 2014); [9](Maric et al., 2015).

Collybistin

Collybistin (CB) is a guanine nucleotide exchange factor for Cdc42, a gephyrin binding partner (Kins et al., 2000) and an important determinant of inhibitory postsynaptic membrane formation and plasticity (Harvey et al., 2004; Tyagarajan et al., 2011a). Robust evidence supports the role of CB in $GABA_AR$ scaffolding with yeast three-hybrid studies (Saiepour et al., 2010) and proteomic studies (Nakamura et al., 2016) suggesting the presence of a tripartite complex between gephyrin, CB and α2 subunit containing $GABA_ARs$. Recently, a thermodynamic analysis revealed that CB binds $GABA_AR$ α2-subunits with high selectivity and affinity (Hines et al., 2018; **Figure 1** and **Table 1**). CB is targeted to the neuronal surface membrane through phosphoinositides (Reddy-Alla et al., 2010; Ludolphs et al., 2016) and interfering human mutations result in cognitive deficits (Long et al., 2016; Chiou et al., 2019). Deficiency in CB reduces gephyrin and $GABA_AR$ clustering and impairs spatial learning (Papadopoulos et al., 2007, 2008). Moreover, mice with a mutation in the α2-subunit binding region of CB display a loss of a distinct subset of inhibitory synapses and a decreased amplitude of inhibitory synaptic currents, which results in a phenotype with increased susceptibility to seizures and early mortality (Hines et al., 2018). Notably, treatment with the α2/α3-selective positive modulator AZD7325 improves the conditions of affected mice, suggesting that compounds targeting the CB-$GABA_AR$ α2 complex could provide an alternative route to specifically affect $GABA_ARs$ containing the α2 subunit.

Neuroligin 2

Proteomic studies (Kang et al., 2014; Nakamura et al., 2016) revealed that the synapse-specific adhesion molecule neuroligin 2 (NL2; Varoqueaux et al., 2004) strongly associates with a subset of $GABA_AR$ subtypes and $GABA_AR$ scaffolds. Neuroligin dysfunction has been implicated in autism (Pettem et al., 2013) and specific intracellular residues in NL1 (Nguyen et al., 2016; Letellier et al., 2018) and NL2 (Poulopoulos et al., 2009; Kang et al., 2014) are critical for proper GABAergic signaling. Yet, the exact molecular interfaces, that mediate the direct or indirect gephyrin or CB dependent (Soykan et al., 2014) interactions of neuroligin with $GABA_AR$, remain uncharacterized. These molecular insights could greatly contribute to our understanding of the development of the inhibitory synapse, as well as the underlying molecular causes of developmental diseases. Neuroligin family members exert distinct roles in the formation and stabilization of inhibitory and excitatory synapses and display distinct cellular and subcellular distributions. Accordingly, molecules that interfere with their isoform-specific interactions could act as highly cell-type selective modulators of neurotransmission.

Gephyrin

Gephyrin is a prime candidate for the role of master regulator of neuronal function at inhibitory sites (Tyagarajan and Fritschy, 2014) and specifically the GABAergic synapses (Choii and Ko, 2015). Initially identified as a glycine receptor binding (Pfeiffer et al., 1982) and scaffolding protein (Feng et al., 1998), gephyrin was later found to be responsible for the

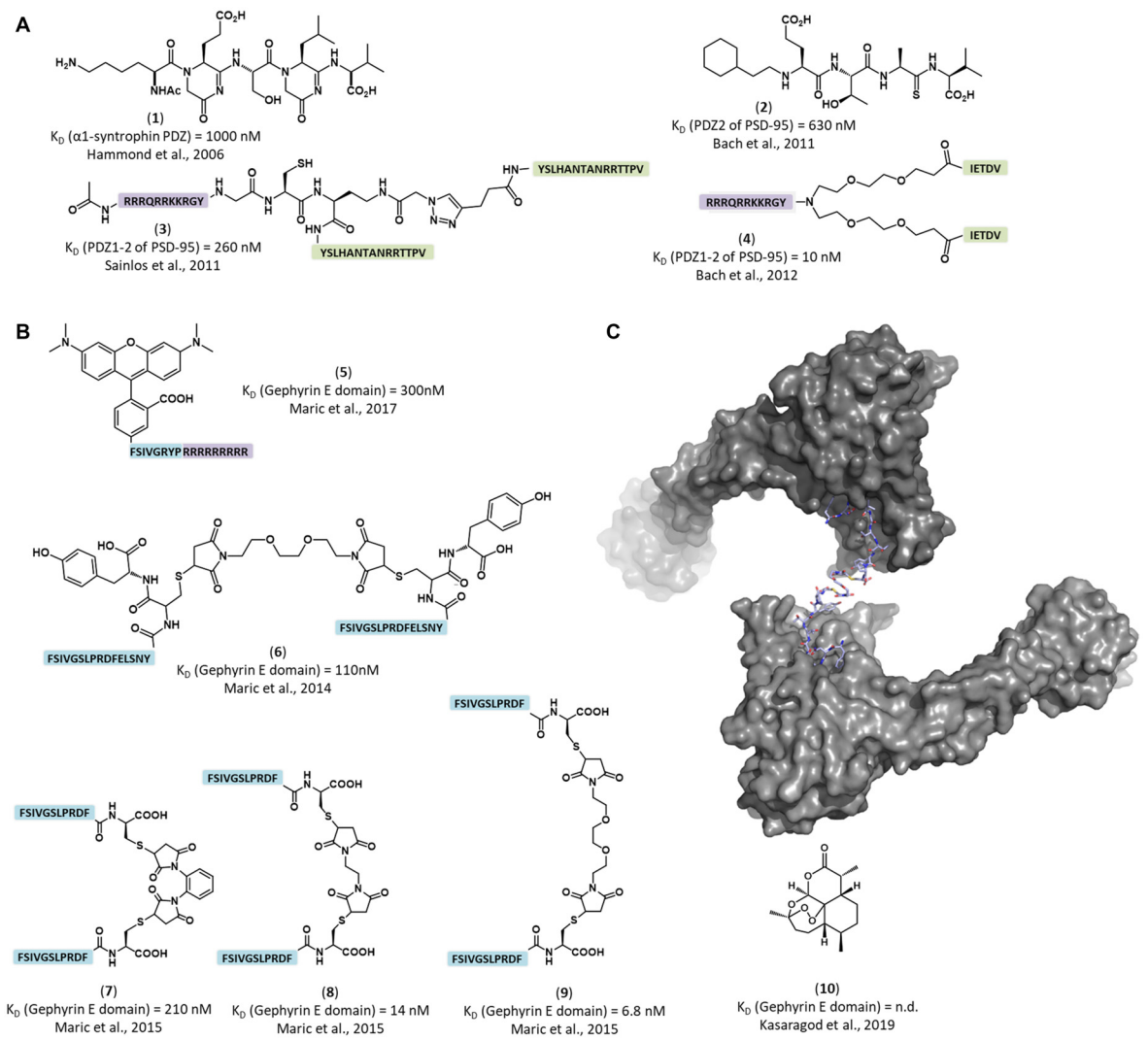

FIGURE 2 | Structure of representative inhibitors and labels of the inhibitory post-synaptic scaffold gephyrin and the excitatory post-synaptic scaffold PSD-95. **(A)** Peptidomimetic and dimeric PSD-95/Discs-large/ZO-1 (PDZ) domain binders. PSD-95 binding peptides (green) were dimerized and combined with cell-penetrating moieties (violet). **(1)** Peptidomimetic ligand for a α-1 syntrophin PDZ domain (Hammond et al., 2006). **(2)** Peptidomimetic inhibitor of the PSD-95 PDZ2 domain (Bach et al., 2011) **(3)** Dimeric inhibitor of PSD-95 PDZ domains, showed strong inhibition of AMPA receptor synaptic currents (Sainlos et al., 2011). **(4)** Dimeric inhibitor of PSD-95 PDZ domains, showed neuroprotective properties in mice with cerebral ischemia (Bach et al., 2012). **(B)** Engineered peptide-based gephyrin inhibitors and fluorescent labels. **(5)** Peptide-based fluorescent gephyrin label (Maric et al., 2017). Tamra dye was directly conjugated to gephyrin binding sequence (blue) combined with cell penetrating peptide (in purple). **(6–9)** Nanomolar affinity dimerized gephyrin peptide binders (blue; Maric et al., 2015). **(10)** The small molecule inhibitor artemisinin competes with the universal engineered peptide-based molecules for receptor binding pocket (Kasaragod et al., 2019). **(C)** Representative crystal structure of a peptide dimer engaging with a gephyrin E domain dimer (PDB-ID 4U90; Maric et al., 2015).

post-synaptic accumulation of $GABA_ARs$. The loss of gephyrin clusters following the loss of the $GABA_AR$ γ2 subunit (Essrich et al., 1998) and the loss of $GABA_AR$ clusters upon gephyrin deficiency (Kneussel et al., 1999) substantiates their critical mutual dependency. Direct binding of gephyrin to α and β $GABA_AR$ subunits could be confirmed (Tretter et al., 2008, 2011; Maric et al., 2011; Mukherjee et al., 2011; Kowalczyk et al., 2013; Brady and Jacob, 2015), but the exact binding interfaces and affinities are still largely undefined. Structural, thermodynamic and high-end microscopic approaches elucidated the complex structure-function relationships between gephyrin and a distinct subgroup of inhibitory neurotransmitter receptors (Kasaragod and Schindelin, 2018) and indicated an important role of the nanoscale arrangement of gephyrin and the associated receptors at post-synaptic sites (Groeneweg et al., 2018; Specht, 2019). Further functional and *in-silico* studies (Pennacchietti et al., 2017) confirmed that gephyrin organizes the receptors in distinct nanoscale structures, which shape GABAergic synaptic potentiation and reduce current variability. The stability, oligomerization and receptor binding of gephyrin are highly regulated and exert tight control over receptor numbers at post-synaptic sites, affecting synaptic strength (Alvarez, 2017;

Groeneweg et al., 2018). Biophysical (Maric et al., 2011) and structural (Maric et al., 2014, 2015) studies provided first insights into the structure and function of the gephyrin-$GABA_AR$ complexes and demonstrated that at least the $GABA_AR$ α1–3 and GlyR β subunits bind to an overlapping site within gephyrin in a mutually exclusive fashion (Maric et al., 2011, 2014; **Figure 1** and **Table 1**). Microscopy studies substantiated that gephyrin acts as a dynamic post-synaptic platform for both, $GABA_ARs$ and GlyRs (Specht et al., 2013), and that receptor residence times at the postsynapse depend on binding affinities and distinct post-translational modifications (Mukherjee et al., 2011; Specht et al., 2011). The concept of ligand competition for gephyrin binding, therefore, lends an elegant explanation for the comparably high diffusion dynamics of high-affinity gephyrin binding receptors. This phenomenon may be the natural solution to the biological requirement to maintain distinct subsets of receptor subtypes in high density at post-synaptic sites, while at the same time allowing for the rapid exchange of these receptors and scalability through diffusion dynamics (Specht, 2019). This model is also consistent with the observation of reciprocal stabilization of receptors, and the underlying protein scaffold, at inhibitory synapses (Essrich et al., 1998).

Gephyrin itself is dynamically regulated, affecting $GABA_AR$ diffusion and contributing to input-specific adaptations at postsynaptic sites (Chen et al., 2012; Villa et al., 2016; Battaglia et al., 2018). Gephyrin phosphorylation at Ser268 and Ser270, located in the intrinsically disordered central region of the protein, directly affects GABAergic signaling (Tyagarajan et al., 2011b, 2013) and induce gephyrin-mediated remodeling of GABAergic synapses in specific neuronal cell-types (Flores et al., 2015). Despite its major functional relevance only a few of the molecular interfaces that engage with the central region of gephyrin could be identified (Groeneweg et al., 2018). The underlying molecular mechanisms for these gephyrin phosphorylation-induced $GABA_AR$ synapse dynamics remain to be explored in a comprehensive approach that includes an extensive alternative splicing and complex post-translational modification patterns of this region. Identification of the targeted binding pockets and insights into the binding affinities of the modified and unmodified peptide regions within the central region of gephyrin could shed light on the enigmatic molecular mechanisms of gephyrin multimerization, degradation and the tuning of its ligand binding affinities. Additionally, gephyrin isoforms are tissue-specific (Paarmann et al., 2006), therefore, molecules targeting distinct gephyrin splice variants may display pronounced effects in distinct tissues or brain regions.

TARGETING THE GEPHYRIN-$GABA_AR$ COMPLEX

Gephyrin's crucial role in glycinergic and GABAergic transmission made it a major pharmacological target. The modulation of synaptic responses *via* gephyrin was achieved more than a decade ago using intrabodies (Zacchi et al., 2008), and a related approach turned out to be useful for acutely removing inhibitory synapses (Gross et al., 2016). Since then, several studies made an impressive progress in the development of agents affecting the intracellular interplay of $GABA_ARs$. One such example is artemisinins [**Figure 2B(10)**]. Li et al. (2017) found that artemisinins, lactones derived from the Qinghao plant, affect pancreatic cells by binding gephyrin and modifying $GABA_AR$ signaling. Kasaragod et al. (2019) identified the artemisinin binding site within gephyrin and showed that application of artemisinins reduces gephyrin and $GABA_ARs$ clustering, making artemisinins the first small molecule lead compounds for a new class of inhibitory neurotransmission modulators. Strikingly, the druggable artemisinin-binding pocket overlaps with the universal receptor binding region of gephyrin, which is critical for the interaction with $GABA_A$ and glycine receptors (Kasaragod et al., 2019). Thermodynamic and structural studies (Maric et al., 2011, 2014) identified the "hotspot" fragments of $GABA_A$ and glycine receptors that bind to gephyrin. Biomimetic optimization of the "hotspots" amino acid sequence, enhanced the affinity of the resulting peptide ligands 46,000-fold compared to the corresponding native peptides (Maric et al., 2015, 2017; **Figures 2B,C**). Further *in vitro* applications of these new super binder peptide reduced $GABA_AR$ α2 conductivity and clustering, providing evidence that $GABA_AR$-associated proteins can be successfully targeted with modified peptides to modulate fast synaptic inhibition (Maric et al., 2017).

TARGETING NON-SCAFFOLD $GABA_AR$ ASSOCIATED PROTEINS

$GABA_AR$ trafficking is pivotal for the plasticity (Luscher et al., 2011) and the development (Lorenz-Guertin and Jacob, 2018) of inhibitory synapses, consequently, dysfunction of the $GABA_AR$ cycling is involved in various neurological disorders (Smith and Kittler, 2010; Mele et al., 2019). Noteworthy, phosphorylation of the intracellular $GABA_AR$ sites, that are involved in the trafficking of the receptors, has been identified to control receptor numbers and their concentration at synaptic sites (Comenencia-Ortiz et al., 2014; Nakamura et al., 2015), a mechanism that proves to be critical for the physiological function of inhibitory synapses (Vien et al., 2015). Therefore, targeting protein-protein interactions (PPIs) that mediate $GABA_AR$ trafficking, endocytosis, degradation or recycling, is a promising pharmacological strategy. The proposed direct protein interactors are numerous, among them are muskelin (Heisler et al., 2011), GABARAP (Wang et al., 1999), the brefeldin-A inhibited GDP/GTP exchange factor 2 (Charych et al., 2004), phospholipase C-related catalytically inactive proteins 1 and 2 (Mizokami et al., 2007), N-ethylmaleimide sensitive factor (Goto et al., 2005), neurobeachin (Nair et al., 2013), Huntingtin-associated protein 1, calcium-modulating cyclophilin ligand (Kittler et al., 2004; Yuan et al., 2008) and the clathrin adaptor protein AP2 (Kittler et al., 2005).

The AP2-$GABA_AR$ interaction rapidly modulates synaptic $GABA_AR$ numbers, inhibitory synaptic strength, neuronal excitability, and notably, affects animal behavior (Kittler et al., 2000, 2005, 2008; Tretter et al., 2009). The μ2 subunit of the clathrin adaptor protein AP2 binds with high affinity to linear and short peptide motifs within the intracellular

regions of specific $GABA_AR$ subunits (**Table 1**). Short $GABA_AR$ derived peptides, that effectively compete with AP2 binding, were successfully used to block the receptor internalization in hippocampal neurons, increasing surface concentration of $GABA_AR$ clusters by 50% (Smith et al., 2012) and enhancing the strength of inhibitory synapses (Kittler et al., 2008). AP2 antagonists demonstrate that the modulation of $GABA_AR$ interactions with its intracellular trafficking partners is an alternative way to influence GABAergic signaling.

PERSPECTIVES

Ongoing research uncovered original, seemingly contrasting, strategies of GABAergic signaling modulation. On the one hand, ligands disrupting gephyrin-$GABA_AR$ clustering, like artemisinins or "super binding peptides," could reduce the $GABA_AR$ synaptic concentration and function. On the other hand, peptides hampering receptor interaction with AP2 trafficking protein increased the synaptic receptor levels. In theory, these approaches could be applied together to achieve bi-directional modulation of inhibitory neurotransmission, promoting a shift in the dynamic equilibrium from phasic to tonic neuronal response.

Those new strategies of GABAergic neurotransmission modulation possess an untapped clinical potential. Agents targeting $GABA_AR$ associated scaffold or trafficking proteins could be applied wherever abnormal GABAergic activity or regulation is involved in pathogenesis. In status epilepticus patients develop a time-dependent pharmacoresistance to GABAergic agents, probably, due to $GABA_AR$ internalization (Naylor et al., 2005). In benzodiazepine tolerance linked to prolonged benzodiazepine use, neurons continuously exposed to diazepam lose postsynaptic $GABA_ARs$ (Nicholson et al., 2018). Both pathologies are related to the reduction of available postsynaptic $GABA_ARs$ and both could potentially be alleviated by targeting $GABA_AR$-associated proteins. Stabilization of the gephyrin-receptor scaffolds at inhibitory postsynapses with molecules that mimic the stabilizing action of CB (Saiepour et al., 2010) could help prevent $GABA_AR$ loss and preserve inhibitory neurotransmission, alternatively, applying AP2 inhibitors could reduce $GABA_AR$ internalization and reverse the loss of postsynaptic $GABA_ARs$. Those examples illustrate the potential of GABAergic modulators as adjuvants ameliorating the effect of existing potent drugs, whereas in epilepsy or other diseases involving deregulation of inhibitory neurotransmission they could be applied as stand-alone therapeutics.

We expect that the study of $GABA_AR$ intracellular interactors, accelerated by *in-silico* predictions and high throughput approaches, will lead to the discovery of novel GABAergic modulators. Affinity, selectivity, bioavailability and immunogenicity of these compounds would have to be optimized for clinical applications, where peptide-based ligands could be further evolved by the introduction of unnatural amino acids, cyclization and other chemical modifications.

Microscopy is an additional intriguing application of these molecules. The enhanced affinity and specificity of the engineered peptide-based compounds allowed to pioneer their use as fluorescent probes [**Figure 2B(5)**], enabling live neuronal staining and visualization of inhibitory post synaptic sites with submicron resolution (Maric et al., 2017). Compact fluorescent peptides, developed from these super binding peptides, bring several advantages over conventional staining agents, namely the antibodies. In contrast to antibodies, peptide probes are live cell compatible and could provide better resolution and localization precision, since the fluorophore, owing to its small size, stays close to the target surface, reducing the linkage error. Moreover, highly affine and selective peptides could achieve stoichiometric labeling, enabling quantification of the target protein.

Here, we discussed how the targeting of $GABA_AR$ associated proteins could prove to be a versatile pharmacological strategy with clinical potential. Further, we suggested that when combined with state-of-the-art super-resolution microscopy methods, the peptide-based fluorescent probes may resolve the nanoscale architecture of synapses in unprecedented detail. We anticipate that the discovery of additional $GABA_AR$ interactors could open the way for the development of new imaging tools and alternative pharmacological approaches.

AUTHOR CONTRIBUTIONS

VK and HM wrote the manuscript and prepared the figures.

FUNDING

This work was supported by Association of the Chemical Industry (Verband der Chemischen Industrie).

ACKNOWLEDGMENTS

We thank Drs Vikram Babu Kasaragod and Hermann Schindelin for helpful suggestions, discussions and editing.

REFERENCES

Ali Rodriguez, R., Joya, C., and Hines, R. M. (2018). Common ribs of inhibitory synaptic dysfunction in the umbrella of neurodevelopmental disorders. *Front. Mol. Neurosci.* 11:132. doi: 10.3389/fnmol.2018.00132

Alvarez, F. J. (2017). Gephyrin and the regulation of synaptic strength and dynamics at glycinergic inhibitory synapses. *Brain Res. Bull.* 129, 50–65. doi: 10.1016/j.brainresbull.2016.09.003

Bach, A., Clausen, B. H., Moller, M., Vestergaard, B., Chi, C. N., Round, A., et al. (2012). A high-affinity, dimeric inhibitor of PSD-95 bivalently interacts with PDZ1–2 and protects against ischemic brain damage. *Proc. Natl. Acad. Sci. U S A* 109, 3317–3322. doi: 10.1073/pnas.1113761109

Bach, A., Eildal, J. N., Stuhr-Hansen, N., Deeskamp, R., Gottschalk, M., Pedersen, S. W., et al. (2011). Cell-permeable and plasma-stable peptidomimetic inhibitors of the postsynaptic density-95/N-methyl-D-aspartate receptor interaction. *J. Med. Chem.* 54, 1333–1346. doi: 10.1021/jm1013924

Battaglia, S., Renner, M., Russeau, M., Come, E., Tyagarajan, S. K., and Lévi, S. (2018). Activity-dependent inhibitory synapse scaling is determined by gephyrin phosphorylation and subsequent regulation of $GABA_A$ receptor

diffusion. *eNeuro* 5:ENEURO.0203-17.2017. doi: 10.1523/eneuro.0203-17.2017

Brady, M. L., and Jacob, T. C. (2015). Synaptic localization of α5 $GABA_A$ receptors *via* gephyrin interaction regulates dendritic outgrowth and spine maturation. *Dev. Neurobiol.* 75, 1241–1251. doi: 10.1002/dneu.22280

Charych, E. I., Yu, W., Li, R., Serwanski, D. R., Miralles, C. P., Li, X., et al. (2004). A four PDZ domain-containing splice variant form of GRIP1 is localized in GABAergic and glutamatergic synapses in the brain. *J. Biol. Chem.* 279, 38978–38990. doi: 10.1074/jbc.m405786200

Chen, J. L., Villa, K. L., Cha, J. W., So, P. T., Kubota, Y., and Nedivi, E. (2012). Clustered dynamics of inhibitory synapses and dendritic spines in the adult neocortex. *Neuron* 74, 361–373. doi: 10.1016/j.neuron.2012.02.030

Chiou, T.-T., Long, P., Schumann-Gillett, A., Kanamarlapudi, V., Haas, S. A., Harvey, K., et al. (2019). Mutation p.R356Q in the collybistin phosphoinositide binding site is associated with mild intellectual disability. *Front. Mol. Neurosci.* 12:60. doi: 10.3389/fnmol.2019.00060

Choii, G., and Ko, J. (2015). Gephyrin: a central GABAergic synapse organizer. *Exp. Mol. Med.* 47:e158. doi: 10.1038/emm.2015.5

Comenencia-Ortiz, E., Moss, S. J., and Davies, P. A. (2014). Phosphorylation of $GABA_A$ receptors influences receptor trafficking and neurosteroid actions. *Psychopharmacology* 231, 3453–3465. doi: 10.1007/s00213-014-3617-z

Darmani, G., Zipser, C. M., Bohmer, G. M., Deschet, K., Muller-Dahlhaus, F., Belardinelli, P., et al. (2016). Effects of the selective α5-GABAAR antagonist S44819 on excitability in the human brain: a TMS-EMG and TMS-EEG phase I study. *J. Neurosci.* 36, 12312–12320. doi: 10.1523/JNEUROSCI.1689-16.2016

Davenport, E. C., Pendolino, V., Kontou, G., Mcgee, T. P., Sheehan, D. F., López-Doménech, G., et al. (2017). An essential role for the tetraspanin LHFPL4 in the cell-type-specific targeting and clustering of synaptic $GABA_A$ receptors. *Cell Rep.* 21, 70–83. doi: 10.1016/j.celrep.2017.09.025

de Jonge, J. C., Vinkers, C. H., Hulshoff Pol, H. E., and Marsman, A. (2017). GABAergic mechanisms in schizophrenia: linking postmortem and *in vivo* studies. *Front. Psychiatry* 8:118. doi: 10.3389/fpsyt.2017.00118

Essrich, C., Lorez, M., Benson, J. A., Fritschy, J. M., and Luscher, B. (1998). Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ 2 subunit and gephyrin. *Nat. Neurosci.* 1, 563–571. doi: 10.1038/2798

Feng, G., Tintrup, H., Kirsch, J., Nichol, M. C., Kuhse, J., Betz, H., et al. (1998). Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. *Science* 282, 1321–1324. doi: 10.1126/science.282.5392.1321

Flores, C. E., Nikonenko, I., Mendez, P., Fritschy, J. M., Tyagarajan, S. K., and Muller, D. (2015). Activity-dependent inhibitory synapse remodeling through gephyrin phosphorylation. *Proc. Natl. Acad. Sci. U S A* 112, E65–E72. doi: 10.1073/pnas.1411170112

Fritschy, J. M., and Panzanelli, P. (2014). GABA receptors and plasticity of inhibitory neurotransmission in the central nervous system. *Eur. J. Neurosci.* 39, 1845–1865. doi: 10.1111/ejn.12534

Goto, H., Terunuma, M., Kanematsu, T., Misumi, Y., Moss, S. J., and Hirata, M. (2005). Direct interaction of N-ethylmaleimide-sensitive factor with $GABA_A$ receptor β subunits. *Mol. Cell. Neurosci.* 30, 197–206. doi: 10.1016/j.mcn.2005.07.006

Govindpani, K., Calvo-Flores Guzman, B., Vinnakota, C., Waldvogel, H. J., Faull, R. L., and Kwakowsky, A. (2017). Towards a better understanding of GABAergic remodeling in Alzheimer's disease. *Int. J. Mol. Sci.* 18:E1813. doi: 10.3390/ijms18081813

Groeneweg, F. L., Trattnig, C., Kuhse, J., Nawrotzki, R. A., and Kirsch, J. (2018). Gephyrin: a key regulatory protein of inhibitory synapses and beyond. *Histochem. Cell Biol.* 150, 489–508. doi: 10.1007/s00418-018-1725-2

Gross, G. G., Straub, C., Perez-Sanchez, J., Dempsey, W. P., Junge, J. A., Roberts, R. W., et al. (2016). An E3-ligase-based method for ablating inhibitory synapses. *Nat. Methods* 13, 673–678. doi: 10.1038/nmeth.3894

Hammond, M. C., Harris, B. Z., Lim, W. A., and Bartlett, P. A. (2006). β strand peptidomimetics as potent PDZ domain ligands. *Chem. Biol.* 13, 1247–1251. doi: 10.1016/j.chembiol.2006.11.010

Harvey, K., Duguid, I. C., Alldred, M. J., Beatty, S. E., Ward, H., Keep, N. H., et al. (2004). The GDP-GTP exchange factor collybistin: an essential determinant of neuronal gephyrin clustering. *J. Neurosci.* 24, 5816–5826. doi: 10.1523/JNEUROSCI.1184-04.2004

Hausrat, T. J., Muhia, M., Gerrow, K., Thomas, P., Hirdes, W., Tsukita, S., et al. (2015). Radixin regulates synaptic $GABA_A$ receptor density and is essential for reversal learning and short-term memory. *Nat. Commun.* 6:6872. doi: 10.1038/ncomms7872

Heisler, F. F., Loebrich, S., Pechmann, Y., Maier, N., Zivkovic, A. R., Tokito, M., et al. (2011). Muskelin regulates actin filament- and microtubule-based $GABA_A$ receptor transport in neurons. *Neuron* 70, 66–81. doi: 10.1016/j.neuron.2011.03.008

Hines, R. M., Maric, H. M., Hines, D. J., Modgil, A., Panzanelli, P., Nakamura, Y., et al. (2018). Developmental seizures and mortality result from reducing $GABA_A$ receptor α2-subunit interaction with collybistin. *Nat. Commun.* 9:3130. doi: 10.1038/s41467-018-05481-1

Kang, Y., Ge, Y., Cassidy, R. M., Lam, V., Luo, L., Moon, K. M., et al. (2014). A combined transgenic proteomic analysis and regulated trafficking of neuroligin-2. *J. Biol. Chem.* 289, 29350–29364. doi: 10.1074/jbc.m114.549279

Kasaragod, V. B., Hausrat, T. J., Schaefer, N., Kuhn, M., Christensen, N. R., Tessmer, I., et al. (2019). Elucidating the molecular basis for inhibitory neurotransmission regulation by artemisinins. *Neuron* 101, 673.e11–689.e11. doi: 10.1016/j.neuron.2019.01.001

Kasaragod, V. B., and Schindelin, H. (2018). Structure-function relationships of glycine and $GABA_A$ receptors and their interplay with the scaffolding protein gephyrin. *Front. Mol. Neurosci.* 11:317. doi: 10.3389/fnmol.2018.00317

Kawaguchi, K., Yoshida, S., Hatano, R., and Asano, S. (2017). Pathophysiological roles of ezrin/radixin/moesin proteins. *Biol. Pharm. Bull.* 40, 381–390. doi: 10.1248/bpb.b16-01011

Kins, S., Betz, H., and Kirsch, J. (2000). Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. *Nat. Neurosci.* 3, 22–29. doi: 10.1038/71096

Kitano, K., Yusa, F., and Hakoshima, T. (2006). Structure of dimerized radixin FERM domain suggests a novel masking motif in C-terminal residues 295-304. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 62, 340–345. doi: 10.1107/s1744309106010062

Kittler, J. T., Arancibia-Carcamo, I. L., and Moss, S. J. (2004). Association of GRIP1 with a $GABA_A$ receptor associated protein suggests a role for GRIP1 at inhibitory synapses. *Biochem. Pharmacol.* 68, 1649–1654. doi: 10.1016/j.bcp.2004.07.028

Kittler, J. T., Chen, G., Honing, S., Bogdanov, Y., Mcainsh, K., Arancibia-Carcamo, I. L., et al. (2005). Phospho-dependent binding of the clathrin AP2 adaptor complex to $GABA_A$ receptors regulates the efficacy of inhibitory synaptic transmission. *Proc. Natl. Acad. Sci. U S A* 102, 14871–14876. doi: 10.1073/pnas.0506653102

Kittler, J. T., Chen, G., Kukhtina, V., Vahedi-Faridi, A., Gu, Z., Tretter, V., et al. (2008). Regulation of synaptic inhibition by phospho-dependent binding of the AP2 complex to a YECL motif in the $GABA_A$ receptor γ2 subunit. *Proc. Natl. Acad. Sci. U S A* 105, 3616–3621. doi: 10.1073/pnas.0707920105

Kittler, J. T., Delmas, P., Jovanovic, J. N., Brown, D. A., Smart, T. G., and Moss, S. J. (2000). Constitutive endocytosis of $GABA_A$ receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. *J. Neurosci.* 20, 7972–7977. doi: 10.1523/jneurosci.20-21-07972.2000

Kneussel, M., Brandstatter, J. H., Laube, B., Stahl, S., Muller, U., and Betz, H. (1999). Loss of postsynaptic $GABA_A$ receptor clustering in gephyrin-deficient mice. *J. Neurosci.* 19, 9289–9297. doi: 10.1523/jneurosci.19-21-09289.1999

Kowalczyk, S., Winkelmann, A., Smolinsky, B., Forstera, B., Neundorf, I., Schwarz, G., et al. (2013). Direct binding of $GABA_A$ receptor β2 and β3 subunits to gephyrin. *Eur. J. Neurosci.* 37, 544–554. doi: 10.1111/ejn.12078

Krueger-Burg, D., Papadopoulos, T., and Brose, N. (2017). Organizers of inhibitory synapses come of age. *Curr. Opin. Neurobiol.* 45, 66–77. doi: 10.1016/j.conb.2017.04.003

Laverty, D., Desai, R., Uchański, T., Masiulis, S., Stec, W. J., Malinauskas, T., et al. (2019). Cryo-EM structure of the human α1β3γ2 $GABA_A$ receptor in a lipid bilayer. *Nature* 565, 516–520. doi: 10.1038/s41586-018-0833-4

Lee, V., and Maguire, J. (2014). The impact of tonic $GABA_A$ receptor-mediated inhibition on neuronal excitability varies across brain region and cell type. *Front. Neural Circuits* 8:3. doi: 10.3389/fncir.2014.00003

Letellier, M., Sziber, Z., Chamma, I., Saphy, C., Papasideri, I., Tessier, B., et al. (2018). A unique intracellular tyrosine in neuroligin-1 regulates AMPA receptor recruitment during synapse differentiation and potentiation. *Nat. Commun.* 9:3979. doi: 10.1038/s41467-018-06220-2

Li, J., Casteels, T., Frogne, T., Ingvorsen, C., Honore, C., Courtney, M., et al. (2017). Artemisinins target $GABA_A$ receptor signaling and impair α cell identity. *Cell* 168, 86.e15–100.e15. doi: 10.1016/j.cell.2016.11.010

Loebrich, S., Bahring, R., Katsuno, T., Tsukita, S., and Kneussel, M. (2006). Activated radixin is essential for $GABA_A$ receptor α5 subunit anchoring at the actin cytoskeleton. *EMBO J.* 25, 987–999. doi: 10.1038/sj.emboj.7600995

Long, P., May, M. M., James, V. M., Granno, S., Johnson, J. P., Tarpey, P., et al. (2016). Missense mutation R338W in ARHGEF9 in a family with X-linked intellectual disability with variable macrocephaly and macro-orchidism. *Front. Mol. Neurosci.* 8:83. doi: 10.3389/fnmol.2015.00083

Lorenz-Guertin, J. M., and Jacob, T. C. (2018). GABA type a receptor trafficking and the architecture of synaptic inhibition. *Dev. Neurobiol.* 78, 238–270. doi: 10.1002/dneu.22536

Löscher, W., and Rogawski, M. A. (2012). How theories evolved concerning the mechanism of action of barbiturates. *Epilepsia* 53, 12–25. doi: 10.1111/epi.12025

Lu, W., Bromley-Coolidge, S., and Li, J. (2017). Regulation of GABAergic synapse development by postsynaptic membrane proteins. *Brain Res. Bull.* 129, 30–42. doi: 10.1016/j.brainresbull.2016.07.004

Ludolphs, M., Schneeberger, D., Soykan, T., Schafer, J., Papadopoulos, T., Brose, N., et al. (2016). Specificity of collybistin-phosphoinositide interactions: impact of the individual protein domains. *J. Biol. Chem.* 291, 244–254. doi: 10.1074/jbc.m115.673400

Luscher, B., Fuchs, T., and Kilpatrick, C. L. (2011). $GABA_A$ receptor trafficking-mediated plasticity of inhibitory synapses. *Neuron* 70, 385–409. doi: 10.1016/j.neuron.2011.03.024

Maher, M. P., Matta, J. A., Gu, S., Seierstad, M., and Bredt, D. S. (2017). Getting a handle on neuropharmacology by targeting receptor-associated proteins. *Neuron* 96, 989–1001. doi: 10.1016/j.neuron.2017.10.001

Maric, H. M., Hausrat, T. J., Neubert, F., Dalby, N. O., Doose, S., Sauer, M., et al. (2017). Gephyrin-binding peptides visualize postsynaptic sites and modulate neurotransmission. *Nat. Chem. Biol.* 13, 153–160. doi: 10.1038/nchembio.2246

Maric, H. M., Kasaragod, V. B., Haugaard-Kedstrom, L., Hausrat, T. J., Kneussel, M., Schindelin, H., et al. (2015). Design and synthesis of high-affinity dimeric inhibitors targeting the interactions between gephyrin and inhibitory neurotransmitter receptors. *Angew. Chem. Int. Ed Engl.* 54, 490–494. doi: 10.1002/anie.201409043

Maric, H. M., Kasaragod, V. B., Hausrat, T. J., Kneussel, M., Tretter, V., Stromgaard, K., et al. (2014). Molecular basis of the alternative recruitment of $GABA_A$ versus glycine receptors through gephyrin. *Nat. Commun.* 5:5767. doi: 10.1038/ncomms6767

Maric, H. M., Mukherjee, J., Tretter, V., Moss, S. J., and Schindelin, H. (2011). Gephyrin-mediated γ-aminobutyric acid type A and glycine receptor clustering relies on a common binding site. *J. Biol. Chem.* 286, 42105–42114. doi: 10.1074/jbc.m111.303412

Masiulis, S., Desai, R., Uchański, T., Serna Martin, I., Laverty, D., Karia, D., et al. (2019). $GABA_A$ receptor signalling mechanisms revealed by structural pharmacology. *Nature* 565, 454–459. doi: 10.1038/s41586-018-0832-5

Mayer, S., Kumar, R., Jaiswal, M., Soykan, T., Ahmadian, M. R., Brose, N., et al. (2013). Collybistin activation by GTP-TC10 enhances postsynaptic gephyrin clustering and hippocampal GABAergic neurotransmission. *Proc. Natl. Acad. Sci. U S A* 110, 20795–20800. doi: 10.1073/pnas.1309078110

Mele, M., Costa, R. O., and Duarte, C. B. (2019). Alterations in $GABA_A$-receptor trafficking and synaptic dysfunction in brain disorders. *Front. Cell. Neurosci.* 13:77. doi: 10.3389/fncel.2019.00077

Miller, P. S., and Aricescu, A. R. (2014). Crystal structure of a human $GABA_A$ receptor. *Nature* 512, 270–275. doi: 10.1038/nature13293

Miller, P. S., Scott, S., Masiulis, S., De Colibus, L., Pardon, E., Steyaert, J., et al. (2017). Structural basis for $GABA_A$ receptor potentiation by neurosteroids. *Nat. Struct. Mol. Biol.* 24, 986–992. doi: 10.1038/nsmb.3484

Mizokami, A., Kanematsu, T., Ishibashi, H., Yamaguchi, T., Tanida, I., Takenaka, K., et al. (2007). Phospholipase C-related inactive protein is involved in trafficking of γ2 subunit-containing $GABA_A$ receptors to the cell surface. *J. Neurosci.* 27, 1692–1701. doi: 10.1523/jneurosci.3155-06.2007

Mody, I. (2019). $GABA_AR$ modulator for postpartum depression. *Cell* 176:1. doi: 10.1016/j.cell.2018.12.016

Mody, I., and Pearce, R. A. (2004). Diversity of inhibitory neurotransmission through $GABA_A$ receptors. *Trends Neurosci.* 27, 569–575. doi: 10.1016/j.tins.2004.07.002

Mukherjee, J., Kretschmannova, K., Gouzer, G., Maric, H. M., Ramsden, S., Tretter, V., et al. (2011). The residence time of $GABA_ARs$ at inhibitory synapses is determined by direct binding of the receptor α1 subunit to gephyrin. *J. Neurosci.* 31, 14677–14687. doi: 10.1523/jneurosci.2001-11.2011

Nair, R., Lauks, J., Jung, S., Cooke, N. E., De Wit, H., Brose, N., et al. (2013). Neurobeachin regulates neurotransmitter receptor trafficking to synapses. *J. Cell Biol.* 200, 61–80. doi: 10.1083/jcb.201207113

Nakamura, Y., Darnieder, L. M., Deeb, T. Z., and Moss, S. J. (2015). Regulation of $GABA_ARs$ by phosphorylation. *Adv. Pharmacol.* 72, 97–146. doi: 10.1016/bs.apha.2014.11.008

Nakamura, Y., Morrow, D. H., Modgil, A., Huyghe, D., Deeb, T. Z., Lumb, M. J., et al. (2016). Proteomic characterization of inhibitory synapses using a novel pHluorin-tagged γ-aminobutyric acid receptor, type A ($GABA_A$), α2 subunit knock-in mouse. *J. Biol. Chem.* 291, 12394–12407. doi: 10.1074/jbc.m116.724443

Naylor, D. E., Liu, H., and Wasterlain, C. G. (2005). Trafficking of $GABA_A$ receptors, loss of inhibition, and a mechanism for pharmacoresistance in status epilepticus. *J. Neurosci.* 25, 7724–7733. doi: 10.1523/jneurosci.4944-04.2005

Nguyen, Q. A., Horn, M. E., and Nicoll, R. A. (2016). Distinct roles for extracellular and intracellular domains in neuroligin function at inhibitory synapses. *Elife* 5:e19236. doi: 10.7554/elife.19236

Nicholson, M. W., Sweeney, A., Pekle, E., Alam, S., Ali, A. B., Duchen, M., et al. (2018). Diazepam-induced loss of inhibitory synapses mediated by PLCdelta/Ca^{2+}/calcineurin signalling downstream of $GABA_A$ receptors. *Mol. Psychiatry* 23, 1851–1867. doi: 10.1038/s41380-018-0100-y

Olsen, R. W. (2018). $GABA_A$ receptor: Positive and negative allosteric modulators. *Neuropharmacology* 136, 10–22. doi: 10.1016/j.neuropharm.2018.01.036

Olsen, R. W., and Sieghart, W. (2009). GABA A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* 56, 141–148. doi: 10.1016/j.neuropharm.2008.07.045

Paarmann, I., Schmitt, B., Meyer, B., Karas, M., and Betz, H. (2006). Mass spectrometric analysis of glycine receptor-associated gephyrin splice variants. *J. Biol. Chem.* 281, 34918–34925. doi: 10.1074/jbc.m607764200

Palma, E., Ruffolo, G., Cifelli, P., Roseti, C., Vliet, E. A. V., and Aronica, E. (2017). Modulation of $GABA_A$ receptors in the treatment of epilepsy. *Curr. Pharm. Des.* 23, 5563–5568. doi: 10.2174/1381612823666170809100230

Papadopoulos, T., Eulenburg, V., Reddy-Alla, S., Mansuy, I. M., Li, Y., and Betz, H. (2008). Collybistin is required for both the formation and maintenance of GABAergic postsynapses in the hippocampus. *Mol. Cell. Neurosci.* 39, 161–169. doi: 10.1016/j.mcn.2008.06.006

Papadopoulos, T., Korte, M., Eulenburg, V., Kubota, H., Retiounskaia, M., Harvey, R. J., et al. (2007). Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. *EMBO J.* 26, 3888–3899. doi: 10.1038/sj.emboj.7601819

Pennacchietti, F., Vascon, S., Nieus, T., Rosillo, C., Das, S., Tyagarajan, S. K., et al. (2017). Nanoscale molecular reorganization of the inhibitory postsynaptic density is a determinant of GABAergic synaptic potentiation. *J. Neurosci.* 37, 1747–1756. doi: 10.1523/JNEUROSCI.0514-16.2016

Pettem, K. L., Yokomaku, D., Takahashi, H., Ge, Y., and Craig, A. M. (2013). Interaction between autism-linked MDGAs and neuroligins suppresses inhibitory synapse development. *J. Cell Biol.* 200, 321–336. doi: 10.1083/jcb.201206028

Pfeiffer, F., Graham, D., and Betz, H. (1982). Purification by affinity chromatography of the glycine receptor of rat spinal cord. *J. Biol. Chem.* 257, 9389–9393.

Poulopoulos, A., Aramuni, G., Meyer, G., Soykan, T., Hoon, M., Papadopoulos, T., et al. (2009). Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* 63, 628–642. doi: 10.1016/j.neuron.2009.08.023

Reddy-Alla, S., Schmitt, B., Birkenfeld, J., Eulenburg, V., Dutertre, S., Bohringer, C., et al. (2010). PH-domain-driven targeting of collybistin but not Cdc42 activation is required for synaptic gephyrin clustering. *Eur. J. Neurosci.* 31, 1173–1184. doi: 10.1111/j.1460-9568.2010.07149.x

Saiepour, L., Fuchs, C., Patrizi, A., Sassoè-Pognetto, M., Harvey, R. J., and Harvey, K. (2010). Complex role of collybistin and gephyrin in $GABA_A$ receptor clustering. *J. Biol. Chem.* 285, 29623–29631. doi: 10.1074/jbc.M110.121368

Sainlos, M., Tigaret, C., Poujol, C., Olivier, N. B., Bard, L., Breillat, C., et al. (2011). Biomimetic divalent ligands for the acute disruption of synaptic AMPAR stabilization. *Nat. Chem. Biol.* 7, 81–91. doi: 10.1038/nchembio.498

Sancar, F., and Czajkowski, C. (2011). Allosteric modulators induce distinct movements at the GABA-binding site interface of the GABA-A receptor. *Neuropharmacology* 60, 520–528. doi: 10.1016/j.neuropharm.2010.11.009

Smith, K. R., and Kittler, J. T. (2010). The cell biology of synaptic inhibition in health and disease. *Curr. Opin. Neurobiol.* 20, 550–556. doi: 10.1016/j.conb.2010.06.001

Smith, K. R., Muir, J., Rao, Y., Browarski, M., Gruenig, M. C., Sheehan, D. F., et al. (2012). Stabilization of $GABA_A$ receptors at endocytic zones is mediated by an AP2 binding motif within the $GABA_A$ receptor β3 subunit. *J. Neurosci.* 32, 2485–2498. doi: 10.1523/JNEUROSCI.1622-11.2011

Soykan, T., Schneeberger, D., Tria, G., Buechner, C., Bader, N., Svergun, D., et al. (2014). A conformational switch in collybistin determines the differentiation of inhibitory postsynapses. *EMBO J.* 33, 2113–2133. doi: 10.15252/embj.201488143

Specht, C. G. (2019). Fractional occupancy of synaptic binding sites and the molecular plasticity of inhibitory synapses. *Neuropharmacology* doi: 10.1016/j.neuropharm.2019.01.008 [Epub ahead of print].

Specht, C. G., Grunewald, N., Pascual, O., Rostgaard, N., Schwarz, G., and Triller, A. (2011). Regulation of glycine receptor diffusion properties and gephyrin interactions by protein kinase C. *EMBO J.* 30, 3842–3853. doi: 10.1038/emboj.2011.276

Specht, C. G., Izeddin, I., Rodriguez, P. C., El Beheiry, M., Rostaing, P., Darzacq, X., et al. (2013). Quantitative nanoscopy of inhibitory synapses: counting gephyrin molecules and receptor binding sites. *Neuron* 79, 308–321. doi: 10.1016/j.neuron.2013.05.013

Takai, Y., Kitano, K., Terawaki, S., Maesaki, R., and Hakoshima, T. (2007). Crystallographic characterization of the radixin FERM domain bound to the cytoplasmic tails of adhesion molecules CD43 and PSGL-1. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 63, 49–51. doi: 10.1107/s1744309106054145

Terawaki, S., Kitano, K., Aoyama, M., and Hakoshima, T. (2008). Crystallographic characterization of the radixin FERM domain bound to the cytoplasmic tail of membrane-type 1 matrix metalloproteinase (MT1-MMP). *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 64, 911–913. doi: 10.1107/s1744309108026869

Terawaki, S., Kitano, K., and Hakoshima, T. (2007). Structural basis for type II membrane protein binding by ERM proteins revealed by the radixin-neutral endopeptidase 24.11 (NEP) complex. *J. Biol. Chem.* 282, 19854–19862. doi: 10.1074/jbc.m609232200

Tretter, V., Jacob, T. C., Mukherjee, J., Fritschy, J. M., Pangalos, M. N., and Moss, S. J. (2008). The clustering of $GABA_A$ receptor subtypes at inhibitory synapses is facilitated *via* the direct binding of receptor α 2 subunits to gephyrin. *J. Neurosci.* 28, 1356–1365. doi: 10.1523/JNEUROSCI.5050-07.2008

Tretter, V., Kerschner, B., Milenkovic, I., Ramsden, S. L., Ramerstorfer, J., Saiepour, L., et al. (2011). Molecular basis of the γ-aminobutyric acid A receptor α3 subunit interaction with the clustering protein gephyrin. *J. Biol. Chem.* 286, 37702–37711. doi: 10.1074/jbc.m111.291336

Tretter, V., Mukherjee, J., Maric, H. M., Schindelin, H., Sieghart, W., and Moss, S. J. (2012). Gephyrin, the enigmatic organizer at GABAergic synapses. *Front. Cell. Neurosci.* 6:23. doi: 10.3389/fncel.2012.00023

Tretter, V., Revilla-Sanchez, R., Houston, C., Terunuma, M., Havekes, R., Florian, C., et al. (2009). Deficits in spatial memory correlate with modified γ-aminobutyric acid type A receptor tyrosine phosphorylation in the hippocampus. *Proc. Natl. Acad. Sci. U S A* 106, 20039–20044. doi: 10.1073/pnas.0908840106

Tyagarajan, S. K., and Fritschy, J. M. (2014). Gephyrin: a master regulator of neuronal function? *Nat. Rev. Neurosci.* 15, 141–156. doi: 10.1038/nrn3670

Tyagarajan, S. K., Ghosh, H., Harvey, K., and Fritschy, J. M. (2011a). Collybistin splice variants differentially interact with gephyrin and Cdc42 to regulate gephyrin clustering at GABAergic synapses. *J. Cell Sci.* 124, 2786–2796. doi: 10.1242/jcs.086199

Tyagarajan, S. K., Ghosh, H., Yevenes, G. E., Nikonenko, I., Ebeling, C., Schwerdel, C., et al. (2011b). Regulation of GABAergic synapse formation and plasticity by GSK3β-dependent phosphorylation of gephyrin. *Proc. Natl. Acad. Sci. U S A* 108, 379–384. doi: 10.1073/pnas.1011824108

Tyagarajan, S. K., Ghosh, H., Yevenes, G. E., Imanishi, S. Y., Zeilhofer, H. U., Gerrits, B., et al. (2013). Extracellular signal-regulated kinase and glycogen synthase kinase 3β regulate gephyrin postsynaptic aggregation and GABAergic synaptic function in a calpain-dependent mechanism. *J. Biol. Chem.* 288, 9634–9647. doi: 10.1074/jbc.m112.442616

Uezu, A., Kanak, D. J., Bradshaw, T. W., Soderblom, E. J., Catavero, C. M., Burette, A. C., et al. (2016). Identification of an elaborate complex mediating postsynaptic inhibition. *Science* 353, 1123–1129. doi: 10.1126/science.aag0821

Varoqueaux, F., Jamain, S., and Brose, N. (2004). Neuroligin 2 is exclusively localized to inhibitory synapses. *Eur. J. Cell Biol.* 83, 449–456. doi: 10.1078/0171-9335-00410

Vien, T. N., Modgil, A., Abramian, A. M., Jurd, R., Walker, J., Brandon, N. J., et al. (2015). Compromising the phosphodependent regulation of the GABAAR β3 subunit reproduces the core phenotypes of autism spectrum disorders. *Proc. Natl. Acad. Sci. U S A* 112, 14805–14810. doi: 10.1073/pnas.1514657112

Villa, K. L., Berry, K. P., Subramanian, J., Cha, J. W., Oh, W. C., Kwon, H. B., et al. (2016). Inhibitory synapses are repeatedly assembled and removed at persistent sites *in vivo*. *Neuron* 89, 756–769. doi: 10.1016/j.neuron.2016.01.010

Wang, H., Bedford, F. K., Brandon, N. J., Moss, S. J., and Olsen, R. W. (1999). $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton. *Nature* 397, 69–72. doi: 10.1038/16264

Wang, Y. C., Dzyubenko, E., Sanchez-Mendoza, E. H., Sardari, M., Silva de Carvalho, T., Doeppner, T. R., et al. (2018). Postacute delivery of $GABA_A$ α5 antagonist promotes postischemic neurological recovery and peri-infarct brain remodeling. *Stroke* 49, 2495–2503. doi: 10.1161/strokeaha.118.021378

Wisden, W., and Seeburg, P. H. (1992). $GABA_A$ receptor channels: from subunits to functional entities. *Curr. Opin. Neurobiol.* 2, 263–269. doi: 10.1016/0959-4388(92)90113-y

Yamasaki, T., Hoyos-Ramirez, E., Martenson, J. S., Morimoto-Tomita, M., and Tomita, S. (2017). GARLH family proteins stabilize $GABA_A$ receptors at synapses. *Neuron* 93, 1138.e6–1152.e6. doi: 10.1016/j.neuron.2017.02.023

Yogesha, S. D., Sharff, A. J., Giovannini, M., Bricogne, G., and Izard, T. (2011). Unfurling of the band 4.1, ezrin, radixin, moesin (FERM) domain of the merlin tumor suppressor. *Protein Sci.* 20, 2113–2120. doi: 10.1002/pro.751

Yuan, X., Yao, J., Norris, D., Tran, D. D., Bram, R. J., Chen, G., et al. (2008). Calcium-modulating cyclophilin ligand regulates membrane trafficking of postsynaptic $GABA_A$ receptors. *Mol. Cell. Neurosci.* 38, 277–289. doi: 10.1016/j.mcn.2008.03.002

Zacchi, P., Dreosti, E., Visintin, M., Moretto-Zita, M., Marchionni, I., Cannistraci, I., et al. (2008). Gephyrin selective intrabodies as a new strategy for studying inhibitory receptor clustering. *J. Mol. Neurosci.* 34, 141–148. doi: 10.1007/s12031-007-9018-6

Zhu, S., Noviello, C. M., Teng, J., Walsh, R. M. Jr., Kim, J. J., and Hibbs, R. E. (2018). Structure of a human synaptic $GABA_A$ receptor. *Nature* 559, 67–72. doi: 10.1038/s41586-018-0255-3

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 Molecular Neuroscience

MINI REVIEW
published: 02 July 2019
doi: 10.3389/fnmol.2019.00161

Subsynaptic Domains in Super-Resolution Microscopy: The Treachery of Images

*Xiaojuan Yang and Christian G. Specht**

École Normale Supérieure, PSL Research University, CNRS, Inserm, Institute of Biology (IBENS), Paris, France

The application of super-resolution optical microscopy to investigating synaptic structures has revealed a highly heterogeneous and variable intra-synaptic organization. Dense subsynaptic protein assemblies named subsynaptic domains or SSDs have been proposed as structural units that regulate the efficacy of neuronal transmission. However, an in-depth characterization of SSDs has been hampered by technical limitations of super-resolution microscopy of synapses, namely the stochasticity of the signals during the imaging procedures and the variability of the synaptic structures. Here, we synthetize the available evidence for the existence of SSDs at central synapses, as well as the possible functional relevance of SSDs. In particular, we discuss the possible regulation of co-transmission at mixed inhibitory synapses as a consequence of the subsynaptic distribution of glycine receptors (GlyRs) and $GABA_A$ receptors ($GABA_A$Rs).

LAY ABSTRACT

Super-resolution imaging strategies bypass the resolution limit of conventional optical microscopy and have given new insights into the distribution of proteins at synapses in the central nervous system. Neurotransmitter receptors and scaffold proteins appear to occupy specialized locations within synapses that we refer to as subsynaptic domains or SSDs. Interestingly, these SSDs are highly dynamic and their formation seems to be related to the remodeling of synapses during synaptic plasticity. It was also shown that SSDs of pre-and post-synaptic proteins are aligned in so-called nanocolumns, highlighting the role of SSDs in the regulation of synaptic transmission. Despite recent advances, however, the detection of SSDs with super-resolution microscopy remains difficult due to the inherent technical limitations of these approaches that are discussed in this review article.

Keywords: subsynaptic domain (SSD), super-resolution microscopy, single molecule localization microscopy (SMLM), inhibitory receptors, gephyrin

OPEN ACCESS

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

Reviewed by:
Carlos B. Duarte,
University of Coimbra, Portugal
Josef Kittler,
University College London, United Kingdom

****Correspondence:***
Christian G. Specht
christian.specht@inserm.fr

Received: *13 April 2019*
Accepted: *12 June 2019*
Published: *02 July 2019*

Citation:
Yang X and Specht CG (2019) Subsynaptic Domains in Super-Resolution Microscopy: The Treachery of Images. Front. Mol. Neurosci. 12:161. doi: 10.3389/fnmol.2019.00161

INTRODUCTION

Single molecule localization microscopy (SMLM) bypasses the diffraction limit by detecting signals from a sparse subset of molecules that are temporally separated, thus achieving a spatial resolution of single molecules of 10–40 nm (Schermelleh et al., 2010; Turkowyd et al., 2016; Sieben et al., 2018). SMLM includes several related techniques, namely STORM, PALM and uPAINT (Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006; Giannone et al., 2010).

In 2010, Dani et al. (2010) measured the laminar distribution of synaptic proteins using multicolor three-dimensional (3D) STORM, demonstrating the capability of SMLM to visualize the ultra-structure of synapses (Specht et al., 2014). This marks the beginning of super-resolution optical imaging of synaptic structures. Numerous studies have since applied SMLM to explore the heterogeneity and complexity of protein assemblies at synapses. Another type of super-resolution optical microscopy achieves sub-diffraction resolution by means of structured excitation, such as stimulated emission depletion (STED; Klar et al., 2000) and structured illumination microscopy (SIM; Gustafsson, 2000). Regardless of the different working principles, super-resolution microscopy techniques have yielded significant insights into the distribution of synaptic proteins on the nanometer scale. Given their wide-field, volumetric imaging strategies, three-dimensional and quantitative information can be gained from a large sample size.

In 2013, several groups reported independently that different synaptic proteins are distributed heterogeneously at synapses (MacGillavry et al., 2013; Nair et al., 2013; Specht et al., 2013). SMLM images showed that the excitatory scaffold protein PSD-95 occupies subdomains within the post-synaptic density (PSD) that regulate AMPAR clustering (MacGillavry et al., 2013; Nair et al., 2013). The existence of PSD-95 subdomains was confirmed with STED microscopy both *in vitro* and *in vivo* (Broadhead et al., 2016; Dzyubenko et al., 2016; Hruska et al., 2018; Masch et al., 2018; Wegner et al., 2018). Likewise, subsynaptic domains of gephyrin were shown to play a role in inhibitory plasticity at GABAergic synapses (Pennacchietti et al., 2017; Crosby et al., 2019). These findings point towards a mechanism whereby subsynaptic domains drive the recruitment of neurotransmitter receptors to specific locations within the PSD, thus regulating synaptic transmission.

SMLM and STED microscopy have also shown that pre-synaptic proteins of the active zone (AZ) and synaptic adhesion proteins display subsynaptic distributions (Perez de Arce et al., 2015; Chamma et al., 2016a,b; Tang et al., 2016; Glebov et al., 2017; Haas et al., 2018). Using multicolor 3D-STORM, Tang et al. (2016) demonstrated that subsynaptic domains of RIM1/2 are aligned with those of PSD-95, an arrangement that is referred to as trans-synaptic nanocolumn. The alignment of pre- and post-synaptic elements appears to be due to neuroligin/neurexin adhesion complexes (Perez de Arce et al., 2015; Haas et al., 2018). These exciting observations not only demonstrate the power of SMLM to visualize the ultra-structures of synapses but also point towards possible roles of subsynaptic domains in synaptic function (reviewed in Biederer et al., 2017; Liu et al., 2017; Chen et al., 2018; Scheefhals and MacGillavry, 2018).

Despite these advances, the concept of subsynaptic domains remains ambiguous, not least because the technical and biological limitations in identifying subsynaptic domains have not been sufficiently scrutinized. Here, we review the available evidence for the existence of subsynaptic domains, highlighting the factors that need to be taken into account in detecting small protein assemblies using SMLM. We then discuss the possible role of subsynaptic domains in the regulation of glycinergic and GABAergic co-transmission based on recent data from inhibitory synapses.

WHAT IS A SUBSYNAPTIC DOMAIN?

Terminology and Definition

A major source of confusion is that different names have been used in the literature to describe subsynaptic domains. Among these, the terms nanodomain, nanocluster, subcluster, subdomain and nanomodule have been used in an interchangeable manner (e.g., MacGillavry et al., 2013; Nair et al., 2013; Broadhead et al., 2016; Haas et al., 2018; Hruska et al., 2018). The lack of a clear and unified terminology has made it difficult to refer to specific molecular structures and to be aware of the differences and similarities between studies. Regarding the choice of words, the term *cluster* should best be avoided, because it can also refer to the clustering algorithms that are widely used for image analysis of SMLM data (Nicovich et al., 2017). The prefix *nano* is redundant because synapses themselves have diameters of only a few hundred nanometers. Furthermore, *nanodomain* has been widely used to describe the high Ca^{2+} ion concentrations in the proximity of an open calcium channel (Augustine et al., 2003; Eggermann et al., 2013; Ghelani and Sigrist, 2018).

We, therefore, refer to these structures as *subsynaptic domain* or *SSD* (Crosby et al., 2019) for the following reasons: (1) the term is self-explanatory, referring to a space that is smaller than the whole synaptic compartment and that is occupied by a given type of molecules; and (2) it is flexible in that it can be equally applied to membrane receptors, scaffold and signaling proteins, whether they are pre-synaptic or post-synaptic. We define SSD as a sub-compartment of the synapse in which the density of a specific synaptic protein is higher than in the surrounding area, and that is typically observed with super-resolution microscopy. We believe that the term SSD could thus provide some clarity in defining specific molecular entities at synapses.

SSD Size and Protein Copy Numbers

The most basic feature of SSDs that holds biologically relevant information is their size and the copy number of proteins that they contain. A wide range of sizes was detected by SMLM and STED microscopy (**Table 1**). For instance, SSDs of excitatory scaffold proteins in cultured hippocampal neurons have a diameter of ∼80 nm as judged by coordinate-based SMLM analysis (MacGillavry et al., 2013), whereas an average diameter of 120 nm was measured in reconstructed super-resolution images (Nair et al., 2013). STED microscopy detected SSDs of PSD-95 with a diameter of 200 nm (Fukata et al., 2013). These differences in SSD size are likely due to the different resolution of the imaging systems and the application of a threshold during image processing. A comparative study of PSD-95 in hippocampal tissue using PALM and STED determined median SSD diameters of 126 nm and 158 nm, respectively, exemplifying the impact of the imaging approach (Broadhead et al., 2016). The typical diameter of the whole PSD in hippocampal neurons ranges from 100 nm to 800 nm, with a mean of about 300 nm (Harris and Stevens, 1989; Arellano et al., 2007). Therefore, the

lower limit of SSD sizes of ∼50 nm reflects the image resolution of the super-resolution imaging techniques, while the upper limit corresponds to the size of the entire synapse. Given that synapse sizes vary substantially across the central nervous system, an interesting question is whether SSDs of different synaptic proteins have stereotypical sizes that are the same at different types of synapses (see Crosby et al., 2019).

Information about protein copy numbers is essential to establish the structural basis of SSD formation. To date, there are hardly any quantitative data about SSD molecule numbers. SSDs of AMPARs have been estimated to contain an average of ∼20 receptor complexes (Nair et al., 2013). Due to the limited accessibility of the epitopes for immunolabeling, however, the actual number of receptors per SSD could be higher. This could have an effect on the role of SSDs in synaptic function since the number of active receptors is directly related to the strength of synaptic transmission (Masugi-Tokita et al., 2007; Tarusawa et al., 2009; Fukazawa and Shigemoto, 2012).

Number of SSDs Per Synapse

Most synapses contain only one SSD or no SSD at all. More specifically, a single SSD was detected in 50% to 80% of synapses imaged with SMLM, SIM or STED microscopy, less than 20% had more than three SSDs, and six SSDs was the upper limit (MacGillavry et al., 2013; Nair et al., 2013; Broadhead et al., 2016; Chamma et al., 2016a,b; Pennacchietti et al., 2017; Hruska et al., 2018; Crosby et al., 2019). It is likely that the different imaging techniques and analyses again have an effect on the detection of multiple SSDs. This raises the question whether the SSD simply reflects the center of mass of the protein assembly, and if so, whether the presence of single or multiple SSDs actually matter for the regulation of synaptic function.

There exists a positive correlation between the number of SSDs and the size of the PSD or the dendritic spine (Fukata et al., 2013; Nair et al., 2013; Hruska et al., 2018; Crosby et al., 2019). EM studies have revealed a large variability in PSD area, ranging from 100 nm to 800 nm in diameter (**Table 1**). More than half of the PSDs are small (<0.05 μm^2), which is similar to the fraction of synapses with only one SSD (Arellano et al., 2007). Moreover, the number of AMPAR molecules is positively correlated with the PSD size, and large complex PSDs have a higher density of AMPARs than small, non-perforated PSDs (Ganeshina et al., 2004; Shinohara et al., 2008; Fukazawa and Shigemoto, 2012). Together, these data indicate that SSDs may only play a role at large PSDs, reflecting the superior strength of these synapses.

Trans-synaptic Nanocolumns

From the viewpoint of neuron connectivity, pre-synaptic and post-synaptic SSDs can be aligned to form trans-synaptic structural units that regulate synaptic function (Biederer et al., 2017; Chen et al., 2018). Such an organization has been observed at excitatory synapses using 3D-SMLM, and was suitably named *trans-synaptic nanocolumn* (Tang et al., 2016). SMLM studies have further shown that synaptic adhesion complexes such as neuroligin and neurexin are also organized in SSDs, suggesting that they contribute to the formation of trans-synaptic nanocolumns (Perez de Arce et al., 2015; Haas et al., 2018). The

TABLE 1 | Size and protein copy numbers of SSDs and PSDs obtained with different experimental techniques.

Structure	Diameter (nm)	Molecule numbers	Technique	Synapse type	References
SSD	50–130*		SMLM	Excitatory, hippocampal	MacGillavry et al. (2013), Nair et al. (2013), Broadhead et al. (2016), Chamma et al. (2016a,b) and Haas et al. (2018)
	130–760*		STED	Excitatory, hippocampal and cortical	Nair et al. (2013), Broadhead et al. (2016) and Hruska et al. (2018)
	∼300*		SIM	Inhibitory, hippocampal	Crosby et al. (2019)
	70*	∼20 AMPARs/SSD*	STORM	Excitatory, hippocampal	Nair et al. (2013)
PSD	300 (100–800)#		EM	Excitatory, hippocampal	Harris and Stevens (1989) and Bourne and Harris (2011)
	290 (110–650)#		EM	Excitatory, cortical	Arellano et al. (2007) and Santuy et al. (2018)
	350 (110–700)#		EM	Inhibitory, hippocampal and cortical	Bourne and Harris (2011) and Santuy et al. (2018)
		50 (0–200) AMPARs#	EM	Excitatory, various CNS regions	Masugi-Tokita et al. (2007), Tarusawa et al. (2009) and Fukazawa and Shigemoto (2012)
		30 (0–200) $GABA_AR$s#	Electrophysiology, EM	Inhibitory, cerebellar and hippocampal	Nusser et al. (1997, 1998)
		30 (40–500) PSD-95#	Biochemistry, TIRF microscopy	Excitatory, various brain regions	Sugiyama et al. (2005) and Sheng and Kim (2011)
		30 (40–500) Gephrin#	SMLM (decay recordings)	Inhibitory, spinal cord	Specht et al. (2013) and Patrizio et al. (2017)

*Mean values obtained in the cited studies; # mean (range in brackets). Values were taken directly or calculated from those reported in the cited studies.

term nanocolumn, therefore, refers to a specific concept, namely the alignment of pre- and post-synaptic SSDs that brings together different functional elements. Future studies are expected to explore the possible role of nanocolumns in synaptic plasticity.

The Dynamics of SSDs

The hypothesis that SSDs regulate synaptic transmission implies that SSDs adapt dynamically to changes in synaptic strength. Indeed, live SMLM in cultured neurons has revealed the mobility and morphological changes of SSDs. Synaptic scaffolds undergo dynamic changes on a timescale of 5–10 min, displaying marked differences in the number, position and shape of SSDs at different time points (Nair et al., 2013; Specht et al., 2013; Rodriguez et al., 2017). STED microscopy further showed that these morphological changes occurred both *in vitro* and *in vivo* (Hruska et al., 2018; Wegner et al., 2018). The dynamics of SSDs are in agreement with the exchange of individual proteins at synaptic and extra-synaptic sites, which is a hallmark of the dynamic synapse (Choquet and Triller, 2013; Delgado and Selvin, 2018). Therefore, SSDs are momentary representations of the protein distribution and need to be viewed as dynamic snapshots rather than rigid structural units.

HOW TO DETECT SUBSYNAPTIC DOMAINS WITH SMLM

The identification of SSDs consists in detecting small numbers of densely packed molecules in a confined space with a high local background from neighboring molecules with lower density. Despite these challenges, SMLM is well suited to resolve the internal organization of small structures such as synapses at single molecule level. In the following, we discuss the relevant factors of the image acquisition and data analysis that have an impact on the identification of SSDs.

Image Acquisition

SMLM techniques aim to record large numbers of single fluorophore detections from densely labeled structures, while ensuring that the signals are sufficiently sparse to be well separated. STORM, PALM and uPAINT have all been employed for detecting SSDs. The three techniques have the same intrinsic challenges when it comes to the ultrastructure of synapses, chief among them being the fluorophore. Most fluorophores are detected repeatedly due to their fluorescence lifetime, photo-switching and blinking. This can create dense clusters of redundant detections that are easily mistaken for SSDs. The blinking behavior of the fluorophores (organic dyes or fluorescent proteins) is dependent on their photo-physical and photo-chemical properties, and it can be modulated by the laser power and the composition of the imaging buffer (Dempsey et al., 2011; Endesfelder et al., 2011; van de Linde et al., 2011; Nahidiazar et al., 2016). Sub-optimal imaging conditions such as inefficient laser illumination or an incompatible buffer system can result in artificial clustering (Annibale et al., 2011; Burgert et al., 2015; Nahidiazar et al., 2016). Even with an optimized imaging protocol, different fluorophores will produce different representations of the analyzed structure (Dempsey et al., 2011; Baddeley and Bewersdorf, 2018). The evaluation of the number and the size of SSDs is therefore strongly dependent on the fluorophores, and control experiments with different fluorophores are crucial to validate the experimental findings (Yang and Specht, in press). In addition to the fluorophores, attention should also be drawn to the labeling strategies used for sample preparation. The distance between the fluorophores and the actual positions of the target molecules (e.g., due to the size of antibodies used for labeling), and under-sampling due to a limited labeling efficiency can add to the uncertainties in the identification of SSDs (Deschout et al., 2014; Maidorn et al., 2016).

Image Segmentation

Depending on the type of SMLM data (pointillist or reconstructed super-resolution images), different algorithms have been adopted for segmenting SSDs. For coordinates-based data, a local density threshold is generally applied. The local density can for instance be defined as the number of detections within a radius of five times the mean nearest neighbor distance of all the detections within each synapse, and SSDs are identified as regions above a certain threshold (MacGillavry et al., 2013; Tang et al., 2016; Pennacchietti et al., 2017). As regards the reconstructed images, an intensity threshold may be adopted instead. For example, wavelet segmentation has been used to identify SSDs at synapses in the whole field of view (Nair et al., 2013; Chamma et al., 2016a,b). Similarly, watershed segmentation can be employed to segment SSDs of individual synapses in reconstructed SMLM images or deconvoluted STED images (Broadhead et al., 2016; Dzyubenko et al., 2016). The difficulty of all these approaches is that the detected size and the number of SSDs are directly dependent on the algorithms and the chosen parameters, which makes an accurate identification of SSDs challenging.

Dealing With Small Molecule Numbers and the Variability of Synapses

Synapses exhibit a large variability not only in size but also in terms of molecule numbers. Neurotransmitter receptors such as AMPARs or $GABA_A$Rs have relatively low copy numbers, with an average of ∼50 receptor complexes per synapse (ranging up to 200 copies; **Table 1**). The main scaffold proteins at excitatory and inhibitory synapses outnumber the receptors by a factor of four to five. PSD-95 and gephyrin molecules amount to 40–500 per synapse, with an average of ∼300 copies (Sugiyama et al., 2005; Sheng and Kim, 2011; Specht et al., 2013; Patrizio et al., 2017). The low copy numbers of synaptic proteins, especially receptors, makes the identification of SSDs with SMLM challenging since the labeling of the structures is often rather faint. At the same time, the high local density of synaptic proteins can further reduce the efficiency of immunolabeling due to epitope masking. The overall receptor density at synapses is in the order of 700 AMPARs/μm^2 for the whole PSD (50 AMPARs/0.07 μm^2). An average SSD with a diameter of 70 nm (area of 0.0038 μm^2) contains about 20 AMPARs, resulting in an estimated density of ∼5,000 AMPAR complexes/μm^2 (Nair et al., 2013). Considering the molecular size of the receptor complexes (10 nm × 20 nm;

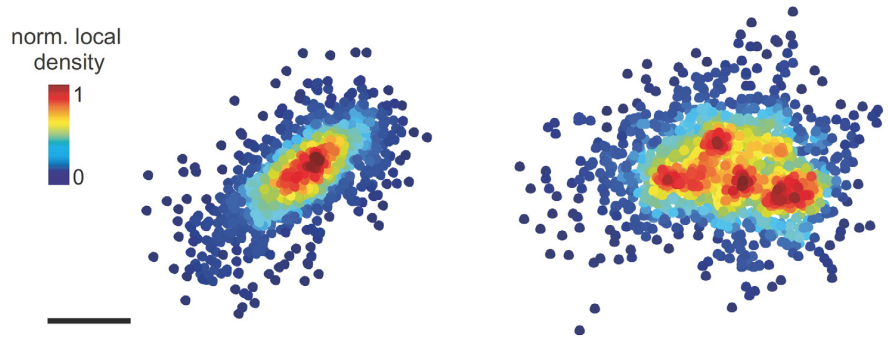

FIGURE 1 | Pointillist images showing synaptic gephyrin clusters with one SSD (left) or four SSDs (right). The points represent the detections of single fluorophores from PALM imaging. Scale bar: 100 nm (adapted with permission from Pennacchietti et al., 2017).

Patriarchi et al., 2018), 20 AMPARs would occupy a membrane area of at least 0.004 μm^2. This means that the receptors are very densely packed inside the SSD, adding to the uncertainties that result from the stochasticity of the immunolabeling and fluorophore detection.

Alternative Approaches

Given the rapid advances in super-resolution imaging technologies, promising alternatives for the investigation of complex structures such as synapses are quickly emerging. Among these, smaller probes such as nanobodies have been produced to bypass the limitations of labeling density and to minimize the distance between the fluorophores and the target proteins (Chamma et al., 2016a; Maidorn et al., 2016). DNA-PAINT allows multi-color SMLM imaging (Nieves et al., 2018). DNA origami standards provide a more precise way for calibrating protein copy numbers given that the absolute quantification of molecules at SSDs is faced with large stochasticity of the imaging technique (Zanacchi et al., 2017). Furthermore, new algorithms are being developed to segment synaptic clusters in coordinates-based datasets more efficiently (Nicovich et al., 2017; Baddeley and Bewersdorf, 2018).

THE EMERGING ROLE OF SSDs IN INHIBITORY SYNAPTIC TRANSMISSION

Electron microscopy of symmetric synapses has revealed a discontinuous network of filaments at the inhibitory PSD and in the synaptic cleft (Linsalata et al., 2014; High et al., 2015). Super-resolution optical microscopy confirmed that the inhibitory scaffold protein gephyrin forms synaptic clusters of variable morphology that can undergo dynamic changes and may contain SSDs (Specht et al., 2013; Dzyubenko et al., 2016; Pennacchietti et al., 2017; Crosby et al., 2019). SMLM imaging in cultured hippocampal neurons further revealed that extra-synaptic gephyrin molecules are recruited to synaptic sites during NMDA-induced inhibitory long-term potentiation (Pennacchietti et al., 2017). The increase in molecule density was accompanied by an increased fraction of gephyrin clusters with multiple SSDs (**Figure 1**). More recently, Crosby et al. (2019) conducted a comprehensive analysis of pre- and postsynaptic components using 3D-SIM, reaching a resolution of ∼120 nm laterally and ∼300 nm axially. It was shown that $GABA_A$Rs form SSDs with an average diameter of ∼300 nm that are closely associated with SSDs of gephyrin and pre-synaptic RIM (Crosby et al., 2019). This implies the existence of trans-synaptic nanocolumns as an organizing principle of inhibitory synapses. Given that the measured size of the SSDs was close to the resolution limit, the concept of nanocolumns at inhibitory synapses will require further validation. Nonetheless, these studies strongly suggest that the internal organization of inhibitory synapses plays an important role in regulating synaptic transmission.

Unlike the cortex and hippocampus where fast neuronal inhibition is mainly mediated by $GABA_A$Rs, both glycine and GABA receptors coexist at synapses in the brainstem and the spinal cord. Gephyrin provides binding sites for the immobilization of both types of receptor (reviewed in Choii and Ko, 2015; Alvarez, 2017; Groeneweg et al., 2018; Specht, 2019). Several $GABA_A$R subunits bind to gephyrin, albeit with a lower affinity than the GlyRβ subunit (e.g., Maric et al., 2011; Kowalczyk et al., 2013). We do not yet know whether GlyRs and $GABA_A$Rs form SSDs at mixed synapses, and if so, how they are related to the SSDs of gephyrin. Mixed inhibitory synapses are activated by the co-release of glycine and GABA from presynaptic vesicles (Jonas et al., 1998; Aubrey and Supplisson, 2018). This creates a situation, where the exact position of GlyRs and $GABA_A$Rs relative to the pre-synaptic release site can have a strong impact on the efficacy of the agonists and thus the activity of the receptors. Through its capacity to resolve the spatial organization of mixed inhibitory synapses, SMLM may provide answers to these open questions.

OUTLOOK

The concept of SSDs as dynamic units underlying synaptic strength provides a new angle to interpret the function of synapses. SMLM and other super-resolution imaging techniques are powerful tools to investigate the internal organization of synapses. Given the intrinsic stochasticity of SMLM and the inherent variability of synaptic protein assemblies, however, the identification and characterization of SSDs demand great

scrutiny in the experimental and analytical procedures. Super-resolution techniques may still have some way to go before we can truly resolve the fast molecular processes at synapses.

AUTHOR CONTRIBUTIONS

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

FUNDING

XY is supported by the China Scholarship Council (CSC).

ACKNOWLEDGMENTS

We thank Andrea Barberis (IIT, Genoa) for the permission to reproduce the pointillist image in **Figure 1**.

REFERENCES

Alvarez, F. J. (2017). Gephyrin and the regulation of synaptic strength and dynamics at glycinergic inhibitory synapses. *Brain Res. Bull.* 129, 50–65. doi: 10.1016/j.brainresbull.2016.09.003

Annibale, P., Vanni, S., Scarselli, M., Rothlisberger, U., and Radenovic, A. (2011). Identification of clustering artifacts in photoactivated localization microscopy. *Nat. Methods* 8, 527–528. doi: 10.1038/nmeth.1627

Arellano, J. I., Benavides-Piccione, R., DeFelipe, J., and Yuste, R. (2007). Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. *Front. Neurosci.* 1, 131–143. doi: 10.3389/neuro.01.1.1.010.2007

Aubrey, K. R., and Supplisson, S. (2018). Heterogeneous signaling at GABA and glycine co-releasing terminals. *Front. Synaptic Neurosci.* 10:40. doi: 10.3389/fnsyn.2018.00040

Augustine, G. J., Santamaria, F., and Tanaka, K. (2003). Local calcium signaling in neurons. *Neuron* 40, 331–346. doi: 10.1016/s0896-6273(03)00639-1

Baddeley, D., and Bewersdorf, J. (2018). Biological insight from super-resolution microscopy: what we can learn from localization-based images. *Annu. Rev. Biochem.* 87, 965–989. doi: 10.1146/annurev-biochem-060815-014801

Betzig, E., Patterson, G. H., Sougrat, R., Lindwasser, O. W., Olenych, S., Bonifacino, J. S., et al. (2006). Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313, 1642–1645. doi: 10.1126/science.1127344

Biederer, T., Kaeser, P. S., and Blanpied, T. A. (2017). Transcellular nanoalignment of synaptic function. *Neuron* 96, 680–696. doi: 10.1016/j.neuron.2017.10.006

Bourne, J. N., and Harris, K. M. (2011). Coordination of size and number of excitatory and inhibitory synapses results in a balanced structural plasticity along mature hippocampal CA1 dendrites during LTP. *Hippocampus* 21, 354–373. doi: 10.1002/hipo.20768

Broadhead, M. J., Horrocks, M. H., Zhu, F., Muresan, L., Benavides-Piccione, R., DeFelipe, J., et al. (2016). PSD95 nanoclusters are postsynaptic building blocks in hippocampus circuits. *Sci. Rep.* 6:24626. doi: 10.1038/srep24626

Burgert, A., Letschert, S., Doose, S., and Sauer, M. (2015). Artifacts in single-molecule localization microscopy. *Histochem. Cell Biol.* 144, 123–131. doi: 10.1007/s00418-015-1340-4

Chamma, I., Letellier, M., Butler, C., Tessier, B., Lim, K. H., Gauthereau, I., et al. (2016a). Mapping the dynamics and nanoscale organization of synaptic adhesion proteins using monomeric streptavidin. *Nat. Commun.* 7:10773. doi: 10.1038/ncomms10773

Chamma, I., Levet, F., Sibarita, J.-B., Sainlos, M., and Thoumine, O. (2016b). Nanoscale organization of synaptic adhesion proteins revealed by single-molecule localization microscopy. *Neurophotonics* 3:041810. doi: 10.1117/1.nph.3.4.041810

Chen, H., Tang, A. H., and Blanpied, T. A. (2018). Subsynaptic spatial organization as a regulator of synaptic strength and plasticity. *Curr. Opin. Neurobiol.* 51, 147–153. doi: 10.1016/j.conb.2018.05.004

Choii, G., and Ko, J. (2015). Gephyrin: a central GABAergic synapse organizer. *Exp. Mol. Med.* 47:e158. doi: 10.1038/emm.2015.5

Choquet, D., and Triller, A. (2013). The dynamic synapse. *Neuron* 80, 691–703. doi: 10.1016/j.neuron.2013.10.013

Crosby, K. C., Gookin, S. E., Garcia, J. D., Hahm, K. M., Dell'Acqua, M. L., and Smith, K. R. (2019). Nanoscale subsynaptic domains underlie the organization of the inhibitory synapse. *Cell Rep.* 26, 3284.e3–3297.e3. doi: 10.1016/j.celrep.2019.02.070

Dani, A., Huang, B., Bergan, J., Dulac, C., and Zhuang, X. (2010). Super-resolution imaging of chemical synapses in the brain. *Neuron* 68, 843–856. doi: 10.1016/j.neuron.2010.11.021

Delgado, J. Y., and Selvin, P. R. (2018). A revised view on the role of surface AMPAR mobility in tuning synaptic transmission: limitations, tools, and alternative views. *Front. Synaptic Neurosci.* 10:21. doi: 10.3389/fnsyn.2018.00021

Dempsey, G. T., Vaughan, J. C., Chen, K. H., Bates, M., and Zhuang, X. (2011). Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging. *Nat. Methods* 8, 1027–1036. doi: 10.1038/nmeth.1768

Deschout, H., Cella Zanacchi, F., Mlodzianoski, M., Diaspro, A., Bewersdorf, J., Hess, S. T., et al. (2014). Precisely and accurately localizing single emitters in fluorescence microscopy. *Nat. Methods* 11, 253–266. doi: 10.1038/nmeth.2843

Dzyubenko, E., Rozenberg, A., Hermann, D. M., and Faissner, A. (2016). Colocalization of synapse marker proteins evaluated by STED-microscopy reveals patterns of neuronal synapse distribution *in vitro*. *J. Neurosci. Methods* 273, 149–159. doi: 10.1016/j.jneumeth.2016.09.001

Eggermann, E., Bucurenciu, I., Goswami, S. P., and Jonas, P. (2013). Europe PMC funders group nanodomain coupling between Ca^{2+} channels and sensors of exocytosis at fast mammalian synapses. *Nat. Rev. Neurosci.* 13, 7–21. doi: 10.1038/nrn3125

Endesfelder, U., Malkusch, S., Flottmann, B., Mondry, J., Liguzinski, P., Verveer, P. J., et al. (2011). Chemically induced photoswitching of fluorescent probes-A general concept for super-resolution microscopy. *Molecules* 16, 3106–3118. doi: 10.3390/molecules16043106

Fukata, Y., Dimitrov, A., Boncompain, G., Vielemeyer, O., Perez, F., and Fukata, M. (2013). Local palmitoylation cycles define activity-regulated postsynaptic subdomains. *J. Cell Biol.* 202, 145–161. doi: 10.1083/jcb.201302071

Fukazawa, Y., and Shigemoto, R. (2012). Intra-synapse-type and inter-synapse-type relationships between synaptic size and AMPAR expression. *Curr. Opin. Neurobiol.* 22, 446–452. doi: 10.1016/j.conb.2012.01.006

Ganeshina, O., Berry, R. W., Petralia, R. S., Nicholson, D. A., and Geinisman, Y. (2004). Synapses with a segmented, completely partitioned postsynaptic density express more AMPA receptors than other axospinous synaptic junctions. *Neuroscience* 125, 615–623. doi: 10.1016/j.neuroscience.2004.02.025

Ghelani, T., and Sigrist, S. J. (2018). Coupling the structural and functional assembly of synaptic release sites. *Front. Neuroanat.* 12:81. doi: 10.3389/fnana.2018.00081

Giannone, G., Hosy, E., Levet, F., Constals, A., Schulze, K., Sobolevsky, A. I., et al. (2010). Dynamic superresolution imaging of endogenous proteins on living cells at ultra-high density. *Biophys. J.* 99, 1303–1310. doi: 10.1016/j.bpj.2010.06.005

Glebov, O. O., Jackson, R. E., Winterflood, C. M., Owen, D. M., Barker, E. A., Doherty, P., et al. (2017). Nanoscale structural plasticity of the active zone matrix modulates presynaptic function. *Cell Rep.* 18, 2715–2728. doi: 10.1016/j.celrep.2017.02.064

Groeneweg, F. L., Trattnig, C., Kuhse, J., Nawrotzki, R. A., and Kirsch, J. (2018). Gephyrin: a key regulatory protein of inhibitory synapses and beyond. *Histochem. Cell Biol.* 150, 489–508. doi: 10.1007/s00418-018-1725-2

Gustafsson, M. G. L. (2000). Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J. Microsc.* 198, 82–87. doi: 10.1046/j.1365-2818.2000.00710.x

Haas, K. T., Compans, B., Letellier, M., Bartol, T. M., Grillo-Bosch, D., Sejnowski, T. J., et al. (2018). Pre-post synaptic alignment through neuroligin-1

tunes synaptic transmission efficiency. *ELife* 7:e31755. doi: 10.7554/elife.31755

Harris, K. M., and Stevens, J. K. (1989). Dendritic spines of CA 1 pyramical cells in the rat hippocampus serial electron microscopy with reference to their biophysical characteristics. *J. Neurosci.* 9, 2982–2997. doi: 10.1523/JNEUROSCI.09-08-02982.1989

Hess, S. T., Girirajan, T. P. K., and Mason, M. D. (2006). Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys. J.* 91, 4256–4272. doi: 10.1529/biophysj.106.091116

High, B., Cole, A. A., Chen, X., and Reese, T. S. (2015). Electron microscopic tomography reveals discrete transcleft elements at excitatory and inhibitory synapses. *Front. Synaptic Neurosci.* 7:9. doi: 10.3389/fnsyn.2015.00009

Hruska, M., Henderson, N., Le Marchand, S. J., Jafri, H., and Dalva, M. B. (2018). Synaptic nanomodules underlie the organization and plasticity of spine synapses. *Nat. Neurosci.* 21, 671–682. doi: 10.1038/s41593-018-0138-9

Jonas, P., Bischofberger, J., and Sandkühler, J. (1998). Corelease of two fast neurotransmitters at a central synapse. *Science* 281, 419–424. doi: 10.1126/science.281.5375.419

Klar, T. A., Jakobs, S., Dyba, M., Egner, A., and Hell, S. W. (2000). Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission Physical Principles and Setup. *Proc. Natl. Acad. Sci. U S A* 97, 8206–8210. doi: 10.1073/pnas.97.15.8206

Kowalczyk, S., Winkelmann, A., Smolinsky, B., Förstera, B., Neundorf, I., Schwarz, G., et al. (2013). Direct binding of $GABA_A$ receptor β2 and β3 subunits to gephyrin. *Eur. J. Neurosci.* 37, 544–554. doi: 10.1111/ejn.12078

Linsalata, A. E., Chen, X., Winters, C. A., and Reese, T. (2014). Electron tomography on γ-aminobutyric acid-ergic synapses reveals a discontinuous postsynaptic network of filaments. *J. Comp. Neurol.* 522, 921–936. doi: 10.1002/cne.23453

Liu, K. K. L., Hagan, M. F., and Lisman, J. E. (2017). Gradation (approx. 10 size states) of synaptic strength by quantal addition of structural modules. *Philos. Trans. R. Soc. B Biol. Sci.* 372:20160328. doi: 10.1098/rstb.2016.0328

MacGillavry, H. D., Song, Y., Raghavachari, S., and Blanpied, T. A. (2013). Nanoscale scaffolding domains within the postsynaptic density concentrate synaptic ampa receptors. *Neuron* 78, 615–622. doi: 10.1016/j.neuron.2013.03.009

Maidorn, M., Rizzoli, S. O., and Opazo, F. (2016). Tools and limitations to study the molecular composition of synapses by fluorescence microscopy. *Biochem. J.* 473, 3385–3399. doi: 10.1042/bcj20160366

Maric, H., Mukherjee, J., Tretter, V., Moss, S. J., and Schindelin, H. (2011). Gephyrin-mediated γ -aminobutyric acid type A and glycine receptor clustering relies on a common binding site. *J. Biol. Chem.* 286, 42105–42114. doi: 10.1074/jbc.M111.303412

Masch, J.-M., Steffens, H., Fischer, J., Engelhardt, J., Hubrich, J., Keller-Findeisen, J., et al. (2018). Robust nanoscopy of a synaptic protein in living mice by organic-fluorophore labeling. *Proc. Natl. Acad. Sci. U S A* 115, E8047–E8056. doi: 10.1073/pnas.1807104115

Masugi-Tokita, M., Tarusawa, E., Watanabe, M., Molnár, E., Fujimoto, K., and Shigemoto, R. (2007). Number and density of AMPA receptors in individual synapses in the rat cerebellum as revealed by SDS-digested freeze-fracture replica labeling. *J. Neurosci.* 27, 2135–2144. doi: 10.1523/JNEUROSCI.2861-06.2007

Nahidiazar, L., Agronskaia, A. V., Broertjes, J., van den Broek, B., and Jalink, K. (2016). Optimizing imaging conditions for demanding multi-color super resolution localization microscopy. *PLoS One* 11:e0158884. doi: 10.1371/journal.pone.0158884

Nair, D., Hosy, E., Petersen, J. D., Constals, A., Giannone, G., Choquet, D., et al. (2013). Super-resolution imaging reveals that AMPA receptors inside synapses are dynamically organized in nanodomains regulated by PSD95. *J. Neurosci.* 33, 13204–13224. doi: 10.1523/JNEUROSCI.2381-12.2013

Nicovich, P. R., Owen, D. M., and Gaus, K. (2017). Turning single-molecule localization microscopy into a quantitative bioanalytical tool. *Nat. Protoc.* 12, 453–460. doi: 10.1038/nprot.2016.166

Nieves, D. J., Gaus, K., and Baker, M. A. B. (2018). DNA-based super-resolution microscopy: DNA-PAINT. *Genes* 9:E621. doi: 10.3390/genes9120621

Nusser, Z., Cull-Candy, S., and Farrant, M. (1997). Differences in synaptic $GABA_A$ receptor number underlie variation in GABA mini amplitude. *Neuron* 19, 697–709. doi: 10.1016/s0896-6273(00)80382-7

Nusser, Z., Hájos, N., Somogyi, P., and Mody, I. (1998). Increased number of synaptic $GABA_A$ receptors underlies potentiation at hippocampal inhibitory synapses. *Nature* 395, 172–177. doi: 10.1038/25999

Patriarchi, T., Buonarati, O. R., and Hell, J. W. (2018). Postsynaptic localization and regulation of AMPA receptors and Cav1.2 by β2 adrenergic receptor/PKA and Ca^{2+}/CaMKII signaling. *EMBO J.* 37:e99771. doi: 10.15252/embj.201899771

Patrizio, A., Renner, M., Pizzarelli, R., Triller, A., and Specht, C. G. (2017). Alpha subunit-dependent glycine receptor clustering and regulation of synaptic receptor numbers. *Sci. Rep.* 7:10899. doi: 10.1038/s41598-017-11264-3

Pennacchietti, F., Vascon, S., Nieus, T., Rosillo, C., Das, S., Tyagarajan, S. K., et al. (2017). Nanoscale molecular reorganization of the inhibitory postsynaptic density is a determinant of gabaergic synaptic potentiation. *J. Neurosci.* 37, 1747–1756. doi: 10.1523/jneurosci.0514-16.2016

Perez de Arce, K., Schrod, N., Metzbower, S. W. R. R., Allgeyer, E., Kong, G. K. W., Tang, A. H., et al. (2015). Topographic mapping of the synaptic cleft into adhesive nanodomains. *Neuron* 88, 1165–1172. doi: 10.1016/j.neuron.2015.11.011

Rodriguez, P. C., Almeida, L. G., and Triller, A. (2017). Continuous rearrangement of the postsynaptic gephyrin scaffolding domain: a super-resolution quantified and energetic approach. *BioRxiv* [Preprint]. 33. doi: 10.1101/193698

Rust, M. J., Bates, M., and Zhuang, X. (2006). Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* 3, 793–795. doi: 10.1038/nmeth929

Santuy, A., Rodríguez, J.-R., DeFelipe, J., and Merchán-Pérez, A. (2018). Study of the size and shape of synapses in the juvenile rat somatosensory cortex with 3d electron microscopy. *eNeuro* 5:ENEURO.0377-17.2017. doi: 10.1523/eneuro.0377-17.2017

Scheefhals, N., and MacGillavry, H. D. (2018). Functional organization of postsynaptic glutamate receptors. *Mol. Cell. Neurosci.* 91, 82–94. doi: 10.1016/j.mcn.2018.05.002

Schermelleh, L., Heintzmann, R., and Leonhardt, H. (2010). A guide to super-resolution fluorescence microscopy. *J. Cell Biol.* 190, 165–175. doi: 10.1083/jcb.201002018

Sheng, M., and Kim, E. (2011). The postsynaptic organization of synapses. *Cold Spring Harb. Perspect. Biol.* 3:a005678. doi: 10.1101/cshperspect.a005678

Shinohara, Y., Hirase, H., Watanabe, M., Itakura, M., Takahashi, M., and Shigemoto, R. (2008). Left-right asymmetry of the hippocampal synapses with differential subunit allocation of glutamate receptors. *Proc. Natl. Acad. Sci. U S A* 105, 19498–19503. doi: 10.1073/pnas.0807461105

Sieben, C., Douglass, K. M., Guichard, P., and Manley, S. (2018). Super-resolution microscopy to decipher multi-molecular assemblies. *Curr. Opin. Struct. Biol.* 49, 169–176. doi: 10.1016/j.sbi.2018.03.017

Specht, C. G. (2019). Fractional occupancy of synaptic binding sites and the molecular plasticity of inhibitory synapses. *Neuropharmacology* doi: 10.1016/j.neuropharm.2019.01.008 [Epub ahead of print].

