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Activity-dependent plasticity of presynaptic GABA_B receptors at parallel fiber synapses

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Abstract

Parallel fiber synapses in the cerebellum express a wide range of presynaptic receptors. However, presynaptic receptor expression at individual parallel fiber synapses is quite heterogeneous, suggesting physiological mechanisms regulate presynaptic receptor expression. We investigated changes in presynaptic GABA_B receptors at parallel fiber-stellate cell synapses in acute cerebellar slices from juvenile mice. GABA_B receptor-mediated inhibition of excitatory postsynaptic currents (EPSCs) is remarkably diverse at these synapses, with transmitter release at some synapses inhibited by >50% and little or no inhibition at others. GABA_B receptor-mediated inhibition was significantly reduced following 4 Hz parallel fiber stimulation but not after stimulation at other frequencies. The reduction in GABA_B receptor-mediated inhibition was replicated by bath application of forskolin and blocked by application of a PKA inhibitor, suggesting activation of adenylyl cyclase and PKA are required. Immunolabeling for an extracellular domain of the GABA_{B2} subunit revealed reduced surface expression in the molecular layer after exposure to forskolin. GABA_B receptor-mediated inhibition of action potential evoked calcium transients in parallel fiber varicosities was also reduced following bath application of forskolin, confirming presynaptic receptors are responsible for the reduced EPSC inhibition. These data demonstrate that presynaptic GABA_B receptor expression can be a plastic property of synapses, which may compliment other forms of synaptic plasticity. This opens the door to novel forms of receptor plasticity previously confined primarily to postsynaptic receptors.

Keywords

cerebellum; GABA_B; parallel fiber; plasticity; presynaptic; stellate; synapse

1 | INTRODUCTION

Over the past several decades many studies have described in detail the composition, trafficking, modulation, and physiological functions of postsynaptic neurotransmitter receptors. However, an increasing number of central synapses are found to also express

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receptors at the presynaptic terminal. Although many studies have examined the expression and function of presynaptic receptors, comparatively few studies have examined activity-dependent plasticity or modulation of these receptors.

Parallel fiber synapses in the cerebellum have been shown to express a remarkable diversity of presynaptic receptors (Delaney & Jahr, 2002; Dittman & Regehr, 1996; Stell, Rostaing, Triller, & Marty, 2007; Zhang & Linden, 2009). However, this doesn't necessarily mean that every parallel fiber synapses expresses every type of receptor. In fact, it has been shown that expression of presynaptic adenosine (A1), metabotropic glutamate (mGluR4), and cannabinoid (Cb1) receptors varies greatly from bouton to bouton, even among consecutive boutons on the same parallel fiber (Zhang & Linden, 2009). One bouton may have high mGluR4 expression, but low Cb1 receptor expression, while a neighboring bouton has high A1 receptor expression, but low mGluR4 expression. This raises the question of which factors determine the level of receptor expression at a particular synapse, and further, is the complement of receptors expressed at a particular synapse a static or dynamic property of the synapse.

One possibility is that presynaptic receptor expression is a plastic property of the synapse that can be modulated by physiological patterns of activity. To test this possibility, we have examined the activity of presynaptic GABA_B receptors (GABA_BRs) at parallel fiber synapses in the cerebellum. Presynaptic GABA_BRs have been identified at a wide range of synapses in the central nervous system and have been implicated in a wide range of diseases including epilepsy (Asproдини, Rainnie, & Shinnick-Gallagher, 1992; Han, Cortez, & Snead, 2012), schizophrenia (Fatemi, Folsom, & Thuras, 2017; Zai et al., 2014), obsessive compulsive disorder (Richter et al., 2012), and ischemia (Cimarosti, Kantamneni, & Henley, 2009). Several studies have shown that presynaptic GABA_BR expression can be altered by pathophysiological conditions. For example, presynaptic GABA_BR expression is reduced in fragile-x syndrome (Kang et al. 2017) and following seizures (Straessle, Loup, Arabadzisz, Ohning, & Fritschy, 2003; Thompson, Ayman, Woodhall, & Jones, 2007) or prolonged exposure to methamphetamine (Padgett et al., 2012). Presynaptic GABA_BR expression is also reduced following long-term activity blockade with tetrodotoxin (Laviv et al., 2010), suggesting homeostatic regulation of receptor expression. However, as far as we are aware, physiological patterns of activity capable of inducing presynaptic GABA_BR plasticity have not been identified.