Specht, C. G., Izeddin, I., and Dahan, M. (2014). "Visualizing the ultrastructures and dynamics of synapses by single-molecule nanoscopy," in *Nanoscale Imaging of Synapses. Neuromethods, Vol 84*, eds U. V. Nägerl and A. Triller (New York, NY: Humana Press), 75–91.

Specht, C. G., Izeddin, I., Rodriguez, P. C., El Beheiry, M., Rostaing, P., Darzacq, X., et al. (2013). Quantitative nanoscopy of inhibitory synapses: counting gephyrin molecules and receptor binding sites. *Neuron* 79, 308–321. doi: 10.1016/j.neuron.2013.05.013

Sugiyama, Y., Kawabata, I., Sobue, K., and Okabe, S. (2005). Determination of absolute protein numbers in single synapses by a GFP-based calibration technique. *Nat. Methods* 2, 677–684. doi: 10.1038/nmeth783

Tang, A. H., Chen, H., Li, T. P., Metzbower, S. R., MacGillavry, H. D., and Blanpied, T. A. (2016). A trans-synaptic nanocolumn aligns neurotransmitter release to receptors. *Nature* 536, 210–214. doi: 10.1038/nature19058

Tarusawa, E., Matsui, K., Budisantoso, T., Molnar, E., Watanabe, M., Matsui, M., et al. (2009). Input-specific intrasynaptic arrangements of ionotropic glutamate receptors and their impact on postsynaptic responses. *J. Neurosci.* 29, 12896–12908. doi: 10.1523/JNEUROSCI.6160-08.2009

Turkowyd, B., Virant, D., and Endesfelder, U. (2016). From single molecules to life: microscopy at the nanoscale. *Anal. Bioanal. Chem.* 408, 6885–6911. doi: 10.1007/s00216-016-9781-8

van de Linde, S., Löschberger, A., Klein, T., Heidbreder, M., Wolter, S., Heilemann, M., et al. (2011). Direct stochastic optical reconstruction microscopy with standard fluorescent probes. *Nat. Protoc.* 6, 991–1009. doi: 10.1038/nprot.2011.336

Wegner, W., Mott, A. C., Grant, S. G. N., Steffens, H., and Willig, K. I. (2018). *In vivo* STED microscopy visualizes PSD95 sub-structures and morphological changes over several hours in the mouse visual cortex. *Sci. Rep.* 8:219. doi: 10.1038/s41598-017-18640-z

Yang, X., and Specht, C. G. (in press). "Practical guidelines for two-color SMLM of synaptic proteins in cultured neurons," in *Single Molecule Microscopy in Neurobiology*, eds Y. Okada and N. Yamamoto (New York, NY: Humana Press).

Zanacchi, F. C., Manzo, C., Alvarez, A. S., Derr, N. D., Garcia-Parajo, M. F., and Lakadamyali, M. (2017). A DNA origami platform for quantifying protein copy number in super-resolution. *Nat. Methods* 14, 789–792. doi: 10.1038/nmeth.4342

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 Molecular Neuroscience

REVIEW
published: 03 July 2019
doi: 10.3389/fnmol.2019.00168

From Hiring to Firing: Activation of Inhibitory Neurons and Their Recruitment in Behavior

Olivia K. Swanson[1,2†] *and Arianna Maffei*[1,2*†]

[1]*Department of Neurobiology and Behavior, SUNY—Stony Brook, Stony Brook, NY, United States,* [2]*Graduate Program in Neuroscience, SUNY—Stony Brook, Stony Brook, NY, United States*

The investigation of GABAergic inhibitory circuits has substantially expanded over the past few years. The development of new tools and technology has allowed investigators to classify many diverse groups of inhibitory neurons by several delineating factors: these include their connectivity motifs, expression of specific molecular markers, receptor diversity, and ultimately their role in brain function. Despite this progress, however, there is still limited understanding of how GABAergic neurons are recruited by their input and how their activity is modulated by behavioral states. This limitation is primarily due to the fact that studies of GABAergic inhibition are mainly geared toward determining how, once activated, inhibitory circuits regulate the activity of excitatory neurons. In this review article, we will outline recent work investigating the anatomical and physiological properties of inputs that activate cortical GABAergic neurons, and discuss how these inhibitory cells are differentially recruited during behavior.

Keywords: GABA, synapses, circuit, excitability, plasticity

OPEN ACCESS

Edited by:
Stephen Brickley,
Imperial College London,
United Kingdom

Reviewed by:
Hiroyuki Hioki,
Juntendo University, Japan
Jamie Maguire,
Tufts University School of Medicine,
United States

***Correspondence:**
Arianna Maffei
arianna.maffei@stonybrook.edu;
olivia.swanson@stonybrook.edu

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

Received: *29 April 2019*
Accepted: *17 June 2019*
Published: *03 July 2019*

Citation:
Swanson OK and Maffei A (2019) From Hiring to Firing: Activation of Inhibitory Neurons and Their Recruitment in Behavior. Front. Mol. Neurosci. 12:168. doi: 10.3389/fnmol.2019.00168

INTRODUCTION

Inhibitory interneurons constitute a small but crucial neuronal class in the cortex. While these cells comprise only 10%–20% of the total neural population, their connectivity and recruitment are essential in sensation, movement, and cognition. One difficulty in synthesizing the role of inhibitory cells lies in their diversity: these neurons express an array of molecular markers and have heterogeneous firing properties as well as distinct synaptic connectivity (Kubota, 2014). However, the diversity of inhibitory neurons allows these cells to provide the appropriate inhibition for a wide variety of stimuli and behaviors. Great strides have been made in identifying clusters of inhibitory interneuron groups based on their varying gene expression (Cauli et al., 2000; Kubota et al., 2011; Tasic et al., 2016; Paul et al., 2017). These data demonstrate that while some genes are expressed to varying degrees across several interneuron types, there are certain non-overlapping markers that can be used to delineate broad groups of inhibitory interneuron groups. This review article will focus on three largely non-overlapping classes of inhibitory interneurons in the rodent cortex that express the following molecular markers: parvalbumin (PV^+), somatostatin (SST^+), and type 3 serotonin receptor ($5HT_3$), particularly focused on $5HT_3{}^+$ neurons that express vasoactive intestinal peptide (VIP^+; Xu et al., 2010; Rudy et al., 2011; Tremblay et al., 2016). PV^+ inhibitory neurons are typically fast-spiking basket cells, found

mainly in layers 4 and 5, that preferentially contact the perisomatic region of pyramidal neurons (Nassar et al., 2015; Neske et al., 2015). SST^+ inhibitory neurons include Martinotti cells found in layers 5 and 6 that contact dendrites of pyramidal neurons (Yavorska and Wehr, 2016). VIP^+ interneurons are bipolar or multipolar inhibitory neurons, found most densely in layer 2/3, that exert disinhibitory control in the cortex by synapsing onto other inhibitory neuron groups (Pronneke et al., 2015). These inhibitory neurons also have a high level of interconnectivity, with each subtype displaying a connection preference to one another, as well as neighboring pyramidal neurons (Jiang et al., 2015; Tremblay et al., 2016). Taken together, inhibitory interneuron classes span all layers of the cortical mantle and thus can powerfully regulate excitatory activity across the cortex. As inhibition is an essential mechanism in maintaining balanced cortical activity (Isaacson and Scanziani, 2011; Tatti et al., 2017), long-range inputs to a given cortical area often simultaneously activate one or more types of inhibitory interneurons as well as pyramidal neurons. This review article will discuss recent results regarding the recruitment of GABAergic neurons by long-range driving and modulating inputs. We will then discuss how the recruitment of cortical interneurons plays a role in the function of cognitive, motor, and sensory cortices.

THALAMIC EXCITATION OF INHIBITORY NEURONS

A major source of excitation to cortical inhibitory neurons arises from the thalamus. Generally, GABAergic neurons receive the largest input from thalamic regions most functionally relevant to their own cortical region, and excitation *via* these pathways is not uniform across interneuron subtype. For example, anatomical studies indicate that PV^+, SST^+, and VIP^+ neurons in somatosensory cortex (S1) receive similar innervation from the ventroposteromedial (VPM) and the posteromedial nucleus (POm) of the thalamus, which are two major thalamic inputs to S1 that are widely known to transmit somatosensory-related signals to the cortex (Landisman and Connors, 2007; Castejon et al., 2016; Wall et al., 2016). However, electrophysiological studies in S1 reveal that thalamocortical (TC) inputs onto these neurons are not congruent: PV^+ neurons respond with a higher connection probability, higher likelihood to spike, and strong synaptic depression to subsequent stimulation, while SST^+ neurons show lower connection probability and facilitating, smaller magnitude responses that have a longer latency from stimulus onset (Cruikshank et al., 2007, 2010; Tan et al., 2008). Additionally, the response of PV^+ interneurons is often comparable to or larger than that of a simultaneously recorded excitatory neurons (Cruikshank et al., 2007, 2010), and they can mediate powerful feedforward inhibition following TC stimulation, particularly in layer 4 (Sun et al., 2006). Input from higher-order thalamic nuclei, like the POm, activates PV^+ and VIP^+ interneurons but suppresses SST^+ neurons (Audette et al., 2018; Williams and Holtmaat, 2019). Inputs to inhibitory neurons from the POm also show laminar specificity, with PV^+ interneurons showing highest connection probability and response amplitude in layer 5 and inhibitory neurons expressing $5HT_3$, including VIP^+ cells, showing largest amplitudes in superficial layers. As VIP^+ interneurons tend to have a disinhibitory action on cortical circuits, their activation by a high order somatosensory thalamic projection may play a role in the recently reported powerful, long-lasting excitation of superficial S1 by the POm (Zhang and Bruno, 2019).

In the primary auditory and visual cortices, TC input (from the medial geniculate body and the lateral geniculate nucleus, respectively), drives excitatory postsynaptic currents (EPSCs) in PV^+, SST^+ and VIP^+ interneurons, however, PV^+ interneurons have a higher connection probability with TC axons, and the input is larger than that of the other interneurons, as well as excitatory neurons (Kloc and Maffei, 2014). In both cortices, TC input to SST^+ and VIP^+ interneurons is largely restricted to layer 4, where these neurons show a low connection probability to this input and the magnitude of current is 1/10 of that onto PV^+ cells (Ji et al., 2016). Similar results were reported in frontal, cognitive-associated cortices: Electron microscopy studies showed that axons from the mediodorsal thalamus synapse onto at least three types of inhibitory neurons, including PV^+, $calretinin^+$, and $calbindin^+$ (Rotaru et al., 2005). Calretinin and calbindin are calcium-binding proteins used to mark interneurons, and each has been shown to colocalize to a considerable degree with VIP and SST, respectively (Gonchar et al., 2007). Despite anatomically defined inputs onto each of these inhibitory neurons, electrophysiological stimulation of this pathway revealed that the mediodorsal thalamus drives feedforward inhibition *via* PV^+ but not SST^+ interneurons (Delevich et al., 2015). An electron microscopy study in the secondary motor cortex (M2) has also shown that motor thalamic input from the ventroanterior and ventromedial nuclei made synapses onto the soma and dendrites of PV^+ interneurons in L2/3 and L5, respectively (Shigematsu et al., 2016). Finally, in the presubiculum, which is a region in the parahippocampal cortex involved in spatial orientation of the head, the anterior thalamic nuclei carrying head direction-related information synapse onto PV^+ but not SST^+ neurons (Nassar et al., 2018). Taken together, these data suggest that TC pathways synapse onto a variety of cortical inhibitory cells, including those expressing PV, SST, and VIP. While anatomical tract tracing studies confirm that TC axons form synapses onto these interneuron subtypes, electrophysiological analyses of these inputs reveal that PV^+ neurons are the most commonly targeted subtype, and they also receive the strongest input. This could possibly be explained by a differential somatodendritic localization of TC synapses onto each inhibitory neuron type. Several studies have investigated the distribution of TC boutons along PV^+ neurons in S1 (Bagnall et al., 2011; Kameda et al., 2012; Hioki, 2015). These studies revealed that TC inputs to PV^+ neurons can show differential anatomical organization that correlates with the power of the connection, where the strongest synaptic input was provided by a concentrated cluster of release sits on the primary dendrites of the GABAergic cell. In contrast, a similar study focused on VIP^+ interneurons showed that these cells mainly receive thalamic input along their distal dendrites

(Sohn et al., 2016). Further studies connecting the anatomic location of TC synapses with physiology data would possibly bridge synapse location and response strength for each inhibitory cell type. For example, in primary visual cortex (V1), TC input to fast-spiking interneurons in the V1 is due to the activation of several powerful release sites (Kloc and Maffei, 2014), but whether the structure/function relationship at these synapses follows this motif, and is generalized to all TC inputs, is unknown. A thorough understanding of the location of all TC synapses on each inhibitory neuron subtype is essential to synthesize these bodies of data.

CORTICOCORTICAL EXCITATION OF INHIBITORY NEURONS

Inhibitory neurons are also excited by inter-areal cortical inputs. While less is known about the anatomy and physiology of long-range cortical inputs onto GABAergic neurons, there have been several interesting trans-synaptic tracing studies of these pathways that suggest that these cells receive a highly diverse set of inputs from many cortical areas. As expected, the source of these cortical inputs depends on the function of the cortex studied: for example, GABAergic neurons in the barrel cortex are contacted by axons from cortical areas including the ipsilateral secondary somatosensory cortex, the contralateral S1, and the primary motor cortex (M1), while the inputs from other sensory or limbic cortices is limited (Wall et al., 2016). When analyzed on an anatomical level, the proportion of input from these cortical sources onto PV^+, SST^+, and VIP^+ neurons was comparable. Optogenetic stimulation of the corticocortical pathway from M1 to S1, however, revealed that this input is strongest onto VIP^+ interneurons (Lee et al., 2013). VIP^+ interneurons showed the largest response to M1 input which exceeded that of simultaneously recorded pyramidal neurons, and these responses showed synaptic depression. PV^+ interneurons also showed depressing responses comparable to that of pyramidal neurons, while SST^+ interneurons had the weakest connection, with facilitating responses that were much smaller than those of excitatory cells. In the visual system, trans-synaptic tracing performed in V1 showed that PV^+ neurons receive input from the secondary visual cortex, auditory cortex (A1), S1, parietal association area, M2, and the contralateral V1 (Lu et al., 2014). Functional study of this excitatory input to V1 PV^+ neurons from M2 and contralateral V1 has revealed that these inputs exhibit strong short-term depression. However just as there is a high level of variety in corticocortical (CC) projections, there is also diversity in the postsynaptic targets of these pathways. For example, the cingulate cortex projects to the ipsilateral V1, and selective inactivation of either PV^+, SST^+, or VIP^+ neurons coinciding with this pathway during a visual discrimination task disrupted normal center-surround modulation (Zhang et al., 2014). This suggests that there are specific motifs for inhibitory interneuron activation dependent on the CC pathway.

A common feature of CC activation of inhibitory neurons is the generation of feedforward inhibition, mediated largely by PV^+ interneurons. In the prefrontal cortex, both PV^+ and SST^+ interneurons receive a monosynaptic, glutamatergic input from the contralateral cortex (Anastasiades et al., 2018). While activation of this pathway can drive both PV^+ and SST^+ neurons to fire, a suprathreshold response is more frequent in PV^+ interneurons, which could indicate they are the primary drivers of feedforward inhibition in this pathway. Feedforward inhibition by PV^+ interneurons was also observed in the callosal input to A1: inputs from the contralateral A1 make synapses onto PV^+ and SST^+ interneurons, and this callosal activation of PV^+ neurons drives selective inhibition of adjacent CC-projecting pyramidal neurons (Rock and Apicella, 2015; Oviedo, 2017). However, this does not appear to be the case regarding callosal input to motor cortices, at least not in deep layers: electron microscopy of these inputs to layer 6 PV^+ interneurons show a direct synaptic connection, however, feedforward inhibition can only be evoked following callosal stimulation in a subset of neighboring pyramidal neurons (Karayannis et al., 2007). This suggests that feedforward inhibition following callosal stimulation may be limited to specific cortical layers or regions.

OTHER SOURCES OF EXCITATION

In addition to thalamic and CC inputs, GABAergic interneurons in cortical circuits can be recruited by the amygdala. While the amygdala is known for processing signals related to emotions and fear memory, the recruitment of cortical inhibitory circuits by amygdalar projections remained controversial until recently. Publications from several groups reported that amygdalocortical pathway activation can have both excitatory and inhibitory effects, suggesting that perhaps the amygdala engages both excitatory and inhibitory circuits in the cortex (Yamamoto et al., 1984; Hanamori, 2009). A recent study demonstrated that the basolateral nucleus of the amygdala (BLA) can evoke feedforward excitatory and inhibitory responses in the insular cortex (Stone et al., 2011), further bolstering this idea. These results were confirmed and expanded by studies examining amygdalocortical projections to a variety of cortical circuits: in the prefrontal cortex, the BLA projects directly onto PV^+ GABAergic neurons, which in turn exert feedforward inhibition onto nearby pyramidal cells (Dilgen et al., 2013; Cheriyan et al., 2016). Subsequent studies using optogenetic approaches to selectively stimulate BLA afferents demonstrated that BLA axons make synapses onto both PV^+ and SST^+ interneurons in the insular and prefrontal cortex (Haley et al., 2016; McGarry and Carter, 2016). This input is robust onto both interneuron types, however, analysis of this synapse's short-term dynamics revealed that BLA input to PV^+ interneurons is depressing, while input onto SST^+ interneurons is stable or facilitating across trains of stimuli. This suggests that excitatory inputs from the BLA to cortical GABAergic interneurons follow the same short-term dynamics as those from thalamic and cortical sources.

MODULATION OF INHIBITORY NEURONS

In addition to being directly recruited by glutamatergic inputs, inhibitory neurons are known to express receptors for neuromodulators, indicating that their activity is also

subject to state changes and the release of a variety of neurotransmitters. Interestingly, while all three inhibitory neuron types express neuromodulatory receptors, the ratio of expression is unique to each (Paul et al., 2017). For example, PV^+ neurons preferentially express the genes for serotonin and opioid receptors, while SST^+ cells express a wider variety of neuromodulatory receptor genes. Results showed that SST^+ interneurons express genes for cholinergic, serotonergic, and oxytocinergic receptors, as well as those that bind substance P and orexin. VIP^+ interneurons showed the highest and most diverse expression for neuromodulatory receptors, including those that bind serotonin, acetylcholine, neuropeptide Y, and catecholamines. Another study found that PV^+ and SST^+ inhibitory neurons in the prefrontal cortex express neurotensin-1 receptors that are activated by neurotensin co-released by dopaminergic afferents in this cortical area (Petrie et al., 2005). Release of neurotensin within the prefrontal cortex increased extracellular GABA, indicating that neuromodulation of these interneurons can directly lead to changes in inhibitory activity.

PV^+, SST^+, and VIP^+ neurons in the barrel cortex receive input from the Basal nucleus of Meynert, which is a source of cholinergic input (Wall et al., 2016). This anatomical work is further supported by transcriptional analysis in M1 and S1 showing that all three GABAergic interneuron subtypes express cholinergic receptors (Paul et al., 2017). Cholinergic modulation of inhibitory interneurons has also been observed in V1, where stimulation of the pathway from the Basal nucleus of Meynert to V1 decorrelates neural response *via* SST^+ interneuron activity (Chen N. et al., 2015). In V1, an *in vivo* calcium imaging study showed that stimulation cholinergic input to the cortex modified the responses of nearly all VIP^+ interneurons studied, and roughly half of PV^+ neurons, while SST^+ interneurons were rarely affected (Alitto and Dan, 2012). Interestingly, cholinergic stimulation consistently increased intracellular calcium in VIP^+ interneurons, while in PV^+ interneurons the responses were heterogeneous.

The noradrenergic system also differentially engages GABAergic neurons. Stimulation of the locus coeruleus, which provides the source of noradrenaline to the cortex, drives an increase in cFos expression in PV^+ and SST^+ and, to a lesser extent, VIP^+ neurons (Toussay et al., 2013). In the rat frontal cortex, noradrenaline depolarizes fast-spiking (putative PV^+) interneurons, while it depolarizes and drives SST^+ interneurons to fire (Kawaguchi and Shindou, 1998).

Together, these studies highlight the complexity of the recruitment structure for inhibitory interneurons (**Figure 1**). These cells are poised at a critical position within cortical circuits: they are often activated by long-range glutamatergic and modulatory inputs alongside excitatory neurons, and thus can act as gating mechanisms for cortical activity. The diversity of means to drive inhibitory neurons also indicates that the function of GABAergic cells goes well beyond simply controlling principal neuron excitability.

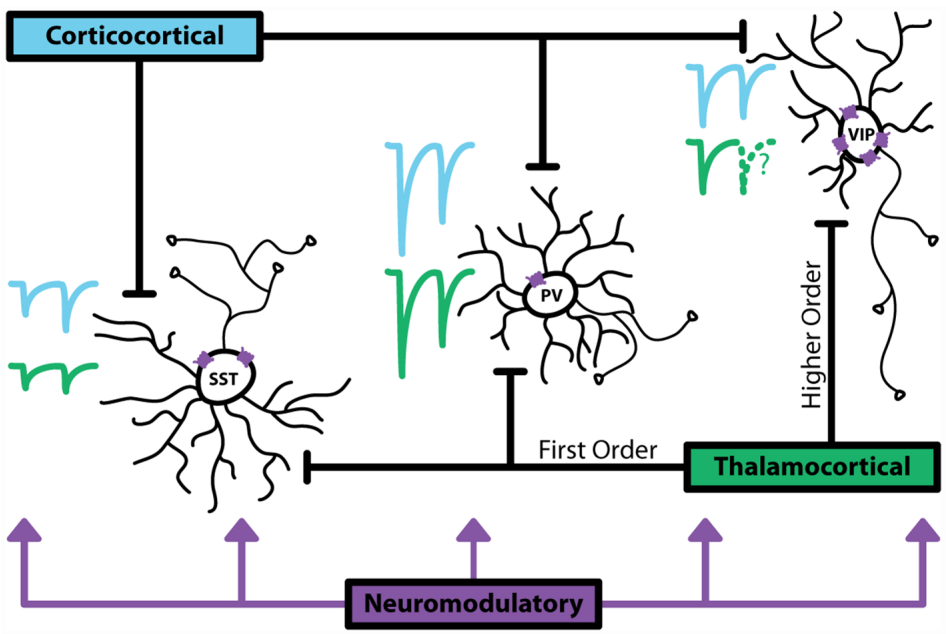

FIGURE 1 | Major sources of activation to inhibitory interneurons. Corticocortical (CC) inputs are represented with blue traces, thalamocortical (TC) inputs are represented with green traces. Relative sizes of excitatory postsynaptic currents (EPSCs) show that generally parvalbumin (PV^+) interneurons receive large, depressing inputs. Somatostatin (SST^+) interneurons receive smaller inputs that facilitate. vasoactive intestinal peptide (VIP^+) interneurons have been shown in anatomical studies to receive synapses from CC and TC pathways, however, data characterizing the magnitude and dynamics of these synapses is limited. VIP^+ express the highest, more diverse levels of neuromodulatory receptors, indicating that these interneurons are a major target for non-glutamatergic or GABAergic activation. Dotted lines indicate lack of data.

INHIBITORY NEURONS AND COGNITION

The recent availability of a variety of experimental tools for the selective activation/inactivation of GABAergic neurons facilitated the investigation of their contribution to complex functions. GABAergic neuron activity in the prefrontal cortex is necessary for several aspects of healthy cognition. Using a selective toxin, PV^+ neuron-specific disruption produced cognitive deficits comparable to those observed following non-specific prefrontal cortex lesion (Murray et al., 2015). Additionally, there is a small population of PV^+ and VIP^+ neurons in the prefrontal cortex that project to the nucleus accumbens (Lee et al., 2014). Stimulation of this long-range GABAergic pathway induced avoidance behavior while the animal performed a place preference task, suggesting that these neurons are involved in conveying aversive signals to the accumbens. Inhibitory activity in the anterior cingulate cortex is also engaged during foraging tasks, with inhibitory neuron subtypes engaged differentially during specific aspects of the behavior (Kvitsiani et al., 2013). PV^+ interneurons were most active when animals were leaving the reward zone, while SST^+ interneurons were highly active until animals entered the reward zone. Subtype-specific recruitment of inhibitory neurons has also been observed during working memory tasks (Kim et al., 2016). SST^+ interneurons showed strong delay period target-dependent activity and only narrow-spiking SST^+ cells were suppressed by reward. Differently, PV^+ interneurons did not show strong activity during the delay period, however, nearly all were strongly suppressed by reward. It is also important to note that these interneurons have been implicated in the generation of synchronized neural firing, specifically that of gamma and theta oscillations (Fanselow et al., 2008; Gonzalez-Burgos and Lewis, 2008; Sohal et al., 2009). In the cortex, PV^+ interneurons appear to be involved in gamma oscillations, while SST^+ interneurons play a role in theta oscillations. Synchrony between brain areas is important for working memory, memory retrieval, cognitive integration, and information processing, thus the proper activity of inhibitory interneurons is integral to the generation of specific brain states and healthy cognition.

How these groups of neurons are recruited by their inputs during executive functions and the mechanisms regulating their responses are still under investigation. Compared to sensory cortices, the activity of specific inhibitory neuron populations in prefrontal areas remains understudied. To fully understand the role of these cells in cognition, it will require a synergistic approach relying on experimental and theoretical efforts that examine these processes at varied levels of resolution to bridge the gap between connectivity and functional recruitment.

INHIBITORY NEURONS AND MOTOR FUNCTION

Motor learning leads to the engagement of inhibitory elements in M1, which leads to plastic changes at both excitatory and inhibitory synapses (**Figure 2**). 2-photon imaging of axon terminal fields in M1 reported an increase in PV^+ boutons during training of a lever-press task in mice, while the training of this task led to a decrease in SST^+ boutons (Chen S. X. et al., 2015). Interestingly, activation or deactivation of SST^+ neurons led to bidirectional changes in spines on neighboring pyramidal neurons. Pyramidal neuron spine reorganization is a common feature of learning, so these results suggest that during motor training activity of SST^+ interneurons is important for learning-dependent plasticity. Additionally, animals trained to run on an accelerating rotarod show a switch in PV expression in M1, as well as inhibitory input onto these cells, across the learning period: during the training period, there is low PV expression in the during training period, with an increase in VIP^+ inhibitory boutons onto PV^+ cells (Donato et al., 2013). Once performance saturates, PV expression in M1 is high, accompanied by a reduction in inhibitory boutons and an increase in excitatory boutons onto these PV^+ neurons. Taken together, these results indicate that PV^+, SST^+ and VIP^+ inhibitory interneurons are differentially engaged during motor learning. GABAergic interneurons are also engaged during the execution of a learned movement: one study trained mice on a sensory stimulation-triggered reaching task and used extracellular recordings and optotagging to selectively monitor regular spiking and fast-spiking cell populations (Estebanez et al., 2017). The results of this study showed that PV^+ interneurons in M1 increased their firing in response to the sensory cue as well as the onset of reaching, suggesting that PV^+ interneurons additionally participate in voluntary movement execution. Another study focused on the role of a small group of PV^+ and SST^+ neurons that project from M1 and M2 to the dorsolateral

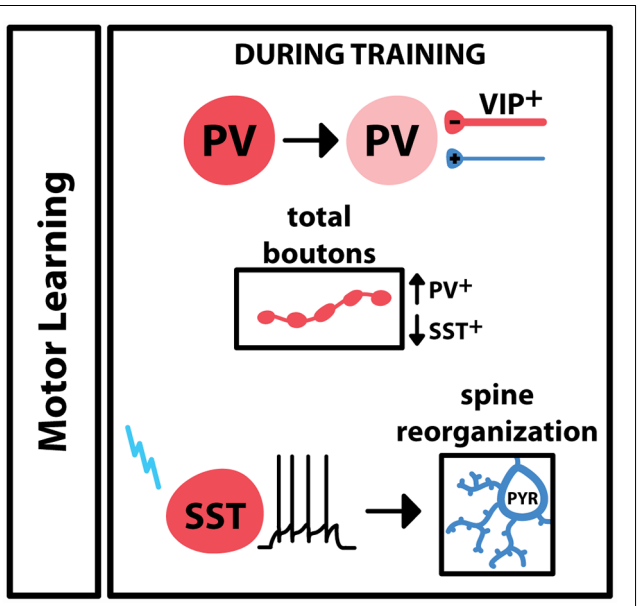

FIGURE 2 | Engagement of GABAergic interneurons during motor learning. In primary motor cortex (M1), motor training drives a decrease in the expression of PV in PV^+ interneurons, and an increase of VIP^+ boutons onto these cells. Overall PV^+ boutons increase during training; SST^+ boutons decrease during training. Motor learning is associated with changes in pyramidal spine dynamics, and these changes have been shown to follow specific activation of the SST^+ interneuron.

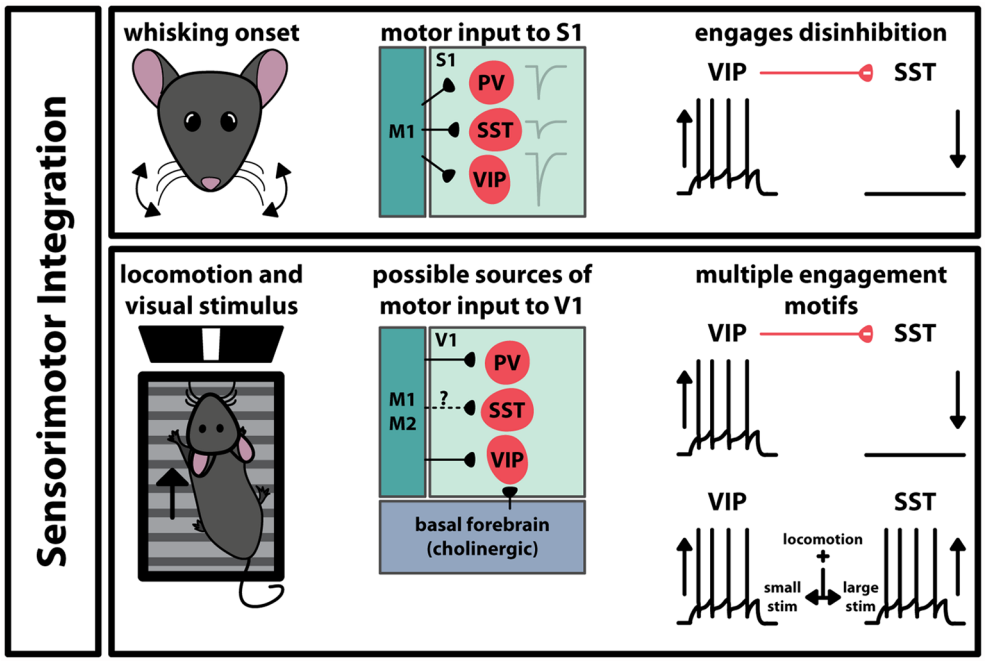

FIGURE 3 | Engagement of GABAergic interneurons during sensorimotor integration. In somatosensory cortex (S1) and primary visual cortex (V1), movement during sensation activates specific pathways that differentially engage interneurons in the respective region. The paths transmitting movement-related signals to these areas differ, however, the coincidence of movement and sensation engages the VIP-SST disinhibitory circuit in both cases. In V1, the type of visual stimulus can influence which interneuron subtype alters its spiking activity.

striatum (Melzer et al., 2017). Transgenic cre-expressing animals and a floxed channelrhodopsin-expressing virus were used to selectively stimulate the axons of these cells within the striatum during spontaneous locomotion. When axons from SST^+-M2 or PV^+-M1 neurons were activated, locomotion decreased, while activation of M1-originating SST^+ neuron axons increased locomotion. Overall, these data point to several different roles for inhibitory neurons in voluntary skilled movement. Specific populations of GABAergic neurons, even within subtypes, differ in activity and recruitment at specific phases or motor activity.

INHIBITORY NEURONS AND SENSORY PROCESSING

Subtype-specific recruitment of inhibitory interneurons for sensation and perception has been observed in several sensory cortices. In V1, recruitment of each inhibitory interneuron subtype has distinct effects on visual processing. Selective activation of PV^+ interneurons using channelrhodopsin expressed exclusively in PV^+ cells resulted in narrowed orientation tuning and increased direction selectivity in neighboring neurons (Lee et al., 2012). Activation of SST^+ or VIP^+ neurons did not recapitulate this effect, suggesting that it is mediated by PV^+ neurons. SST^+ interneurons, on the other hand, have been implicated in sensory integration and gating cross-modal signals reaching V1 (Scheyltjens et al., 2018). Finally, recruitment of VIP^+ and SST^+ neurons *via* a CC pathway from the cingulate cortex to V1 plays a role in center-surround modulation (Zhang et al., 2014). Behavioral states can also modulate the gain of excitatory neurons. Locomotion, in particular, increases the gain of V1 neurons with no effect on their spontaneous activity or tuning properties (Niell and Stryker, 2010). *In vivo* calcium imaging during locomotion and visual stimulation has shown that the activity VIP^+ interneurons increases during locomotion, leading to an augmented response to visual stimuli in non-VIP^+ neurons (Fu et al., 2014). SST^+ interneurons consistently showed suppression of activity during locomotion, while PV^+ interneurons had heterogeneous responses, consistent with the strong inhibitory connection between VIP^+ and SST^+ interneurons (Pfeffer et al., 2013). Furthermore, pharmacological blockade of nicotinic acetylcholine receptors attenuated the locomotion-induced response of VIP^+ neurons, suggesting a functional role for subtype-specific cholinergic modulation of inhibitory neurons in V1 (Alitto and Dan, 2012). The VIP-SST inhibitory circuit is necessary for cortical plasticity in adults after a change in the level of visual stimulus: either activating VIP^+ interneurons, or silencing SST^+ neurons, is sufficient to increase visual cortical plasticity (Fu et al., 2015). Response features of VIP^+ neurons are distinct depending on cortical area: in V1 they behave similarly to PV^+ neurons in that they are broadly tuned to stimulation, while in A1, VIP^+ interneurons behave unlike PV^+ interneurons or pyramidal neurons, with a strong selectivity to sound intensity (Mesik et al., 2015). In A1, context switching from passive tone perception to active tone perception in a

decision-based task differentially modulates GABAergic neurons by type (Kuchibhotla et al., 2017). VIP^+ interneurons show the largest change in activity following the context switch, and they show the highest level of activity during the passive perception period. In contrast, PV^+ and SST^+ neurons increase their activity from the passive to active context.

INTERNEURONS AND SENSORIMOTOR INTEGRATION

Interneurons also play a role in the interaction between functionally connected brain regions. Movement is intrinsic to many sensory processes, so it stands to reason that there are pathways between motor and sensory cortices (**Figure 3**). For example, M1 projects to S1, making synaptic contact with all three interneuron subtypes (Kinnischtzke et al., 2016; Wall et al., 2016). This input is strongest onto VIP^+ interneurons, which in turn inhibit SST^+ interneurons (Lee et al., 2013). Interestingly, VIP^+ interneurons increase their spiking probability during active whisking, while SST^+ interneurons decrease their activity. Acute inactivation of vibrissal M1 with tetrodotoxin had no overall effect on local field potentials in S1, however, it did significantly reduce the correlation between whisking and increase VIP^+ interneuron activity. This suggests that motor input to S1 engages a disinhibitory circuit that involves the activation of VIP^+ interneurons and the suppression of SST^+ interneurons. A similar circuit has been observed in V1: PV^+, SST^+, and VIP^+ interneurons have been shown to receive input from M2 and to a lesser extent M1 (Lu et al., 2014; Leinweber et al., 2017). Locomotion has been shown to enhance visual perception, and studies have shown that GABAergic cells in V1 modulate their activity during locomotion while animals experience visual stimulus (**Figure 3**). In one study, VIP^+ interneurons increase their activity during locomotion, and SST^+ interneurons decreased activity (Fu et al., 2014). The increase in VIP^+ interneuron activity is tied to nicotinic acetylcholine receptor activity, activated by an input from the basal forebrain. However, it is important to note that these results are controversial. Subsequent studies have shown that GABAergic neuron modulation depends on the context and magnitude of the visual stimulus and that this disinhibitory circuit model may not be the only way that locomotion can engage interneurons in V1 (Pakan et al., 2016; Dipoppa et al., 2018). These results suggest that even within a specific function, the brain may employ different interneuron subtypes depending on the characteristics of the sensory stimulus.

CONCLUSIONS

While there are commonalities in the engagement of GABAergic cells across sensory regions, these data suggest that the activation of inhibitory interneurons is customized to the function of the region in which they reside. There have been great advancements in the development of tools for the investigation of inhibitory circuits and for the identification of specific GABAergic neuron groups. Anatomical and *in vitro* studies have made strides in elucidating the sources of activation for these cells, demonstrating that these cells receive diverse synaptic input from many thalamic, cortical, amygdalar, and neuromodulatory regions. Additionally, studies performed in behaving animals have shed light on the active roles these interneurons play in sensation, cognition, and movement. The next step in understanding the full picture of inhibitory function is to determine how the recruitment structure of these cells is used to drive inhibition during behavior. Synthesizing common roles of inhibitory cells across areas is difficult, in part because the behavioral paradigms used to engage one cortex may be difficult to compare to a task used in another region. Furthermore, while we did not extensively discuss GABAergic plasticity in this review article, it is well known that inhibitory circuits are dynamically regulated, and the efficacy of inhibitory synaptic transmission is activity-dependent. Thus, the functional engagement of cortical GABAergic neurons can powerfully expand the computational capacity of other neuron types and the neural network. We have examined the overarching similarities of inhibitory circuits across cortical regions while pointing out that some properties of these cells may be tailored to the area's specific function. Expanding our knowledge of how each inhibitory neuron is recruited and the role that they play in shaping behavioral output remains a fundamental step to understand how the brain functions in health and disease.

AUTHOR CONTRIBUTIONS

Both authors were involved in writing and commenting the manuscript.

FUNDING

This work was supported by National Institutes of Health-National Institute on Deafness and Other Communication Disorders (NIH-NIDCD) awards DC013770, DC015234, and the Thomas Hartman Foundation for Parkinson's disease.

REFERENCES

Alitto, H. J., and Dan, Y. (2012). Cell-type-specific modulation of neocortical activity by basal forebrain input. *Front. Syst. Neurosci.* 6:79. doi: 10.3389/fnsys.2012.00079

Anastasiades, P. G., Marlin, J. J., and Carter, A. G. (2018). Cell-type specificity of callosally evoked excitation and feedforward inhibition in the prefrontal cortex. *Cell Rep.* 22, 679–692. doi: 10.1016/j.celrep.2017.12.073

Audette, N. J., Urban-Ciecko, J., Matsushita, M., and Barth, A. L. (2018). POm thalamocortical input drives layer-specific microcircuits in somatosensory cortex. *Cereb. Cortex* 28, 1312–1328. doi: 10.1093/cercor/bhx044

Bagnall, M. W., Hull, C., Bushong, E. A., Ellisman, M. H., and Scanziani, M. (2011). Multiple clusters of release sites formed by individual thalamic afferents onto cortical interneurons ensure reliable transmission. *Neuron* 71, 180–194. doi: 10.1016/j.neuron.2011.05.032

Castejon, C., Barros-Zulaica, N., and Nuñez, A. (2016). Control of somatosensory cortical processing by thalamic posterior medial nucleus: a new role of thalamus

in cortical function. *PLoS One* 11:e0148169. doi: 10.1371/journal.pone.0148169

Cauli, B., Porter, J. T., Tsuzuki, K., Lambolez, B., Rossier, J., Quenet, B., et al. (2000). Classification of fusiform neocortical interneurons based on unsupervised clustering. *Proc. Natl. Acad. Sci. U S A* 97, 6144–6149. doi: 10.1073/pnas.97.11.6144

Chen, S. X., Kim, A. N., Peters, A. J., and Komiyama, T. (2015). Subtype-specific plasticity of inhibitory circuits in motor cortex during motor learning. *Nat. Neurosci.* 18, 1109–1115. doi: 10.1038/nn.4049

Chen, N., Sugihara, H., and Sur, M. (2015). An acetylcholine-activated microcircuit drives temporal dynamics of cortical activity. *Nat. Neurosci.* 18, 892–902. doi: 10.1038/nn.4002

Cheriyan, J., Kaushik, M. K., Ferreira, A. N., and Sheets, P. L. (2016). Specific targeting of the basolateral amygdala to projectionally defined pyramidal neurons in prelimbic and infralimbic cortex. *eNeuro* 3:ENEURO.0002-16.2016. doi: 10.1523/eneuro.0002-16.2016

Cruikshank, S. J., Lewis, T. J., and Connors, B. W. (2007). Synaptic basis for intense thalamocortical activation of feedforward inhibitory cells in neocortex. *Nat. Neurosci.* 10, 462–468. doi: 10.1038/nn1861

Cruikshank, S. J., Urabe, H., Nurmikko, A. V., and Connors, B. W. (2010). Pathway-specific feedforward circuits between thalamus and neocortex revealed by selective optical stimulation of axons. *Neuron* 65, 230–245. doi: 10.1016/j.neuron.2009.12.025

Delevich, K., Tucciarone, J., Huang, Z. J., and Li, B. (2015). The mediodorsal thalamus drives feedforward inhibition in the anterior cingulate cortex *via* parvalbumin interneurons. *J. Neurosci.* 35, 5743–5753. doi: 10.1523/JNEUROSCI.4565-14.2015

Dilgen, J., Tejeda, H. A., and O'Donnell, P. (2013). Amygdala inputs drive feedforward inhibition in the medial prefrontal cortex. *J. Neurophysiol.* 110, 221–229. doi: 10.1152/jn.00531.2012

Dipoppa, M., Ranson, A., Krumin, M., Pachitariu, M., Carandini, M., and Harris, K. D. (2018). Vision and locomotion shape the interactions between neuron types in mouse visual cortex. *Neuron* 98, 602.e8–615.e8. doi: 10.1016/j.neuron.2018.03.037

Donato, F., Rompani, S. B., and Caroni, P. (2013). Parvalbumin-expressing basket-cell network plasticity induced by experience regulates adult learning. *Nature* 504, 272–276. doi: 10.1038/nature12866

Estebanez, L., Hoffmann, D., Voigt, B. C., and Poulet, J. F. A. (2017). Parvalbumin-expressing GABAergic neurons in primary motor cortex signal reaching. *Cell Rep.* 20, 308–318. doi: 10.1016/j.celrep.2017.06.044

Fanselow, E. E., Richardson, K. A., and Connors, B. W. (2008). Selective, state-dependent activation of somatostatin-expressing inhibitory interneurons in mouse neocortex. *J. Neurophysiol.* 100, 2640–2652. doi: 10.1152/jn.90691.2008

Fu, Y., Kaneko, M., Tang, Y., Alvarez-Buylla, A., and Stryker, M. P. (2015). A cortical disinhibitory circuit for enhancing adult plasticity. *Elife* 4:e05558. doi: 10.7554/elife.05558

Fu, Y., Tucciarone, J. M., Espinosa, J. S., Sheng, N., Darcy, D. P., Nicoll, R. A., et al. (2014). A cortical circuit for gain control by behavioral state. *Cell* 156, 1139–1152. doi: 10.1016/j.cell.2014.01.050

Gonchar, Y., Wang, Q., and Burkhalter, A. (2007). Multiple distinct subtypes of GABAergic neurons in mouse visual cortex identified by triple immunostaining. *Front. Neuroanat.* 1:3. doi: 10.3389/neuro.05.003.2007

Gonzalez-Burgos, G., and Lewis, D. A. (2008). GABA neurons and the mechanisms of network oscillations: implications for understanding cortical dysfunction in schizophrenia. *Schizophr. Bull.* 34, 944–961. doi: 10.1093/schbul/sbn070

Haley, M. S., Fontanini, A., and Maffei, A. (2016). Laminar- and target-specific amygdalar inputs in rat primary gustatory cortex. *J. Neurosci.* 36, 2623–2637. doi: 10.1523/JNEUROSCI.3224-15.2016

Hanamori, T. (2009). Effects of electrical and chemical stimulation of the amygdala on the spontaneous discharge in the insular cortex in rats. *Brain Res.* 1276, 91–102. doi: 10.1016/j.brainres.2009.04.024

Hioki, H. (2015). Compartmental organization of synaptic inputs to parvalbumin-expressing GABAergic neurons in mouse primary somatosensory cortex. *Anat. Sci. Int.* 90, 7–21. doi: 10.1007/s12565-014-0264-8

Isaacson, J. S., and Scanziani, M. (2011). How inhibition shapes cortical activity. *Neuron* 72, 231–243. doi: 10.1016/j.neuron.2011.09.027

Ji, X. Y., Zingg, B., Mesik, L., Xiao, Z., Zhang, L. I., and Tao, H. W. (2016). Thalamocortical innervation pattern in mouse auditory and visual cortex: laminar and cell-type specificity. *Cereb. Cortex* 26, 2612–2625. doi: 10.1093/cercor/bhv099

Jiang, X., Shen, S., Cadwell, C. R., Berens, P., Sinz, F., Ecker, A. S., et al. (2015). Principles of connectivity among morphologically defined cell types in adult neocortex. *Science* 350:aac9462 . doi: 10.1126/science.aac9462

Kameda, H., Hioki, H., Tanaka, Y. H., Tanaka, T., Sohn, J., Sonomura, T., et al. (2012). Parvalbumin-producing cortical interneurons receive inhibitory inputs on proximal portions and cortical excitatory inputs on distal dendrites. *Eur. J. Neurosci.* 35, 838–854. doi: 10.1111/j.1460-9568.2012.08027.x

Karayannis, T., Huerta-Ocampo, I., and Capogna, M. (2007). GABAergic and pyramidal neurons of deep cortical layers directly receive and differently integrate callosal input. *Cereb. Cortex* 17, 1213–1226. doi: 10.1093/cercor/bhl035

Kawaguchi, Y., and Shindou, T. (1998). Noradrenergic excitation and inhibition of GABAergic cell types in rat frontal cortex. *J. Neurosci.* 18, 6963–6976. doi: 10.1523/JNEUROSCI.18-17-06963.1998

Kim, D., Jeong, H., Lee, J., Ghim, J. W., Her, E. S., Lee, S. H., et al. (2016). Distinct roles of parvalbumin- and somatostatin-expressing interneurons in working memory. *Neuron* 92, 902–915. doi: 10.1016/j.neuron.2016.09.023

Kinnischtzke, A. K., Fanselow, E. E., and Simons, D. J. (2016). Target-specific M1 inputs to infragranular S1 pyramidal neurons. *J. Neurophysiol.* 116, 1261–1274. doi: 10.1152/jn.01032.2015

Kloc, M., and Maffei, A. (2014). Target-specific properties of thalamocortical synapses onto layer 4 of mouse primary visual cortex. *J. Neurosci.* 34, 15455–15465. doi: 10.1523/JNEUROSCI.2595-14.2014

Kubota, Y., Shigematsu, N., Karube, F., Sekigawa, A., Kato, S., Yamaguchi, N., et al. (2011). Selective coexpression of multiple chemical markers defines discrete populations of neocortical GABAergic neurons. *Cereb. Cortex* 21, 1803–1817. doi: 10.1093/cercor/bhq252

Kubota, Y. (2014). Untangling GABAergic wiring in the cortical microcircuit. *Curr. Opin. Neurobiol.* 26, 7–14. doi: 10.1016/j.conb.2013.10.003

Kuchibhotla, K. V., Gill, J. V., Lindsay, G. W., Papadoyannis, E. S., Field, R. E., Sten, T. A., et al. (2017). Parallel processing by cortical inhibition enables context-dependent behavior. *Nat. Neurosci.* 20, 62–71. doi: 10.1038/nn.4436

Kvitsiani, D., Ranade, S., Hangya, B., Taniguchi, H., Huang, J. Z., and Kepecs, A. (2013). Distinct behavioural and network correlates of two interneuron types in prefrontal cortex. *Nature* 498, 363–366. doi: 10.1038/nature12176

Landisman, C. E., and Connors, B. W. (2007). VPM and PoM nuclei of the rat somatosensory thalamus: intrinsic neuronal properties and corticothalamic feedback. *Cereb. Cortex* 17, 2853–2865. doi: 10.1093/cercor/bhm025

Lee, A. T., Vogt, D., Rubenstein, J. L., and Sohal, V. S. (2014). A class of GABAergic neurons in the prefrontal cortex sends long-range projections to the nucleus accumbens and elicits acute avoidance behavior. *J. Neurosci.* 34, 11519–11525. doi: 10.1523/JNEUROSCI.1157-14.2014

Lee, S., Kruglikov, I., Huang, Z. J., Fishell, G., and Rudy, B. (2013). A disinhibitory circuit mediates motor integration in the somatosensory cortex. *Nat. Neurosci.* 16, 1662–1670. doi: 10.1038/nn.3544

Lee, S. H., Kwan, A. C., Zhang, S., Phoumthipphavong, V., Flannery, J. G., Masmanidis, S. C., et al. (2012). Activation of specific interneurons improves V1 feature selectivity and visual perception. *Nature* 488, 379–383. doi: 10.1038/nature11312

Leinweber, M., Ward, D. R., Sobczak, J. M., Attinger, A., and Keller, G. B. (2017). A sensorimotor circuit in mouse cortex for visual flow predictions. *Neuron* 95, 1420.e5–1432.e5. doi: 10.1016/j.neuron.2017.08.036

Lu, J. T., Tucciarone, J., Lin, Y., and Huang, Z. J. (2014). Input-specific maturation of synaptic dynamics of parvalbumin interneurons in primary visual cortex. *Proc. Natl. Acad. Sci. U S A* 111, 16895–18900. doi: 10.1073/pnas.1400694111

McGarry, L. M., and Carter, A. G. (2016). Inhibitory gating of basolateral amygdala inputs to the prefrontal cortex. *J. Neurosci.* 36, 9391–9406. doi: 10.1523/JNEUROSCI.0874-16.2016

Melzer, S., Gil, M., Koser, D. E., Michael, M., Huang, K. W., and Monyer, H. (2017). Distinct corticostriatal GABAergic neurons modulate striatal output neurons and motor activity. *Cell Rep.* 19, 1045–1055. doi: 10.1016/j.celrep.2017.04.024

Mesik, L., Ma, W. P., Li, L. Y., Ibrahim, L. A., Huang, Z. J., Zhang, L. I., et al. (2015). Functional response properties of VIP-expressing inhibitory neurons in mouse visual and auditory cortex. *Front. Neural Circuits* 9:22. doi: 10.3389/fncir.2015.00022

Murray, A. J., Woloszynowska-Fraser, M. U., Ansel-Bollepalli, L., Cole, K. L., Foggetti, A., Crouch, B., et al. (2015). Parvalbumin-positive interneurons of the prefrontal cortex support working memory and cognitive flexibility. *Sci. Rep.* 5:16778. doi: 10.1038/srep16778

Nassar, M., Simonnet, J., Huang, L. W., Mathon, B., Cohen, I., Bendels, M. H. K., et al. (2018). Anterior thalamic excitation and feedforward inhibition of presubicular neurons projecting to medial entorhinal cortex. *J. Neurosci.* 38, 6411–6425. doi: 10.1523/JNEUROSCI.0014-18.2018

Nassar, M., Simonnet, J., Lofredi, R., Cohen, I., Savary, E., Yanagawa, Y., et al. (2015). Diversity and overlap of parvalbumin and somatostatin expressing interneurons in mouse presubiculum. *Front. Neural Circuits* 9:20. doi: 10.3389/fncir.2015.00020

Neske, G. T., Patrick, S. L., and Connors, B. W. (2015). Contributions of diverse excitatory and inhibitory neurons to recurrent network activity in cerebral cortex. *J. Neurosci.* 35, 1089–1105. doi: 10.1523/JNEUROSCI.2279-14.2015

Niell, C. M., and Stryker, M. P. (2010). Modulation of visual responses by behavioral state in mouse visual cortex. *Neuron* 65, 472–479. doi: 10.1016/j.neuron.2010.01.033

Oviedo, H. V. (2017). Connectivity motifs of inhibitory neurons in the mouse auditory cortex. *Sci. Rep.* 7:16987. doi: 10.1038/s41598-017-16904-2

Pakan, J. M., Lowe, S. C., Dylda, E., Keemink, S. W., Currie, S. P., Coutts, C. A., et al. (2016). Behavioral-state modulation of inhibition is context-dependent and cell type specific in mouse visual cortex. *Elife* 5:e14985. doi: 10.7554/eLife.14985

Paul, A., Crow, M., Raudales, R., He, M., Gillis, J., and Huang, Z. J. (2017). Transcriptional architecture of synaptic communication delineates GABAergic neuron identity. *Cell* 171, 522.e20–539.e20. doi: 10.1016/j.cell.2017.08.032

Petrie, K. A., Schmidt, D., Bubser, M., Fadel, J., Carraway, R. E., and Deutch, A. Y. (2005). Neurotensin activates GABAergic interneurons in the prefrontal cortex. *J. Neurosci.* 25, 1629–1636. doi: 10.1523/JNEUROSCI.3579-04.2005

Pfeffer, C. K., Xue, M., He, M., Huang, Z. J., and Scanziani, M. (2013). Inhibition of inhibition in visual cortex: the logic of connections between molecularly distinct interneurons. *Nat. Neurosci.* 16, 1068–1076. doi: 10.1038/nn.3446

Pronneke, A., Scheuer, B., Wagener, R. J., Möck, M., Witte, M., and Staiger, J. F. (2015). Characterizing VIP neurons in the barrel cortex of VIPcre/tdTomato mice reveals layer-specific differences. *Cereb. Cortex* 25, 4854–4868. doi: 10.1093/cercor/bhv202

Rock, C., and Apicella, A. J. (2015). Callosal projections drive neuronal-specific responses in the mouse auditory cortex. *J. Neurosci.* 35, 6703–6713. doi: 10.1523/JNEUROSCI.5049-14.2015

Rotaru, D. C., Barrionuevo, G., and Sesack, S. R. (2005). Mediodorsal thalamic afferents to layer III of the rat prefrontal cortex: synaptic relationships to subclasses of interneurons. *J. Comp. Neurol.* 490, 220–238. doi: 10.1002/cne.20661

Rudy, B., Fishell, G., Lee, S., and Hjerling-Leffler, J. (2011). Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. *Dev. Neurobiol.* 71, 45–61. doi: 10.1002/dneu.20853

Scheyltjens, I., Vreysen, S., Van den Haute, C., Sabanov, V., Balschun, D., Baekelandt, V., et al. (2018). Transient and localized optogenetic activation of somatostatin-interneurons in mouse visual cortex abolishes long-term cortical plasticity due to vision loss. *Brain Struct. Funct.* 223, 2073–2095. doi: 10.1007/s00429-018-1611-7

Shigematsu, N., Ueta, Y., Mohamed, A. A., Hatada, S., Fukuda, T., Kubota, Y., et al. (2016). Selective thalamic innervation of rat frontal cortical neurons. *Cereb. Cortex* 26, 2689–2704. doi: 10.1093/cercor/bhv124

Sohal, V. S., Zhang, F., Yizhar, O., and Deisseroth, K. (2009). Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. *Nature* 459, 698–702. doi: 10.1038/nature07991

Sohn, J., Okamoto, S., Kataoka, N., Kaneko, T., Nakamura, K., and Hioki, H. (2016). Differential inputs to the perisomatic and distal-dendritic compartments of VIP-positive neurons in layer 2/3 of the mouse barrel cortex. *Front. Neuroanat.* 10:124. doi: 10.3389/fnana.2016.00124

Stone, M. E., Maffei, A., and Fontanini, A. (2011). Amygdala stimulation evokes time-varying synaptic responses in the gustatory cortex of anesthetized rats. *Front. Integr. Neurosci.* 5:3. doi: 10.3389/fnint.2011.00003

Sun, Q. Q., Huguenard, J. R., and Prince, D. A. (2006). Barrel cortex microcircuits: thalamocortical feedforward inhibition in spiny stellate cells is mediated by a small number of fast-spiking interneurons. *J. Neurosci.* 26, 1219–1230. doi: 10.1523/JNEUROSCI.4727-04.2006

Tan, Z. J., Hu, H., Huang, Z. J., and Agmon, A. (2008). Robust but delayed thalamocortical activation of dendritic-targeting inhibitory interneurons. *Proc. Natl. Acad. Sci. U S A* 105, 2187–2192. doi: 10.1073/pnas.0710628105

Tasic, B., Menon, V., Nguyen, T. N., Kim, T. K., Jarsky, T., Yao, Z., et al. (2016). Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nat. Neurosci.* 19, 335–346. doi: 10.1038/nn.4216

Tatti, R., Haley, M. S., Swanson, O. K., Tselha, T., and Maffei, A. (2017). Neurophysiology and regulation of the balance between excitation and inhibition in neocortical circuits. *Biol. Psychiatry* 81, 821–831. doi: 10.1016/j.biopsych.2016.09.017

Toussay, X., Basu, K., Lacoste, B., and Hamel, E. (2013). Locus coeruleus stimulation recruits a broad cortical neuronal network and increases cortical perfusion. *J. Neurosci.* 33, 3390–3401. doi: 10.1523/JNEUROSCI.3346-12.2013

Tremblay, R., Lee, S., and Rudy, B. (2016). GABAergic interneurons in the neocortex: from cellular properties to circuits. *Neuron* 91, 260–292. doi: 10.1016/j.neuron.2016.06.033

Wall, N. R., De La Parra, M., Sorokin, J. M., Taniguchi, H., Huang, Z. J., and Callaway, E. M. (2016). Brain-wide maps of synaptic input to cortical interneurons. *J. Neurosci.* 36, 4000–4009. doi: 10.1523/JNEUROSCI.3967-15.2016

Williams, L. E., and Holtmaat, A. (2019). Higher-order thalamocortical inputs gate synaptic long-term potentiation *via* disinhibition. *Neuron* 101, 91.e4–102.e4. doi: 10.1016/j.neuron.2018.10.049

Xu, X. M., Roby, K. D., and Callaway, E. M. (2010). Immunochemical characterization of inhibitory mouse cortical neurons: three chemically distinct classes of inhibitory cells. *J. Comp. Neurol.* 518, 389–404. doi: 10.1002/cne.22229

Yamamoto, T., Azuma, S., and Kawamura, Y. (1984). Functional relations between the cortical gustatory area and the amygdala: electrophysiological and behavioral studies in rats. *Exp. Brain Res.* 56, 23–31. doi: 10.1007/bf00237438

Yavorska, I., and Wehr, M. (2016). Somatostatin-expressing inhibitory interneurons in cortical circuits. *Front. Neural Circuits* 10:76. doi: 10.3389/fncir.2016.00076

Zhang, W., and Bruno, R. M. (2019). High-order thalamic inputs to primary somatosensory cortex are stronger and longer lasting than cortical inputs. *Elife* 8:e44158. doi: 10.7554/elife.44158

Zhang, S., Xu, M., Kamigaki, T., Hoang Do, J. P., Chang, W. C., Jenvay, S., et al. (2014). Selective attention. Long-range and local circuits for top-down modulation of visual cortex processing. *Science* 345, 660–665. doi: 10.1126/science.1254126

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 Molecular Neuroscience

MINI REVIEW
published: 24 July 2019
doi: 10.3389/fnmol.2019.00179

Neurobiology and Therapeutic Potential of α5-GABA Type A Receptors

*Tija C. Jacob**

Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States

α5 subunit containing GABA type A receptors ($GABA_ARs$) have long been an enigmatic receptor subtype of interest due to their specific brain distribution, unusual surface localization and key role in synaptic plasticity, cognition and memory. These receptors are uniquely positioned to sculpt both the developing and mature hippocampal circuitry due to high overall expression and a distinct peak within the critical synapse formation period during the second postnatal week. Unlike the majority of other $GABA_ARs$, they exhibit both receptor clustering at extrasynaptic sites *via* interactions with the radixin scaffold as well as synaptic sites *via* gephyrin, thus contributing respectively to tonic currents and synaptic GABAergic neurotransmission. α5 $GABA_AR$ signaling can be altered in neurodevelopmental disorders including autism and mental retardation and by inflammation in CNS injury and disease. Due to the unique physiology and pharmacology of α5 $GABA_ARs$, drugs targeting these receptors are being developed and tested as treatments for neurodevelopmental disorders, depression, schizophrenia, and mild cognitive impairment. This review article focuses on advances in understanding how the α5 subunit contributes to $GABA_AR$ neurobiology. In particular, I discuss both recent insights and remaining knowledge gaps for the functional role of these receptors, pathologies associated with α5 $GABA_AR$ dysfunction, and the effects and potential therapeutic uses of α5 receptor subtype targeted drugs.

Keywords: GABA A receptor, alpha 5 subunit, autism, cognition, memory, development, negative and positive allosteric modulators

OPEN ACCESS

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

Reviewed by:
Bernhard Lüscher,
Pennsylvania State University, United States
Marianne Renner,
Sorbonne Universités, France

***Correspondence:**
Tija C. Jacob
tcj11@pitt.edu

Received: *04 June 2019*
Accepted: *08 July 2019*
Published: *24 July 2019*

Citation:
Jacob TC (2019) Neurobiology and Therapeutic Potential of α5-GABA Type A Receptors. Front. Mol. Neurosci. 12:179. doi: 10.3389/fnmol.2019.00179

INTRODUCTION

Structure, Distribution and Composition

GABA type A receptors ($GABA_ARs$) are heteropentameric ligand-gated chloride (Cl^-) ion channels typically composed of two α (α1–6), two β (β1–3), and one γ (γ1–3) or δ subunit (**Figure 1A**). The common structure of individual subunits consists of a large extracellular N-terminus (NT), four transmembrane α-helices (M1–4) and a barely extruding extracellular C-terminus (CT). The conserved hydrophobic M domains are connected by small regions with a larger cytoplasmic domain between M3 and M4 (CD) that mediates interactions with intracellular proteins critical for receptor trafficking and surface localization (**Figure 1B**). Receptors can contain two different α or β subunits that are arranged in a counterclockwise configuration of γ-β-α-β-α (**Figure 1C**). The two αβ NT interfaces form GABA binding sites composed of the

principal (+) side of the β subunit and the complementary α subunit (−) side, while a single α+(1, 2, 3 or 5)/γ2- interface generates the primary binding site for benzodiazepines, which are allosteric positive modulators of the GABA$_A$R and an important clinical sedative-hypnotic-anxiolytic drug class. Several recent high resolution cryo-electron microscopy studies have provided unprecedented structural information for GABA$_A$R (Phulera et al., 2018; Zhu et al., 2018; Laverty et al., 2019; Masiulis et al., 2019), advancing understanding of receptor architecture, principles of assembly, and binding of various ligands: GABA, bicuculline (antagonist), picrotoxin (channel blocker), and benzodiazepines. The channel properties, subcellular localization and pharmacological sensitivity of a GABA$_A$R are defined by the subunit composition. While α5 containing GABA$_A$Rs makeup only approximately 5% of the total receptor population in the brain, they are highly expressed in both the hippocampus and olfactory bulb. They represent close to 25% of all hippocampal GABA$_A$R (Olsen and Sieghart, 2009) and are particularly abundant in CA1 and CA3. In the olfactory bulb, over a third of the neurons in the internal granule cell layer have α5 GABA$_A$Rs (Sur et al., 1999), although the function here is unknown. α5 GABA$_A$Rs are also expressed in the spinal cord, where they contribute to presynaptic inhibitory control over sensory-motor transmission (Lucas-Osma et al., 2018) and are also implicated in resolution of hyperalgesia (Perez-Sanchez et al., 2017). Other brain regions where these receptors are found at lower levels include the cortex, subiculum, hypothalamus, sympathetic preganglionic neurons, and amygdala (Martin et al., 2009a).

Early pharmacological analysis indicated rat and human hippocampal α5 GABA$_A$Rs have α5β3γ2 characteristics (Sur et al., 1998). However, sequential immunoprecipitation from hippocampal tissue identified that α1/α5 heteromers constitute approximately 9% of the α1 GABA$_A$Rs and α2/α5 heteromers constitute about 20% of the α2 population in the hippocampus (Araujo et al., 1999; del Río et al., 2001). More recent mass spectrometry analysis of affinity purified α5 GABA$_A$Rs from mouse hippocampus supported association of α5 with α1–3, β1–3 and both γ2S and γ2L isoforms (Ju et al., 2009). A recent comparison of α5β1–3γ2L GABA$_A$Rs in HEK cells co-cultured with neurons revealed robust inhibitory postsynaptic currents (IPSCs) with slow decay rates and isoform-specific effects of pharmacological inhibitors (Chen et al., 2017). Importantly, in mixed alpha subunit GABA$_A$Rs there appears to be preferential assembly of α5 and γ2 together, generating a benzodiazepine binding site with α5 subunit pharmacology (Araujo et al., 1999; del Río et al., 2001). Thus for a mixed α5 GABA$_A$R, the other alpha subunit is essentially pharmacologically inactive for benzodiazepines and other alpha/gamma subunit interface binding drugs (i.e., the "Z-drugs" for insomnia treatment zolpidem, zopiclone, zaleplon). Mutation of the α5 subunit H105 residue, a key alpha subunit residue required for forming the benzodiazepine binding site with the γ2 subunit, led to repositioning of α5 H105R subunits into the pharmacologically inactive alpha subunit location (Balic et al., 2009). Interestingly, our recent mass spectrometry analysis identified a specific increase in α5βγ2 containing receptors in the cortex following diazepam injection, consistent with benzodiazepine exposure leading to modification of GABA$_A$R composition and potentially drug effects through α5 plasticity (Lorenz-Guertin et al., 2019).

CELLULAR AND CIRCUIT LOCALIZATION

Subcellular Localization

Controversies regarding α5 GABA$_A$R subcellular localization in the literature have mirrored debates about its functional impact on GABAergic neurotransmission. Due to their initial identification as a key generator of hippocampal tonic current (Caraiscos et al., 2004; Glykys and Mody, 2006; Bonin et al., 2007), α5 GABA$_A$Rs were generally considered extrasynaptic receptors, despite earlier evidence for synaptic clustering on dendrites and the axon initial segment (Brünig et al., 2002; Christie and de Blas, 2002; Serwanski et al., 2006). α5 GABA$_A$Rs predominantly mediate tonic inhibition in hippocampal CA3 and CA1 pyramidal neurons, cortical neurons (layer 5) and are contributors to tonic inhibition in dentate gyrus granule cells (Glykys et al., 2008; Herd et al., 2008). Immunocytochemistry indicates an extensive extrasynaptic presence of α5 GABA$_A$Rs (Brünig et al., 2002; Crestani et al., 2002). However, this receptor subtype is unique in displaying surface clustering at extrasynaptic locations rather than a uniformly diffuse extrasynaptic distribution. Regions within the large cytoplasmic domain between M3 and M4 regulate subcellular clustering of α5 GABA$_A$Rs *via* interactions with radixin and gephyrin scaffolds (**Figure 1D**). Extrasynaptic clustering is mediated by radixin, an ezrin/radixin/moesin (ERM) family member that links actin to the plasma membrane (Loebrich et al., 2006). Phosphorylated radixin scaffolds α5βγ2 receptors to the actin cytoskeleton, ultimately reducing diffusion rates and concentrating channel activity away from axon terminals (Hausrat et al., 2015). Treatment with GABA promotes radixin phosphorylation and retention of α5 GABA$_A$Rs extrasynaptically, while AMPA, a ligand for ionotropic glutamatergic GluA type receptors, leads to dephosphorylation, an increase in synaptic α5-subunit receptors and an increase in slowly decaying miniature IPSCs (mIPSCs). Further support for the specific contribution of α5 GABA$_A$Rs to slowly decaying IPSCs is seen in early neurodevelopment during the switch from α5 to α1 and α3 subunit expression (Pangratz-Fuehrer et al., 2016). Important areas of further investigation include assessment of the level and role of α5 GABA$_A$Rs associated with radixin or gephyrin in the developing and adult brain and plasticity mechanisms regulating these interactions.

Functional studies indicate the α5 subunit is also important for phasic events including: spontaneous inhibitory postsynaptic currents (sIPSCs), evoked IPSCs (eIPSCs) and GABA$_{slow}$ IPSCs (Collinson et al., 2002; Prenosil et al., 2006; Zarnowska et al., 2009; Vargas-Caballero et al., 2010). Consistent with a synaptic role for α5 GABA$_A$Rs, we demonstrated that the α5 subunit directly interacts with the gephyrin synaptic scaffold, with approximately half of surface α5 GABA$_A$Rs being synaptically

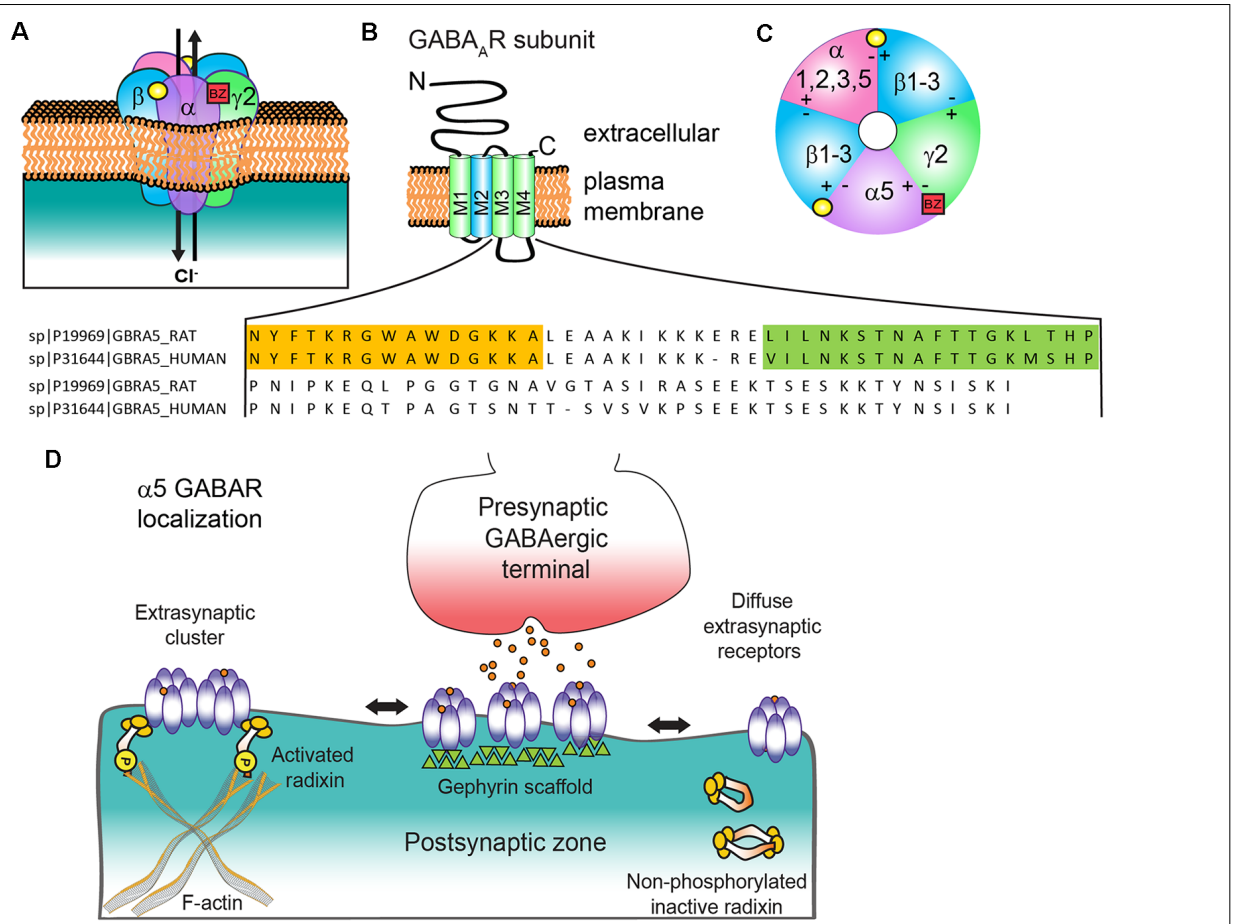

FIGURE 1 | α_5 subunit containing GABA type A receptor (α5 GABA$_A$R) structure and subunit topology. **(A)** Generic synaptic GABA$_A$R heteropentamer. Binding of the neurotransmitter GABA (yellow circle) at the αβ interface triggers ion channel opening and allows the rapid Cl^- influx and membrane hyperpolarization. Benzodiazepines (BZ, red box) bind at the interface of an α1/2/3/5 and γ2 subunit. **(B)** All subunits have a common topology including an extracellular N-terminal domain (NT), short C-terminal tail (CT), and four transmembrane regions (M1–4) which compose the transmembrane domain. M2 (blue) contributes to formation of the receptor ion channel pore, while the large cytoplasmic domain between M3 and M4 (CD) contains sites for protein interactions and post translational modifications that modulate channel function and/or trafficking: amino acid residue alignment of rat and human α5 CD with radixin binding domain (orange highlighted residues, from Loebrich et al., 2006) and gephyrin interacting region (green highlighted residues, from Brady and Jacob, 2015). **(C)** α5 GABA$_A$R extracellular representation with potential subunit combinations. **(D)** Schematic of α5 GABA$_A$R clustering mechanisms at extrasynaptic and synaptic locations with radixin and gephyrin. Phosphorylated radixin interacts with receptors and actin, while with dephosphorylation radixin N-terminal FERM and C-terminal F-actin binding domains interact and form inactive monomers or dimers.

localized throughout the first 3 weeks of circuit development (Brady and Jacob, 2015). Single particle tracking studies measured reduced diffusion of surface α5 GABA$_A$Rs at synapses (Renner et al., 2012) and similar to other synaptic receptors, α5 GABA$_A$Rs showed an increase in diffusion with negative modulator DMCM treatment (Lévi et al., 2015). Further studies are needed to determine both acute and prolonged effects of α5 preferring GABA$_A$R drugs on receptor diffusive properties and surface stability.

Cell Type and Input-Specific Expression

α5 GABA$_A$Rs show input-specific synaptic localization and function in different brain regions both for pyramidal cells and interneurons. Recent work demonstrates preferential localization of α5 GABA$_A$Rs to inhibitory synapses on dendrites of somatostatin-expressing interneurons in CA1 that are targeted by vasoactive intestinal peptide and calretinin-positive interneurons (Magnin et al., 2019). Somatostatin interneurons and NO-synthase-positive neurogliaform cells target α5 GABA$_A$Rs on dendrites of hippocampal CA1 pyramidal neurons to generate slow IPSCs (Schulz et al., 2018). Importantly, these outward-rectifying α5-GABA$_A$Rs generate a greater hyperpolarizing current at slightly depolarized membrane potentials, thereby having a large impact on NMDA-receptor-activation and action potential firing in pyramidal neurons. In the cortex, pyramidal cells exhibit dendritically localized α5 GABA$_A$Rs at sites innervated by bitufted interneurons (an SST positive neuron class; Ali and Thomson, 2008). A recent human and mouse prefrontal cortex gene expression study determined that the majority of α5 GABA$_A$Rs are in pyramidal

cells, followed by parvalbumin interneurons (Hu et al., 2018). Interestingly, α5 GABA$_A$R mRNA was uniquely expressed in human SST interneurons, albeit at a low level. As deficits in both GABAergic signaling and SST signaling (Fuchs et al., 2017) have been identified as contributors to major depressive disorder, this data suggests positive modulation of α5 GABA$_A$R could be therapeutic by multiple mechanisms. It is clear that improving understanding of GABA$_A$R subtype subcellular (extrasynaptic vs. synaptic) and circuit-specific localization and function are critical areas of current research and future pharmacological development (reviewed in Engin et al., 2018).

FUNCTIONAL ROLE OF α5 GABA$_A$Rs

Neuronal Excitability, Learning and Memory

Genetic and pharmacological studies in rodents demonstrate that α5 GABA$_A$Rs are key in learning and memory processes (reviewed in Martin et al., 2009a). The two primary mouse models used in studying the α5 GABA$_A$R contribution to cognitive processes are the α5 subunit knockout mice (*Gabra5*$^{-/-}$) and the α5H105R point mutation mice. Although originally generated to render α5 receptors insensitive to benzodiazepines, α5H105R mice also have a 25% decrease in hippocampal α5 protein level (Crestani et al., 2002). As described earlier, *Gabra5*$^{-/-}$ mice showed a reduction in diverse types of phasic GABA$_A$R currents and the tonic current. Behaviorally, the increased excitability of *Gabra5*$^{-/-}$ hippocampal pyramidal neurons was correlated with improved performance in a spatial learning behavior (Collinson et al., 2002), though later studies were not able to replicate this result (Cheng et al., 2006; Martin et al., 2009b). However, both *Gabra5*$^{-/-}$ and α5H105R mice show enhanced trace fear conditioning, a hippocampal learning task, while performing similarly to wild-type mice in a cued fear conditioning assay, which relies on the amygdala, hippocampus, and cortex (Crestani et al., 2002; Martin et al., 2009b). Long-term potentiation (LTP), the cellular correlate of learning and memory, is constrained by GABA$_A$R-mediated inhibition. *Gabra5*$^{-/-}$ mice showed a reduced threshold for LTP induction with 10–20 Hz stimulation (Martin et al., 2010). In addition, *Gabra5*$^{-/-}$ mice showed greater power of kainate-induced gamma frequency oscillations (Towers et al., 2004), and knockout of delta and α5 subunits led to spontaneous gamma oscillations in CA3 (Glykys et al., 2008). Gamma oscillations occur in a range of cognitive states including memory processing, are thought to support neural coding of environmental information and are disturbed in some psychiatric disorders (reviewed in Lisman and Buzsáki, 2008). In summary, a reduction in α5 inhibition may improve learning and memory through enhanced neuronal firing and network oscillatory activity.

Development

In contrast to their inhibitory role in the mature nervous system, GABA$_A$Rs can promote excitation in newly forming circuits, allowing chloride efflux to produce membrane depolarization which promotes calcium entry, dendritic outgrowth, synaptogenesis and unsilencing of glutamatergic synapses (reviewed in Ben-Ari et al., 2007). α5 GABA$_A$Rs are particularly well positioned to sculpt early hippocampal circuit development due to exceptionally high expression that peaks in the first two postnatal weeks (Liu et al., 1998; Ramos et al., 2004; Yu et al., 2014; Bader et al., 2017), and receptor localization at both extrasynaptic and synaptic sites. During the first postnatal week, tonic α5 currents enhance cell excitability and synaptic activity, facilitating the induction of giant depolarizing potentials, which are important for early network maturation (Ben-Ari, 2002; Marchionni et al., 2007). Importantly, GABAergic activation of circuit formation also occurs with newborn neurons integrating into networks in the adult mammalian brain *in vivo* (Ge et al., 2006). A few *in vitro* pharmacological and genetic studies have supported the role of α5 GABA$_A$Rs in dendritic development. Cultured hippocampal neurons treated with an α5-specific negative allosteric modulator (NAM; RY-80) exhibited decreased dendritic arborization and reduced expression of the AMPA type glutamate receptor GluA2 subunit (Giusi et al., 2009). To investigate the role of α5 GABA$_A$Rs in emerging circuits, we genetically manipulated α5 binding to gephyrin, increasing or decreasing the ratio of extrasynaptic/synaptic α5 GABA$_A$Rs (Brady and Jacob, 2015). Interestingly, reducing synaptic α5 GABA$_A$Rs promoted dendritic outgrowth at the expense of dendritic spine maturation in hippocampal neurons. Consistent with these findings, recent work showed that single-cell deletion of *Gabra5* in adult-born dentate gyrus granule cells caused severe alterations of migration and dendrite development (Deprez et al., 2016). Further research is needed to elucidate the specific role of the α5 subunit in dendritic architecture, both during development and in adult neurogenesis.