We examined changes in GABA_BR activity at parallel fiber to stellate cell synapses in the cerebellum of juvenile mice. We found that GABA_BR-mediated inhibition of synaptic responses was transiently reduced following 4 Hz parallel fiber stimulation, but not by other stimulus frequencies tested. This effect was replicated by bath application of forskolin, an adenylyl cyclase activator, and blocked by KT5720, a PKA inhibitor. Immunolabeling revealed reduced surface expression of GABA_BRs immediately following exposure to forskolin. We found a similar reduction in GABA_BR-mediated inhibition of action potential evoked calcium transients in parallel fiber varicosities following application of forskolin using two-photon calcium imaging, consistent with a presynaptic change. These data demonstrate activity-dependent plasticity of presynaptic GABA_BRs.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center San Antonio (protocol 14013x).

2.2 | Slice preparation

Acute parasagittal brain slices were prepared from the cerebella of male and female P14-P25 (average P16.7) C57BL/6 mice (Charles River). Mice were deeply anesthetized with isoflurane before rapid dissection of the cerebellum in accordance with University protocols and guidelines. The cerebellum was immediately placed in ice-cold (0–4°C), oxygenated (95% O₂ and 5% CO₂) low calcium/high magnesium artificial cerebrospinal fluid (aCSF) containing (in mM): 110 NaCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 0.5 CaCl₂, 3 MgCl₂, 15 glucose. Slices (300 µm) were cut from the vermis of the cerebellum using a vibratome (Leica Biosystems) and transferred to a submerged recovery chamber filled with standard oxygenated aCSF (in mM: 115 NaCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 2 CaCl₂, 1.3 MgCl₂, 25 glucose), and maintained at 35°C for 30 min. Slices were allowed to return from 35°C to room temperature and subsequently kept under continuous oxygenation until being transferred to the recording chamber.

2.3 | Electrophysiology

During all recordings, slices were superfused with room temperature aCSF containing either 100 µM picrotoxin (PTX, Abcam) or 10 µM gabazine (SR 95531, Sigma) to block GABA_A receptors at a flow rate of ~2 mL/min. For GABA uncaging experiments, 10 mL of aCSF containing 60 µM RuBi-GABA (Tocris) was recirculated using a fluid pump (Masterflex). Where indicated, 20 µM Forskolin (Tocris) and 1 µM KT5720 (Tocris) were diluted in aCSF and bath applied.

Putative stellate cells were visually identified with gradient contrast infrared optics (Dodt, Eder, Schierloh, & Zieglängsberger, 2002) in the outer third of the molecular layer and patched with borosilicate pipettes (4–6 MΩ) filled with internal solution containing the following (in mM): 125 CsMeSO₃ 125, 2 MgCl₂, 10 NaCl, 10 HEPES, 10 EGTA, 4 Na-ATP, 0.5 Na-GTP, 2 Qx-314 (pH 7.3–7.4). Electrophysiological currents were recorded with a Multiclamp 700B amplifier (Molecular Devices), filtered at 5 kHz and digitized at 50 kHz. Data were collected using pClamp software (Axon Instruments). Excitatory postsynaptic currents (EPSCs) were evoked by stimulation of parallel fibers with 1–3 pulses (100 µs, 10–60 V) at 50 Hz through a patch pipette filled with aCSF. On alternating sweeps, GABA was photolytically uncaged by fullfield 5 ms light pulses from a 470 nm LED (Cool LED; 20% power) 350 ms prior to parallel fiber stimulation (Fig 1a). Five millisecond light pulses were used to maximize activation of axonal GABA receptors and to mimic the relatively slow “spillover” GABA transient experienced by parallel fiber GABA receptors (Dittman & Regehr, 1997; Pugh & Jahr, 2011). GABA_BR-mediated inhibition was calculated by comparing the average EPSC amplitude in control sweeps and following GABA uncaging ($1 - \text{EPSC}_{\text{GABA}} / \text{EPSC}_{\text{ctrl}}$). Cells that did not display GABA_BR inhibition (~5% of cells) or

the level of inhibition was not stable during the baseline period (>35% change in inhibition over 5 min; ~30% of cells) were excluded from the analysis.