Genetic Disorders with Altered α5 GABA$_A$R Neurotransmission

While acute reduction in α5 GABA$_A$Rs has shown potential for improving cognition and memory, further studies both in mouse models and human patients link long term reduction with significant pathologies. Reduced α5 GABA$_A$R levels, function or protein interactions have been observed in patients with neurodevelopmental disorders including intellectual disability, epilepsy and autism. Common conditions among these disorders include cognitive impairments, increased anxiety, autism-related behaviors, sleep disorders and epilepsy susceptibility. Analogous behavioral changes and pathologies are observed in mouse models including *Gabra5*$^{-/-}$mice (Zurek et al., 2016; Mesbah-Oskui et al., 2017), Fragile X syndrome model mice (*Fmr1*$^{-/-}$mice, Bakker and Oostra, 2003), and other mouse models of ASD (reviewed in Kazdoba et al., 2016). *Fmr1*$^{-/-}$ mice show downregulation of α5 GABA$_A$R and a deficit in tonic inhibition (Curia et al., 2009). Subsequent studies of α5H105R mice identified behavioral changes including hyperactivity and impaired encoding of object location memories (Hauser et al., 2005; Prut et al., 2010), although some behavioral changes may be attributed to subunit ordering rearrangements in a mixed alpha subunit GABA$_A$R (see earlier, Composition).

The most commonly reported loci of chromosomal abnormalities in ASD patients are found in the q11.2–13 region on chromosome 15 (Hogart et al., 2010). Among the genes in this region are the α5, β3, and γ3 subunits. An autism patient exome study identified mutations including α5G113A (NT), α5V204I (NT) and mutations in the extrasynaptic anchor radixin: T516I, P471T, D197H, A496V (Zurek et al., 2016). Exome sequencing of sporadic genetic epilepsy patients identified α5V204I (NT), α5W280R (M1), α5S402A (CD) and α5P453L (CT) mutations (Hernandez et al., 2016). Recombinant studies of these mutant α5β3γ2 GABA$_A$Rs indicated no pronounced changes in surface or total α5 levels, while functional deficiencies ranged from reduced currents and gating defects to altered channel activation and deactivation. A V294L (M2, pore-lining helix) mutation identified in a patient with severe early-onset epilepsy and developmental delay showed receptors with 10 times greater GABA sensitivity, although maximal GABA currents were reduced by increased receptor desensitization (Butler et al., 2018). An autism patient pilot PET imaging study with the α5 preferring tracer [11C]Ro15-4513 identified reduced α5 binding across multiple brain regions (Mendez et al., 2013), while another recent study showed changes in a GABA-sensitive perceptual task without differences in binding (Horder et al., 2018). As both studies were without genetic information, this suggests further testing with patient stratification by exome data could provide greater insight. Despite being a genetically heterogeneous disorder, the potential utility for mechanism-based GABA$_A$R pharmacologic treatment with ASDs is supported by shared pathologies both in patients and related mouse models.

α5 GABA$_A$R THERAPEUTICS

NAMs that selectively reduce α5 GABA$_A$R function have been heavily pursued for the potential development of cognitive enhancing or "smart" drugs. The following are a selection of α5 GABA$_A$R NAMs: L-655,708, α5IA, Ro15-4513, MRK-016, RO4938581, and RY-80 (reviewed in Clayton et al., 2015; Sieghart and Savic, 2018). Importantly, α5 NAMs did not exhibit the convulsant or pro-convulsant activity of more general alpha subunit NAMs, had good oral bioavailability and easily crossed the blood brain barrier (reviewed in Atack, 2011). In contrast to NAMs which act *via* the GABA$_A$R benzodiazepine binding site, S44819 was recently identified as a competitive antagonist of GABA at α5 GABA$_A$R and showed similar pro-cognitive effects as NAMs: blocking α5-GABA$_A$R tonic current, enhancing LTP, reversing scopolamine-induced impairment of spatial working memory and enhancing object recognition memory (Ling et al., 2015; Etherington et al., 2017). Finally, recent evidence for beneficial effects of positive allosteric modulators (PAMs) in aged brain cognition, autism, depression and schizophrenia has bolstered α5 PAM drug development. A selection of α5 preferring PAMs includes SH-053-R-CH3-2′F, MP-III-022, and GL-II-73 (Sieghart and Savic, 2018; Prevot et al., 2019). Potential therapeutic applications for α5 preferring NAMs and PAMs are discussed below with a focus on CNS specific uses (**Table 1**), although important remaining questions exist for both *in vivo* specificity and receptor subtype selectivity as recently reviewed (Sieghart and Savic, 2018).

TABLE 1 | Summary table of α_5 subunit containing GABA type A receptor (α5 GABA$_A$R) targeted drugs and potential utility.

Drug type	Reduce α5 GABA$_A$R activity (NAM or competetive antagonist)	Increase α5 GABA$_A$R activity (PAM)
Compound	L-655, 708, α5IA, Ro15-4513, MRK-016, RO4938581, RY-80, S44819 (competetive antagonist)	SH-053-R-CH3-2′F, MP-III-022, Compound 44, GL-II-73
Therapeutic potential	Procognition/smart drugs	Mild cognitive impairment in aging
	Neurodevelopmental disorders with excessive GABAergic neurotransmission	Neurodevelopmental disorders with insufficient inhibitory tone
	Inflammation induced mild cognitive impairment	Depression
	Post-anesthesia memory blockade	Schizophrenia

This includes drugs that can reduce α5 GABA$_A$R activity [negative allosteric modulators (NAMs) and the competitive antagonist S44819] and positive allosteric modulators (PAMs) that enhance α5 GABA$_A$R activity. Representative compounds and therapeutic potential are listed.

NAM α5 GABA$_A$R Therapeutic Applications

Pro-cognition

The ability of α5 preferring NAMs to enhance learning and memory in rodents provided crucial evidence for the importance of α5 GABA$_A$Rs in these processes (Chambers et al., 2002, 2003; Street et al., 2004). The α5 NAM L-655,708, which shows approximately 50–100-fold selectivity for α5 GABA$_A$Rs, reduced tonic inhibition, enhanced LTP, improved performance in the Morris water maze and generated spontaneous gamma oscillations in the CA3 region of the hippocampus (Caraiscos et al., 2004; Atack et al., 2006; Glykys et al., 2008). However anxiogenic activity and pharmacokinetics (reviewed in Atack, 2011) prevented its use in humans. Although α5IA was non-anxiogenic and reduced ethanol-induced learning impairment in young volunteers, prolonged use was prevented by high dose renal toxicity (Atack, 2010). MRK-016 showed pro-cognitive efficacy and was non-anxiogenic; poor compound tolerance in the elderly stopped further clinical development (Atack et al., 2009). Efforts to develop clinically successful α5 NAM are ongoing.

Developmental Disorders

Down syndrome mice (Ts65Dn) show cognitive impairment due to excessive GABAergic inhibition. Acute treatment with α5IA reversed deficits in novel object recognition and spatial learning and was able to restore deficits of immediate early genes expression during memory processing (Braudeau et al., 2011). Although Ts65Dn mice show no major changes in α5 GABA$_A$R levels (Deidda et al., 2015), growing evidence indicates increased α5 GABA$_A$R activity is an important

pathological component, as genetic ablation of α5 GABA$_A$Rs partially rescues learning, LTP and neuromorphological changes (Vidal et al., 2018). Furthermore, a recent study revealed a specific increase in GABA$_A$R dendritic inhibition in Ts65Dn mice that led to reduced NMDAR activation and impaired LTP that could be restored with α5 NAM treatment (Schulz et al., 2019). *Rdx*$^{-/-}$ mice have increased GABAergic inhibition *via* enhanced α5 synaptic levels, impaired short-term memory and a reversal learning deficit, with the latter being improved with α5IA treatment (Hausrat et al., 2015). The subsequently identified α5 NAM RO4938581, with high affinity and efficacy at α5 GABA$_A$Rs vs. α1–3 GABA$_A$Rs (Ballard et al., 2009), demonstrated efficacy in Ts65Dn mice at improving spatial memory, reversing LTP deficits, and restoring neurogenesis while reducing both hyperactivity and the enhanced density of hippocampal GABAergic boutons (Martínez-Cué et al., 2013). Although these pharmacological successes led to a Phase II clinical trial for a related compound RG1662 (Hoffman-La Roche) in Down syndrome patients, the trial did not meet the primary and secondary endpoints of improved cognition and function.

Inflammation Induced Mild Cognitive Impairment and Post Anesthesia Memory Blockade

Increased systemic inflammation caused by pathological events such as stroke, infection, and traumatic brain injury is associated with memory problems during recovery from the initial insult. In an acute inflammation model, increased tonic α5 GABA$_A$R current and surface levels *via* P38 MAPK signaling was central to generating inflammation induced memory deficits (Wang et al., 2012). Importantly, these inflammation induced memory impairments were absent in *Gabra5*$^{-/-}$ mice and could be blocked by treatment with the α5 NAMs L-655,708 or MRK-016. Similarly, following stroke injury, tonic inhibition is increased in the peri-infarct zone, and L-655,708 treatment from 3-days post-stroke increases functional recovery (Clarkson et al., 2010). Gabra5$^{-/-}$ mice also exhibited improved motor recovery post-stroke. Sustained upregulation of α5 GABA$_A$Rs is also indicated in memory blockade following anesthesia (Zurek et al., 2014). Both the injectable anesthetic etomidate and the inhaled anesthetic isoflurane increase α5 GABA$_A$R tonic conductance, promoting the amnesic properties of these drugs (Cheng et al., 2006; Martin et al., 2009b; Saab et al., 2010). Pharmacological inhibition of α5 GABA$_A$Rs reduces anesthetic potentiation of GABA$_A$Rs (Lecker et al., 2013) and restores recognition memory in mice after anesthesia. Recent investigation of age-dependent efficacy of L-655,708 showed that α5 NAM treatment prior or following anesthesia restored spatial learning and memory in young rats, while aged rats only showed improvement with α5 NAM treatment prior to anesthesia (Zhao et al., 2019). Importantly, low dose isoflurane downregulated α5 mRNA in aging hippocampal neurons but upregulated α5 mRNA in neurons from young animals. This suggests different approaches will be needed to improve post anesthesia memory blockade in young vs. aged populations.

PAM α5 GABA$_A$R Therapeutic Applications

Neurodevelopmental Disorders

Mouse models of neurodevelopmental disorders that present with insufficient inhibitory tone show improvement with positive modulators of GABA$_A$R signaling. In the Scn1a+/− mouse model of Dravet syndrome, a severe childhood epileptic encephalopathy syndrome with hyperactivity and autism behaviors, abnormal social behaviors and fear memory deficits were rescued following treatment with a benzodiazepine, clonazepam (Han et al., 2014). In an ASD mouse model with reduced GABA$_A$R-mediated inhibition, the BTBR T+tf/J mouse, the α2,3 and 5 PAM L-838,417, improved deficits in social interaction, repetitive behaviors, and spatial learning (Han et al., 2014).

Mild Cognitive Impairment in Aging

Although α5 GABA$_A$R NAMs enhance memory in young rodents, it appears positive modulation may be more therapeutic in aging brains impaired by excess activity. Particularly in disorders such as Alzheimer's which are hallmarked by overexcitation (Ambrad Giovannetti and Fuhrmann, 2019), enhanced cognition may be achieved with reducing pathological excitability, as observed with the FDA approved NMDAR antagonist memantine. Furthermore, there is growing evidence for a general decline in GABAergic inhibitory tone in aging humans, monkeys and rodents (Rozycka and Liguz-Lecznar, 2017; Lissemore et al., 2018). From this newer perspective, an α5 GABA$_A$R PAM focused approach (Compound 44) identified improved hippocampal-dependent memory in aged rats with cognitive impairment (Koh et al., 2013).

Depression and Schizophrenia

Another important unmet need where α5 GABA$_A$Rs PAM pharmacotherapy may be applicable is in the development of new fast-acting anti-depressant drugs. Most current antidepressants act on the monoaminergic systems, and are only moderately therapeutically efficacious after dosing for several weeks. Significant evidence links GABAergic deficits with major depressive disorders (MDD) (Luscher et al., 2011). Investigation of anti-depressant activity of the α5 PAM SH-053-2′F-R-CH3 showed stress reduction in female mice both as an acute and chronic treatment (Piantadosi et al., 2016). Although male mice did not respond to PAM treatment, they also failed to show the upregulation of Gabra5 gene expression following unpredictable chronic mild stress seen in female mice. This particular PAM was also able to reverse pathological increases in dopaminergic activity in the MAM-model of schizophrenia (Gill et al., 2011). GL-II-73 a recently developed α5 preferring PAM showed anxiolytic and antidepressant efficacy, reversing stress-induced and age-related working memory deficits both in male and female mice (Prevot et al., 2019). Somewhat contradictory to this data and the GABA deficit hypothesis of MDD, α5 NAM have also shown rapid antidepressant actions in mice, potentially via ketamine like mechanisms of disinhibition (Fischell et al., 2015; Zanos et al., 2017).

CONCLUSION

Due to the unique physiology and pharmacology of α5 GABA$_A$Rs, these receptors are being targeted and tested as treatments for neurodevelopmental disorders, mild cognitive impairment, depression and schizophrenia. The recent cryo-EM studies of heteropentameric synaptic GABA$_A$Rs and binding of GABA, antagonists, and benzodiazepines should further advance α5 subtype specific structure-based drug design. Despite the progress in understanding of α5 GABA$_A$R neurobiology, comparatively little is understood regarding mechanisms that regulate α5 GABA$_A$R trafficking, stability, and both synaptic and extrasynaptic clustering. Furthermore, understanding of α5 GABA$_A$R plasticity occurring from endogenous signaling mechanisms and from drug treatments in the developing, mature and aging brain will be needed to effectively and safely advance therapeutic application of α5 GABA$_A$R preferring drugs.

AUTHOR CONTRIBUTIONS

TJ prepared the figure, table and wrote the manuscript.

FUNDING

This work was supported by National Institute of Mental Health (NIMH) 1R01MH114908-01A1.

REFERENCES

Ali, A. B., and Thomson, A. M. (2008). Synaptic α5 subunit-containing GABA$_A$ receptors mediate IPSPs elicited by dendrite-preferring cells in rat neocortex. *Cereb. Cortex* 18, 1260–1271. doi: 10.1093/cercor/bhm160

Ambrad Giovannetti, E., and Fuhrmann, M. (2019). Unsupervised excitation: GABAergic dysfunctions in Alzheimer's disease. *Brain Res.* 1707, 216–226. doi: 10.1016/j.brainres.2018.11.042

Araujo, F., Ruano, D., and Vitorica, J. (1999). Native γ-aminobutyric acid type A receptors from rat hippocampus, containing both α 1 and α 5 subunits, exhibit a single benzodiazepine binding site with α 5 pharmacological properties. *J. Pharmacol. Exp. Ther.* 290, 989–997.

Atack, J. R. (2010). Preclinical and clinical pharmacology of the GABA$_A$ receptor α5 subtype-selective inverse agonist α5IA. *Pharmacol. Ther.* 125, 11–26. doi: 10.1016/j.pharmthera.2009.09.001

Atack, J. R. (2011). GABA$_A$ receptor subtype-selective modulators: II. α5-selective inverse agonists for cognition enhancement. *Curr. Top. Med. Chem.* 11, 1203–1214. doi: 10.2174/156802611795371314

Atack, J. R., Bayley, P. J., Seabrook, G. R., Wafford, K. A., Mckernan, R. M., and Dawson, G. R. (2006). L-655,708 enhances cognition in rats but is not proconvulsant at a dose selective for α5-containing GABA$_A$ receptors. *Neuropharmacology* 51, 1023–1029. doi: 10.1016/j.neuropharm.2006.04.018

Atack, J. R., Maubach, K. A., Wafford, K. A., O'Connor, D., Rodrigues, A. D., Evans, D. C., et al. (2009). *In vitro* and *in vivo* properties of 3-tert-butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1H-1,2,4-triazol-5-ylmethoxy)-pyrazolo[1,5-d]-[1,2,4]triazine (MRK-016), a GABA$_A$ receptor α5 subtype-selective inverse agonist. *J. Pharmacol. Exp. Ther.* 331, 470–484. doi: 10.1124/jpet.109.157636

Bader, B. M., Steder, A., Klein, A. B., Frølund, B., Schroeder, O. H. U., and Jensen, A. A. (2017). Functional characterization of GABA$_A$ receptor-mediated modulation of cortical neuron network activity in microelectrode array recordings. *PLoS One* 12:e0186147. doi: 10.1371/journal.pone.0186147

Bakker, C. E., and Oostra, B. A. (2003). Understanding fragile X syndrome: insights from animal models. *Cytogenet. Genome Res.* 100, 111–123. doi: 10.1159/000072845

Balic, E., Rudolph, U., Fritschy, J.-M., Mohler, H., and Benke, D. (2009). The α5(H105R) mutation impairs α5 selective binding properties by altered positioning of the α5 subunit in GABA$_A$ receptors containing two distinct types of α subunits. *J. Neurochem.* 110, 244–254. doi: 10.1111/j.1471-4159.2009.06119.x

Ballard, T. M., Knoflach, F., Prinssen, E., Borroni, E., Vivian, J. A., Basile, J., et al. (2009). RO4938581, a novel cognitive enhancer acting at GABA$_A$ α5 subunit-containing receptors. *Psychopharmacology* 202, 207–223. doi: 10.1007/s00213-008-1357-7

Ben-Ari, Y. (2002). Excitatory actions of gaba during development: the nature of the nurture. *Nat. Rev. Neurosci.* 3, 728–739. doi: 10.1038/nrn920

Ben-Ari, Y., Gaiarsa, J. L., Tyzio, R., and Khazipov, R. (2007). GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol. Rev.* 87, 1215–1284. doi: 10.1152/physrev.00017.2006

Bonin, R. P., Martin, L. J., Macdonald, J. F., and Orser, B. A. (2007). α5GABA$_A$ receptors regulate the intrinsic excitability of mouse hippocampal pyramidal neurons. *J. Neurophysiol.* 98, 2244–2254. doi: 10.1152/jn.00482.2007

Brady, M. L., and Jacob, T. C. (2015). Synaptic localization of α5 GABA$_A$ receptors *via* gephyrin interaction regulates dendritic outgrowth and spine maturation. *Dev. Neurobiol.* 75, 1241–1251. doi: 10.1002/dneu.22280

Braudeau, J., Dauphinot, L., Duchon, A., Loistron, A., Dodd, R. H., Hérault, Y., et al. (2011). Chronic treatment with a promnesiant GABA-A α5-selective inverse agonist increases immediate early genes expression during memory processing in mice and rectifies their expression levels in a down syndrome mouse model. *Adv. Pharmacol. Sci.* 2011:153218. doi: 10.1155/2011/153218

Brünig, I., Scotti, E., Sidler, C., and Fritschy, J. M. (2002). Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons *in vitro*. *J. Comp. Neurol.* 443, 43–55. doi: 10.1002/cne.10102

Butler, K. M., Moody, O. A., Schuler, E., Coryell, J., Alexander, J. J., Jenkins, A., et al. (2018). *De novo* variants in *GABRA2* and *GABRA5* alter receptor function and contribute to early-onset epilepsy. *Brain* 141, 2392–2405. doi: 10.1093/brain/awy171

Caraiscos, V. B., Elliott, E. M., You-Ten, K. E., Cheng, V. Y., Belelli, D., Newell, J. G., et al. (2004). Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. *Proc. Natl. Acad. Sci. U S A* 101, 3662–3667. doi: 10.1073/pnas.0307231101

Chambers, M. S., Atack, J. R., Bromidge, F. A., Broughton, H. B., Cook, S., Dawson, G. R., et al. (2002). 6,7-Dihydro-2-benzothiophen-4(5H)-ones: a novel class of GABA-A α5 receptor inverse agonists. *J. Med. Chem.* 45, 1176–1179. doi: 10.1021/jm010471b

Chambers, M. S., Atack, J. R., Broughton, H. B., Collinson, N., Cook, S., Dawson, G. R., et al. (2003). Identification of a novel, selective GABA$_A$ α5 receptor inverse agonist which enhances cognition. *J. Med. Chem.* 46, 2227–2240. doi: 10.1021/jm020582q

Chen, X., Keramidas, A., and Lynch, J. W. (2017). Physiological and pharmacological properties of inhibitory postsynaptic currents mediated by α5β1γ2, α5β2γ2 and α5β3γ2 GABA$_A$ receptors. *Neuropharmacology* 125, 243–253. doi: 10.1016/j.neuropharm.2017.07.027

Cheng, V. Y., Martin, L. J., Elliott, E. M., Kim, J. H., Mount, H. T., Taverna, F. A., et al. (2006). α5GABA$_A$ receptors mediate the amnestic but not sedative-hypnotic effects of the general anesthetic etomidate. *J. Neurosci.* 26, 3713–3720. doi: 10.1523/JNEUROSCI.5024-05.2006

Christie, S. B., and de Blas, A. L. (2002). α5 subunit-containing GABA$_A$ receptors form clusters at GABAergic synapses in hippocampal cultures. *Neuroreport* 13, 2355–2358. doi: 10.1097/00001756-200212030-00037

Clarkson, A. N., Huang, B. S., Macisaac, S. E., Mody, I., and Carmichael, S. T. (2010). Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. *Nature* 468, 305–309. doi: 10.1038/nature09511

Clayton, T., Poe, M. M., Rallapalli, S., Biawat, P., Savic, M. M., Rowlett, J. K., et al. (2015). A review of the updated pharmacophore for the α 5 GABA$_A$ benzodiazepine receptor model. *Int. J. Med. Chem.* 2015:430248. doi: 10.1155/2015/430248

Collinson, N., Kuenzi, F. M., Jarolimek, W., Maubach, K. A., Cothliff, R., Sur, C., et al. (2002). Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the α5 subunit of the GABA$_A$ receptor. *J. Neurosci.* 22, 5572–5580. doi: 10.1523/jneurosci.22-13-05572.2002

Crestani, F., Keist, R., Fritschy, J. M., Benke, D., Vogt, K., Prut, L., et al. (2002). Trace fear conditioning involves hippocampal α_5 GABA$_A$ receptors. *Proc. Natl. Acad. Sci. U S A* 99, 8980–8985. doi: 10.1073/pnas.142288699

Curia, G., Papouin, T., Séguéla, P., and Avoli, M. (2009). Downregulation of tonic GABAergic inhibition in a mouse model of fragile X syndrome. *Cereb. Cortex* 19, 1515–1520. doi: 10.1093/cercor/bhn159

Deidda, G., Parrini, M., Naskar, S., Bozarth, I. F., Contestabile, A., and Cancedda, L. (2015). Reversing excitatory GABA$_A$ signaling restores synaptic plasticity and memory in a mouse model of Down syndrome. *Nat. Med.* 21, 318–326. doi: 10.1038/nm.3827

del Río, J. C., Araujo, F., Ramos, B., Ruano, D., and Vitorica, J. (2001). Prevalence between different α subunits performing the benzodiazepine binding sites in native heterologous GABA$_A$ receptors containing the α2 subunit. *J. Neurochem.* 79, 183–191. doi: 10.1046/j.1471-4159.2001.00551.x

Deprez, F., Vogt, F., Floriou-Servou, A., Lafourcade, C., Rudolph, U., Tyagarajan, S. K., et al. (2016). Partial inactivation of GABA$_A$ receptors containing the α5 subunit affects the development of adult-born dentate gyrus granule cells. *Eur. J. Neurosci.* 44, 2258–2271. doi: 10.1111/ejn.13329

Engin, E., Benham, R. S., and Rudolph, U. (2018). An emerging circuit pharmacology of GABA$_A$ receptors. *Trends Pharmacol. Sci.* 39, 710–732. doi: 10.1016/j.tips.2018.04.003

Etherington, L. A., Mihalik, B., Pálvölgyi, A., Ling, I., Pallagi, K., Kertesz, S., et al. (2017). Selective inhibition of extra-synaptic α5-GABA$_A$ receptors by S44819, a new therapeutic agent. *Neuropharmacology* 125, 353–364. doi: 10.1016/j.neuropharm.2017.08.012

Fischell, J., Van Dyke, A. M., Kvarta, M. D., LeGates, T. A., and Thompson, S. M. (2015). Rapid antidepressant action and restoration of excitatory synaptic strength after chronic stress by negative modulators of alpha5-containing GABA$_A$ receptors. *Neuropsychopharmacology* 40, 2499–2509. doi: 10.1038/npp.2015.112

Fuchs, T., Jefferson, S. J., Hooper, A., Yee, P. H., Maguire, J., and Luscher, B. (2017). Disinhibition of somatostatin-positive GABAergic interneurons results in an anxiolytic and antidepressant-like brain state. *Mol. Psychiatry* 22, 920–930. doi: 10.1038/mp.2016.188

Ge, S., Goh, E. L. K., Sailor, K. A., Kitabatake, Y., Ming, G.-L., and Song, H. (2006). GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature* 439, 589–593. doi: 10.1038/nature04404

Gill, K. M., Lodge, D. J., Cook, J. M., Aras, S., and Grace, A. A. (2011). A novel α5GABA$_A$R-positive allosteric modulator reverses hyperactivation of the dopamine system in the MAM model of schizophrenia. *Neuropsychopharmacology* 36, 1903–1911. doi: 10.1038/npp.2011.76

Giusi, G., Facciolo, R. M., Rende, M., Alo, R., Di Vito, A., Salerno, S., et al. (2009). Distinct α subunits of the GABA$_A$ receptor are responsible for early hippocampal silent neuron-related activities. *Hippocampus* 19, 1103–1114. doi: 10.1002/hipo.20584

Glykys, J., Mann, E. O., and Mody, I. (2008). Which GABA$_A$ receptor subunits are necessary for tonic inhibition in the hippocampus? *J. Neurosci.* 28, 1421–1426. doi: 10.1523/jneurosci.4751-07.2008

Glykys, J., and Mody, I. (2006). Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABA$_A$ receptor α5 subunit-deficient mice. *J. Neurophysiol.* 95, 2796–2807. doi: 10.1152/jn.01122.2005

Han, S., Tai, C., Jones, C. J., Scheuer, T., and Catterall, W. A. (2014). Enhancement of inhibitory neurotransmission by GABA$_A$ receptors having α2,3-subunits ameliorates behavioral deficits in a mouse model of autism. *Neuron* 81, 1282–1289. doi: 10.1016/j.neuron.2014.01.016

Hauser, J., Rudolph, U., Keist, R., Möhler, H., Feldon, J., and Yee, B. K. (2005). Hippocampal α5 subunit-containing GABA$_A$ receptors modulate the expression of prepulse inhibition. *Mol. Psychiatry* 10, 201–207. doi: 10.1038/sj.mp.4001554

Hausrat, T. J., Muhia, M., Gerrow, K., Thomas, P., Hirdes, W., Tsukita, S., et al. (2015). Radixin regulates synaptic GABA$_A$ receptor density and is essential for reversal learning and short-term memory. *Nat. Commun.* 6:6872. doi: 10.1038/ncomms7872

Herd, M. B., Haythornthwaite, A. R., Rosahl, T. W., Wafford, K. A., Homanics, G. E., Lambert, J. J., et al. (2008). The expression of GABA$_A$β subunit isoforms in synaptic and extrasynaptic receptor populations of mouse dentate gyrus granule cells. *J. Physiol.* 586, 989–1004. doi: 10.1113/jphysiol.2007.146746

Hernandez, C. C., Klassen, T. L., Jackson, L. G., Gurba, K., Hu, N., Noebels, J. L., et al. (2016). Deleterious rare variants reveal risk for loss of GABA$_A$ receptor function in patients with genetic epilepsy and in the general population. *PLoS One* 11:e0162883. doi: 10.1371/journal.pone.0162883

Hogart, A., Wu, D., Lasalle, J. M., and Schanen, N. C. (2010). The comorbidity of autism with the genomic disorders of chromosome 15q11.2-q13. *Neurobiol. Dis.* 38, 181–191. doi: 10.1016/j.nbd.2008.08.011

Horder, J., Andersson, M., Mendez, M. A., Singh, N., Tangen, A., Lundberg, J., et al. (2018). GABA$_A$ receptor availability is not altered in adults with autism spectrum disorder or in mouse models. *Sci. Transl. Med.* 10:eaam8434. doi: 10.1126/scitranslmed.aam8434

Hu, X., Rocco, B. R., Fee, C., and Sibille, E. (2018). Cell type-specific gene expression of α 5 subunit-containing γ-aminobutyric acid subtype A receptors in human and mouse frontal cortex. *Mol. Neuropsychiatry* 4, 204–215. doi: 10.1159/000495840

Ju, Y. H., Guzzo, A., Chiu, M. W., Taylor, P., Moran, M. F., Gurd, J. W., et al. (2009). Distinct properties of murine α5 γ-aminobutyric acid type a receptors revealed by biochemical fractionation and mass spectroscopy. *J. Neurosci. Res.* 87, 1737–1747. doi: 10.1002/jnr.21991

Kazdoba, T. M., Leach, P. T., Yang, M., Silverman, J. L., Solomon, M., and Crawley, J. N. (2016). Translational mouse models of autism: advancing toward pharmacological therapeutics. *Curr. Top. Behav. Neurosci.* 28, 1–52. doi: 10.1007/7854_2015_5003

Koh, M. T., Rosenzweig-Lipson, S., and Gallagher, M. (2013). Selective GABA$_A$ α5 positive allosteric modulators improve cognitive function in aged rats with memory impairment. *Neuropharmacology* 64, 145–152. doi: 10.1016/j.neuropharm.2012.06.023

Laverty, D., Desai, R., Uchanski, T., Masiulis, S., Stec, W. J., Malinauskas, T., et al. (2019). Cryo-EM structure of the human α1β3γ2 GABA$_A$ receptor in a lipid bilayer. *Nature* 565, 516–520. doi: 10.1038/s41586-018-0833-4

Lecker, I., Yin, Y., Wang, D. S., and Orser, B. A. (2013). Potentiation of GABA$_A$ receptor activity by volatile anaesthetics is reduced by α5GABA$_A$ receptor-preferring inverse agonists. *Br. J. Anaesth.* 110, i73–i81. doi: 10.1093/bja/aet038

Lévi, S., Le Roux, N., Eugène, E., and Poncer, J. C. (2015). Benzodiazepine ligands rapidly influence GABA$_A$ receptor diffusion and clustering at hippocampal inhibitory synapses. *Neuropharmacology* 88, 199–208. doi: 10.1016/j.neuropharm.2014.06.002

Ling, I., Mihalik, B., Etherington, L. A., Kapus, G., Pálvölgyi, A., Gigler, G., et al. (2015). A novel GABA$_A$ α 5 receptor inhibitor with therapeutic potential. *Eur. J. Pharmacol.* 764, 497–507. doi: 10.1016/j.ejphar.2015.07.005

Lisman, J., and Buzsáki, G. (2008). A neural coding scheme formed by the combined function of gamma and theta oscillations. *Schizophr. Bull.* 34, 974–980. doi: 10.1093/schbul/sbn060

Lissemore, J. I., Bhandari, A., Mulsant, B. H., Lenze, E. J., Reynolds, C. F. III., Karp, J. F., et al. (2018). Reduced GABAergic cortical inhibition in aging and depression. *Neuropsychopharmacology* 43, 2277–2284. doi: 10.1038/s41386-018-0093-x

Liu, Z. F., Kamatchi, G. L., Moreira, T., Mu, W., and Burt, D. R. (1998). The α5 subunit of the murine type A GABA receptor. *Mol. Brain Res.* 59, 84–89. doi: 10.1016/s0169-328x(98)00144-2

Loebrich, S., Bähring, R., Katsuno, T., Tsukita, S., and Kneussel, M. (2006). Activated radixin is essential for GABA$_A$ receptor α5 subunit anchoring at the actin cytoskeleton. *EMBO J.* 25, 987–999. doi: 10.1038/sj.emboj.7600995

Lorenz-Guertin, J. M., Bambino, M. J., Das, S., Weintraub, S. T., and Jacob, T. C. (2019). Diazepam accelerates GABA$_A$R synaptic exchange and alters intracellular trafficking. *Cell. Neurosci.* 13:163. doi: 10.3389/fncel.2019.00163

Lucas-Osma, A. M., Li, Y., Lin, S., Black, S., Singla, R., Fouad, K., et al. (2018). Extrasynaptic α5GABA$_A$ receptors on proprioceptive afferents produce a tonic depolarization that modulates sodium channel function in the rat spinal cord. *J. Neurophysiol.* 120, 2953–2974. doi: 10.1152/jn.00499.2018

Luscher, B., Shen, Q., and Sahir, N. (2011). The GABAergic deficit hypothesis of major depressive disorder. *Mol. Psychiatry* 16, 383–406. doi: 10.1038/mp.2010.120

Magnin, E., Francavilla, R., Amalyan, S., Gervais, E., David, L. S., Luo, X., et al. (2019). Input-specific synaptic location and function of the α5 GABA$_A$ receptor subunit in the mouse CA1 hippocampal neurons. *J. Neurosci.* 39, 788–801. doi: 10.1523/JNEUROSCI.0567-18.2018

Marchionni, I., Omrani, A., and Cherubini, E. (2007). In the developing rat hippocampus a tonic GABA$_A$-mediated conductance selectively enhances the glutamatergic drive of principal cells. *J. Physiol.* 581, 515–528. doi: 10.1113/jphysiol.2006.125609

Martin, L. J., Bonin, R. P., and Orser, B. A. (2009a). The physiological properties and therapeutic potential of α5-GABA$_A$ receptors. *Biochem. Soc. Trans.* 37, 1334–1337. doi: 10.1042/BST0371334

Martin, L. J., Oh, G. H., and Orser, B. A. (2009b). Etomidate targets α5 γ-aminobutyric acid subtype A receptors to regulate synaptic plasticity and memory blockade. *Anesthesiology* 111, 1025–1035. doi: 10.1097/ALN.0b013e3181bbc961

Martin, L. J., Zurek, A. A., MacDonald, J. F., Roder, J. C., Jackson, M. F., and Orser, B. A. (2010). α5GABA$_A$ receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. *J. Neurosci.* 30, 5269–5282. doi: 10.1523/JNEUROSCI.4209-09.2010

Martínez-Cué, C., Martínez, P., Rueda, N., Vidal, R., García, S., Vidal, V., et al. (2013). Reducing GABA$_A$ α5 receptor-mediated inhibition rescues functional and neuromorphological deficits in a mouse model of down syndrome. *J. Neurosci.* 33, 3953–3966. doi: 10.1523/JNEUROSCI.1203-12.2013

Masiulis, S., Desai, R., Uchanski, T., Serna Martin, I., Laverty, D., Karia, D., et al. (2019). GABA$_A$ receptor signalling mechanisms revealed by structural pharmacology. *Nature* 565, 454–459. doi: 10.1038/s41586-018-0832-5

Mendez, M. A., Horder, J., Myers, J., Coghlan, S., Stokes, P., Erritzoe, D., et al. (2013). The brain GABA-benzodiazepine receptor α-5 subtype in autism spectrum disorder: a pilot [^{11}C]Ro15–4513 positron emission tomography study. *Neuropharmacology* 68, 195–201. doi: 10.1016/j.neuropharm.2012.04.008

Mesbah-Oskui, L., Penna, A., Orser, B. A., and Horner, R. L. (2017). Reduced expression of α5GABA$_A$ receptors elicits autism-like alterations in EEG patterns and sleep-wake behavior. *Neurotoxicol. Teratol.* 61, 115–122. doi: 10.1016/j.ntt.2016.10.009

Olsen, R. W., and Sieghart, W. (2009). GABA$_A$ receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* 56, 141–148. doi: 10.1016/j.neuropharm.2008.07.045

Pangratz-Fuehrer, S., Sieghart, W., Rudolph, U., Parada, I., and Huguenard, J. R. (2016). Early postnatal switch in GABA$_A$ receptor α-subunits in the reticular thalamic nucleus. *J. Neurophysiol.* 115, 1183–1195. doi: 10.1152/jn.00905.2015

Perez-Sanchez, J., Lorenzo, L. E., Lecker, I., Zurek, A. A., Labrakakis, C., Bridgwater, E. M., et al. (2017). α5GABA$_A$ receptors mediate tonic inhibition in the spinal cord dorsal horn and contribute to the resolution of hyperalgesia. *J. Neurosci. Res.* 95, 1307–1318. doi: 10.1002/jnr.23981

Phulera, S., Zhu, H., Yu, J., Claxton, D. P., Yoder, N., Yoshioka, C., et al. (2018). Cryo-EM structure of the benzodiazepine-sensitive α1β1γ2S tri-heteromeric GABA$_A$ receptor in complex with GABA. *eLife* 7:e39383. doi: 10.7554/elife.39383

Piantadosi, S. C., French, B. J., Poe, M. M., Timic, T., Markovic, B. D., Pabba, M., et al. (2016). Sex-dependent anti-stress effect of an α5 subunit containing GABA$_A$ receptor positive allosteric modulator. *Front. Pharmacol.* 7:446. doi: 10.3389/fphar.2016.00446

Prenosil, G. A., Schneider Gasser, E. M., Rudolph, U., Keist, R., Fritschy, J. M., and Vogt, K. E. (2006). Specific subtypes of GABA$_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. *J. Neurophysiol.* 96, 846–857. doi: 10.1152/jn.01199.2006

Prevot, T. D., Li, G., Vidojevic, A., Misquitta, K. A., Fee, C., Santrac, A., et al. (2019). Novel benzodiazepine-like ligands with various anxiolytic, antidepressant, or pro-cognitive profiles. *Mol. Neuropsychiatry* 5, 84–97. doi: 10.1159/000496086

Prut, L., Prenosil, G., Willadt, S., Vogt, K., Fritschy, J. M., and Crestani, F. (2010). A reduction in hippocampal GABA$_A$ receptor α5 subunits disrupts the memory for location of objects in mice. *Genes Brain Behav.* 9, 478–488. doi: 10.1111/j.1601-183x.2010.00575.x

Ramos, B., Lopez-Tellez, J. F., Vela, J., Baglietto-Vargas, D., Del Rio, J. C., Ruano, D., et al. (2004). Expression of α 5 GABA$_A$ receptor subunit in developing rat hippocampus. *Dev. Brain Res.* 151, 87–98. doi: 10.1111/j.0953-816x.2004.03349.x

Renner, M., Schweizer, C., Bannai, H., Triller, A., and Lévi, S. (2012). Diffusion barriers constrain receptors at synapses. *PLoS One* 7:e43032. doi: 10.1371/journal.pone.0043032

Rozycka, A., and Liguz-Lecznar, M. (2017). The space where aging acts: focus on the GABAergic synapse. *Aging Cell* 16, 634–643. doi: 10.1111/acel.12605

Saab, B. J., Maclean, A. J., Kanisek, M., Zurek, A. A., Martin, L. J., Roder, J. C., et al. (2010). Short-term memory impairment after isoflurane in mice is prevented by the α5 γ-aminobutyric acid type A receptor inverse agonist L-655,708. *Anesthesiology* 113, 1061–1071. doi: 10.1097/ALN.0b013e3181f56228

Schulz, J. M., Knoflach, F., Hernandez, M.-C., and Bischofberger, J. (2018). Dendrite-targeting interneurons control synaptic NMDA-receptor activation via nonlinear α5-GABA$_A$ receptors. *Nat. Commun.* 9:3576. doi: 10.1038/s41467-018-06004-8

Schulz, J. M., Knoflach, F., Hernandez, M.-C., and Bischofberger, J. (2019). Enhanced dendritic inhibition and impaired NMDAR activation in a mouse model of down syndrome. *J. Neurosci.* 39, 5210–5221. doi: 10.1523/JNEUROSCI.2723-18.2019

Serwanski, D. R., Miralles, C. P., Christie, S. B., Mehta, A. K., Li, X., and De Blas, A. L. (2006). Synaptic and nonsynaptic localization of GABA$_A$ receptors containing the α5 subunit in the rat brain. *J. Comp. Neurol.* 499, 458–470. doi: 10.1002/cne.21115

Sieghart, W., and Savic, M. M. (2018). International union of basic and clinical pharmacology. CVI: GABA$_A$ receptor subtype- and function-selective ligands: key issues in translation to humans. *Pharmacol. Rev.* 70, 836–878. doi: 10.1124/pr.117.014449

Street, L. J., Sternfeld, F., Jelley, R. A., Reeve, A. J., Carling, R. W., Moore, K. W., et al. (2004). Synthesis and biological evaluation of 3-Heterocyclyl-7,8,9,10-tetrahydro-(7,10-ethano)-1,2,4-triazolo[3,4-a]phthalazines and analogues as subtype-selective inverse agonists for the GABA$_A$α5 benzodiazepine binding site. *J. Med. Chem.* 47, 3642–3657.

Sur, C., Fresu, L., Howell, O., McKernan, R. M., and Atack, J. R. (1999). Autoradiographic localization of α5 subunit-containing GABA$_A$ receptors in rat brain. *Brain Res.* 822, 265–270. doi: 10.1016/s0006-8993(99)01152-x

Sur, C., Quirk, K., Dewar, D., Atack, J., and McKernan, R. (1998). Rat and human hippocampal α5 subunit-containing γ-aminobutyric AcidA receptors have α5 β3 γ2 pharmacological characteristics. *Mol. Pharmacol.* 54, 928–933. doi: 10.1124/mol.54.5.928

Towers, S. K., Gloveli, T., Traub, R. D., Driver, J. E., Engel, D., Fradley, R., et al. (2004). α 5 subunit-containing GABA$_A$ receptors affect the dynamic range of mouse hippocampal kainate-induced γ frequency oscillations *in vitro*. *J. Physiol.* 559, 721–728. doi: 10.1113/jphysiol.2004.071191

Vargas-Caballero, M., Martin, L. J., Salter, M. W., Orser, B. A., and Paulsen, O. (2010). α5 Subunit-containing GABA$_A$ receptors mediate a slowly decaying inhibitory synaptic current in CA1 pyramidal neurons following Schaffer collateral activation. *Neuropharmacology* 58, 668–675. doi: 10.1016/j.neuropharm.2009.11.005

Vidal, V., Garcia-Cerro, S., Martínez, P., Corrales, A., Lantigua, S., Vidal, R., et al. (2018). Decreasing the expression of GABA$_A$ α5 subunit-containing receptors partially improves cognitive, electrophysiological, and morphological hippocampal defects in the Ts65Dn model of down syndrome. *Mol. Neurobiol.* 55, 4745–4762. doi: 10.1007/s12035-017-0675-3

Wang, D.-S., Zurek, A. A., Lecker, I., Yu, J., Abramian, A. M., Avramescu, S., et al. (2012). Memory deficits induced by inflammation are regulated by α5-subunit-containing GABA$_A$ receptors. *Cell Rep.* 2, 488–496. doi: 10.1016/j.celrep.2012.08.022

Yu, Y., Fuscoe, J. C., Zhao, C., Guo, C., Jia, M., Qing, T., et al. (2014). A rat RNA-Seq transcriptomic BodyMap across 11 organs and 4 developmental stages. *Nat. Commun.* 5:3230. doi: 10.1038/ncomms4230

Zanos, P., Nelson, M. E., Highland, J. N., Krimmel, S. R., Georgiou, P., Gould, T. D., et al. (2017). A negative allosteric modulator for alpha5 subunit-containing GABA receptors exerts a rapid and persistent antidepressant-like action without the side effects of the NMDA receptor antagonist ketamine in mice. *eNeuro* 4. doi: 10.1523/ENEURO.0285-16.2017

Zarnowska, E. D., Keist, R., Rudolph, U., and Pearce, R. A. (2009). GABA$_A$ receptor α5 subunits contribute to GABA$_A$,slow synaptic inhibition in mouse hippocampus. *J. Neurophysiol.* 101, 1179–1191. doi: 10.1152/jn.91203.2008

Zhao, Z.-F., Du, L., Gao, T., Bao, L., Luo, Y., Yin, Y.-Q., et al. (2019). Inhibition of α5 GABA$_A$ receptors has preventive but not therapeutic effects on isoflurane-induced memory impairment in aged rats. *Neural Regen. Res.* 14, 1029–1036. doi: 10.4103/1673-5374.250621

Zhu, S., Noviello, C. M., Teng, J., Walsh, R. M. Jr., Kim, J. J., and Hibbs, R. E. (2018). Structure of a human synaptic GABA$_A$ receptor. *Nature* 559, 67–72. doi: 10.1038/s41586-018-0255-3

Zurek, A. A., Kemp, S. W., Aga, Z., Walker, S., Milenkovic, M., Ramsey, A. J., et al. (2016). α5GABA$_A$ receptor deficiency causes autism-like behaviors. *Ann. Clin. Transl. Neurol.* 3, 392–398. doi: 10.1002/acn3.303

Zurek, A. A., Yu, J., Wang, D. S., Haffey, S. C., Bridgwater, E. M., Penna, A., et al. (2014). Sustained increase in α5GABA$_A$ receptor function impairs memory after anesthesia. *J. Clin. Invest.* 124, 5437–5441. doi: 10.1172/JCI76669

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.

frontiers in Molecular Neuroscience

MINI REVIEW
published: 07 August 2019
doi: 10.3389/fnmol.2019.00191

Structure of Heteropentameric $GABA_A$ Receptors and Receptor-Anchoring Properties of Gephyrin

*Vikram Babu Kasaragod *† and Hermann Schindelin **

Institute of Structural Biology, Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg, Würzburg, Germany

γ-Aminobutyric acid type A receptors ($GABA_A$Rs) mediate the majority of fast synaptic inhibition in the central nervous system (CNS). $GABA_A$Rs belong to the Cys-loop superfamily of pentameric ligand-gated ion channels (pLGIC) and are assembled from 19 different subunits. As dysfunctional GABAergic neurotransmission manifests itself in neurodevelopmental disorders including epilepsy and anxiety, $GABA_A$Rs are key drug targets. The majority of synaptic $GABA_A$Rs are anchored at the inhibitory postsynaptic membrane by the principal scaffolding protein gephyrin, which acts as the central organizer in maintaining the architecture of the inhibitory postsynaptic density (iPSD). This interaction is mediated by the long intracellular loop located in between transmembrane helices 3 and 4 (M3–M4 loop) of the receptors and a universal receptor-binding pocket residing in the C-terminal domain of gephyrin. In 2014, the crystal structure of the β3-homopentameric $GABA_A$R provided crucial information regarding the architecture of the receptor; however, an understanding of the structure and assembly of heteropentameric receptors at the atomic level was lacking. This review article will highlight recent advances in understanding the structure of heteropentameric synaptic $GABA_A$Rs and how these structures have provided fundamental insights into the assembly of these multi-subunit receptors as well as their modulation by diverse ligands including the physiological agonist GABA. We will further discuss the role of gephyrin in the anchoring of synaptic $GABA_A$Rs and glycine receptors (GlyRs), which are crucial for maintaining the architecture of the iPSD. Finally, we will also summarize how anti-malarial artemisinin drugs modulate gephyrin-mediated inhibitory neurotransmission.

Keywords: $GABA_A$ receptors, gephyrin, diazepam, GABA, PIP_2, artemisinin, Cryo-EM, inhibitory neurotransmission

OPEN ACCESS

Edited by:
Shiva K. Tyagarajan, University of Zurich, Switzerland

Reviewed by:
Tija Jacob, University of Pittsburgh, United States
Gonzalo E. Yevenes, Universidad de Concepción, Chile

****Correspondence:***
Vikram Babu Kasaragod vikram.kasaragod@virchow.uni-wuerzburg.de
Hermann Schindelin hermann.schindelin@virchow.uni-wuerzburg.de

†Present address:
Vikram Babu Kasaragod, Department of Pharmacology, University of Cambridge, Cambridge, United Kingdom

Received: *14 February 2019*
Accepted: *22 July 2019*
Published: *07 August 2019*

Citation:
Kasaragod VB and Schindelin H (2019) Structure of Heteropentameric $GABA_A$ Receptors and Receptor-Anchoring Properties of Gephyrin. Front. Mol. Neurosci. 12:191. doi: 10.3389/fnmol.2019.00191

INTRODUCTION

Complex macromolecular interplays at excitatory and inhibitory synapses contribute in a fundamental way to the incredible functional capabilities of the human brain. Inhibition in the central nervous system (CNS) is mediated by key members of the Cys-loop receptor superfamily, in particular, the γ-aminobutyric acid type A receptors ($GABA_A$Rs), and, to a smaller extent,

the glycine receptors (GlyRs). Synaptic GABA$_A$Rs are pentameric ligand-gated ion channels (pLGICs) mainly composed of two α, two β and a single γ subunit, which are selected from a diverse pool of 19 different subunit types (Sigel and Steinmann, 2012). Each subunit consists of an extracellular domain (ECD) rich in β-sheet architecture, a four α-helical bundle transmembrane domain (TMD) and two intracellular, unstructured loops, the short M1–2 and the long M3–4 loop, connecting these helices. The ECDs harbor the sites for the natural agonist GABA and drugs, in particular the benzodiazepines, while the binding site for allosteric modulators such as endogenous neurosteroids reside in the TMD (Miller and Aricescu, 2014; Laverty et al., 2017; Miller et al., 2017; Phulera et al., 2018; Zhu et al., 2018).

The majority of synaptic GABA$_A$Rs, as well as GlyRs, are recruited to and anchored at the inhibitory postsynaptic membrane by the principal scaffolding protein gephyrin (Kirsch et al., 1991; Kneussel et al., 1999). This multi-domain protein consists of two terminal domains; the N-terminal G domain (GephG) and the C-terminal E domain (GephE), which are connected by a highly unstructured linker region (Kirsch et al., 1991; Prior et al., 1992; Schwarz et al., 2001; Kim et al., 2006; Sander et al., 2013). The interaction of gephyrin with postsynaptic receptors is mediated by a continuous segment within the large intracellular M3–4 loop and a universal receptor-binding pocket residing in GephE. In addition to the interactions with inhibitory neurotransmitter receptors, gephyrin also interacts with a diverse set of macromolecules, thus playing an essential role in establishing and maintaining the architecture of the inhibitory postsynaptic density (iPSD; Tyagarajan and Fritschy, 2014; Kasaragod and Schindelin, 2018). Besides its anchoring function, gephyrin also catalyzes the two terminal steps in the evolutionarily conserved molybdenum cofactor (Moco) biosynthesis pathway (Kuper et al., 2004; Kasaragod and Schindelin, 2016), a critical active site component of almost all Mo-containing enzymes.

Small molecules such as benzodiazepines, which target synaptic α-subunit containing GABA$_A$Rs, have been in clinical use for decades for the treatment of neurological disorders (for a detailed review see Rudolph and Knoflach, 2011). Since dysfunctional inhibitory neurotransmission triggered by defects residing in either the receptors or gephyrin has been implicated in a diverse set of neurodevelopmental disorders including anxiety and epilepsy (Agarwal et al., 2008; Hales et al., 2013; Dejanovic et al., 2014, 2015), these macromolecules may be suitable targets of future structure-based drug discovery processes.

In this review article, we will highlight recent advances in the structural elucidation of heteromeric GABA$_A$Rs and how these structures have helped us to understand the assembly and also regulation of these ion channels by diverse ligands (Laverty et al., 2019; Masiulis et al., 2019). Besides, we will also briefly discuss the alternative GABA$_A$R/GlyR recruitment to the iPSD and finally, summarize our recent contribution on the elucidation of the modulation of inhibitory neurotransmission by artemisinins.

STRUCTURAL INSIGHTS INTO SYNAPTIC HETEROPENTAMERIC GABA$_A$Rs

Until recently, knowledge regarding the atomic architectures of GABA$_A$Rs and their modulation by ligands was derived solely from structural studies performed with either homopentameric receptors or homopentameric receptor chimeras. While the crystal structure of the β3 homopentameric GABA$_A$R described the architecture of the receptor for the first time (Miller and Aricescu, 2014), studies with chimeric versions of the GABA$_A$Rs receptors provided atomic insights into the neurosteroid (e.g., pregnanolone and pregnenolone) binding site in the TMD and the modulation of GABA$_A$Rs by these compounds (Laverty et al., 2017; Miller et al., 2017). Nevertheless, structures of heteropentameric receptors had remained elusive until recently, when several independent studies (Phulera et al., 2018; Zhu et al., 2018; Laverty et al., 2019; Masiulis et al., 2019), which were aided by recent developments in the field of cryo-electron microscopy (Cryo-EM), provided crucial insights into the structure of heteropentameric receptors.

The first Cryo-EM structure of a heteromeric GABA$_A$R, in this case, composed of the human α1β2γ2 subunits, was determined by Hibbs and colleagues (Zhu et al., 2018). Subsequently, Gouaux and coworkers (Phulera et al., 2018) solved the Cryo-EM structure of the rat α1β1γ2 heteropentamer. Although both structures provided valuable insights into the binding of the agonist GABA and also the modulation of these receptors by flumazenil, which targets the benzodiazepine binding site, these structures were somewhat incomplete with respect to the overall architecture of the receptors. The first study (Zhu et al., 2018) described a structure in which the pore had collapsed due to an unusual arrangement of the γ2-subunit (PDB: 6D6U) while the other structure (Phulera et al., 2018) featured fragmented density in the TMD (PDB: 6DW0). A common denominator of these structures is that they were solved in the presence of detergents. Whereas Phulera et al. (2018) determined the structure by using the shorter splice variant of the γ2 subunit, it is unclear which γ2 subunit splice variant was used by Zhu et al. (2018). In addition, for the structural studies, Zhu et al. (2018) replaced the intracellular loop connecting the M3–4 helices with a seven-residue artificial linker, whereas Phulera et al. (2018) introduced a fluorescent tag in the M3–4 loop of the γ2 subunit in addition to shortening the M3–4 loops of the other subunits. In this review article, we will mainly focus on the structures of the human α1β3γ2 receptor published recently (Laverty et al., 2019; Masiulis et al., 2019) in which full-length GABA$_A$R subunits were used and the structures were solved by reconstituting the receptors in discoidal membranes (nanodiscs) composed of a double layer of lipid molecules surrounded by a membrane scaffold protein. These structures yielded unprecedented insights not only into the overall architecture of heteropentameric GABA$_A$Rs but also into the binding of diverse ligands including the agonist GABA. Finally, these structures also demonstrated how membrane lipids interact with the TMD (**Figures 1A–D**).

All structures revealed that the subunits are arranged in an α–β–α–β–γ arrangement in a clockwise manner when viewed

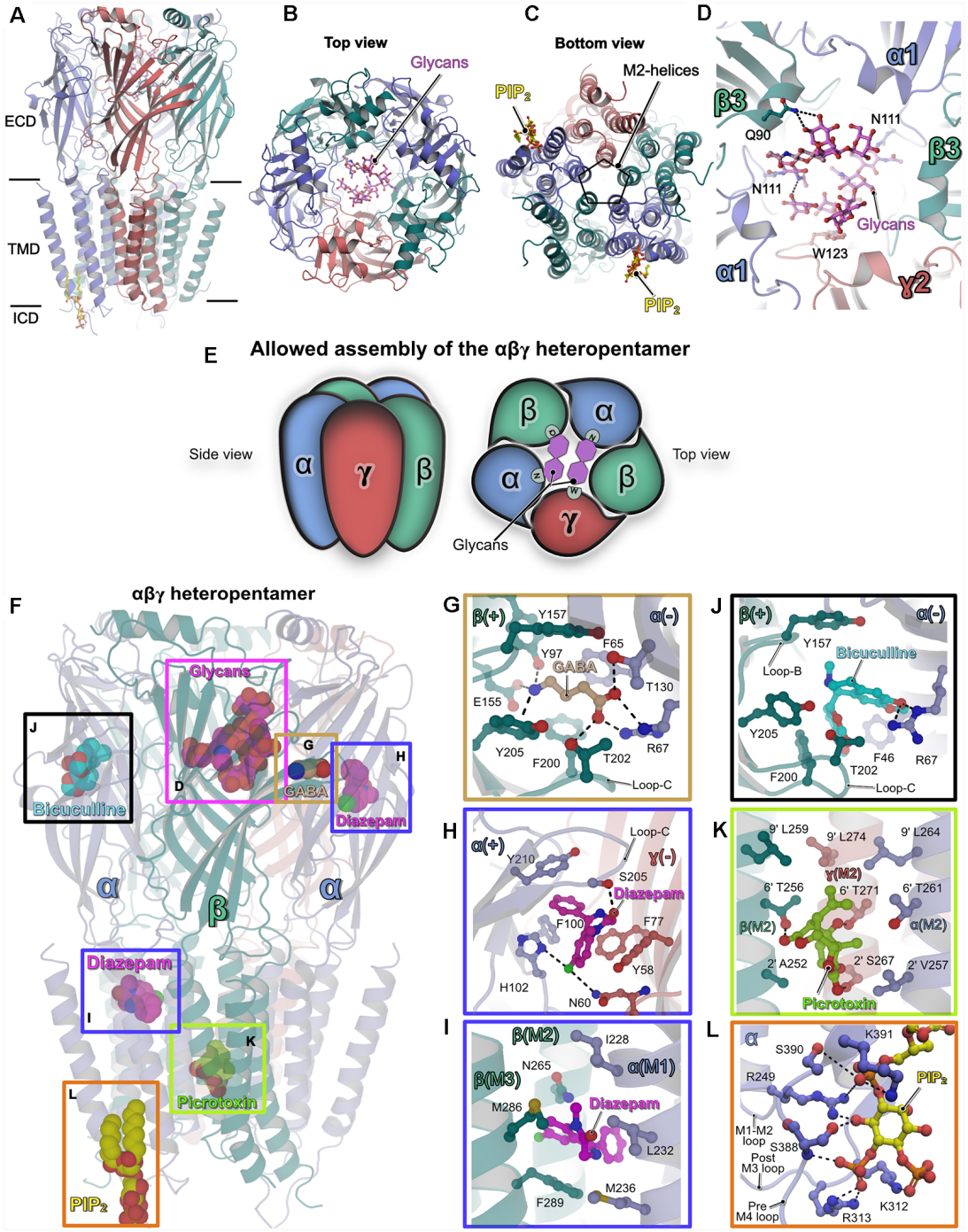

FIGURE 1 | Structures of heteropentameric γ-aminobutyric acid type A receptors (GABA$_A$Rs). **(A)** Side view of the overall structure of the heteropentameric GABA$_A$R as determined by cryo-electron microscopy (Cryo-EM). **(B)** Architecture of the receptor viewed from the extracellular side (top view) with the receptor subunits in cartoon representation and the glycans in ball and stick representation. **(C)** View of the receptor from the intracellular side into the ion-conducting pore (bottom view). **(D)** Close-up view of the glycosylation sites in the extracellular vestibule. The glycans and critical residues mediating their binding are shown in ball and stick representation. **(E)** Schematic representation of the underlying principle governing the assembly of synaptic heteropentameric GABA$_A$Rs. The scheme demonstrates how glycosylation of the conserved Asn111 plays a crucial structural role in receptor assembly, which in turn also determines the order in which the subunits are arranged. **(F–L)** Structures of GABA$_A$Rs bound to various ligands. **(F)** The heteropentameric GABA$_A$R is shown in cartoon representation along with structurally validated ligands in space-filling representation. Enlarged views of the binding pockets of the natural agonist GABA (PDB: 6HUJ, **G**), the positive allosteric modulator (PAM) diazepam (PDB: 6HUP, **H–I**), the competitive antagonist bicuculline (PDB: 6HUK, **J**), the channel blocker picrotoxin (PDB: 6HUG, **K**) and the lipid PIP$_2$ (PDB: 6I53, **L**). Enlarged views are shown according to the color of the box in the overall structure displayed in **(F)**. In **(F–L)** all ligands and the critical residues which mediate binding are shown in ball and stick and the protein chains in cartoon representation.

from the extracellular side, consistent with previous biochemical studies (Tretter et al., 1997; Baumann et al., 2002). Although the earlier structural analyses (Phulera et al., 2018; Zhu et al., 2018) and the more recent ones (Laverty et al., 2019; Masiulis et al., 2019) differed in receptor subunit composition and structural organization of the TMD, a common denominator amongst all of them was the observation of two unique glycosylation sites in the extracellular vestibule. These glycosylations originate from residue Asn111 which is present in all α-subunits and hence all heteropentameric GABA$_A$Rs. In addition to several inter-glycan interactions, Gln90 in the β-subunit mediates interactions with these glycans *via* hydrogen bonds which are augmented by a critical hydrophobic π-π stacking interaction with the conserved residue Trp123 residing in the γ2 subunit (**Figure 1D**). Depending on their occupancies, these glycans may have critical implications on the assembly and subunit arrangement in heteropentameric GABA$_A$Rs. Interestingly, a recent study (Hannan and Smart, 2018) showed that α1 homopentamer formation is controlled by two TMD residues (Gln241 and Ala290); if either residue is mutated (Q241W or A290W), α1 forms functional homopentamers on the surface of HEK cells. In addition, future research will also be required to understand the mechanism of assembly of heterodimeric receptors and the impact of glycosylation of Asn111 on receptor assembly. Nevertheless, this post-translational modification (PTM) is unique to heteropentameric GABA$_A$Rs and may have critical implications for receptor permeability while also critically contributing to subunit composition and arrangement within the heteropentamer (**Figure 1E**). In addition to this crucial information regarding the assembly of the heteropentamers, a series of structures of the α1β3γ2-GABA$_A$R in complex with diverse ligands provided valuable insights into their interactions with these receptors as briefly described below (**Figures 1F–L**).

GABA

The agonist GABA only occupied the two orthosteric binding sites created by the contribution of the principal β-subunit and complementary α-subunit as already reported in one of the earlier structures (Zhu et al., 2018), however, this is in contrast to the three GABA binding sites proposed by the Gouaux group (Phulera et al., 2018). The binding of GABA is mediated by residues from the "aromatic box" created by Tyr157, Phe200, Tyr205 from the β3-subunit and Phe65 from the α1-subunit, which are located in the ECD at the β–α subunit interface. The agonist is stabilized by an extensive hydrogen-bonding network between GABA and Tyr97, Glu155 of the principal β-subunit along with Arg67 and Thr130 from the complimentary α-subunit. The contribution from loop-C, through Thr202 *via* a hydrogen bond with the GABA carboxylate, additionally stabilizes the agonist (PDB: 6HUJ; **Figure 1G**).

Diazepam

Diazepam, which acts as a positive allosteric modulator (PAMs), has been used clinically for decades in the treatment of anxiety disorders and also epilepsy (Rudolph and Knoflach, 2011). The structure of the GABA$_A$R-diazepam complex (PDB: 6HUP) revealed that the drug molecule not only binds to the "classical diazepam binding pocket" created by the principal α-subunit and the complementary γ-subunit, but, in addition, a strong density feature was observed in the TMD. The binding at the ECD (**Figure 1H**) is mediated mainly by hydrophobic π-π stacking interactions with Phe100, His102 from the principal α-subunit and Phe77 and Tyr58 from the complementary γ-subunit. In addition, hydrogen bonds from His102 (α-subunit) and Asn60 (γ-subunit) augment diazepam binding at the ECD. Strikingly, His102 has been shown to be critical for the binding of benzodiazepine. Heteropentameric receptors composed of the αβγ subunits and containing either the α1-α3 or α5 subunits possess this histidine and are benzodiazepine-sensitive. In contrast, in the α4 and α6-subunits an arginine is present at this position and the corresponding receptors are non-responsive to benzodiazepine (Wieland et al., 1992; Davies et al., 1998; Dunn et al., 1999).

In contrast, the binding of diazepam in the TMD is mediated by the M2 and M3 helices from the β-subunit as well as the M1 helix from the α-subunit. Previous studies have proposed this site as target area of anesthetics such as azietomidate (Forman and Miller, 2011). The binding is mediated purely by hydrophobic interactions involving Met286 and Phe289 from M3 of the β-subunit as well as Leu232 and also Met236 from M1 of the α-subunit. In addition, the drug molecule comes into close proximity of Asn265 from the M2 helix of the β-subunit, which, in turn, will have a direct impact on the gating properties of the GABA$_A$R pore (**Figure 1I**). The two diazepam binding sites may provide an explanation for the biphasic potentiation of these receptors by diazepams as observed in electrophysiological experiments (Walters et al., 2000). Nevertheless, future research will be required to fully understand the properties of the secondary diazepam-binding site located in the TMD.

Bicuculline

The action of the competitive antagonist bicuculline is achieved by its binding into the aromatic box with contributions from loop-B and loop-C of the principal β-subunit (PDB: 6HUK). Bicuculline is sandwiched between the aromatic Tyr157 from loop-B of the principal β-subunit and Phe46 from the complementary α-subunit. In addition, hydrogen bonds to the guanidinium group of Arg67, which is also critical for agonist-binding, mediate binding of this antagonist (**Figure 1J**).

Picrotoxin

The structural analyses also revealed the binding site and blocking mechanism of GABA$_A$Rs by the classical channel blocker picrotoxin (**Figure 1K**). The picrotoxin-binding pocket resides in the channel and is lined by the Leu at the 9′ position (Leu264, Leu259 and Leu274 from the α, β and γ-subunit, respectively) and the respective variable 2′ residues (Val257, Ala252 and Ser267 from the α, β and γ-subunits, respectively) of the M2 helices in each subunit. In addition, hydrogen bonds mediated by the 6′ residues (Thr261 Thr256 and Thr271 from the α, β and γ-subunits, respectively), with principal contributions from the β and γ subunits, strengthen picrotoxin-binding (PDB: 6HUG).This is in contrast to the glutamate-gated chloride channel (GluCl), in which the picrotoxin-induced channel block

is achieved by its binding into a pocket created by the 2′-Thr and -2′-Pro residues (Hibbs and Gouaux, 2011).

Phosphatidylinositol Phosphates

The GABA$_A$R structure embedded in a lipid bilayer also revealed binding sites for phosphatidylinositol 4,5 bisphosphate (PDB: 6I53). The lipid occupies an electropositive area exclusive to the α-subunits and its binding is mediated by extensive hydrogen bonds from Lys312 and Arg313 from the post-M3 loop as well as Ser388, Ser390 and Lys391 from the pre-M4 loop with the inositol head group. PIP_2 binding is also complemented by Arg249 from the M1–2 loop (**Figure 1L**). Interestingly, while Lys312 and Arg313 are conserved in all synaptic α-subunits, the remaining residues mediating PIP_2-binding are conserved only in synaptic α-subunits (α1–3 and α5) and not in extrasynaptic α-subunits (α4 and α6). Thus, this specificity of synaptic GABA$_A$Rs towards PIP_2 may have critical implications for receptor trafficking at the iPSDs and on the channel gating properties as seen in the structurally validated cases of the transient receptor potential vanilloid 5 (TRPV5; Hughes et al., 2018), TRP mucolipin 1 (TRPML1; Fine et al., 2018) and also inward rectifier potassium channels (Hansen et al., 2011).

ARTEMISININS—GEPHYRIN-SPECIFIC MODULATORS OF INHIBITORY NEUROTRANSMISSION

The central scaffolding protein gephyrin anchors a large subset of postsynaptic GABA$_A$Rs (mainly those containing the α1-3 subunits) and also heteropentameric GlyRs, *via* their β-subunit, to the iPSD. This interaction is mediated by the universal receptor-binding pocket residing in the C-terminally located GephE domain and the M3–4 loop of the cognate inhibitory receptor (Maric et al., 2011). Common determinants between GABA$_A$Rs and the GlyR are the presence of an aromatic Phe/Tyr at the first position of the core binding pocket and a conserved Tyr at position 8 in the cognate GABA$_A$R subunits (Kim et al., 2006; Tretter et al., 2008, 2011; Maric et al., 2011, 2014a,b, 2015; Mukherjee et al., 2011; **Figure 2A**). Both types of receptors bind to a hydrophobic groove in GephE generated by contributions from subdomains III and IV. Although these receptors bind to an overlapping binding pocket and engage in similar interactions at the N-terminus of the core-binding motif, a receptor-specific interaction is present at the C-terminus. As could be only derived from the crystal structures (GephE-GlyRβ-49, Kim et al., 2006 and GephE-GABA$_A$R α3, Maric et al., 2014a), the Tyr at the +8 position of GABA$_A$R α3 subunits correspond to a Phe located at the last position of the GlyR β-subunit.

Recently, the anti-malarial drug artemisinin and its semi-synthetic derivatives, collectively referred to as artemisinins, were discovered to target GABA$_A$R signaling by interacting with gephyrin in pancreatic cells. While one study concluded that this interaction mediates the trans-differentiation of glucagon-producing Tα cells into insulin-secreting Tβ cells, thus ascribing an anti-diabetic nature to these compounds (Li et al., 2017), subsequent studies (van der Meulen et al., 2018; Ackermann et al., 2018) failed to reproduce the induction of trans-differentiation in pancreas-derived cells. Chemically, artemisinins are sesquiterpene lactones with an unusual endoperoxide bridge. In traditional Chinese medicine, artemisinins have been used for centuries to treat malaria and artemisinin-based combination therapies (ACTs) such as artesunate, the succinate derivative of artemisinin, with lumefantrine and artemether together with mefloquine are recommended by the World Health Organization (WHO, 2015) as standard drug regiment to treat malaria caused by *Plasmodium falciparum*. In addition to their anti-parasitic activity, artemisinins have additionally been implicated in regulating the activity of multiple cellular pathways, including the modulation of a variety of cancers (Crespo-Ortiz and Wei, 2012; Tu, 2016). Despite the widespread applications of these compounds as drugs and effectors of cellular pathways, the molecular basis of their regulatory properties including their target recognition mechanisms has so far remained elusive.

Studies from our lab deciphered the molecular basis for the interaction between gephyrin and artemisinins by determining the first structure of a protein-artemisinin complex (Kasaragod et al., 2019; **Figure 2B**). Specifically, we determined crystal structures of GephE with the artemisinin derivatives artesunate and artemether. The structures revealed that artemisinin-binding is mediated by a hydrophobic pocket formed by contributions from subdomains III and IV of GephE (**Figure 2C**). More importantly, these structures revealed that these compounds target the N-terminal region of the universal receptor-binding pocket in GephE and inhibit important hydrophobic interactions (^{368}FNI^{370} of the GABA$_A$R α3 subunit and ^{398}FSI^{400} of the GlyR β subunit), which represent critical determinants of the gephyrin-receptor interactions containing the aromatic residues at the first position of the consensus binding motif (**Figures 2D,E**). Displacement isothermal titration calorimetry (ITC) measurements and a supported membrane sheet assay (SCMS) demonstrated that these compounds negatively affect the gephyrin-receptor interaction. Electrophysiological experiments revealed a significant decrease in glycinergic currents in the presence of these compounds, with a strict dependence on gephyrin. Furthermore, receptor and gephyrin clustering studies displayed a strong and time-dependent decrease in GABA$_A$R and gephyrin cluster sizes. In addition, our analyses also revealed a time-dependent neurotoxic effect of these compounds, in line with previous observations of cytotoxic effects of these compounds when administered in high doses (Brewer et al., 1994; Wesche et al., 1994). Since artemisinins have been shown to be capable of crossing the blood brain barrier (Davis et al., 2003) and as dysfunctions in gephyrin-mediated neurotransmission have been implicated in severe neurological disorders such as Alzheimer's disease, autism, schizophrenia, epilepsy and also in hyperekplexia (Agarwal et al., 2008; Fang et al., 2011; Hales et al., 2013; Dejanovic et al., 2014, 2015), the gephyrin-artemisinin co-crystal structures

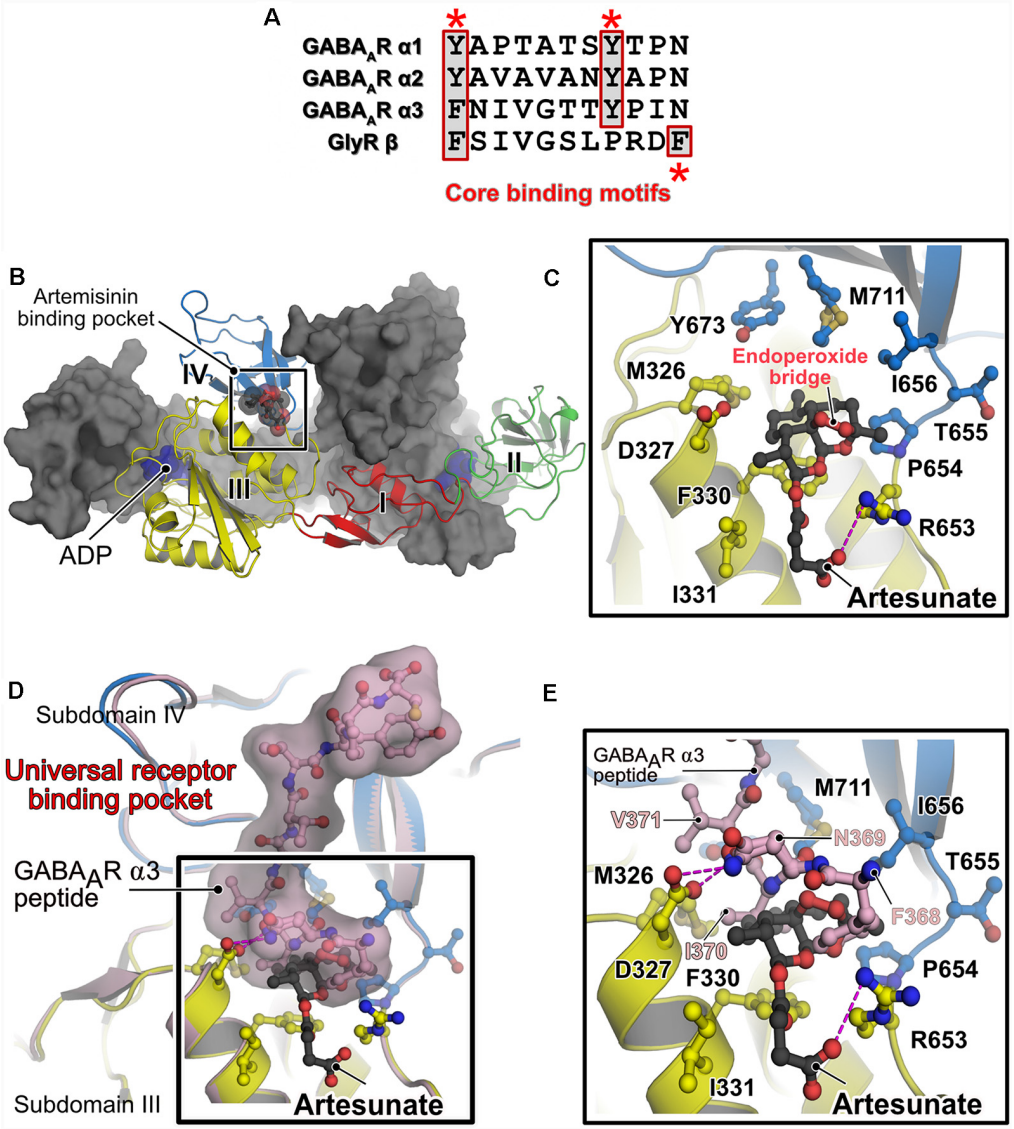

FIGURE 2 | Alternative receptor clustering of the GABA$_A$Rs by gephyrin and modulation by artemisinins. **(A)** Sequence alignment of the core binding motifs located in the M3–M4 loops of the glycine receptor (GlyR) β and GABA$_A$Rs α1, α2 and α3 subunits. Structurally conserved aromatic residues are highlighted with red asterisks. The multiple sequence alignment is represented by using the ESPript server (Robert and Gouet, 2014). **(B)** Crystal structure of GephE in complex with the anti-malarial drug artesunate (PDB: 6FGC). One protomer of the dimeric E domain is shown in cartoon representation, with the four subdomains (indicated by Roman numerals) being colored differently. The second protomer is shown in surface representation in gray. The bound artesunate is shown in space-filling representation. **(C)** Enlarged view of the artesunate-binding pocket demonstrating that binding is mediated by residues present in subdomains III and IV of GephE. The bound artesunate and residues which mediate binding are shown in ball and stick representation. **(D)** Superimposition of the crystal structures of GephE in complex with artesunate (PDB: 6FGC) and the GephE-GABA$_A$R-α3 subunit-derived peptide complex (PDB:4U90). **(E)** An enlarged view of the binding pocket of artemisinin or the N-terminal end of the peptide demonstrates that artesunate inhibits critical contacts (^{368}FNI^{370}) between the receptor and GephE.

may serve as a starting point for future drug development efforts against these disorders. In addition, the discovery of the artemisinin-binding pocket may serve as the basis for the future identification of additional cellular artemisinin-targets *via in silico* approaches. This study also established artemisinins as a tool for impairing inhibitory neurotransmission, which could eventually help to better understand the physiology of the human brain.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite a plethora of high-resolution structures of GABA$_A$Rs these receptors, initially homopentameric, but recently, driven by Cryo-EM, also heteropentameric receptors, a complete understanding of the multiple architecture and function of the iPSD still remains elusive. First and foremost, will be

to address the lack of structures of extrasynaptic GABA$_A$Rs. The structural elucidation of such a variant will certainly reveal whether these receptors also follow the same assembly principle as that observed for synaptic GABA$_A$Rs. Furthermore, all currently available structural information on inhibitory neurotransmitter receptors was determined for receptors in the absence of any binding partners. In the context of the iPSD, one should take into consideration that these receptors are closely associated with scaffolding proteins such as gephyrin (Kneussel et al., 1999) and collybistin (Kins et al., 2000; Saiepour et al., 2010) as well as with the auxiliary subunit GARLH (Davenport et al., 2017; Yamasaki et al., 2017). While most receptor structures were determined by shortening the unstructured M3–M4 loop (Miller and Aricescu, 2014; Phulera et al., 2018; Zhu et al., 2018), the most recent studies were performed with full-length heteropentameric GABA$_A$Rs including the native M3–M4 loop (Laverty et al., 2019; Masiulis et al., 2019). Nevertheless, even in these latest structures, these residues could not be resolved. At the iPSD, this region serves as the interaction hub for intracellular binding partners and hence the full-length heteropentameric receptors provide the necessary framework for structural studies with intracellular binding partners such as gephyrin and collybistin. The elucidation of the macromolecular complexes involving the receptors and their intracellular binding partners will provide crucial information not only regarding the structural organization of the intracellular loops but will also generate a molecular understanding of receptor clustering by scaffolding proteins at the iPSD. Hence, future research should be directed towards achieving a holistic, high-resolution view of the iPSD.

Another critical aspect is that, although the structure of the GephE-GABA$_A$R α3-derived peptide complex provides critical information about the alternative receptor recruitment by gephyrin, high-resolution structural data describing how different types of GABA$_A$Rs are recruited and anchored at the iPSD is still missing. The membrane sheet assay employed to study the inhibitory effect of artemisinins can also be adopted to analyze these uncharacterized GABA$_A$Rs as it will take into consideration possible avidity effects triggered by the presence of two gephyrin-binding α-subunits in the heterotrimeric GABA$_A$Rs and the oligomeric state of gephyrin as well as membrane contributions to the gephyrin-receptor interaction. With respect to the function of gephyrin, crucial information regarding the mechanism of the oligomeric organization of this scaffolding protein is still missing.

Although our structures of GephE-artemisinin complexes provide valuable insights into the modulation of inhibitory neurotransmission by gephyrin, multiple aspects of the regulation still remain to be deciphered; (a) What are possible effects of artemisinins on presynaptic terminals? (b) How does the balance of inhibitory and excitatory neurotransmission counteract the administration of artemisinins in human patients? (c) Are artemisinin metabolites equally potent as their parental compounds in modulating inhibitory neurotransmission? Although our structures can be used for the development of gephyrin-specific regulators of neurotransmission, one has to bear in mind that artemisinins influence a variety of cellular pathways possibly targeting multiple proteins. Thus, future structure-based drug design studies to optimize this lead compound with the aim of increasing its specificity towards gephyrin should be conducted. At the same time, structures of these compounds with other cellular targets would be desirable to better understand the molecular mechanism underlying target recognition and the pharmacological action of these anti-malarials.

AUTHOR CONTRIBUTIONS

VK prepared the figures and illustrations. VK and HS wrote the manuscript.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (DFG, SCHI425/8-2) and the Rudolf Virchow Center of Experimental Biomedicine to HS.

REFERENCES

Ackermann, A. M., Moss, N. G., and Kaestner, K. H. (2018). GABA and artesunate do not induce pancreatic α-to-β cell transdifferentiation *in vivo*. *Cell Metab.* 28, 787.e3–792.e3. doi: 10.1016/j.cmet.2018.07.002

Agarwal, S., Tannenberg, R. K., and Dodd, P. R. (2008). Reduced expression of the inhibitory synapse scaffolding protein gephyrin in Alzheimer's disease. *J. Alzheimers Dis.* 14, 313–321. doi: 10.3233/jad-2008-14305

Baumann, S. W., Baur, R., and Sigel, E. (2002). Forced subunit assembly in $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors. Insight into the absolute arrangement. *J. Biol. Chem.* 277, 46020–46025. doi: 10.1074/jbc.m207663200

Brewer, T. G., Grate, S. J., Peggins, J. O., Weina, P. J., Petras, J. M., Levine, B. S., et al. (1994). Fatal neurotoxicity of arteether and artemether. *Am. J. Trop. Med. Hyg.* 51, 251–259. doi: 10.4269/ajtmh.1994.51.251

Crespo-Ortiz, M. P., and Wei, M. Q. (2012). Antitumor activity of artemisinin and its derivatives: from a well-known antimalarial agent to a potential anticancer drug. *J. Biomed. Biotechnol.* 2012:247597. doi: 10.1155/2012/247597

Davenport, E. C., Pendolino, V., Kontou, G., Mcgee, T. P., Sheehan, D. F., López-Doménech, G., et al. (2017). An essential role for the tetraspanin LHFPL4 in the cell-type-specific targeting and clustering of synaptic GABA$_A$ receptors. *Cell Rep.* 21, 70–83. doi: 10.1016/j.celrep.2017.09.025

Davies, M., Bateson, A. N., and Dunn, S. M. (1998). Structural requirements for ligand interactions at the benzodiazepine recognition site of the GABA$_A$ receptor. *J. Neurochem.* 70, 2188–2194. doi: 10.1046/j.1471-4159.1998.70052188.x

Davis, T. M. E., Binh, T. Q., Ilett, K. F., Batty, K. T., Phuong, H. L., Chiswell, G. M., et al. (2003). Penetration of dihydroartemisinin into cerebrospinal fluid after administration of intravenous artesunate in severe falciparum malaria. *Antimicrob. Agents Chemother.* 47, 368–370. doi: 10.1128/aac.47.1.368-370.2003

Dejanovic, B., Djemie, T., Grunewald, N., Suls, A., Kress, V., Hetsch, F., et al. (2015). Simultaneous impairment of neuronal and metabolic function of mutated gephyrin in a patient with epileptic encephalopathy. *EMBO Mol. Med.* 7, 1580–1594. doi: 10.15252/emmm.201505323

Dejanovic, B., Lal, D., Catarino, C. B., Arjune, S., Belaidi, A. A., Trucks, H., et al. (2014). Exonic microdeletions of the gephyrin gene impair GABAergic synaptic inhibition in patients with idiopathic generalized epilepsy. *Neurobiol. Dis.* 67, 88–96. doi: 10.1016/j.nbd.2014.02.001

Dunn, S. M., Davies, M., Muntoni, A. L., and Lambert, J. J. (1999). Mutagenesis of the rat α1 subunit of the γ-aminobutyric acid(A) receptor reveals the importance of residue 101 in determining the allosteric effects of benzodiazepine site ligands. *Mol. Pharmacol.* 56, 768–774.

Fang, M., Shen, L., Yin, H., Pan, Y. M., Wang, L., Chen, D., et al. (2011). Downregulation of gephyrin in temporal lobe epilepsy neurons in humans and a rat model. *Synapse* 65, 1006–1014. doi: 10.1002/syn.20928

Fine, M., Schmiege, P., and Li, X. (2018). Structural basis for PtdInsP_2-mediated human TRPML1 regulation. *Nat. Commun.* 9:4192. doi: 10.1038/s41467-018-06493-7

Forman, S. A., and Miller, K. W. (2011). Anesthetic sites and allosteric mechanisms of action on Cys-loop ligand-gated ion channels. *Can. J. Anaesth.* 58, 191–205. doi: 10.1007/s12630-010-9419-9

Hales, C. M., Rees, H., Seyfried, N. T., Dammer, E. B., Duong, D. M., Gearing, M., et al. (2013). Abnormal gephyrin immunoreactivity associated with Alzheimer disease pathologic changes. *J. Neuropathol. Exp. Neurol.* 72, 1009–1015. doi: 10.1097/01.jnen.0000435847.59828.db

Hannan, S., and Smart, T. G. (2018). Cell surface expression of homomeric GABA$_A$ receptors depends on single residues in subunit transmembrane domains. *J. Biol. Chem.* 293, 13427–13439. doi: 10.1074/jbc.ra118.002792

Hansen, S. B., Tao, X., and Mackinnon, R. (2011). Structural basis of PIP_2 activation of the classical inward rectifier K^+ channel Kir2.2. *Nature* 477, 495–498. doi: 10.1038/nature10370

Hibbs, R. E., and Gouaux, E. (2011). Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature* 474, 54–60. doi: 10.1038/nature10139

Hughes, T. E. T., Pumroy, R. A., Yazici, A. T., Kasimova, M. A., Fluck, E. C., Huynh, K. W., et al. (2018). Structural insights on TRPV5 gating by endogenous modulators. *Nat. Commun.* 9:4198. doi: 10.1038/s41467-018-06753-6

Kasaragod, V. B., Hausrat, T. J., Schaefer, N., Kuhn, M., Christensen, N. R., Tessmer, I., et al. (2019). Elucidating the molecular basis for inhibitory neurotransmission regulation by artemisinins. *Neuron* 101, 673.e11–689.e11. doi: 10.1016/j.neuron.2019.01.001

Kasaragod, V. B., and Schindelin, H. (2016). Structural framework for metal incorporation during molybdenum cofactor biosynthesis. *Structure* 24, 782–788. doi: 10.1016/j.str.2016.02.023

Kasaragod, V. B., and Schindelin, H. (2018). Structure-function relationships of glycine and GABA$_A$ receptors and their interplay with the scaffolding protein gephyrin. *Front. Mol. Neurosci.* 11:317. doi: 10.3389/fnmol.2018.00317

Kim, E. Y., Schrader, N., Smolinsky, B., Bedet, C., Vannier, C., Schwarz, G., et al. (2006). Deciphering the structural framework of glycine receptor anchoring by gephyrin. *EMBO J.* 25, 1385–1395. doi: 10.1038/sj.emboj.7601029

Kins, S., Betz, H., and Kirsch, J. (2000). Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. *Nat. Neurosci.* 3, 22–29. doi: 10.1038/71096

Kirsch, J., Langosch, D., Prior, P., Littauer, U. Z., Schmitt, B., and Betz, H. (1991). The 93-kDa glycine receptor-associated protein binds to tubulin. *J. Biol. Chem.* 266, 22242–22245.