2.4 | Calcium imaging

Granule cells were visually identified and patched using borosilicate pipettes (4–6 M Ω) containing the following (in mM): 142 KGlu, 2 KCl, 4 MgCl₂, 10 HEPES, 4 Na-ATP, and 0.5 Na-GTP (pH 7.3–7.4, osmolality ~315 mosmol kg⁻¹). Pipette solutions also contained the fluorescent dye Alexa 594 (50 μ M, Invitrogen) and the calcium-sensitive fluorescent dye Fluo-5 F (200 μ M, Invitrogen). Calcium imaging was performed using a two-photon laser scanning microscope (Scientifica) with a Ti:sapphire laser (Coherent, Santa Clara, CA) tuned to 810 nm. Photomultipliers (Hamamatsu, Hamamatsu City, Japan) collected red and green light in both the epi- and transfluorescence pathways. ScanImage software (Pologruto, Sabatini, & Svoboda, 2003) was used for acquisition. Line scans were performed at 500 Hz. Fluorescence changes were calculated by subtracting the baseline from the green trace and dividing by the red trace (G/R). Single action potentials were evoked at the soma and AP-dependent calcium transients were measured at parallel fiber varicosities before and after 10–30 s pressure application (PDES-02TX; NPI electronic) of baclofen (100 μ M, Abcam) from a patch-clamp pipette.

2.5 | Analysis

Data were analyzed using IgorPro (Wavemetrics), Clampfit 10 (Molecular Device) and ImageJ (NIH). Statistical significance was determined using paired Student's *t* tests in Excel (Microsoft). A value of *p* < .05 was considered significant. Data are reported as mean $\pm$ SE. Stimulus artifacts have been digitally removed in all figures.

2.6 | Immunolabeling

After slicing and recovery for 30 min at 35°C, acute sagittal cerebellar slices (200 μ m) were transferred to 12-well plates containing either oxygenated control aCSF or oxygenated aCSF containing forskolin (10 μ M) for 6 min and then rapidly incubated in 4% paraformaldehyde in 0.1 M PB for 20 min and immediately used for immunohistochemistry. Following one hour incubation in the blocking solution containing 4% goat serum, slices were incubated overnight at 4°C with the anti-GABA_{B2} antibody (1:100, sc-393286 Santa Cruz) directed against the extracellular domain of the GABA_{B2} subunit. Slices were then washed in PBS 0.1 M and incubated for 1 h 30 minutes with the secondary antibody conjugated to AlexaFluor 488 (1:400; Life Technologies). For each experiment cerebellar slices were obtained from two sibling animals of the same gender. Slices from one animal were used as the control group (exposed only to aCSF) and slices from the other animal were used as the treatment group (exposed to forskolin). We repeated this process in four independent labeling experiments. For each experiment the control and forskolin slices were processed together.

2.7 | Image acquisition

Sections were observed on a confocal laser scanning microscope (CLSM; Zeiss LSM700) equipped with solid state fiber optic lasers and single plane images were acquired with a $1\times$

digital zoom using a 20× objective. Three non-consecutive sections were analyzed and an average of 10 images per section was acquired for each animal from control and forskolin groups. All images have been acquired at a resolution of $1,024 \times 1,024$ pixels with the same parameters.

2.8 | Analysis

We first created a region of interest (ROI) using ImageJ used to measure the background and the average intensity of fluorescence in the molecular layer of each image. Out of the values obtained for each image of a section, we then calculated the mean background and signal intensity and subtract the background value to the mean fluorescence intensity signal.

Normalized fluorescence intensity was obtained by divided mean value in forskolin to mean control value for each control/forskolin pair.

3 | RESULTS

3.1 | Heterogeneity of presynaptic GABA_BR-mediated inhibition

As we have done previously in Howell and Pugh (2016), we measured presynaptic GABA_BR-mediated inhibition of glutamate release by performing whole cell patch-clamp recordings from molecular layer interneurons (MLIs) in acute cerebellar slices and recorded evoked EPSCs induced by paired stimulation of parallel fibers (20 ms ISI; Figure 1a). On alternating sweeps, GABA_BRs were activated by photolytic uncaging of Rubi-GABA 350 ms prior to parallel fibers stimulation. GABA_ARs were blocked by 100 μM picrotoxin or 10 μM gabazine in the bath solution. As has been shown previously (Dittman & Regehr, 1996, 1997; Howell & Pugh, 2016), GABA_BR activation decreased EPSC peak amplitudes ($40.3 \pm 1.8\%$ inhibition, $n = 115$, $p < .0001$) and increased the paired-pulse ratio (PPR) (2.23 ± 0.07 vs. 2.98 ± 0.15 , $n = 122$, $p < .0001$), suggesting a presynaptic inhibition. Interestingly, we found the extent of presynaptic GABA_BR-mediated inhibition varied widely across cells, from little or no inhibition in some cells to ~80% inhibition of postsynaptic responses at others (Figure 1b,c). As can be seen in the frequency histogram (Figure 1c) GABA_BR-mediated inhibition was $< 30\%$ in ~1/3 of cells (44/133) and $> 50\%$ in ~1/3 of cells (40/133). The average GABA_BR-mediated inhibition and variability of inhibition across cells did not change over the age range tested (P15: %inhibition $= 36.5\%$, standard deviation $= 17.9\%$; P23: %inhibition $= 34.8\%$, $SD = 18.4\%$) and the GABA_BR-mediated inhibition did not correlated with the age of the animal ($r^2 = .003$).

We hypothesized that the variability in presynaptic inhibition reflected a functional plasticity of GABA_B receptors and tested whether physiological patterns of activity could induce changes in GABA_BR-mediated inhibition.

3.2 | 4 Hz stimulation reduces presynaptic GABA_BR-mediated inhibition

For each cell, we first measured GABA_BR-mediated inhibition of glutamate release over a 10-min period (using the same experimental paradigm shown in Figure 1a) to confirm that the level of inhibition was stable (Figure 2a left, b). We then stimulated parallel fibers at 4 Hz for 15 s, a stimulus protocol previously shown to induce long-term potentiation (LTP) at parallel fiber synapses (Qiu & Knopfel, 2007; Salin, Malenka, & Nicoll, 1996; Storm,

Hansel, Hacker, Parent, & Linden, 1998), and again measured GABA_BR-mediated inhibition for an additional ~20 min. We found that GABA_BR-mediated inhibition was significantly reduced immediately after 4 Hz stimulation ($43.8 \pm 5.2\%$ vs. $23.7 \pm 7.9\%$ after 4 Hz stimulation; $n=14$, $p=.02$; Figure 2a,b). Furthermore, GABA_BR activation increased the PPR during the baseline period (2.0 ± 0.2 vs. 2.7 ± 0.4 ; $n=10$; $p=.02$), but did not alter the PPR after the 4 Hz stimulation (2.5 ± 0.4 vs. 2.5 ± 0.4 , $p=.9$; Figure 2c,d). Likewise, the increase in CV² of EPSC amplitudes by GABA was greater during the baseline ($CV_{\text{cnt}}^2/CV_{\text{GABA}}^2 = 0.50 \pm 0.07$) than following 4 Hz stimulation (0.79 ± 0.12 , $n=13$, $p=.04$; Figure 2e), consistent with a reduction of presynaptic inhibition. In approximately two-thirds of the cells (9/14), GABA_BR-mediated inhibition was biphasic, returning to the baseline level of inhibition after 5–10 min, while in the remaining cells (5/14) inhibition was reduced throughout the duration of the recording. As shown in Figure 1, the level of GABA_BR-mediated inhibition during the baseline period was quite variable; however, this did not correlate with the subsequent change in EPSC amplitude ($r^2=.02$) or the change in GABA_BR-mediated inhibition ($r^2=.1$) following 4 Hz stimulation. There was also no change in the decay kinetics of EPSCs (τ -decay: 2.30 ± 0.25 ms vs. 2.13 ± 0.23 ms, $n=10$, $p=.3$).