Kneussel, M., Brandstätter, J. H., Laube, B., Stahl, S., Müller, U., and Betz, H. (1999). Loss of postsynaptic GABA$_A$ receptor clustering in gephyrin-deficient mice. *J. Neurosci.* 19, 9289–9297. doi: 10.1523/jneurosci.19-21-09289.1999

Kuper, J., Llamas, A., Hecht, H. J., Mendel, R. R., and Schwarz, G. (2004). Structure of the molybdopterin-bound Cnx1G domain links molybdenum and copper metabolism. *Nature* 430, 803–806. doi: 10.1038/nature02681

Laverty, D., Desai, R., Uchanski, T., Masiulis, S., Stec, W. J., Malinauskas, T., et al. (2019). Cryo-EM structure of the human α1β3γ2 GABA$_A$ receptor in a lipid bilayer. *Nature* 565, 516–520. doi: 10.1038/s41586-018-0833-4

Laverty, D., Thomas, P., Field, M., Andersen, O. J., Gold, M. G., Biggin, P. C., et al. (2017). Crystal structures of a GABA$_A$-receptor chimera reveal new endogenous neurosteroid-binding sites. *Nat. Struct. Mol. Biol.* 24, 977–985. doi: 10.1038/nsmb.3477

Li, J., Casteels, T., Frogne, T., Ingvorsen, C., Honore, C., Courtney, M., et al. (2017). Artemisinins target GABA$_A$ receptor signaling and impair α cell identity. *Cell* 168, 86.e15–100.e15. doi: 10.1016/j.cell.2016.11.010

Maric, H. M., Kasaragod, V. B., Haugaard-Kedstrom, L., Hausrat, T. J., Kneussel, M., Schindelin, H., et al. (2015). Design and synthesis of high-affinity dimeric inhibitors targeting the interactions between gephyrin and inhibitory neurotransmitter receptors. *Angew. Chem. Int. Ed Engl.* 54, 490–494. doi: 10.1002/anie.201409043

Maric, H. M., Kasaragod, V. B., Hausrat, T. J., Kneussel, M., Tretter, V., Stromgaard, K., et al. (2014a). Molecular basis of the alternative recruitment of GABA$_A$ versus glycine receptors through gephyrin. *Nat. Commun.* 5:5767. doi: 10.1038/ncomms6767

Maric, H. M., Kasaragod, V. B., and Schindelin, H. (2014b). Modulation of gephyrin-glycine receptor affinity by multivalency. *ACS Chem. Biol.* 9, 2554–2562. doi: 10.1021/cb500303a

Maric, H. M., Mukherjee, J., Tretter, V., Moss, S. J., and Schindelin, H. (2011). Gephyrin-mediated γ-aminobutyric acid type A and glycine receptor clustering relies on a common binding site. *J. Biol. Chem.* 286, 42105–42114. doi: 10.1074/jbc.m111.303412

Masiulis, S., Desai, R., Uchanski, T., Serna Martin, I., Laverty, D., Karia, D., et al. (2019). GABA$_A$ receptor signalling mechanisms revealed by structural pharmacology. *Nature* 565, 454–459. doi: 10.1038/s41586-018-0832-5

Miller, P. S., and Aricescu, A. R. (2014). Crystal structure of a human GABA$_A$ receptor. *Nature* 512, 270–275. doi: 10.1038/nature13293

Miller, P. S., Scott, S., Masiulis, S., De Colibus, L., Pardon, E., Steyaert, J., et al. (2017). Structural basis for GABA$_A$ receptor potentiation by neurosteroids. *Nat. Struct. Mol. Biol.* 24, 986–992. doi: 10.1038/nsmb.3484

Mukherjee, J., Kretschmannova, K., Gouzer, G., Maric, H. M., Ramsden, S., Tretter, V., et al. (2011). The residence time of GABA$_A$Rs at inhibitory synapses is determined by direct binding of the receptor α1 subunit to gephyrin. *J. Neurosci.* 31, 14677–14687. doi: 10.1523/jneurosci.2001-11.2011

Phulera, S., Zhu, H., Yu, J., Claxton, D. P., Yoder, N., Yoshioka, C., et al. (2018). Cryo-EM structure of the benzodiazepine-sensitive α1β1γ2S tri-heteromeric GABA$_A$ receptor in complex with GABA. *Elife* 7:e39383. doi: 10.7554/eLife.39383

Prior, P., Schmitt, B., Grenningloh, G., Pribilla, I., Multhaup, G., Beyreuther, K., et al. (1992). Primary structure and alternative splice variants of gephyrin, a putative glycine receptor-tubulin linker protein. *Neuron* 8, 1161–1170. doi: 10.1016/0896-6273(92)90136-2

Robert, X., and Gouet, P. (2014). Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res.* 42, W320–W324. doi: 10.1093/nar/gku316

Rudolph, U., and Knoflach, F. (2011). Beyond classical benzodiazepines: novel therapeutic potential of GABA$_A$ receptor subtypes. *Nat. Rev. Drug Discov.* 10, 685–697. doi: 10.1038/nrd3502

Saiepour, L., Fuchs, C., Patrizi, A., Sassoe-Pognetto, M., Harvey, R. J., and Harvey, K. (2010). Complex role of collybistin and gephyrin in GABA$_A$ receptor clustering. *J. Biol. Chem.* 285, 29623–29631. doi: 10.1074/jbc.m110.121368

Sander, B., Tria, G., Shkumatov, A. V., Kim, E. Y., Grossmann, J. G., Tessmer, I., et al. (2013). Structural characterization of gephyrin by AFM and SAXS reveals a mixture of compact and extended states. *Acta Crystallogr. D Biol. Crystallogr.* 69, 2050–2060. doi: 10.1107/s0907444913018714

Schwarz, G., Schrader, N., Mendel, R. R., Hecht, H. J., and Schindelin, H. (2001). Crystal structures of human gephyrin and plant Cnx1 G domains: comparative analysis and functional implications. *J. Mol. Biol.* 312, 405–418. doi: 10.1006/jmbi.2001.4952

Sigel, E., and Steinmann, M. E. (2012). Structure, function, and modulation of GABA$_A$ receptors. *J. Biol. Chem.* 287, 40224–40231. doi: 10.1074/jbc.R112.386664

Tretter, V., Ehya, N., Fuchs, K., and Sieghart, W. (1997). Stoichiometry and assembly of a recombinant GABA$_A$ receptor subtype. *J. Neurosci.* 17, 2728–2737. doi: 10.1523/JNEUROSCI.17-08-02728.1997

Tretter, V., Jacob, T. C., Mukherjee, J., Fritschy, J. M., Pangalos, M. N., and Moss, S. J. (2008). The clustering of GABA$_A$ receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor α2 subunits to gephyrin. *J. Neurosci.* 28, 1356–1365. doi: 10.1523/jneurosci.5050-07.2008

Tretter, V., Kerschner, B., Milenkovic, I., Ramsden, S. L., Ramerstorfer, J., Saiepour, L., et al. (2011). Molecular basis of the γ-aminobutyric acid A receptor α3 subunit interaction with the clustering protein gephyrin. *J. Biol. Chem.* 286, 37702–37711. doi: 10.1074/jbc.m111.291336

Tu, Y. (2016). Artemisinin-A gift from traditional chinese medicine to the world (nobel lecture). *Angew. Chem. Int. Ed Engl.* 55, 10210–10226. doi: 10.1002/anie.201601967

Tyagarajan, S. K., and Fritschy, J. M. (2014). Gephyrin: a master regulator of neuronal function? *Nat. Rev. Neurosci.* 15, 141–156. doi: 10.1038/nrn3670

van der Meulen, T., Lee, S., Noordeloos, E., Donaldson, C. J., Adams, M. W., Noguchi, G. M., et al. (2018). Artemether does not turn α cells into β cells. *Cell Metab.* 27, 218.e4–225.e4. doi: 10.1016/j.cmet.2017.10.002

Walters, R. J., Hadley, S. H., Morris, K. D., and Amin, J. (2000). Benzodiazepines act on GABA$_A$ receptors *via* two distinct and separable mechanisms. *Nat. Neurosci.* 3, 1274–1281. doi: 10.1038/81800

Wesche, D. L., Decoster, M. A., Tortella, F. C., and Brewer, T. G. (1994). Neurotoxicity of artemisinin analogs *in vitro*. *Antimicrob. Agents Chemother.* 38, 1813–1819. doi: 10.1128/aac.38.8.1813

WHO. (2015). *Guidelines for the Treatment of Malaria.* 3rd Edn. Geneva: World Health Organization.

Wieland, H. A., Lüddens, H., and Seeburg, P. H. (1992). A single histidine in GABA$_A$ receptors is essential for benzodiazepine agonist binding. *J. Biol. Chem.* 267, 1426–1429.

Yamasaki, T., Hoyos-Ramirez, E., Martenson, J. S., Morimoto-Tomita, M., and Tomita, S. (2017). GARLH family proteins stabilize GABA$_A$ receptors at synapses. *Neuron* 93, 1138.e6–1152.e6. doi: 10.1016/j.neuron.2017.02.023

Zhu, S., Noviello, C. M., Teng, J., Walsh, R. M. Jr., Kim, J. J., and Hibbs, R. E. (2018). Structure of a human synaptic GABA$_A$ receptor. *Nature* 559, 67–72. doi: 10.1038/s41586-018-0255-3

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 Molecular Neuroscience

MINI REVIEW
published: 13 August 2019
doi: 10.3389/fnmol.2019.00187

Cellular Mechanisms Contributing to the Functional Heterogeneity of GABAergic Synapses

*Benjamin F. N. Campbell and Shiva K. Tyagarajan**

Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland

GABAergic inhibitory neurotransmission contributes to diverse aspects of brain development and adult plasticity, including the expression of complex cognitive processes. This is afforded for in part by the dynamic adaptations occurring at inhibitory synapses, which show great heterogeneity both in terms of upstream signaling and downstream effector mechanisms. Single-particle tracking and live imaging have revealed that complex receptor-scaffold interactions critically determine adaptations at GABAergic synapses. Super-resolution imaging studies have shown that protein interactions at synaptic sites contribute to nano-scale scaffold re-arrangements through post-translational modifications (PTMs), facilitating receptor and scaffold recruitment to synaptic sites. Additionally, plasticity mechanisms may be affected by the protein composition at individual synapses and the type of pre-synaptic input. This mini-review article examines recent discoveries of plasticity mechanisms that are operational within GABAergic synapses and discusses their contribution towards functional heterogeneity in inhibitory neurotransmission.

Keywords: homeostatic plasticity, postsynaptic density, interneurons, gephyrin, post-transcriptional regulation, post-translational modifications

OPEN ACCESS

Edited by:
Stephen Moss,
Tufts University School of Medicine, United States

Reviewed by:
Wei Lu,
National Institute of Neurological Disorders and Stroke (NINDS), United States
Carlos B. Duarte,
University of Coimbra, Portugal

****Correspondence:***
Shiva K. Tyagarajan
tyagarajan@pharma.uzh.ch

Received: *30 April 2019*
Accepted: *19 July 2019*
Published: *13 August 2019*

Citation:
Campbell BFN and Tyagarajan SK (2019) Cellular Mechanisms Contributing to the Functional Heterogeneity of GABAergic Synapses. Front. Mol. Neurosci. 12:187. doi: 10.3389/fnmol.2019.00187

INTRODUCTION

The plasticity of individual synapses occurs downstream of activity or neuro-modulatory signaling and must be reconciled with homeostatic mechanisms to maintain overall network function (Abbott and Nelson, 2000). The inherent variability in functional connectivity between different neuronal cell types within or between brain regions is becoming apparent. However, even at the post-synaptic compartment level, individual synapses themselves exhibit functional diversity, and the cellular processes that facilitate this heterogeneity of function is currently an exciting topic of research. Unlike the mechanisms that have been described to influence specific aspects of excitatory postsynaptic plasticity, mechanisms operational at GABAergic postsynaptic terminals are relatively unexplored. Recent technological developments including single-particle tracking and super-resolution imaging demonstrate that the inhibitory post-synapse is subject to dynamic activity-dependent reorganization. Therefore, understanding the cellular mechanisms that contribute to dynamics at GABAergic synapses will help to explain emergent functional heterogeneity.

PRE-SYNAPTIC SPECIFICATION OF GABAergic PLASTICITY

Pre-synaptically, a diverse pool of inhibitory interneurons provides GABAergic input onto post-synaptic cells. These interneurons differ in their spatial innervation patterns, firing properties, and pre-synaptic release mechanisms (Pelkey et al., 2017). Interestingly, recent data suggest that GABAergic plasticity occurs differentially between synapses innervated by distinct classes of interneurons. Pre-synaptic plasticity importantly involves regulation of neurotransmitter release onto the post-synaptic cell, often *via* modification of vesicular release (McBain and Kauer, 2009). How this released GABA is sensed and transduced to the target cell then depends on post-synaptic signaling.

Distinct interneuron subclasses differentially target specific neurons and sub-cellular compartments (e.g., soma, dendritic shaft, dendritic spines, axon-initial segment, et cetera; **Figures 1A,A′**). For example, cholecystokinin-positive (CCK+) and parvalbumin-positive (PV+) basket cells target the soma and proximal dendrites of neurons, whereas somatostatin-positive (SST+) interneurons preferentially target both the shafts and spines of dendrites. The mechanisms specifying different innervation patterns are in part provided by the expression of specific synaptic organizers by the post-synaptic cell. At hippocampal perisomatic synapses, the dystrophin-glycoprotein complex specifically organizes inputs from CCK+ interneurons which target the peri-somatic domain (Früh et al., 2016; Panzanelli et al., 2017). This complex is absent from distal dendrites or the axon-initial segment, and genetic deletion of this complex specifically affects CCK+ terminals. In contrast, trans-synaptic organizers like L1CAM-AnkyrinG interactions specify axo-axonic synapses onto the axon initial segment (AIS), and organize the input-specific synaptic properties of chandelier cells (Tai et al., 2019). Neuroligins which mediate trans-synaptic interactions control spatial input specificity and synaptic strength depending on the neuroligin isoform expressed. While neuroligin 2 is required to form both PV+ and SST+ synapses, neuroligin 3 can selectively regulate the strength of SST+ synapses dependent on its expression level (Horn and Nicoll, 2018). Moreover, PV+ and SST+ synapses are regulated by distinct upstream signaling, with PV+ synapses being more affected by cell-autonomous firing and SST+ synapses affected by NMDA receptor (NMDAR)-driven glutamatergic input (Horn and Nicoll, 2018). In another example, activation of post-synaptic NMDARs signal downstream to the kinase CaMKIIα, which then specifically drives inhibitory long-term potentiation (iLTP) at SST+, but not PV+ synapses (Chiu et al., 2018). The subunit composition of post-synaptic $GABA_A$Rs may also act as a substrate for synapse-specific plasticity between these interneuron types, as post-synaptic loss of the β3 subunit specifically affects PV+ driven input (Nguyen and Nicoll, 2018). Interneuron-specific plasticity is also represented at CCK+ synapses onto pyramidal cells, which are regulated by retrograde signaling *via* cannabinoid type-1 (CB1) receptors. These CB1 receptors are pre-synaptically enriched at CCK+ synapses and participate in the depolarization-induced suppression of inhibition (DSI; Busquets-Garcia et al., 2018). Interestingly pyramidal neuron activation was shown to affect the expression of the intermediate early gene and transcription factor NPAS4 to enhance inputs from CCK+ neurons to drive DSI but failed to enhance PV+ neuron input (Hartzell et al., 2018). This study provides a link between neuron activation status and interneuron-specific inhibition *via* transcriptional control, although which NPAS4-regulated synaptogenic targets couple activity to synapse-specific recruitment are currently undetermined. While the generality of input-specific plasticity and description of underlying mechanisms remains to be elaborated, it is clear that variation in synaptic protein composition facilitates at least some forms of pre-synaptic input specificity (Chiu et al., 2018).

IMPORTANCE OF RECEPTOR-SCAFFOLD INTERACTIONS

The GABAergic post-synapse contains $GABA_A$ receptors ($GABA_A$Rs), post-synaptic scaffolding and signaling proteins, and trans-synaptic adhesion molecules which facilitate effective communication between the pre- and post-synapse for efficient neurotransmission. $GABA_A$Rs are composed of pentamers from a family of subunits encoded by 19 distinct genes (subunits α1–6, β1–3, γ1–3, δ, ε, π, ρ1–3, and τ). Although it has been recently shown that many receptor subunits can access the synaptic space (Hannan et al., 2019), the select interactions between receptors and post-synaptic scaffolds such as gephyrin encourage the retention of $GABA_A$Rs composed of the combination of α1–3 subunits along with β1–3 and γ2 subunits, whereas those containing the subunits α4–6 and δ tend to be extra-synaptic (Fritschy and Panzanelli, 2014; Hannan et al., 2019). $GABA_A$Rs are trafficked to the plasma membrane from cytoplasmic pools, or diffuse laterally within the membrane in and out of synapses to alter the local concentration of receptors and therefore synaptic strength (Flores and Méndez, 2014; Petrini and Barberis, 2014). Thus, control over the diffusion dynamics of $GABA_A$Rs is an important mechanism by which inhibitory plasticity is achieved (Petrini and Barberis, 2014). In gephyrin-containing GABAergic synapses, the magnitude of retention of $GABA_A$Rs scales with the size of gephyrin clusters (Specht et al., 2013; Flores et al., 2015; Crosby et al., 2019). Consequently, knockdown of gephyrin leads to a reduction in synaptic receptors *via* decreased confinement of $GABA_A$Rs (Jacob, 2005; Thomas et al., 2005). Similarly, signaling which induces gephyrin clustering is often coupled to increase in $GABA_A$R clustering. For example, activity induction in hippocampal slices leads to inhibitory potentiation that is correlated to increases in gephyrin cluster size concordant with mIPSC amplitude (Flores et al., 2015). Additionally, during long-term potentiation of GABAergic synapses (iLTP), synaptic gephyrin clusters show increases in the number of gephyrin molecules at the same time that extra-synaptic clusters shrink (Pennacchietti et al., 2017). Due to the close and interrelated changes between gephyrin clustering and those of GABAergic transmission (Petrini et al., 2014; Flores et al., 2015; Specht, 2019), the analysis of changes in both gephyrin and $GABA_A$R synaptic

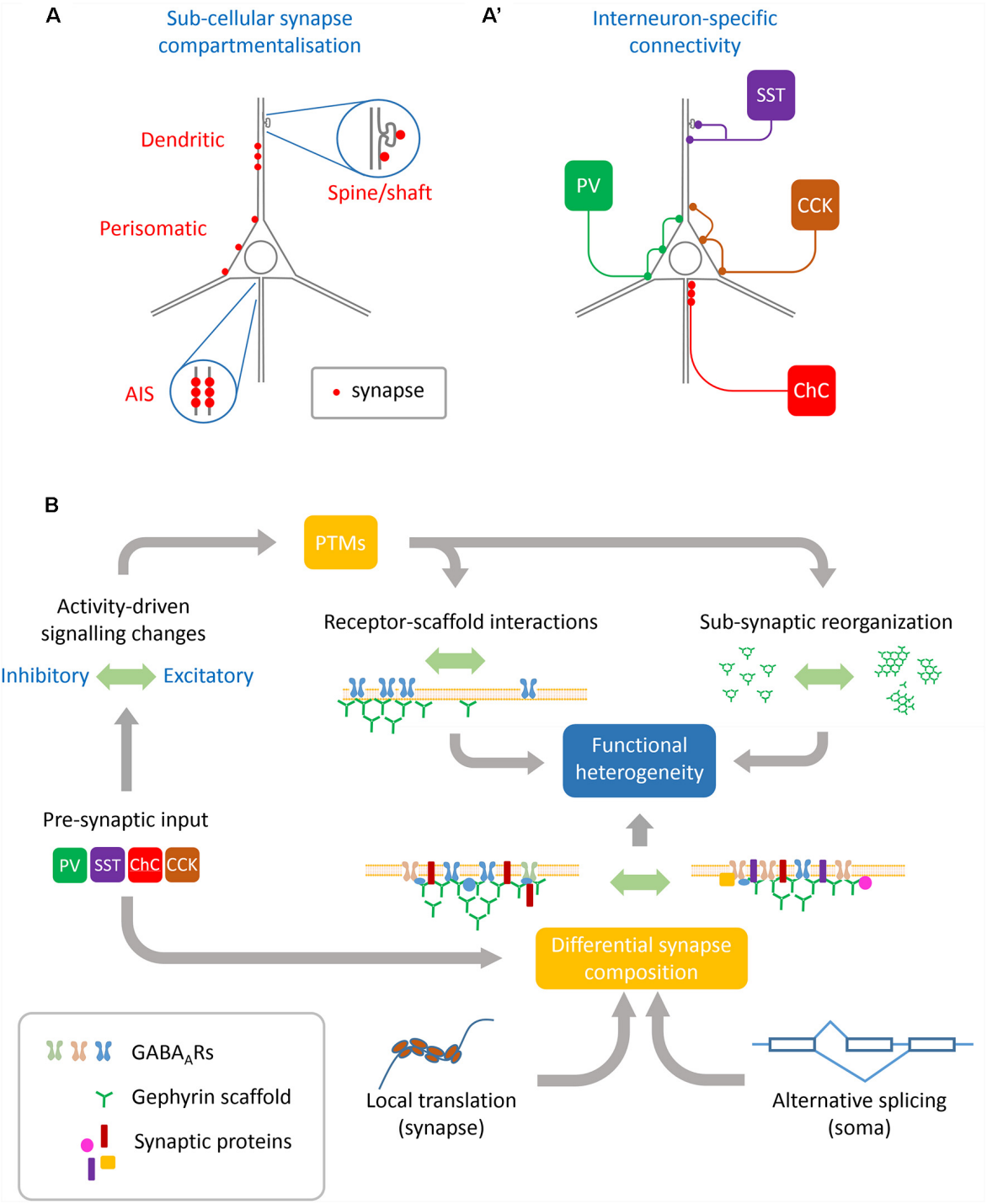

FIGURE 1 | Sources of heterogeneity contributing to GABAergic synapse remodeling. **(A)** Basal synapse dynamics and responses to activity are distinct between different neuronal sub-compartments such as the axon initial segment (AIS), perisomatic and dendritic synapses, and even between inhibitory synapses situated on dendritic shafts vs. spines. **(A')** Pre-synaptic interneuron subtypes innervate different neuronal sub-compartments. Interneuron subtypes innervating similar compartments can also differ in their functional modulation, such as between synapses innervated by PV+ or CCK+ basket cells which both target the perisomatic domain. **(B)** Many cellular mechanisms converge to achieve functional heterogeneity at GABAergic synapses: pre-synaptic interneurons specify some aspects of synaptic protein composition as well as determine pre-synaptic GABA release and plasticity. These along with other upstream signals including neuronal activation and intracellular calcium concentration can regulate post-translational modifications (PTMs) on both receptors and scaffolds which alter their dynamics as well as sub-synaptic organization. This synaptic organization is further defined by local translation of nascent proteins as well as alternate splicing of transcripts conferring specific properties to the synapse.

organization can be used to understand mechanistic bases for synapse alterations.

HETEROGENEITY OF GABAergic POST-SYNAPTIC REMODELING

While plasticity occurs at all synapses, basal synapse characteristics such as size, strength, and composition are variable, and therefore the extent of induced synaptic plasticity is also variable. For example, spinal cord synapses contain over four times as many gephyrin molecules per synapse and at a higher density than cortical synapses (Specht et al., 2013). GABAergic synaptic dynamics can even vary between compartments within the same neuron, where spine synapses are more dynamic than shaft synapses (Villa et al., 2016). Critically, the manner in which inhibitory synapses remodel depends on the valency of signaling received, where activity increases or decreases can have similar or opposing effects on synaptic dynamics. A strong pharmacological network activity increase can lead to a reduction in the clustering of gephyrin, resulting in a decrease of inhibitory synaptic currents in a NMDAR- and calcineurin-dependent manner (Bannai et al., 2009). This contrasts with data suggesting that activity increases lead to enhanced gephyrin clustering and $GABA_AR$ synaptic accumulation through CaMKII signaling (Flores et al., 2015). These differences have been explained by the degree of activity-induction triggering distinct calcium signaling pathways: whereas low calcium can act to stabilize gephyrin and $GABA_ARs$ at synapses, large increases in calcium leads to reduced retention of $GABA_ARs$ (Petrini and Barberis, 2014; Bannai et al., 2015). Moreover, after induction of activity paradigms such as iLTP, some but not all synapses show re-arrangement of their nano-domains (Pennacchietti et al., 2017), suggesting that even synaptic plasticity itself can only occur where synapse-specific mechanisms allow for it. How signaling then is organized to effect plasticity can only be understood once upstream signaling effectors or downstream signaling targets are identified.

MULTIPLE SIGNAL TRANSDUCTION PATHWAYS MODULATE RECEPTOR-SCAFFOLD INTERACTIONS

Direct modification of $GABA_ARs$, the interaction between $GABA_ARs$ and post-synaptic scaffolds, or the dynamics of the post-synaptic scaffolds themselves could all contribute to modulating synaptic receptor retention and therefore the function of inhibitory synapses (Choquet and Triller, 2003; Petrini and Barberis, 2014; Specht, 2019). Post-translational modifications (PTMs) including protein phosphorylation, SUMOylation, acetylation, palmitoylation, and nitrosylation, are known to occur at the inhibitory post-synapse (Tyagarajan and Fritschy, 2014) where they can effectively function *via* altered receptor-scaffold interactions. Of these, modification of $GABA_ARs$ (Comenencia-Ortiz et al., 2014; Petrini and Barberis, 2014) and gephyrin (Tyagarajan and Fritschy, 2014; Zacchi et al., 2014; Kasaragod and Schindelin, 2018) are best described. Palmitoylation of both $GABA_ARs$ and gephyrin result in enhanced surface localization (Matt et al., 2019), conversely ubiquitination (Luscher et al., 2011) or SUMOylation (Ghosh et al., 2016) of these proteins results in decreased synaptic accumulation. While phosphorylation of $GABA_ARs$ controls both surface trafficking and removal (Comenencia-Ortiz et al., 2014), it also influences receptor diffusion in and out of synapses *via* gephyrin-dependent (Mukherjee et al., 2011) or independent mechanisms (Lévi et al., 2015). Gephyrin itself is importantly regulated by phosphorylation, which can lead to either reduced gephyrin clustering (Tyagarajan et al., 2013), or enhanced gephyrin clustering (Flores et al., 2015) depending on the specific amino acid residue phosphorylated. Still, the molecular and biophysical mechanisms transducing these phosphorylation events to effect function are poorly understood.

Recent efforts towards describing post-synaptic dynamics have employed live-imaging and super-resolution microscopy to determine real-time and nano-scale re-organization of the post-synapse (Specht et al., 2013; Pennacchietti et al., 2017; Battaglia et al., 2018; Crosby et al., 2019). These studies demonstrate that gephyrin is arranged in nano-domains within the post-synapse, and also that it can cluster at extra-synaptic sites previously overlooked by conventional microscopy (reviewed by Specht, 2019). Recently, gephyrin nano-domains were directly shown to overlap with the nano-domains of $GABA_ARs$ as well as those of pre-synaptic vesicle release sites clearly demonstrating that synaptic gephyrin nano-domains represent functional organizational units (Crosby et al., 2019). In this context, the impact of gephyrin upon $GABA_ARs$ has been shown by perturbing gephyrin clustering *via* overexpression of dominant-negative gephyrin, which causes a reduction in the number and size of $GABA_AR$ nano-domains (Crosby et al., 2019) and functionally reduces the dwell time of $GABA_ARs$ at synaptic sites (Battaglia et al., 2018).

PTMs have now been shown to control gephyrin nano-domain structure and $GABA_AR$ retention at synapses. A recent study has found that phosphorylation of gephyrin at serine 268 (regulated by ERK1/2; Tyagarajan et al., 2013) results in increased nano-domain compaction and a reduction in $GABA_AR$ synaptic dwell time (Battaglia et al., 2018). Conversely preventing phosphorylation at residue serine 270 (regulated by GSK3β or CDK5; Tyagarajan et al., 2011; Kuhse et al., 2012) causes a decrease in gephyrin scaffold compaction, while also increasing the scaffold size. Interestingly gephyrin mutations additionally altered $GABA_AR$ dynamics outside of synaptic sites, suggesting that gephyrin is involved in extra-synaptic receptor scaffolding regulated by phosphorylation of distinct serine residues (Battaglia et al., 2018). Taken together PTMs such as phosphorylation provide a link between upstream signaling cascades and functional plasticity at the post-synapse *via* receptor-scaffold interactions. Phospho-proteomic analyses of synaptic proteins indicate that more than just gephyrin and $GABA_ARs$ are dynamically phosphorylated, and that altered brain states such as sleep deprivation (Wang et al., 2018) or induction of learning lead to broad phosphorylation changes (Kähne et al., 2016). Learning paradigms can alter the abundance

of kinases and phosphatases which regulate the phospho-status of synaptic proteins including those which signal to $GABA_ARs$ and gephyrin (Šmidák et al., 2016). Therefore, differential phosphorylation of inhibitory synaptic protein networks may serve as a substrate underlying synapse-specific or broader network form of plasticity.

SYNAPTIC COMPOSITION CHANGES MAY DRIVE SYNAPSE REMODELING

Models for receptor-scaffold interactions propose that modifying the number of scaffolds or the affinity of receptor-scaffold binding will define the equilibrium governing immobilization of receptors at the synapse (Choquet and Triller, 2003; Specht, 2019). Therefore, heterogeneity in synaptic protein composition between areas of the nervous system, within microcircuits, and even within the same cell may explain resulting differences in synaptic plasticity. While the contribution of a handful of inhibitory synaptic proteins such as collybistin, gephyrin, and neuroligins to $GABA_ARs$ dynamics and inhibitory synapse function have been identified (Fritschy et al., 2012; Tyagarajan and Fritschy, 2014; Groeneweg et al., 2018), recent unbiased screens have greatly expanded the pool of potential regulatory proteins. Immunoprecipitation or proximity ligation-based detection of the protein identity of post-synaptic interacting complexes has been performed for gephyrin, collybistin, InSyn1 (Uezu et al., 2016), neuroligin 2 (Kang et al., 2014), GABA receptors (Nakamura et al., 2016; Ge et al., 2018), as well as for the inhibitory synaptic cleft (Loh et al., 2016). These efforts have uncovered hundreds of novel inhibitory synaptic proteins including scaffolding proteins, kinases, and components of signal transduction cascades. For example, the tetraspanin protein LHFPL4 was identified as a novel binding partner of neuroligin 2 (Yamasaki et al., 2017), disruption of which results in severe inhibitory synapse deficits leading to death (Wu et al., 2018). Interestingly this protein was shown to mediate cell-types-specific regulation, affecting synapses in pyramidal cells but not interneurons (Davenport et al., 2017). Comparative analysis of proteomes between excitatory synapses have shown regional (Roy et al., 2018), activity-, and state-dependent alterations in plasticity proteins (Lautz et al., 2018). Currently, similar condition-dependent information specific to GABAergic synapses is lacking, and moreover how the protein composition of these synapse is modified dynamically is only starting to be understood.

POST-TRANSCRIPTIONAL CONTROL OVER GABAergic SYNAPSES

Recent data suggests that local translation of mRNA coding for synaptic proteins could offer a way to acutely modify synaptic composition in a synapse-specific manner (Rangaraju et al., 2017). In fact, a plethora of inhibitory synaptic mRNA transcripts have been identified as present at the synapse including those coding for $GABA_ARs$ and adaptor proteins (Cajigas et al., 2012; Zappulo et al., 2017). Recently, it was found that 75% of inhibitory synaptic terminals possess translational machinery, and 40% of these terminals exhibit active translation at a given time (Hafner et al., 2019), although the identity and inhibitory synapse specificity of these newly-translated proteins are unknown. Functionally, disruption of the localization of synaptic mRNA transcripts can affect synapse organization. For example, synaptic accumulation of mRNA coding for the α2 $GABA_AR$ subunit is disrupted in a loss-of-function mouse model null for the RNA binding protein NONO, leading to a reduction in synaptic $GABA_ARs$ and gephyrin clustering (Mircsof et al., 2015). Alternative splicing of mRNA coding for synaptic proteins provides an additional mechanism to generate heterogeneity in synaptic signaling. Splicing of neurexins has been shown to be important for excitatory synapse specification, differentially affecting NMDAR or AMPAR driven transmission (Dai et al., 2019), and leading to synaptic and behavioral dysfunction when splicing is disrupted (Traunmüller et al., 2016). Recently, alternative splicing of inhibitory synaptic proteins was shown to coordinate spatial GABAergic synapse organization. Splice isoforms of collybistin, a core component of inhibitory synapses was found to control dendritic inhibitory synapse patterning along the proximal-distal axis (de Groot et al., 2017). Collybistin was later identified as a target for alternative splicing by the RNA binding protein Sam68, which was also shown to control splicing of gephyrin mRNA at the C4 splice cassette known to control post-synaptic clustering (Witte et al., 2019). Whether splicing of mRNA coding for inhibitory proteins occurs locally at individual synaptic sites and contributes to synapse-specific protein composition is currently unknown.

CONCLUSION

The findings highlighted in this mini-review article (summarized in **Figure 1B**) reveals a shift in thinking about how inhibitory synaptic plasticity occurs. Beyond simple measurements of changes in post-synaptic currents, advances in microscopic imaging technology, RNA sequencing, mass spectrometry, and molecular visualization tools enable the investigation of how plasticity manifests within and between individual synapses. While future interrogation of plasticity will undoubtedly uncover new mechanisms underlying synapse remodeling, they also allow us to fully appreciate the heterogeneity in synaptic function, between different brain circuits, neuronal compartments, individual synapses, and now even within sub-synaptic nano-domains.

AUTHOR CONTRIBUTIONS

BC and ST contributed to the organization and writing of this manuscript.

FUNDING

This study was supported by the Swiss National Science Foundation grant to ST (Grant No. 31003A-159867) and internal funding to ST from the University of Zurich and University of Zurich Forschungskredit grant to BC.

REFERENCES

Abbott, L. F., and Nelson, S. (2000). Synaptic plasticity: taming the beast–nature neuroscience. *Nat. Neurosci.* 3, 1178–1183. doi: 10.1038/81453

Bannai, H., Lévi, S., Schweizer, C., Inoue, T., Launey, T., Racine, V., et al. (2009). Activity-dependent tuning of inhibitory neurotransmission based on $GABA_AR$ diffusion dynamics. *Neuron* 62, 670–682. doi: 10.1016/j.neuron.2009.04.023

Bannai, H., Niwa, F., Sherwood, M. W., Shrivastava, A. N., Arizono, M., Miyamoto, A., et al. (2015). Bidirectional control of synaptic $GABA_AR$ clustering by glutamate and calcium. *Cell Rep.* 13, 2768–2780. doi: 10.1016/j.celrep.2015.12.002

Battaglia, S., Renner, M., Russeau, M., Côme, E., Tyagarajan, S. K., and Lévi, S. (2018). Activity-dependent inhibitory synapse scaling is determined by gephyrin phosphorylation and subsequent regulation of $GABA_A$ receptor diffusion. *eNeuro* 5:ENEURO.0203-17.2017. doi: 10.1523/ENEURO.0203-17.2017

Busquets-Garcia, A., Bains, J., and Marsicano, G. (2018). CB 1 receptor signaling in the brain: extracting specificity from ubiquity. *Neuropsychopharmacology* 43, 4–20. doi: 10.1038/npp.2017.206

Cajigas, I. J., Tushev, G., Will, T. J., Tom Dieck, S., Fuerst, N., and Schuman, E. M. (2012). The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. *Neuron* 74, 453–466. doi: 10.1016/j.neuron.2012.02.036

Chiu, C. Q., Martenson, J. S., Yamazaki, M., Natsume, R., Sakimura, K., Tomita, S., et al. (2018). Input-specific NMDAR-dependent potentiation of dendritic GABAergic inhibition. *Neuron* 97, 368.e3–377.e3. doi: 10.1016/j.neuron.2017.12.032

Choquet, D., and Triller, A. (2003). The role of receptor diffusion in the organization of the postsynaptic membrane. *Nat. Rev. Neurosci.* 4, 251–265. doi: 10.1038/nrn1077

Comenencia-Ortiz, E., Moss, S. J., and Davies, P. A. (2014). Phosphorylation of $GABA_A$ receptors influences receptor trafficking and neurosteroid actions. *Psychopharmacology* 231, 3453–3465. doi: 10.1007/s00213-014-3617-z

Crosby, K. C., Gookin, S. E., Garcia, J. D., Hahm, K. M., Dell'Acqua, M. L., and Smith, K. R. (2019). Nanoscale subsynaptic domains underlie the organization of the inhibitory synapse. *Cell Rep.* 26, 3284.e3–3297.e3. doi: 10.1016/j.celrep.2019.02.070

Dai, J., Aoto, J., and Südhof, T. C. (2019). Alternative splicing of presynaptic neurexins differentially controls postsynaptic NMDA and AMPA receptor responses. *Neuron* 102, 993.e5–1008.e5. doi: 10.1016/j.neuron.2019.03.032

Davenport, E. C., Pendolino, V., Kontou, G., McGee, T. P., Sheehan, D. F., López-Doménech, G., et al. (2017). An essential role for the tetraspanin LHFPL4 in the cell-type-specific targeting and clustering of synaptic $GABA_A$ receptors. *Cell Rep.* 21, 70–83. doi: 10.1016/j.celrep.2017.09.025

de Groot, C., Floriou-Servou, A., Tsai, Y. C., Früh, S., Kohler, M., Parkin, G., et al. (2017). RhoGEF9 splice isoforms influence neuronal maturation and synapse formation downstream of α2 $GABA_A$receptors. *PLoS Genet.* 13:e1007073. doi: 10.1371/journal.pgen.1007073

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

Flores, C. E., Nikonenko, I., Mendez, P., Fritschy, J.-M., Tyagarajan, S. K., and Muller, D. (2015). Activity-dependent inhibitory synapse remodeling through gephyrin phosphorylation. *Proc. Natl. Acad. Sci. U S A* 112, E65–E72. doi: 10.1073/pnas.1411170112

Fritschy, J.-M., and Panzanelli, P. (2014). $GABA_A$ receptors and plasticity of inhibitory neurotransmission in the central nervous system. *Eur. J. Neurosci.* 39, 1845–1865. doi: 10.1111/ejn.12534

Fritschy, J.-M., Panzanelli, P., and Tyagarajan, S. K. (2012). Molecular and functional heterogeneity of GABAergic synapses. *Cell. Mol. Life Sci.* 69, 2485–2499. doi: 10.1007/s00018-012-0926-4

Früh, S., Romanos, J., Panzanelli, P., Bürgisser, D., Tyagarajan, S. K., Campbell, K. P., et al. (2016). Neuronal dystroglycan is necessary for formation and maintenance of functional CCK-positive basket cell terminals on pyramidal cells. *J. Neurosci.* 36, 10296–10313. doi: 10.1523/JNEUROSCI.1823-16.2016

Ge, Y., Kang, Y., Cassidy, R. M., Moon, K., Lewis, R., Wong, R. O. L., et al. (2018). Clptm1 limits forward trafficking of $GABA_A$ receptors to scale inhibitory synaptic strength. *Neuron* 97, 596.e8–610.e8. doi: 10.1016/j.neuron.2017.12.038

Ghosh, H., Auguadri, L., Battaglia, S., Simone Thirouin, Z., Zemoura, K., Messner, S., et al. (2016). Several posttranslational modifications act in concert to regulate gephyrin scaffolding and GABAergic transmission. *Nat. Commun.* 7:13365. doi: 10.1038/ncomms13365

Groeneweg, F. L., Trattnig, C., Kuhse, J., Nawrotzki, R. A., and Kirsch, J. (2018). Gephyrin: a key regulatory protein of inhibitory synapses and beyond. *Histochem. Cell Biol.* 150, 489–508. doi: 10.1007/s00418-018-1725-2

Hafner, A.-S., Donlin-Asp, P. G., Leitch, B., Herzog, E., and Schuman, E. M. (2019). Local protein synthesis is a ubiquitous feature of neuronal pre- and postsynaptic compartments. *Science* 364:eaau3644. doi: 10.1126/science.aau3644

Hannan, S., Minere, M., Harris, J., Izquierdo, P., Thomas, P., Tench, B., et al. (2019). $GABA_AR$ isoform and subunit structural motifs determine synaptic and extrasynaptic receptor localisation. *Neuropharmacology*. doi: 10.1016/j.neuropharm.2019.02.022 [Epub ahead of print].

Hartzell, A. L., Martyniuk, K. M., Brigidi, G. S., Heinz, D. A., Djaja, N. A., Payne, A., et al. (2018). NPAS4 recruits CCK basket cell synapses and enhances cannabinoid-sensitive inhibition in the mouse hippocampus. *Elife* 7:e35927. doi: 10.7554/eLife.35927

Horn, M. E., and Nicoll, R. A. (2018). Somatostatin and parvalbumin inhibitory synapses onto hippocampal pyramidal neurons are regulated by distinct mechanisms. *Proc. Natl. Acad. Sci. U S A* 115, 589–594. doi: 10.1073/pnas.1719523115

Jacob, T. C. (2005). Gephyrin regulates the cell surface dynamics of synaptic $GABA_A$ receptors. *J. Neurosci.* 25, 10469–10478. doi: 10.1523/jneurosci.2267-05.2005

Kähne, T., Richter, S., Kolodziej, A., Smalla, K.-H., Pielot, R., Engler, A., et al. (2016). Proteome rearrangements after auditory learning: high-resolution profiling of synapse-enriched protein fractions from mouse brain. *J. Neurochem.* 138, 124–138. doi: 10.1111/jnc.13636

Kang, Y., Ge, Y., Cassidy, R. M., Lam, V., Luo, L., Moon, K., et al. (2014). A combined transgenic proteomic analysis and regulated trafficking of neuroligin-2. *J. Biol. Chem.* 289, 29350–29364. doi: 10.1074/jbc.m114.549279

Kasaragod, V. B., and Schindelin, H. (2018). Structure-function relationships of glycine and $GABA_A$ receptors and their interplay with the scaffolding protein gephyrin. *Front. Mol. Neurosci.* 11:317. doi: 10.3389/fnmol.2018.00317

Kuhse, J., Kalbouneh, H., Schlicksupp, A., Mükusch, S., Nawrotzki, R., and Kirsch, J. (2012). Phosphorylation of gephyrin in hippocampal neurons by cyclin-dependent kinase CDK5 at Ser-270 is dependent on collybistin. *J. Biol. Chem.* 287, 30952–30966. doi: 10.1074/jbc.m112.349597

Lautz, J. D., Brown, E. A., Williams VanSchoiack, A. A., and Smith, S. E. P. (2018). Synaptic activity induces input-specific rearrangements in a targeted synaptic protein interaction network. *J. Neurochem.* 146, 540–559. doi: 10.1111/jnc.14466

Lévi, S., Le Roux, N., Eugène, E., and Poncer, J. C. (2015). Benzodiazepine ligands rapidly influence $GABA_A$ receptor diffusion and clustering at hippocampal inhibitory synapses. *Neuropharmacology* 88, 199–208. doi: 10.1016/j.neuropharm.2014.06.002

Loh, K. H., Stawski, P. S., Draycott, A. S., Stevens, B., Carr, S. A., Ting, A. Y., et al. (2016). Proteomic analysis of unbounded cellular compartments: synaptic clefts resource proteomic analysis of unbounded cellular compartments: synaptic clefts. *Cell* 166, 1295.e21–1307.e21. doi: 10.1016/j.cell.2016.07.041

Luscher, B., Fuchs, T., and Kilpatrick, C. L. (2011). $GABA_A$ receptor trafficking-mediated plasticity of inhibitory synapses. *Neuron* 70, 385–409. doi: 10.1016/j.neuron.2011.03.024

Matt, L., Kim, K., Chowdhury, D., and Hell, J. W. (2019). Role of palmitoylation of postsynaptic proteins in promoting synaptic plasticity. *Front. Mol. Neurosci.* 12:8. doi: 10.3389/fnmol.2019.00008

McBain, C. J., and Kauer, J. A. (2009). Presynaptic plasticity: targeted control of inhibitory networks. *Curr. Opin. Neurobiol.* 19, 254–262. doi: 10.1016/j.conb.2009.05.008

Mircsof, D., Langouët, M., Rio, M., Moutton, S., Siquier-Pernet, K., Bole-Feysot, C., et al. (2015). Mutations in NONO lead to syndromic intellectual disability and inhibitory synaptic defects. *Nat. Neurosci.* 18, 1731–1736. doi: 10.1038/nn.4169

Mukherjee, J., Kretschmannova, K., Gouzer, G., Maric, H.-M., Ramsden, S., Tretter, V., et al. (2011). The residence time of $GABA_A$Rs at inhibitory synapses is determined by direct binding of the receptor 1 subunit to gephyrin. *J. Neurosci.* 31, 14677–14687. doi: 10.1523/JNEUROSCI.2001-11.2011

Nakamura, Y., Morrow, D. H., Modgil, A., Huyghe, D., Deeb, T. Z., Lumb, M. J., et al. (2016). Proteomic characterization of inhibitory synapses using a novel pHluorin-tagged γ-aminobutyric acid receptor, type A ($GABA_A$), α2 subunit knock-in mouse. *J. Biol. Chem.* 291, 12394–12407. doi: 10.1074/jbc.m116.724443

Nguyen, Q. A., and Nicoll, R. A. (2018). The $GABA_A$ receptor β subunit is required for inhibitory transmission. *Neuron* 98, 718.e3–725.e3. doi: 10.1016/j.neuron.2018.03.046

Panzanelli, P., Früh, S., and Fritschy, J.-M. M. (2017). Differential role of $GABA_A$ receptors and neuroligin 2 for perisomatic GABAergic synapse formation in the hippocampus. *Brain Struct. Funct.* 222, 4149–4161. doi: 10.1007/s00429-017-1462-7

Pelkey, K. A., Chittajallu, R., Craig, M. T., Tricoire, L., Wester, J. C., and McBain, C. J. (2017). Hippocampal GABAergic inhibitory interneurons. *Physiol. Rev.* 97, 1619–1747. doi: 10.1152/physrev.00007.2017

Pennacchietti, F., Vascon, S., Nieus, T., Rosillo, C., Das, S., Tyagarajan, S. K., et al. (2017). Nanoscale molecular reorganization of the inhibitory postsynaptic density is a determinant of gabaergic synaptic potentiation. *J. Neurosci.* 37, 1747–1756. doi: 10.1523/JNEUROSCI.0514-16.2016

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

Petrini, E. M., Ravasenga, T., Hausrat, T. J., Iurilli, G., Olcese, U., Racine, V., et al. (2014). Synaptic recruitment of gephyrin regulates surface $GABA_A$ receptor dynamics for the expression of. *Nat. Commun.* 5:3921. doi: 10.1038/ncomms4921

Rangaraju, V., Tom Dieck, S., and Schuman, E. M. (2017). Local translation in neuronal compartments: how local is local? *EMBO Rep.* 18, 693–711. doi: 10.15252/embr.201744045

Roy, M., Sorokina, O., McLean, C., Tapia-González, S., DeFelipe, J., Armstrong, J., et al. (2018). Regional diversity in the postsynaptic proteome of the mouse brain. *Proteomes* 6:31. doi: 10.3390/proteomes6030031

Traunmüller, L., Gomez, A. M., Nguyen, T. M., and Scheiffele, P. (2016). Control of neuronal synapse specification by a highly dedicated alternative splicind program. *Science* 352, 982–986. doi: 10.1126/science.aaf2397

Šmidák, R., Mayer, R. L., Bileck, A., Gerner, C., Mechtcheriakova, D., Stork, O., et al. (2016). Quantitative proteomics reveals protein kinases and phosphatases in the individual phases of contextual fear conditioning in the C57BL/6J mouse. *Behav. Brain Res.* 303, 208–217. doi: 10.1016/j.bbr.2015.12.033

Specht, C. G. (2019). Neuropharmacology fractional occupancy of synaptic binding sites and the molecular plasticity of inhibitory synapses. *Neuropharmacology* doi: 10.1016/j.neuropharm.2019.01.008 [Epub ahead of print].

Specht, C. G., Izeddin, I., Rodriguez, P. C., ElBeheiry, M., Rostaing, P., Darzacq, X., et al. (2013). Quantitative nanoscopy of inhibitory synapses: counting gephyrin molecules and receptor binding sites. *Neuron* 79, 308–321. doi: 10.1016/j.neuron.2013.05.013

Tai, Y., Gallo, N. B., Wang, M., Yu, J.-R., and Van Aelst, L. (2019). Axo-axonic innervation of neocortical pyramidal neurons by GABAergic chandelier cells requires ankyring-associated L1CAM. *Neuron* 102, 358.e9–372.e9. doi: 10.1016/j.neuron.2019.02.009

Thomas, P., Mortensen, M., Hosie, A. M., and Smart, T. G. (2005). Dynamic mobility of functional $GABA_A$ receptors at inhibitory synapses. *Nat. Neurosci.* 8, 889–897. doi: 10.1038/nn1483

Tyagarajan, S. K., and Fritschy, J.-M. (2014). Gephyrin: a master regulator of neuronal function? *Nat. Rev. Neurosci.* 15, 141–156. doi: 10.1038/nrn3670

Tyagarajan, S. K., Ghosh, H., Yévenes, G. E., Imanishi, S. Y., Zeilhofer, H. U., Gerrits, B., et al. (2013). Extracellular signal-regulated kinase and glycogen synthase kinase 3β regulate gephyrin postsynaptic aggregation and GABAergic synaptic function in a calpain-dependent mechanism. *J. Biol. Chem.* 288, 9634–9647. doi: 10.1074/jbc.M112.442616

Tyagarajan, S. K., Ghosh, H., Yévenes, G. E., Nikonenko, I., Ebeling, C., Schwerdel, C., et al. (2011). Regulation of GABAergic synapse formation and plasticity by GSK3beta-dependent phosphorylation of gephyrin. *Proc. Natl. Acad. Sci. U S A* 108, 379–384. doi: 10.1073/pnas.1011824108

Uezu, A., Kanak, D. J., Bradshaw, T. W. A. A., Soderblom, E. J., Catavero, C. M., Burette, A. C., et al. (2016). Identification of an elaborate complex mediating postsynaptic inhibition. *Science* 353, 1123–1129. doi: 10.1126/science.aag0821

Villa, K. L., Berry, K. P., Subramanian, J., Cha, J. W., Oh, W. C., Kwon, H. B., et al. (2016). Inhibitory synapses are repeatedly assembled and removed at persistent sites *in vivo*. *Neuron* 89, 756–769. doi: 10.1016/j.neuron.2016.01.010

Wang, Z., Ma, J., Miyoshi, C., Li, Y., Sato, M., Ogawa, Y., et al. (2018). Quantitative phosphoproteomic analysis of the molecular substrates of sleep need. *Nature* 558, 435–439. doi: 10.1038/s41586-018-0218-8

Witte, H., Schreiner, D., and Scheiffele, P. (2019). A Sam68-dependent alternative splicing program shapes postsynaptic protein complexes. *Eur. J. Neurosci.* doi: 10.1111/ejn.14332 [Epub ahead of print].

Wu, M., Tian, H., Liu, X., Lai, J. H. C., Du, S., and Xia, J. (2018). Impairment of inhibitory synapse formation and motor behavior in mice lacking the NL2 binding partner LHFPL4/GARLH4. *Cell Rep.* 23, 1691–1705. doi: 10.1016/j.celrep.2018.04.015

Yamasaki, T., Hoyos-Ramirez, E., Martenson, J. S., Morimoto-Tomita, M., and Tomita, S. (2017). GARLH family proteins stabilize $GABA_A$ receptors at synapses. *Neuron* 93, 1138.e6–1152.e6. doi: 10.1016/j.neuron.2017.02.023

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

Zappulo, A., Van Den Bruck, D., Ciolli Mattioli, C., Franke, V., Imami, K., McShane, E., et al. (2017). RNA localization is a key determinant of neurite-enriched proteome. *Nat. Commun.* 8:583. doi: 10.1038/s41467-017-00690-6

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 Molecular Neuroscience

ORIGINAL RESEARCH
published: 10 September 2019
doi: 10.3389/fnmol.2019.00213

A Conserved Tyrosine Residue in Slitrk3 Carboxyl-Terminus Is Critical for GABAergic Synapse Development

*Jun Li, Wenyan Han, Kunwei Wu, Yuping Derek Li, Qun Liu and Wei Lu**

Synapse and Neural Circuit Research Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, United States

Single-passing transmembrane protein, Slitrk3 (Slit and Trk-like family member 3, ST3), is a synaptic cell adhesion molecule highly expressed at inhibitory synapses. Recent studies have shown that ST3, through its extracellular domain, selectively regulates inhibitory synapse development via the trans-synaptic interaction with presynaptic cell adhesion molecule, receptor protein tyrosine phosphatase δ (PTPδ) and the *cis*-interaction with postsynaptic cell adhesion molecule, Neuroligin 2 (NL2). However, little is known about the physiological function of ST3 intracellular, carboxyl (C)-terminal region. Here we report that in heterologous cells, ST3 C-terminus is not required for ST3 homo-dimerization and trafficking to the cell surface. In contrast, in hippocampal neurons, ST3 C-terminus, more specifically, the conserved tyrosine Y969 (in mice), is critical for GABAergic synapse development. Indeed, overexpression of ST3 Y969A mutant markedly reduced the gephyrin puncta density and GABAergic transmission in hippocampal neurons. In addition, single-cell genetic deletion of ST3 strongly impaired GABAergic transmission. Importantly, wild-type (WT) ST3, but not the ST3 Y969A mutant, could fully rescue GABAergic transmission deficits in neurons lacking endogenous ST3, confirming a critical role of Y969 in the regulation of inhibitory synapses. Taken together, our data identify a single critical residue in ST3 C-terminus that is important for GABAergic synapse development and function.

Keywords: Slitrk3, GABAergic synapse, gephyrin, cell adhesion molecule, GABAergic synapse development, inhibition, tyrosine, hippocampus

OPEN ACCESS

Edited by:
Shiva K. Tyagarajan, University of Zurich, Switzerland

Reviewed by:
Katsuhiko Tabuchi, Shinshu University, Japan
Josef Kittler, University College London, United Kingdom

***Correspondence:**
Wei Lu
luw4@mail.nih.gov

Received: *30 April 2019*
Accepted: *20 August 2019*
Published: *10 September 2019*

Citation:
Li J, Han W, Wu K, Li YD, Liu Q and Lu W (2019) A Conserved Tyrosine Residue in Slitrk3 Carboxyl-Terminus Is Critical for GABAergic Synapse Development. Front. Mol. Neurosci. 12:213. doi: 10.3389/fnmol.2019.00213

INTRODUCTION

Synapses, the highly specialized cellular junctions, are essential for rapid chemical communication between neurons. Glutamate is the predominant excitatory neurotransmitter in the central nervous system and mainly acts on ionotropic glutamate receptors to mediate excitatory transmission. On the other hand, GABA is the dominant inhibitory neurotransmitter in the adult brain and fast inhibitory transmission is largely mediated by $GABA_A$ receptors, a process that provides inhibitory balance to glutamatergic excitation and controls neuronal output. Accumulating evidence has shown that perturbations of synapse development and function are associated with a variety of neurological and psychiatric disorders, such as autism spectrum disorders, schizophrenia, and epilepsy (Dani et al., 2005; Scharfman, 2007; Eichler and Meier, 2008; Kehrer et al., 2008; Dudek, 2009; Gogolla et al., 2009; Markram and Markram, 2010; Rubenstein, 2010; Vattikuti and Chow, 2010; Paluszkiewicz et al., 2011; Yizhar et al., 2011; Lisman, 2012; Sheng et al., 2012). Thus, it is

critical to understand the molecular mechanisms for synaptogenesis and synaptic function. While development of glutamatergic synapses has been extensively studied (Waites et al., 2005; McAllister, 2007; Kelsch et al., 2010; Clarke and Barres, 2013; Hanse et al., 2013), much less is known about the mechanisms underlying GABAergic synapse development.

Synaptic cell adhesion molecules are a class of cell surface proteins that are key players in instructing various steps of both excitatory and inhibitory synaptogenesis (Waites et al., 2005; Sudhof, 2008; Siddiqui and Craig, 2011; Lu et al., 2016; Krueger-Burg et al., 2017). Among these molecules, Slit- and Trk-like (Slitrk) proteins have been implicated in synapse development and function (Proenca et al., 2011; Won et al., 2019). Slitrks constitute a family of six members, and, among them, ST3 plays a specific role in the regulation of GABAergic synapse development, whereas other Slitrks are critical for excitatory synaptogenesis and function (Takahashi et al., 2012; Yim et al., 2013; Beaubien et al., 2016; Li et al., 2017). Molecularly, Slitrks contain two clusters of the leucine-rich repeat (LRR) domain (LRR1 and LRR2) in the amino-terminal (N-terminal) extracellular region with each cluster consisting of six LRR motif repeats, a single transmembrane domain, and a carboxyl-terminal (C-terminal) domain (Aruga and Mikoshiba, 2003). The LRR1 domain of these postsynaptic cell adhesion molecules mediates the trans-synaptic interaction with presynaptic cell adhesion molecules, receptor protein tyrosine phosphatases (PTPs), to regulate synapse development (Proenca et al., 2011; Um et al., 2014; Won et al., 2019). In addition, the LRR2 domain of ST3 has been shown to bind to another synaptic cell adhesion molecule, NL2, to regulate GABAergic synapse development (Li et al., 2017), and Slitrk1 LRR2 domain is critical for protein oligomerization (Beaubien et al., 2016). Recent studies have also identified a number of missense mutations in Slitrk N-termini that are associated with neuropsychiatric disorders (Proenca et al., 2011; Kang et al., 2016), highlighting the importance of Slitrk extracellular domains in brain development and function. However, the role of Slitrk C-termini in synapse development and transmission remains largely unclear. One prominent feature of Slitrk C-termini is that they contain several conserved tyrosine (Tyr or Y) residues (Aruga and Mikoshiba, 2003). Among them, a tyrosine residue in the distal C-termini of Slitrks, conserved between Slitrks and Trk neurotrophin receptor proteins (Y791 in human TrkA), is intriguing. In Trk receptors, neurotrophin binding leads to Tyr phosphorylation at Y791 (Reichardt, 2006), which in turn recruits phospholipase C-γ (PLC-γ) that can generate second messengers, such as IP3 and diacylglycerol (DAG), for intracellular signaling (Huang and Reichardt, 2003). However, the role of this conserved tyrosine residue in Slitrks in the regulation of synapse development and function remains unknown.

Here we have investigated the function of the C-terminus of inhibitory synaptic cell adhesion molecule, ST3, in regulating GABAergic synapses. We have found that the conserved tyrosine residue, Y969 in ST3 C-terminus, is critical for GABAergic synapse development and transmission. Mutation at this tyrosine residue impaired GABAergic synapse development and reduced inhibitory transmission, demonstrating an important role of ST3 C-terminus in the regulation of inhibitory synapses.

MATERIALS AND METHODS

Animals

Animal housing and procedures were performed in accordance with the guidelines of the Animal Care and Use Committee (ACUC) at National Institute of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH), and were approved by the NINDS ACUC at NIH. Adult C57BL/6 mice were purchased from Charles River, housed and bred with standard laboratory chow and water under a 12-h light/dark cycle. Mice of either sex were used in this study.

Plasmids

Full length mouse cDNA encoding Slitrk3 (ST3) in this study was purchased from OriGene (Cat #: MR211375). Flag- or Myc-tagged full length or truncation mutants of ST3 were generated by overlapping PCR and were subcloned into pcDNA3.0 expression vector, respectively. Y969A point mutation (TAC→GCA) in ST3 was generated by overlapping PCR and subcloned into pcDNA3.0 expression vector. To screen the ST3 single-guidance RNA (sgRNA) sequences for single-cell knockout experiment, we have designed 3 sgRNA sequence candidates using online tools[1]. The primer sequences are as shown below:

ST3 #1: forward, 5′-CACCgAGCTGTTTCCTTAACGCATC-3′;
reverse, 5′-AAACGATGCGTTAAGGAAACAGCTc-3;
ST3 #2: forward 5′-CACCgACGAAGGTCCAGATGCGTTA-3′;
reverse 5′-AAACTAACGCATCTGGACCTTCGTc-3′;
ST3 #3: forward 5-CACCgCAATAGTGCGCACATCACGG-3;
reverse 5-AAACCCGTGATGTGCGCACTATTGc-3.

The human codon-optimized Cas9 and chimeric sgRNA expression plasmid (pSpCas9 BB-2A-GFP, or pX458) was purchased from Addgene (#48138, Ran et al., 2013). To generate sgRNA plasmids, a pair of annealed oligos were ligated into the sgRNA scaffold of pX458. To examine the specificity of the single-cell knockout effect on GABAergic synapses, sgRNA resistant ST3 plasmids were constructed for rescue experiments. The constructs of Flag- or Myc-tagged ST3, which were resistant to ST3 sgRNA#2, were generated by overlapping PCR to make five-point mutations in the ST3 sgRNA#2-targeting site (mutation region: ACGAAGGTCCAGATGCGTTA to ACGATGGACCTGAAGCATTA; amino acids: Asn-Ala-Ser-Gly-Pro-Ser) and then subcloned into the pcDNA3.0 plasmid, respectively. All constructs were verified by DNA sequencing.

Cell Culture and Transfection

HEK293T and COS7 cells were grown in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO),

[1]http://crispr.mit.edu; http://www.e-crisp.org

1% Pen/Strep, 1% Glutamine, and 1% sodium pyruvate, in a humidified atmosphere in a 37°C incubator with 5% CO_2. Transfection was performed in 6 cm dishes with indicated cDNAs using CalPhos Mammalian Transfection Kit (Clontech, 631312) or Lipofectamine 3000 Transfection Reagent (Invitrogen, L3000015), following the manufacturer's instructions.

Dissociated Hippocampal Neuronal Culture

Hippocampal neuronal cultures were prepared from E18 time-pregnant C57BL/6 mice as previously described (Gu et al., 2016). Briefly, the embryonic mouse hippocampi were dissected out in ice-cold Hank's balanced salt solution, and digested in papain (Worthington, LK003176) solution at 37°C for 45 min. After centrifugation for 5 min at 800 rpm, the pellet was resuspended in DNase I-containing Hank's solution, and then mechanically dissociated into single cells by gentle trituration using a pipette. Digestion was stopped by adding trypsin inhibitor (10 mg/ml, Sigma T9253) and BSA (10 mg/ml, Sigma A9647), and then centrifuged at 800 rpm for 10 min. The pellet was resuspended in Neurobasal media containing 2% B27 supplements and L-glutamine (2 mM). Dissociated neurons were plated at a density of 1.5∼2.0 × 10^5 cells/well on poly-D-lysine (Sigma P7886)-coated 12 mm glass coverslips residing in 24-well plates for electrophysiology recording, and a lower plating density (1.0∼1.5 × 10^5 cells/well) was adopted when neurons were used for immunocytochemistry. Culture media were changed by a half volume once a week.

Neuronal Transfection

For sgRNA transfection, hippocampal neurons were transfected at day 2–3 *in vitro* (DIV2-3) using a modified calcium phosphate transfection as described previously (Li et al., 2019). Briefly, 5 μg total cDNA was used to generate 200 μL total precipitates, which was added to each well at a 40 μL volume (five coverslips/group). After 2-h incubation in a 37°C incubator, the transfected cells were incubated with pre-warmed, 10% CO_2 pre-equilibrated Neurobasal medium, and placed in a 37°C, 5% CO_2 incubator for 20 min to dissolve the calcium-phosphate particles. The coverslips were then transferred back to the original conditioned medium. The cells were cultured to DIV 14-16 before experiments. For overexpression experiments for both staining and electrophysiological recordings, neuronal transfection was performed at DIV12-13 in 24-well plate with indicated cDNAs using Lipofectamine 3000 Reagent following the manufacturer's instructions. Neurons after transfection were analyzed at DIV 14-16.

Immunocytochemistry

The cells grown on coverslips were rinsed with PBS twice and fixed in 4% paraformaldehyde (PFA)/4% sucrose/1× PBS solution for 15 min at RT, followed by permeabilization with 0.2% TritonX-100/1× PBS for 15 min. Subsequently, cells were blocked with 5% normal goat serum in 1× PBS for 1 h. Cells were incubated with primary antibodies as follows: anti-Myc (1:1,000, ab18185, Abcam), anti-Flag M2 (1:1,000, F3165, Sigma), anti-Flag (1:1,000, F7425, Sigma), anti-Gephyrin (1:500, 147018, Synaptic Systems), anti-Gephyrin (1:500, 147021, Synaptic Systems), anti-Slitrk3 (1:1,000, ABN356, Sigma) and anti-MAP2 (1:1,000, MAP, Aves Labs) in 1× PBS solutions overnight at 4°C. Cells were washed three times with 1× PBS and then incubated with Alexa Fluor 488, 555, or 647-conjugated IgG for 30 min. Coverslips were washed for three times with 1× PBS and mounted with Fluoromount-G (Southern Biotech) for imaging acquisition.

Co-immunoprecipitation and Western Blot

For co-immunoprecipitation (Co-IP) experiments, the indicated constructs were transfected into HEK293T cells by calcium phosphate transfection. After transfection for 48 h, cells were homogenized in ice-cold lysis buffer containing 25 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 5% glycerol, 1 mM EDTA, and EDTA-free protease inhibitors (Roche, 5892791001). Equal amounts of cell lysates were incubated with anti-Flag M2 affinity gel (Sigma, A2220) overnight at 4°C. Beads were washed three times with 500 μl lysis buffer and diluted in an equal amount of 2 × loading buffer (Bio-Rad 161-0737) containing 10% β-mercaptoethanol (Fisher Scientific BP176100) and denatured for 5 min at 95°C. Proteins were separated on 10% SDS-PAGE (Bio-Rad), and transferred onto PVDF membrane for immunoblotting with indicated antibodies. For the dimerization experiment, Flag-ST3 and Myc-ST3 or Myc-ST3 ΔCT plasmids were co-transfected into HEK293T cells for 48 h. Anti-Myc (1:1,000, 2278, Cell Signaling Technology) and anti-Flag (1:1,000, F2555, Sigma) antibodies were used in this experiment. For ST3 sgRNA screening and resistant plasmid verification experiments, Myc-ST3 or resistant mutants and sgRNA candidates were co-transfected in a ratio at 1:2 to HEK293T cells (2 × 10^6 cells/well on transfection day in 6-well plate), while empty pcDNA3.0 vector was added to balance the total amount of DNA in single transfection conditions. Proteins were detected with anti-Slitrk3 antibody (1:1,000, ABN356, Sigma) or anti-α-tubulin antibody (1:5,000, T8203, Sigma) by enhanced chemiluminescence (ECL) method.

Image Acquisition and Analysis

Fluorescence images were acquired with a Zeiss LSM 880 laser scanning confocal microscope using a 63× oil-immersion objective lens (numerical aperture 1.4). For fluorescent intensity analysis in both of COS7 cells and neurons, the maximal intensity projected images were generated by ZEN software (Zeiss) from seven serial optical sections, and the mean fluorescent intensity of region of interest (ROI) was measured following the subtraction for off-cell background with ImageJ software. For gephyrin and vGAT puncta density analysis, confocal images from 1 to 3 secondary or tertiary dendrites (35 μm in length) per neuron from at least ten neurons in each group were collected and quantified by counting the number of puncta per 10 μm dendrites with ImageJ puncta analyzer program. Thresholds were set at 3 SDs above the mean staining intensity of six nearby regions in the same visual field. Thresholded images present a fixed intensity for all pixels above threshold after having

removed all of those below. Labeled puncta were defined as areas containing at least four contiguous pixels after thresholding. For co-localization analysis, the gephyrin-positive Myc clusters indicate the number of Myc clusters exhibiting at least partial pixel overlapping with thresholded gephyrin clusters, and co-localization percentage was quantified by the measurement of gephyrin-positive Myc clusters compared to the total number of thresholded Myc clusters.

Electrophysiology

For mIPSC recording in dissociated hippocampal neuronal cultures, neurons grown on coverslips were transferred to a submersion chamber on an upright Olympus microscope, and perfused with ACSF solution supplemented with TTX (0.5 μM), DNQX (20 μM), and strychnine (1 μM). GFP fluorescent positive neurons in neuronal cultures were identified by epifluorescence microscopy. Neurons were voltage-clamped at −70 mV for detection of mIPSC events. The intracellular solution for GABAergic mIPSC recording contained (in mM) $CsMeSO_4$ 70, CsCl 70, NaCl 8, EGTA 0.3, HEPES 20, MgATP 4, and Na_3GTP 0.3. Osmolality was adjusted to 285–290 mOsm and pH was buffered at 7.25–7.35. Series resistance was monitored and not compensated, and cells in which series resistance varied by 25% during a recording session were discarded. Synaptic responses were collected with a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA, United States), filtered at 2 kHz, and digitized at 10 kHz. All recordings were performed at RT. 100–300 consecutive miniature events were semi automatically detected by off-line analysis using customized software Igor Pro (Wavemetrics) as described before (Milstein et al., 2007; Lu et al., 2009, 2013; Herring et al., 2013), using a threshold of 6 pA. All mIPSC events were visually inspected to ensure that they were mIPSCs during analysis, and non-mIPSC traces were discarded. All pharmacological reagents were purchased from Abcam, and other chemicals were purchased from Sigma.

Statistical Analysis

Statistical analysis was performed in GraphPad Prism 7.0. Direct comparisons between two groups were made using two-tailed, unpaired Student's *t*-test. Multiple group comparisons were made using one-way analysis of variance (ANOVA) with *post hoc* Fisher's LSD test. The significance of cumulative probability distributions was assessed by Kolmogorov–Smirnov (K–S) test. The difference was considered significant at levels of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****), respectively. *p*-values ≥ 0.05 were considered not significant. All data *n* in the text and figures were presented as Mean ± SEM (standard error of mean).

RESULTS

We first examined whether ST3 could form homo-dimers in heterologous cells and whether ST3 C-terminus was important in this process, as dimerization is a common feature for transmembrane protein-mediated signaling (Maruyama, 2015) and is important for cell adhesion molecules in promoting synapse development and function (Ko et al., 2009; Fogel et al., 2011; Shipman and Nicoll, 2012). Toward this end, we generated Flag or Myc tagged ST3 WT at its N-terminus (Flag-ST3 WT, Myc-ST3 WT) and Myc tagged ST3 C-terminal deletion mutant (Myc-ST3 ΔCT), as shown in **Figure 1A**. We then performed immunocytochemical experiments in COS7 cells expressing both Flag-ST3 WT and Myc-ST3 WT and examined the distribution of surface ST3. We found that surface Flag-ST3 WT co-localized with Myc-ST3 WT at distinct puncta (**Figure 1B**), indicating that these two molecules localize at the same subcellular compartments. Interestingly, ST3 Myc-ST3 ΔCT lacking the majority of C-terminus also co-localized with Flag-ST3 WT at the cell surface (**Figure 1C**), indicating that ST3 C-terminus is not critical for the co-localization of tagged ST3 in heterologous cells. We also conducted co-immunoprecipitation assays in HEK293T cells expressing both Flag-ST3 WT and Myc-ST3 WT or expressing either plasmids on its own. We found that a Flag antibody could pull down Myc-ST3 WT from cells expressing both constructs, but not from control cells expressing either one (**Figure 1D**), showing that ST3 can form homo-dimers in heterologous cells. To probe whether ST3 C-terminus was important for homo-dimerization, we co-transfected Myc-ST3 ΔCT together with Flag-ST3 WT in HEK293T cells. Co-immunoprecipitation assays showed that both Myc-ST3 and Myc-ST3 ΔCT were co-immunoprecipitated with Flag-ST3 (**Figure 1E**), indicating that ST3 C-terminus is not required for ST3 homo-dimerization. Together, these data show that ST3 forms dimers in a C-terminus independent manner in heterologous cells.

To determine whether ST3 C-terminus was important for trafficking of ST3 to the cell surface in heterologous cells, we generated a series of mutants of Flag-ST3 in its C-terminus (**Figure 2D**) and expressed them individually in COS7 cells. We then performed immunocytochemical experiments to examine the surface expression of WT and truncated Flag-ST3 mutants by measuring the ratio of surface Flag fluorescence to total Flag fluorescence. As shown in the **Figures 2E,F**, all Flag-ST3 truncation mutants showed similar expression levels on the cell surface as compared to WT Flag-ST3. In fact, the surface expression levels of Flag-ST3ΔCT, lacking the majority of C-terminus (truncation at 680), was comparable to WT Flag-ST3 (**Figures 2E,F**). We noticed that in the C-terminus of ST3, there is an evolutionarily conserved tyrosine residue at the position of 969 (**Figures 2A–C**), which shows homology with Trk receptors. We mutated the conserved tyrosine residue in Y969 to alanine (Flag-ST3 Y969A) and found that expression of this mutant on the cell surface was similar to WT Flag-ST3 (**Figures 2E,F**). Taken together, **Figures 1**, **2** show that, while ST3 can form dimers and traffic to the cell surface in heterologous cells, its C-terminus is not critical in these processes.

To further examine the role of ST3 C-terminus, we investigated the function of ST3 C-terminus in the regulation of GABAergic synapses in hippocampal neuronal cultures, as ST3 is a key inhibitory synaptic cell adhesion molecule (Takahashi et al., 2012; Yim et al., 2013; Li et al., 2017). We first overexpressed WT Myc-ST3 and Myc-ST3 ΔCT in dissociated hippocampal cultures, and examined the density of gephyrin, an inhibitory

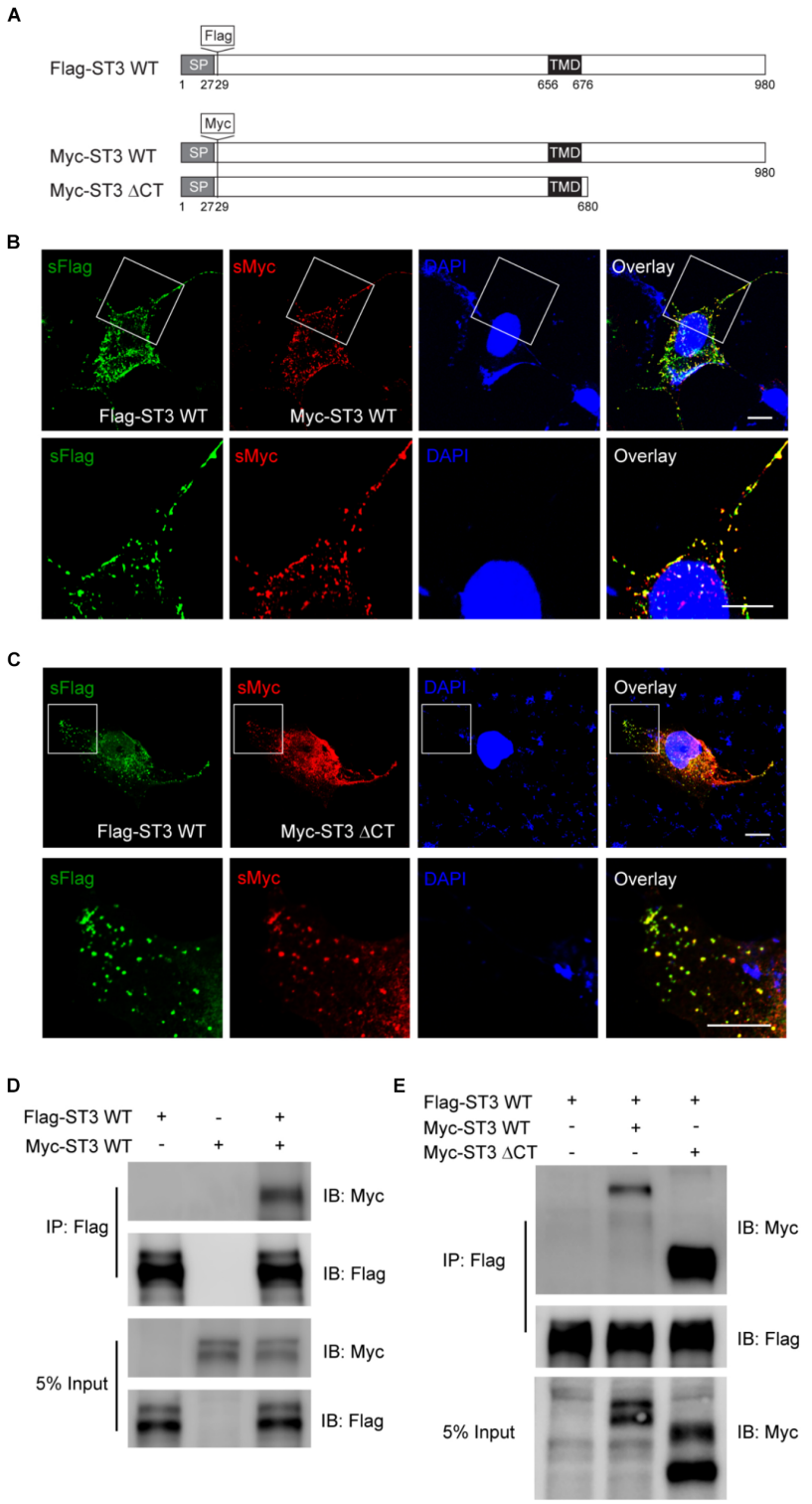

FIGURE 1 | ST3 C-terminus is not required for ST3 homo-dimerization in heterologous cells. **(A)** Schematic of Flag-ST3 WT, Myc-ST3 WT, and Myc-ST3 ΔCT. Flag or Myc tag was inserted at amino acid 29. Signal peptide (SP). **(B,C)** Representative images (top panel, low magnification; bottom panel, high magnification of the boxed area at the top) showing surface (s) Flag-ST3 co-localized with surface Myc-ST3 **(B)** or surface Myc-ST3 ΔCT **(C)** in COS7 cells. Scale bar, 10 μm. **(D,E)** Co-IP assay of Flag-ST3 with Myc-ST3 **(D)** or Myc-ST3 ΔCT **(E)** in HEK293T cells. Cell lysates from HEK293T cells transfected with Flag-ST3, Myc-ST3, or Flag-ST3 together with Myc-ST3 or Myc-ST3 ΔCT, were immunoprecipitated with agarose beads conjugated with anti-Flag antibody, and then probed with indicated antibodies. IB, immunoblotting. Both Myc-ST3 **(D)** or Myc-ST3 ΔCT **(E)** were co-IPed with Flag-ST3. *N* = 3 independent repeats.

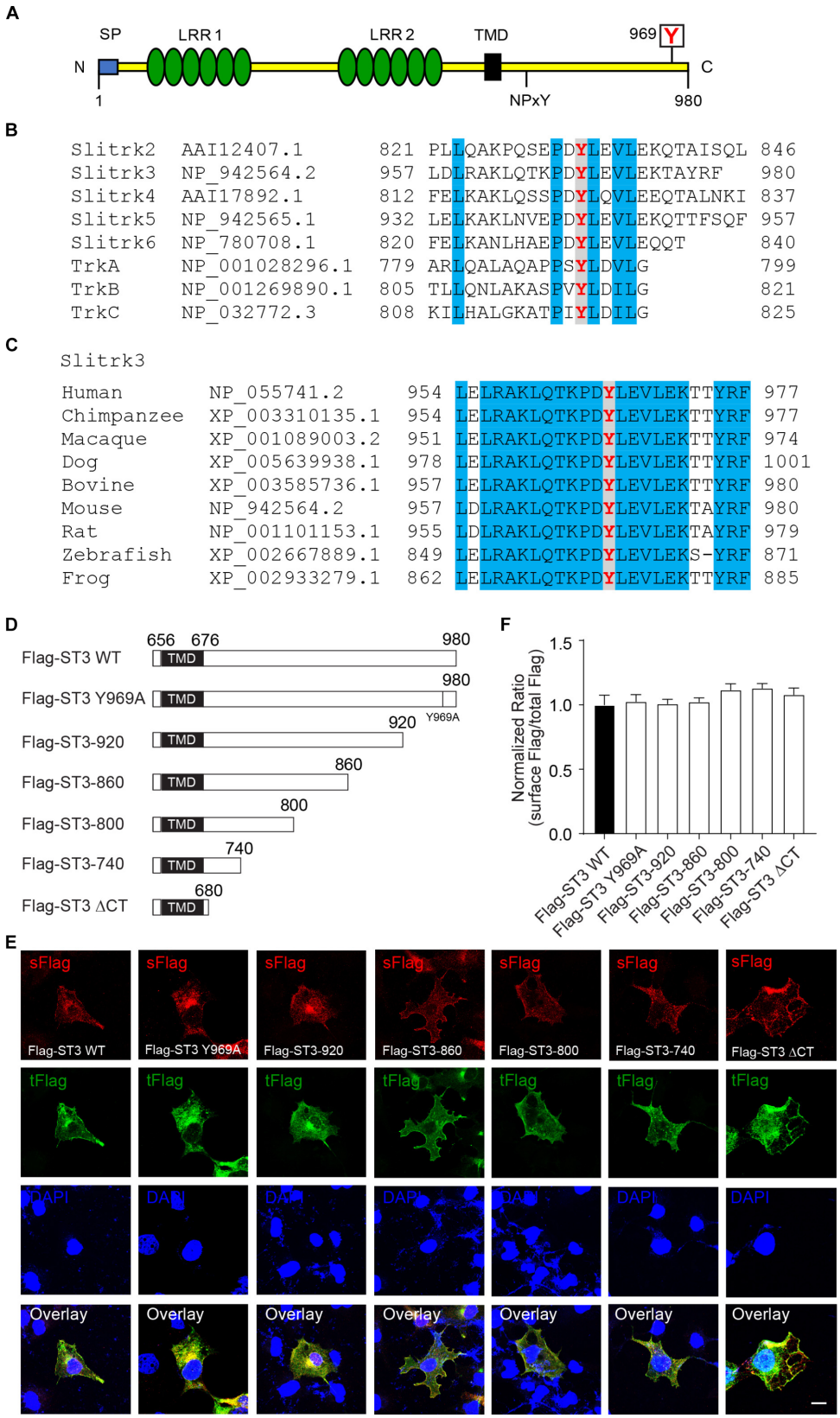

FIGURE 2 | Continued

FIGURE 2 | ST3 C-terminus is not necessary for surface expression of ST3 in heterologous cells. **(A)** Schematic of ST3 showing LRR1 and LRR2 clusters in its extracellular region, and the intracellular conserved tyrosine residues. N, N-terminus; C, C-terminus; SP, signal peptide; LRR1, leucine-rich repeats cluster 1; LRR2, leucine-rich repeats cluster 2; TMD, transmembrane domain; NPxY, NPxY motif; boxed Y, a conserved tyrosine residue in Slitrks and Trk receptors. **(B)** Amino acid sequence alignment of the C-termini of mouse Slitrk and Trk proteins. The tyrosine in red indicates the conserved residues (Y969 in mouse ST3) in Slitrks and Trk receptors, and residues in blue indicate other conserved amino acid residues between Slitrks and Trk receptors. **(C)** Cross species alignment of the ST3 C-termini. The tyrosine in red indicates the conserved residues (Y969 in mouse ST3) in ST3 C-termini from nine different vertebrate species, and residues in blue indicate other conserved amino acid residues across different species in the distal Slitrk3 C-termini. **(D)** Schematic of WT and C-terminal mutant forms of Flag-ST3. TMD, transmembrane domain. **(E,F)** Representative images showing surface (*s*) and total (*t*) Flag expressions of Flag-ST3 WT or Flag-ST3 mutants in COS7 cells. The ratios of surface to total fluorescent intensity were calculated and showed that ST3 C-terminus was not required for ST3 expression at the cell surface ($n \geq 14$ for each group, One-way ANOVA test, $p > 0.05$, $N = 3$ independent experiments). Scale bar, 10 μm.

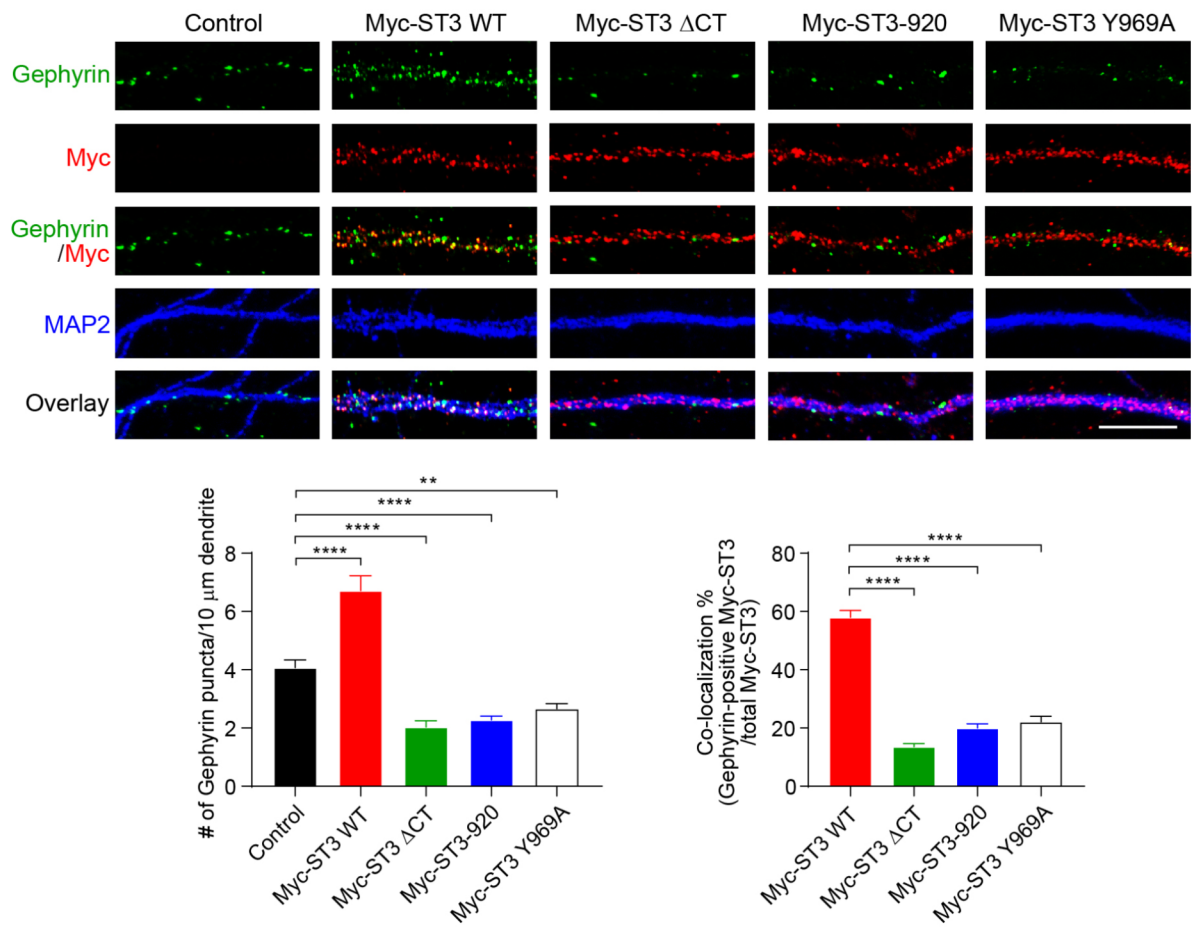

FIGURE 3 | Identification of a critical residue Y969 in ST3 C-terminus that is important for GABAergic synapse development in hippocampal neurons. Representative images of dendrites (top) and quantification analysis (bottom) showed that overexpression of WT Myc-ST3 significantly increased gephyrin puncta density in cultured hippocampal neurons, whereas overexpression of Myc-ST3 ΔCT, Myc-ST3-920, in which the last 60 amino acids of ST3 were deleted, or the Myc-ST3 Y969A mutant significantly decreased gephyrin puncta density (Control, 4.07 ± 0.27, $n = 12$; Myc-ST3 WT, 6.72 ± 0.52, $n = 12$; Myc-ST3 ΔCT, 2.03 ± 0.22, $n = 9$; Myc-ST3-920, 2.28 ± 0.13, $n = 18$; Myc-ST3 Y969A, 2.66 ± 0.18, $n = 11$. One-way ANOVA test, $^{****}p < 0.0001$, $^{**}p < 0.01$. $N = 3$ independent experiments). Overexpression of Myc-ST3 Y969A also significantly decreased co-localization between ST3 and gephyrin (percentage of co-localization: Myc-ST3 WT, 57.96 ± 2.38, $n = 12$; Myc-ST3 ΔCT, 13.59 ± 1.03, $n = 9$; Myc-ST3-920, 19.98 ± 1.45, $n = 18$; Myc-ST3 Y969A, 22.04 ± 1.98, $n = 11$. One-way ANOVA test, $^{****}p < 0.0001$. $N = 3$ independent experiments). Scale bar, 10 μm.

postsynaptic marker and scaffold protein (Tretter et al., 2012; Tyagarajan and Fritschy, 2014), in neuronal dendrites. We found that, in neurons overexpressing WT Myc-ST3, the gephyrin density was significantly increased (**Figure 3**), which is in agreement with a previous study (Yim et al., 2013). However, in neurons overexpressing Myc-ST3 ΔCT that lacked the majority of C-terminus, the density of gephyrin puncta was significantly reduced, as compared to control neurons (**Figure 3**), suggesting that ST3 C-terminus is critical for the regulation of GABAergic synapse density by ST3. Interestingly, overexpression of a ST3 mutant, Myc-ST3-920, in which the last 60 amino acids after the residue 920 (including the conserved Y969) were deleted,

also strongly decreased the gephyrin density (**Figures 2D**, **3**). This indicates that the sequence after amino acid 920 harbors the functional domain important for ST3 to regulate neuronal gephyrin density. Furthermore, to determine whether the Y969 residue in the distal C-terminus was critical (**Figures 2A–C**), we expressed the Myc-ST3 Y969A mutant in hippocampal cultures. We found that the gephyrin density was significantly reduced in neurons expressing this mutant (**Figure 3**), similar to Myc-ST3 ΔCT and Myc-ST3-920. In addition, compared to Myc-ST3 WT, co-localization of Myc-ST3 ΔCT, Myc-ST3-920, or Myc-ST3 Y969A with gephyrin was significantly impaired (**Figure 3**).