In order to determine the specificity of this phenomenon to 4 Hz stimulation, we tested whether other stimulus frequencies (1, 8, 20, 50, and 100 Hz) could also induce changes in GABA_BR-mediated inhibition. We did not observe a significant change in inhibition for any of the other frequencies used (for all frequencies $p>.2$, Figure 2f). However, following 8 Hz stimulation GABA uncaging no longer increased the PPR (baseline PPR: 1.5 ± 0.1 vs. 1.8 ± 0.2 , $p=0.03$; 8 Hz stim PPR: 1.4 ± 0.1 vs. 1.5 ± 0.2 , $p=.7$), suggesting there may be a partial loss of presynaptic GABA_BR-mediated inhibition that was not observed by measuring inhibition of EPSC amplitudes alone. These data suggest that GABA_BR-mediated inhibition can be suppressed specifically by 4 Hz stimulation.

3.3 | Signaling mechanism

Previous studies have shown that 4 or 8 Hz stimulation of parallel fibers can activate adenylyl cyclase and increase cAMP in parallel fiber boutons (Salin et al., 1996, Rancillac & Crpel 2004). To test whether adenylyl cyclase and cAMP are sufficient for the suppression of GABA_BR-mediated inhibition, we replaced the 4 Hz stimulation with bath application of the adenylyl cyclase activator, forskolin. We found that application of forskolin significantly reduced GABA_BR-mediated inhibition of EPSCs (baseline: $47.2 \pm 4.1\%$, forskolin: $18.5 \pm 6.6\%$, $n=14$, $p=.002$; Figure 3a,c). Unlike 4 Hz stimulation, following forskolin application the majority of cells (9/14) showed persistent reduction in GABA_BR-mediated inhibition for the duration of the recording, while inhibition recovered to baseline after 5–10 min in only five cells. The increased portion of cells showing a long-term reduction of inhibition compared with cells given 4 Hz stimulation was likely due to stronger or longer activation of adenylyl cyclase by forskolin. Furthermore, activation of GABA_BRs increased the PPR during the baseline period (1.9 ± 0.1 vs. 3.0 ± 0.3 , $p=.001$), but not in the presence of forskolin (2.0 ± 0.3 vs. 1.8 ± 0.2 , $p=.58$; Figure 3b), consistent with the loss of presynaptic inhibition. These results suggested that activation of adenylyl cyclase was sufficient to suppress the inhibitory effect of presynaptic GABA_BRs on vesicular release.

One of the main downstream targets of cAMP is PKA, and PKA has been shown to phosphorylate GABA_BRs (Adelfinger et al., 2014; Fairfax et al., 2004). We therefore tested whether PKA was necessary for suppression of GABA_BR-mediated inhibition. In the presence of the specific PKA inhibitor KT5720 there was no change in GABA_BR-mediated inhibition following application of forskolin ($47.3 \pm 4.5\%$ vs. $47.4 \pm 4.5\%$, $n=11$; $p=.38$; Figure 3d1). Furthermore, in the presence of KT5720, the PPR was increased by GABA uncaging during the baseline ($p=.02$) and in the presence of forskolin ($p=.001$, $n=11$; Figure 3d2), consistent with continued presynaptic GABA_BR-mediated inhibition in forskolin. These data suggest that activation of adenylyl cyclase and PKA is sufficient to reduce presynaptic GABA_BR-mediated inhibition of transmitter release.

Previous studies have shown that multiple phosphorylation sites on GABA_BRs control the membrane and intracellular trafficking of GABA_BRs (Fairfax et al., 2004; Kuramoto et al., 2007). To investigate the possibility that GABA_BRs are internalized, we used immunofluorescence to label surface expression of GABA_{B2} subunits in acute cerebellar slices bathed in either control aCSF or 10 μ M forskolin 6 minutes prior to fixation. As expected, we found GABA_{B2} antibody labeling in all cerebellar cortical layers. In the molecular layer, which is primarily composed of parallel fibers and Purkinje cell dendrites, forskolin treatment significantly decreased mean fluorescence intensity obtained from GABA_{B2} immunostaining ($75.6 \pm 4.0\%$ of control, $p=.01$; $n=4$, Figure 3e1,e2). Although we were not able to distinguish GABA_BR expression on Purkinje cell dendrites and parallel fibers, these results are consistent with a decrease in GABA_BR surface expression in parallel fiber boutons following forskolin application.

3.4 | Inhibition of presynaptic calcium influx

In the preceding experiments, we measured activity of presynaptic GABA_BRs by inhibition of postsynaptic EPSCs and changes in the PPR. To more directly measure presynaptic GABA_BR activity and to rule out the possibility that postsynaptic changes are responsible for reduced GABA_BR-mediated inhibition, we used two-photon laser scanning microscopy to measure action potential dependent calcium influx in the presynaptic terminal. We made patch-clamp recordings from cerebellar granule cells and filled them with Alexa594 to visualize the morphology of the cell, and fluo5F to measure changes in intracellular calcium. This allowed us to identify the axon and follow it out to the molecular layer where it branches to form parallel fibers (Figure 4a). We then performed a line scan over a parallel fiber varicosity to measure calcium-dependent changes in fluorescence in response to action potentials evoked at the soma (Figure 4a right). We measured calcium responses either in control conditions or during pressure application of baclofen (a GABA_BR agonist) through a pipette placed above the surface of the slice. Consistent with previous studies (Dittman & Regehr, 1997; Sakaba & Neher, 2003), we found that baclofen reduced the calcium response (Figure 4b top, 4c), while puffing aCSF had no effect ($p=.5$, $n=3$). However, with the addition of forskolin, the effect of baclofen on the calcium response was reduced ($44.9 \pm 4.5\%$ inhibition vs. $28.1 \pm 5.8\%$ inhibition; $n=11$; $p=.005$; Figure 4b bottom, 4c). Likewise, applying depolarizing currents at the granule cell soma at 4 Hz to evoke firing also reduced the effect of baclofen on calcium responses in the axon ($44.5 \pm 10.0\%$ inhibition vs. $33.8 \pm 10.3\%$ inhibition, $n=3$, $p=.05$; Figure 4c). These data demonstrate that the change in

GABA_BR activity can be observed directly in calcium influx in the presynaptic bouton, although they do not rule out changes in the postsynaptic cell as well.

4 | DISCUSSION

In this study, we find that presynaptic GABA_BR expression is a plastic property of parallel fiber synapses in juvenile mice. GABA_BR-mediated inhibition of vesicular release was reduced following 4 Hz parallel fiber stimulation, while all other stimulus frequencies tested did not alter inhibition. GABA_BR-mediated inhibition was also suppressed by direct pharmacological activation of adenylyl cyclase and this effect is blocked by inhibiting PKA. Using immunohistochemistry, we showed that surface expression of GABA_BRs in the molecular layer of the cerebellum was reduced following exposure to forskolin, consistent with reduced expression in parallel fiber boutons. We also observed a reduction in GABA_BR-mediated inhibition of presynaptic calcium influx using two-photon calcium imaging, consistent with the loss of presynaptic inhibition. These data demonstrate for the first time that physiological patterns of activity can alter presynaptic GABA_BR activity.