The reduction of gephyrin puncta in neurons expressing ST3 mutants, as shown in **Figure 3**, suggested impairment of GABAergic transmission in these neurons. To examine this, we performed whole-cell recordings to measure miniature inhibitory postsynaptic currents (mIPSCs) in hippocampal neurons overexpressing WT ST3 or the ST3 Y969A mutant in C-terminus (also simultaneously expressing GFP). In neurons overexpressing WT ST3, the frequency, but not amplitude, of mIPSCs was significantly increased (**Figure 4A**). In contrast, in neurons overexpressing ST3 Y969A, GABAergic transmission was strongly reduced (**Figure 4A**). Specifically, mIPSC frequency was decreased by ∼50% (**Figure 4A**), indicating a key role of ST3 Y969 in the regulation of inhibitory transmission. In addition, we found that vGAT density was reduced in neurons overexpressing Myc-ST3 Y969A (**Figure 4B**), indicating a reduction of GABAergic synapse density, consistent with the decrease of mIPSC frequency in these cells (**Figure 4A**).

To further characterize the role of ST3 Y969 in the regulation of GABAergic synapses, we performed single-cell knockout (KO) and rescue experiments. To this end, we employed the CRISPR-Cas9 system to develop three single-guide RNAs (sgRNAs) to target *ST3* gene loci in the mouse genome (**Figure 5A**). In HEK293T cells, Western blot experiments showed that,

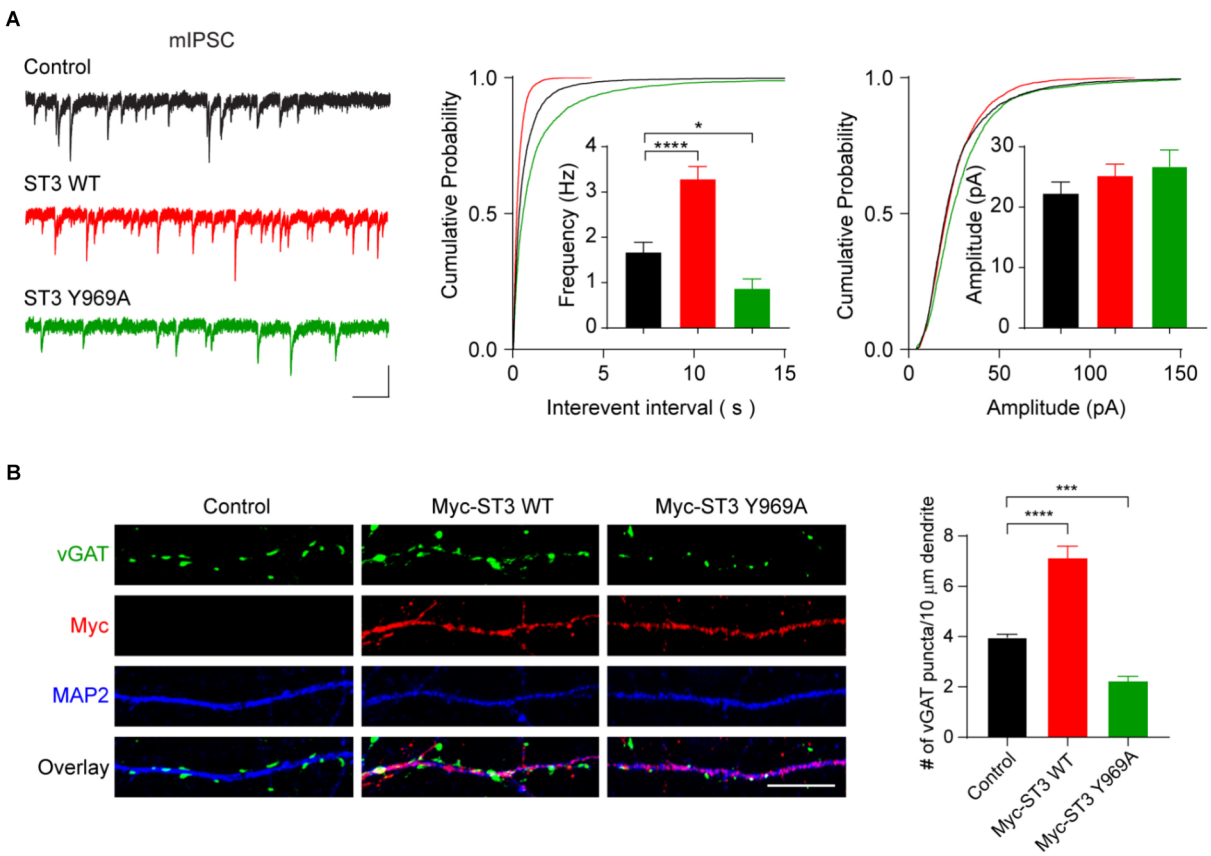

FIGURE 4 | Y969 in ST3 C-terminus is critical for GABAergic synaptic transmission. **(A)** mIPSC recording showed that overexpression of WT Myc-ST3 (co-expressed with GFP) significantly increased mIPSC frequency, whereas overexpression of the Myc-ST3 Y969A mutant significantly reduced the frequency of mIPSCs in hippocampal cultured neurons. Insets showed the mean ± SEM of mIPSC frequency and amplitude, respectively [Frequency (Hz): Control, 1.67 ± 0.22, n = 15; ST3 WT, 3.29 ± 0.28, n = 13; ST3 Y969A, 0.87 ± 0.21, n = 12. One-way ANOVA test, ****$p < 0.0001$, *$p < 0.05$. Kolmogorov–Smirnov (K–S) test, $p < 0.0001$ between Control and ST3 WT or ST3 Y969A for interevent interval. Amplitude (pA): Control, 22.29 ± 1.87, n = 15; ST3 WT, 25.18 ± 1.96, n = 13; ST3 Y969A, 26.78 ± 2.67, n = 12. One-way ANOVA test, $p > 0.05$. K–S test, $p < 0.05$ between Control and ST3 WT for amplitude, $p < 0.0001$ between Control and ST3 Y969A for amplitude. N = 3 independent experiments]. Scale bar, 20 pA and 1 s. **(B)** Representative images of dendrites (left) and quantification analysis (right) showed that overexpression of Myc-ST3 WT significantly increased vGAT puncta density in cultured hippocampal neurons, whereas overexpression of Myc-ST3 Y969A mutant significantly decreased vGAT puncta density (Control, 3.97 ± 0.13, n = 14; Myc-ST3 WT, 7.13 ± 0.46, n = 11; Myc-ST3 Y969A, 2.23 ± 0.19, n = 12. One-way ANOVA test, ****$p < 0.0001$, ***$p < 0.001$. N = 3 independent experiments), Scale bar, 10 μm.

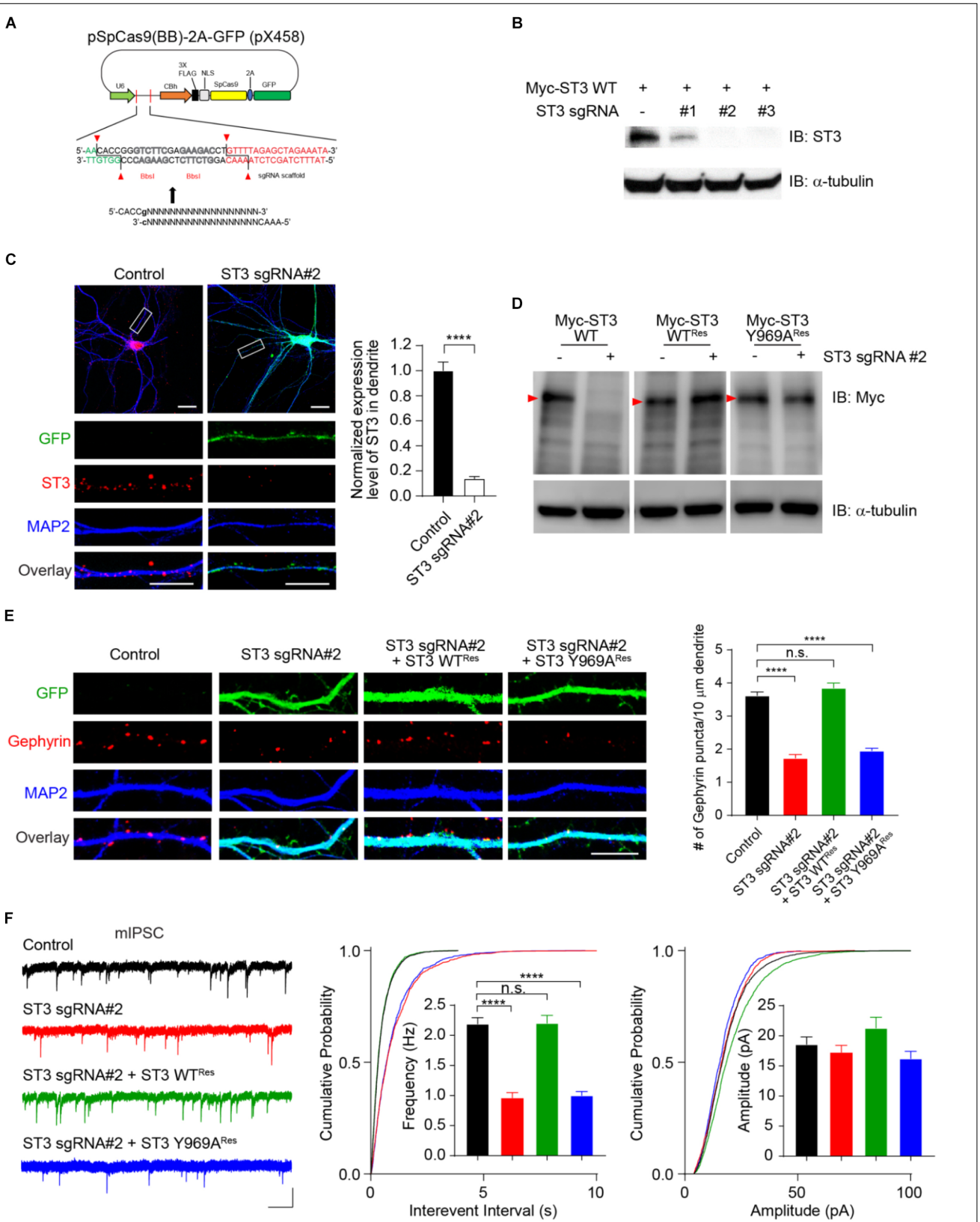

FIGURE 5 | Continued

FIGURE 5 | Single-cell genetic deletion and rescue of ST3 reveal the importance of Y969 in the regulation of GABAergic transmission. **(A)** Schematic diagram of CRISPR/Cas9 vector (simultaneously expresses GFP) targeting *ST3* gene loci in mouse genome. **(B)** Screening of knockout effect of candidate sgRNAs in HEK293T cells. Western blot analysis showed that sgRNA#2 and sgRNA#3, but not sgRNA#1, strongly reduced ST3 expression in HEK293T cells. α-tubulin was used as an internal control. $N = 3$ independent repeats. **(C)** Confocal images and quantification analysis showed a significant decrease of ST3 expression in the dendritic region of hippocampal neurons expressing sgRNA#2 (Control, 1.0 ± 0.07, $n = 12$; ST3 sgRNA#2, 0.14 ± 0.02, $n = 12$, unpaired *t*-test, $^{****}p < 0.0001$. $N = 3$ independent repeats). Scale bar, 20 mm (top) and 10 mm (bottom). **(D)** Western blot analysis validated the expression of sgRNA#2 resistant WT ST3 (ST3 WT^{Res}) and the Y969A mutant (ST3 $Y969A^{Res}$) in HEK293T cells. α-tubulin was used as an internal control. Red arrow heads indicated Myc-ST3 protein bands. $N = 3$ independent repeats. **(E)** Representative images and quantification analysis showed that the decrease of gephyrin puncta density in hippocampal neurons expressing ST3 sgRNA#2 could be rescued by co-expressing ST3 WT^{Res}, but not ST3 $Y969A^{Res}$ (Control, 3.62 ± 0.11, $n = 15$; ST3 sgRNA#2, 1.73 ± 0.10, $n = 13$; ST3 sgRNA#2 + ST3 WT^{Res}, 3.85 ± 0.14, $n = 13$; ST3 sgRNA#2 + ST3 $Y969A^{Res}$, 1.96 ± 0.07, $n = 14$. One-way ANOVA test, $^{****}p < 0.0001$. $N = 3$ independent repeats). Scale bar, 10 mm. **(F)** mIPSC recording data showed that sgRNA#2 resistant WT ST3, but not ST3 Y969A, could fully rescue GABAergic transmission deficits in hippocampal cultured neurons expressing sgRNA#2. Insets displayed the mean ± SEM frequency and amplitude, respectively [Frequency (Hz): Control, 2.19 ± 0.11, $n = 12$; ST3 sgRNA#2, 0.96 ± 0.09, $n = 10$; ST3 sgRNA#2 + ST3 WT^{Res}, 2.2 ± 0.13, $n = 10$; ST3 sgRNA#2 + ST3 $Y969A^{Res}$, 1.0 ± 0.06, $n = 10$. One-way ANOVA test, $^{****}p < 0.0001$. K–S test, $p < 0.0001$ between Control and ST3 sgRNA#2 or ST3 sgRNA#2 + ST3 $Y969A^{Res}$ for interevent interval. Amplitude (pA): Control, 18.59 ± 1.23, $n = 12$; ST3 sgRNA#2, 17.25 ± 1.18, $n = 10$; ST3 sgRNA#2 + ST3 WT^{Res}, 21.31 ± 1.79, $n = 10$; ST3 sgRNA#2 + ST3 $Y969A^{Res}$, 16.21 ± 1.21, $n = 10$. One-way ANOVA test, $p > 0.05$. K–S test, $p < 0.0001$ between Control and ST3 sgRNA#2 + ST3 WT^{Res} for amplitude, $p < 0.001$ between Control and ST3 sgRNA#2 + ST3 $Y969A^{Res}$ for amplitude. $N = 3$ independent repeats]. Scale bar, 20 pA and 1 s.

among the three candidates, sgRNA candidate 1 (sgRNA#1) only partially reduced the expression of co-transfected Myc-ST3, while both sgRNA#2 and sgRNA#3 strongly decreased the expression levels of Myc-ST3 (**Figure 5B**). We further probed the effectiveness of sgRNA#2 in hippocampal neuronal cultures by performing immunocytochemical assays. We found that expression of sgRNA#2 (the vector also simultaneously expresses GFP) strongly diminished endogenous ST3 in neurons, as compared to control cells, showing that sgRNA#2 was an effective candidate in targeting endogenous ST3 (**Figure 5C**). To study the specificity of the effect of sgRNA#2-mediated KO on GABAergic synapses, we also developed sgRNA#2-resistant ST3 mutants (ST3 WT^{Res} and ST3 $Y969A^{Res}$) (**Figure 5D**).

We found that, in hippocampal cultured neurons expressing ST3 sgRNA#2, gephyrin puncta density was significantly reduced (**Figure 5E**), consistent with previous studies using ST3 knockdown approaches (Takahashi et al., 2012; Yim et al., 2013; Li et al., 2017). Importantly, co-expression of sgRNA#2 with ST3 WT^{Res} fully restored the gephyrin puncta deficits (**Figure 5E**), showing that the effect of sgRNA#2 on gephyrin puncta is due to the loss of ST3 protein. Strikingly, co-expression of sgRNA#2 with ST3 $Y969A^{Res}$ could not rescue the deficits of gephyrin puncta density (**Figure 5E**), demonstrating the critical importance of Y969 in determining the function of ST3 in the regulation of gephyrin puncta density in hippocampal neurons.

Electrophysiological measurement of mIPSCs in hippocampal cultured neurons expressing sgRNA#2 further demonstrated that there was a strong reduction of mIPSC frequency, but not amplitude (**Figure 5F**), in agreement with previous studies using ST3 shRNA knockdown or germline ST3 KO approaches (Takahashi et al., 2012; Yim et al., 2013; Li et al., 2017). To examine the specificity of single-cell ST3 KO on GABAergic transmission, we performed rescue experiments by co-expressing sgRNA#2 and ST3 WT^{Res} and measured mIPSCs. We found that ST3 WT^{Res} fully rescued deficits of mIPSC frequency (**Figure 5F**). To determine the role of ST3 Y969 in the regulation of GABAergic transmission, we co-expressed sgRNA#2 and ST3 $Y969A^{Res}$ and examined mIPSCs. We found that ST3 $Y969A^{Res}$ could not rescue inhibitory transmission in neurons expressing sgRNA#2 (**Figure 5F**), consistent with the cell biological data of gephyrin puncta density (**Figure 5E**). Taken together, these data show that Y969A mutation abolishes ST3 function in regulating GABAergic synapses and reveal a novel mechanism for ST3-mediated synapse development.

DISCUSSION

Previous studies have demonstrated that, among the six Slitrk family members Slitrk1-6, ST3 is an inhibitory postsynaptic adhesion molecule critical for GABAergic synapse development and function (Takahashi et al., 2012; Yim et al., 2013). Recently, we have further shown that ST3 plays a temporal specific role in the regulation of GABAergic synaptogenesis in hippocampal neurons (Li et al., 2017). Indeed, ST3 is important for GABAergic synapse development in more mature, but not in developing, hippocampal neurons in culture (Li et al., 2017). Importantly, the regulation of GABAergic synapse development by ST3 requires ST3 C-terminus (Li et al., 2017), highlighting the importance of ST3 C-terminus-mediated signaling in development of GABAergic connections. However, the molecular analysis and functional dissection of ST3 C-terminus in ST3 function and synapse development have not been investigated.

Our data demonstrate that, while ST3 C-terminus is not critical for a variety of ST3 functions in heterologous cells, it is important for GABAergic synapse development in hippocampal neurons. Indeed, we found that ST3 forms homodimers in a C-terminus independent manner in heterologous cells, suggesting that other domains of ST3 are critical for its dimerization. This is consistent with a recent report that the second LRR cluster in the extracellular region of Slitrk1 is necessary for Slitrk1 homo-dimerization (Beaubien et al., 2016). In addition, in heterologous cells, ST3 C-terminus is dispensable for its trafficking to the cell surface. In contrast, through both overexpression and molecular replacement approaches in hippocampal neurons, we have identified a single, evolutionarily conserved amino acid, Y969, in the ST3 C-terminus, which is crucial for ST3 function in the regulation of inhibitory synapse development. Specifically, overexpression of WT ST3 increases gephyrin or vGAT puncta and enhances inhibitory

transmission, but overexpression of ST3 Y969A mutant in hippocampal neuronal cultures strongly reduces the density of gephyrin or vGAT puncta and significantly decreases GABAergic transmission. This suggests that Y969A acts as a dominant negative mutant of ST3. Furthermore, while co-expression of WT ST3 could rescue gephyrin puncta density and GABAergic transmission deficits in ST3 KO neurons, the Y969A mutant could not, showing that the mutation at Y969 inactivates ST3 function in promoting GABAergic synapse development. Thus, Y969-mediated signaling is critical for the regulation of GABAergic synapse development by ST3.

Currently, the molecular mechanisms underlying ST3 Y969-mediated signaling for inhibitory synapse development remain unclear. Y969 is a conserved tyrosine residue in Slitrk2-6 and Trk neurotrophin receptor proteins (**Figures 2A–C**; Aruga and Mikoshiba, 2003). In TrkA receptors, ligand binding leads to Tyr phosphorylation at Y791, the homologous conserved tyrosine in human TrkA (Reichardt, 2006). Functionally, Y791 phosphorylation in TrkA can recruit the signaling molecule, PLC-γ, which in turn generates second messengers such as IP3 and DAG for intracellular signaling (Reichardt, 2006). Similarly, analogous sites in TrkB and TrkC also undergo phosphorylation and initiate PLC-γ-mediated signaling (Reichardt, 2006). It remains unknown as to whether ST3 Y969 can be phosphorylated and whether PLC-γ-mediated signaling is involved in ST3 function in neurons. Interestingly, a recent study has shown that Slitrk5 can interact with TrkB in a BDNF-dependent manner (Song et al., 2015), raising the possibility that Slitrks, including ST3, might be substrates of Trk tyrosine kinases.

Recent structure and function analysis have identified several functional domains in ST3 that are important for ST3 in the regulation of inhibitory synapse development. For instance, ST3 extracellular LRR domains, likely the LRR1 cluster, mediate the interaction with presynaptic cell adhesion molecule, PTPδ, for induction of inhibitory synapse differentiation (Takahashi et al., 2012; Yim et al., 2013; Um et al., 2014). The LRR9 in the LRR2 cluster of ST3 binds to NL2 and the ST3-NL2 interaction is critical for development of GABAergic innervations at the late developmental stages (Li et al., 2017). We have now shown that a single conserved residue, Y969 in the ST3 C-terminus, is critical for the function of ST3 in the regulation of inhibitory synapse development. Understanding the regulatory mechanisms underlying the ST3 Y969-mediated signaling in the future will help reveal the molecular pathways for constructing inhibitory neural circuits in the brain.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

Animal housing and procedures were performed in accordance with the guidelines of the Animal Care and Use Committee (ACUC) at National Institute of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH), and were approved by the NINDS ACUC at NIH.

AUTHOR CONTRIBUTIONS

JL and WL designed the experiments and wrote the manuscript. JL, YL, and QL cloned and characterized sgRNA constructs and other plasmids. JL performed the biochemical and immunocytochemical experiments. JL, WH, and KW performed the electrophysiological assays. All authors read and commented on the manuscript.

ACKNOWLEDGMENTS

We thank Q. J. Tian for technical assistance and all members from the Lu Laboratory for helpful discussions. WL was supported by the NINDS Intramural Research Program.

REFERENCES

Aruga, J., and Mikoshiba, K. (2003). Identification and characterization of slitrk, a novel neuronal transmembrane protein family controlling neurite outgrowth. *Mol. Cell. Neurosci.* 24, 117–129. doi: 10.1016/s1044-7431(03)00129-5

Beaubien, F., Raja, R., Kennedy, T. E., Fournier, A. E., and Cloutier, J. F. (2016). Slitrk1 is localized to excitatory synapses and promotes their development. *Sci. Rep.* 6:27343. doi: 10.1038/srep27343

Clarke, L. E., and Barres, B. A. (2013). Emerging roles of astrocytes in neural circuit development. *Nat. Rev. Neurosci.* 14, 311–321. doi: 10.1038/nrn3484

Dani, V. S., Chang, Q., Maffei, A., Turrigiano, G. G., Jaenisch, R., and Nelson, S. B. (2005). Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of rett syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12560–12565. doi: 10.1073/pnas.0506071102

Dudek, F. E. (2009). Epileptogenesis: a new twist on the balance of excitation and inhibition. *Epilepsy Curr.* 9, 174–176. doi: 10.1111/j.1535-7511.2009.01334.x

Eichler, S. A., and Meier, J. C. (2008). E-I balance and human diseases - from molecules to networking. *Front. Mol. Neurosci.* 1:2. doi: 10.3389/neuro.02.002.2008

Fogel, A. I., Stagi, M., Perez De Arce, K., and Biederer, T. (2011). Lateral assembly of the immunoglobulin protein SynCAM 1 controls its adhesive function and instructs synapse formation. *EMBO J.* 30, 4728–4738. doi: 10.1038/emboj.2011.336

Gogolla, N., Leblanc, J. J., Quast, K. B., Sudhof, T. C., Fagiolini, M., and Hensch, T. K. (2009). Common circuit defect of excitatory-inhibitory balance in mouse models of autism. *J. Neurodev. Disord.* 1, 172–181. doi: 10.1007/s11689-009-9023-x

Gu, X., Mao, X., Lussier, M. P., Hutchison, M. A., Zhou, L., Hamra, F. K., et al. (2016). GSG1L suppresses AMPA receptor-mediated synaptic transmission and uniquely modulates AMPA receptor kinetics in hippocampal neurons. *Nat. Commun.* 7:10873. doi: 10.1038/ncomms10873

Hanse, E., Seth, H., and Riebe, I. (2013). AMPA-silent synapses in brain development and pathology. *Nat. Rev. Neurosci.* 14, 839–850. doi: 10.1038/nrn3642

Herring, B. E., Shi, Y., Suh, Y. H., Zheng, C. Y., Blankenship, S. M., Roche, K. W., et al. (2013). Cornichon proteins determine the subunit composition of synaptic AMPA receptors. *Neuron* 77, 1083–1096. doi: 10.1016/j.neuron.2013.01.017

Huang, E. J., and Reichardt, L. F. (2003). Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem.* 72, 609–642. doi: 10.1146/annurev.biochem.72.121801.161629

Kang, H., Han, K. A., Won, S. Y., Kim, H. M., Lee, Y. H., Ko, J., et al. (2016). Slitrk missense mutations associated with neuropsychiatric disorders distinctively impair slitrk trafficking and synapse formation. *Front. Mol. Neurosci.* 9:104. doi: 10.3389/fnmol.2016.00104

Kehrer, C., Maziashvili, N., Dugladze, T., and Gloveli, T. (2008). Altered excitatory-inhibitory balance in the nmda-hypofunction model of schizophrenia. *Front. Mol. Neurosci.* 1:6. doi: 10.3389/neuro.02.006.2008

Kelsch, W., Sim, S., and Lois, C. (2010). Watching synaptogenesis in the adult brain. *Annu. Rev. Neurosci.* 33, 131–149. doi: 10.1146/annurev-neuro-060909-153252

Ko, J., Zhang, C., Arac, D., Boucard, A. A., Brunger, A. T., and Sudhof, T. C. (2009). Neuroligin-1 performs neurexin-dependent and neurexin-independent functions in synapse validation. *EMBO J.* 28, 3244–3255. doi: 10.1038/emboj.2009.249

Krueger-Burg, D., Papadopoulos, T., and Brose, N. (2017). Organizers of inhibitory synapses come of age. *Curr. Opin. Neurobiol.* 45, 66–77. doi: 10.1016/j.conb.2017.04.003

Li, J., Han, W., Pelkey, K. A., Duan, J., Mao, X., Wang, Y. X., et al. (2017). Molecular dissection of neuroligin 2 and slitrk3 reveals an essential framework for GABAergic synapse development. *Neuron* 96, 808–826. doi: 10.1016/j.neuron.2017.10.003

Li, J., Sekine-Aizawa, Y., Ebrahimi, S., Tanaka, S., and Okabe, S. (2019). Tumor suppressor protein CYLD regulates morphogenesis of dendrites and spines. *Eur. J. Neurosci.* doi: 10.1111/ejn.14421 [Epub ahead of print].

Lisman, J. (2012). Excitation, inhibition, local oscillations, or large-scale loops: what causes the symptoms of schizophrenia? *Curr. Opin. Neurobiol.* 22, 537–544. doi: 10.1016/j.conb.2011.10.018

Lu, W., Bromley-Coolidge, S., and Li, J. (2016). Regulation of GABAergic synapse development by postsynaptic membrane proteins. *Brain Res. Bull.* 129, 30–42. doi: 10.1016/j.brainresbull.2016.07.004

Lu, W., Bushong, E. A., Shih, T. P., Ellisman, M. H., and Nicoll, R. A. (2013). The cell-autonomous role of excitatory synaptic transmission in the regulation of neuronal structure and function. *Neuron* 78, 433–439. doi: 10.1016/j.neuron.2013.02.030

Lu, W., Shi, Y., Jackson, A. C., Bjorgan, K., During, M. J., Sprengel, R., et al. (2009). Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. *Neuron* 62, 254–268. doi: 10.1016/j.neuron.2009.02.027

Markram, K., and Markram, H. (2010). The intense world theory - a unifying theory of the neurobiology of autism. *Front. Hum. Neurosci.* 4:224. doi: 10.3389/fnhum.2010.00224

Maruyama, I. N. (2015). Activation of transmembrane cell-surface receptors via a common mechanism? The "rotation model". *Bioessays* 37, 959–967. doi: 10.1002/bies.201500041

McAllister, A. K. (2007). Dynamic aspects of CNS synapse formation. *Annu. Rev. Neurosci.* 30, 425–450. doi: 10.1146/annurev.neuro.29.051605.112830

Milstein, A. D., Zhou, W., Karimzadegan, S., Bredt, D. S., and Nicoll, R. A. (2007). TARP subtypes differentially and dose-dependently control synaptic AMPA receptor gating. *Neuron* 55, 905–918. doi: 10.1016/j.neuron.2007.08.022

Paluszkiewicz, S. M., Martin, B. S., and Huntsman, M. M. (2011). Fragile X syndrome: the GABAergic system and circuit dysfunction. *Dev. Neurosci.* 33, 349–364. doi: 10.1159/000329420

Proenca, C. C., Gao, K. P., Shmelkov, S. V., Rafii, S., and Lee, F. S. (2011). Slitrks as emerging candidate genes involved in neuropsychiatric disorders. *Trends Neurosci.* 34, 143–153. doi: 10.1016/j.tins.2011.01.001

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308. doi: 10.1038/nprot.2013.143

Reichardt, L. F. (2006). Neurotrophin-regulated signalling pathways. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 1545–1564. doi: 10.1098/rstb.2006.1894

Rubenstein, J. L. (2010). Three hypotheses for developmental defects that may underlie some forms of autism spectrum disorder. *Curr. Opin. Neurol.* 23, 118–123. doi: 10.1097/WCO.0b013e328336eb13

Scharfman, H. E. (2007). The neurobiology of epilepsy. *Curr. Neurol. Neurosci. Rep.* 7, 348–354.

Sheng, M., Sabatini, B. L., and Sudhof, T. C. (2012). Synapses and Alzheimer's disease. *Cold Spring Harb. Perspect. Biol.* 4:a005777. doi: 10.1101/cshperspect.a005777

Shipman, S. L., and Nicoll, R. A. (2012). Dimerization of postsynaptic neuroligin drives synaptic assembly via transsynaptic clustering of neurexin. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19432–19437. doi: 10.1073/pnas.1217633109

Siddiqui, T. J., and Craig, A. M. (2011). Synaptic organizing complexes. *Curr. Opin. Neurobiol.* 21, 132–143. doi: 10.1016/j.conb.2010.08.016

Song, M., Giza, J., Proenca, C. C., Jing, D., Elliott, M., Dincheva, I., et al. (2015). Slitrk5 mediates BDNF-dependent TrkB receptor trafficking and signaling. *Dev. Cell.* 33, 690–702. doi: 10.1016/j.devcel.2015.04.009

Sudhof, T. C. (2008). Neuroligins and neurexins link synaptic function to cognitive disease. *Nature* 455, 903–911. doi: 10.1038/nature07456

Takahashi, H., Katayama, K., Sohya, K., Miyamoto, H., Prasad, T., Matsumoto, Y., et al. (2012). Selective control of inhibitory synapse development by Slitrk3-PTPdelta trans-synaptic interaction. *Nat. Neurosci.* 15, S381–S382. doi: 10.1038/nn.3040

Tretter, V., Mukherjee, J., Maric, H. M., Schindelin, H., Sieghart, W., and Moss, S. J. (2012). Gephyrin, the enigmatic organizer at GABAergic synapses. *Front. Cell. Neurosci.* 6:23. doi: 10.3389/fncel.2012.00023

Tyagarajan, S. K., and Fritschy, J. M. (2014). Gephyrin: a master regulator of neuronal function? *Nat. Rev. Neurosci.* 15, 141–156. doi: 10.1038/nrn3670

Um, J. W., Kim, K. H., Park, B. S., Choi, Y., Kim, D., Kim, C. Y., et al. (2014). Structural basis for LAR-RPTP/Slitrk complex-mediated synaptic adhesion. *Nat. Commun.* 5:5423. doi: 10.1038/ncomms6423

Vattikuti, S., and Chow, C. C. (2010). A computational model for cerebral cortical dysfunction in autism spectrum disorders. *Biol. Psychiatry* 67, 672–678. doi: 10.1016/j.biopsych.2009.09.008

Waites, C. L., Craig, A. M., and Garner, C. C. (2005). Mechanisms of vertebrate synaptogenesis. *Annu. Rev. Neurosci.* 28, 251–274. doi: 10.1146/annurev.neuro.27.070203.144336

Won, S. Y., Lee, P., and Kim, H. M. (2019). Synaptic organizer: slitrks and type IIa receptor protein tyrosine phosphatases. *Curr. Opin. Struct. Biol.* 54, 95–103. doi: 10.1016/j.sbi.2019.01.010

Yim, Y. S., Kwon, Y., Nam, J., Yoon, H. I., Lee, K., Kim, D. G., et al. (2013). Slitrks control excitatory and inhibitory synapse formation with LAR receptor protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4057–4062. doi: 10.1073/pnas.1209881110

Yizhar, O., Fenno, L. E., Prigge, M., Schneider, F., Davidson, T. J., O'shea, D. J., et al. (2011). Neocortical excitation/inhibition balance in information processing and social dysfunction. *Nature* 477, 171–178. doi: 10.1038/nature10360

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 Molecular Neuroscience

MINI REVIEW
published: 05 November 2019
doi: 10.3389/fnmol.2019.00266

Inhibitory Synapse Formation at the Axon Initial Segment

Anna J. Nathanson[1], *Paul A. Davies*[1] *and Stephen J. Moss*[1,2,3]*

[1]*Department of Neuroscience, Tufts University School of Medicine, Boston, MA, United States,* [2]*AstraZeneca Tufts Laboratory for Basic and Translational Neuroscience, Boston, MA, United States,* [3]*Department of Neuroscience, Physiology and Pharmacology, University College, London, United Kingdom*

The axon initial segment (AIS) is the site of action potential (AP) initiation in most neurons and is thus a critical site in the regulation of neuronal excitability. Normal function within the discrete AIS compartment requires intricate molecular machinery to ensure the proper concentration and organization of voltage-gated and ligand-gated ion channels; in humans, dysfunction at the AIS due to channel mutations is commonly associated with epileptic disorders. In this review, we will examine the molecular mechanisms underlying the formation of the only synapses found at the AIS: synapses containing γ-aminobutyric type A receptors ($GABA_A$Rs). $GABA_A$Rs are heteropentamers assembled from 19 possible subunits and are the primary mediators of fast synaptic inhibition in the brain. Although the total $GABA_A$R population is incredibly heterogeneous, only one specific $GABA_A$R subtype—the α2-containing receptor—is enriched at the AIS. These AIS synapses are innervated by GABAergic chandelier cells, and this inhibitory signaling is thought to contribute to the tight control of AP firing. Here, we will summarize the progress made in understanding the regulation of $GABA_A$R synapse formation, concentrating on post-translational modifications of subunits and on interactions with intracellular proteins. We will then discuss subtype-specific synapse formation, with a focus on synapses found at the AIS, and how these synapses influence neuronal excitation.

Keywords: $GABA_A$ receptor, axon initial segment, collybistin, gephyrin, inhibition, synapse formation

OPEN ACCESS

Edited by:
Shiva K. Tyagarajan, University of Zurich, Switzerland

Reviewed by:
Jaewon Ko, Daegu Gyeongbuk Institute of Science and Technology (DGIST), South Korea
Hansjürgen Volkmer, University of Tübingen, Germany
Joachim Kirsch, Heidelberg University, Germany

***Correspondence:**
Stephen J. Moss
stephen.moss@tufts.edu

Received: *14 August 2019*
Accepted: *17 October 2019*
Published: *05 November 2019*

Citation:
Nathanson AJ, Davies PA and Moss SJ (2019) Inhibitory Synapse Formation at the Axon Initial Segment. Front. Mol. Neurosci. 12:266. doi: 10.3389/fnmol.2019.00266

INTRODUCTION

The firing of glutamatergic pyramidal cells is tightly controlled by inhibitory interneurons (INs). By precisely directing pyramidal cell activity, INs are able to regulate network activity, generate oscillations, and even terminate pathological hyperexcitability (Fritschy, 2008; Roux and Buzsáki, 2015). On a molecular level, INs regulate pyramidal cell firing through GABAergic neurotransmission: releasing the neurotransmitter γ-aminobutyric acid (GABA) onto inhibitory postsynaptic specializations containing GABA type A receptors ($GABA_A$Rs) on pyramidal neuron dendrites, soma, and axon initial segments (AISs). Thus, the construction and maintenance of GABAergic synapses are essential for normal inhibitory neurotransmission and brain function. However, relatively little is known about inhibitory synaptogenesis compared to glutamatergic synapses. To complicate the picture, there are many $GABA_A$R subtypes composed of different subunits, which confer distinct physiological properties on the receptors. In addition, different $GABA_A$R subtypes are selectively stabilized at different types of synapses; the AIS, for example,

contains primarily one kind of $GABA_AR$. Thus, the type of receptor present at a given synapse determines the type of inhibition that takes place. Again, little is known about how neurons direct different types of $GABA_ARs$ to different synapses. The following review will briefly summarize what is known about the formation and trafficking of $GABA_AR$ subtypes and the construction of inhibitory synapses overall and specifically at the AIS.

$GABA_A$ RECEPTOR STRUCTURE AND FUNCTION

In the adult mammalian central nervous system, most fast, synaptic inhibitory neurotransmission is mediated by $GABA_ARs$, a group of heteropentameric, ligand-gated anion channels (Connolly and Wafford, 2004). When the neurotransmitter GABA binds to the receptor, the intrinsic ion pore opens and allows permeable ions to pass through (Bormann et al., 1987). $GABA_ARs$ are primarily permeable to chloride (Cl^-) anions (Fatima-Shad and Barry, 1993), and in the mature brain—where the Cl^- reversal potential is more negative than the resting membrane potential—the opening of the $GABA_AR$ channel allows Cl^- ions to flow down their electrochemical gradient into the neuron, lowering the neuron's membrane potential and producing a hyperpolarizing response that reduces the probability of action potential (AP) firing (Busch and Sakmann, 1990; Blaesse et al., 2009).

Structurally, $GABA_ARs$ are diverse. The receptors are assembled from 19 different known subunits: α(1–6), β(1–3), γ(1–3), δ, ε, θ, π, and ρ(1–3; Olsen and Sieghart, 2008), putting the number of possible subunit combinations in the thousands; however, only certain subtypes are expressed in the brain. For synaptic $GABA_ARs$, which this review will focus on, the typical stoichiometric ratio is as follows: 2α:2β:1γ (Wisden et al., 1992; Baumann et al., 2003). $GABA_AR$ subunits possess a similar amino acid sequence and protein structure, with each subunit composed of an extracellular N-terminal domain, four transmembrane domains (TM1–4), an intracellular loop domain (ICD) between TM3 and TM4, and an extracellular C-terminal domain (Schofield et al., 1987; Miller and Aricescu, 2014). The ICD is important for regulating $GABA_AR$ activity, as it is the site of phosphorylation and protein-protein interactions that alter receptor trafficking and plasma membrane (PM) expression (Moss et al., 1992; Nymann-Andersen et al., 2002; O'Toole and Jenkins, 2011). In addition, the ICD is the site of greatest sequence variability between subunits, making it an attractive candidate for a locus of subtype-specific $GABA_AR$ regulation (Arancibia-Cárcamo and Kittler, 2009). It seems likely that such differential regulation occurs, as different types of synaptic $GABA_ARs$ are restricted to certain synapses. For instance, within pyramidal neurons in the cortex and hippocampus, $GABA_ARs$ that contain the α1 subunit tend to be found at synapses in the soma and dendrites, while α2-containing $GABA_ARs$ are enriched at synapses on the AIS (Nusser et al., 1996).

The subunit composition of a given $GABA_AR$ not only influences receptor localization, but also determines the physiological properties of that receptor (see **Table 1** for summary). In addition, the specific α subunit composition of $GABA_ARs$ determines receptor kinetics. α1-$GABA_ARs$ mediate an inhibitory current with a longer decay time than α2-$GABA_ARs$ (Goldstein et al., 2002). Thus, $GABA_AR$ subtypes mediate specific kinds of inhibition; restricting $GABA_AR$ subtypes to different spatial domains allows INs to control pyramidal neuron firing in a precise but dynamic manner.

TABLE 1 | The distribution and synaptic roles of γ-aminobutyric acid type A receptor ($GABA_AR$) α subunits.

Subunit	Brain distribution	Subcellular localization	Synaptic role
α1	60% of all $GABA_ARs$ Widely expressed	Synaptic in somatodendritic compartments	Phasic inhibition
α2	15–20% of $GABA_ARs$ Cerebral cortex (layers 1–4), hippocampus, striatum	Primarily synaptic; enriched in perisomatic regions and at the AIS of cortical and hippocampal pyramidal neurons	Phasic inhibition
α3	10%–15% of $GABA_ARs$ Cerebral cortex (layers 5–6), amyddala, thalamus	Primarily synaptic; found in some AIS	Phasic inhibition
α4	<5% of $GABA_AR$ Dentate gyrus, thalamuss	Extrasynaptic	Tonic inhibition
α5	<5% of $GABA_ARs$ Hippocampus	Extrasynaptic	Tonic inhibition
α6	<5% of $GABA_ARs$ Cerebellum	Primarily extrasynaptic	Tonic inhibition

$GABA_A$ RECEPTOR OLIGOMERIZATION AND TRAFFICKING

$GABA_AR$ subunits are assembled into receptors in the endoplasmic reticulum (ER; Kittler et al., 2002). Oligomerization is controlled by the subunits' N-terminal domains, with assistance from resident ER chaperone proteins to ensure appropriate protein assembly and folding (Connolly et al., 1996; Moss and Smart, 2001). Only those receptors that are conformationally mature are permitted to exit the ER and continue along the $GABA_AR$ lifecycle; receptors that are found to be incomplete or composed of inappropriate subunit combinations are retained in the ER and degraded (Gorrie et al., 1997; Saliba et al., 2007).

Conformationally mature $GABA_ARs$ travel from the ER to the Golgi apparatus, where receptors are segregated into vesicles and transported to the PM (Vithlani et al., 2011). This forward trafficking delivers $GABA_ARs$ to and insert them into the PM, primarily in extrasynaptic areas (Bogdanov et al., 2006). $GABA_AR$ surface expression is also regulated by receptor internalization *via* clathrin-mediated endocytosis (Lorenz-Guertin and Jacob, 2018). The clathrin adaptor protein (AP)-2 binds $GABA_AR$ subunits—the ICD of the $GABA_AR$ β1–3 and γ2 subunits both contain AP2 binding motifs—and clathrin, anchoring receptors in endocytotic pits.

INHIBITORY SYNAPSE CONSTRUCTION

$GABA_ARs$ are inserted into the PM at extrasynaptic locations (Bogdanov et al., 2006). At the surface, $GABA_ARs$ are highly dynamic and diffuse laterally within the PM, where they continually move between the synaptic and extrasynaptic space (Thomas et al., 2005). Recent single-particle trafficking experiments show that both synaptic (α1–3-containing) and extrasynaptic (α4–6-containing) receptors can access the inhibitory synapse; however, when within the synaptic domain, the diffusion rate of synaptic $GABA_ARs$ was reduced relative to extrasynaptic receptors, suggesting that $GABA_ARs$ with "synaptic" subunit compositions are selectively stabilized at synapses (Hannan et al., 2019).

How are these receptors stabilized in the inhibitory synapse? Research to date suggests that protein-protein interactions play an essential role in this process: structural proteins present at the inhibitory synapse bind to $GABA_ARs$, reducing their lateral diffusion rate and effectively anchoring them at the synapse (Hannan et al., 2019). Though the composition of the multimolecular protein complexes present at the inhibitory synapse remains relatively unknown, a number of proteins that reside at the inhibitory synapse and appear to regulate $GABA_AR$ clustering have been identified.

GEPHYRIN

One of the first inhibitory synaptic proteins described was gephyrin (GPN), which is still considered to be an integral structural component of the inhibitory postsynaptic domain (Tyagarajan and Fritschy, 2014). The most common splice variant of GPN is composed of three domains: an N-terminal G domain, a linker C domain, and a C-terminal E domain (Feng et al., 1998; Schwarz et al., 2001). The E and G domains of GPN self-aggregate, leading to the hypothesized formation of hexameric macromolecular GPN complexes that could serve as a lattice to stabilize receptors at the synapse (Saiyed et al., 2007). GPN was first identified as a binding partner of glycine receptors, which mediate inhibition in the spine (Prior et al., 1992). Constitutive knock-out of GPN in the mouse leads to a complete loss of glycine receptor clusters in the periphery, resulting in early postnatal death (Feng et al., 1998). However, it was also found that GPN knock-out mice show a dramatic reduction in the presence of $GABA_ARs$ at brain synapses, providing the first evidence that GPN is also crucial for inhibitory synapse formation in the central nervous system (Kneussel et al., 1999; Fischer et al., 2000).

More recent experiments have shown that GPN co-localizes with $GABA_ARs$ containing α1–3 subunits at synapses (Sassoè-Pognetto et al., 2000). Isothermal titration calorimetry experiments performed with the GPN E domain and the ICDs of $GABA_AR$ α1–3 subunits have demonstrated that GPN interacts directly with the ICD of $GABA_AR$ α subunits at an amino acid stretch between ICD residues 360–375 (Hines et al., 2018). The amino acid sequence in this region is not well conserved between α subunit subtypes, thus it follows that GPN binds α1–3 with differing affinities: the α1 and α3 ICDs formed tight complexes with the GPN E domain, while the α2 ICD formed a comparatively weaker complex (Hines et al., 2018). These data suggest a $GABA_AR$ subtype-specific affinity for GPN, dependent on the amino acid composition of the 360–375 ICD motif of the α subunit and raise the possibility that GPN, or other proteins that bind the 360–375 motif, can selectively stabilize $GABA_AR$ subtypes at certain synapses.

COLLYBISTIN

A more recently identified inhibitory synapse protein is collybistin (CB), a guanine nucleotide exchange factor (Reid et al., 1999). Most functional CB isoforms are composed of three domains: a catalytic double homology domain, a PM-binding pleckstrin homology domain, and an N-terminal Src homology (SH)-3 domain (Harvey et al., 2004). CB was first identified as a GPN interacting protein (Kins et al., 2000). Indeed, the GPN E domain directly binds CB's double homology domain, and co-expression of CB with GPN in heterologous cells causes the translocation of GPN clusters to the PM (Kins et al., 2000; Grosskreutz et al., 2001). CB knock-out mice show a loss of GPN clustering at inhibitory synapses in certain brain regions, such as the hippocampus, suggesting that CB plays a role in postsynaptic GPN clustering at a subset of inhibitory synapses (Papadopoulos et al., 2007, 2008).

Recent evidence showed that CB also directly interacts with certain $GABA_AR$ subtypes. Yeast tri-hybrid screens revealed that the $GABA_AR$ α2 subunit interacts with the CB SH3 domain, and in fact the GPN/CB interaction is strengthened by the addition of α2, suggesting that these three proteins can act synergistically (Saiepour et al., 2010). *In vitro* isothermal titration calorimetry showed that the CB SH3 domain preferentially binds the α2 ICD, over either the α1 or α3 ICD, at residues 360–375, suggesting that this ICD motif is integral to $GABA_AR$ subtype-specific protein-protein interactions (Hines et al., 2018). Supporting this hypothesis, knocking the α2 360–375 motif into the α1 subunit in mice leads to increased immunoprecipitation of endogenous CB with the chimeric α1 subunit (Nathanson et al., 2019). This same study also showed an increase in the pull-down of GPN with mutant α1, demonstrating a possible synergistic interaction between CB/GPN/α2 ICD that is overall strengthened when the interaction between two partner proteins is enhanced (Nathanson et al., 2019). The overarching question becomes: does this α2 ICD motif and its preferential protein interactions play a role in subtype-specific synapse formation in the brain, particularly in the construction of α2-enriched synapses at the AIS?

THE AXON INITIAL SEGMENT

At the interface between the somatodendritic and axonal compartments lies the AIS. This discrete region is composed of unique molecular machinery and maintains a barrier between the somatodendritic and axonal environments, sustaining the neuronal anatomical asymmetry necessary for the unidirectional propagation of information (Leterrier, 2018). Morphologically,

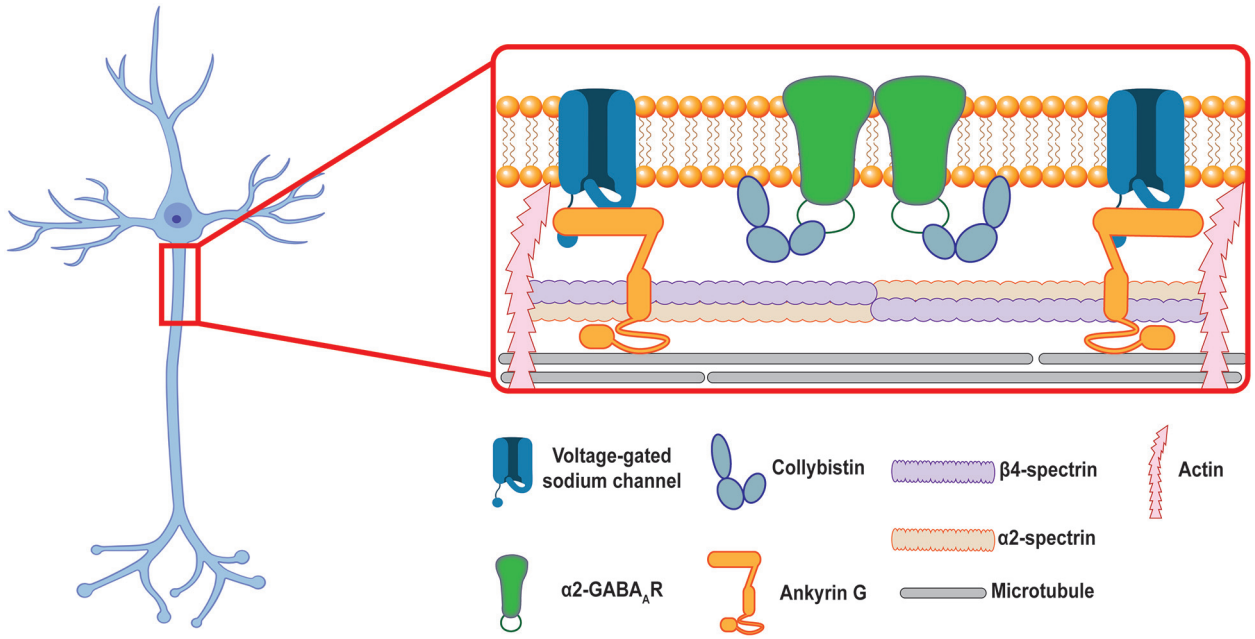

FIGURE 1 | The inhibitory postsynaptic specialization at the axon initial segment (AIS). A cartoon showing a putative model of the postsynaptic inhibitory synapse at the AIS in a hippocampal pyramidal neuron. Ankyrin G and the β4/α2-spectrin tetramer associate to stabilize voltage-gated ion channels and link the periodic domain to the actin and microtubule cytoskeleton. α2-GABA$_A$Rs are enriched at inhibitory synapses at the AIS, where they are selectively stabilized by protein-protein interactions at their intracellular loop domain (ICD). This review proposes that collybistin is a candidate for an AIS selective stabilizer, linking the α2-GABA$_A$R to the AIS plasma membrane (PM).

the AIS displays an electron-dense submembranous granular layer composed of a high density of voltage-gated ion channels and the highly organized, periodic protein scaffold that supports them (Xu et al., 2013). A number of electrophysiological studies established that the AIS is not only a barrier but is also the site of AP generation, as belied by its high resident concentrations of voltage-gated sodium and potassium channels, which are essential for the propagation of APs (Araki and Otani, 1955; Coombs et al., 1957; Fuortes et al., 1957).

Giant Ankyrin G is the key scaffolding protein and master organizer at the AIS; it recruits other essential AIS components, such as βIV-spectrin and voltage-gated ion channels, through either direct or indirect interactions (Zhou et al., 1998; Jenkins and Bennett, 2001; Han et al., 2017). Ankyrin G also interacts with microtubules, anchoring the entire complex in place (Leterrier et al., 2011). The AIS protein scaffold is dense and super-stable, maintaining axonal integrity and serving as a barrier to the entry of inappropriate somatodendritic proteins: the expression and/or stabilization of proteins at the AIS is tightly controlled (Albrecht et al., 2016; Huang and Rasband, 2016).

INHIBITION AT THE AXON INITIAL SEGMENT

To current knowledge, the only ligand-gated ion channels mediating neurotransmission at the AIS are GABA$_A$Rs (Leterrier, 2018). The AIS of certain cell types—pyramidal cells of the forebrain, for instance—contain inhibitory synapses that are exclusively innervated by one type of IN: the chandelier cell (Somogyi et al., 1983; Wang et al., 2016). Given that the AIS is the site of AP firing, any inhibitory signaling in this domain has an outsize effect on neuronal excitability (Zhu et al., 2004; Glickfeld et al., 2009). As previously discussed, α2-GABA$_A$Rs are specifically enriched at the AIS (Nusser et al., 1996; Nyíri et al., 2001); since different GABA$_A$R subtypes have their own kinetics and mediate distinct types of inhibition, it follows that the enrichment of a particular GABA$_A$R subtype in a restricted domain like the AIS would have functional relevance.

To investigate the above hypothesis, mice in which residues 360–375 of the GABA$_A$R α1 subunit have been knocked-in to the α2 subunit (*Gabra*2–1 mice) were generated. This mutation abolished α2's preferential interaction with CB and led to loss of α2+ synapses at the AIS. Strikingly, *Gabra*2–1 animals display postnatal spontaneous seizures; these seizures are often lethal, causing death around postnatal day 20 (Hines et al., 2018). These data demonstrate that the localization of α2-GABA$_A$Rs to the AIS is essential to inhibitory control of pathological excitation.

INHIBITORY SYNAPSE FORMATION AT THE AXON INITIAL SEGMENT

Clearly then, inhibition at the AIS is integral to maintaining the dynamic balance between inhibition and excitation.

However, the manner in which $GABA_AR$ subtype-specific axo-axonic synapses are constructed and maintained remains unclear. Although α2-$GABA_ARs$ are enriched at the AIS, live imaging of α1- and α2-$GABA_ARs$ coupled to quantum dots showed that both subtypes can enter the AIS: the AIS diffusion barrier does not seem to select for α2-$GABA_ARs$ (Muir and Kittler, 2014). However, these same studies demonstrated that α2-$GABA_ARs$ were less mobile at the AIS than α1-$GABA_ARs$, indicating that while both subtypes can access the AIS compartment, α2-$GABA_ARs$ are somehow preferentially anchored at there. Given that inhibitory synapse formation in other neuronal compartments has been shown to depend on protein-protein interactions, it stands to reason that synapse formation at the AIS would follow the same principles.

Indeed, GPN is expressed at the AIS, forming co-clusters with α2-$GABA_ARs$ (Panzanelli et al., 2011), although GPN's association with $GABA_ARs$ at the AIS is relatively weaker than its association with $GABA_ARs$ at the soma and dendrites (Gao and Heldt, 2016), suggesting that another protein present in the AIS multimolecular scaffold could play a more important role. CB is also present at AIS inhibitory synapses in cortical and hippocampal neurons (Panzanelli et al., 2011), and its specific interactions with α2-$GABA_ARs$ provide a putative model for inhibitory synapse formation at the AIS: removing the 360–375 motif from the α2 subunit ICD prevents the accumulation of α2-$GABA_ARs$ at axo-axonic synapses, suggesting that this motif, and the preferential protein interactions it mediates—such as that with CB—is *necessary* for $GABA_AR$ stabilization at the AIS (Hines et al., 2018). Experiments performed in another mutant mouse, in which residues 360–375 of the α2 subunit are knocked-in to the α1 subunit (the *Gabra*1–2 mouse), increases the affinity of the α1 subunit for CB and leads to an increase in α1-$GABA_ARs$ expression at axo-axonic synapses. These data show that residues 360–375 of the α2 subunit are *sufficient* for $GABA_AR$ stabilization at the AIS (Nathanson et al., 2019). Given that CB has a relatively stronger association with the α2 360–375 motif and is present at the AIS, it stands to reason that CB interactions selectively stabilize α2-$GABA_ARs$ at inhibitory AIS synapses.

Together, these data provide a potential model for axo-axonic synapse formation: after $GABA_ARs$ are inserted into the extrasynaptic PM at the AIS those receptors that contain the α2 ICD motif are able to bind intracellular scaffolding proteins, such as CB, to form stable complexes that anchor the receptor at axo-axonic synapses. Receptors that do not contain the α2 ICD motif are not stabilized at synapses and diffuse back into the extrasynaptic space (see **Figure 1**). Other proteins in the AIS scaffold, especially Ankyrin G, might also play a role in the selective stabilization of $GABA_ARs$ at the AIS. Future experiments utilizing the *Gabra*1–2 and *Gabra*2–1 mice could provide more information about the importance of these proteins in $GABA_AR$ stabilization. In addition, the above model only describes the *postsynaptic* side of inhibitory synapse formation. Additional mechanisms regulate the formation of presynaptic chandelier cell boutons apposing the AIS. Most recently, a transsynaptic mechanism was described: the cell adhesion molecule L1CAM, localized to the AIS of neocortical pyramidal neurons, was found to be necessary for the targeting of chandelier cell boutons to the AIS (Tai et al., 2019). Although the presynaptic interactor of L1CAM remains unidentified, such transsynaptic interactions provide an intriguing path for future research into synapse formation at the AIS.

CONCLUSIONS

Despite the progress made in understanding the formation of inhibitory synapses, little is known about how neurons direct $GABA_AR$ subtype-specific synapse formation. This subtype specificity is important for the maintenance of neuronal excitability. α2-$GABA_AR$-enriched synapse formation at the AIS is an especially intriguing case, as AIS inhibition is essential for normal brain function. Better understanding axo-axonic synapse formation will not only shed light on the molecular mechanisms of subtype-specific inhibitory synapse formation but may also provide new avenues of research into treatment for neurological disorders like epilepsy, which result from pathological hyperexcitability. It appears that protein-protein interactions between the ICD of $GABA_AR$ subunits and intracellular scaffolding proteins at inhibitory synapses play an important role in this process. The make-up of the inhibitory synaptic scaffold is variable depending on cell type and subcellular domain, making such interactions good candidates for synapse-specific $GABA_AR$ subtype enrichment. Further research will need to be done to fully explore the "interactome" of each $GABA_AR$ subtype and the importance of each interaction at the many different types of synapses present in even one neuron.

AUTHOR CONTRIBUTIONS

AN wrote the manuscript, with input from PD and SM.

FUNDING

This work was supported by funding from the National Institute of Mental Health (NIMH) grant MH097466.

REFERENCES

Albrecht, D., Winterflood, C. M., Sadeghi, M., Tschager, T., Noé, F., and Ewers, H. (2016). Nanoscopic compartmentalization of membrane protein motion at the axon initial segment. *J. Cell Biol.* 215, 37–46. doi: 10.1083/jcb.201603108

Araki, T., and Otani, T. (1955). Response of single motoneurons to direct stimulation in toad's spinal cord. *J. Neurophysiol.* 18, 472–485. doi: 10.1152/jn.1955.18.5.472

Arancibia-Cárcamo, I. L., and Kittler, J. T. (2009). Regulation of $GABA_A$ receptor membrane trafficking and synaptic localization. *Pharmacol. Ther.* 123, 17–31. doi: 10.1016/j.pharmthera.2009.03.012

Baumann, S. W., Baur, R., and Sigel, E. (2003). Individual properties of the two functional agonist sites in $GABA_A$ receptors. *J. Neurosci.* 23, 11158–11166. doi: 10.1523/JNEUROSCI.23-35-11158.2003

Blaesse, P., Airaksinen, M. S., Rivera, C., and Kaila, K. (2009). Cation-chloride cotransporters and neuronal function. *Neuron* 61, 820–838. doi: 10.1016/j.neuron.2009.03.003

Bogdanov, Y., Michels, G., Armstrong-Gold, C., Haydon, P. G., Lindstrom, J., Pangalos, M., et al. (2006). Synaptic $GABA_A$ receptors are directly recruited from their extrasynaptic counterparts. *EMBO J.* 25, 4381–4389. doi: 10.1038/sj.emboj.7601309

Bormann, J., Hamill, O. P., and Sakmann, B. (1987). Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured spinal neurones. *J. Physiol.* 385, 243–286. doi: 10.1113/jphysiol.1987.sp016493

Busch, C., and Sakmann, B. (1990). Synaptic transmission in hippocampal neurons: numerical reconstruction of quantal IPSCs. *Cold Spring Harb. Symp. Quant. Biol.* 55, 69–80. doi: 10.1101/sqb.1990.055.01.009

Connolly, C. N., Krishek, B. J., McDonald, B. J., Smart, T. G., and Moss, S. J. (1996). Assembly and cell surface expression of heteromeric and homomeric γ-aminobutyric acid type A receptors. *J. Biol. Chem.* 271, 89–96. doi: 10.1074/jbc.271.1.89

Connolly, C. N., and Wafford, K. A. (2004). The Cys-Loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. *Biochem. Soc. Trans.* 32, 529–534. doi: 10.1042/bst0320529

Coombs, J. S., Curtis, D. R., and Eccles, J. C. (1957). The generation of impulses in motoneurones. *J. Physiol.* 139, 232–249. doi: 10.1113/jphysiol.1957.sp005888

Fatima-Shad, K., and Barry, P. H. (1993). Anion permeation in GABA- and glycine-gated channels of mammalian cultured hippocampal neurons. *Proc. R. Soc. Lond. B Biol. Sci.* 253, 69–75. doi: 10.1098/rspb.1993.0083

Feng, G., Tintrup, H., Kirsch, J., Nichol, M. C., Kuhse, J., Betz, H., et al. (1998). Dual-requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. *Science* 282, 1321–1324. doi: 10.1126/science.282.5392.1321

Fritschy, J.-M. (2008). Epilepsy, E/I balance and $GABA_A$ receptor plasticity. *Front. Mol. Neurosci.* 1:5. doi: 10.3389/neuro.02.005.2008

Fischer, F., Kneussel, M., Tintrup, H., Haverkamp, S., Rauen, T., Betz, H., et al. (2000). Reduced synaptic clustering of GABA and glycine receptors in the retina of the gephyrin null mutant mouse. *J. Comp. Neurol.* 427, 634–648. doi: 10.1002/1096-9861(20001127)427:4<634::aid-cne10>3.0.co;2-x

Fuortes, M. G. F., Frank, K., and Becker, M. C. (1957). Steps in the production of motoneuron spikes. *J. Gen. Physiol.* 40, 735–752. doi: 10.1085/jgp.40.5.735

Gao, Y., and Heldt, S. A. (2016). Enrichment of $GABA_A$ receptor α-subunits on the axonal initial segment shows regional differences. *Front. Cell. Neurosci.* 10:39. doi: 10.3389/fncel.2016.00039

Glickfeld, L. L., Roberts, J., Somogyi, P., and Scanziani, M. (2009). Interneurons hyperpolarize pyramidal cells along their entire somatodendritic axis. *Nat. Neurosci.* 12, 21–23. doi: 10.1038/nn.2230

Goldstein, P. A., Elsen, F. P., Ying, S.-W., Ferguson, C., Homanics, G. E., and Harrison, N. L. (2002). Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the $GABA_A$ receptor α1 subunit. *J. Neurophysiol.* 88, 3208–3217. doi: 10.1152/jn.00885.2001

Gorrie, G. H., Vallis, Y., Stephenson, A., Whitfield, J., Browning, B., Smart, T. G., et al. (1997). Assembly of $GABA_A$ receptors composed of A1 and B2 subunits in both cultured neurons and fibroblasts. *J. Neurosci.* 17, 6587–6596. doi: 10.1523/JNEUROSCI.17-17-06587.1997

Grosskreutz, Y., Hermann, A., Kins, S., Fuhrmann, J. C., Betz, H., and Kneussel, M. (2001). Identification of a gephyrin-binding motif in the GDP/GTP exchange factor collybistin. *Biol. Chem.* 382, 1455–1462. doi: 10.1515/bc.2001.179

Han, B., Zhou, R., Xia, C., and Zhuang, X. (2017). Structural organization of the actin-spectrin-based membrane skeleton in dendrites and soma of neurons. *Proc. Natl. Acad. Sci. U S A* 114, E6678–E6685. doi: 10.1073/pnas.1705043114

Hannan, S., Minere, M., Harris, J., Izquierdo, P., Thomas, P., Tench, B., et al. (2019). $GABA_A$R isoform and subunit structural motifs determine synaptic and extrasynaptic receptor localisation. *Neuropharmacology* doi: 10.1016/j.neuropharm.2019.02.022 [Epub ahead of print].

Harvey, K., Duguid, I. C., Alldred, M. J., Beatty, S. E., Ward, H., Keep, N. H., et al. (2004). The GDP-GTP exchange factor collybistin: an essential determinant of neuronal gephyrin clustering. *J. Neurosci.* 24, 5816–5826. doi: 10.1523/JNEUROSCI.1184-04.2004

Hines, R. M., Maric, H. M., Hines, D. J., Modgil, A., Panzanelli, P., Nakamura, Y., et al. (2018). Developmental seizures and mortality result from reducing $GABA_A$ receptor A2-subunit interaction with collybistin. *Nat. Commun.* 9:3130. doi: 10.1038/s41467-018-05481-1

Huang, Y.-M., and Rasband, M. N. (2016). Organization of the axon initial segment: actin like a fence. *J. Cell Biol.* 215, 9–11. doi: 10.1083/jcb.201609084

Jenkins, S. M., and Bennett, V. (2001). Ankyrin-G coordinates assembly of the spectrin-based membrane skeleton, voltage-gated sodium channels, and L1 CAMs at purkinje neuron initial segments. *J. Cell Biol.* 155, 739–746. doi: 10.1083/jcb.200109026

Kins, S., Betz, H., and Kirsch, J. (2000). Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. *Nat. Neurosci.* 3, 22–29. doi: 10.1038/71096

Kittler, J. T., McAinsh, K., and Moss, S. J. (2002). Mechanisms of $GABA_A$ receptor assembly and trafficking: implications for the modulation of inhibitory neurotransmission. *Mol. Neurobiol.* 26, 251–268. doi: 10.1385/mn:26:2-3:251

Kneussel, M., Brandstätter, J. H., Laube, B., Stahl, S., Müller, U., and Betz, H. (1999). Loss of postsynaptic $GABA_A$ receptor clustering in gephyrin-deficient mice. *J. Neurosci.* 19, 9289–9297. doi: 10.1523/JNEUROSCI.19-21-09289.1999

Leterrier, C. (2018). The axon initial segment: an updated viewpoint. *J. Neurosci.* 38, 2135–2145. doi: 10.1523/JNEUROSCI.1922-17.2018

Leterrier, C., Vacher, H., Fache, M.-P., d'Angles, S. A., Castets, F., Autillo-Touati, A., et al. (2011). End-binding proteins EB3 and EB1 link microtubules to ankyrin G in the axon initial segment. *Proc. Natl. Acad. Sci. U S A* 108, 8826–8831. doi: 10.1073/pnas.1018671108

Lorenz-Guertin, J. M., and Jacob, T. C. (2018). GABA type a receptor trafficking and the architecture of synaptic inhibition. *Dev. Neurobiol.* 78, 238–270. doi: 10.1002/dneu.22536

Miller, P. S., and Aricescu, A. R. (2014). Crystal structure of a human $GABA_A$ receptor. *Nature* 512, 270–275. doi: 10.1038/nature13293

Moss, S. J., Doherty, C. A., and Huganir, R. L. (1992). Identification of the CAMP-dependent protein kinase and protein kinase C phosphorylation sites within the major intracellular domains of the β 1, γ 2S, and γ 2L subunits of the γ-aminobutyric acid type A receptor. *J. Biol. Chem.* 267, 14470–14476.

Moss, S. J., and Smart, T. G. (2001). Constructing inhibitory synapses. *Nat. Rev. Neurosci.* 2, 240–250. doi: 10.1038/35067500

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

Nathanson, A. J., Zhang, Y., Smalley, J. L., Ollerhead, T. A., Rodriguez Santos, M. A., Andrews, P. M., et al. (2019). Identification of a core amino acid motif within the α subunit of $GABA_A$Rs that promotes inhibitory synaptogenesis and resilience to seizures. *Cell Rep.* 28, 670.e8–681.e8. doi: 10.1016/j.celrep.2019.06.014

Nusser, Z., Sieghart, W., Benke, D., Fritschy, J. M., and Somogyi, P. (1996). Differential synaptic localization of two major γ-aminobutyric acid type A receptor α subunits on hippocampal pyramidal cells. *Proc. Natl. Acad. Sci. U S A* 93, 11939–11944. doi: 10.1073/pnas.93.21.11939

Nyíri, G., Freund, T. F., and Somogyi, P. (2001). Input-dependent synaptic targeting of A2-subunit-containing $GABA_A$ receptors in synapses of hippocampal pyramidal cells of the rat. *Eur. J. Neurosci.* 13, 428–442. doi: 10.1046/j.1460-9568.2001.01407.x

Nymann-Andersen, J., Sawyer, G. W., and Olsen, R. W. (2002). Interaction between $GABA_A$ receptor subunit intracellular loops: implications for higher order complex formation. *J. Neurochem.* 83, 1164–1171. doi: 10.1046/j.1471-4159.2002.01222.x

O'Toole, K. K., and Jenkins, A. (2011). Discrete M3–M4 intracellular loop subdomains control specific aspects of γ-aminobutyric acid type A receptor function. *J. Biol. Chem.* 286, 37990–37999. doi: 10.1074/jbc.M111.258012

Olsen, R. W., and Sieghart, W. (2008). International union of pharmacology. LXX. Subtypes of γ-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol. Rev.* 60, 243–260. doi: 10.1124/pr.108.00505

Panzanelli, P., Gunn, B. G., Schlatter, M. C., Benke, D., Tyagarajan, S. K., Scheiffele, P., et al. (2011). Distinct mechanisms regulate $GABA_A$ receptor

and gephyrin clustering at perisomatic and axo-axonic synapses on CA1 pyramidal cells. *J. Physiol.* 589, 4959–4980. doi: 10.1113/jphysiol.2011.216028

Papadopoulos, T., Eulenburg, V., Reddy-Alla, S., Mansuy, I. M., Li, Y., and Betz, H. (2008). Collybistin is required for both the formation and maintenance of GABAergic postsynapses in the hippocampus. *Mol. Cell. Neurosci.* 39, 161–169. doi: 10.1016/j.mcn.2008.06.006

Papadopoulos, T., Korte, M., Eulenburg, V., Kubota, H., Retiounskaia, M., Harvey, R. J., et al. (2007). Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. *EMBO J.* 26, 3888–3899. doi: 10.1038/sj.emboj.7601819

Prior, P., Schmitt, B., Grenningloh, G., Pribilla, I., Multhaup, G., Beyreuther, K., et al. (1992). Primary structure and alternative splice variants of gephyrin, a putative glycine receptor-tubulin linker protein. *Neuron* 8, 1161–1170. doi: 10.1016/0896-6273(92)90136-2

Reid, T., Bathoorn, A., Ahmadian, M. R., and Collard, J. G. (1999). Identification and characterization of HPEM-2, a guanine nucleotide exchange factor specific for Cdc42. *J. Biol. Chem.* 274, 33587–33593. doi: 10.1074/jbc.274.47.33587

Roux, L., and Buzsáki, G. (2015). Tasks for inhibitory interneurons in intact brain circuits. *Neuropharmacology* 88, 10–23. doi: 10.1016/j.neuropharm.2014.09.011

Saiepour, L., Fuchs, C., Patrizi, A., Sassoè-Pognetto, M., Harvey, R. J., and Harvey, K. (2010). Complex role of collybistin and gephyrin in $GABA_A$ receptor clustering. *J. Biol. Chem.* 285, 29623–29631. doi: 10.1074/jbc.m110.121368

Saiyed, T., Paarmann, I., Schmitt, B., Haeger, S., Sola, M., Schmalzing, G., et al. (2007). Molecular basis of gephyrin clustering at inhibitory synapses: role of G- and E-domain interactions. *J. Biol. Chem.* 282, 5625–5632. doi: 10.1074/jbc.m610290200

Saliba, R. S., Michels, G., Jacob, T. C., Pangalos, M. N., and Moss, S. J. (2007). Activity-dependent ubiquitination of $GABA_A$ receptors regulates their accumulation at synaptic sites. *J. Neurosci.* 27, 13341–13351. doi: 10.1523/JNEUROSCI.3277-07.2007

Sassoè-Pognetto, M., Panzanelli, P., Sieghart, W., and Fritschy, J. M. (2000). Colocalization of multiple $GABA_A$ receptor subtypes with gephyrin at postsynaptic sites. *J. Comp. Neurol.* 420, 481–498. doi: 10.1002/(sici)1096-9861(20000515)420:4<481::aid-cne6>3.0.co;2-5

Schofield, P. R., Darlison, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., et al. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature* 328, 221–227. doi: 10.1038/328221a0

Schwarz, G., Schrader, N., Mendel, R. R., Hecht, H. J., and Schindelin, H. (2001). Crystal structures of human gephyrin and plant Cnx1 G domains: comparative analysis and functional implications. *J. Mol. Biol.* 312, 405–418. doi: 10.2210/pdb1eav/pdb

Somogyi, P., Nunzi, M. G., Gorio, A., and Smith, A. D. (1983). A new type of specific interneuron in the monkey hippocampus forming synapses exclusively with the axon initial segments of pyramidal cells. *Brain Res.* 259, 137–142. doi: 10.1016/0006-8993(83)91076-4

Tai, Y., Gallo, N. B., Wang, M., Yu, J.-R., and Van Aelst, L. (2019). Axo-axonic innervation of neocortical pyramidal neurons by GABAergic chandelier cells requires AnkyrinG-associated L1CAM. *Neuron* 102, 358.e9–372.e9. doi: 10.1016/j.neuron.2019.02.009

Thomas, P., Mortensen, M., Hosie, A. M., and Smart, T. G. (2005). Dynamic mobility of functional $GABA_A$ receptors at inhibitory synapses. *Nat. Neurosci.* 8, 889–897. doi: 10.1038/nn1483

Tyagarajan, S. K., and Fritschy, J.-M. (2014). Gephyrin: a master regulator of neuronal function? *Nat. Rev. Neurosci.* 15, 141–156. doi: 10.1038/nrn3670

Vithlani, M., Terunuma, M., and Moss, S. J. (2011). The dynamic modulation of $GABA_A$ receptor trafficking and its role in regulating the plasticity of inhibitory synapses. *Physiol. Rev.* 91, 1009–1022. doi: 10.1152/physrev.00015.2010

Wang, Y., Zhang, P., and Wyskiel, D. R. (2016). Chandelier cells in functional and dysfunctional neural circuits. *Front. Neural Circuits* 10:33. doi: 10.3389/fncir.2016.00033

Wisden, W., Laurie, D. J., Monyer, H., and Seeburg, P. H. (1992). The distribution of 13 $GABA_A$ receptor subunit MRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.* 12, 1040–1062. doi: 10.1523/JNEUROSCI.12-03-01040.1992

Xu, K., Zhong, G., and Zhuang, X. (2013). Actin, spectrin, and associated proteins form a periodic cytoskeletal structure in axons. *Science* 339, 452–456. doi: 10.1126/science.1232251

Zhou, D., Lambert, S., Malen, P. L., Carpenter, S., Boland, L. M., and Bennett, V. (1998). AnkyrinG is required for clustering of voltage-gated na channels at axon initial segments and for normal action potential firing. *J. Cell Biol.* 143, 1295–1304. doi: 10.1083/jcb.143.5.1295

Zhu, Y., Stornetta, R. L., and Zhu, J. J. (2004). Chandelier cells control excessive cortical excitation: characteristics of whisker-evoked synaptic responses of layer 2/3 nonpyramidal and pyramidal neurons. *J. Neurosci.* 24, 5101–5108. doi: 10.1523/JNEUROSCI.0544-04.2004

Conflict of Interest: SM serves as a consultant for AstraZeneca, Bain Capital, and Sage Therapeutics, relationships that are regulated by Tufts University. SM is also a shareholder of SAGE Therapeutics.

The remaining 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.

The handling Editor declared a past co-authorship with one of the authors SM.

frontiers in Molecular Neuroscience

REVIEW
published: 07 November 2019
doi: 10.3389/fnmol.2019.00267

Diverse Neuron Properties and Complex Network Dynamics in the Cerebellar Cortical Inhibitory Circuit

Francesca Prestori[1]*, *Lisa Mapelli*[1] and *Egidio D'Angelo*[1,2]*

[1]Department of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy, [2]IRCCS Mondino Foundation, Pavia, Italy

Neuronal inhibition can be defined as a spatiotemporal restriction or suppression of local microcircuit activity. The importance of inhibition relies in its fundamental role in shaping signal processing in single neurons and neuronal circuits. In this context, the activity of inhibitory interneurons proved the key to endow networks with complex computational and dynamic properties. In the last 50 years, the prevailing view on the functional role of cerebellar cortical inhibitory circuits was that excitatory and inhibitory inputs sum spatially and temporally in order to determine the motor output through Purkinje cells (PCs). Consequently, cerebellar inhibition has traditionally been conceived in terms of restricting or blocking excitation. This assumption has been challenged, in particular in the cerebellar cortex where all neurons except granule cells (and unipolar brush cells in specific lobules) are inhibitory and fire spontaneously at high rates. Recently, a combination of electrophysiological recordings *in vitro* and *in vivo*, imaging, optogenetics and computational modeling, has revealed that inhibitory interneurons play a much more complex role in regulating cerebellar microcircuit functions: inhibition *shapes* neuronal response dynamics in the whole circuit and eventually regulate the PC output. This review elaborates current knowledge on cerebellar inhibitory interneurons [Golgi cells, Lugaro cells (LCs), basket cells (BCs) and stellate cells (SCs)], starting from their ontogenesis and moving up to their morphological, physiological and plastic properties, and integrates this knowledge with that on the more renown granule cells and PCs. We will focus on the circuit loops in which these interneurons are involved and on the way they generate feed-forward, feedback and lateral inhibition along with complex spatio-temporal response dynamics. In this perspective, inhibitory interneurons emerge as the real controllers of cerebellar functioning.

Keywords: cerebellar cortex, inhibitory interneurons, dynamic properties, cellular neurophysiology, synaptic inhibition

OPEN ACCESS

Edited by:
Arianna Maffei,
Stony Brook University, United States

Reviewed by:
Christian Hansel,
University of Chicago, United States
Marylka Yoe Uusisaari,
Okinawa Institute of Science and Technology Graduate University, Japan

***Correspondence:**
Francesca Prestori
francesca.prestori@unipv.it
Egidio D'Angelo
dangelo@unipv.it

Received: *16 August 2019*
Accepted: *17 October 2019*
Published: *07 November 2019*

Citation:
Prestori F, Mapelli L and D'Angelo E (2019) Diverse Neuron Properties and Complex Network Dynamics in the Cerebellar Cortical Inhibitory Circuit. Front. Mol. Neurosci. 12:267. doi: 10.3389/fnmol.2019.00267

CEREBELLAR INTERNEURONS CLASSIFICATION

Circuit Microanatomy

The cerebellar cortex consists of three layers, namely the molecular layer (ML), the Purkinje cell layer (PCL) and the granular layer (GL; **Figure 1**). The primary input systems enter the cerebellum *via* mossy fibers (MFs) and climbing fibers (CFs). Both are excitatory and use glutamate as neurotransmitter. The MF input originates from several nuclei in the brain stem and spinal cord. In the GL, MFs make excitatory synapses onto granule cells,

whose axons rise vertically to the ML, where they divide to form T-shaped branches called parallel fibers (PFs; Pijpers et al., 2006; Oberdick and Sillitoe, 2011). Each PF makes excitatory contacts with hundreds of Purkinje cells (PCs) that, in turn, make inhibitory synapses onto deep cerebellar nuclei (DCN) neurons. At the same time, DCN sends excitatory and inhibitory fibers to the cerebellar cortex generating a positive internal feedback (Ankri et al., 2015; Gao et al., 2016). The PCs provide the only output of the cerebellar cortex. Since PCs are GABAergic, the control exerted on DCN neurons is inhibitory. In addition, DCN neurons receive excitatory synaptic contacts from mossy and CFs collaterals. The CF input originates from the inferior olive (IO). Each PC receives a strong excitatory input *via* a single CF (Ito, 2013; Ito et al., 2014). The inhibitory control exerted by PCs on DCN neurons can be powerfully modulated by local inhibitory circuits formed by basket and stellate cells (SCs). These latter receive excitatory synapses from PFs and inhibitory synapses from PC axon collaterals (Crook et al., 2007; Witter et al., 2016). Basket cells (BCs) are found in the deep ML and provide a powerful inhibitory input to PC bodies and axonal initial segments. SCs are located in the upper ML and make synaptic contacts on PC dendrites, determining a weaker inhibitory influence since they contact the PCs more distally compared to BCs. In the GL, there are two types of interneurons, characterized by a mixed glycinergic/GABAergic phenotype, which do not directly regulate the efferent activity of PCs: Lugaro cells (LCs) and Golgi cells. The LCs are located just beneath the PCL and are the primary target of serotonin released from extracerebellar fibers (Lainé and Axelrad, 1998). Their axons contact basket and SC soma and dendrites in the ML and, through collaterals, form a major input to Golgi cells (Dieudonné and Dumoulin, 2000). In addition, LC soma and dendrites appear to be densely innervated by PC axon collaterals (Lainé and Axelrad, 2002; Crook et al., 2007; Witter et al., 2016). Golgi cell bodies lay in the GL. They receive a double excitatory input: on the basal dendrites from MFs and ascending granule

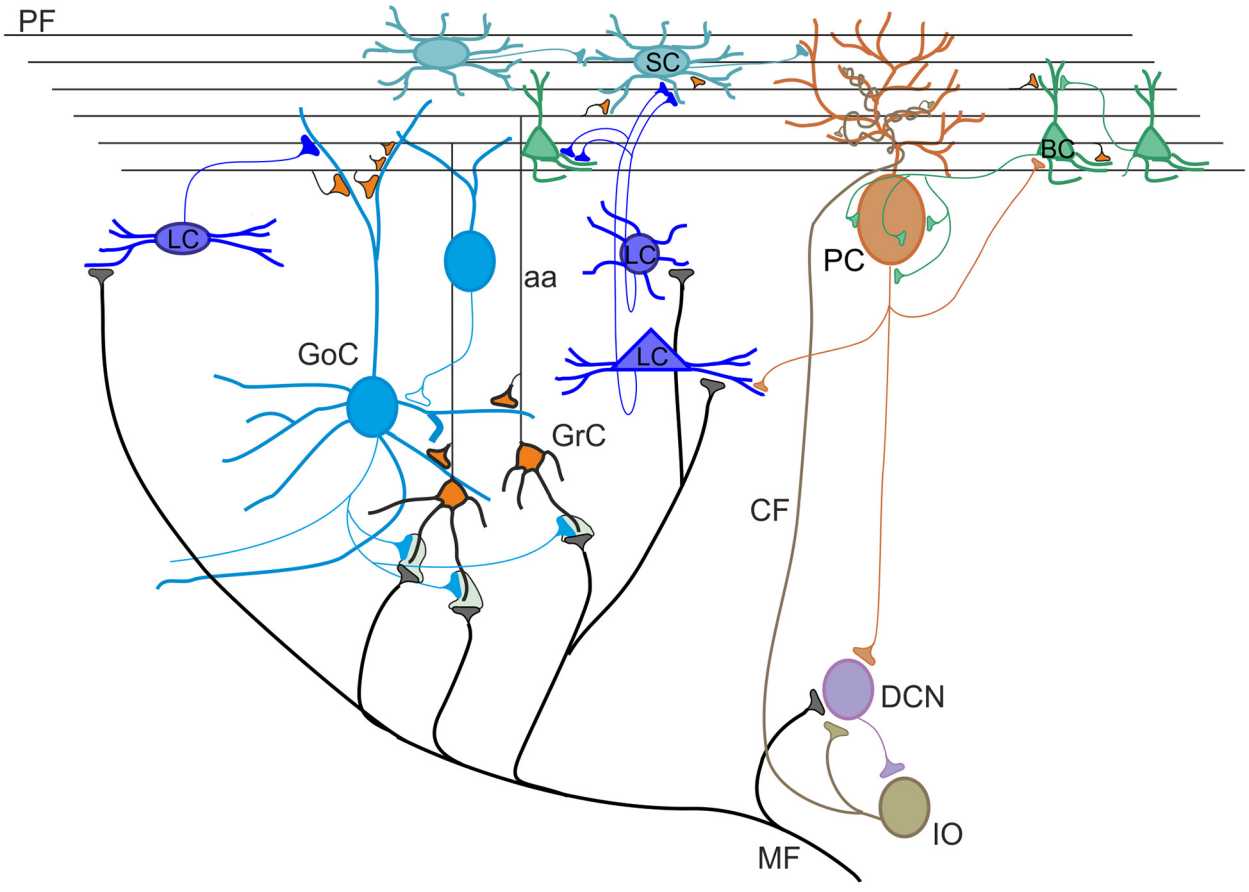

FIGURE 1 | Schematic view of the cerebellar circuit. All cells in the cerebellar cortex are inhibitory except granule cells and unipolar brush cells (not shown). The cerebellar cortex receives two excitatory inputs from mossy fibers (MF) originating in various brain stem and spinal cord nuclei and from climbing fibers (CF) originating from the inferior olive (IO). Mossy fibers contact the granular layer [GL; containing granule cells (GrC), Golgi cells (GoC) and Lugaro cells (LC)] and the deep cerebellar nuclei (DCN). Climbing fibers contact Purkinje cells (PC) and DCN. The ascending axon (aa) of the GrC bifurcates in the molecular layer (ML) forming the parallel fibers (PF), which synapse onto PCs and ML interneurons [stellate cells (SCs) and BCs]. The only output of the cortex is provided by PCs, which project to the DCN. The activity of PCs is under inhibitory control by SC and BC. SC and BC mutually inhibit each other and are coupled through gap junctions. Modified from D'Angelo et al. (2016).

cell axons, and on the apical dendrites from PFs (Chan-Palay et al., 1977; Dieudonné, 1998; Vos et al., 1999). Recently, several lines of evidence for functional gap junctions and chemical synapses among Golgi cells were provided (Dugué et al., 2009; Vervaeke et al., 2010; Hull and Regehr, 2012; Eyre and Nusser, 2016; Szoboszlay et al., 2016). Golgi cell axon occupies the GL and inhibits, in turn, granule cell dendrites (Hámori and Szentágothai, 1966). Lastly, the candelabrum cells, first described in 1994 by Lainé and Axelrad (1994) in the rat, are located within the PCL. They have one or two thick dendrites, dividing into few branches, which run almost vertically into the ML, and several short dendrites which spread for a short distance into the granule cell layer. The connectivity and the function of candelabrum cells have not been investigated yet, though their dendritic structure suggests that PFs and CFs might provide afferent inputs. Current evidences indicate that these cells use GABA and glycine as transmitters (Flace et al., 2004; Tanaka and Ezure, 2004; Crook et al., 2006).