Despite a wealth of studies on activity-dependent trafficking of postsynaptic receptors (Ehlers, 2000; Ju et al., 2004), few studies have observed or described this process in presynaptic terminals. Several studies have shown changes in presynaptic GABA_BRs in pathophysiological conditions. For example, presynaptic GABA_BR expression is reduced in the ventral tegmental area following prolonged opioid use (Padgett et al., 2012), and at multiple synapses in the hippocampus and entorhinal cortex following epileptic activity (Sperk, Furtinger, Schwarzer, & Pirker, 2004; Straessle et al., 2003; Thompson et al., 2007). A handful of reports describe activity-dependent plasticity of other types of presynaptic receptors. Presynaptic kainate receptors are upregulated during long-term depression (LTD) at Schaffer collateral synapses, though this was only observed at immature synapses (Clarke, Molchanova, Hirvonen, Taira, & Lauri, 2014). Presynaptic inhibition through cannabinoid receptors is down regulated at basket cell synapses in the hippocampus during high-frequency (20 Hz) activity (Foldy, Neu, Jones, & Soltesz, 2006), though this appears to be due to changes in presynaptic voltage-gated calcium channels rather than the receptor itself. This study demonstrates activity-dependent plasticity of presynaptic inhibition mediated by GABA_BRs following physiological patterns of stimulation. Although the plasticity shown here is transient in many cells (5–10 min), this demonstrates that presynaptic GABA_BR activity is a regulated property of the synapse. This extends the field of receptor plasticity from the postsynaptic side of the synapse, where the signaling and trafficking mechanisms of receptor plasticity are well known, to the presynaptic side of the synapse, which may employ unique signaling and trafficking mechanisms (Huang et al., 2012; Pennock, Dicken, & Hentges, 2012).

4.1 | Mechanism of GABA_BR plasticity

We considered several mechanisms that could produce a loss of GABA_BR-mediated inhibition. An increase in activity of GABA transporters could reduce the concentration or duration of GABA reaching the receptors. However, in experiments using baclofen, a non-transported GABA_BR agonist, instead of the transported molecule GABA, we observed the

same reduction in inhibition (Figure 4), indicating that a change in transporter activity is not sufficient to explain this phenomenon. An increase in presynaptic calcium channel number or activation, such that the vesicular release machinery is nearly saturated by calcium even during GABA_BR activation, could also explain the reduced inhibition. However, we did not observe a consistent increase in EPSC amplitude in control sweeps following 4 Hz stimulation or in action potential evoked calcium transients after forskolin application (Chen & Regehr, 1997), arguing against a change in presynaptic calcium channels. Our data suggests a mechanism dependent on phosphorylation by PKA. Several studies have shown that PKA can phosphorylate GABA_BRs at multiple sites (Adelfinger et al., 2014; Kuramoto et al., 2007) and that phosphorylation regulates receptor trafficking (Fairfax et al., 2004; Terunuma et al., 2010). However, PKA phosphorylation generally increases surface expression by stabilizing GABA_BRs at the plasma membrane, while our results indicate decreased expression. One possible explanation is that that PKA does not directly phosphorylate GABA_BRs, but acts on other proteins that then modify GABA_BR activity and/or trafficking. Activation of NMDA receptors has been shown to transiently reduce GABA_BR surface expression by altering postendocytic sorting with a similar time course to the loss of GABA_BR inhibition seen in this study (Terunuma et al., 2010). It has been suggested that parallel fibers express presynaptic NMDA receptors (Bouvier et al., 2016; Casado, Dieudonné, & Ascher, 2000; but see Shin & Linden, 2005) and several studies have shown that NMDA receptor function is directly modulated by PKA (Aman, Maki, Ruffino, Kasperek, & Popescu, 2014; Skeberdis et al., 2006). Finally, we find that surface expression of GABA_BRs in the molecular layer is reduced following exposure to forskolin, consistent with internalization (or reduced recycling) of presynaptic GABA_BRs. However, it is not possible to distinguish whether this represents a change in presynaptic or postsynaptic GABA_BRs, or both. Although we cannot rule out the possibility of changes in G-protein signaling downstream of GABA_BR activation, our data is most consistent with PKA dependent internalization of presynaptic GABA_B receptors.

4.2 | Sources of synapse heterogeneity

We propose that activity-dependent changes in presynaptic GABA_BR expression contribute to the variability in GABA_BR-mediated inhibition observed at parallel fiber synapses. However, other sources of variability may also contribute to the differences in presynaptic inhibition. Release probability at parallel fiber synapses is highly variable (Nahir & Jahr, 2013; van Beugen, Gao, Boele, Hoebeek, & De Zeeuw, 2013) and likely determined by the potentiated/depressed state of the synapse. Several previous studies have shown presynaptic forms of LTP and LTD at parallel fiber synapses resulting in changes in release probability (Bender, Pugh, & Jahr, 2009; Qui and Knopfel, 2009; Salin et al., 1996). Although it is tempting to link changes in GABA_BR-mediated inhibition to presynaptic LTP/LTD at these synapses, especially considering both changes are induced by 4 or 8 Hz stimulation, we did not find a correlation between the change in GABA_BR-mediated inhibition and change in the EPSC amplitude ($r^2 = .004$).

Another possible source of variability is the identity of the postsynaptic target neuron. Parallel fibers synapse onto stellate, basket, Golgi, and Purkinje cells. Previous work has shown that short-term plasticity at parallel fiber synapses (a presynaptic property) depends

on the postsynaptic target neuron (Bao, Reim, & Sakaba, 2010). In our experiments we recorded exclusively from the outer third of the molecular layer where the majority of interneurons are stellate cells. However, we cannot exclude the possibility that some of the cells we recorded from were basket cells. In addition, it has also been suggested that MLIs form a continuum of cells ranging from stellate-like to basket-like cells (Sultan & Bower, 1998; Sotelo, 2015). In this case, there may be variability in the postsynaptic cells we recorded and a small part of the variability we observed is due to differences in the postsynaptic target neurons.

4.3 | Physiological consequences

Earlier studies suggest two likely forms of presynaptic GABA receptor activation. GABA_BRs on Golgi cell terminals (Mapelli, Rossi, Nieuwenhuis, & D'Angelo, 2009) and GABA_ARs on granule cells show tonic activation by ambient GABA in the granule cell layer of the cerebellum (Brickley, Cull-Candy, & Farrant, 1996). Ambient GABA concentration in the molecular layer has not been explicitly measured; however, Berglund, Wen, Dunbar, Feng, and Augustine (2016) suggest that GABA_ARs in parallel fibers are also tonically active. Given that presynaptic GABA_BRs respond to lower GABA concentrations than GABA_ARs at parallel fiber synapses (Howell & Pugh, 2016), it is possible that presynaptic GABA_BRs are also tonically activated by ambient GABA in the molecular layer. If so, then down-regulation of GABA_BRs would be expected to increase the release probability of the synapse and may underlie or complement presynaptic LTP at these synapses.

Other studies have suggested that presynaptic GABA_BRs are primarily activated by spillover of GABA from nearby inhibitory synapses (Dittman & Regehr, 1997). Recent work has shown that activation of MLIs and release of GABA from their terminals is sufficient to activate GABA_ARs expressed on parallel fibers (Pugh & Jahr, 2011, 2013, Stell et al., 2007). In this case, loss of GABA_BR-mediated inhibition would not increase release probability directly, but change how parallel fiber synapses respond to GABA release. Parallel fiber GABA_BRs likely provide negative feedback regulation in the cerebellar circuit. Increased activity of parallel fibers can activate MLIs causing release of GABA. Spillover of GABA from these synapses likely activates parallel fiber GABA_BRs and reduces glutamate release (Dittman & Regehr, 1997). In this model of GABA_BR function, loss of presynaptic GABA_BR-mediated inhibition would temporally remove this feedback and allow greater transmission through the parallel fiber pathway, possibly enhancing induction or extending the window for LTD at parallel fiber-Purkinje cell synapses. This would suggest that patterns of activity that down-regulate presynaptic GABA_BRs (such as 4 Hz activity) transiently prime the circuit (or subsets of parallel fiber synapses within the circuit) for enhanced associative learning.

4.4 | Future directions

This work demonstrates that expression of presynaptic GABA_BRs can be modulated by physiological patterns of activity, opening up a range of interesting new questions. Parallel fibers express a number of other presynaptic receptors, including mGluRs, CB1, A1, kainate, and GABA_A receptors; do these other receptors also experience activity-dependent

changes in expression? In particular, presynaptic GABA_A receptors oppose the effects of GABA_B receptors, despite being activated by the same ligand (Howell & Pugh, 2016); are presynaptic GABA_A receptors regulated in concert with, or in opposition to GABA_B receptors, if at all?

It will be especially interesting to discover whether this form of plasticity is specific