Embryological Origin and Development

All cerebellar neurons arise from two primary germinal epithelia: the ventricular zone (VZ) gives origin to GABAergic neurons, whereas the rhombic lip (RL) generates glutamatergic types (Altman and Bayer, 1997; **Figure 2**). GABAergic DCN neurons are produced first, followed by PCs. Within the VZ, these projection neurons proliferate and acquire specific mature phenotypes through cell-autonomous programs (Florio et al., 2012). By contrast, cortical interneurons, including basket, stellate, Golgi and LCs, derive from precursors that continue their neurogenic activity in a secondary germinative zone i.e., the prospective white matter (pWM) or the deep white matter (dWM), where they differentiate in mature identities under the influence of specific extracellular signals (Leto et al., 2006, 2009, 2012; Leto and Rossi, 2012). In regard to gene expression, the basic helix-loop-helix (bHLH) transcription factor *Ptf1a* is crucial for the initial specification of the GABAergic lineage, ensuring the appropriate cell number production and their subsequent survival (Hoshino et al., 2005; Hoshino, 2006; Pascual et al., 2007; Dennis et al., 2019). Recent studies have shown that, in *Ptf1a* KO mice, GABAergic interneurons adopt a glutamatergic fate, characteristic of external granular layer (EGL) cell precursors (Glasgow et al., 2005; Pascual et al., 2007; Hori et al., 2008), indicating that *Ptf1a* is also necessary for the suppression of the granule cell phenotype in VZ-derived progenitors. While it is clear that *Ptf1a* expression directly specifies GABAergic or glutamatergic neural fate, it has not yet been understood whether all GABAergic types are actually generated from a single pool of VZ progenitors. Several reports have established that the VZ includes different microdomains, characterized by specific gene expression profiles, which are believed to be the origin of different populations of GABAergic neurons (Chizhikov et al., 2006; Sillitoe and Joyner, 2007; Zordan et al., 2008; Lundell et al., 2009; Sudarov et al., 2011). Among the VZ microdomains, some are known to give rise to different classes of PCs, while others are associated with the generation of interneurons (Zordan et al., 2008; Lundell et al., 2009). To date, the only cerebellar GABAergic interneuron-specific marker is *Pax-2* (Maricich and Herrup, 1999; Weisheit et al., 2006). The first *Pax-2*-positive cells appear at E12.5, continue to proliferate up to the birth and then originate major types of GABAergic interneurons through a precise inside-out sequence (Zhang and Goldman, 1996a,b; Altman and Bayer, 1997; Schilling, 2000; Leto et al., 2006): first in the GL (Golgi and LCs), then in the ML (basket and SCs). In the mouse, the generation of 75% of all the interneurons occurs prior to P7 while, in the rat cerebellum, it is completed within the second postnatal week (Weisheit et al., 2006; Leto et al., 2008). The mechanism by which *Pax-2* regulates the GABAergic fate of cerebellar neurons is not completely clarified. *Ptf1a*-positive progenitors promote distinct genetic cascades to express other proneural genes (Zordan et al., 2008; Consalez and Hawkes, 2012; Dastjerdi et al., 2012) which are suitable candidates to operate as determinants of GABAergic identity. Indeed, VZ cells are characterized by the expression of *neurogenin-1* (*Ngn-1*), *neurogenin-2* (*Ngn-2*), and *Ascl-1* genes (Zordan et al., 2008). Precursors expressing *Ngn-1* give rise to PCs (Kim et al., 2008; Lundell et al., 2009; Leto and Rossi, 2012) while only a defined subset of PCs and GABAergic DCN neurons derive from *Ngn-2*-positive progenitors (Florio et al., 2012). Finally, all GABAergic interneurons of cerebellar cortex derive from *Ascl-1* precursors (Kim et al., 2008; Grimaldi et al., 2009; Sudarov et al., 2011). During development, a four-layered organization is evident in the cerebellar cortex: the EGL, the ML, the PCL and

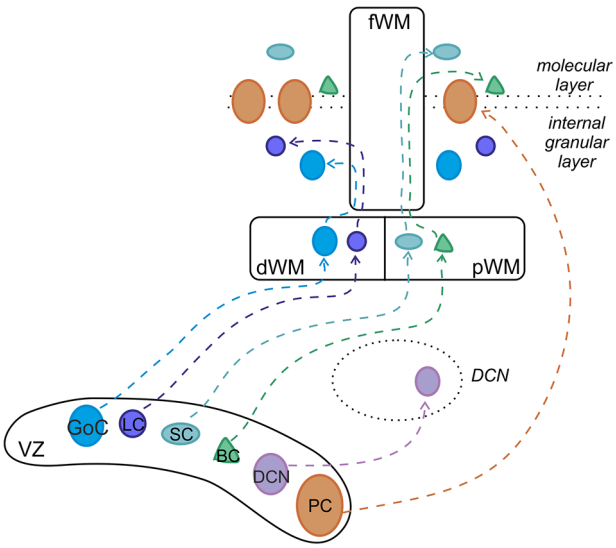

FIGURE 2 | Developmental origin of cerebellar GABAergic neurons. *Ptf1-a*-positive progenitors in the ventricular zone (VZ) generate the entire repertory of GABAergic projection neurons and interneurons through different neurogenic strategies. While projection neurons proliferate and become specified within the VZ, cortical interneurons derive from precursors that originate in the VZ but continue their neurogenic activity in the pWM or dWM, where they acquire mature identities under the influence of specific extracellular cues. Afterward, the interneurons move to their final destination through the folial white matter (fWM). pWM, prospective white matter; dWM, deep white matter; fWM, folial white matter; DCN, deep cerebellar nuclei; PC, Purkinje cell; BC, basket cell; SC, stellate cell; LC, Lugaro cell; GoC, Golgi cell. Modified from Leto et al. (2012).

the internal granular layer (IGL; Sillitoe and Joyner, 2007). By the third postnatal week, the EGL completely disappears and the IGL becomes the GL (Zhang and Goldman, 1996a; Maricich and Herrup, 1999). From VZ, Golgi cells progenitors migrate to reach the dWM while continuing to undergo cell division. Afterward, through the folial white matter (fWM), they move to their final destination within the IGL until around P4 (Zhang and Goldman, 1996a; Maricich and Herrup, 1999; Weisheit et al., 2006; Galas et al., 2017). Additionally, a specific population of Golgi cells, indentified as *Zac1*-positive, are derived from EGL overlying posterior lobules IX and X. Compared to Golgi cells migrating from VZ, they show a different birthdate time window. LCs derive from progenitors in the dWM, and move through the fWM to reach their final location at the top of the IGL (Galas et al., 2017). At this time, there are contradictory results concerning birthdate time and differentiation of LCs. In rats, mature LCs were suggested to appear towards the end of the second postnatal week (Altman, 1972; Altman and Bayer, 1997) but Lainé et al. (1992) have shown that LCs differentiate at a much earlier age (around P5) suggesting a postnatal migration to the IGL. Basket and SCs migrate from VZ to pWM and postnatally through the fWM while continuing to divide until the second postnatal week (Zhang and Goldman, 1996a; Carletti and Rossi, 2008; Cameron et al., 2009; Galas et al., 2017; Wefers et al., 2018). Subsequently, they move radially to accumulate at the inner border of EGL and then tangentially to reach their final location within the ML.

Localization and Distribution of Neurochemical Markers

Since the 1980s, with the advent of immunohistochemical strategies for identifying cell types, researcher had begun correlating cell-specific neurochemical marker expression (typically Ca^{2+}-binding proteins, neuropeptides and certain receptors) with morphological and electrophysiological characterization in order to distinguish several subtypes of GABAergic interneurons (Kubota et al., 1993, 2011; Kubota and Kawaguchi, 1994, 1997; Cauli et al., 2014). Recent advances suggest that neurons expressing an unidentified cytoplasmic antigen Rat-303 (Hockfield, 1987; Hockberger et al., 1994; Geurts et al., 2001), the metabotropic glutamate and serotonin receptors (Neki et al., 1996; Geurts et al., 2001, 2002; Simat et al., 2007; Sillitoe et al., 2008), the neuropeptide somatostatin (SOM; Johansson et al., 1984; Geurts et al., 2001; Galas et al., 2017) and Ca^{2+}-binding proteins such as parvalbumin and calretinin, respectively (PRV and CRT; Schneeberger et al., 1985; Rogers, 1989; Geurts et al., 2001; Schwaller et al., 2002; Bastianelli, 2003; Pibiri et al., 2017), account for nearly 100% of cerebellar GABAergic interneurons. Rat-303 antibody selectively stains Golgi cells and LCs (Hockfield, 1987; Rogers, 1989; Dieudonné and Dumoulin, 2000; Geurts et al., 2001, 2002). Large Rat-303-positive cells displaying also mGluR2, 5-HT2A/5HT5A, SOM and neurogranin immunostaining were identified as Golgi cells based on their location and morphology (typically spherical soma with fan-shaped dendritic arborization located in the depth of the GL). Conversely, large cells, located just underneath the PCL and characterized by a fusiform soma, displayed both Rat-303 and CRT-immunoreactivity. These latter were identified as LCs. However, Rat-303 staining in LC was less pronounced that in Golgi cells (Geurts et al., 2001). Moreover, LCs have been shown to be immunopositive for mGluR1α and mGluR5 in rat (Baude et al., 1993; Hámori et al., 1996; Négyessy et al., 1997; Víg et al., 2003), respectively. Besides LCs, mGluR1α immunoreactivity has been described also for Golgi cells and ML interneurons (Baude et al., 1993; Gorcs et al., 1993; Hámori et al., 1996) while CRT antibody, especially in rat and macaque, could stain Golgi cells, although in much lower numbers (Diño et al., 1999; Geurts et al., 2001). Finally, PRV was found in two subpopulations of GABAergic interneurons within the ML, SCs and BCs (Celio, 1990; Kosaka et al., 1993; Geurts et al., 2002). PRV immunolabeling of BCs revealed the staining of "pinceau" formation, i.e., ramified axons of BCs embracing the axon initial segment (AIS) of PCs. Double immunohistochemistry for PRV and 5HT5A showed 5HT5A immunoreactivity in PRV-positive SCs and BCs (Geurts et al., 2002). Developing of new Cre-driver mouse lines, together with viral vector tools, could provide a very useful support to unravel the complexity of GABAergic interneurons, concurrently contributing to promoting considerable advances in the entire field (Taniguchi et al., 2011; Madisen et al., 2012).

Morphology and Intrinsic Properties

Given the heterogeneity of GABAergic interneurons, it is crucial to convey the diversity into functional specificity (Gupta et al., 2000; Ascoli et al., 2008; DeFelipe et al., 2013; Kepecs and Fishell, 2014; Zeng and Sanes, 2017). In order to better describe the diverse population of interneurons, several parameters defining axonal and dendritic geometry and intrinsic properties have been used in classification studies. Therefore, the morphological and electrophysiological characterization of GABAergic interneurons reviewed here, together with their synaptic connections, is an important step towards understanding information processing in the cerebellum.

Golgi Cells

Golgi cells were first characterized through the pioneering histological studies of Camillo Golgi (Golgi, 1874; Galliano et al., 2010; see **Box 1**). Golgi cells are the large and primary interneurons located throughout the GL. The majority of them use both GABA and glycine as neurotransmitters (80%) but some use specifically GABA (20%) or glycine (5%; Ottersen et al., 1988; Voogd and Glickstein, 1998; Simat et al., 2007). Immunostainings for different neurochemical markers have underlined the heterogeneity of Golgi cells. Surprisingly, neurogranin labeled GABAergic Golgi cells selectively, whereas mGluR2 was expressed in all Golgi cells with a double neurotransmitter profile (Simat et al., 2007). Golgi cells are characterized by round or polygonal soma emitting from 4 to 10 dendrites (Palay and Chan-Palay, 1974). Golgi cell dendrites can be divided into two classes: basal and apical dendrites. Typically, basal dendrites remain into the GL where they ramify

BOX 1 | How the Concept of Cerebellar Inhibition Evolved.

The cerebellum has always been considered as a distinct subdivision of the brain. Aristotle in the 4th century BC wrote: "Behind, right at the back, comes what is termed the cerebellum, differing in form from the brain as we may both feel and see" (Thompson, 1908). Over the years, there was an increasingly accurate description of its structural entity and major subdivisions. By the beginning of the 19th century, the classic anatomical studies were completed and experimental investigation of the cerebellar function began. Three researchers, Rolando (1773–1831), Flourens (1794–1867) and Luciani (1840–1919) helped shaping our understanding of the cerebellum through animal studies. Rolando first demonstrated that, following cerebellar injuries, disturbances of voluntary movements occurred (Rolando, 1809). Flourens observed that cerebellar ablation altered the "harmony of coordinated movements" (Flourens, 1824). Luciani described the three classical symptoms (atonia, asthenia and astasia) of cerebellar diseases (Luciani, 1907). The first description of the functional organization of the cerebellar cortex was proposed in 1906 by the Dutch anatomist Lodewijk Bolk, who divided the cerebellum into four main regions: the anterior lobe, the posterior vermis, and the paired cerebellar hemispheres. He was the first to conceive a functional localization for the coordinating action of the cerebellum in the motor system (Bolk, 1906; Voogd and Koehler, 2018). It was in the later parts of the 20th century that neuronal mechanisms of cerebellar functions were extensively investigated, employing the vestibulo-ocular reflex (VOR) adaptation, eye-blink conditioning and learning in arm movements as experimental paradigms (Ito, 2002), in association with a careful electrophysiological characterization of neurons. The main concepts emerged that *the cerebellar cortex was dominated by inhibitory neurons, whose function was regulated by long-term synaptic plasticity*. The enormous successes reported by these researchers has determined a significant shift in our knowledge of cerebellar cortex circuitry, leading to characterize all inhibitory interneurons and their functional connections and plasticities (for comprehensive reviews, see Hansel et al., 2001; D'Angelo, 2014; Mapelli et al., 2015; D'Angelo et al., 2016; Gao et al., 2016).

It is impossible to discuss the history of cerebellar interneurons research without referring to Camillo Golgi (**Figure 3**) and Santiago Ramon y Cajal. In 1873, Golgi (1843–1926) described two distinct categories of neurons in the granular layer (GL), which were named Golgi Type I and Golgi Type II (Golgi, 1873). It is probable that the first type was the Lugaro Cell [LC; this name derives from the first detailed description of these cells by Lugaro (1894)], whereas, type II corresponded to a neuronal population that later was called by Cajal (1894) the Golgi cells. Still today, the main feature to identify the Golgi cell is the broad extension of its considerable axonal plexus (Dieudonné, 1998; Forti et al., 2006), so well illustrated by Golgi himself. Moreover, he tried to identify a role for these cells: since Golgi cell axonal plexus do not extend beyond the cerebellar cortex, Golgi speculated that they were connectional elements in the network (Golgi, 1873). During the first 60 years of the 20th century, no additional findings were added to clarify Golgi cell physiological function. An important advancement occurred in the 1964 when Eccles (1903–1997) discovered that Golgi cells operate a double feedforward and feedback inhibition of the granule cells (Eccles et al., 1964; Eccles, 1967), contributing to provide the first evidence of a central inhibitory neuron. This result led to the definition of two theories: John Eccles elaborated the Beam Theory (Eccles, 1967, 1973) and Marr (1945–1980) the Motor Learning Theory (Marr, 1969; Albus, 1971). Eccles proposed that Golgi cells, causing a strong inhibition in granule cells, would improve the spatial discrimination of the inputs reaching the cerebellar cortex. Instead, Marr predicted that Golgi cells would be capable to regulate GL excitability and, thus, the amount of information that can be elaborated, transmitted and learned (Marr, 1969). Although both theories were quite appealing and appeared to provide an exhaustive explanation for the whole cerebellum and Golgi cells functions, electrophysiological recordings *in vitro* and *in vivo*, in the 1990s redefined the connectivity of these neurons and their histochemical and functional properties (see below and for review D'Angelo, 2016, 2018). Cajal (1854–1934), by applying Golgi staining to the cerebellum, confirmed the cell types that Golgi had identified and added a detailed morphological characterization of all the elements of the cerebellar cortex, including stellate cells (SCs) and basket cells (BCs) as we know them today (Cajal, 1888). His great contribution was not properly exploited until the 1960s when Rodolfo Llinas characterized and defined the excitatory and inhibitory nature of all synaptic interactions within the cerebellar cortex (Eccles et al., 1966a). He demonstrated that all connectivities in the cerebellar cortex were inhibitory with the exception of the mossy fiber (MF)-granule cell-parallel fiber (PF) system and climbing fiber (CF) input (Eccles et al., 1966b) Moreover, Llinas's experiments showed that the stimulation of PFs excited molecular layer (ML) interneurons and evoked in Purkinje cells (PCs) an early excitatory postsynaptic potential (EPSP) followed by disynaptic and prolonged inhibitory postsynaptic potentials (IPSPs) that were strongly dendritic as well as somatic (Eccles, 1967). These results were against the prevailing dogma that the soma is considered to be the only location for inhibition. Thus, dendritic inhibition started to represent a different view of neuronal integration. It can therefore be safely concluded that *the study of cerebellar inhibitory mechanisms as contributed not just to understand the functional mechanisms of the cerebellum but also inhibition in brain circuits as a whole*.

several times acquiring a characteristic curvy appearance. They receive excitatory inputs from MFs and ascending granule cell axons (Cesana et al., 2013). The initial statement that CF collaterals make synaptic contacts on Golgi cell basal dendrites (Hámori and Szentágothai, 1966, 1980; Sugihara et al., 1999; Shinoda et al., 2000) has not been confirmed (Galliano et al., 2013). Although some electrophysiological studies showed that stimulation of CFs caused depression of Golgi cell firing,

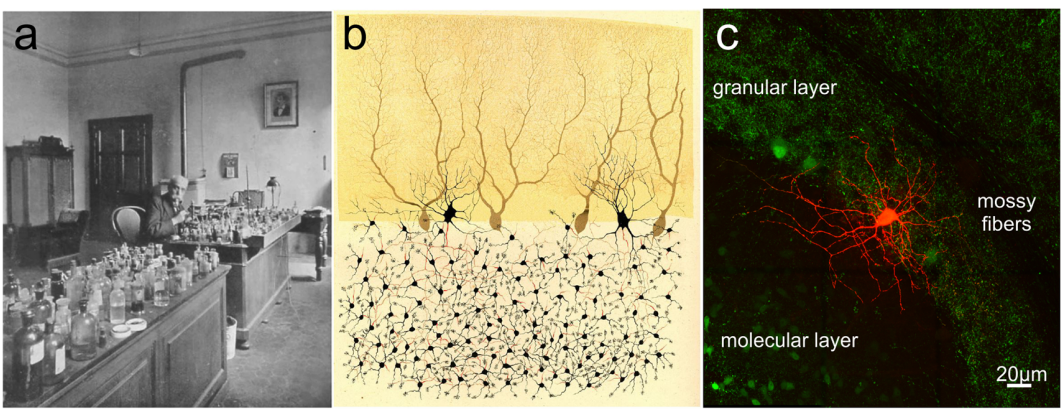

FIGURE 3 | Camillo Golgi and the cerebellar cortex. **(A)** Camillo Golgi in his laboratory at the University of Pavia. **(B)** Illustration by Camillo Golgi of a Golgi impregnated preparation of the cerebellum. Taken from Golgi (1883; available *via* license CC BY 4.0). **(C)** The current high-resolution rendering of a Golgi cell filled with a fluorescent dye and imaged with a two-photon microscope (courtesy of J. DeFelipe).

the nature of this effect still remains to be determined (Schulman and Bloom, 1981; Xu and Edgley, 2008). Apical dendrites, in general stout and straight, ascend towards the ML where they branch extensively forming a distinctive fan-shaped dendritic tree. They receive excitatory synapses *via* PFs of distant granule cells (Chan-Palay et al., 1977; Dieudonné, 1998; Vos et al., 1999; Cesana et al., 2013) and mixed GABA/glycine inputs from LCs (Dumoulin et al., 2001). Pure GABAergic synapses between stellate/BCs and apical Golgi cell dendrites have been also suggested (Palay and Chan-Palay, 1974) but this finding has not been confirmed. Recently, optogenetic studies have shown that axons of stellate/BCs do not functionally innervate Golgi cells and that Golgi cells inhibit each other through reciprocal GABAergic synapses (Hull and Regehr, 2012; Eyre and Nusser, 2016). Finally, apical Golgi cell dendrites are known to form a highly interconnected network using gap junctions endowing Golgi cells with a further level of complexity (Dugué et al., 2009; Vervaeke et al., 2010). Golgi cell axons, composed by very thin beaded fibers, extend profusely into the GL originating widespread neuritic plexi (Dieudonné, 1998; Geurts et al., 2001; Sillitoe et al., 2008). They contribute to the glomerular synapses on granule cell dendrites (Eccles et al., 1966a; Hámori and Szentágothai, 1966; Fox et al., 1967). In the vestibulo-cerebellum, Golgi cell axons make synaptic contact with the unipolar brush cells, in addition to granule cells. Whereas inhibitory postsynaptic responses in granule cells are purely mediated by $GABA_A$ receptors, those in unipolar brush cells display a mixed GABAergic/glycinergic component (Dugué et al., 2005; Rousseau et al., 2012). The physiology of Golgi cells has been extensively explored by electrophysiological recordings *in vitro* and *in vivo*. Patch-clamp recordings *in vitro* have reported that (Dieudonné, 1998; Forti et al., 2006; Solinas et al., 2007a,b; **Figure 4A**):

- Golgi cells are autorhythmic, generating spikes in the range of 1–10 Hz in the absence of synaptic input; occasionally they can be silent during cell-attached recordings.
- they show discharge adaptation during depolarizing current pulses;
- the application of hyperpolarizing current steps determines sagging inward rectification followed by a large rebound depolarization after pulse offset;
- following a sequence of spikes, Golgi cells can reset the phase of their own spontaneous rhythmic firing. After a silent pause lasting exactly as long as the oscillatory period, they restart to discharge;
- Golgi cells are resonant for input frequencies of about 4 Hz.

Rhythmic activity is also observed *in vivo* both in awake and anesthetized animals (Edgley and Lidierth, 1987; Vos et al., 1999; Holtzman et al., 2006a,b; Duguid et al., 2015). It has been recently suggested that gap junction communication between Golgi cells might be essential to allow low-frequency pacemaking and at the same time, to synchronize oscillations in neighboring Golgi cells (Dugué et al., 2009). However, experimental results and simulation with detailed network models have shown that sparse synaptic inputs can tonically and transiently desynchronize Golgi cell networks by triggering a gap junction mediated inhibition (Vervaeke et al., 2010, 2012; Szoboszlay et al., 2016).

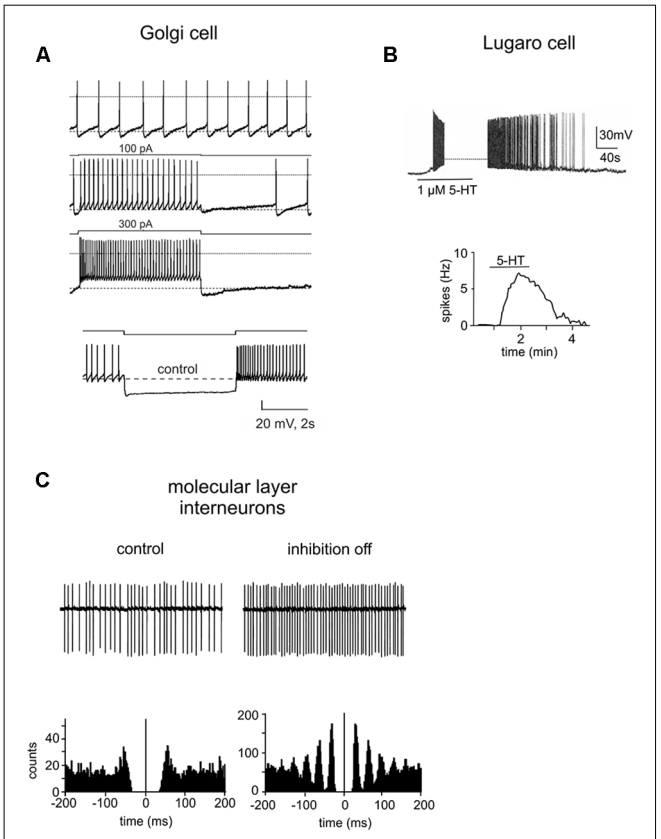

FIGURE 4 | Intrinsic properties of cerebellar GABAergic neurons. **(A)** Electroresponsiveness of a Golgi cell. The neuron shows low-frequency pacemaking activity and, upon depolarizing current injection, high-frequency spike discharge. Spike discharges are followed by an afterhyperpolarization and a silent pause. Upon hyperpolarizing current injection, the Golgi cell shows sagging inward rectification, followed by a post-inhibitory rebound. Adapted from Forti et al. (2006). **(B)** Whole-cell current-clamp recording in rat cerebellar slices demonstrated that the spontaneously inactive LC was reversibly excited by serotonin (1 μM). Below, summary of the effect of serotonin on the firing frequency of a LC. Adapted from Dieudonné and Dumoulin (2000). **(C)** Cell-attached patch-clamp recording from a ML interneuron under control conditions and during the block of inhibition. Below, autocorrelation of action potential trains from the corresponding recordings. Note that the block of inhibition causes a marked increase in the regularity of firing. Adapted from Hausser and Clark (1997).

Lugaro Cells

LCs were first described by Ernesto Lugaro more than a 100 years ago in the cat cerebellum (Lugaro, 1894). They have been characterized as a morphologically distinct GABAergic/glycinergic interneurons with unique physiological features. LCs are mainly distributed in the posterior lobules (VII to X; Lainé and Axelrad, 1996, 1998; Dieudonné and Dumoulin, 2000). On the basis of shape and location of the soma, LCs can be divided into two groups: the first group consists of large-sized LCs characterized by a fusiform or triangular soma which occupy the deeper GL (Lugaro, 1894; Geurts et al., 2001; Melik-Musyan and Fanardzhyan, 2004; Crook et al., 2006). The second group

consists of small-sized LCs marked by a fusiform soma located underneath the PCs layer (Lainé and Axelrad, 2002; Simat et al., 2007; Schilling et al., 2008; Hirono et al., 2012). Globular cells, likely a subtype of LCs, have been recently described; they have a small rounded soma and are distributed throughout the GL (Lainé and Axelrad, 2002). From the opposite extremities of the LC soma, two pairs of thick, horizontal, rarely ramified dendrites emerge, running parallel to the PCL in the parasagittal plane (Lainé and Axelrad, 2002; Geurts et al., 2003). These dendrites are very long (from 100 μm to 700 μm) and can ascend obliquely towards the ML and/or extend down more or less vertically through the GL (Lainé and Axelrad, 1996). Conversely, globular cells exhibit radiating dendrites that spread mostly in the PCL (Lainé and Axelrad, 2002; Hirono et al., 2012). LC axons are myelinated and can be divided into two groups, in accordance with their route to the ML (Lainé and Axelrad, 1996). In the first case, the axon is parasagittally oriented and heads downwards in the GL before ascending back and ending inside the ML. This parasagittal plexus makes synaptic junctions with stellate and BC soma. The second axon type enters directly the ML where it generates a local and transversal plexus running parallel to the PFs. These transverse fibers preferentially contact apical dendrites of Golgi cells (Lainé and Axelrad, 1996; Dieudonné and Dumoulin, 2000; Dumoulin et al., 2001). Although LCs have also been suggested to inhibit PCs (Dean et al., 2003), the location of these synapses remains debated (Lainé and Axelrad, 1998, 2002; Simat et al., 2007). Globular cell axons project into the ML following the directly or indirectly ascending trajectory (Lainé and Axelrad, 2002). LCs in the rat cerebellum are normally completely silent (**Figure 4B**). In the presence of serotonin they become intensively active showing a robust firing (5–15 Hz), determining the inhibition of BCs and SCs, as well as Golgi cells and PCs (Dieudonné and Dumoulin, 2000; Dumoulin et al., 2001; Dean et al., 2003; Hirono et al., 2012). In the cerebellum, the LC-Golgi cell synapse was the first functional evidence of mixed GABA/glycine co-release (Dieudonné, 1995; Dumoulin et al., 2001). Since glycine receptors are not expressed at LC-stellate/basket/PC synapses, LCs can perform target-specific synaptic transmission. High sensitivity to both serotonin and norepinephrine differentiate globular cells from other LCs. Moreover, they receive strong inhibitory synaptic inputs through PC axon collaterals and probably also excitatory synaptic inputs through MFs (Colin et al., 2002; Hirono et al., 2012).

Basket Cells and Stellate Cells

BCs and SCs, first described by Golgi (1883) and Cajal (1888) are GABAergic interneurons located in the ML of the adult cerebellum (see **Box 1**). BCs and SCs have distinctive morphology, positioning and axonal arborization (Lemeky-Johnston and Larramendi, 1968; Chan-Palay and Palay, 1972; Palay and Chan-Palay, 1974; Llinas et al., 2004). BCs have a pyramidal or oval soma with a diameter of 20 μm and are usually found in the inner third of the ML or between PCs. BCs have 4–10 straight dendrites that expand vertically producing a fan-shaped field in the parasagittal plane. In general, the dendrites are large, thick and smooth although some have spines. Many of them, before curving upwards, extend in the horizontal plane above the PCs for millimeters, originating relatively few branches in their course (DeFelipe et al., 1986). SCs, which have a small and fusiform soma about 7–10 μm in diameter, are located in the upper two-third of the ML. They are characterized by long, contorted, aspinous, frequently branching dendrites radiating in all directions. Some cells appear bipolar with dendrites originating from opposite sides of the soma (Palay and Chan-Palay, 1974; Jacobs et al., 2014). The longitudinally arranged PFs cross the dendritic tree of both cell types at right angles, providing the major excitatory synaptic input. In addition, several studies have reported excitatory synapses between basket/SCs and CF collaterals acting exclusively *via* spillover of glutamate from nearby release sites. CFs stimulation resulted in an increased spike firing in both interneurons both *in vivo* (Jörntell and Ekerot, 2002, 2003) and *in vitro* (Hámori and Szentágothai, 1980; Szapiro and Barbour, 2009). Finally, both molecular interneurons receive inhibitory GABAergic contacts, mainly on their soma. BC soma is contacted by PC axon collaterals and axons from other BCs (O'Donoghue et al., 1989; O'Donoghue and Bishop, 1990; Hausser and Clark, 1997) whereas SC soma receives axonal contacts from other SCs (Kondo and Marty, 1998). BC axon emerges either from the soma or from one of the major dendrites extending horizontally in the parasagittal plane above the PC soma for the distance of 500–600 μm (Chan-Palay et al., 1974; Castejon et al., 2001). It emits a succession of collaterals: ascending collaterals headed to the ML; descending collaterals that envelop the soma of several PCs forming the pericellular "basket" that gives the cells their name. Some terminate their course surrounding the initial axon segment of PCs establishing a very complicated axo-axonic synapse called *pinceau*. The axons of SCs are less characteristic: they branch immediately generating short and circumscribed collaterals. This simple arborization contacts the dendrites of PCs (Palay and Chan-Palay, 1974; Ito, 1984). Both ML interneurons fire spontaneously in the range of 1–35 Hz, both *in vitro*, in the absence of external inputs, and *in vivo* (Hausser and Clark, 1997; Carter and Regehr, 2002; Jörntell and Ekerot, 2003; Barmack and Yakhnitsa, 2008). The excitability of BCs and SCs is shown to be modulated by several molecular mechanisms. For example, the firing rate of SCs is dynamically regulated by T-type channel-mediated Ca^{2+} transient through A-type K^+ channel modulation (Molineux et al., 2005; Anderson et al., 2013; Alexander et al., 2019). Moreover, ML interneurons firing patterns are typically irregular, characterized by a shift toward a more regular rate when inhibitory synaptic currents are blocked (**Figure 4C**; Hausser and Clark, 1997; Lachamp et al., 2009). This irregularity is presumably due to spontaneous CF activities occurring in irregular patterns. Interestingly, CFs, especially terminating in the same parasagittal bands, tend to display synchrony (De Zeeuw et al., 1997; Lang et al., 1999) determining a more synchronized spiking activity of the interneurons localized in the same microzone. This tendency may be further enhanced by mutual inhibitory contacts (see above) and gap junction communication (Mann-Metzer and Yarom, 1999).

CEREBELLAR INTERNEURONS FUNCTIONAL CONNECTIVITY

Although the functional connections of principal neurons (PCs and granule cells) have been described in detail for the cerebellar cortex (for critical reviews, see Tank et al., 1988; D'Angelo et al., 1995, 2009, 2016; Silver et al., 1996; Hansel et al., 2001; D'Angelo and De Zeeuw, 2009; D'Angelo, 2014, 2016, 2018; Masoli et al., 2015), attention is only recently starting to shift toward the dynamical interactions among inhibitory interneurons. The correlated morphological and functional analysis of inhibitory interneurons is still representing a major challenge. Indeed, GABAergic interneurons differ in molecular, structural and firing properties, making their functional roles in the microcircuits more complex to understand. The inhibitory interneurons appear to be organized in multineuronal inhibitory chains generating feed-forward, feed-back and lateral inhibition, which are likely to cooperate in determining cerebellar signal processing in a way that is not yet fully elucidated. For the sake of simplicity, in the following section, the functional connectivity of cerebellar cortical interneurons will be elucidated on the basis of these three "classical" types of inhibition mediated by GABAergic interneurons in the cerebellum. We will focus on the recruitment of BCs, SCs, LCs and Golgi cells into these circuit mechanisms.

Feed-Forward Inhibitory Circuits

In a feed-forward inhibitory circuit, principal cells and inhibitory interneurons simultaneously receive are the same excitatory input. The interneurons inhibits the principal cell with disynaptic delay, thereby narrowing the window for suprathreshold summation of excitatory inputs (Buzsáki, 1984; Pouille and Scanziani, 2001; Blitz and Regehr, 2005; Gabernet et al., 2005; Mittmann et al., 2005; Cruikshank et al., 2007; D'Angelo and De Zeeuw, 2009; Torborg et al., 2010; Najac et al., 2011). Feed-forward inhibition (FFI) is a way of regulating the timing of neuronal responses in many brain regions, enhancing network performance.

Parallel Fiber–Molecular Layer Interneurons–Purkinje Cell

In the cerebellum, basket and SCs activated by PFs control the rate and temporal precision of PC spike output using FFI (Eccles, 1967; Hausser and Clark, 1997; Jaeger and Bower, 1999; Mittmann et al., 2005; Barmack and Yakhnitsa, 2008). PCs generate complex spikes in response to CF activity (Davie et al., 2008) and simple spikes which occur spontaneously (Hausser and Clark, 1997; Raman and Bean, 1997) or are driven by PF input (Eccles et al., 1967). Thus, the PC spike output reflects a complex interaction between spontaneous activity, excitatory and inhibitory synaptic inputs from PFs and ML interneurons (Hausser and Clark, 1997; Jörntell and Ekerot, 2002; Santamaria et al., 2007). FFI is mediated by two distinct pathways involving different subcellular segments of PCs. Experimental and computational studies suggest that stellate (dendritic)-type inhibition and basket (somatic)-type inhibition play diverse functional roles and have different postsynaptic effects on PCs (Santamaria et al., 2002, 2007; Santamaria and Bower, 2005; Bower, 2010; Masoli and D'Angelo, 2017). Dendritic FFI contributes to compensate PF excitation in local segments of the PC dendrite resulting in indirect influence on spike output. Specifically, the interaction of PF and SC synaptic inputs on PC spike output is mediated by large intrinsic calcium and calcium-activated dendritic currents which, counterbalancing each other, affect the excitability of the PC dendrite. Consistent with simulation-based studies, experimental results suggest that the temporal balance between dendritic FFI and PF input results in a compensation of calcium currents by calcium-activated potassium currents. This, in turn, does not determine any net current flow and thus no effect of PF activity on the PC spike output (Jaeger and Bower, 1999). In conclusion, dendritic FFI is involved in modulating the "state" of the PC dendrite and this regulation of dynamic balance between voltage-dependent conductances is the explanation of how the PC soma activity typically functions (Jaeger et al., 1997; Jaeger and Bower, 1999; Santamaria et al., 2002; Womack and Khodakhah, 2003; Santamaria and Bower, 2005; Bower, 2010). Conversely, somatic FFI is very effective, rapid and powerful in controlling PC responses. Specifically, this type of inhibition: (i) reduces the time window for summation of independent input pathways; (ii) increases the temporal spike precision of; and (iii) suppresses the response to subsequent inputs (Vincent and Marty, 1996; Mittmann et al., 2005; Barmack and Yakhnitsa, 2008). Recently, the BC *pinceau* has been proposed to cause ephaptic inhibition *via* the current flow surrounding the PC AIS (Blot and Barbour, 2014), confirming the prediction derived by analogy with the Mauthner cell axon cap (Chan-Palay and Palay, 1970; Sotelo and Llinás, 1972). Through the ephaptic *pinceau* effect, BCs, once activated by PFs, determine an extremely fast inhibition of PCs, thus without synaptic delay. This is a very effective mechanism to prevent the simultaneous on-beam exciting action of PFs and to reduce off-beam PC firing through a pure inhibition (Blot and Barbour, 2014). The influence of dendritic and somatic FFI on PCs are predicted to be different along the course of PFs (Santamaria et al., 2007).

Despite the long history of works dedicated to understand cerebellar cellular circuitry and function (Cajal, 1908; Eccles, 1967; Voogd and Glickstein, 1998; Voogd, 2014), the last decade of cerebellar research has discovered additional afferent and efferent contacts that could impact on the ML interneuron processing. The high majority of granule cells ascending axon synapses on PCs (Sultan and Bower, 1998; Apps and Garwicz, 2005) are located in the inner ML (Gundappa-Sulur et al., 1999). A recent hypothesis is that ascending axon synapses can also end on BCs, though this is not confirmed by either physiological or anatomical (Gundappa-Sulur et al., 1999) data. If this was true, then an interesting scenario would appear. The ascending axon of granule cell is invaded very rapidly by the spike (Diwakar et al., 2009; Dover et al., 2016), which then run more slowly along the PFs (0.1 m/s). Thus, excitatory inputs arriving at PCs through ascending axons would be too early to undergo the FFI influence. By contrast, at longer distances, PF inputs can be counterbalanced by dendritic FFI. In conclusion, the fundamental features of the connectivity and therefore

the function of the cortical network are rigidly determined by the spatial relationship between neuronal components. Different form of plasticity at PF–ML interneuron and ML interneuron–PC synapses are assumed to increase the variability of PC spike output (Albus, 1971; Dean and Porrill, 2010). Several arguments of evidence converge to sustain the concept that ML interneuron FFI plays a role in controlling the gain and timing of motor learning (Wulff et al., 2009; Heiney et al., 2014; Yamazaki et al., 2015; Jelitai et al., 2016). PC $GABA_A$ receptor knock-out mice show significant motor coordination deficits (ataxia). Moreover, the modulation of the firing rate of ML interneurons using optogenetics proved able to drive movement kinematics in awake mice. In eyeblink conditioning, a pause in PC firing occurs after training (Jirenhed et al., 2007). However, PC spontaneous activity has been reported to be independent of PF input (Hausser and Clark, 1997; Cerminara and Rawson, 2004). Consequently, the well-timed reduction of PC spontaneous activity could be explained by an acquired increase in molecular interneuron FFI, which could, therefore, contribute to the mechanisms underlying consolidation of the learned eyeblink response. In conclusion, plastic changes in ML interneuron FFI onto PCs could play a crucial role in controlling the temporal aspects of learned output of the cerebellar cortex (Attwell et al., 2002; Cooke et al., 2004; Jörntell et al., 2010; see also below).

Parallel Fiber–Molecular Layer Interneuron–Molecular Layer Interneuron

Since ML interneurons are highly interconnected *via* electrical and chemical synapses, in addition to providing FFI to PCs they also receive FFI from each other (Mittmann et al., 2005; Rieubland et al., 2014). Interestingly, the effect of FFI in interneurons appears to be less powerful than that observed in PCs. This could depend on differences in the intrinsic conductances in interneurons or PCs or, as an alternative, PFs could activate fewer feed-forward inhibitory connections among interneurons than onto PCs. Modeling studies investigating the functional role of ML interneuron mutual inhibition revealed a significant influence on activity of the network by regulating the firing rate and variability of spike timing of ML interneurons and PCs. Lennon et al. (2014) simulated a scenario where synapses between ML interneurons were removed. Following decreased mutual inhibition, ML interneuron firing rates increased. The consequence of the increased ML interneuron firing is an increase of FFI onto PCs, resulting in decreased PC firing rates thus preventing DCN neurons from firing appropriately. Thus, FFI onto ML interneurons could be needed to assure effective motor performance and learning (Walter et al., 2006; Wulff et al., 2009).

Parallel Fiber–Molecular Layer Interneuron–Golgi Cell

Anatomical and physiological (Dumoulin et al., 2001) lines of evidence have proposed that, in addition to PCs, ML interneurons recruited by PFs inhibit Golgi cells (**Figure 4**). However, recent studies reported that Golgi cells are synaptically inhibited by other Golgi cells (Dugué et al., 2009; Galliano et al., 2010; Simões de Souza and De Schutter, 2011) rather than by ML interneurons (Hull and Regehr, 2012), and that they are also connected by gap junctions (Isope and Barbour, 2002; Geurts et al., 2003; D'Angelo and De Zeeuw, 2009; Galliano et al., 2010; Jörntell et al., 2010). At present, the existence of ML interneuron–Golgi cell synapses issue remains controversial.

Mossy Fiber–Golgi Cell–Granule Cell

In the GL, Golgi cells generate synaptic inhibition onto granule cells. Granule cells receive excitatory inputs from the MFs which, in turn, excite Golgi cells providing FFI to granule cells (Pouille and Scanziani, 2001; Kanichay and Silver, 2008; D'Angelo and De Zeeuw, 2009; D'Angelo et al., 2013). FFI from Golgi cells acts through two main mechanisms, phasic and tonic. Phasic inhibition consist of synaptic $GABA_A$-mediated inhibitory post-synaptic currents and potentials (Wall and Usowicz, 1997; Rossi and Hamann, 1998; Armano et al., 2000), while tonic inhibition is mediated by extrasynaptic $GABA_A$ receptors activated by low GABA levels in the extracellular space (Brickley et al., 1996; Wall and Usowicz, 1997; Hamann et al., 2002; Rossi et al., 2003; Farrant and Nusser, 2005; Glykys and Mody, 2007). FFI mediated by phasic inhibition enhances granule cell spike timing precision by narrowing the time window for synaptic integration. In response to a single MF input or brief bursts, phasic inhibition generated by the feed-forward circuit lasts about 4–5 ms and limits the duration of granule cell responses to 1–2 spikes. Interestingly, since MF–granule cell LTP tends to anticipate the emission of the first spike, while LTD does the opposite (Nieus et al., 2006; Mapelli et al., 2014; Nieus et al., 2014), synaptic plasticity contributes with the time window mechanism in regulating information transfer (D'Angelo et al., 2013). Extending a prediction from theoretical network analysis (Medina and Mauk, 2000; De Schutter and Bjaalie, 2001), Golgi cells endow the GL with the properties of a temporal filter determining how bursts are conveyed toward ML and how PFs activate PCs and interneurons (Bower, 2002; Lu et al., 2005). Furthermore, at the PF synapses Golgi cells could also regulate the short- and long-term synaptic plasticity induction by controlling the temporal pattern of spikes generated by granule cells (Isope and Barbour, 2002; Sims and Hartell, 2005). During MF high-frequency activity, like that generated in response to sensory stimulation (Chadderton et al., 2004; Rancz et al., 2007), the time window effect can be momentarily abolished, due to diverse possible mechanisms including: (i) presynaptic decrease of GABA release through $GABA_B$ autoreceptors or mGlu receptors expressed on Golgi cell terminals (Mitchell and Silver, 2000a,b; Mapelli et al., 2009); (ii) postsynaptic down-regulation of $GABA_A$ currents mediated by $GABA_B$ activation (Brandalise et al., 2012); (iii) postsynaptic reduction of an inward rectifier potassium current through $GABA_B$ receptors which determines an enhancement of granule cell responsiveness (Rossi et al., 2006); and (iv) reduction of Golgi cell firing through dendritic activation of mGlu2 receptors which enhances an inward rectifier potassium current (Watanabe and Nakanishi, 2003). Additional *in vitro* and *in vivo* studies are needed to clarify the significance of these mechanisms. Otherwise, FFI mediated by tonic inhibition determines a shift of input/output

(I/O) relationship by decreasing the membrane resistance of granule cells, leading to a reduction of the excitability (Hamann et al., 2002). This holds confirmed only for constant excitatory inputs. When excitation is mediated by time-varying synaptic inputs, tonic inhibition changes the slope (gain) of the I/O relationship (Chance et al., 2002; Mitchell and Silver, 2003). Thus, FFI mediated by tonic inhibition has a double effect: the shift in I/O relationship configure the level of granule cell excitability, while gain regulation makes the neuron less sensitive to changes in its inputs. In this manner, tonic inhibition would allow providing the appropriate excitability of granule cells and discriminating significant information from background activity eventually reducing signal-to-noise ratio of information transmission in granule cells and of PF input to the molecular interneurons and PCs (Duguid et al., 2012; Mapelli et al., 2014).

Feed-Back Inhibitory Circuits

In a feed-back inhibitory circuit, the principal cell provides the excitatory input onto the inhibitory interneurons, which, in turn, further inhibit the principal cell. Therefore, feedback inhibition (FBI) plays a general role in locally controlling the excitatory-inhibitory (E/I) balance within a neural circuit (Dieudonné, 1998; D'Angelo and De Zeeuw, 2009; D'Angelo et al., 2013; Feldmeyer et al., 2018).

Parallel Fiber–Golgi Cell–Granule Cell

Electrophysiological and morphological experimental results indicate that Golgi cells feedback onto the granule cells (**Figure 4**). Actually, previous *in vivo* researches reported that an intense stimulation of the PFs caused a decrease of the MF excitatory input transmission to PCs, possibly through the excitation of Golgi cells and successive inhibition of granule cells (Eccles et al., 1964, 1966a; Dieudonné, 1998). Considering PF lengths, a granule cell could excite a Golgi cell at a long distance along the transverse section. Conversely, a Golgi cell will only inhibit the granule cells mostly located in the narrow parasagittal zone occupied by its axon. FBI from Golgi cells onto granule cells mediated by phasic inhibition is critical for generating and sustaining *coherent oscillations* (Maex and De Schutter, 1998; Solinas et al., 2010; Mapelli et al., 2014). Following MF input, Golgi cell and granule cell populations become entrained in a synchronous oscillatory activity, whose basic frequency range from 10 to 40 Hz (Maex and De Schutter, 1998). This could account for the large-amplitude oscillation recorded in the GL of freely moving rats (Pellerin and Lamarre, 1997; Hartmann and Bower, 1998; Courtemanche et al., 2002; Courtemanche and Lamarre, 2005) and monkeys. Furthermore, Golgi cell autorhythmic activity (Forti et al., 2006), SC-Golgi cell synapses (Casado et al., 2000), and Golgi cell-Golgi cell synapses and gap junctions (Vervaeke et al., 2010, 2012; Hull and Regehr, 2012) also participate to originate circuit oscillations (Maex and De Schutter, 1998; D'Angelo and De Zeeuw, 2009; Solinas et al., 2010; D'Angelo et al., 2013). Recently, FBI mediated by phasic inhibition has been shown to be implicated in the phenomenon of resonance in the GL (Gandolfi et al., 2013). The authors showed that the blockade of phasic inhibition prevented oscillations but not resonance (which is just modulated), indicating that the two processes have a complicated and only partially mechanistic relationship with the inhibitory circuit. Finally, a computational model of the GL suggested that tonic inhibition generated by FBI desynchronizes the network, but this effect could be counterbalanced completely by enhancing MF firing rate (Maex and De Schutter, 1998). Thus, tonic inhibition could further have an effect on coherence of distributed signal processing (Singer and Gray, 1995; Semyanov et al., 2004).

Lugaro Cell–Molecular Layer Interneurons–Purkinje Cell

A LC makes synaptic contacts preferentially with ML interneurons in the sagittal axonal plexus and Golgi cells in the transversal axonal plexus (Simat et al., 2007; Schilling et al., 2008), while soma and dendrites receive massive innervation from PC axon collaterals (Colin et al., 2002; Hirono et al., 2012). LCs, once activated by MFs or monoaminergic inputs, can increase the PC activity through ML disinhibition. Thus, the PC–LC feedback circuit proceeds and could silence LCs. LC activity is able to synchronize the firing of PC clusters in different microzones, likely contributing to motor learning and coordination (Hirono et al., 2012). A previous *in vivo* study described a correlation between the spontaneous firing rate of PCs and the effect of serotonin (Strahlendorf et al., 1984). Specifically, PCs that responded to serotonin with increases in discharge rate showed significantly lower basal firing frequencies than those cells that were silenced by serotonin. This correlation can be explained by FBI circuit. When PCs fire high-frequency action potentials, LCs are allowed to generate only a few spikes even in the presence of serotonin, and PCs firing is no longer facilitated by serotonin, but rather in some cases decreased by the direct effects of serotonin on the PCs (Bishop and Kerr, 1992; Li et al., 1993). Conversely, when PCs fire at low frequencies, serotonin can induce robust firing in LCs, which lead to facilitation of PC firing. At the behavioral level, the pharmacological depletion of brain serotonin in the rabbit causes a loss of vision-guided adaptation of vestibulo-ocular reflex (VOR; Miyashita and Watanabe, 1984). Moreover, a serotonin precursor was used for cerebellar ataxia therapy (Trouillas et al., 1988, 1995). Recent clinical research in patients with ADHD, some of whom show abnormal activity in monoaminergic systems, showed that the timing of conditioned eyeblink responses is impaired (Oades et al., 2008; Frings et al., 2010). In conclusion, since LCs also contact Golgi cells, they may be considered as a key node in modulating inhibition levels both in the molecular and GL (**Figure 5**).

Lateral Inhibition

Lateral inhibition is considered a consequence of FBI where a principal neuron response to a stimulus is inhibited by the excitation of a neighboring interneuron by other principal cells nearby. This type of neural network was first discovered by Hartline and Ratliff (1957) in their studies of the compound eye of the horseshoe crab. In general, lateral inhibition enhances neurons responsiveness to spatially varying stimuli than to spatially uniform ones. That is, a neuron stimulated by a spatially uniform stimulus is also inhibited by its surrounding

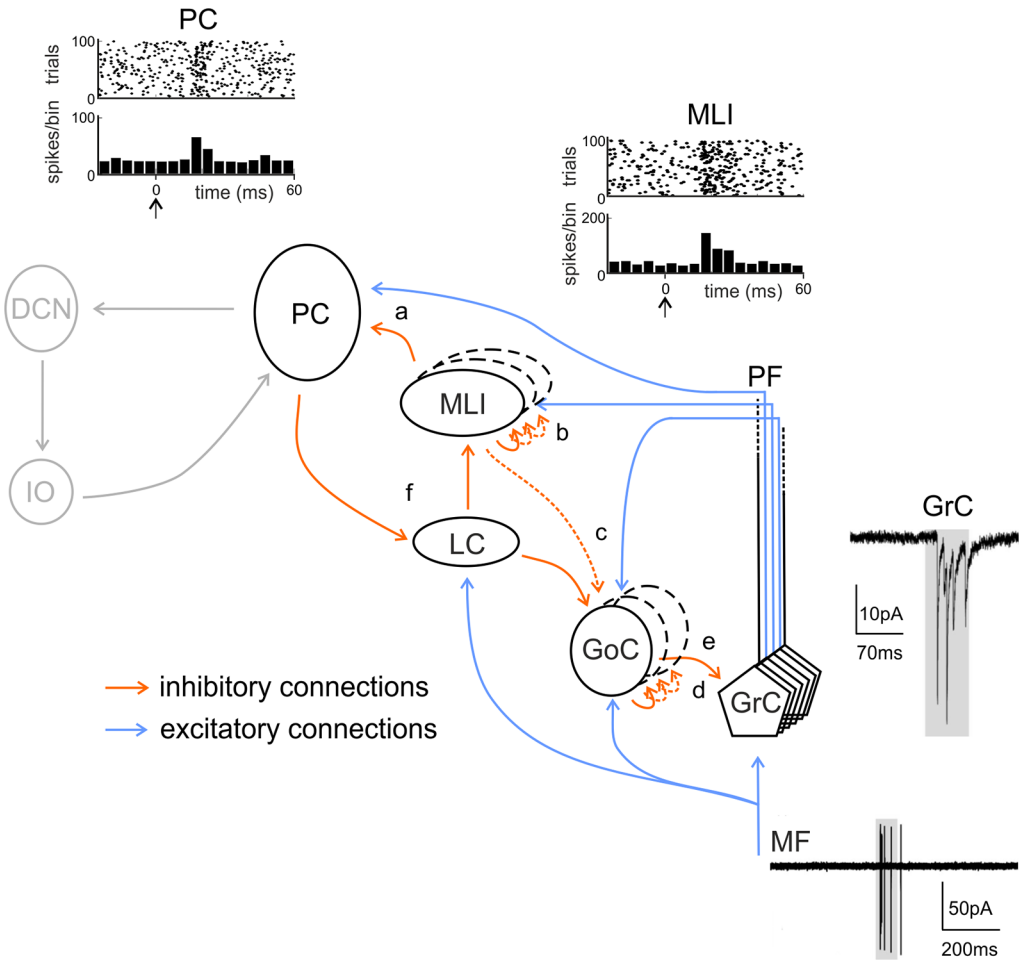

FIGURE 5 | Inhibitory chains in the cerebellar cortex. The scheme shows the main excitatory and inhibitory connections in the cerebellar cortical circuit. Note that feed-forward (FFI) and feed-back (FBI) inhibitory loops are integrated in a complex inhibitory chain. FFI: (a) A PC may be both directly excited and then inhibited with disynaptic delay *via* ML interneurons (MLIs) activated by the same set of active PFs. (b) MLIs receive feed-forward inhibition (FFI) from each other. (c) MLIs, activated by PFs, inhibit Golgi cells (GoCs) in the same manner as PCs. This issue is somewhat controversial (see main text, dashed line). (d) Granule cells (GrCs) receive excitatory inputs from MFs. MFs also excite GoCs which provide FFI to GrCs. FBI: (e) through the PFs, GrCs activate GoCs that, in turn, inhibited GrCs in a feedback loop. (f) PC collaterals are known to inhibit LCs, which in turn inhibit MLIs. Activation of LCs by excitatory MF or monoaminergic inputs would lead to enhanced PC activity through disinhibition. In gray, non-cortical circuits involving DCN and IO. Traces show the activity of MF, granule cells, ML interneurons and PCs during tactile sensory stimulation in rodents. Adapted from Rancz et al. (2007) and Ramakrishnan et al. (2016). Noteworthy, the complex regulatory mechanisms brought about by the inhibitory interneuron chain remain to be investigated during dynamic signal processing.

interneurons, thus suppressing its response. By contrast, a neuron subjected to a spatially varying stimulus is less inhibited by its neighbors that are not excited, thus producing stronger response (Bakshi and Ghosh, 2017).

Golgi Cell Lateral Inhibition

Golgi cell primary axonal plexus is confined in the GL and send collaterals originating secondary plexuses in the same or even in nearby laminae (Eccles, 1967; Barmack and Yakhnitsa, 2008). In the case of the Golgi cell, the origin of lateral inhibition phenomena relies on the larger extension of the axonal plexus compared to that of basal dendrites. The functional relevance of this inhibitory organization has recently been described through multi-electrode array recordings and voltage-sensitive dye imaging (Mapelli and D'Angelo, 2007; Mapelli et al., 2009; D'Angelo et al., 2013). Previous investigations *in vitro* have shown that lateral inhibition in the GL originates a *center-surround* organization of granule cell activity (Mapelli and D'Angelo, 2007; D'Angelo, 2008; Soda et al., 2019), characterized by prevailing excitation in the core, surrounded by an inhibited area. The *center-surround* pattern is generated as follows: when the MFs discharge in bursts, both granule cells and Golgi cells are activated in the same region. The resulting E/I balance is characterized by excitation prevailing in the core and inhibition prevailing in the surround, by virtue of the broader inhibitory territory of Golgi cells, with granule cell excitation that decreases radially from the excitation core (**Figure 6A**; Mapelli and D'Angelo, 2007). A detailed multicompartmental model has

calculated that an excited core can contain 260 granule cells with a probability of generating spikes up to 35%; by contrast, this probability is almost zero in the surround. Switching off inhibition in this context increases the probability of firing in the core up to 50% (Diwakar et al., 2009). Therefore, Golgi cells are pivotal players in determining the center-surround organization of granule cell activity following MFs stimulation. Three main functional consequence can be pointed out:

1. Organization of information transmission and processing along channels activating granule cell ascending axons running toward the ML and contacting overlying PCs. Coherently with high excitation levels in the core, the E/I organization in the GL facilitates the transmission of high-frequency burst along the channel. The prevailing inhibition in the surround acts as a filter preventing the transmission of low-frequency discharges (Mapelli et al., 2010). As a consequence, Golgi cells are able to define, converge and refine information transmission to PCs originating transmission channels running vertically to the ML, as suggested by previous experiments (Bower and Woolston, 1983).
2. Dynamic configuration of network topology by controlling the distribution of long-term synaptic plasticity. In particular, the higher excitation level in the core facilitates LTP, while the weaker excitation levels in the surround facilitate LTD. The center-surround organization of the E/I balance determines, in condition of suitable high-frequency stimulation, a matching center-surround distribution of LTP and LTD. This further sharpens the topological organization of signal transmission (**Figure 6a**). In the perspective of the transmission channeling mentioned above, regions showing LTP and LTD are likely to represent these channels by processing MFs incoming activity in different ways. Relying on the available data on synaptic plasticity modification of MF–granule cell synaptic properties, the LTP channel would be characterized by reduced response latency and increased post-synaptic firing frequency; the opposite is expected in the LTD channel (Nieus et al., 2006). Interestingly, considering electrophysiological and simulation modeling data (Mapelli et al., 2010; Solinas et al., 2010), the LTP channel is expected to display a heightened high-frequency transmission gain than the LTD channel. This prediction has not yet been experimentally confirmed.
3. A third and distinct effect of Golgi cell feed-back inhibition is the transformation of asynchronous granule cell activity into synchronous low-frequency GL oscillations. When asynchronous granule cell activity is received on their dendrites, it is summed up until the Golgi cells make a spike, which inhibits a large GL area. This results in self-sustained oscillations. And since Golgi cells, thanks to reciprocal connection through gap-junctions and inhibitory synapses, tend to form a functional syncytium, their pulsation tends to synchronize. Finally, the circuit time constant and the intrinsic resonant frequency of granule and Golgi cells will phase-lock the oscillation toward the theta band. These oscillations have been observed *in vivo* (Pellerin and Lamarre, 1997; Hartmann and Bower, 1998) and their mechanism has been predicted by computational models (**Figure 8**; Maex and De Schutter, 1998; Solinas et al., 2010; Casali et al., 2019).

Molecular Layer Interneuron Lateral Inhibition

Over 50 years ago, Szentágothai (1965) proposed that ML interneurons could laterally inhibit PCs by virtue the anatomical arrangement of excitation and inhibition onto PCs: the PFs (axons of granule cells) run coronally, whereas the axons of ML interneurons run sagittally (**Figure 6b**). Szentagothai's suggestion give rise to the beam hypothesis: activation of a beam of lead to excitation a long row of PCs in the coronal plane and inhibition in laterally located PCs (Andersen et al., 1964; Szentágothai, 1965; Eccles, 1967, 1973; Palay and Chan-Palay, 1974). Several experiments supported this idea (Cohen and Yarom, 2000; Sullivan et al., 2005). Consistent with lateral inhibition, recent advances have reported that the activation of granule cells immediately underlying a PC evoked pure excitation in the sagittal orientation, while the activation of granule cells positioned more laterally—as far as 480 μm away—provide pure inhibition (Dizon and Khodakhah, 2011; Valera et al., 2016; **Figure 6c**). These findings are also in agreement with *in vivo* studies reporting that sensory stimulation excited a patch of PCs and simultaneously inhibited neighboring PCs (Gao et al., 2006). Given that voluntary movement requires the coordinated activity of muscles that have opposite functions (agonist and antagonist), one function of this lateral inhibition might be to efficiently generate reciprocal signals from the same MF synaptic input (Dizon and Khodakhah, 2011). In this perspective, the role of FFI in enhancing the temporal precision of PCs must be considered. Whether these roles are fundamental for cerebellar functions remains to be demonstrated.

PLASTICITY IN THE INHIBITORY INTERNEURON NETWORK

Different forms of plastic changes in connection properties and/or in intrinsic excitability have been observed in inhibitory interneurons. Theoretical modeling of the cerebellar circuit suggested that plasticity in Golgi cells and ML interneurons would critically impact cerebellar circuit processing (e.g., affecting temporal precision, strength of excitatory transmission and filtering). To date, the main forms of plasticity involving cerebellar inhibitory interneurons are the following. A form of LTD has been observed at the connection between PFs and Golgi cells, following high-frequency activation (Robberechts et al., 2010). Golgi cells have also been described to undergo an increase in intrinsic excitability, as an increase in spontaneous firing, following hyper-polarization (Hull et al., 2013). In the ML, a recent study *in vivo* showed a long-lasting decrease in spontaneous firing in MLIs after theta-sensory stimulation (a pattern that is able to induce plasticity *in vivo* in the cerebellar network; Ramakrishnan et al., 2016). Other forms of LTP and LTD had been previously described *in vitro*, namely a postsynaptic PFs–ML interneurons LTD following high

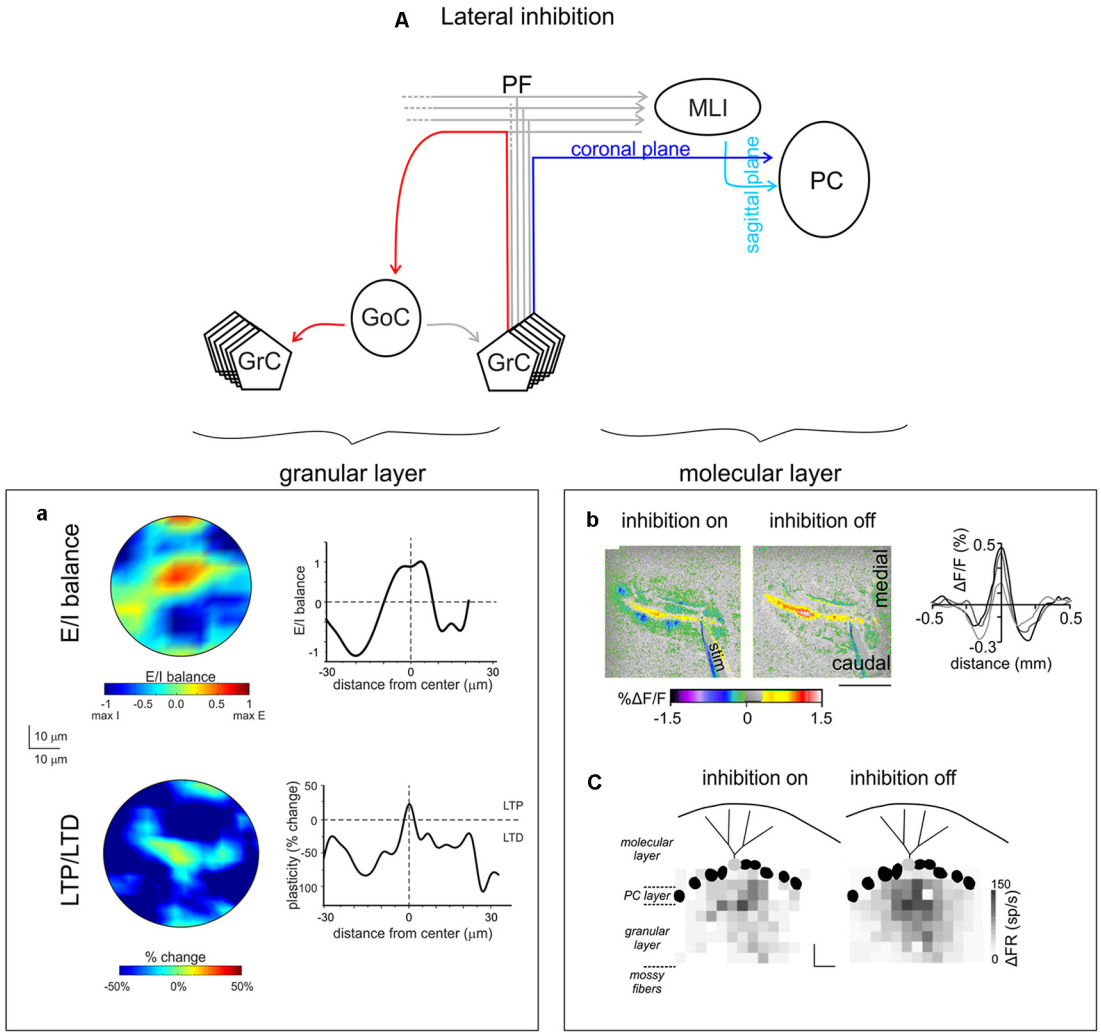

FIGURE 6 | Lateral inhibition in the cerebellar cortex. **(A)** Schematic of the cerebellar circuit of lateral inhibition, with the relevant granular and ML connectivity highlighted at left and at right, respectively. **(a)** GoCs generate a broad lateral inhibition that extends beyond the afferent synaptic field. The center-surround effect is generated by lateral inhibition. After excitation, GrCs in the core are more activated than those in the surrounding area. Below, long-term synaptic plasticity is controlled by Golgi cell synaptic inhibition at the MF-granule cell synapse. The result is LTP in the center and LTD in the surround, so that LTP and LTD assume a center-surround organization. Modified from Soda et al. (2019). **(b)** MLIs provide the substrate for lateral inhibition of PCs by virtue of the orthogonal arrangement of excitation and inhibition onto PCs: the PFs run coronally, whereas the axons of MLIs run sagittally. Surface stimulation of the cerebellar cortex evokes a large on-beam increase in fluorescence attributable to PF excitation of its postsynaptic targets (PCs and MLIs) and a narrow off-beam decrease in fluorescence due to postsynaptic inhibition generated by MLIs. Blocking inhibition using bicuculline application abolishes off-beam decrease in fluorescence and enhances the on-beam increase in fluorescence (scale bar 1 mm). Right, intensity profiles of the fluorescence change perpendicular to the beam. Adapted from Gao et al. (2006). **(c)** Response maps of a single PC (light gray) in terms of change in firing rate (gray scale) with inhibition on or off, while stimulating different regions in the GL. The probability that pure and net inhibitory responses are elicited by granule cells increased as a function of lateral distance from the PC (scale bars 80 µm × 80 µm). Adapted from Dizon and Khodakhah (2011).

frequency activation of the terminal (Soler-Llavina and Sabatini, 2006), a PFs–ML interneurons LTP requiring SCs depolarization during terminal activation (Rancillac and Crépel, 2004); and *in vivo*, as a PFs–ML interneurons LTP depending on CFs simultaneous activation (Jörntell and Ekerot, 2002). For a comprehensive review of the plasticity sites in the cerebellar network and the effects of distributed plasticity on circuit processing, see Mapelli et al. (2015). Further forms of plasticity at synapses impinging Golgi cells have been predicted by theory (Garrido et al., 2013) but remain to be demonstrated experimentally.

INSIGHT FROM DETAILED CEREBELLAR MICROCIRCUIT MODELS

Modeling of cerebellar function has its roots back to the work of Eccles, Marr and Albus in the second half of the 20th century (Eccles et al., 1967; Marr, 1969; Albus, 1971). The initial

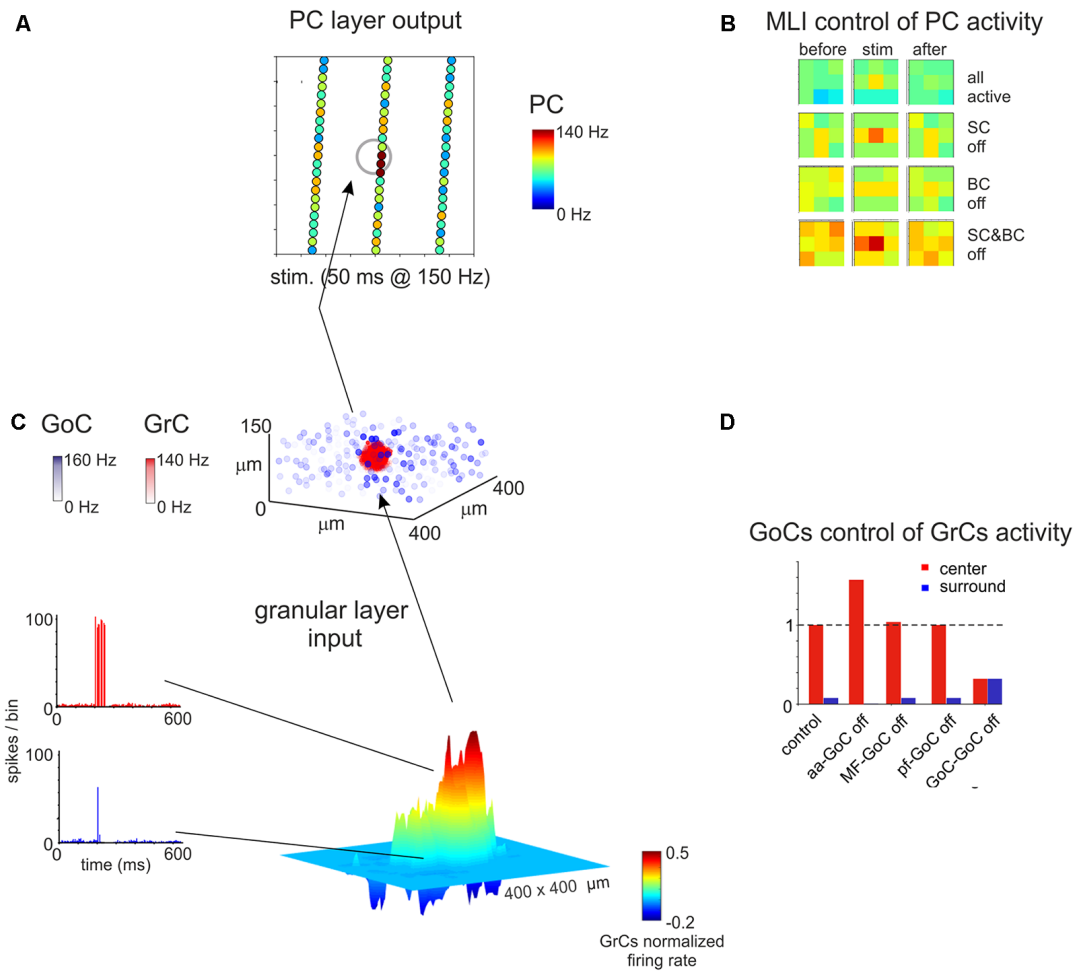

FIGURE 7 | Spatial organization of the granular and ML activity predicted by computational modeling. **(A)** The map shows the activity change of PCs in response to a MF burst. **(B)** The PC activity was averaged into 3 × 3 matrices in order to better appreciate when alternative patterns are generated. **(C)** In response to a MF burst, the GL responds with a core (red area) of activity surrounded by inhibition (blue area). The upper plot represents the activity of GoCs (blue) and GrCs (red) before and after the stimulus burst. **(D)** Peri-stimulus time histograms (PSTH) of GrCs in the center-surround. The activity in the core is characterized by robust spike bursts, while just sporadic spikes are generated in the surround. No activity changes are observed outside the center-surround structure. Adapted from Casali et al. (2019).

models were not realistic, also because available information about neuronal mechanisms was very limited. More recently, the availability of a huge amount of literature on cerebellar neuron properties and the development of high-performance computing and modeling platforms has allowed generating models incorporating fine details on intrinsic electroresponsive properties of neurons and synapses (D'Angelo et al., 2016). In the last decades, different models of cerebellar neurons have been proposed, mainly for principal cells [as granule cells (Solinas et al., 2010); and PCs (Masoli and D'Angelo, 2017)], but also for interneurons. Modeling of interneurons is indeed necessary to understand microcircuit dynamics and reproduce complex network behaviors in large-scale simulations (**Figure 7**; Casali et al., 2019).

Golgi Cell Models

The first realistic model of the Golgi cell was proposed in 2007 (Solinas et al., 2007a,b) and its properties were incorporated in a detailed model of the GL later on Solinas et al. (2010). These models were able to reproduce complex non linear Golgi cell properties, as pace-making activity, phase-reset and resonance, and to help explain how these features play a role in emerging microcircuit properties (e.g., the center-surround and synchronous low-frequency oscillations in the GL; Maex and De Schutter, 1998; Solinas et al., 2010; Casali et al., 2019). The implementation of dendritic gap junctions in the realistic model showed that depolarization of one Golgi cell had an impact on the firing of neighboring Golgi cells (Dugué et al., 2009; Vervaeke et al., 2010) and enabled synchronization between Golgi cells (van Welie et al., 2016), prompting further investigation on Golgi cells mutual inhibition. For a comprehensive review of this subject see (D'Angelo et al., 2013). Recently, a simplified model of Golgi cell was obtained, maintaining the crucial firing dynamics shown in the previous models, making it feasible to integrate these properties in large-scale simulations (Geminiani et al., 2018). The 3D connectivity

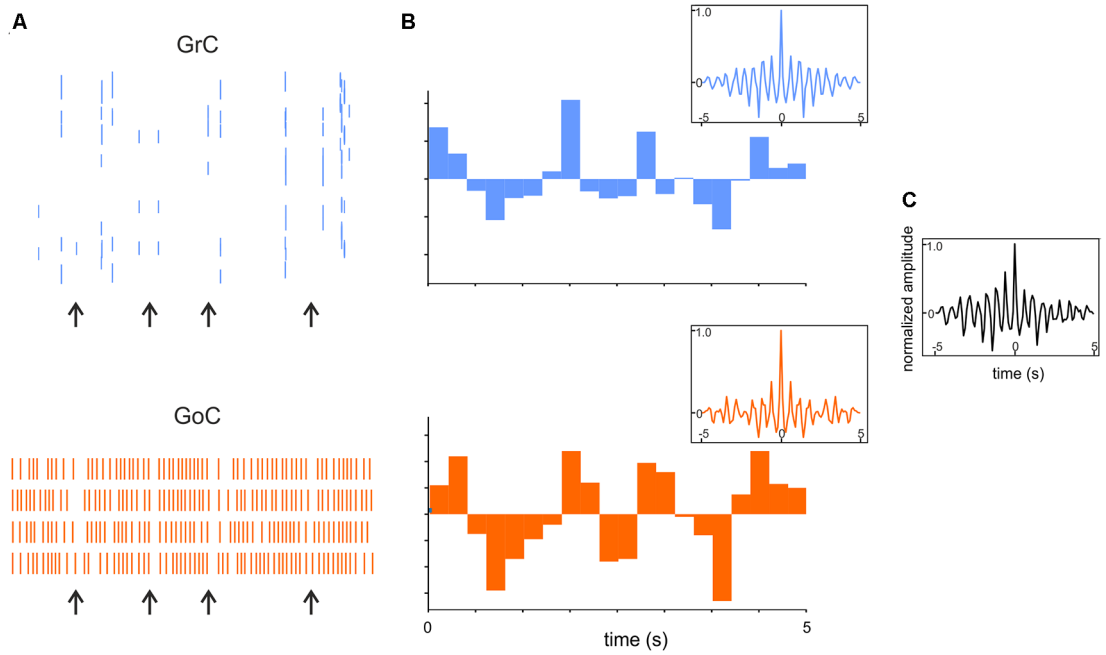

FIGURE 8 | Synchronous low-frequency oscillations in the GL revealed by computational modeling. **(A)** Raster plots of representative GrC and GoC during 5 Hz random MF input. Note that synchronous patterns are visible (arrows). **(B)** Cumulative PSTH of the whole GrCs and GoCs population. Insets show the autocorrelograms of the activity in corresponding population. **(C)** Crosscorrelogram of GrC and GoC populations activity. Adapted from Casali et al. (2019).

of Golgi cells within the granular and ML has allowed to explain also the center-surround organization of responses emerging following MF bundle stimulation (Solinas et al., 2010; Casali et al., 2019).

Molecular Layer Interneurons Models

Realistic models of stellate and BCs, based on their experimentally-measured passive properties, are not available yet. Simplified models have been used to explain the role of these interneurons in determining PCs firing within the feed-forward loops, mimicking the irregular firing observed *in vitro* for both PCs and ML interneurons (Santamaria et al., 2007; Lennon et al., 2014). These models also suggested the importance of ML interneurons mutual inhibition to reproduce experimental data. Recently, a more detailed model of ML interneurons (but lacking a distinction between stellate and BCs (Maex and Gutkin, 2017) has been proposed in order to investigate the role of these interneurons in the timing of cerebellar processing. Interestingly, they found that synaptic and electric coupling of these neurons provides the reciprocal inhibition that allows the time constant necessary for temporal integration. Electrical coupling has been suggested to reduce ML interneurons response heterogeneity, improving the signal-to-noise ratio (Maex and Gutkin, 2017). While modeling the molecular interneuron network, the para-sagittal or medio-lateral orientation of the cerebellar cortex strip reconstructed should be taken into account. Morphological details suggested that SCs and BCs show different axonal orientations, so that SCs are expected to have an impact mostly in the transversal axis (then evident in medio-lateral strip, also referred to as "on beam") and BCs should affect PCs processing in the sagittal axis (also referred to as "off beam"). The effect of disconnecting selectively SCs or BCs from the circuit has been described in a recent work, in a reconstruction of the scaffold model of the whole cerebellar cortex, using simplified models for single-cell neurons (**Figure 7**). Predictably, switching off SCs determined a spread of excitation along the PCL, prevalently along the transverse axis, while switching off BCs excitation extended mainly along the para-sagittal axis (Casali et al., 2019).

CONCLUSIONS

The last decades have been characterized by considerable progress in understanding the diversity of cerebellar inhibitory interneurons, in terms of embryological and developmental origin, localization and distribution of neurochemical markers, morphological and intrinsic properties. The concept has emerged that neuron properties and network dynamics in the cerebellar inhibitory circuit are more complex than originally thought. Both in the granular and in the ML, cerebellar interneurons are involved in complex inhibitory chains generating feedback, feedforward and lateral inhibition that regulate spatio-temporal dynamics of fundamental importance to determine the processing capabilities of the cerebellar cortex. Interneuron inhibition leads to the emergence of: *(1) center-surround organization in the GL; (2) gain and timing regulation in the GL; (3) synchronous low-frequency oscillations*

in the GL; (4) beam organization in the ML; (5) burst-pause regulation in PCs; and (6) gating of synaptic plasticity. Since the spatiotemporal pattern of cerebellar cortical activity is *de facto* controlled by cerebellar cortical interneurons, these cannot anymore be considered "subordinates" to excitatory cells (i.e., just maintaining the E/I balance) but rather integral parts of diverse microcircuits for multimodal information processing (Casali et al., 2019). This emerging view prompts for further investigations on these inhibitory interneurons in cerebellar physiology and pathology. It has already been shown that perturbing inhibitory interneurons functions results in altered cerebellar computation and motor behavior both in the GL (Watanabe et al., 1998) and in the ML (Rowan et al., 2018). Moreover, synaptic inhibition has been proposed to play a key role in neurodevelopmental disorders, such as autism, where the correct balance between excitation and inhibition might be disheveled by a malfunctioning of inhibition [as already reported for other brain regions (Pizzarelli and Cherubini, 2011)]. Future investigations should aim at dissecting the role of cerebellar cortical interneurons in specific processing features, thus shedding new light on the understanding of cerebellar processing and the generation of a unified theory of cerebellar functioning. In this perspective, the development of large-scale theoretical models will be fundamental to integrate the different neuronal types in a scaffold of the cerebellar cortex (D'Angelo et al., 2016; Casali et al., 2019). This will not only help identifying the contribution of inhibitory interneurons to local and global network dynamics but also to make predictions about their contribution to cerebellar processing and about the effects of their alterations in cerebellar pathology.

AUTHOR CONTRIBUTIONS

FP organized and wrote the manuscript. LM prepared the figures and contributed to writing the text. ED'A contributed to the final version of the manuscript.

FUNDING

This work has received funding from: the European Union's Horizon 2020 Framework Programme for Research and Innovation under the Specific Grant Agreement No. 785907 (Human Brain Project SGA2) to ED'A; Blue-Sky Research Grant of the University of Pavia (Università degli Studi di Pavia; BSR77992) to LM.

REFERENCES

Albus, J. S. (1971). The theory of cerebellar function. *Math. Biosci.* 10, 25–61. doi: 10.1016/0025-5564(71)90051-4

Alexander, R. P. D., Mitry, J., Sareen, V., Khadra, A., and Bowie, D. (2019). Cerebellar stellate cell excitability is coordinated by shifts in the gating behavior of voltage-gated Na^+ and A-type K^+ channels. *eNeuro* 6:ENEURO.0126-19.2019. doi: 10.1523/eneuro.0126-19.2019

Altman, J. (1972). Postnatal development of the cerebellar cortex in the rat. *J. Comp. Neurol.* 145, 465–513. doi: 10.1002/cne.901450403

Altman, J., and Bayer, S. (1997). *Development of the Cerebellar System in Relation to its Evolution, Structures and Functions.* Boca Raton, FL: CRC Press.

Andersen, P., Eccles, J. C., and Voorhoeve, P. E. (1964). Postsynaptic inhibition of cerebellar purkinje cells. *J. Neurophysiol.* 27, 1138–1153. doi: 10.1152/jn.1964.27.6.1138

Anderson, D., Engbers, J. D., Heath, N. C., Bartoletti, T. M., Mehaffey, W. H., Zamponi, G. W., et al. (2013). The Ca_v3-K_v4 complex acts as a calcium sensor to maintain inhibitory charge transfer during extracellular calcium fluctuations. *J. Neurosci.* 33, 7811–7824. doi: 10.1523/JNEUROSCI.5384-12.2013

Ankri, L., Husson, Z., Pietrajtis, K., Proville, R., Léna, C., Yarom, Y., et al. (2015). A novel inhibitory nucleo-cortical circuit controls cerebellar Golgi cell activity. *Elife* 4:e06262. doi: 10.7554/elife.06262

Apps, R., and Garwicz, M. (2005). Anatomical and physiological foundations of cerebellar information processing. *Nat. Rev. Neurosci.* 6, 297–311. doi: 10.1038/nrn1646

Armano, S., Rossi, P., Taglietti, V., and D'Angelo, E. (2000). Long-term potentiation of intrinsic excitability at the mossy fiber-granule cell synapse of rat cerebellum. *J. Neurosci.* 20, 5208–5216. doi: 10.1523/JNEUROSCI.20-14-05208.2000

Ascoli, G. A., Alonso-Nanclares, L., Anderson, S. A., Barrionuevo, G., Benavides-Piccione, R., Burkhalter, A., et al. (2008). Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat. Rev. Neurosci.* 9, 557–568. doi: 10.1038/nrn2402

Attwell, P. J., Ivarsson, M., Millar, L., and Yeo, C. H. (2002). Cerebellar mechanisms in eyeblink conditioning. *Ann. N Y Acad. Sci.* 978, 79–92. doi: 10.1111/j.1749-6632.2002.tb07557.x

Bakshi, A., and Ghosh, K. (2017). "A neural model of attention and feedback for computing perceived brightness in vision," in *Handbook of Neural Computation*, eds P. Samui, S. Sekhar and V. Balas (Cambridge, MA: Academic Press), 487–513.

Barmack, N. H., and Yakhnitsa, V. (2008). Functions of interneurons in mouse cerebellum. *J. Neurosci.* 28, 1140–1152. doi: 10.1523/JNEUROSCI.3942-07.2008

Bastianelli, E. (2003). Distribution of calcium-binding proteins in the cerebellum. *Cerebellum* 2, 242–262. doi: 10.1080/14734220310022289

Baude, A., Nusser, Z., Roberts, J. D., Mulvihill, E., McIlhinney, R. A., and Somogyi, P. (1993). The metabotropic glutamate receptor (mGluR1 α) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron* 11, 771–787. doi: 10.1016/0896-6273(93)90086-7

Bishop, G. A., and Kerr, C. W. (1992). The physiological effects of peptides and serotonin on Purkinje cell activity. *Prog. Neurobiol.* 39, 475–492. doi: 10.1016/0301-0082(92)90002-v

Blitz, D. M., and Regehr, W. G. (2005). Timing and specificity of feed-forward inhibition within the LGN. *Neuron* 45, 917–928. doi: 10.1016/j.neuron.2005.01.033

Blot, A., and Barbour, B. (2014). Ultra-rapid axon-axon ephaptic inhibition of cerebellar Purkinje cells by the pinceau. *Nat. Neurosci.* 17, 289–295. doi: 10.1038/nn.3624

Bolk, L. (1906). *Cerebellum der Saugetiere.* Haarlem: Fisher.

Bower, J. M. (2002). The organization of cerebellar cortical circuitry revisited: implications for function. *Ann. N Y Acad. Sci.* 978, 135–155. doi: 10.1111/j.1749-6632.2002.tb07562.x

Bower, J. M. (2010). Model-founded explorations of the roles of molecular layer inhibition in regulating purkinje cell responses in cerebellar cortex: more trouble for the beam hypothesis. *Front. Cell. Neurosci.* 4:27. doi: 10.3389/fncel.2010.00027

Bower, J. M., and Woolston, D. C. (1983). Congruence of spatial organization of tactile projections to granule cell and Purkinje cell layers of cerebellar hemispheres of the albino rat: vertical organization of cerebellar cortex. *J. Neurophysiol.* 49, 745–766. doi: 10.1152/jn.1983.49.3.745

Brandalise, F., Gerber, U., and Rossi, P. (2012). Golgi cell-mediated activation of postsynaptic $GABA_B$ receptors induces disinhibition of the Golgi cell-granule

cell synapse in rat cerebellum. *PLoS One* 7:e43417. doi: 10.1371/journal.pone.0043417

Brickley, S. G., Cull-Candy, S. G., and Farrant, M. (1996). Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. *J. Physiol.* 497, 753–759. doi: 10.1113/jphysiol.1996.sp021806

Buzsáki, G. (1984). Feed-forward inhibition in the hippocampal formation. *Prog. Neurobiol.* 22, 131–153. doi: 10.1016/0301-0082(84)90023-6

Cajal, S. R. (1888). Sobre las fibras nerviosas de la capa molecular del cerebelo. *Rev. Trim. Histol. Norm. Patol.* 1, 33–49.

Cajal, S. R. (1894). The Croonian Lecture.—La fine structure des centres nerveux. *Proc. R. Soc. Lond.* 55, 444–468. doi: 10.1098/rspl.1894.0063

Cajal, S. R. (1908). L'hypothèse de la continuité d'Apathy. Réponse aux objections de cet auteur contre la doctrine neuronale. *Trav. Labor. Réch. Biol. l'Univ. Madr.* 6, 21–90.

Cameron, D. B., Kasai, K., Jiang, Y., Hu, T., Saeki, Y., and Komuro, H. (2009). Four distinct phases of basket/stellate cell migration after entering their final destination (the molecular layer) in the developing cerebellum. *Dev. Biol.* 332, 309–324. doi: 10.1016/j.ydbio.2009.05.575

Carletti, B., and Rossi, F. (2008). Neurogenesis in the cerebellum. *Neuroscientist* 14, 91–100. doi: 10.1177/1073858407304629

Carter, A. G., and Regehr, W. G. (2002). Quantal events shape cerebellar interneuron firing. *Nat. Neurosci.* 5, 1309–1318. doi: 10.1038/nn970

Casado, M., Dieudonne, S., and Ascher, P. (2000). Presynaptic N-methyl-D-aspartate receptors at the parallel fiber-Purkinje cell synapse. *Proc. Natl. Acad. Sci. U S A* 97, 11593–11597. doi: 10.1073/pnas.200354297

Casali, S., Marenzi, E., Medini, C., Casellato, C., and D'Angelo, E. (2019). Reconstruction and simulation of a scaffold model of the cerebellar network. *Front. Neuroinform.* 13:37. doi: 10.3389/fninf.2019.00037

Castejon, O. J., Castejon, H. V., and Sims, P. (2001). Light microscopy, confocal laser scanning microscopy, scanning and transmission electron microscopy of cerebellar basket cells. *J. Submicrosc. Cytol. Pathol.* 33, 23–32.

Cauli, B., Zhou, X., Tricoire, L., Toussay, X., and Staiger, J. F. (2014). Revisiting enigmatic cortical calretinin-expressing interneurons. *Front. Neuroanat.* 8:52. doi: 10.3389/fnana.2014.00052

Celio, M. R. (1990). Calbindin D-28k and parvalbumin in the rat nervous system. *Neuroscience* 35, 375–475. doi: 10.1016/0306-4522(90)90091-h

Cerminara, N. L., and Rawson, J. A. (2004). Evidence that climbing fibers control an intrinsic spike generator in cerebellar Purkinje cells. *J. Neurosci.* 24, 4510–4517. doi: 10.1523/JNEUROSCI.4530-03.2004

Cesana, E., Pietrajtis, K., Bidoret, C., Isope, P., D'Angelo, E., Dieudonné, S., et al. (2013). Granule cell ascending axon excitatory synapses onto Golgi cells implement a potent feedback circuit in the cerebellar granular layer. *J. Neurosci.* 33, 12430–12446. doi: 10.1523/JNEUROSCI.4897-11.2013

Chadderton, P., Margrie, T. W., and Häusser, M. (2004). Integration of quanta in cerebellar granule cells during sensory processing. *Nature* 428, 856–860. doi: 10.1038/nature02442

Chance, F. S., Abbott, L. F., and Reyes, A. D. (2002). Gain modulation from background synaptic input. *Neuron* 35, 773–782. doi: 10.1016/s0896-6273(02)00820-6

Chan-Palay, V., and Palay, S. L. (1970). Interrelations of basket cell axons and climbing fibers in the cerebellar cortex of the rat. *Z. Anat. Entwicklungsgesch.* 132, 191–227. doi: 10.1007/bf00523377

Chan-Palay, V., and Palay, S. L. (1972). The stellate cells of the rat's cerebellar cortex. *Z. Anat. Entwicklungsgesch.* 136, 224–248. doi: 10.1007/BF00519180

Chan-Palay, V., Palay, S. L., and Billings-Gagliardi, S. M. (1974). Meynert cells in the primate visual cortex. *J. Neurocytol.* 3, 631–658. doi: 10.1007/bf01097628

Chan-Palay, V., Palay, S. L., Brown, J. T., and Van Itallie, C. (1977). Sagittal organization of olivocerebellar and reticulocerebellar projections: autoradiographic studies with 35S-methionine. *Exp. Brain Res.* 30, 561–576. doi: 10.1007/bf00237645

Chizhikov, V. V., Lindgren, A. G., Currle, D. S., Rose, M. F., Monuki, E. S., and Millen, K. J. (2006). The roof plate regulates cerebellar cell-type specification and proliferation. *Development* 133, 2793–2804. doi: 10.1242/dev.02441

Cohen, D., and Yarom, Y. (2000). Cerebellar on-beam and lateral inhibition: two functionally distinct circuits. *J. Neurophysiol.* 83, 1932–1940. doi: 10.1152/jn.2000.83.4.1932

Colin, F., Ris, L., and Godaux, E. (2002). "Neuroanatomy of cerebellum," in *The Cerebellum and Its Disorders*, eds M. Manto and M. Pandolfo (Cambridge, MA: Cambridge University Press), 6–29.

Consalez, G. G., and Hawkes, R. (2012). The compartmental restriction of cerebellar interneurons. *Front. Neural Circuits* 6:123. doi: 10.3389/fncir.2012.00123

Cooke, S. F., Attwell, P. J., and Yeo, C. H. (2004). Temporal properties of cerebellar-dependent memory consolidation. *J. Neurosci.* 24, 2934–2941. doi: 10.1523/JNEUROSCI.5505-03.2004

Courtemanche, R., and Lamarre, Y. (2005). Local field potential oscillations in primate cerebellar cortex: synchronization with cerebral cortex during active and passive expectancy. *J. Neurophysiol.* 93, 2039–2052. doi: 10.1152/jn.00080.2004

Courtemanche, R., Pellerin, J. P., and Lamarre, Y. (2002). Local field potential oscillations in primate cerebellar cortex: modulation during active and passive expectancy. *J. Neurophysiol.* 88, 771–782. doi: 10.1152/jn.2002.88.2.771

Crook, J. D., Hendrickson, A., Erickson, A., Possin, D., and Robinson, F. R. (2007). Purkinje cell axon collaterals terminate on Cat-301+ neurons in *Macaca* monkey cerebellum. *Neuroscience* 149, 834–844. doi: 10.1016/j.neuroscience.2007.08.030

Crook, J., Hendrickson, A., and Robinson, F. R. (2006). Co-localization of glycine and gaba immunoreactivity in interneurons in Macaca monkey cerebellar cortex. *Neuroscience* 141, 1951–1959. doi: 10.1016/j.neuroscience.2006.05.012

Cruikshank, S. J., Lewis, T. J., and Connors, B. W. (2007). Synaptic basis for intense thalamocortical activation of feedforward inhibitory cells in neocortex. *Nat. Neurosci.* 10, 462–468. doi: 10.1038/nn1861

D'Angelo, E. (2008). The critical role of Golgi cells in regulating spatio-temporal integration and plasticity at the cerebellum input stage. *Front. Neurosci.* 2, 35–46. doi: 10.3389/neuro.01.008.2008

D'Angelo, E. (2014). The organization of plasticity in the cerebellar cortex: from synapses to control. *Prog. Brain Res.* 210, 31–58. doi: 10.1016/b978-0-444-63356-9.00002-9

D'Angelo, E. (2016). "Challenging Marr's theory of the cerebellum," in *Computational Theories and Their Implementation in the Brain*, eds L. Vaina and R. Passingham (Oxford, UK: Oxford University Press), 62–78.

D'Angelo, E. (2018). "Physiology of cerebellum," in *The Cerebellum: From Embriology to Diagnostic Investigations*, eds M. Manto and T. Huisman (The Netherlands: Elsevier).

D'Angelo, E., Antonietti, A., Casali, S., Casellato, C., Garrido, J. A., Luque, N. R., et al. (2016). Modeling the cerebellar microcircuit: new strategies for a long-standing issue. *Front. Cell. Neurosci.* 10:176. doi: 10.3389/fncel.2016.00176

D'Angelo, E., De Filippi, G., Rossi, P., and Taglietti, V. (1995). Synaptic excitation of individual rat cerebellar granule cells *in situ*: evidence for the role of NMDA receptors. *J. Physiol.* 484, 397–413. doi: 10.1113/jphysiol.1995.sp020673

D'Angelo, E., and De Zeeuw, C. I. (2009). Timing and plasticity in the cerebellum: focus on the granular layer. *Trends Neurosci.* 32, 30–40. doi: 10.1016/j.tins.2008.09.007

D'Angelo, E., Koekkoek, S. K., Lombardo, P., Solinas, S., Ros, E., Garrido, J., et al. (2009). Timing in the cerebellum: oscillations and resonance in the granular layer. *Neuroscience* 162, 805–815. doi: 10.1016/j.neuroscience.2009.01.048

D'Angelo, E., Solinas, S., Mapelli, J., Gandolfi, D., Mapelli, L., and Prestori, F. (2013). The cerebellar Golgi cell and spatiotemporal organization of granular layer activity. *Front. Neural Circuits* 7:93. doi: 10.3389/fncir.2013.00093

Dastjerdi, F. V., Consalez, G. G., and Hawkes, R. (2012). Pattern formation during development of the embryonic cerebellum. *Front. Neuroanat.* 6:10. doi: 10.3389/fnana.2012.00010

Davie, J. T., Clark, B. A., and Häusser, M. (2008). The origin of the complex spike in cerebellar Purkinje cells. *J. Neurosci.* 28, 7599–7609. doi: 10.1523/JNEUROSCI.0559-08.2008

De Schutter, E., and Bjaalie, J. G. (2001). Coding in the granular layer of the cerebellum. *Prog. Brain Res.* 130, 279–296. doi: 10.1016/s0079-6123(01)30019-5

De Zeeuw, C. I., Koekkoek, S. K., Wylie, D. R., and Simpson, J. I. (1997). Association between dendritic lamellar bodies and complex spike synchrony

in the olivocerebellar system. *J. Neurophysiol.* 77, 1747–1758. doi: 10.1152/jn.1997.77.4.1747

Dean, P., and Porrill, J. (2010). The cerebellum as an adaptive filter: a general model? *Funct. Neurol.* 25, 173–180.

Dean, I., Robertson, S. J., and Edwards, F. A. (2003). Serotonin drives a novel GABAergic synaptic current recorded in rat cerebellar purkinje cells: a Lugaro cell to Purkinje cell synapse. *J. Neurosci.* 23, 4457–4469. doi: 10.1523/JNEUROSCI.23-11-04457.2003

DeFelipe, J., Hendry, S. H., and Jones, E. G. (1986). A correlative electron microscopic study of basket cells and large GABAergic neurons in the monkey sensory-motor cortex. *Neuroscience* 17, 991–1009. doi: 10.1016/0306-4522(86)90075-8

DeFelipe, J., López-Cruz, P. L., Benavides-Piccione, R., Bielza, C., Larrañaga, P., Anderson, S., et al. (2013). New insights into the classification and nomenclature of cortical GABAergic interneurons. *Nat. Rev. Neurosci.* 14, 202–216. doi: 10.1038/nrn3444

Dennis, D. J., Han, S., and Schuurmans, C. (2019). bHLH transcription factors in neural development, disease, and reprogramming. *Brain Res.* 1705, 48–65. doi: 10.1016/j.brainres.2018.03.013

Dieudonné, S. (1995). Glycinergic synaptic currents in Golgi cells of the rat cerebellum. *Proc. Natl. Acad. Sci. U S A* 92, 1441–1445. doi: 10.1073/pnas.92.5.1441

Dieudonné, S. (1998). Submillisecond kinetics and low efficacy of parallel fibre-Golgi cell synaptic currents in the rat cerebellum. *J. Physiol.* 510, 845–866. doi: 10.1111/j.1469-7793.1998.845bj.x

Dieudonné, S., and Dumoulin, A. (2000). Serotonin-driven long-range inhibitory connections in the cerebellar cortex. *J. Neurosci.* 20, 1837–1848. doi: 10.1523/JNEUROSCI.20-05-01837.2000

Diño, M. R., Willard, F. H., and Mugnaini, E. (1999). Distribution of unipolar brush cells and other calretinin immunoreactive components in the mammalian cerebellar cortex. *J. Neurocytol.* 28, 99–123. doi: 10.1023/a:1007072105919

Diwakar, S., Magistretti, J., Goldfarb, M., Naldi, G., and D'Angelo, E. (2009). Axonal Na^+ channels ensure fast spike activation and back-propagation in cerebellar granule cells. *J. Neurophysiol.* 101, 519–532. doi: 10.1152/jn.90382.2008

Dizon, M. J., and Khodakhah, K. (2011). The role of interneurons in shaping Purkinje cell responses in the cerebellar cortex. *J. Neurosci.* 31, 10463–10473. doi: 10.1523/JNEUROSCI.1350-11.2011

Dover, K., Marra, C., Solinas, S., Popovic, M., Subramaniyam, S., Zecevic, D., et al. (2016). FHF-independent conduction of action potentials along the leak-resistant cerebellar granule cell axon. *Nat. Commun.* 7:12895. doi: 10.1038/ncomms12895

Dugué, G. P., Brunel, N., Hakim, V., Schwartz, E., Chat, M., Lévesque, M., et al. (2009). Electrical coupling mediates tunable low-frequency oscillations and resonance in the cerebellar Golgi cell network. *Neuron* 61, 126–139. doi: 10.1016/j.neuron.2008.11.028

Dugué, G. P., Dumoulin, A., Triller, A., and Dieudonné, S. (2005). Target-dependent use of co-released inhibitory transmitters at central synapses. *J. Neurosci.* 25, 6490–6498. doi: 10.1523/JNEUROSCI.1500-05.2005

Duguid, I., Branco, T., Chadderton, P., Arlt, C., Powell, K., and Häusser, M. (2015). Control of cerebellar granule cell output by sensory-evoked Golgi cell inhibition. *Proc. Natl. Acad. Sci. U S A* 112, 13099–13104. doi: 10.1073/pnas.1510249112

Duguid, I., Branco, T., London, M., Chadderton, P., and Häusser, M. (2012). Tonic inhibition enhances fidelity of sensory information transmission in the cerebellar cortex. *J. Neurosci.* 32, 11132–11143. doi: 10.1523/JNEUROSCI.0460-12.2012

Dumoulin, A., Triller, A., and Dieudonné, S. (2001). IPSC kinetics at identified GABAergic and mixed GABAergic and glycinergic synapses onto cerebellar Golgi cells. *J. Neurosci.* 21, 6045–6057. doi: 10.1523/JNEUROSCI.21-16-06045.2001

Eccles, J. C. (1967). Circuits in the cerebellar control of movement. *Proc. Natl. Acad. Sci. U S A* 58, 336–343. doi: 10.1073/pnas.58.1.336

Eccles, J. C. (1973). The cerebellum as a computer: patterns in space and time. *J. Physiol.* 229, 1–32. doi: 10.1113/jphysiol.1973.sp010123

Eccles, J. C., Ito, M., and Szentagothai, J. (1967). *The Cerebellum as a Neuronal Machine.* Berlin, Heidelberg, New York, NY: Springer-Verlag, 337–347.

Eccles, J., Llinas, R., and Sasaki, K. (1964). Golgi cell inhibition in the cerebellar cortex. *Nature* 204, 1265–1266. doi: 10.1038/2041265a0

Eccles, J. C., Llinás, R., and Sasaki, K. (1966a). The inhibitory interneurones within the cerebellar cortex. *Exp. Brain Res.* 1, 1–16. doi: 10.1007/bf00235206

Eccles, J. C., Llinás, R., Sasaki, K., and Voorhoeve, P. E. (1966b). Interaction experiments on the responses evoked in Purkinje cells by climbing fibres. *J. Physiol.* 182, 297–315. doi: 10.1113/jphysiol.1966.sp007825

Edgley, S. A., and Lidierth, M. (1987). The discharges of cerebellar Golgi cells during locomotion in the cat. *J. Physiol.* 392, 315–332. doi: 10.1113/jphysiol.1987.sp016782

Eyre, M. D., and Nusser, Z. (2016). Only a minority of the inhibitory inputs to cerebellar golgi cells originates from local GABAergic cells. *eNeuro* 3:ENEURO.0055-16.2016. doi: 10.1523/ENEURO.0055-16.2016

Farrant, M., and Nusser, Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat. Rev. Neurosci.* 6, 215–229. doi: 10.1038/nrn1625

Feldmeyer, D., Qi, G., Emmenegger, V., and Staiger, J. F. (2018). Inhibitory interneurons and their circuit motifs in the many layers of the barrel cortex. *Neuroscience* 368, 132–151. doi: 10.1016/j.neuroscience.2017.05.027

Flace, P., Benagiano, V., Lorusso, L., Girolamo, F., Rizzi, A., Virgintino, D., et al. (2004). Glutamic acid decarboxylase immunoreactive large neuron types in the granular layer of the human cerebellar cortex. *Anat. Embryol.* 208, 55–64. doi: 10.1007/s00429-003-0374-x

Florio, M., Leto, K., Muzio, L., Tinterri, A., Badaloni, A., Croci, L., et al. (2012). Neurogenin 2 regulates progenitor cell-cycle progression and Purkinje cell dendritogenesis in cerebellar development. *Development* 139, 2308–2320. doi: 10.1242/dev.075861

Flourens, P. (1824). *Recherchers Experimentales Sur le Proprietes et les Functions du Systeme Nerveux Dans les Animaux Vertebres.* Paris: Crevot.

Forti, L., Cesana, E., Mapelli, J., and D'Angelo, E. (2006). Ionic mechanisms of autorhythmic firing in rat cerebellar Golgi cells. *J. Physiol.* 574, 711–729. doi: 10.1113/jphysiol.2006.110858

Fox, C. A., Hillman, D. E., Siegesmund, K. A., and Dutta, C. R. (1967). The primate cerebellar cortex: a Golgi and electron microscopic study. *Prog. Brain Res.* 25, 174–225. doi: 10.1016/s0079-6123(08)60965-6

Frings, M., Gaertner, K., Buderath, P., Gerwig, M., Christiansen, H., Schoch, B., et al. (2010). Timing of conditioned eyeblink responses is impaired in children with attention-deficit/hyperactivity disorder. *Exp. Brain Res.* 201, 167–176. doi: 10.1007/s00221-009-2020-1

Gabernet, L., Jadhav, S. P., Feldman, D. E., Carandini, M., and Scanziani, M. (2005). Somatosensory integration controlled by dynamic thalamocortical feed-forward inhibition. *Neuron* 48, 315–327. doi: 10.1016/j.neuron.2005.09.022

Galas, L., Bénard, M., Lebon, A., Komuro, Y., Schapman, D., Vaudry, H., et al. (2017). Postnatal migration of cerebellar interneurons. *Brain Sci.* 7:E62. doi: 10.3390/brainsci7060062

Galliano, E., Baratella, M., Sgritta, M., Ruigrok, T. J., Haasdijk, E. D., Hoebeek, F. E., et al. (2013). Anatomical investigation of potential contacts between climbing fibers and cerebellar Golgi cells in the mouse. *Front. Neural Circuits* 7:59. doi: 10.3389/fncir.2013.00059

Galliano, E., Mazzarello, P., and D'Angelo, E. (2010). Discovery and rediscoveries of Golgi cells. *J. Physiol.* 588, 3639–3655. doi: 10.1113/jphysiol.2010.189605

Gandolfi, D., Lombardo, P., Mapelli, J., Solinas, S., and D'Angelo, E. (2013). θ-frequency resonance at the cerebellum input stage improves spike timing on the millisecond time-scale. *Front. Neural Circuits* 7:64. doi: 10.3389/fncir.2013.00064

Gao, W., Chen, G., Reinert, K., and Ebner, T. (2006). Cerebellar cortical molecular layer inhibition is organized in parasagittal zones. *J. Neurosci.* 26, 8377–8387. doi: 10.1523/JNEUROSCI.2434-06.2006

Gao, Z., Proietti-Onori, M., Lin, Z., Ten Brinke, M. M., Boele, H. J., Potters, J. W., et al. (2016). Excitatory cerebellar nucleocortical circuit provides internal amplification during associative conditioning. *Neuron* 89, 645–657. doi: 10.1016/j.neuron.2016.01.008

Garrido, J. A., Ros, E., and D'Angelo, E. (2013). Spike timing regulation on the millisecond scale by distributed synaptic plasticity at the cerebellum input stage:

a simulation study. *Front. Comput. Neurosci.* 7:64. doi: 10.3389/fncom.2013.00064

Geminiani, A., Casellato, C., Locatelli, F., Prestori, F., Pedrocchi, A., and D'Angelo, E. (2018). Complex dynamics in simplified neuronal models: reproducing golgi cell electroresponsiveness. *Front. Neuroinform.* 12:88. doi: 10.3389/fninf.2018.00088

Geurts, F. J., De Schutter, E., and Dieudonné, S. (2003). Unraveling the cerebellar cortex: cytology and cellular physiology of large-sized interneurons in the granular layer. *Cerebellum* 2, 290–299. doi: 10.1080/14734220310011948

Geurts, F. J., De Schutter, E., and Timmermans, J. P. (2002). Localization of 5-HT2A, 5-HT3, 5-HT5A and 5-HT7 receptor-like immunoreactivity in the rat cerebellum. *J. Chem. Neuroanat.* 24, 65–74. doi: 10.1016/s0891-0618(02)00020-0

Geurts, F. J., Timmermans, J., Shigemoto, R., and De Schutter, E. (2001). Morphological and neurochemical differentiation of large granular layer interneurons in the adult rat cerebellum. *Neuroscience* 104, 499–512. doi: 10.1016/s0306-4522(01)00058-6

Glasgow, S. M., Henke, R. M., Macdonald, R. J., Wright, C. V., and Johnson, J. E. (2005). Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. *Development* 132, 5461–5469. doi: 10.1242/dev.02167

Glykys, J., and Mody, I. (2007). Activation of $GABA_A$ receptors: views from outside the synaptic cleft. *Neuron* 56, 763–770. doi: 10.1016/j.neuron.2007.11.002

Golgi, C. (1873). *Sulla Struttura Della Grigia del Cervello.* Lombardia: Gazzetta Medica Italiana.

Golgi, C. (1874). Sulla fina anatomia del cervelletto umano. *Arch. Ital. Malat. Nerv. Part. Alienaz. Ment.* 11, 90–107.

Golgi, C. (1883). Sulla fina anatomia degli organi centrali del sistema nervoso IV. Sulla fina anatomia delle circomvoluzioni cerebellari. *Riv. Sper. Freniatr. Med. Leg. Alien. Ment.* 9, 1–17.

Gorcs, T. J., Penke, B., Boti, Z., Katarova, Z., and Hamori, J. (1993). Immunohistochemical visualization of a metabotropic glutamate receptor. *Neuroreport* 4, 283–286. doi: 10.1097/00001756-199303000-00014

Grimaldi, P., Parras, C., Guillemot, F., Rossi, F., and Wassef, M. (2009). Origins and control of the differentiation of inhibitory interneurons and glia in the cerebellum. *Dev. Biol.* 328, 422–433. doi: 10.1016/j.ydbio.2009.02.008

Gundappa-Sulur, G., De Schutter, E., and Bower, J. M. (1999). Ascending granule cell axon: an important component of cerebellar cortical circuitry. *J. Comp. Neurol.* 408, 580–596. doi: 10.1002/(sici)1096-9861(19990614)408:4<580::aid-cne11>3.0.co;2-o

Gupta, A., Wang, Y., and Markram, H. (2000). Organizing principles for a diversity of GABAergic interneurons and synapses in the neocortex. *Science* 287, 273–278. doi: 10.1126/science.287.5451.273

Hamann, M., Rossi, D. J., and Attwell, D. (2002). Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron* 33, 625–633. doi: 10.1016/s0896-6273(02)00593-7

Hámori, J., and Szentágothai, J. (1966). Participation of Golgi neuron processes in the cerebellar glomeruli: an electron microscope study. *Exp. Brain Res.* 2, 35–48. doi: 10.1007/bf00234359

Hámori, J., and Szentágothai, J. (1980). Lack of evidence of synaptic contacts by climbing fibre collaterals to basket and stellate cells in developing rat cerebellar cortex. *Brain Res.* 186, 454–457. doi: 10.1016/0006-8993(80)90990-7

Hámori, J., Takács, J., and Görcs, T. J. (1996). Immunocytochemical localization of mGluR1a metabotropic glutamate receptor in inhibitory interneurons of the cerebellar cortex. *Acta Biol. Hung.* 47, 181–194.

Hansel, C., Linden, D. J., and D'Angelo, E. (2001). Beyond parallel fiber LTD: the diversity of synaptic and non-synaptic plasticity in the cerebellum. *Nat. Neurosci.* 4, 467–475. doi: 10.1038/87419

Hartline, H. K., and Ratliff, F. (1957). Inhibitory interaction of receptor units in the eye of Limulus. *J. Gen. Physiol.* 40, 357–376. doi: 10.1085/jgp.40.3.357

Hartmann, M. J., and Bower, J. M. (1998). Oscillatory activity in the cerebellar hemispheres of unrestrained rats. *J. Neurophysiol.* 80, 1598–1604. doi: 10.1152/jn.1998.80.3.1598

Hausser, M., and Clark, B. A. (1997). Tonic synaptic inhibition modulates neuronal output pattern and spatiotemporal synaptic integration. *Neuron* 19, 665–678. doi: 10.1016/s0896-6273(00)80379-7

Heiney, S. A., Wohl, M. P., Chettih, S. N., Ruffolo, L. I., and Medina, J. F. (2014). Cerebellar-dependent expression of motor learning during eyeblink conditioning in head-fixed mice. *J. Neurosci.* 34, 14845–14853. doi: 10.1523/JNEUROSCI.2820-14.2014

Hirono, M., Saitow, F., Kudo, M., Suzuki, H., Yanagawa, Y., Yamada, M., et al. (2012). Cerebellar globular cells receive monoaminergic excitation and monosynaptic inhibition from Purkinje cells. *PLoS One* 7:e29663. doi: 10.1371/journal.pone.0029663

Hockberger, P. E., Yousif, L., and Nam, S. C. (1994). Identification of acutely isolated cells from developing rat cerebellum. *Neuroimage* 1, 276–287. doi: 10.1006/nimg.1994.1012

Hockfield, S. (1987). A Mab to a unique cerebellar neuron generated by immunosuppression and rapid immunization. *Science* 237, 67–70. doi: 10.1126/science.3603010

Holtzman, T., Mostofi, A., Phuah, C. L., and Edgley, S. A. (2006a). Cerebellar Golgi cells in the rat receive multimodal convergent peripheral inputs *via* the lateral funiculus of the spinal cord. *J. Physiol.* 577, 69–80. doi: 10.1113/jphysiol.2006.117218

Holtzman, T., Rajapaksa, T., Mostofi, A., and Edgley, S. A. (2006b). Different responses of rat cerebellar Purkinje cells and Golgi cells evoked by widespread convergent sensory inputs. *J. Physiol.* 574, 491–507. doi: 10.1113/jphysiol.2006.108282

Hori, K., Cholewa-Waclaw, J., Nakada, Y., Glasgow, S. M., Masui, T., Henke, R. M., et al. (2008). A nonclassical bHLH Rbpj transcription factor complex is required for specification of GABAergic neurons independent of Notch signaling. *Genes Dev.* 22, 166–178. doi: 10.1101/gad.1628008

Hoshino, M. (2006). Molecular machinery governing GABAergic neuron specification in the cerebellum. *Cerebellum* 5, 193–198. doi: 10.1080/14734220600589202

Hoshino, M., Nakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y. V., et al. (2005). Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. *Neuron* 47, 201–213. doi: 10.1016/j.neuron.2005.06.007

Hull, C. A., Chu, Y., Thanawala, M., and Regehr, W. G. (2013). Hyperpolarization induces a long-term increase in the spontaneous firing rate of cerebellar Golgi cells. *J. Neurosci.* 33, 5895–5902. doi: 10.1523/JNEUROSCI.4052-12.2013

Hull, C., and Regehr, W. G. (2012). Identification of an inhibitory circuit that regulates cerebellar Golgi cell activity. *Neuron* 73, 149–158. doi: 10.1016/j.neuron.2011.10.030

Isope, P., and Barbour, B. (2002). Properties of unitary granule cell-->Purkinje cell synapses in adult rat cerebellar slices. *J. Neurosci.* 22, 9668–9678. doi: 10.1523/JNEUROSCI.22-22-09668.2002

Ito, M. (1984). The modifiable neuronal network of the cerebellum. *Jpn. J. Physiol.* 34, 781–792. doi: 10.2170/jjphysiol.34.781

Ito, M. (2002). Hopes for cerebellar research in the 21st century. *Cerebellum* 1, 93–94. doi: 10.1080/147342202753671213

Ito, M. (2013). Error detection and representation in the olivo-cerebellar system. *Front. Neural Circuits* 7:1. doi: 10.3389/fncir.2013.00001

Ito, M., Yamaguchi, K., Nagao, S., and Yamazaki, T. (2014). Long-term depression as a model of cerebellar plasticity. *Prog. Brain Res.* 210, 1–30. doi: 10.1016/b978-0-444-63356-9.00001-7

Jacobs, B., Johnson, N. L., Wahl, D., Schall, M., Maseko, B. C., Lewandowski, A., et al. (2014). Comparative neuronal morphology of the cerebellar cortex in afrotherians, carnivores, cetartiodactyls and primates. *Front. Neuroanat.* 8:24. doi: 10.3389/fnana.2014.00024

Jaeger, D., and Bower, J. M. (1999). Synaptic control of spiking in cerebellar Purkinje cells: dynamic current clamp based on model conductances. *J. Neurosci.* 19, 6090–6101. doi: 10.1523/JNEUROSCI.19-14-06090.1999

Jaeger, D., De Schutter, E., and Bower, J. M. (1997). The role of synaptic and voltage-gated currents in the control of Purkinje cell spiking: a modeling study. *J. Neurosci.* 17, 91–106. doi: 10.1523/JNEUROSCI.17-01-00091.1997

Jelitai, M., Puggioni, P., Ishikawa, T., Rinaldi, A., and Duguid, I. (2016). Dendritic excitation-inhibition balance shapes cerebellar output during motor behaviour. *Nat. Commun.* 7:13722. doi: 10.1038/ncomms13722

Jirenhed, D. A., Bengtsson, F., and Hesslow, G. (2007). Acquisition, extinction, and reacquisition of a cerebellar cortical memory trace. *J. Neurosci.* 27, 2493–2502. doi: 10.1523/JNEUROSCI.4202-06.2007

Johansson, O., Hokfelt, T., and Elde, R. P. (1984). Immunohistochemical distribution of somatostatin-like immunoreactivity in the central nervous

system of the adult rat. *Neuroscience* 13, 265–339. doi: 10.1016/0306-4522(84)90233-1

Jörntell, H., Bengtsson, F., Schonewille, M., and De Zeeuw, C. I. (2010). Cerebellar molecular layer interneurons—computational properties and roles in learning. *Trends Neurosci.* 33, 524–532. doi: 10.1016/j.tins.2010.08.004

Jörntell, H., and Ekerot, C. F. (2002). Reciprocal bidirectional plasticity of parallel fiber receptive fields in cerebellar Purkinje cells and their afferent interneurons. *Neuron* 34, 797–806. doi: 10.1016/s0896-6273(02)00713-4

Jörntell, H., and Ekerot, C. F. (2003). Receptive field plasticity profoundly alters the cutaneous parallel fiber synaptic input to cerebellar interneurons *in vivo*. *J. Neurosci.* 23, 9620–9631. doi: 10.1523/jneurosci.23-29-09620.2003

Kanichay, R. T., and Silver, R. A. (2008). Synaptic and cellular properties of the feedforward inhibitory circuit within the input layer of the cerebellar cortex. *J. Neurosci.* 28, 8955–8967. doi: 10.1523/jneurosci.5469-07.2008

Kepecs, A., and Fishell, G. (2014). Interneuron cell types are fit to function. *Nature* 505, 318–326. doi: 10.1038/nature12983

Kim, S. S., Yoo, S. W., Park, T. S., Ahn, S. C., Jeong, H. S., Kim, J. W., et al. (2008). Neural induction with neurogenin1 increases the therapeutic effects of mesenchymal stem cells in the ischemic brain. *Stem Cells* 26, 2217–2228. doi: 10.1634/stemcells.2008-0108

Kondo, S., and Marty, A. (1998). Synaptic currents at individual connections among stellate cells in rat cerebellar slices. *J. Physiol.* 509, 221–232. doi: 10.1111/j.1469-7793.1998.221bo.x

Kosaka, T., Kosaka, K., Nakayama, T., Hunziker, W., and Heizmann, C. W. (1993). Axons and axon terminals of cerebellar Purkinje cells and basket cells have higher levels of parvalbumin immunoreactivity than somata and dendrites: quantitative analysis by immunogold labeling. *Exp. Brain Res.* 93, 483–491. doi: 10.1007/bf00229363

Kubota, Y., and Kawaguchi, Y. (1994). Three classes of GABAergic interneurons in neocortex and neostriatum. *Jpn. J. Physiol.* 44, S145–S148.

Kubota, Y., and Kawaguchi, Y. (1997). Two distinct subgroups of cholecystokinin-immunoreactive cortical interneurons. *Brain Res.* 752, 175–183. doi: 10.1016/s0006-8993(96)01446-1

Kubota, Y., Mikawa, S., and Kawaguchi, Y. (1993). Neostriatal GABAergic interneurones contain NOS, calretinin or parvalbumin. *Neuroreport* 5, 205–208. doi: 10.1097/00001756-199312000-00004

Kubota, Y., Shigematsu, N., Karube, F., Sekigawa, A., Kato, S., Yamaguchi, N., et al. (2011). Selective coexpression of multiple chemical markers defines discrete populations of neocortical GABAergic neurons. *Cereb. Cortex* 21, 1803–1817. doi: 10.1093/cercor/bhq252

Lachamp, P. M., Liu, Y., and Liu, S. J. (2009). Glutamatergic modulation of cerebellar interneuron activity is mediated by an enhancement of GABA release and requires protein kinase A/RIM1α signaling. *J. Neurosci.* 29, 381–392. doi: 10.1523/jneurosci.2354-08.2009

Lainé, J., and Axelrad, H. (1994). The candelabrum cell: a new interneuron in the cerebellar cortex. *J. Comp. Neurol.* 339, 159–173. doi: 10.1002/cne.903390202

Lainé, J., and Axelrad, H. (1996). Morphology of the Golgi-impregnated Lugaro cell in the rat cerebellar cortex: a reappraisal with a description of its axon. *J. Comp. Neurol.* 375, 618–640. doi: 10.1002/(sici)1096-9861(19961125)375:4<618::aid-cne5>3.0.co;2-4

Lainé, J., and Axelrad, H. (1998). Lugaro cells target basket and stellate cells in the cerebellar cortex. *Neuroreport* 9, 2399–2403. doi: 10.1097/00001756-199807130-00045

Lainé, J., and Axelrad, H. (2002). Extending the cerebellar Lugaro cell class. *Neuroscience* 115, 363–374. doi: 10.1016/s0306-4522(02)00421-9

Lainé, J., Axelrad, H., and Rahbi, N. (1992). Intermediate cells of Lugaro are present in the immature rat cerebellar cortex at an earlier stage than previously thought. *Neurosci. Lett.* 145, 225–228. doi: 10.1016/0304-3940(92)90028-6

Lang, E. J., Sugihara, I., Welsh, J. P., and Llinas, R. (1999). Patterns of spontaneous purkinje cell complex spike activity in the awake rat. *J. Neurosci.* 19, 2728–2739. doi: 10.1523/jneurosci.19-07-02728.1999

Lemeky-Johnston, N., and Larramendi, L. M. (1968). Morphological characteristics of mouse stellate and basket cells and their neuroglial envelope: an electron microscopic study. *J. Comp. Neurol.* 134, 39–72. doi: 10.1002/cne.901340105

Lennon, W., Hecht-Nielsen, R., and Yamazaki, T. (2014). A spiking network model of cerebellar Purkinje cells and molecular layer interneurons exhibiting irregular firing. *Front. Comput. Neurosci.* 8:157. doi: 10.3389/fncom.2014.00157

Leto, K., Bartolini, A., and Rossi, F. (2008). Development of cerebellar GABAergic interneurons: origin and shaping of the "minibrain" local connections. *Cerebellum* 7, 523–529. doi: 10.1007/s12311-008-0079-z

Leto, K., Bartolini, A., Yanagawa, Y., Obata, K., Magrassi, L., Schilling, K., et al. (2009). Laminar fate and phenotype specification of cerebellar GABAergic interneurons. *J. Neurosci.* 29, 7079–7091. doi: 10.1523/jneurosci.0957-09.2009

Leto, K., Carletti, B., Williams, I. M., Magrassi, L., and Rossi, F. (2006). Different types of cerebellar GABAergic interneurons originate from a common pool of multipotent progenitor cells. *J. Neurosci.* 26, 11682–11694. doi: 10.1523/jneurosci.3656-06.2006

Leto, K., Rolando, C., and Rossi, F. (2012). The genesis of cerebellar GABAergic neurons: fate potential and specification mechanisms. *Front. Neuroanat.* 6:6. doi: 10.3389/fnana.2012.00006

Leto, K., and Rossi, F. (2012). Specification and differentiation of cerebellar GABAergic neurons. *Cerebellum* 11, 434–435. doi: 10.1007/s12311-011-0324-8

Li, S. J., Wang, Y., Strahlendorf, H. K., and Strahlendorf, J. C. (1993). Serotonin alters an inwardly rectifying current (Ih) in rat cerebellar Purkinje cells under voltage clamp. *Brain Res.* 617, 87–95. doi: 10.1016/0006-8993(93)90617-v

Llinas, R., Leznik, E., and Makarenko, V. (2004). The olivo-cerebellar circuit as a universal control system. *IEEE J. Ocean. Eng.* 29, 631–639. doi: 10.1109/joe.2004.833212

Lu, H., Hartmann, M. J., and Bower, J. M. (2005). Correlations between purkinje cell single-unit activity and simultaneously recorded field potentials in the immediately underlying granule cell layer. *J. Neurophysiol.* 94, 1849–1860. doi: 10.1152/jn.01275.2004

Luciani, L. (1907). *Physiologie des Menschen.* Jena: Fisher.

Lugaro, E. (1894). Sulle connessioni tra gli elementi nervosi della corteccia cerebellare con considerazioni generali sul significato fisiologico dei rapporti tra gli elementi nervosi. *Riv. Sper. Freniat. Med. Leg.* 20, 297–331.

Lundell, T. G., Zhou, Q., and Doughty, M. L. (2009). Neurogenin1 expression in cell lineages of the cerebellar cortex in embryonic and postnatal mice. *Dev. Dyn.* 238, 3310–3325. doi: 10.1002/dvdy.22165

Madisen, L., Mao, T., Koch, H., Zhuo, J. M., Berenyi, A., Fujisawa, S., et al. (2012). A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. *Nat. Neurosci.* 15, 793–802. doi: 10.1038/nn.3078

Maex, R., and De Schutter, E. (1998). Synchronization of golgi and granule cell firing in a detailed network model of the cerebellar granule cell layer. *J. Neurophysiol.* 80, 2521–2537. doi: 10.1152/jn.1998.80.5.2521

Maex, R., and Gutkin, B. (2017). Temporal integration and 1/f power scaling in a circuit model of cerebellar interneurons. *J. Neurophysiol.* 118, 471–485. doi: 10.1152/jn.00789.2016

Mann-Metzer, P., and Yarom, Y. (1999). Electrotonic coupling interacts with intrinsic properties to generate synchronized activity in cerebellar networks of inhibitory interneurons. *J. Neurosci.* 19, 3298–3306. doi: 10.1523/jneurosci.19-09-03298.1999

Mapelli, J., and D'Angelo, E. (2007). The spatial organization of long-term synaptic plasticity at the input stage of cerebellum. *J. Neurosci.* 27, 1285–1296. doi: 10.1523/jneurosci.4873-06.2007

Mapelli, J., Gandolfi, D., and D'Angelo, E. (2010). High-pass filtering and dynamic gain regulation enhance vertical bursts transmission along the mossy fiber pathway of cerebellum. *Front. Cell. Neurosci.* 4:14. doi: 10.3389/fncel.2010.00014

Mapelli, L., Pagani, M., Garrido, J. A., 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

Mapelli, L., Rossi, P., Nieus, T., and D'Angelo, E. (2009). Tonic activation of GABAB receptors reduces release probability at inhibitory connections in the cerebellar glomerulus. *J. Neurophysiol.* 101, 3089–3099. doi: 10.1152/jn.91190.2008

Mapelli, L., Solinas, S., and D'Angelo, E. (2014). Integration and regulation of glomerular inhibition in the cerebellar granular layer circuit. *Front. Cell. Neurosci.* 8:55. doi: 10.3389/fncel.2014.00055

Maricich, S. M., and Herrup, K. (1999). Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. *J. Neurobiol.* 41, 281–294. doi: 10.1002/(sici)1097-4695(19991105)41:2<281::aid-neu10>3.0.co;2-5

Marr, D. (1969). A theory of cerebellar cortex. *J. Physiol.* 202, 437–470. doi: 10.1113/jphysiol.1969.sp008820

Masoli, S., and D'Angelo, E. (2017). Synaptic activation of a detailed purkinje cell model predicts voltage-dependent control of burst-pause responses in active dendrites. *Front. Cell. Neurosci.* 11:278. doi: 10.3389/fncel.2017.00278

Masoli, S., Solinas, S., and D'Angelo, E. (2015). Action potential processing in a detailed Purkinje cell model reveals a critical role for axonal compartmentalization. *Front. Cell. Neurosci.* 9:47. doi: 10.3389/fncel.2015.00047

Medina, J. F., and Mauk, M. D. (2000). Computer simulation of cerebellar information processing. *Nat. Neurosci.* 3, 1205–1211. doi: 10.1038/81486

Melik-Musyan, A. B., and Fanardzhyan, V. V. (2004). Morphological characteristics of Lugaro cells in the cerebellar cortex. *Neurosci. Behav. Physiol.* 34, 633–638. doi: 10.1023/b:neab.0000028297.30474.f9

Mitchell, S. J., and Silver, R. A. (2000a). Glutamate spillover suppresses inhibition by activating presynaptic mGluRs. *Nature* 404, 498–502. doi: 10.1038/35006649

Mitchell, S. J., and Silver, R. A. (2000b). GABA spillover from single inhibitory axons suppresses low-frequency excitatory transmission at the cerebellar glomerulus. *J. Neurosci.* 20, 8651–8658. doi: 10.1523/jneurosci.20-23-08651.2000

Mitchell, S. J., and Silver, R. A. (2003). Shunting inhibition modulates neuronal gain during synaptic excitation. *Neuron* 38, 433–445. doi: 10.1016/s0896-6273(03)00200-9

Mittmann, W., Koch, U., and Häusser, M. (2005). Feed-forward inhibition shapes the spike output of cerebellar Purkinje cells. *J. Physiol.* 563, 369–378. doi: 10.1113/jphysiol.2004.075028

Miyashita, Y., and Watanabe, E. (1984). Loss of vision-guided adaptation of the vestibulo-ocular reflex after depletion of brain serotonin in the rabbit. *Neurosci. Lett.* 51, 177–182. doi: 10.1016/0304-3940(84)90547-0

Molineux, M. L., Fernandez, F. R., Mehaffey, W. H., and Turner, R. W. (2005). A-type and T-type currents interact to produce a novel spike latency-voltage relationship in cerebellar stellate cells. *J. Neurosci.* 25, 10863–10873. doi: 10.1523/jneurosci.3436-05.2005

Najac, M., De Saint Jan, D., Reguero, L., Grandes, P., and Charpak, S. (2011). Monosynaptic and polysynaptic feed-forward inputs to mitral cells from olfactory sensory neurons. *J. Neurosci.* 31, 8722–8729. doi: 10.1523/jneurosci.0527-11.2011

Négyessy, L., Vidnyanszky, Z., Kuhn, R., Knopfel, T., Gorcs, T. J., and Hamori, J. (1997). Light and electron microscopic demonstration of mGluR5 metabotropic glutamate receptor immunoreactive neuronal elements in the rat cerebellar cortex. *J. Comp. Neurol.* 385, 641–650. doi: 10.1002/(sici)1096-9861(19970908)385:4<641::aid-cne9>3.0.co;2-3

Neki, A., Ohishi, H., Kaneko, T., Shigemoto, R., Nakanishi, S., and Mizuno, N. (1996). Metabotropic glutamate receptors mGluR2 and mGluR5 are expressed in two non-overlapping populations of Golgi cells in the rat cerebellum. *Neuroscience* 75, 815–826. doi: 10.1016/0306-4522(96)00316-8

Nieus, T. R., Mapelli, L., and D'Angelo, E. (2014). Regulation of output spike patterns by phasic inhibition in cerebellar granule cells. *Front. Cell. Neurosci.* 8:246. doi: 10.3389/fncel.2014.00246

Nieus, T., Sola, E., Mapelli, J., Saftenku, E., Rossi, P., and D'Angelo, E. (2006). LTP regulates burst initiation and frequency at mossy fiber-granule cell synapses of rat cerebellum: experimental observations and theoretical predictions. *J. Neurophysiol.* 95, 686–699. doi: 10.1152/jn.00696.2005

Oades, R. D., Lasky-Su, J., Christiansen, H., Faraone, S. V., Sonuga-Barke, E. J., Banaschewski, T., et al. (2008). The influence of serotonin- and other genes on impulsive behavioral aggression and cognitive impulsivity in children with attention-deficit/hyperactivity disorder (ADHD): findings from a family-based association test (FBAT) analysis. *Behav. Brain Funct.* 4:48. doi: 10.1186/1744-9081-4-48

Oberdick, J., and Sillitoe, R. V. (2011). Cerebellar zones: history, development and function. *Cerebellum* 10, 301–306. doi: 10.1007/s12311-011-0306-x

O'Donoghue, D. L., and Bishop, G. A. (1990). A quantitative analysis of the distribution of Purkinje cell axonal collaterals in different zones of the cat's cerebellum: an intracellular HRP study. *Exp. Brain Res.* 80, 63–71. doi: 10.1007/bf00228848

O'Donoghue, D. L., King, J. S., and Bishop, G. A. (1989). Physiological and anatomical studies of the interactions between Purkinje cells and basket cells in the cat's cerebellar cortex: evidence for a unitary relationship. *J. Neurosci.* 9, 2141–2150. doi: 10.1523/jneurosci.09-06-02141.1989

Ottersen, O. P., Storm-Mathisen, J., and Somogyi, P. (1988). Colocalization of glycine-like and GABA-like immunoreactivities in Golgi cell terminals in the rat cerebellum: a postembedding light and electron microscopic study. *Brain Res.* 450, 342–353. doi: 10.1016/0006-8993(88)91573-9

Palay, S. L., and Chan-Palay, V. (1974). *Cerebellar Cortex: Cytology and Organization*. New York, NY: Springer-Verlag.

Pascual, M., Abasolo, I., Mingorance-Le Meur, A., Martinez, A., Del Rio, J. A., Wright, C. V., et al. (2007). Cerebellar GABAergic progenitors adopt an external granule cell-like phenotype in the absence of Ptf1a transcription factor expression. *Proc. Natl. Acad. Sci. U S A* 104, 5193–5198. doi: 10.1073/pnas.0605699104

Pellerin, J. P., and Lamarre, Y. (1997). Local field potential oscillations in primate cerebellar cortex during voluntary movement. *J. Neurophysiol.* 78, 3502–3507. doi: 10.1152/jn.1997.78.6.3502

Pibiri, V., Gerosa, C., Vinci, L., Faa, G., and Ambu, R. (2017). Immunoreactivity pattern of calretinin in the developing human cerebellar cortex. *Acta Histochem.* 119, 228–234. doi: 10.1016/j.acthis.2017.01.005

Pijpers, A., Apps, R., Pardoe, J., Voogd, J., and Ruigrok, T. J. (2006). Precise spatial relationships between mossy fibers and climbing fibers in rat cerebellar cortical zones. *J. Neurosci.* 26, 12067–12080. doi: 10.1523/jneurosci.2905-06.2006

Pizzarelli, R., and Cherubini, E. (2011). Alterations of GABAergic signaling in autism spectrum disorders. *Neural Plast.* 2011:297153. doi: 10.1155/2011/297153

Pouille, F., and Scanziani, M. (2001). Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. *Science* 293, 1159–1163. doi: 10.1126/science.1060342

Ramakrishnan, K. B., Voges, K., De Propris, L., De Zeeuw, C. I., and D'Angelo, E. (2016). Tactile stimulation evokes long-lasting potentiation of purkinje cell discharge *in vivo*. *Front. Cell. Neurosci.* 10:36. doi: 10.3389/fncel.2016.00036

Raman, I. M., and Bean, B. P. (1997). Resurgent sodium current and action potential formation in dissociated cerebellar Purkinje neurons. *J. Neurosci.* 17, 4517–4526. doi: 10.1523/jneurosci.17-12-04517.1997

Rancillac, A., and Crépel, F. (2004). Synapses between parallel fibres and stellate cells express long-term changes in synaptic efficacy in rat cerebellum. *J. Physiol.* 554, 707–720. doi: 10.1113/jphysiol.2003.055871

Rancz, E. A., Ishikawa, T., Duguid, I., Chadderton, P., Mahon, S., and Häusser, M. (2007). High-fidelity transmission of sensory information by single cerebellar mossy fibre boutons. *Nature* 450, 1245–1248. doi: 10.1038/nature05995

Rieubland, S., Roth, A., and Häusser, M. (2014). Structured connectivity in cerebellar inhibitory networks. *Neuron* 81, 913–929. doi: 10.1016/j.neuron.2013.12.029

Robberechts, Q., Wijnants, M., Giugliano, M., and De Schutter, E. (2010). Long-term depression at parallel fiber to Golgi cell synapses. *J. Neurophysiol.* 104, 3413–3423. doi: 10.1152/jn.00030.2010

Rogers, J. H. (1989). Immunoreactivity for calretinin and other calcium-binding proteins in cerebellum. *Neuroscience* 31, 711–721. doi: 10.1016/0306-4522(89)90435-1

Rolando, L. (1809). *Saggio Sopra la Vera Struttura del Cervello Dell'uomo e Degli Animali e Sopra le Funzioni del Sistema Nervoso*. Sassari: Stamperia da S.S.R.M Previlegiata.

Rossi, D. J., and Hamann, M. (1998). Spillover-mediated transmission at inhibitory synapses promoted by high affinity α6 subunit $GABA_A$ receptors and glomerular geometry. *Neuron* 20, 783–795. doi: 10.1016/s0896-6273(00)81016-8

Rossi, D. J., Hamann, M., and Attwell, D. (2003). Multiple modes of GABAergic inhibition of rat cerebellar granule cells. *J. Physiol.* 548, 97–110. doi: 10.1113/jphysiol.2002.036459

Rossi, P., Mapelli, L., Roggeri, L., Gall, D., de Kerchove d'Exaerde, A., Schiffmann, S. N., et al. (2006). Inhibition of constitutive inward rectifier currents in cerebellar granule cells by pharmacological and synaptic activation

of GABA receptors. *Eur. J. Neurosci.* 24, 419–432. doi: 10.1111/j.1460-9568.2006.04914.x

Rousseau, C. V., Dugué, G. P., Dumoulin, A., Mugnaini, E., Dieudonné, S., and Diana, M. A. (2012). Mixed inhibitory synaptic balance correlates with glutamatergic synaptic phenotype in cerebellar unipolar brush cells. *J. Neurosci.* 32, 4632–4644. doi: 10.1523/jneurosci.5122-11.2012

Rowan, M. J. M., Bonnan, A., Zhang, K., Amat, S. B., Kikuchi, C., Taniguchi, H., et al. (2018). Graded control of climbing-fiber-mediated plasticity and learning by inhibition in the cerebellum. *Neuron* 99, 999.e6–1015.e6. doi: 10.1016/j.neuron.2018.07.024

Santamaria, F., and Bower, J. M. (2005). Background synaptic activity modulates the response of a modeled purkinje cell to paired afferent input. *J. Neurophysiol.* 93, 237–250. doi: 10.1152/jn.00458.2004

Santamaria, F., Jaeger, D., De Schutter, E., and Bower, J. M. (2002). Modulatory effects of parallel fiber and molecular layer interneuron synaptic activity on purkinje cell responses to ascending segment input: a modeling study. *J. Comput. Neurosci.* 13, 217–235. doi: 10.1023/A:1020266315730

Santamaria, F., Tripp, P. G., and Bower, J. M. (2007). Feedforward inhibition controls the spread of granule cell-induced Purkinje cell activity in the cerebellar cortex. *J. Neurophysiol.* 97, 248–263. doi: 10.1152/jn.01098.2005

Schilling, K. (2000). Lineage, development and morphogenesis of cerebellar interneurons. *Prog. Brain Res.* 124, 51–68. doi: 10.1016/s0079-6123(00)24007-7

Schilling, K., Oberdick, J., Rossi, F., and Baader, S. L. (2008). Besides Purkinje cells and granule neurons: an appraisal of the cell biology of the interneurons of the cerebellar cortex. *Histochem. Cell Biol.* 130, 601–615. doi: 10.1007/s00418-008-0483-y

Schneeberger, P. R., Norman, A. W., and Heizmann, C. W. (1985). Parvalbumin and vitamin D-dependent calcium-binding protein (Mr 28,000): comparison of their localization in the cerebellum of normal and rachitic rats. *Neurosci. Lett.* 59, 97–103. doi: 10.1016/0304-3940(85)90221-6

Schulman, J. A., and Bloom, F. E. (1981). Golgi cells of the cerebellum are inhibited by inferior olive activity. *Brain Res.* 210, 350–355. doi: 10.1016/0006-8993(81)90908-2

Schwaller, B., Meyer, M., and Schiffmann, S. (2002). 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. *Cerebellum* 1, 241–258. doi: 10.1080/147342202320883551

Semyanov, A., Walker, M. C., Kullmann, D. M., and Silver, R. A. (2004). Tonically active $GABA_A$ receptors: modulating gain and maintaining the tone. *Trends Neurosci.* 27, 262–269. doi: 10.1016/j.tins.2004.03.005

Shinoda, Y., Sugihara, I., Wu, H. S., and Sugiuchi, Y. (2000). The entire trajectory of single climbing and mossy fibers in the cerebellar nuclei and cortex. *Prog. Brain Res.* 124, 173–186. doi: 10.1016/s0079-6123(00)24015-6

Sillitoe, R. V., Chung, S. H., Fritschy, J. M., Hoy, M., and Hawkes, R. (2008). Golgi cell dendrites are restricted by Purkinje cell stripe boundaries in the adult mouse cerebellar cortex. *J. Neurosci.* 28, 2820–2826. doi: 10.1523/JNEUROSCI.4145-07.2008

Sillitoe, R. V., and Joyner, A. L. (2007). Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. *Annu. Rev. Cell Dev. Biol.* 23, 549–577. doi: 10.1146/annurev.cellbio.23.090506.123237

Silver, R. A., Cull-Candy, S. G., and Takahashi, T. (1996). Non-NMDA glutamate receptor occupancy and open probability at a rat cerebellar synapse with single and multiple release sites. *J. Physiol.* 494, 231–250. doi: 10.1113/jphysiol.1996.sp021487

Simat, M., Parpan, F., and Fritschy, J. M. (2007). Heterogeneity of glycinergic and gabaergic interneurons in the granule cell layer of mouse cerebellum. *J. Comp. Neurol.* 500, 71–83. doi: 10.1002/cne.21142

Simões de Souza, F. M., and De Schutter, E. (2011). Robustness effect of gap junctions between Golgi cells on cerebellar cortex oscillations. *Neural Syst. Circuits* 1:7. doi: 10.1186/2042-1001-1-7

Sims, R. E., and Hartell, N. A. (2005). Differences in transmission properties and susceptibility to long-term depression reveal functional specialization of ascending axon and parallel fiber synapses to Purkinje cells. *J. Neurosci.* 25, 3246–3257. doi: 10.1523/JNEUROSCI.0073-05.2005

Singer, W., and Gray, C. M. (1995). Visual feature integration and the temporal correlation hypothesis. *Annu. Rev. Neurosci.* 18, 555–586. doi: 10.1146/annurev.ne.18.030195.003011

Soda, T., Mapelli, L., Locatelli, F., Botta, L., Goldfarb, M., Prestori, F., et al. (2019). Hyperexcitability and hyperplasticity disrupt cerebellar signal transfer in the IB2 KO mouse model of autism. *J. Neurosci.* 39, 2383–2397. doi: 10.1523/JNEUROSCI.1985-18.2019

Soler-Llavina, G. J., and Sabatini, B. L. (2006). Synapse-specific plasticity and compartmentalized signaling in cerebellar stellate cells. *Nat. Neurosci.* 9, 798–806. doi: 10.1038/nn1698

Solinas, S., Forti, L., Cesana, E., Mapelli, J., De Schutter, E., and D'Angelo, E. (2007a). Fast-reset of pacemaking and theta-frequency resonance patterns in cerebellar golgi cells: simulations of their impact *in vivo*. *Front. Cell. Neurosci.* 1:4. doi: 10.3389/neuro.03.004.2007

Solinas, S., Forti, L., Cesana, E., Mapelli, J., De Schutter, E., and D'Angelo, E. (2007b). Computational reconstruction of pacemaking and intrinsic electroresponsiveness in cerebellar Golgi cells. *Front. Cell. Neurosci.* 1:2. doi: 10.3389/neuro.03.002.2007

Solinas, S., Nieus, T., and D'Angelo, E. (2010). A realistic large-scale model of the cerebellum granular layer predicts circuit spatio-temporal filtering properties. *Front. Cell. Neurosci.* 4:12. doi: 10.3389/fncel.2010.00012

Sotelo, C., and Llinás, R. (1972). Specialized membrane junctions between neurons in the vertebrate cerebellar cortex. *J. Cell Biol.* 53, 271–289. doi: 10.1083/jcb.53.2.271

Strahlendorf, J. C., Lee, M., and Strahlendorf, H. K. (1984). Effects of serotonin on cerebellar Purkinje cells are dependent on the baseline firing rate. *Exp. Brain Res.* 56, 50–58. doi: 10.1007/bf00237441

Sudarov, A., Turnbull, R. K., Kim, E. J., Lebel-Potter, M., Guillemot, F., and Joyner, A. L. (2011). Ascl1 genetics reveals insights into cerebellum local circuit assembly. *J. Neurosci.* 31, 11055–11069. doi: 10.1523/JNEUROSCI.0479-11.2011

Sugihara, I., Wu, H., and Shinoda, Y. (1999). Morphology of single olivocerebellar axons labeled with biotinylated dextran amine in the rat. *J. Comp. Neurol.* 414, 131–148. doi: 10.1002/(sici)1096-9861(19991115)414:2<131::aid-cne1>3.0.co;2-f

Sullivan, M. R., Nimmerjahn, A., Sarkisov, D. V., Helmchen, F., and Wang, S. S. (2005). *In vivo* calcium imaging of circuit activity in cerebellar cortex. *J. Neurophysiol.* 94, 1636–1644. doi: 10.1152/jn.01013.2004

Sultan, F., and Bower, J. M. (1998). Quantitative Golgi study of the rat cerebellar molecular layer interneurons using principal component analysis. *J. Comp. Neurol.* 393, 353–373. doi: 10.1002/(sici)1096-9861(19980413)393:3<353::aid-cne7>3.3.co;2-7

Szapiro, G., and Barbour, B. (2009). Parasynaptic signalling by fast neurotransmitters: the cerebellar cortex. *Neuroscience* 162, 644–655. doi: 10.1016/j.neuroscience.2009.03.077

Szentágothai, J. (1965). The use of degeneration methods in the investigation of short neuronal connexions. *Prog. Brain Res.* 14, 1–32. doi: 10.1016/s0079-6123(08)63737-1

Szoboszlay, M., Lörincz, A., Lanore, F., Vervaeke, K., Silver, R. A., and Nusser, Z. (2016). Functional properties of dendritic gap junctions in cerebellar golgi cells. *Neuron* 90, 1043–1056. doi: 10.1016/j.neuron.2016.03.029

Tanaka, I., and Ezure, K. (2004). Overall distribution of GLYT2 mRNA-containing versus GAD67 mRNA-containing neurons and colocalization of both mRNAs in midbrain, pons, and cerebellum in rats. *Neurosci. Res.* 49, 165–178. doi: 10.1016/j.neures.2004.02.007

Taniguchi, H., He, M., Wu, P., Kim, S., Paik, R., Sugino, K., et al. (2011). A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. *Neuron* 71, 995–1013. doi: 10.1016/j.neuron.2011.07.026

Tank, D. W., Sugimori, M., Connor, J. A., and Llinas, R. R. (1988). Spatially resolved calcium dynamics of mammalian Purkinje cells in cerebellar slice. *Science* 242, 773–777. doi: 10.1126/science.2847315

Thompson, A. (1908). *The Oxford Translation of Aristotle*. Oxford: Clarendon Press.

Torborg, C. L., Nakashiba, T., Tonegawa, S., and McBain, C. J. (2010). Control of CA3 output by feedforward inhibition despite developmental changes in the excitation-inhibition balance. *J. Neurosci.* 30, 15628–15637. doi: 10.1523/jneurosci.3099-10.2010

Trouillas, P., Brudon, F., and Adeleine, P. (1988). Improvement of cerebellar ataxia with levorotatory form of 5-hydroxytryptophan. *Arch. Neurol.* 45, 1217–1222. doi: 10.1001/archneur.1988.00520350055016

Trouillas, P., Xie, J., Getenet, J. C., Adeleine, P., Nighoghossian, N., Honnorat, J., et al. (1995). [Effect of buspirone, a serotonergic 5-HT-1A agonist in cerebellar ataxia: a pilot study. Preliminary communication]. *Rev. Neurol.* 151, 708–713.

Valera, A. M., Binda, F., Pawlowski, S. A., Dupont, J. L., Casella, J. F., Rothstein, J. D., et al. (2016). Stereotyped spatial patterns of functional synaptic connectivity in the cerebellar cortex. *Elife* 5:e09862. doi: 10.7554/elife.09862

van Welie, I., Roth, A., Ho, S. S., Komai, S., and Hausser, M. (2016). Conditional spike transmission mediated by electrical coupling ensures millisecond precision-correlated activity among interneurons *in vivo*. *Neuron* 90, 810–823. doi: 10.1016/j.neuron.2016.04.013

Vervaeke, K., Lorincz, A., Gleeson, P., Farinella, M., Nusser, Z., and Silver, R. A. (2010). Rapid desynchronization of an electrically coupled interneuron network with sparse excitatory synaptic input. *Neuron* 67, 435–451. doi: 10.1016/j.neuron.2010.06.028

Vervaeke, K., Lorincz, A., Nusser, Z., and Silver, R. A. (2012). Gap junctions compensate for sublinear dendritic integration in an inhibitory network. *Science* 335, 1624–1628. doi: 10.1126/science.1215101

Víg, J., Takács, J., Vastagh, C., Baldauf, Z., Veisenberger, E., and Hámori, J. (2003). Distribution of mGluR1α and SMI 311 immunoreactive Lugaro cells in the kitten cerebellum. *J. Neurocytol.* 32, 217–227. doi: 10.1023/b:neur.0000010081.54613.94

Vincent, P., and Marty, A. (1996). Fluctuations of inhibitory postsynaptic currents in Purkinje cells from rat cerebellar slices. *J. Physiol.* 494, 183–199. doi: 10.1113/jphysiol.1996.sp021484

Voogd, J. (2014). What we do not know about cerebellar systems neuroscience. *Front. Syst. Neurosci.* 8:227. doi: 10.3389/fnsys.2014.00227

Voogd, J., and Glickstein, M. (1998). The anatomy of the cerebellum. *Trends Neurosci.* 21, 370–375. doi: 10.1016/s0166-2236(98)01318-6

Voogd, J., and Koehler, P. J. (2018). Historic notes on anatomic, physiologic, and clinical research on the cerebellum. *Handb. Clin. Neurol.* 154, 3–26. doi: 10.1016/b978-0-444-63956-1.00001-1

Vos, B. P., Volny-Luraghi, A., and De Schutter, E. (1999). Cerebellar Golgi cells in the rat: receptive fields and timing of responses to facial stimulation. *Eur. J. Neurosci.* 11, 2621–2634. doi: 10.1046/j.1460-9568.1999.00678.x

Wall, M. J., and Usowicz, M. M. (1997). Development of action potential-dependent and independent spontaneous $GABA_A$ receptor-mediated currents in granule cells of postnatal rat cerebellum. *Eur. J. Neurosci.* 9, 533–548. doi: 10.1111/j.1460-9568.1997.tb01630.x

Walter, J. T., Alviña, K., Womack, M. D., Chevez, C., and Khodakhah, K. (2006). Decreases in the precision of Purkinje cell pacemaking cause cerebellar dysfunction and ataxia. *Nat. Neurosci.* 9, 389–397. doi: 10.1038/nn1648

Watanabe, D., Inokawa, H., Hashimoto, K., Suzuki, N., Kano, M., Shigemoto, R., et al. (1998). Ablation of cerebellar Golgi cells disrupts synaptic integration involving GABA inhibition and NMDA receptor activation in motor coordination. *Cell* 95, 17–27. doi: 10.1016/s0092-8674(00)81779-1

Watanabe, D., and Nakanishi, S. (2003). mGluR2 postsynaptically senses granule cell inputs at Golgi cell synapses. *Neuron* 39, 821–829. doi: 10.1016/s0896-6273(03)00530-0

Wefers, A. K., Haberlandt, C., Surchev, L., Steinhauser, C., Jabs, R., and Schilling, K. (2018). Migration of interneuron precursors in the nascent cerebellar cortex. *Cerebellum* 17, 62–71. doi: 10.1007/s12311-017-0900-7

Weisheit, G., Gliem, M., Endl, E., Pfeffer, P. L., Busslinger, M., and Schilling, K. (2006). Postnatal development of the murine cerebellar cortex: formation and early dispersal of basket, stellate and Golgi neurons. *Eur. J. Neurosci.* 24, 466–478. doi: 10.1111/j.1460-9568.2006.04915.x

Witter, L., Rudolph, S., Pressler, R. T., Lahlaf, S. I., and Regehr, W. G. (2016). Purkinje cell collaterals enable output signals from the cerebellar cortex to feed back to purkinje cells and interneurons. *Neuron* 91, 312–319. doi: 10.1016/j.neuron.2016.05.037

Womack, M. D., and Khodakhah, K. (2003). Somatic and dendritic small-conductance calcium-activated potassium channels regulate the output of cerebellar Purkinje neurons. *J. Neurosci.* 23, 2600–2607. doi: 10.1523/jneurosci.23-07-02600.2003

Wulff, P., Schonewille, M., Renzi, M., Viltono, L., Sassoe-Pognetto, M., Badura, A., et al. (2009). Synaptic inhibition of Purkinje cells mediates consolidation of vestibulo-cerebellar motor learning. *Nat. Neurosci.* 12, 1042–1049. doi: 10.1038/nn.2348

Xu, W., and Edgley, S. A. (2008). Climbing fibre-dependent changes in Golgi cell responses to peripheral stimulation. *J. Physiol.* 586, 4951–4959. doi: 10.1113/jphysiol.2008.160879

Yamazaki, T., Nagao, S., Lennon, W., and Tanaka, S. (2015). Modeling memory consolidation during posttraining periods in cerebellovestibular learning. *Proc. Natl. Acad. Sci. U S A* 112, 3541–3546. doi: 10.1073/pnas.1413798112

Zeng, H., and Sanes, J. R. (2017). Neuronal cell-type classification: challenges, opportunities and the path forward. *Nat. Rev. Neurosci.* 18, 530–546. doi: 10.1038/nrn.2017.85

Zhang, L., and Goldman, J. E. (1996a). Developmental fates and migratory pathways of dividing progenitors in the postnatal rat cerebellum. *J. Comp. Neurol.* 370, 536–550. doi: 10.1002/(SICI)1096-9861(19960708)370:4<536::AID-CNE9>3.0.CO;2-5

Zhang, L., and Goldman, J. E. (1996b). Generation of cerebellar interneurons from dividing progenitors in white matter. *Neuron* 16, 47–54. doi: 10.1016/s0896-6273(00)80022-7

Zordan, P., Croci, L., Hawkes, R., and Consalez, G. G. (2008). Comparative analysis of proneural gene expression in the embryonic cerebellum. *Dev. Dyn.* 237, 1726–1735. doi: 10.1002/dvdy.21571

Conflict of Interest: 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.

The reviewer CH is currently the co-Field Chief Editor of Frontiers in Cellular Neuroscience with one of the authors, ED'A, and confirms absence of any collaboration to the handling editor.

frontiers in Molecular Neuroscience

MINI REVIEW
published: 12 December 2019
doi: 10.3389/fnmol.2019.00304

Molecular Architecture of Genetically-Tractable GABA Synapses in *C. elegans*

*Xin Zhou and Jean-Louis Bessereau**

Univ Lyon, Université Claude Bernard Lyon 1, CNRS UMR 5310, INSERM U 1217, Institut NeuroMyoGène, Lyon, France

Inhibitory synapses represent a minority of the total chemical synapses in the mammalian brain, yet proper tuning of inhibition is fundamental to shape neuronal network properties. The neurotransmitter γ-aminobutyric acid (GABA) mediates rapid synaptic inhibition by the activation of the type A GABA receptor ($GABA_AR$), a pentameric chloride channel that governs major inhibitory neuronal transduction in the nervous system. Impaired GABA transmission leads to a variety of neuropsychiatric diseases, including schizophrenia, autism, epilepsy or anxiety. From an evolutionary perspective, $GABA_AR$ shows remarkable conservations, and are found in all eukaryotic clades and even in bacteria and archaea. Specifically, *bona fide* $GABA_ARs$ are found in the nematode *Caenorhabditis elegans*. Because of the anatomical simplicity of the nervous system and its amenability to genetic manipulations, *C. elegans* provide a powerful system to investigate the molecular and cellular biology of GABA synapses. In this mini review article, we will introduce the structure of the *C. elegans* GABAergic system and describe recent advances that have identified novel proteins controlling the localization of $GABA_ARs$ at synapses. In particular, Ce-Punctin/MADD-4 is an evolutionarily-conserved extracellular matrix protein that behaves as an anterograde synaptic organizer to instruct the excitatory or inhibitory identity of postsynaptic domains.

Keywords: $GABA_A$ receptor, *C. elegans*, neuromuscular junction, punctin, neuroligin

OPEN ACCESS

Edited by:
Shiva K. Tyagarajan,
University of Zurich, Switzerland

Reviewed by:
Jasmina N. Jovanovic,
UCL School of Pharmacy,
United Kingdom
Angelo Keramidas,
University of Queensland, Australia

****Correspondence:***
Jean-Louis Bessereau
jean-louis.bessereau@univ-lyon1.fr

Received: *05 August 2019*
Accepted: *26 November 2019*
Published: *12 December 2019*

Citation:
Zhou X and Bessereau J-L (2019) Molecular Architecture of Genetically-Tractable GABA Synapses in C. elegans. Front. Mol. Neurosci. 12:304. doi: 10.3389/fnmol.2019.00304

INTRODUCTION

Neurochemical synapses are the elementary structures that process the directional transfer of electrical signals in neural circuits. Based on their molecular composition, synapses probably emerged early during evolution before the divergence of Cnidarians and Bilaterians, more than 1.2 billion years ago (Sakarya et al., 2007; Emes and Grant, 2012). The molecular composition of the synapse shows high conservation. For example, among Bilaterians, a comparison of mouse genes encoding the postsynaptic proteome indicates that ≈45% have detectable orthologs in the ecdysozoans *Caenorhabditis elegans* or *Drosophila melanogaster* (Ryan and Grant, 2009). Although synapses were further diversified in the chordate lineage, it is possible to interrogate the general organization and function of chemical synapses in simple invertebrate organisms, and thereby take advantage of their ease of manipulation and the power of their genetic toolkits. In this mini review article, we outline how this strategy was successful in the nematode *C. elegans* to identify a novel organizer of inhibitory γ-aminobutyric acid (GABA)ergic synapses.

C. elegans is an anatomically-simple, 1 mm-long, non-parasitic nematode. Stereotyped divisions of the zygote, in combination with fixed programmed cell-death events, generate 959 somatic cells in the adult hermaphrodite and 1,033 in the adult male. The adult hermaphrodite contains 302 neurons, most of which are morphologically simple, extending only a few unbranched neurites. The connectivity of the *C. elegans* nervous system was reconstructed in the 1970s from serial EM sections (White et al., 1986). Connectivity is relatively sparse since the entire network contains less than 10,000 chemical synapses, including 1,500 neuromuscular junctions (NMJs), and about 800 gap junctions. Based on the reconstruction of few independent specimens and the visualization of specific synapses with fluorescent markers, the overall connectivity of the system appears strikingly reproducible among individuals, yet data are currently being generated using modern connectomic techniques to get a better sense of interindividual variability with single-synapse resolution (Mulcahy et al., 2018; Cook et al., 2019). This anatomical simplicity contrasts with the complexity of the molecular repertoire expressed in the nervous system. Although *C. elegans* contains 10^8 times fewer neurons than humans, its genome contains about 22,000 genes, which is very comparable with the human gene content. All classes of neurotransmitter systems found in mammals are present within *C. elegans* (Hobert, 2018), with a remarkable diversity of peptidergic transmission and the expansion of some receptor families, such as nicotinic and olfactory receptors. Specifically, the machinery to synthesize, release and sense the neurotransmitter GABA is remarkably conserved within mammals (Schuske et al., 2004).

GABAergic NEUROTRANSMISSION IN *C. elegans*

Early mapping of the GABAergic system by anti-GABA immunostaining identified 26 neurons in the *C. elegans* nervous system: 19 motoneurons (D-class) that establish NMJs on body-wall muscles, four motoneurons (RMEs) that control head muscles, two neurons (AVL and DVB) that innervate intestinal muscles and the interneuron RIS (McIntire et al., 1993b). A recent study identified 10 additional GABA-positive neurons, out of which three express the glutamic acid decarboxylase (GAD)/UNC-25, while the others might accumulate GABA by re-uptake using the plasma membrane transporter GAT/SNF-11 or some uncharacterized mechanisms (Gendrel et al., 2016).

The prominent phenotype caused by impairing GABA neurotransmission in *C. elegans* is an abnormal locomotion. Unlike mammals, *C. elegans* body-wall muscles receive both excitatory input from cholinergic motoneurons and inhibitory input from GABAergic motoneurons. When a cholinergic motoneuron releases acetylcholine (ACh), it triggers both muscle contraction and the activation of a downstream GABAergic motoneuron that projects to the opposite muscles, causing their relaxation (**Figure 1A**). This ensures local out of phase dorsal/ventral contraction/relaxation, the elementary component of sinusoidal locomotion (Jorgensen and Nonet, 1995). Laser ablation of GABAergic motoneurons causes a specific "shrinker" phenotype due to concomitant hyper contraction of both ventral and dorsal muscles when animals try to move backward. Similarly, RME motoneurons relax head muscles during foraging and impairment of GABA neurotransmission impacts head movements. By contrast, ablation of the AVL and DVB neurons causes a "constipated" phenotype because these neurons directly activate (rather than inhibit) the enteric muscles required for expulsion of the intestinal content (McIntire et al., 1993b). GABA-dependent excitation depends on EXP-1, a GABA-sensitive cation channel with the hallmarks of the Cys-loop receptor superfamily (Thomas, 1990; Beg and Jorgensen, 2003).

The "shrinker" phenotype was used in genetic screens as a proxy to recognize "Uncoordinated" (*unc*-) mutants with impaired GABA neurotransmission among the initial collection of mutants isolated by *Sydney Brenner* (Brenner, 1974; Hodgkin, 1983; McIntire et al., 1993a). These included mutants in *unc-25*, the single gene encoding the GABA-synthetizing enzyme GAD, *unc-47*, the first gene identified in any species to encode the vesicular GABA Transporter vGAT (McIntire et al., 1997), and *unc-49*, which codes for the $GABA_A$ receptors present at NMJs (Bamber et al., 1999). Interestingly, complete inactivation of GABAergic neurotransmission produces viable mutants that can reproduce under laboratory conditions.

In addition to UNC-49, the *C. elegans* genome encodes three canonical $GABA_A$ receptor subunits, the two alpha-subunit type LGC-36 and LGC-37 and the beta-subunit GAB-1, that are orthologous to $GABA_AR$ subunits in mammals (Tsang et al., 2007). UNC-49 and the related receptor LGC-38 are phylogenetically closer to the Drosophila receptor RDL (**Figure 1C**). Moreover, there are at least two additional *bona fide* ionotropic GABA receptors, EXP-1 and LGC-35, that are permeable to cations due to specific amino-acid composition of the channel selectivity filter (Beg and Jorgensen, 2003; Jobson et al., 2015). Of the 118 anatomically defined neuron classes of the *C. elegans* hermaphrodite, 47 neuron classes are innervated by GABAergic neurons (White et al., 1986). Twenty one of these neuron classes express at least one of the aforementioned receptors based on transcriptional reporters. The apparent inability to detect $GABA_AR$ expression in the rest of the GABA-innervated neurons might be due to technical limitations. However, it is also likely that additional GABA receptors remain to be characterized because the *C. elegans* genome contains up to 39 GABA/Glycine receptor-like genes, including Glutamate- and Acetylcholine-gated anion channels (Jones and Sattelle, 2008; Hobert, 2018). Finally, *C. elegans* expresses two metabotropic GABA receptors for which a comprehensive expression pattern remains to be described (Dittman and Kaplan, 2008; Schultheis et al., 2011). Interestingly, a number of neurons that do not receive direct GABAergic inputs still express $GABA_A$ receptors. These receptors may mediate GABA spillover transmission as demonstrated for LGC-35, which activates cholinergic motoneurons when GABA is released by GABAergic motoneurons (Jobson et al., 2015). Notably, our knowledge of the roles of $GABA_ARs$ beyond the NMJ in *C. elegans* is rudimentary since the cellular localization and function of every canonical $GABA_ARs$ still remains to be characterized.

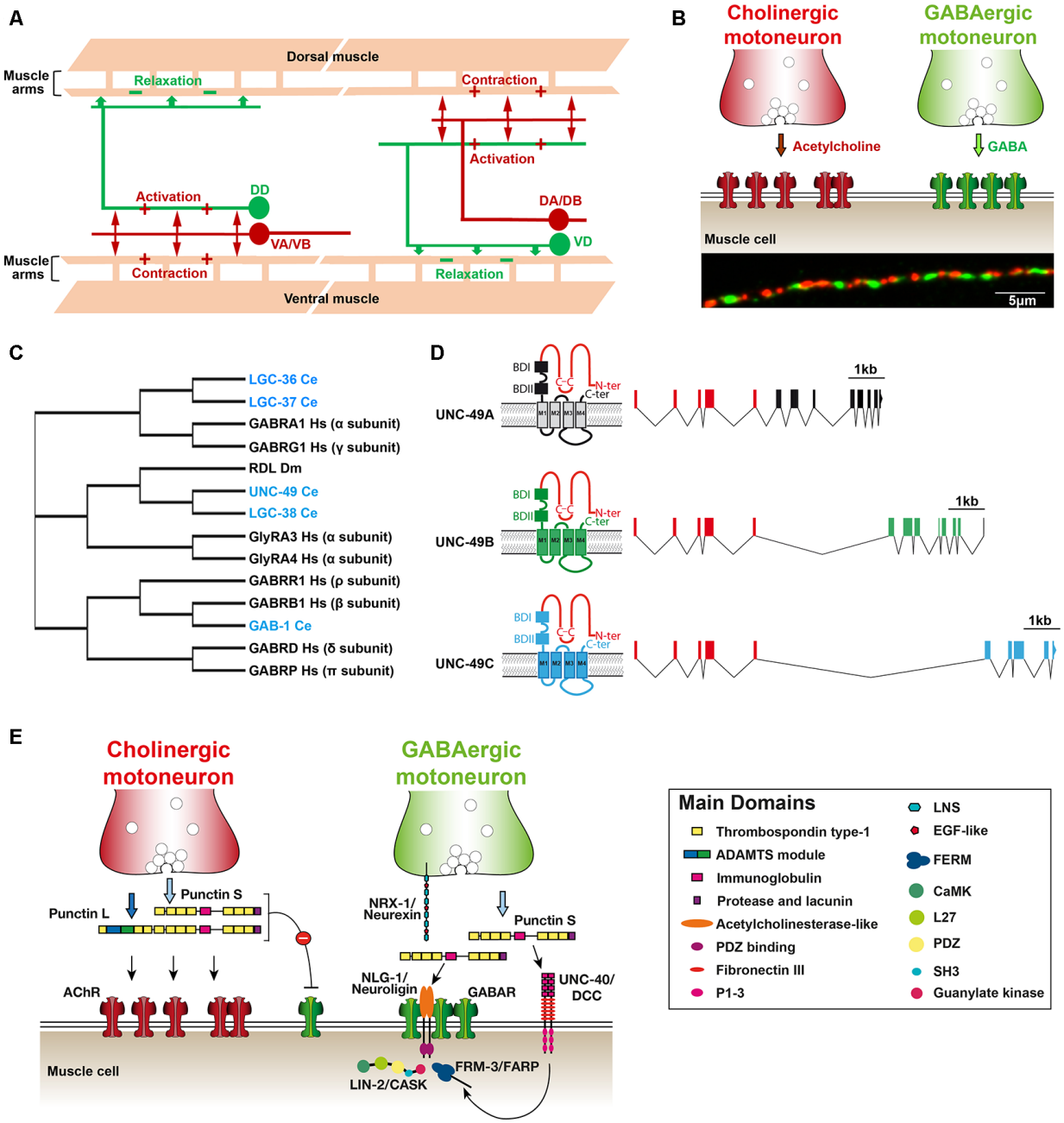

FIGURE 1 | (A) Schematic organization of the *C. elegans* neuromuscular network. Mononucleated body-wall muscle cells on the ventral and dorsal sides of the worm extend ≈5 muscle arms to contact the axon of cholinergic (red) and γ-aminobutyric acid (GABA)ergic (green) motoneurons along the ventral and dorsal nerve cords, respectively. Cholinergic neurons (VA/VB and DA/DB) form dyadic synapses activating muscle cells and GABAergic motoneurons (DD and VD) that form inhibitory neuromuscular junctions (NMJs) on opposite muscle cells. **(B)** Distribution of excitatory and inhibitory NMJs along the ventral nerve cord. Upper panel: a schematic drawing showing that each muscle cell receives both cholinergic and GABAergic inputs. Lower panel: immunostaining of cholinergic boutons (anti-UNC-17/VAChT; red) and $GABA_A$Rs (anti-UNC-49; green) at the dorsal nerve cord. **(C)** Cladogram showing the phylogenic relationships of the *C. elegans* genes encoding $GABA_A$ receptor subunits (blue). The tree was adapted from Tsang et al. (2007) and Gendrel et al. (2016). Dm, Drosophila melanogaster; Hs, Homo sapiens. **(D)** Schematic structure of the *unc-49* locus encoding the $GABA_A$R present at inhibitory NMJs (adapted from Bamber et al., 1999). The locus generates three distinct subunits by alternative splicing. The first five exons encode most of the extracellular N-terminal, which is common to the three subunits (red). Alternative splicing of 3′ blocks of exons encode the C-terminal part of the **A,B** and **C** subunits (black, green and blue, respectively). Putative GABA binding sites (BD) and transmembrane segments are distinct between the different subunits. **(E)** Working model of $GABA_A$R clustering at NMJ in *C. elegans*. See the main text for discussion of the model. ADAMTS, a disintegrin and metalloproteinase with thrombospondin; P1-3, protein binding domain 1, 2 and 3; LNS, laminin-neurexin/sex hormone-binding globulin; EGF, epidermal growth factor; FERM, (4.1, ezrin, radixin, moesin) family; SH3, src homology 3 domain; FARP, FERM, ARH/RhoGEF and pleckstrin domain protein; CASK, calcium/calmodulin-dependent serine protein kinase.

THE GABAergic NEUROMUSCULAR JUNCTION, A GENETICALLY-TRACTABLE MODEL OF INHIBITORY SYNAPSE

Paradoxically, the best characterized inhibitory synapse in *C. elegans* is the GABAergic NMJ, which might relate more closely to neuro-neuronal synapses than to "standard" NMJs. Invertebrates or Drosophila, motoneurons establish a single NMJ with myofibers containing hundreds to thousands of nuclei. This differs from neuronal innervation, where a single neuron typically receives thousands of excitatory and inhibitory inputs, building a mosaic of specialized domains concentrating receptors to match presynaptic inputs. Interestingly, the anatomical organization of the *C. elegans* neuromuscular system provides a means to interrogate a number of questions which may more closely relate to the innervation of vertebrate neurons.

First, *C. elegans* body-wall muscle cells do not fuse and remain mononucleated. Second, they send dendrite-like extensions that contact and extend along the motoneurons that run in the ventral and dorsal cords and form "en-passant" synapses. Third, as presented above, each muscle cell receives both excitatory cholinergic and inhibitory GABAergic inputs from distinct classes of motoneurons (**Figures 1A,B**). Based on functional (Liu et al., 2007) and EM data (White et al., 1986), each synaptic bouton likely activates receptors present on more than one postsynaptic muscle arm facing the presynaptic active zone. Hence, the *C. elegans* neuromuscular arrangement represents a very simple poly-neuronal innervation system. Specifically, it can be used to interrogate how specific compartments are built on the plasma membrane to concentrate on different neurotransmitter receptors in front of the corresponding neurotransmitter release sites.

C. elegans development is fast. Fourteen hours after fertilization, eggs hatch as the first larval stage. Development then proceeds through four larval stages (called L1 to L4), each separated by a molt, and reach adulthood within 2.5 days at 20°C. At hatching, only six dorsal D-class (DD) motoneurons have been generated and innervate ventral body-wall muscles. The 13 ventral D-class (VD) motoneurons differentiate during the first larval stage. DD neurons rewire at the end of the L1 stage to innervate dorsal muscles while VD neurons innervate ventral muscles (White et al., 1978; Kurup and Jin, 2015). Although the adult is about 10× bigger than L1 larvae, the number of inhibitory NMJs does not increase. Rather, additional active zones form in presynaptic boutons to scale up inhibition (Yeh et al., 2005). Because of the relatively sparse distribution of presynaptic boutons and their highly reproducible patterns across animals, forward genetic screens were successful in identifying mutants with abnormal synapses using fluorescently-tagged presynaptic proteins expressed in GABA motoneurons. These screens were extremely powerful and identified multiple proteins required for the general organization of active zones in neurons such as SYD-2/Liprin, SYD-1, and RPM-1, a founding member of the PHR (Phr1/MYCBP2, highwire and RPM-1) family of proteins (Zhen and Jin, 1999; Zhen et al., 2000; Hallam et al., 2002). However, these screens did not identify proteins specifically involved in the differentiation of inhibitory synapses.

The UNC-49 GABAA receptors are generated from a single complex locus, which generates at least three different subunits (A, B and C) by alternative splicing (**Figure 1D**). A block of exons encodes most of the extracellular N-terminal domain, which is shared by all subunits, while exons coding for transmembrane regions is specific to each subunit. In *Xenopus* oocytes, functional GABA receptors can be reconstituted by expressing the B-subunit either alone or in combination with the C-subunit (Bamber et al., 1999). UNC-49B and UNC-49B/C have distinct pharmacology. The positive allosteric regulator diazepam, instead of activating $GABA_A$Rs, inhibits the GABA-evoked UNC-49B/C current while it has no obvious effect on the UNC-49B homomer. Neurosteroids such as pregnenolone sulfate, that enhances GABA-evoked currents in mammals, have a strong inhibitory effect on the UNC-49B receptor and much weaker effects on UNC-49-B/C. UNC-49B homomers were also found to be sensitive to the broadly-active inhibitor picrotoxin, while UNC-49B/C heteromers are resistant to it (Bamber et al., 2003).

The development of a dissection technique for adult *C. elegans* enabled stable, whole-cell voltage-clamp recording from ventral medial muscle cells and gave access to native $GABA_A$Rs (Richmond and Jorgensen, 1999). Spontaneous GABAergic synaptic currents can be isolated either after the pharmacological block of AChRs (Richmond et al., 1999) or by using recording solutions that discriminate excitatory and inhibitory postsynaptic currents (Vashlishan et al., 2008). The total amount of $GABA_A$R present at the muscle cell surface is usually probed by measuring the response to pressure-application of the general agonist muscimol, and the synaptic pool can be activated after optogenetic stimulation of GABA motoneurons (Liewald et al., 2008). *in vivo* recordings and the pharmacological analyses of endogenous $GABA_A$Rs indicate that they are likely composed of UNC-49B/C heteromers.

UNC-49 $GABA_A$ receptors are clustered in register with presynaptic GABAergic boutons (**Figure 1B**). Clustering depends on presynaptic innervation and occurs concomitantly with presynaptic differentiation based on the visualization of fluorescently-tagged synaptic proteins (Gally and Bessereau, 2003). However, a detailed longitudinal analysis is still missing to ascertain the precise temporal relationship between pre- and postsynaptic differentiation. Remarkably, in mutants that do not synthesize GABA, both pre- and postsynaptic structures are indistinguishable from wild type, demonstrating that "inhibitory" synapses differentiate in the absence of neurotransmission (Gally and Bessereau, 2003). This situation is not unique since various synaptic types were also reported to differentiate in mammalian cell cultures and in mice in the absence of neurotransmitter release (Misgeld et al., 2002; Varoqueaux et al., 2002; Sigler et al., 2017). Fluorescently-tagged UNC-49 receptors remain functional. Again, because the distribution of these receptors is stereotyped, screens for mutants with abnormal fluorescence distribution identified factors specifically required for the differentiation and organization of GABA NMJs, as described below.

MOLECULES BASIS OF UNC-49 RECEPTOR CLUSTERING IN *C. elegans*

In mammalian neurons, $GABA_AR$s clustering mostly relies on the scaffolding protein gephyrin that is hypothesized to form an intracellular lattice providing anchoring sites for synaptic $GABA_AR$s (Fritschy et al., 2008; Tyagarajan and Fritschy, 2014). Collybistin, a GTP/GDP exchange factor (GEF) interacts with the synaptic adhesion protein Neuroligin-2 and promotes the clustering of gephyrin and $GABA_AR$s (Kins et al., 2000; Tyagarajan et al., 2011). Although Gephyrin acts as a prominent player for $GABA_AR$s synaptic clustering, gephyrin-independent $GABA_AR$ clustering can occur and the requirement of gephyrin for $GABA_AR$ clustering is dependent on neuronal and synapse type (Kneussel et al., 2001; Tretter et al., 2012). Interestingly, gephyrin and collybistin are not conserved in *C. elegans*, giving access to a different molecular organization for $GABA_AR$ clustering.

Ce-Punctin/MADD-4

The postsynaptic assembly of cholinergic and GABAergic NMJs in *C. elegans* relies on a recently-identified anterograde synaptic organizer Ce-Punctin/MADD-4 (Muscle Arm Development Defective-4). Ce-Punctin belongs to a family of poorly characterized extracellular matrix proteins, the ADAMTS-like proteins, that contain multiple thrombospondin-repeat, immunoglobulin, and structurally-unsolved domains (Apte, 2009). There are two *madd-4* orthologs in vertebrates, *Punctin1/ADAMTSL1* and *Punctin2/ADAMTSL3*. The precise function of these genes is unknown. However, a variant of *Punctin1* was recently shown to cause a complex phenotype including congenital glaucoma, craniofacial and other systemic features (Hendee et al., 2017). *Punctin2* is expressed in the brain and was identified as a susceptibility gene for schizophrenia (Dow et al., 2011). Whether these proteins are involved in synaptic organization has not been determined.

Ce-punctin generates long (Punctin L) and short (Punctin S) isoforms by the use of alternative promoters. Punctin S was initially found to attract muscle arm growth and be required for midline-oriented guidance in *C. elegans* (Seetharaman et al., 2011). The role of *Ce-punctin* in synaptic organization was subsequently identified in a visual screen for mutants with abnormal positioning of fluorescently-tagged AChRs at NMJs (Pinan-Lucarré et al., 2014). Punctin L is only expressed in cholinergic motoneurons and secreted in the synaptic cleft where it triggers postsynaptic clustering of AChRs. Punctin S is expressed in both cholinergic and GABAergic neurons (**Figure 1E**). At cholinergic synapses, Punctin S inhibits the attraction of $GABA_AR$s by Punctin L, possibly following heterodimerization of the L and S isoforms. At GABAergic synapses, Punctin S promotes the clustering of $GABA_AR$s in front of presynaptic GABA boutons. Genetic inactivation of Punctin S does not alter presynaptic GABA boutons, but $GABA_AR$s relocalize at cholinergic synapses. Conversely, forced expression of Punctin L in GABAergic motoneurons in a *punctin* null mutant triggers the colocalization of AChRs and $GABA_AR$s opposed to GABAergic boutons (Pinan-Lucarré et al., 2014). These results demonstrated that the identity of pre- and post-synaptic domains can be genetically uncoupled *in vivo*.

Interestingly, the expression of Punctin is under direct regulation of the transcription factors that specify the terminal identity of motoneurons. The phylogenetically conserved transcription factor UNC-3 controls the expression of numerous genes required for the cholinergic neurotransmission. It also directly activates the transcription of *punctin L* and *S* isoforms in cholinergic motoneurons (Kratsios et al., 2015). Similarly, the homeobox transcription factor UNC-30 controls the GABAergic identity of D-type motoneurons and regulates the expression of *punctin S* (P. Kratsios and O. Hobert, personal communication). Therefore, coordinated control of motoneuron identity and Punctin expression provides a means to ensure proper coupling between presynaptic identity and postsynaptic differentiation.

NLG-1/NEUROLIGIN

The clustering of $GABA_AR$s at *C. elegans* NMJs requires the synaptic adhesion molecule neuroligin NLG-1. Neuroligins (NLs) are evolutionary ancient proteins that are readily detected in Bilaterians (Lenfant et al., 2014). The human genome encodes 5 NLs that support trans-synaptic adhesive functions at excitatory and inhibitory synapses and contribute to postsynaptic receptor clustering (for review see Südhof, 2008). The *C. elegans* genome contains only one NL-coding gene, *nlg-1*, which is expressed in multiple types of neurons and in the muscle (Hunter et al., 2010). NLG-1 shares about 25% identity with human NLs and cannot be related to one specific paralog. However, the core protein organization is conserved between mammals and the nematode (Calahorro, 2014). Three main NLG-1 isoforms are generated by alternative splicing of exons encoding cytoplasmic domains of the protein (Calahorro et al., 2015). This splicing seems developmentally regulated but the precise complement of NLG-1 isoforms expressed in neurons and muscle and its functional relevance remains to be analyzed.

In muscle, NLG-1 is only found at GABAergic NMJs and strictly colocalizes with the UNC-49 $GABA_AR$s (Maro et al., 2015; Tu et al., 2015). Disruption of *nlg-1* causes a redistribution of the $GABA_AR$s out of the GABA receptor domains and a reduction of the frequency and amplitude of spontaneous miniature inhibitory postsynaptic currents (mIPSCs). The synaptic localization of NLG-1 depends on Punctin S, which directly binds the NLG-1 ectodomain. The intracellular moiety of NLG-1 is dispensable for its synaptic localization but is required for its ability to cluster $GABA_AR$s (Maro et al., 2015; Tu et al., 2015).

GABA motoneurons also express NRX-1, the sole ortholog of the mammalian neurexins that are presynaptic ligands of neuroligins (reviewed in Südhof, 2008). NRX-1 is present at presynaptic sites of GABAergic NMJs but is not required for the synaptic localization of NLG-1. Based on genetic evidence, NRX-1 was proposed to work in parallel with Punctin to promote the clustering of $GABA_AR$s (Maro et al., 2015). The *nrx-1*

mutant used in this study no longer expressed the NRX-1 γ isoform, which was recently shown to be important for the presynaptic organization (Kurshan et al., 2018). However, most of the ectodomain of the NRX-1 α isoform potentially remained synthesized. Therefore, the positive interaction between NRX-1 and Punctin at GABA synapses remains to be further investigated in this system.

UNC-40/DCC

At the *C. elegans* NMJ, the synaptic content of $GABA_A$Rs depends on the netrin receptor UNC-40/DCC (deleted in colorectal cancer; Tu et al., 2015). This receptor has been implicated in a wide range of developmental events involving cellular migration and axonal navigation (Chan et al., 1996; Keino-Masu et al., 1996). It is a single transmembrane domain protein that does not contain any obvious catalytic domain. Upon netrin binding, UNC-40 is believed to dimerize, causing the intracellular domains to serve as a signaling platform to recruit or activate numerous downstream targets, including several signal transduction molecules that regulate cytoskeletal dynamics (for reviews see Finci et al., 2015; Boyer and Gupton, 2018).

In *C. elegans*, UNC-40 plays a specific role in the neuromuscular system. First, it promotes the growth of muscle arms (Alexander et al., 2009). At the early larval stage, the Punctin S localizes UNC-40 at the tip of the muscle arms and, together with the guidance cue UNC-6/netrin, activates UNC-40. Thus, the number of muscle arms that project to the ventral and dorsal nerve cords is drastically reduced in *unc-40* mutants. However, the number of GABAergic boutons is unaffected and NLG-1 postsynaptic clusters remain readily detected. In addition, UNC-40 controls the amount of $GABA_A$Rs at synapses. In *unc-40* mutants, there is a 60% reduction of receptors at GABAergic NMJs. A constitutively-activated version of UNC-40, which only contains the intracellular moiety of UNC-40 targeted to the plasma membrane, rescues the synaptic clustering of $GABA_A$Rs (Tu et al., 2015). This suggests that upon activation by Punctin, UNC-40 promotes the recruitment of $GABA_A$Rs onto NLG-1 clusters.

FRM-3/FARP AND LIN-2/CASK

Recently, the intracellular proteins FRM-3 and LIN-2 were reported to regulate $GABA_A$Rs at NMJs (Tong et al., 2015). FRM-3 was initially described as a band 4.1 (EPB4.1) paralog (Tong et al., 2015), but the recently annotated FRM-3B isoform appears to be the unambiguous ortholog of the mammalian FARP1 and FARP2 proteins. FARPs are able to modulate F-actin assembly and regulate neuronal development and synaptogenesis by interacting with cell-surface proteins such as SynCAM1 and class A Plexins (Cheadle and Biederer, 2012, 2014). In *C. elegans*, FRM-3 binds the intracellular loop of the UNC-49B subunit (Tong et al., 2015). It also binds LIN-2, the ortholog of CASK, a membrane-associated guanylate kinase (MAGUK) multidomain scaffolding molecule (Hata et al., 1996) that plays important role in presynaptic assembly at mammalian synapses. At GABAergic NMJs, disruption of *frm-3* or *lin-2* causes about 25% reduction of mIPSC amplitude, while pressure-applied response to the $GABA_A$Rs agonist muscimol was unchanged, indicating that receptors were expressed but not properly clustered at synapses. FRM-3 and LIN-2 were proposed to stabilize $GABA_A$Rs at the synapse, in parallel to a Neuroligin-dependent scaffold (Tong et al., 2015). The precise interplay between these different proteins remains to be characterized further.

CONCLUSION

GABAergic inhibitory neurotransmission is an evolutionarily ancient system that has been conserved over ≈1,000 million years of evolution. Genetic analysis in *C. elegans* identified a novel anterograde synaptic organizer, Ce-Punctin, which specifies the position of post-synaptic domains by localizing neuroligin in register with synaptic boutons, and controls the number of postsynaptic receptors through the activation of UNC-40/DCC (**Figure 1E**). The conservation of this system still remains to be tested in mammals. Even at the *C. elegans* NMJ, several questions remain unanswered: how is Punctin secreted and confined at synapses? What are the mechanisms that differentiate Punctin function at cholinergic and GABAergic synapses? To what extent is this system regulated by synaptic activity? Most surprisingly, it is amazing to see that the cellular and molecular basis of synaptic neuro-neuronal GABA transmission in *C. elegans* remains *terra incognita*. Lots remain to be learned.

AUTHOR CONTRIBUTIONS

XZ and J-LB wrote the manuscript.

FUNDING

This work was supported by the Programme Avenir Lyon Saint-Etienne, the Agence Nationale de la Recherche (ANR) grant GABAREL ANR-15-CE11-0016-01 and the European Research Council (ERC_Adg C.NAPSE #695295).

ACKNOWLEDGMENTS

We thank Maëlle Jospin and Thomas Boulin for critical reading of the manuscript and Camilla Luccardini for helping in figure design.

REFERENCES

Alexander, M., Chan, K. K. M., Byrne, A. B., Selman, G., Lee, T., Ono, J., et al. (2009). An UNC-40 pathway directs postsynaptic membrane extension in *Caenorhabditis elegans*. *Development* 136, 911–922. doi: 10.1242/dev.030759

Apte, S. S. (2009). A disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif (ADAMTS) superfamily: functions and mechanisms. *J. Biol. Chem.* 284, 31493–31497. doi: 10.1074/jbc.r109.052340

Bamber, B. A., Beg, A. A., Twyman, R. E., and Jorgensen, E. M. (1999). The *Caenorhabditis elegans* unc-49 locus encodes multiple subunits

of a heteromultimeric GABA receptor. *J. Neurosci.* 19, 5348–5359. doi: 10.1523/JNEUROSCI.19-13-05348.1999

Bamber, B. A., Twyman, R. E., and Jorgensen, E. M. (2003). Pharmacological characterization of the homomeric and heteromeric UNC-49 GABA receptors in *C. elegans*. *Br. J. Pharmacol.* 138, 883–893. doi: 10.1038/sj.bjp.0705119

Beg, A. A., and Jorgensen, E. M. (2003). EXP-1 is an excitatory GABA-gated cation channel. *Nat. Neurosci.* 6, 1145–1152. doi: 10.1038/nn1136

Boyer, N. P., and Gupton, S. L. (2018). Revisiting netrin-1: one who guides (Axons). *Front. Cell. Neurosci.* 12:221. doi: 10.3389/fncel.2018.00221

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94.

Calahorro, F. (2014). Conserved and divergent processing of neuroligin and neurexin genes: from the nematode *C. elegans* to human. *Invert. Neurosci.* 14, 79–90. doi: 10.1007/s10158-014-0173-5

Calahorro, F., Holden-Dye, L., and O'Connor, V. (2015). Analysis of splice variants for the *C. elegans* orthologue of human neuroligin reveals a developmentally regulated transcript. *Gene Expr. Patterns* 17, 69–78. doi: 10.1016/j.gep.2015.02.002

Chan, S. S.-Y., Zheng, H., Su, M.-W., Wilk, R., Killeen, M. T., Hedgecock, E. M., et al. (1996). UNC-40, a *C. elegans* homolog of DCC (deleted in colorectal cancer), is required in motile cells responding to UNC-6 netrin cues. *Cell* 87, 187–195. doi: 10.1016/s0092-8674(00)81337-9

Cheadle, L., and Biederer, T. (2012). The novel synaptogenic protein Farp1 links postsynaptic cytoskeletal dynamics and transsynaptic organization. *J. Cell Biol.* 199, 985–1001. doi: 10.1083/jcb.201205041

Cheadle, L., and Biederer, T. (2014). Activity-dependent regulation of dendritic complexity by semaphorin 3A through Farp1. *J. Neurosci.* 34, 7999–8009. doi: 10.1523/jneurosci.3950-13.2014

Cook, S. J., Jarrell, T. A., Brittin, C. A., Wang, Y., Bloniarz, A. E., Yakovlev, M. A., et al. (2019). Whole-animal connectomes of both *Caenorhabditis elegans* sexes. *Nature* 571, 63–71. doi: 10.1038/s41586-019-1352-7

Dittman, J. S., and Kaplan, J. M. (2008). Behavioral impact of neurotransmitter-activated GPCRs: muscarinic and GABAB receptors regulate *C. elegans* locomotion. *J. Neurosci.* 28, 7104–7112. doi: 10.1523/jneurosci.0378-08.2008

Dow, D. J., Huxley-Jones, J., Hall, J. M., Francks, C., Maycox, P. R., Kew, J. N. C., et al. (2011). ADAMTSL3 as a candidate gene for schizophrenia: gene sequencing and ultra-high density association analysis by imputation. *Schizophr. Res.* 127, 28–34. doi: 10.1016/j.schres.2010.12.009

Emes, R. D., and Grant, S. G. N. (2012). Evolution of synapse complexity and diversity. *Ann. Rev. Neurosci.* 35, 111–131. doi: 10.1146/annurev-neuro-062111-150433

Finci, L., Zhang, Y., Meijers, R., and Wang, J.-H. (2015). Signaling mechanism of the netrin-1 receptor DCC in axon guidance. *Prog. Biophys. Mol. Biol.* 118, 153–160. doi: 10.1016/j.pbiomolbio.2015.04.001

Fritschy, J.-M., Harvey, R. J., and Schwarz, G. (2008). Gephyrin: where do we stand, where do we go? *Trends Neurosci.* 31, 257–264. doi: 10.1016/j.tins.2008.02.006

Gally, C., and Bessereau, J.-L. (2003). GABA is dispensable for the formation of junctional GABA receptor clusters in *Caenorhabditis elegans*. *J. Neurosci.* 23, 2591–2599. doi: 10.1523/jneurosci.23-07-02591.2003

Gendrel, M., Atlas, E. G., and Hobert, O. (2016). A cellular and regulatory map of the GABAergic nervous system of *C. elegans*. *Elife* 5:e17686. doi: 10.7554/eLife.17686

Hallam, S. J., Goncharov, A., McEwen, J., Baran, R., and Jin, Y. (2002). SYD-1, a presynaptic protein with PDZ, C2 and rhoGAP-like domains, specifies axon identity in *C. elegans*. *Nat. Neurosci.* 5, 1137–1146. doi: 10.1038/nn959

Hata, Y., Butz, S., and Südhof, T. C. (1996). CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. *J. Neurosci.* 16, 2488–2494. doi: 10.1523/jneurosci.16-08-02488.1996

Hendee, K., Wang, L. W., Reis, L. M., Rice, G. M., Apte, S. S., and Semina, E. V. (2017). Identification and functional analysis of an ADAMTSL1 variant associated with a complex phenotype including congenital glaucoma, craniofacial and other systemic features in a three generation human pedigree. *Hum. Mutat.* 38, 1485–1490. doi: 10.1002/humu.23299

Hobert, O. (2018). The neuronal genome of *Caenorhabditis elegans*. *WormBook* 13, 1–106. doi: 10.1895/wormbook.1.161.1

Hodgkin, J. (1983). Male phenotypes and mating efficiency in *Caenorhabditis elegans*. *Genetics* 103, 43–64.

Hunter, J. W., Mullen, G. P., McManus, J. R., Heatherly, J. M., Duke, A., and Rand, J. B. (2010). Neuroligin-deficient mutants of *C. elegans* have sensory processing deficits and are hypersensitive to oxidative stress and mercury toxicity. *Dis. Model. Mech.* 3, 366–376. doi: 10.1242/dmm.003442

Jobson, M. A., Valdez, C. M., Gardner, J., Garcia, L. R., Jorgensen, E. M., and Beg, A. A. (2015). Spillover transmission is mediated by the excitatory GABA receptor LGC-35 in *C. elegans*. *J. Neurosci.* 35, 2803–2816. doi: 10.1523/jneurosci.4557-14.2015

Jones, A., and Sattelle, D. (2008). The cys-loop ligand-gated ion channel gene superfamily of the nematode, *Caenorhabditis elegans*. *Invert. Neurosci.* 8, 41–47. doi: 10.1007/s10158-008-0068-4

Jorgensen, E. M., and Nonet, M. L. (1995). Neuromuscular junctions in the nematode *C. elegans*. *Semin. Dev. Biol.* 6, 207–220. doi: 10.1016/s1044-5781(06)80030-7

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S.-Y., Culotti, J. G., et al. (1996). Deleted in colorectal cancer (DCC) encodes a netrin receptor. *Cell* 87, 175–185. doi: 10.1016/s0092-8674(00)81336-7

Kins, S., Betz, H., and Kirsch, J. (2000). Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. *Nat. Neurosci.* 3, 22–29. doi: 10.1038/71096

Kneussel, M., Helmut Brandstätter, J., Gasnier, B., Feng, G., Sanes, J. R., and Betz, H. (2001). Gephyrin-independent clustering of postsynaptic GABAA receptor subtypes. *Mol. Cell. Neurosci.* 17, 973–982. doi: 10.1006/mcne.2001.0983

Kratsios, P., Pinan-Lucarré, B., Kerk, S. Y., Weinreb, A., Bessereau, J.-L., and Hobert, O. (2015). Transcriptional coordination of synaptogenesis and neurotransmitter signaling. *Curr. Biol.* 25, 1282–1295. doi: 10.1016/j.cub.2015.03.028

Kurshan, P. T., Merrill, S. A., Dong, Y., Ding, C., Hammarlund, M., Bai, J., et al. (2018). γ-neurexin and frizzled mediate parallel synapse assembly pathways antagonized by receptor endocytosis. *Neuron* 100, 150.e4–166.e4. doi: 10.1016/j.neuron.2018.09.007

Kurup, N., and Jin, Y. (2015). Neural circuit rewiring: insights from DD synapse remodeling. *Worm* 5:e1129486. doi: 10.1080/21624054.2015.1129486

Lenfant, N., Hotelier, T., Bourne, Y., Marchot, P., and Chatonnet, A. (2014). Tracking the origin and divergence of cholinesterases and neuroligins: the evolution of synaptic proteins. *J. Mol. Neurosci.* 53, 362–369. doi: 10.1007/s12031-013-0194-2

Liewald, J. F., Brauner, M., Stephens, G. J., Bouhours, M., Schultheis, C., Zhen, M., et al. (2008). Optogenetic analysis of synaptic function. *Nat. Methods* 5, 895–902. doi: 10.1038/nmeth.1252

Liu, Q., Chen, B., Hall, D. H., and Wang, Z.-W. (2007). A quantum of neurotransmitter causes minis in multiple postsynaptic cells at the *Caenorhabditis elegans* neuromuscular junction. *Dev. Neurobiol.* 67, 123–128. doi: 10.1002/dneu.20307

Maro, G. S., Gao, S., Olechwier, A. M., Hung, W. L., Liu, M., Özkan, E., et al. (2015). MADD-4/punctin and neurexin Organize *C. elegans* GABAergic postsynapses through neuroligin. *Neuron* 86, 1420–1432. doi: 10.1016/j.neuron.2015.05.015

McIntire, S. L., Jorgensen, E., and Horvitz, H. R. (1993a). Genes required for GABA function in *Caenorhabditis elegans*. *Nature* 364, 334–337. doi: 10.1038/364334a0

McIntire, S. L., Jorgensen, E., Kaplan, J., and Horvitz, H. R. (1993b). The GABAergic nervous system of *Caenorhabditis elegans*. *Nature* 364, 337–341. doi: 10.1038/364337a0

McIntire, S. L., Reimer, R. J., Schuske, K., Edwards, R. H., and Jorgensen, E. M. (1997). Identification and characterization of the vesicular GABA transporter. *Nature* 389, 870–876. doi: 10.1038/39908

Misgeld, T., Burgess, R. W., Lewis, R. M., Cunningham, J. M., Lichtman, J. W., and Sanes, J. R. (2002). Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. *Neuron* 36, 635–648. doi: 10.1016/s0896-6273(02)01020-6

Mulcahy, B., Witvliet, D., Holmyard, D., Mitchell, J., Chisholm, A. D., Meirovitch, Y., et al. (2018). A pipeline for volume electron microscopy of the *Caenorhabditis elegans* nervous system. *Front. Neural Circuits* 12:94. doi: 10.3389/fncir.2018.00094

Pinan-Lucarré, B., Tu, H., Pierron, M., Cruceyra, P. I., Zhan, H., Stigloher, C., et al. (2014). *C. elegans* Punctin specifies cholinergic versus GABAergic

identity of postsynaptic domains. *Nature* 511, 466–470. doi: 10.1038/nature13313

Richmond, J. E., Davis, W. S., and Jorgensen, E. M. (1999). UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat. Neurosci.* 2, 959–964. doi: 10.1038/14755

Richmond, J. E., and Jorgensen, E. M. (1999). One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat. Neurosci.* 2, 791–797. doi: 10.1038/12160

Ryan, T. J., and Grant, S. G. N. (2009). The origin and evolution of synapses. *Nat. Rev. Neurosci.* 10, 701–712. doi: 10.1038/nrn2717

Sakarya, O., Armstrong, K. A., Adamska, M., Adamski, M., Wang, I.-F., Tidor, B., et al. (2007). A post-synaptic scaffold at the origin of the animal kingdom. *PLoS One* 2:e506. doi: 10.1371/journal.pone.0000506

Schultheis, C., Brauner, M., Liewald, J. F., and Gottschalk, A. (2011). Optogenetic analysis of GABAB receptor signaling in *Caenorhabditis elegans* motor neurons. *J. Neurophysiol.* 106, 817–827. doi: 10.1152/jn.00578.2010

Schuske, K., Beg, A. A., and Jorgensen, E. M. (2004). The GABA nervous system in *C. elegans*. *Trends in Neurosci.* 27, 407–414. doi: 10.1016/j.tins.2004.05.005

Seetharaman, A., Selman, G., Puckrin, R., Barbier, L., Wong, E., D'Souza, S. A., et al. (2011). MADD-4 is a secreted cue required for midline-oriented guidance in *Caenorhabditis elegans*. *Dev. Cell* 21, 669–680. doi: 10.1016/j.devcel.2011.07.020

Sigler, A., Oh, W. C., Imig, C., Altas, B., Kawabe, H., Cooper, B. H., et al. (2017). Formation and maintenance of functional spines in the absence of presynaptic glutamate release. *Neuron* 94, 304.e4–311.e4. doi: 10.1016/j.neuron.2017.03.029

Südhof, T. C. (2008). Neuroligins and neurexins link synaptic function to cognitive disease. *Nature* 455, 903–911. doi: 10.1038/nature07456

Thomas, J. H. (1990). Genetic analysis of defecation in *Caenorhabditis elegans*. *Genetics* 124, 855–872.

Tong, X.-J., Hu, Z., Liu, Y., Anderson, D., and Kaplan, J. M. (2015). A network of autism linked genes stabilizes two pools of synaptic GABAA receptors. *ELife* 4:e09648. doi: 10.7554/eLife.09648

Tretter, V. E., Mukherjee, J., Maric, H. M., Schindelin, H., Sieghart, W., and Moss, S. J. (2012). Gephyrin, the enigmatic organizer at GABAergic synapses. *Front. Cell. Neurosci.* 6:23. doi: 10.3389/fncel.2012.00023

Tsang, S.-Y., Ng, S.-K., Xu, Z., and Xue, H. (2007). The evolution of GABAA receptor-like genes. *Mol. Biol. Evol.* 24, 599–610. doi: 10.1093/molbev/msl188

Tu, H., Pinan-Lucarré, B., Ji, T., Jospin, M., and Bessereau, J.-L. (2015). *C. elegans* punctin clusters $GABA_A$ receptors *via* neuroligin binding and UNC-40/DCC recruitment. *Neuron* 86, 1407–1419. doi: 10.1016/j.neuron.2015.05.013

Tyagarajan, S. K., and Fritschy, J.-M. (2014). Gephyrin: a master regulator of neuronal function? *Nat. Rev. Neurosci.* 15, 141–156. doi: 10.1038/nrn3670

Tyagarajan, S. K., Ghosh, H., Harvey, K., and Fritschy, J.-M. (2011). Collybistin splice variants differentially interact with gephyrin and Cdc42 to regulate gephyrin clustering at GABAergic synapses. *J. Cell Sci.* 124, 2786–2796. doi: 10.1242/jcs.086199

Varoqueaux, F., Sigler, A., Rhee, J.-S., Brose, N., Enk, C., Reim, K., et al. (2002). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc. Natl. Acad. Sci. U S A* 99, 9037–9042. doi: 10.1073/pnas.122623799

Vashlishan, A. B., Madison, J. M., Dybbs, M., Bai, J., Sieburth, D., Ch'ng, Q., et al. (2008). An RNAi screen identifies genes that regulate GABA synapses. *Neuron* 58, 346–361. doi: 10.1016/j.neuron.2008.02.019

White, J. G., Albertson, D. G., and Anness, M. A. R. (1978). Connectivity changes in a class of motoneurone during the development of a nematode. *Nature* 271, 764–766. doi: 10.1038/271764a0

White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 314, 1–340. doi: 10.1098/rstb.1986.0056

Yeh, E., Kawano, T., Weimer, R. M., Bessereau, J.-L., and Zhen, M. (2005). Identification of genes involved in synaptogenesis using a fluorescent active zone marker in *Caenorhabditis elegans*. *J. Neurosci.* 25, 3833–3841. doi: 10.1523/JNEUROSCI.4978-04.2005

Zhen, M., Huang, X., Bamber, B., and Jin, Y. (2000). Regulation of presynaptic terminal organization by *C. elegans* RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. *Neuron* 26, 331–343. doi: 10.1016/s0896-6273(00)81167-8

Zhen, M., and Jin, Y. (1999). The liprin protein SYD-2 regulates the differentiation of presynaptic termini in *C. elegans*. *Nature* 401, 371–375. doi: 10.1038/43886

Conflict of Interest: 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 Molecular Neuroscience

REVIEW
published: 19 December 2019
doi: 10.3389/fnmol.2019.00313

Inhibitory Receptor Diffusion Dynamics

*Stephanie A. Maynard and Antoine Triller**

Institute of Biology of Ecole Normale Supérieure (IBENS), PSL Research University, CNRS, Inserm, Paris, France

The dynamic modulation of receptor diffusion-trapping at inhibitory synapses is crucial to synaptic transmission, stability, and plasticity. In this review article, we will outline the progression of understanding of receptor diffusion dynamics at the plasma membrane. We will discuss how regulation of reversible trapping of receptor-scaffold interactions in combination with theoretical modeling approaches can be used to quantify these chemical interactions at the postsynapse of living cells.

Keywords: diffusion-trapping, inhibitory synapse, $GABA_A$ receptor, glycine receptor, gephyrin, single-particle tracking

OPEN ACCESS

Edited by:
Shiva K. Tyagarajan, University of Zurich, Switzerland

Reviewed by:
Enrica Maria Petrini, Italian Institute of Technology (IIT), Italy
Jasmina N. Jovanovic, UCL School of Pharmacy, United Kingdom

****Correspondence:***
Antoine Triller
triller@biologie.ens.fr

Received: *06 August 2019*
Accepted: *04 December 2019*
Published: *19 December 2019*

Citation:
Maynard SA and Triller A (2019) Inhibitory Receptor Diffusion Dynamics. Front. Mol. Neurosci. 12:313. doi: 10.3389/fnmol.2019.00313

INTRODUCTION

Synaptic organization is a dynamic multiscale process in neuronal cell networks. The role of receptor diffusion-trapping in the plasma membrane is now understood to be a molecular mechanism resulting from chemical interactions and is crucial for synapse formation, stability, and plasticity in neurons.

The fluid mosaic model postulated by Singer and Nicolson (1972) reflects the thermodynamics and interactions of the individual components which have a critical role in the composition and organization of biological membranes. This idea was further enforced when, in 1974, the receptor protein rhodopsin was shown to be in constant motion in the lipid bilayer (Poo and Cone, 1974). Beside molecular turnover, it became obvious that synaptic molecular components were dynamic, enabling activity-dependent regulation of synaptic functions. The importance of molecular turnover at the synapse, as a regulator of synaptic strength and memory, was suggested by Crick (1984). Crick noted that the individual molecular components of the synapse were subject to characteristic times of turnover shorter than that of memory. Crick postulated that post-translational modifications of the molecules at the synapse could explain a longer-term persistent state of synaptic strength contributing to memory, despite a molecular turnover within days. Since then, there has been a concentrated effort into uncoupling membrane composition, diffusion dynamics and activity-dependent synaptic regulation with long-term structural stability.

This review will focus on the recent advances in our understanding of molecule dynamics in inhibitory synapses, covering technological advancements that have enabled probing of receptor and scaffold protein dynamics, organization and regulation.

RECEPTOR DIFFUSION-TRAPPING DYNAMICS IN THE INHIBITORY SYNAPSE

Membrane Receptor Insertion

Underlying fundamental processes controlling synaptic receptor delivery and removal, and the implications of these in synaptic strength have been of intense interest over the last 20 years. It was previously known that regulation of receptor number at the post synapse influenced plasticity at both excitatory and inhibitory synapses (e.g., Nusser et al., 1998; Hayashi et al., 2000; reviewed in Turrigiano, 2000). It was originally postulated that the dynamic turnover was driven exclusively by endocytosis and exocytosis of receptors and scaffold molecules to the membrane following *de novo* receptor synthesis or recycling. $GABA_A$ receptor ($GABA_AR$) exocytosis and endocytosis *via* a clathrin-mediated pathway demonstrated exchange between the surface and intracellular compartments of the synapse (Kittler et al., 2000). Further, it was shown glycine receptor (GlyR) exocytosis occurred predominantly at extrasynaptic sites in the cell body and initial portion of dendrites in spinal cord neurons, and that this exocytosis was not directed or synapse-specific (Rosenberg et al., 2001). $GABA_AR$ exocytosis was also shown to be extrasynaptic followed by recruitment to synapses *via* lateral diffusion in the membrane in hippocampal neurons (Thomas et al., 2005; Bogdanov et al., 2006). Studies of excitatory synapses have showed AMPAR GluR1 subunits are initially inserted at extrasynaptic sites, whereas the GluR2 subunit is inserted in spines closer to synapses (Passafaro et al., 2001) and thus subunit specificity may further regulate receptor delivery. Further, in hippocampal pyramidal neurons, AMPARs were shown to enter spines preferentially following membrane insertion in the adjoining dendritic shaft (Yudowski et al., 2007). The balance of exocytosis and endocytosis regulates the number of postsynaptic receptors and has long been regarded as the main cellular mechanism underlying long-term potentiation (LTP) and long-term depression (LTD) (Mammen et al., 1997; Nishimune et al., 1998; Lüthi et al., 1999; Song and Huganir, 2002; Park et al., 2004; Tanaka and Hirano, 2012; Fujii et al., 2018).

Membrane Receptor Diffusion

However, in addition to receptor exocytosis and endocytosis, lateral receptor diffusion and trapping within the postsynaptic membrane has since been established as a key mediator of synaptic strength and plasticity. In 2001, Meier et al. (2001) demonstrated the lateral diffusion of the GlyR at the cell surface *via* the use of 500 nm latex beads. Additionally, they confirmed GlyR diffusion alternated between diffusive and confined states, with confinement spatially associated with the scaffold protein gephyrin. This led them to propose a dynamic equilibrium between pools of stabilized and freely mobile receptors (**Figure 1**). This lateral diffusion was then directly demonstrated *via* the tracking of quantum dots (QDs) bound to surface GlyRs (Dahan et al., 2003). This lateral movement from extrasynaptic pools and switching from free to confined Brownian motion has since been generalized for most neurotransmitter receptors (Thomas et al., 2005; Bogdanov et al., 2006; Pooler and McIlhinney, 2007; Lévi et al., 2008; Bannai et al., 2009; Choquet, 2010; Renner et al., 2017). Differences in diffusion of receptors at extrasynaptic and synaptic sites vary up to 10-fold, as shown for the $GABA_AR$ (Bannai et al., 2009; de Luca et al., 2017; Hannan et al., 2019) and the GlyR (Dahan et al., 2003; Lévi et al., 2008; Calamai et al., 2009). The characteristic time for receptor exchange by lateral receptor movement is much faster than that related to receptor recycling from internal stores or *de novo* receptor synthesis (Renner et al., 2008).

Multiple Factors Influence Receptor Diffusion

At the postsynaptic membrane, there are multiple aspects that may influence receptor lateral diffusion. The transient trapping at synapses of laterally diffusing molecules can result from interactions of receptors with other proteins at the membrane such as scaffold molecules, acting as diffusion traps, or from non-specific obstacles, such as molecular crowding, lipid composition and the sub-membrane cytoskeleton (**Figure 1**).

Interaction of receptors with scaffold molecules represents one of the primary effectors of synaptic diffusion. At the inhibitory synapse, gephyrin interactions have been analyzed for their influence on $GABA_AR$ (e.g., Jacob et al., 2005; Petrini et al., 2014) and GlyR (e.g., Meier et al., 2001; Meier and Grantyn, 2004) mobility. GlyRs and $GABA_ARs$ diffuse far more freely at extrasynaptic sites than when confined in inhibitory synapses at gephyrin clusters. Specifically, gephyrin interaction with receptors at synapses causes transient receptor retention (Meier et al., 2001; Dahan et al., 2003; Calamai et al., 2009; Specht et al., 2011). Furthermore, the binding of the $GABA_AR$ to gephyrin and subsequent increased dwell time of $GABA_AR$ at gephyrin-positive synaptic sites affected the synaptic strength of inhibition (Mukherjee et al., 2011). A comparable decrease in diffusion of metabotropic- and AMPA-type glutamate receptors upon binding to their respective scaffold molecules has also been observed (Borgdorff and Choquet, 2002; Sergé et al., 2002).

Competition between receptors, including their subunit composition, may further regulate lateral movement and accumulation into synapses. Lateral diffusion of $GABA_ARs$ containing α5 or α2 subunits were reported to be modulated by $GABA_BRs$ for binding to scaffold proteins (Gerrow and Triller, 2014). It was recently shown that $GABA_ARs$ comprised of different subunit combinations have variable diffusion and synaptic retention rates (Hannan et al., 2019). Additional regulation of receptor diffusion may hence be inferred through subunit-specific regulations, leading to coordinated molecular and functional specificity. Likewise, different diffusion properties arise from contrasting affinities of $GABA_AR$ and GlyR subunits for gephyrin (Tretter et al., 2008; Maric et al., 2011; Kowalczyk et al., 2013). Finally, the multivalency of the gephyrin scaffold network is also likely to further regulate the molecular organization and diffusion of receptors at the membrane (Specht et al., 2013).

Physical barriers such as cholesterol, phospholipids, other receptors and the cytoskeleton can also regulate the

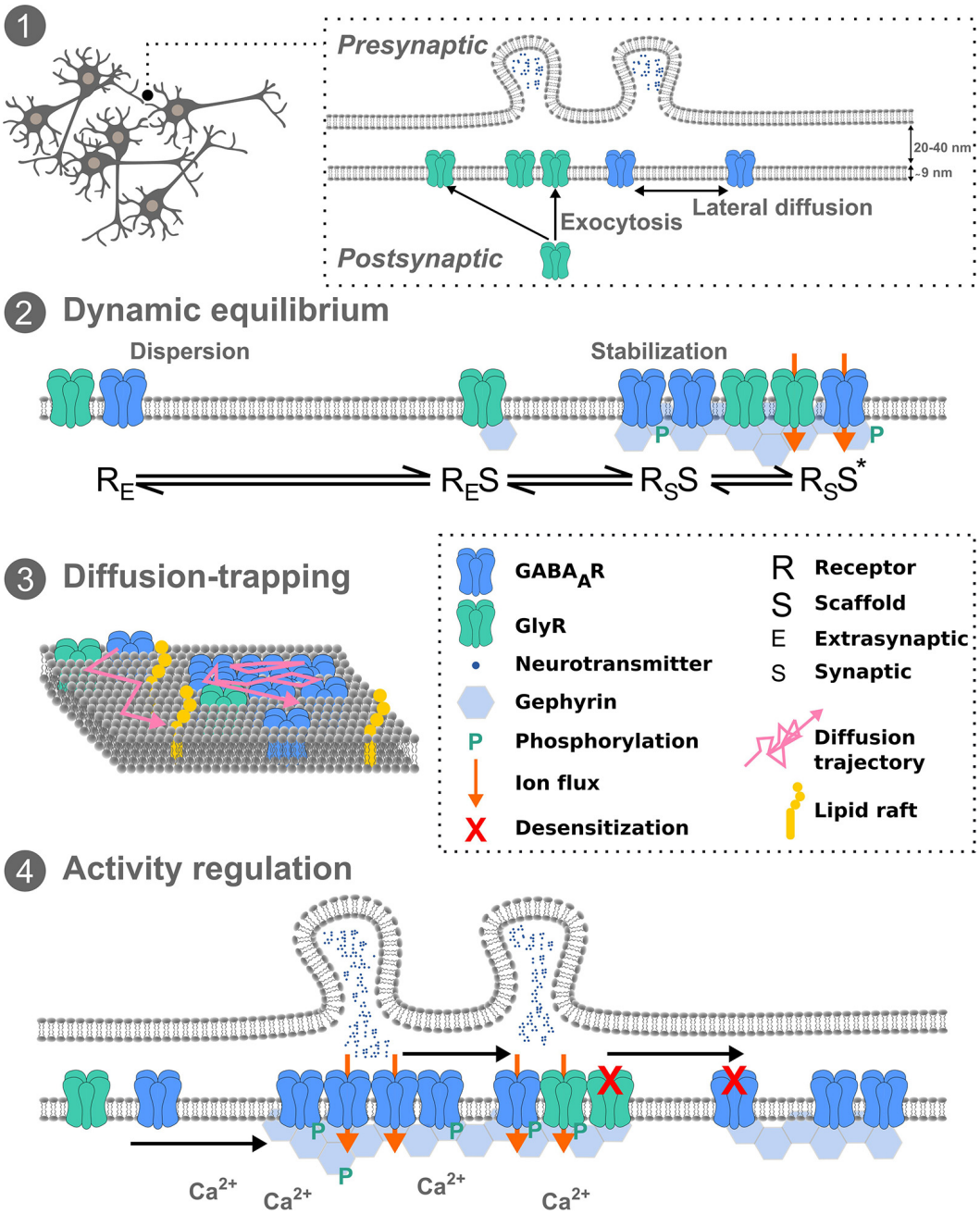

FIGURE 1 | Inhibitory receptor diffusion-trapping. (1) Overview schematic of pre- and postsynaptic inhibitory neuronal membranes, exocytosis, and lateral diffusion. (2) The dynamic equilibrium between stabilized and freely mobile receptors, at the synapse and extrasynaptically, respectively. (3) Receptor diffusion-trapping depends not only on chemical interactions with synaptic components but also on non-specific obstacles, such as lipid rafts, leading to molecular crowding. (4) Activity regulation of receptor mobility can affect post-translational modifications of receptors and scaffold proteins and subsequently their immobilization at synapses. Exchange of receptors between synapses can fine-tune network activity.

diffusion-trap mechanism. The physical properties of the plasma membrane, including surface geometry, curvature and viscosity determine the flux of receptors (for review, see Marguet et al., 2006). Lipid raft domains can reduce lateral mobility of receptors (Allen et al., 2007), while cholesterol depletion affects apparent membrane viscosity and subsequently receptor diffusion properties (Renner et al., 2009). Thus controlling membrane lipid composition, including cholesterol, can have consequences on molecular flow in and out of the postsynapse. Furthermore, phospholipids within the membrane themselves can act as local messengers in neurotransmission (García-Morales et al., 2015). The tuning of GlyR lateral diffusion has been shown at

synaptic and extrasynaptic sites upon F-actin and microtubule disruption respectively (Charrier et al., 2006). Increased lateral diffusion upon dissociation of $GABA_A$Rs from their actin anchor, radixin, lead to increased synaptic expression (Loebrich et al., 2006; Hausrat et al., 2015). Additionally, gephyrin interacts with actin filaments *via* several proteins including profilin, Mena/Vasp (Mammoto et al., 1998; Giesemann et al., 2003). Thus the regulation of scaffold trafficking by the cytoskeleton can also affect receptor lateral diffusion and synapse accumulation. Furthermore, activity-dependent extracellular matrix (ECM) modifications may also have structural and functional consequences on receptor lateral mobility (Dityatev et al., 2010). In fact, the secreted ECM molecule Reelin has been shown to regulate the surface distribution and diffusion of NMDA receptors in hippocampal neurons (Groc et al., 2007). The ECM protein thrombospondin-1 increased the lateral diffusion and endocytosis of AMPARs and increased synaptic accumulation of GlyRs in rat spinal cord neurons (Hennekinne et al., 2013). These effects on GlyRs are also dependent on increased excitation as well as the presence of β-1 integrins. Gephyrin clustering itself has been shown to be tuned by integrin-mediated interactions leading to GlyR trapping at the synapse (Charrier et al., 2010). Consequently, the presence of other trans-membrane proteins, in particular those involved in ECM binding such as integrins, can affect neurotransmitter diffusion and synaptic trapping.

Hence surface availability is governed by a combination of processes, such as receptor exocytosis, lateral mobility, diffusion-trapping, dynamic interactions with membrane components, molecular crowding.

ANALYZING RECEPTOR DIFFUSION DYNAMICS

Several approaches have been utilized with the aim of quantifying molecular-scale dynamics in cells. Single-molecule fluorescence imaging *via* low-density antibody labeling of GluR2-containing AMPARs enabled visualization of receptor entry and exit at synapses (Tardin et al., 2003). Advances in super-resolution imaging techniques, such as single-particle tracking (SPT) using QDs or coupled with photoactivated localization microscopy (PALM), or universal point accumulation for imaging in nanoscale topography (uPAINT) have enabled further analysis within synapses. uPAINT relies on the binding of fluorescently-labeled ligands or dye-coupled antibodies to the molecule of interest (Sharonov and Hochstrasser, 2006; Giannone et al., 2010). SPT using QDs relies on QD-conjugated antibodies, whereas sptPALM relies on endogenous expression of fluorescent proteins tagged to the target molecule. SPT and uPAINT techniques produce thousands of trajectories generating dense diffusion information with high spatiotemporal resolution. Although it depends on the length of the trajectories, this enables differentiation of active, confined or random movements. These movements can be followed on the cell surface and the landscape of the diffusion dynamics mapped. The most common parameters calculated from these techniques include the diffusion coefficient (D) reflecting the area explored and the mean squared displacement (MSD), the function ($f_{(t)}$) of which, describes the diffusion behavior over time i.e., Brownian, confined or directed (e.g., Kusumi et al., 1993; Saxton and Jacobson, 1997). QDs bound to receptors and their diffusion provided the first direct demonstration that receptors enter and exit the postsynapse *via* lateral diffusion (Dahan et al., 2003). Although they blink, QD fluorescence is more stable than that of conventional fluorophores with an average size of ∼10–15 nm (or bigger if one includes the binding components). Multiple exchanges of GlyRs between extrasynaptic and synaptic domains were observed, with free and confined states respectively. GlyRs were also tracked from one synaptic site to another 4–5 μm away demonstrating synaptic exchange by lateral diffusion of receptors. The D was ∼0.1 $\mu m^2/s$ outside of the synapses, matching that expected for free Brownian diffusion in a lipid bilayer. The D then decreased to ∼0.02 $\mu m^2/s$ (or lower) as it entered the synapse, corresponding to confined movement. QD tracking allows for relatively long acquisition times, yielding long trajectories where changes in diffusion properties can be mapped, however labeling density is low. In comparison, sptPALM, due to the fast bleaching of the fluorophores used, produces much shorter trajectories, but in far larger numbers. Thus, multiple-target tracing (MTT) has been employed to reconnect the single-molecule trajectories and extract their molecular dynamics (Sergé et al., 2008).

Recently, methods to analyze the movement of single molecule trajectories have been advanced with the aim to describe more accurately kinetics of individual interactions in native cell environments. Measuring the D of a whole trajectory does not take into account transient stabilizations *via* interactions with other molecules at given locations. Instead, the localized effective binding energy is more ideally suited to analyzing such biochemical interactions (Masson et al., 2014). Therefore, Masson et al. (2014) suggested an approach using Bayesian inference and overdamped Langevin equations to analyze the molecular motion. This generates an energy landscape which takes into account the heterogeneous diffusivity in the cell membrane. The depth of the energy trap is modulated by biochemical interactions between the receptor and scaffold proteins (Masson et al., 2014). This spatial cartography demonstrated that the presence of gephyrin clusters coincided with energy minima and hence was consistent with transient stabilization of receptors at synapses (El Beheiry et al., 2015). Consequently, the neuronal membrane has to be considered as a statistical field with constantly moving and transiently trapped molecules rather than formed by the juxtaposition of domains with fixed compositions. Using computer simulations and mathematical modeling, insight into molecular dynamics and the relationship between transient receptor trapping and local chemical reactions has been estimated. Moreover, using a Markovian approach, synaptic weight could be expressed as fluctuations in the number of bound receptors in the postsynapse (Holcman and Triller, 2006). Langevin equation models have enabled an analysis of molecular interactions of AMPARs (Hoze et al., 2012) and GlyRs (Masson et al., 2014; El Beheiry et al., 2015)

in the postsynaptic membrane. Additionally, the geometrical effect of membrane curvature on the 2D projected stochastic trajectory of a molecule affects diffusion properties (Domanov et al., 2011; Renner et al., 2011). The introduction of another parameter different from MTT based tracking and reconnection, the packing coefficient (Pc), allows characterization of the movement of a given molecule along its trajectory, thus as a function of time, independently of its overall diffusivity (Renner et al., 2017). It can also be utilized to derivate the effective K_{on} and K_{off} of a receptor to its scaffold. A cooperative mesoscopic model of the reciprocal stabilization of synaptic receptors and scaffolding proteins allowed accounting for the synapse "stability" as a quasi-equilibrium (Sekimoto and Triller, 2009). Then, using an out-of-equilibrium model, it has been proposed that the size of the scaffold clusters can be explained by the aggregation of gephyrin proteins diffusing in the sub-membrane space whilst bound to the GlyR, balanced against membrane turnover "aggregation-removal model" (Ranft et al., 2017).

The combination of theoretical modeling with single-molecule experimental data can now provide a quantification of synapse receptor dynamics in relation to the chemical modulation of these dynamics in the live cell environment, thus the concept of "chemistry *in-cellulo*" (Salvatico et al., 2015). This mixed experimental and theoretical approach will enable comprehension of how the dynamic movement of receptors and their interactions with other proteins can lead to longer-term stabilizations as well as the chemical determinants of receptor number and synapse function.

ACTIVITY REGULATION OF INHIBITORY RECEPTOR DIFFUSION

Many studies in recent years have concentrated on molecular mechanisms of inhibitory neurotransmission and synaptic scaffold protein modification that influence the local interactions and diffusion events underlying synaptic plasticity. It is now established that receptor diffusion-trapping at synapses can be affected by neuronal activity (**Figure 1**). Increased mobility of QD-labeled GlyRs and $GABA_A$Rs has been seen upon increased excitatory neuronal activity (Lévi et al., 2008; Bannai et al., 2009; Muir et al., 2010). Application of TTX to spinal cord neurons demonstrated reduced lateral diffusion of GlyR with an increase in receptor cluster number, but not for $GABA_A$Rs (Lévi et al., 2008). However the same effect was not seen for $GABA_A$Rs in hippocampal cells, instead, TTX application reduced $GABA_A$R lateral diffusion by means of an NMDA-calcineurin-dependent mechanism (Bannai et al., 2009), suggesting cell type and receptor type plays an additional regulatory role. In fact the involvement of NMDARs in the exocytosis of $GABA_A$Rs is CaMKII-dependent, and consequently potentiates inhibitory transmission (iLTP) (Marsden et al., 2007). It should be noted NMDAR-induced iLTP leads to a moderate intracellular calcium recruitment and activation of CaMKII (Lucchesi et al., 2011; Petrini et al., 2014). Conversely, iLTD *via* NMDARs and voltage-gated calcium channels leads to a massive increase in intracellular calcium and subsequent recruitment of calcineurin to inhibitory synapses (Bannai et al., 2009; Muir et al., 2010). These converging pathways constitute a fine-tuning of activity-dependent $GABA_A$R diffusion dynamics and thus inhibition. More precisely, calcineurin-induced phosphorylation of $GABA_A$Rs following NMDA activation confirmed the $GABA_A$R dispersal with important implications for activity-dependent control of synaptic inhibition (Muir et al., 2010). Conversely, $GABA_A$R cluster promotion at the postsynapse and enhanced GABAergic signaling *via* a metabotropic glutamate receptor-induced IP3 and PKC signaling pathway show spatiotemporal signaling patterns of calcium can fine-tune $GABA_A$R availability (Bannai et al., 2015). As shown in spinal cord neurons, PKC also phosphorylates the GlyR β-subunit at residue S403 (Specht et al., 2011). Consequently cross talk and competition between $GABA_A$Rs and GlyRs, at mixed GABA-Gly synapses in the spinal cord, adds an additional layer of complexity to the regulation of synaptic inhibition. Combining experimental work and theory it has been hypothesized that the long-term stability of synaptic cluster size obeys a dynamic equilibrium between the attraction of scaffold molecules to each other and the repulsion of receptor-receptor interactions (Haselwandter et al., 2011). Other synaptic components also impact these interactions. Upon chemical iLTP, $GABA_A$Rs are immobilized at synapses following active gephyrin recruitment in hippocampal neurons, the mechanism of which requires phosphorylation of $GABA_A$R-β3 by CaMKIIα (Petrini et al., 2014). Whilst gephyrin plays a critical role in $GABA_A$R membrane clustering, gephyrin-independent mechanisms of $GABA_A$R stabilization also exist. Following sustained excitatory activity $GABA_A$R mobility and clustering was shown to be independent of gephyrin clustering in hippocampal neurons (Niwa et al., 2012). More recently, QD-SPT combined with optogenetics to control calcium flow with high temporal precision showed inter-synaptic lateral diffusion of $GABA_A$Rs in a desensitized state in hippocampal neurons (de Luca et al., 2017). Synapses were typically 2–4 μm apart, with intersynaptic diffusion occurring in ∼15% trajectories at a D of 0.07 $\mu m^2 S^{-1}$. Further, they showed that glutamatergic activity limits this inter-synaptic diffusion *via* trapping $GABA_A$Rs at excitatory synapses. They suggested this might present a mechanism by which a memory of recent activation is transmitted to neighboring synapses. In addition to regulation of inhibitory synaptic receptors *via* direct neuronal activity, microglia have also been implicated in receptor dynamics. Prostaglandin E2 from microglia was recently shown to regulate GlyR diffusion dynamics and synaptic trapping but not GABAergic synapses (Cantaut-Belarif et al., 2017). Importantly, this demonstrated that microglia could regulate the plasticity of glycinergic synapses by tuning GlyR diffusion-trapping. Hence diffusion-trapping is not a cell-autonomous event. Additional fine-tuning of receptor diffusion dynamics may further occur in certain inflammatory states.

Recent work into the organization within synaptic clusters of receptor proteins and scaffold molecules have revealed the existence of subsynaptic domains in both excitatory (e.g., MacGillavry et al., 2013; Nair et al., 2013) and inhibitory

(e.g., Specht et al., 2013; Crosby et al., 2019) synapses. In spinal cord neurons the stoichiometry of gephyrin to GlyR binding sites was estimated to be approximately 1:1 (Specht et al., 2013). Incorporating super-resolution microscopy and model simulations, gephyrin stabilization in nano-domains was visualized upon iLTP which in turn stabilized the number of $GABA_A$Rs in mouse hippocampal neurons (Pennacchietti et al., 2017). In a separate study, QD-SPT of $GABA_A$R diffusion in rat hippocampal neurons showed GSK-3β and ERK1/2 differentially altered the gephyrin scaffold mesh, which as a result affected $GABA_A$R surface dynamics (Battaglia et al., 2018). They found that gephyrin microdomain compaction was regulated by phosphorylation in an activity-dependent way. Future work into this nano-organization and its control on intrasynaptic diffusion will allow understanding of long-term synaptic stability and $GABA_A$R/GlyR competition at inhibitory synapses.

FUNCTIONAL CONSEQUENCES OF DIFFUSION REGULATION

Diffusion trapping of receptors at synapses tunes receptor number, hence regulating neuronal activity with functional consequences on synaptic plasticity (Choquet and Triller, 2013; Petrini and Barberis, 2014). Plasticity associated changes in lateral mobility have been shown in inhibitory (e.g., Bannai et al., 2009; Petrini et al., 2014) and excitatory synapses (e.g., Ehlers et al., 2007; Makino and Malinow, 2009). In one such example, tracking surface $GABA_A$Rs on cultured hippocampal neurons during chemical iLTP showed synaptic recruitment of gephyrin from extrasynaptic regions was promoted by CamKII-dependent phosphorylation of $GABA_A$R-β3 at Ser838 (Petrini et al., 2014). Further, they showed that impairment of gephyrin assembly prevented chemical iLTP with an associated decrease in $GABA_A$R immobilization at synapses. Concurrently, changes in the exocytosis of inhibitory receptors can also occur upon neuronal activation, but over slower time courses (Marsden et al., 2007). This activity-dependent plasticity is hence determined by diffusion of the molecular synaptic components and the underlying mechanisms that regulate receptor availability across multi-time scales.

The link between lateral diffusion of receptors and their confinement at synapses with behavior is not yet understood. However, one mechanism has recently been described, linking RhoA/ROCK activity-dependent phosphorylation of radixin which in turn uncouples $GABA_A$R-α5 from extrasynaptic sites enabling their enrichment at synapses (Hausrat et al., 2015). This radixin phosphorylation was shown to occur in wild-type mice during short-term memory and reversal learning. In excitatory synapses, interfering with AMPAR surface diffusion impaired synaptic potentiation of Schaffer collaterals and commissural inputs to the CA1 of the mouse hippocampus in cultured slices and *in vivo* (Penn et al., 2017). Moreover, they showed AMPAR immobilization in the hippocampus *in vivo* inhibited fear conditioning. Thus, lateral diffusion of receptors and their temporal confinement at both excitatory and inhibitory synapses is likely to be a fundamental mechanism involved in learning and memory.

Affecting local and network-wide activity, diffusion dynamics may be implicated in certain neuropathologies. Benzodiazepines (BZDs) are widely used to treat many neurological and psychiatric diseases. It is now thought that in addition to their effects on receptor gating, membrane dynamics are also affected. SPT experiments of $GABA_A$Rs in mouse hippocampal neurons upon addition of the $GABA_A$R agonist muscimol showed accelerated $GABA_A$R diffusion, which was subsequently abolished upon addition of the BZD agonist diazepam (Gouzer et al., 2014). Using SPT in hippocampal neurons, diazepam was shown to increase synaptic stabilization and clustering of $GABA_A$Rs and decreased their lateral diffusion upon sustained neuronal activity but not at rest (Gouzer et al., 2014; Lévi et al., 2015). Acute estradiol treatment has also been demonstrated to decrease the confinement of $GABA_A$Rs, reducing their dwell time in synaptic compartments and increasing the D at extrasynaptic sites (Mukherjee et al., 2017). These results have a direct impact on the design of therapeutic compounds for diseases arising from dysregulation of inhibition.

CONCLUSIONS AND PERSPECTIVES

The plasma membrane is dynamic and trans-membrane molecules such as receptors diffuse laterally. These processes provide mechanisms for regulation of receptor number at synapses and thus function and plasticity. Recent results have isolated various pathways involved in receptor diffusion control, however, there are many important questions still to be answered. The contribution of receptor dynamics in synapse development, maturation, and refinement in both health and disease is yet to be fully explored. The interplay of $GABA_A$R and GlyR competition within inhibitory synapses in different brain regions, alongside distance and distribution of inhibitory and excitatory receptors is likely to underpin activity-dependent modification of synapse strength.

Whilst there have been huge technological advancements over a relatively short period of time, there remains inherent limitations in the currently used techniques for analyzing lateral membrane diffusion. QDs are a popular choice due to their photostability, bright fluorescence, long trajectories and ability to multiplex (Cutler et al., 2013; Kakizuka et al., 2016; Renner et al., 2017). However, their large size complexed with antibodies can sterically hinder lateral mobility (Abraham et al., 2017) and low-density labeling strategies mean only a fraction of the molecules are probed. The use of sptPALM enables direct genetic tagging of target molecules with a fluorescent protein, either by lentiviral expression or knock-in animal models, allowing tracking of all target molecules and analysis of endogenous molecule copy number (Lee et al., 2012; Specht et al., 2013). However incorrect protein folding and targeted degradation of the fluorescent protein-target complex can occur (Tanudji et al., 2002; Stepanenko et al., 2013; Guo et al., 2014) and trajectories are shorter than those of QDs. uPAINT relies on binding of high affinity fluorescently tagged antibodies or ligands to the target (Giannone et al., 2010).

The main drawback of this technique is the saturation of the target with bleached ligands. Future technical developments will include the manipulation of fluorescent proteins and organic probes to be smaller and brighter, improvements in microscope set-ups to track multiple proteins simultaneously, improved resolution in 3D imaging and tracking, and use of brain slices and *in vivo* set-ups will provide additional comprehension of diffusion dynamics within a biologically relevant microenvironment.

AUTHOR CONTRIBUTIONS

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

FUNDING

SM is supported by a Fondation pour la Recherche Médicale (FRM) postdoctoral fellowship (SPF201809007132).

REFERENCES

Abraham, L., Lu, H. Y., Falcão, R. C., Scurll, J., Jou, T., Irwin, B., et al. (2017). Limitations of Qdot labelling compared to directly-conjugated probes for single particle tracking of B cell receptor mobility. *Sci. Rep.* 7:11379. doi: 10.1038/s41598-017-11563-9

Allen, J. A., Halverson-Tamboli, R. A., and Rasenick, M. M. (2007). Lipid raft microdomains and neurotransmitter signalling. *Nat. Rev. Neurosci.* 8, 128–140. doi: 10.1038/nrn2059

Bannai, H., Lévi, S., Schweizer, C., Inoue, T., Launey, T., Racine, V., et al. (2009). Activity-dependent tuning of inhibitory neurotransmission based on $GABA_AR$ diffusion dynamics. *Neuron* 62, 670–682. doi: 10.1016/j.neuron.2009.04.023

Bannai, H., Niwa, F., Sherwood, M. W., Shrivastava, A. N., Arizono, M., Miyamoto, A., et al. (2015). Bidirectional control of synaptic $GABA_AR$ clustering by glutamate and calcium. *Cell Rep.* 13, 2768–2780. doi: 10.1016/j.celrep.2015.12.002

Battaglia, S., Renner, M., Russeau, M., Côme, E., Tyagarajan, S. K., and Lévi, S. (2018). Activity-dependent inhibitory synapse scaling is determined by gephyrin phosphorylation and subsequent regulation of $GABA_A$ receptor diffusion. *eNeuro* 5:ENEURO.0203-17.2017. doi: 10.1523/eneuro.0203-17.2017

Bogdanov, Y., Michels, G., Armstrong-Gold, C., Haydon, P. G., Lindstrom, J., Pangalos, M., et al. (2006). Synaptic $GABA_A$ receptors are directly recruited from their extrasynaptic counterparts. *EMBO J.* 25, 4381–4389. doi: 10.1038/sj.emboj.7601309

Borgdorff, A. J., and Choquet, D. (2002). Regulation of AMPA receptor lateral movements. *Nature* 417, 649–653. doi: 10.1038/nature00780

Calamai, M., Specht, C. G., Heller, J., Alcor, D., Machado, P., Vannier, C., et al. (2009). Gephyrin oligomerization controls GlyR mobility and synaptic clustering. *J. Neurosci.* 29, 7639–7648. doi: 10.1523/JNEUROSCI.5711-08.2009

Cantaut-Belarif, Y., Antri, M., Pizzarelli, R., Colasse, S., Vaccari, I., Soares, S., et al. (2017). Microglia control the glycinergic but not the GABAergic synapses *via* prostaglandin E2 in the spinal cord. *J. Cell Biol.* 216, 2979–2989. doi: 10.1083/jcb.201607048

Charrier, C., Ehrensperger, M.-V., Dahan, M., Lévi, S., and Triller, A. (2006). Cytoskeleton regulation of glycine receptor number at synapses and diffusion in the plasma membrane. *J. Neurosci.* 26, 8502–8511. doi: 10.1523/JNEUROSCI.1758-06.2006

Charrier, C., Machado, P., Tweedie-Cullen, R. Y., Rutishauser, D., Mansuy, I. M., and Triller, A. (2010). A crosstalk between β1 and β3 integrins controls glycine receptor and gephyrin trafficking at synapses. *Nat. Neurosci.* 13, 1388–1395. doi: 10.1038/nn.2645

Choquet, D. (2010). Fast AMPAR trafficking for a high-frequency synaptic transmission. *Eur. J. Neurosci.* 32, 250–260. doi: 10.1111/j.1460-9568.2010.07350.x

Choquet, D., and Triller, A. (2013). The dynamic synapse. *Neuron* 80, 691–703. doi: 10.1016/j.neuron.2013.10.013

Crick, F. (1984). Neurobiology: memory and molecular turnover. *Nature* 312:101. doi: 10.1038/312101a0

Crosby, K. C., Gookin, S. E., Garcia, J. D., Hahm, K. M., Dell'Acqua, M. L., and Smith, K. R. (2019). Nanoscale subsynaptic domains underlie the organization of the inhibitory synapse. *Cell Rep.* 26, 3284.e3–3297.e3. doi: 10.1016/j.celrep.2019.02.070

Cutler, P. J., Malik, M. D., Liu, S., Byars, J. M., Lidke, D. S., and Lidke, K. A. (2013). Multi-color quantum dot tracking using a high-speed hyperspectral line-scanning microscope. *PLoS One* 8:e64320. doi: 10.1371/journal.pone.0064320

Dahan, M., Lévi, S., Luccardini, C., Rostaing, P., Riveau, B., and Triller, A. (2003). Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. *Science* 302, 442–445. doi: 10.1126/science.1088525

de Luca, E., Ravasenga, T., Petrini, E. M., Polenghi, A., Nieus, T., Guazzi, S., et al. (2017). Inter-synaptic lateral diffusion of $GABA_A$ receptors shapes inhibitory synaptic currents. *Neuron* 95, 63.e5–69.e5. doi: 10.1016/j.neuron.2017.06.022

Dityatev, A., Schachner, M., and Sonderegger, P. (2010). The dual role of the extracellular matrix in synaptic plasticity and homeostasis. *Nat. Rev. Neurosci.* 11, 735–746. doi: 10.1038/nrn2898

Domanov, Y. A., Aimon, S., Toombes, G. E. S., Renner, M., Quemeneur, F., Triller, A., et al. (2011). Mobility in geometrically confined membranes. *Proc. Natl. Acad. Sci. U S A* 108, 12605–12610. doi: 10.1073/pnas.1102646108

Ehlers, M. D., Heine, M., Groc, L., Lee, M.-C., and Choquet, D. (2007). Diffusional trapping of GluR1 AMPA receptors by input-specific synaptic activity. *Neuron* 54, 447–460. doi: 10.1016/j.neuron.2007.04.010

El Beheiry, M., Dahan, M., and Masson, J.-B. (2015). InferenceMAP: mapping of single-molecule dynamics with Bayesian inference. *Nat. Methods* 12, 594–595. doi: 10.1038/nmeth.3441

Fujii, S., Tanaka, H., and Hirano, T. (2018). Suppression of AMPA receptor exocytosis contributes to hippocampal LTD. *J. Neurosci.* 38, 5523–5537. doi: 10.1523/JNEUROSCI.3210-17.2018

García-Morales, V., Montero, F., González-Forero, D., Rodríguez-Bey, G., Gómez-Pérez, L., Medialdea-Wandossell, M. J., et al. (2015). Membrane-derived phospholipids control synaptic neurotransmission and plasticity. *PLoS Biol.* 13:e1002153. doi: 10.1371/journal.pbio.1002153

Gerrow, K., and Triller, A. (2014). $GABA_A$ receptor subunit composition and competition at synapses are tuned by $GABA_B$ receptor activity. *Mol. Cell. Neurosci.* 60, 97–107. doi: 10.1016/j.mcn.2014.04.001

Giannone, G., Hosy, E., Levet, F., Constals, A., Schulze, K., Sobolevsky, A. I., et al. (2010). Dynamic superresolution imaging of endogenous proteins on living cells at ultra-high density. *Biophys. J.* 99, 1303–1310. doi: 10.1016/j.bpj.2010.06.005

Giesemann, T., Schwarz, G., Nawrotzki, R., Berhörster, K., Rothkegel, M., Schlüter, K., et al. (2003). Complex formation between the postsynaptic scaffolding protein gephyrin, profilin, and Mena: a possible link to the microfilament system. *J. Neurosci.* 23, 8330–8339. doi: 10.1523/JNEUROSCI.23-23-08330.2003

Gouzer, G., Specht, C. G., Allain, L., Shinoe, T., and Triller, A. (2014). Benzodiazepine-dependent stabilization of $GABA_A$ receptors at synapses. *Mol. Cell. Neurosci.* 63, 101–113. doi: 10.1016/j.mcn.2014.10.004

Groc, L., Choquet, D., Stephenson, F. A., Verrier, D., Manzoni, O. J., and Chavis, P. (2007). NMDA receptor surface trafficking and synaptic subunit composition are developmentally regulated by the extracellular matrix protein reelin. *J. Neurosci.* 27, 10165–10175. doi: 10.1523/JNEUROSCI.1772-07.2007

Guo, M., Gelman, H., and Gruebele, M. (2014). Coupled protein diffusion and folding in the cell. *PLoS One* 9:e113040. doi: 10.1371/journal.pone.0113040

Hannan, S., Minere, M., Harris, J., Izquierdo, P., Thomas, P., Tench, B., et al. (2019). $GABA_AR$ isoform and subunit structural motifs determine synaptic and extrasynaptic receptor localisation. *Neuropharmacology* doi: 10.1016/j.neuropharm.2019.02.022 [Epub ahead of print].

Haselwandter, C. A., Calamai, M., Kardar, M., Triller, A., and da Silveira, R. A. (2011). Formation and stability of synaptic receptor domains. *Phys. Rev. Lett.* 106:238104. doi: 10.1103/physrevlett.106.238104

Hausrat, T. J., Muhia, M., Gerrow, K., Thomas, P., Hirdes, W., Tsukita, S., et al. (2015). Radixin regulates synaptic $GABA_A$ receptor density and is essential for reversal learning and short-term memory. *Nat. Commun.* 6:6872. doi: 10.1038/ncomms7872

Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C., and Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* 287, 2262–2267. doi: 10.1126/science.287.5461.2262

Hennekinne, L., Colasse, S., Triller, A., and Renner, M. (2013). Differential control of thrombospondin over synaptic glycine and AMPA receptors in spinal cord neurons. *J. Neurosci.* 33, 11432–11439. doi: 10.1523/JNEUROSCI.5247-12.2013

Holcman, D., and Triller, A. (2006). Modeling synaptic dynamics driven by receptor lateral diffusion. *Biophys. J.* 91, 2405–2415. doi: 10.1529/biophysj.106.081935

Hoze, N., Nair, D., Hosy, E., Sieben, C., Manley, S., Herrmann, A., et al. (2012). Heterogeneity of AMPA receptor trafficking and molecular interactions revealed by superresolution analysis of live cell imaging. *Proc. Natl. Acad. Sci. U S A* 109, 17052–17057. doi: 10.1073/pnas.1204589109

Jacob, T. C., Bogdanov, Y. D., Magnus, C., Saliba, R. S., Kittler, J. T., Haydon, P. G., et al. (2005). Gephyrin regulates the cell surface dynamics of synaptic $GABA_A$ receptors. *J. Neurosci.* 25, 10469–10478. doi: 10.1523/JNEUROSCI.2267-05.2005

Kakizuka, T., Ikezaki, K., Kaneshiro, J., Fujita, H., Watanabe, T. M., and Ichimura, T. (2016). Simultaneous nano-tracking of multiple motor proteins *via* spectral discrimination of quantum dots. *Biomed. Opt. Express* 7, 2475–2493. doi: 10.1364/boe.7.002475

Kittler, J. T., Delmas, P., Jovanovic, J. N., Brown, D. A., Smart, T. G., and Moss, S. J. (2000). Constitutive endocytosis of $GABA_A$ receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. *J. Neurosci.* 20, 7972–7977. doi: 10.1523/JNEUROSCI.20-21-07972.2000

Kowalczyk, S., Winkelmann, A., Smolinsky, B., Förstera, B., Neundorf, I., Schwarz, G., et al. (2013). Direct binding of $GABA_A$ receptor β2 and β3 subunits to gephyrin. *Eur. J. Neurosci.* 37, 544–554. doi: 10.1111/ejn.12078

Kusumi, A., Sako, Y., and Yamamoto, M. (1993). Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. *Biophys. J.* 65, 2021–2040. doi: 10.1016/s0006-3495(93)81253-0

Lee, S.-H., Shin, J. Y., Lee, A., and Bustamante, C. (2012). Counting single photoactivatable fluorescent molecules by photoactivated localization microscopy (PALM). *Proc. Natl. Acad. Sci. U S A* 109, 17436–17441. doi: 10.1073/pnas.1215175109

Lévi, S., Le Roux, N., Eugène, E., and Poncer, J. C. (2015). Benzodiazepine ligands rapidly influence $GABA_A$ receptor diffusion and clustering at hippocampal inhibitory synapses. *Neuropharmacology* 88, 199–208. doi: 10.1016/j.neuropharm.2014.06.002

Lévi, S., Schweizer, C., Bannai, H., Pascual, O., Charrier, C., and Triller, A. (2008). Homeostatic regulation of synaptic GlyR numbers driven by lateral diffusion. *Neuron* 59, 261–273. doi: 10.1016/j.neuron.2008.05.030

Loebrich, S., Bähring, R., Katsuno, T., Tsukita, S., and Kneussel, M. (2006). Activated radixin is essential for $GABA_A$ receptor α5 subunit anchoring at the actin cytoskeleton. *EMBO J.* 25, 987–999. doi: 10.1038/sj.emboj.7600995

Lucchesi, W., Mizuno, K., and Giese, K. P. (2011). Novel insights into CaMKII function and regulation during memory formation. *Brain Res. Bull.* 85, 2–8. doi: 10.1016/j.brainresbull.2010.10.009

Lüthi, A., Chittajallu, R., Duprat, F., Palmer, M. J., Benke, T. A., Kidd, F. L., et al. (1999). Hippocampal LTD expression involves a pool of AMPARs regulated by the NSF-GluR2 interaction. *Neuron* 24, 389–399. doi: 10.1016/s0896-6273(00)80852-1

MacGillavry, H. D., Song, Y., Raghavachari, S., and Blanpied, T. A. (2013). Nanoscale scaffolding domains within the postsynaptic density concentrate synaptic AMPA receptors. *Neuron* 78, 615–622. doi: 10.1016/j.neuron.2013.03.009

Makino, H., and Malinow, R. (2009). AMPA receptor incorporation into synapses during LTP: the role of lateral movement and exocytosis. *Neuron* 64, 381–390. doi: 10.1016/j.neuron.2009.08.035

Mammen, A., Huganir, R., and O'Brien, R.-J. (1997). Redistribution and stabilization of cell surface glutamate receptors during synapse formation. *J. Neurosci.* 17, 7351–7358. doi: 10.1523/JNEUROSCI.17-19-07351.1997

Mammoto, A., Sasaki, T., Asakura, T., Hotta, I., Imamura, H., Takahashi, K., et al. (1998). Interactions of drebrin and gephyrin with profilin. *Biochem. Biophys. Res. Commun.* 243, 86–89. doi: 10.1006/bbrc.1997.8068

Marguet, D., Lenne, P.-F., Rigneault, H., and He, H.-T. (2006). Dynamics in the plasma membrane: how to combine fluidity and order. *EMBO J.* 25, 3446–3457. doi: 10.1038/sj.emboj.7601204

Maric, H.-M., Mukherjee, J., Tretter, V., Moss, S. J., and Schindelin, H. (2011). Gephyrin-mediated γ-aminobutyric acid type A and glycine receptor clustering relies on a common binding site. *J. Biol. Chem.* 286, 42105–42114. doi: 10.1074/jbc.M111.303412

Marsden, K. C., Beattie, J. B., Friedenthal, J., and Carroll, R. C. (2007). NMDA receptor activation potentiates inhibitory transmission through GABA receptor-associated protein-dependent exocytosis of $GABA_A$ receptors. *J. Neurosci.* 27, 14326–14337. doi: 10.1523/JNEUROSCI.4433-07.2007

Masson, J.-B., Dionne, P., Salvatico, C., Renner, M., Specht, C. G., Triller, A., et al. (2014). Mapping the energy and diffusion landscapes of membrane proteins at the cell surface using high-density single-molecule imaging and bayesian inference: application to the multiscale dynamics of glycine receptors in the neuronal membrane. *Biophys. J.* 106, 74–83. doi: 10.1016/j.bpj.2013.10.027

Meier, J., and Grantyn, R. (2004). A gephyrin-related mechanism restraining glycine receptor anchoring at GABAergic synapses. *J. Neurosci.* 24, 1398–1405. doi: 10.1523/JNEUROSCI.4260-03.2004

Meier, J., Vannier, C., Sergé, A., Triller, A., and Choquet, D. (2001). Fast and reversible trapping of surface glycine receptors by gephyrin. *Nat. Neurosci.* 4, 253–260. doi: 10.1038/85099

Muir, J., Arancibia-Carcamo, I. L., MacAskill, A. F., Smith, K. R., Griffin, L. D., and Kittler, J. T. (2010). NMDA receptors regulate $GABA_A$ receptor lateral mobility and clustering at inhibitory synapses through serine 327 on the 2 subunit. *Proc. Natl. Acad. Sci. U S A* 107, 16679–16684. doi: 10.1073/pnas.1000589107

Mukherjee, J., Cardarelli, R. A., Cantaut-Belarif, Y., Deeb, T. Z., Srivastava, D. P., Tyagarajan, S. K., et al. (2017). Estradiol modulates the efficacy of synaptic inhibition by decreasing the dwell time of $GABA_A$ receptors at inhibitory synapses. *Proc. Natl. Acad. Sci. U S A* 114, 11763–11768. doi: 10.1073/pnas.1705075114

Mukherjee, J., Kretschmannova, K., Gouzer, G., Maric, H.-M., Ramsden, S., Tretter, V., et al. (2011). The residence time of $GABA_A$Rs at inhibitory synapses is determined by direct binding of the receptor 1 subunit to gephyrin. *J. Neurosci.* 31, 14677–14687. doi: 10.1523/JNEUROSCI.2001-11.2011

Nair, D., Hosy, E., Petersen, J. D., Constals, A., Giannone, G., Choquet, D., et al. (2013). Super-resolution imaging reveals that AMPA receptors inside synapses are dynamically organized in nanodomains regulated by PSD95. *J. Neurosci.* 33, 13204–13224. doi: 10.1523/JNEUROSCI.2381-12.2013

Nishimune, A., Isaac, J. T., Molnar, E., Noel, J., Nash, S. R., Tagaya, M., et al. (1998). NSF binding to GluR2 regulates synaptic transmission. *Neuron* 21, 87–97. doi: 10.1016/s0896-6273(00)80517-6

Niwa, F., Bannai, H., Arizono, M., Fukatsu, K., Triller, A., and Mikoshiba, K. (2012). Gephyrin-independent $GABA_A$R mobility and clustering during plasticity. *PLoS One* 7:e36148. doi: 10.1371/journal.pone.0036148

Nusser, Z., Hájos, N., Somogyi, P., and Mody, I. (1998). Increased number of synaptic $GABA_A$ receptors underlies potentiation at hippocampal inhibitory synapses. *Nature* 395, 172–177. doi: 10.1038/25999

Park, M., Penick, E. C., Edwards, J. G., Kauer, J. A., and Ehlers, M. D. (2004). Recycling endosomes supply AMPA receptors for LTP. *Science* 305, 1972–1975. doi: 10.1126/science.1102026

Passafaro, M., Piëch, V., and Sheng, M. (2001). Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. *Nat. Neurosci.* 4, 917–926. doi: 10.1038/nn0901-917

Penn, A. C., Zhang, C. L., Georges, F., Royer, L., Breillat, C., Hosy, E., et al. (2017). Hippocampal LTP and contextual learning require surface diffusion of AMPA receptors. *Nature* 549, 384–388. doi: 10.1038/nature23658

Pennacchietti, F., Vascon, S., Nieus, T., Rosillo, C., Das, S., Tyagarajan, S. K., et al. (2017). Nanoscale molecular reorganization of the inhibitory postsynaptic

density is a determinant of GABAergic synaptic potentiation. *J. Neurosci.* 37, 1747–1756. doi: 10.1523/JNEUROSCI.0514-16.2016

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

Petrini, E. M., Ravasenga, T., Hausrat, T. J., Iurilli, G., Olcese, U., Racine, V., et al. (2014). Synaptic recruitment of gephyrin regulates surface $GABA_A$ receptor dynamics for the expression of inhibitory LTP. *Nat. Commun.* 5:3921. doi: 10.1038/ncomms4921

Poo, M., and Cone, R. A. (1974). Lateral diffusion of rhodopsin in the photoreceptor membrane. *Nature* 247, 438–441. doi: 10.1038/247438a0

Pooler, A. M., and McIlhinney, R. A. J. (2007). Lateral diffusion of the $GABA_B$ receptor is regulated by the $GABA_{B2}$ C terminus. *J. Biol. Chem.* 282, 25349–25356. doi: 10.1074/jbc.M702358200

Ranft, J., Almeida, L. G., Rodriguez, P. C., Triller, A., and Hakim, V. (2017). An aggregation-removal model for the formation and size determination of post-synaptic scaffold domains. *PLoS Comput. Biol.* 13:e1005516. doi: 10.1371/journal.pcbi.1005516

Renner, M., Choquet, D., and Triller, A. (2009). Control of the postsynaptic membrane viscosity. *J. Neurosci.* 29, 2926–2937. doi: 10.1523/JNEUROSCI.4445-08.2009

Renner, M., Specht, C. G., and Triller, A. (2008). Molecular dynamics of postsynaptic receptors and scaffold proteins. *Curr. Opin. Neurobiol.* 18, 532–540. doi: 10.1016/j.conb.2008.09.009

Renner, M., Domanov, Y., Sandrin, F., Izeddin, I., Bassereau, P., and Triller, A. (2011). Lateral diffusion on tubular membranes: quantification of measurements bias. *PLoS One* 6:e25731. doi: 10.1371/journal.pone.0025731

Renner, M., Wang, L., Levi, S., Hennekinne, L., and Triller, A. (2017). A simple and powerful analysis of lateral subdiffusion using single particle tracking. *Biophys. J.* 113, 2452–2463. doi: 10.1016/j.bpj.2017.09.017

Rosenberg, M., Meier, J., Triller, A., and Vannier, C. (2001). Dynamics of glycine receptor insertion in the neuronal plasma membrane. *J. Neurosci.* 21, 5036–5044. doi: 10.1523/jneurosci.21-14-05036.2001

Salvatico, C., Specht, C. G., and Triller, A. (2015). Synaptic receptor dynamics: from theoretical concepts to deep quantification and chemistry in cellulo. *Neuropharmacology* 88, 2–9. doi: 10.1016/j.neuropharm.2014.09.020

Saxton, M. J., and Jacobson, K. (1997). Single-particle tracking: applications to membrane dynamics. *Annu. Rev. Biophys. Biomol. Struct.* 26, 373–399. doi: 10.1146/annurev.biophys.26.1.373

Sekimoto, K., and Triller, A. (2009). Compatibility between itinerant synaptic receptors and stable postsynaptic structure. *Phys. Rev. E Stat. Nonlin. Soft Matter Phys.* 79:031905. doi: 10.1103/physreve.79.031905

Sergé, A., Bertaux, N., Rigneault, H., and Marguet, D. (2008). Dynamic multiple-target tracing to probe spatiotemporal cartography of cell membranes. *Nat. Methods* 5, 687–694. doi: 10.1038/nmeth.1233

Sergé, A., Fourgeaud, L., Hémar, A., and Choquet, D. (2002). Receptor activation and homer differentially control the lateral mobility of metabotropic glutamate receptor 5 in the neuronal membrane. *J. Neurosci.* 22, 3910–3920. doi: 10.1523/jneurosci.22-10-03910.2002

Sharonov, A., and Hochstrasser, R. M. (2006). Wide-field subdiffraction imaging by accumulated binding of diffusing probes. *Proc. Natl. Acad. Sci. U S A* 103, 18911–18916. doi: 10.1073/pnas.0609643104

Singer, S. J., and Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Science* 175, 720–731. doi: 10.1126/science.175.4023.720

Song, I., and Huganir, R. L. (2002). Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci.* 25, 578–588. doi: 10.1016/s0166-2236(02)02270-1

Specht, C. G., Grünewald, N., Pascual, O., Rostgaard, N., Schwarz, G., and Triller, A. (2011). Regulation of glycine receptor diffusion properties and gephyrin interactions by protein kinase C. *EMBO J.* 30, 3842–3853. doi: 10.1038/emboj.2011.276

Specht, C. G., Izeddin, I., Rodriguez, P. C., El Beheiry, M., Rostaing, P., Darzacq, X., et al. (2013). Quantitative nanoscopy of inhibitory synapses: counting gephyrin molecules and receptor binding sites. *Neuron* 79, 308–321. doi: 10.1016/j.neuron.2013.05.013

Stepanenko, O. V., Stepanenko, O. V., Kuznetsova, I. M., Verkhusha, V. V., and Turoverov, K. K. (2013). Beta-barrel scaffold of fluorescent proteins: folding, stability and role in chromophore formation. *Int. Rev. Cell Mol. Biol.* 302, 221–278. doi: 10.1016/B978-0-12-407699-0.00004-2

Tanaka, H., and Hirano, T. (2012). Visualization of subunit-specific delivery of glutamate receptors to postsynaptic membrane during hippocampal long-term potentiation. *Cell Rep.* 1, 291–298. doi: 10.1016/j.celrep.2012.02.004

Tanudji, M., Hevi, S., and Chuck, S. L. (2002). Improperly folded green fluorescent protein is secreted *via* a non-classical pathway. *J. Cell Sci.* 115, 3849–3857. doi: 10.1242/jcs.00047

Tardin, C., Cognet, L., Bats, C., Lounis, B., and Choquet, D. (2003). Direct imaging of lateral movements of AMPA receptors inside synapses. *EMBO J.* 22, 4656–4665. doi: 10.1093/emboj/cdg463

Thomas, P., Mortensen, M., Hosie, A. M., and Smart, T. G. (2005). Dynamic mobility of functional $GABA_A$ receptors at inhibitory synapses. *Nat. Neurosci.* 8, 889–897. doi: 10.1038/nn1483

Tretter, V., Jacob, T. C., Mukherjee, J., Fritschy, J.-M., Pangalos, M. N., and Moss, S. J. (2008). The clustering of $GABA_A$ receptor subtypes at inhibitory synapses is facilitated *via* the direct binding of receptor 2 subunits to gephyrin. *J. Neurosci.* 28, 1356–1365. doi: 10.1523/JNEUROSCI.5050-07.2008

Turrigiano, G. G. (2000). AMPA receptors unbound: membrane cycling and synaptic plasticity. *Neuron* 26, 5–8. doi: 10.1016/s0896-6273(00)81131-9

Yudowski, G. A., Puthenveedu, M. A., Leonoudakis, D., Panicker, S., Thorn, K. S., Beattie, E. C., et al. (2007). Real-time imaging of discrete exocytic events mediating surface delivery of AMPA receptors. *J. Neurosci.* 27, 11112–11121. doi: 10.1523/JNEUROSCI.2465-07.2007

Conflict of Interest: 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.

The handling Editor declared a past co-authorship with one of the authors AT.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership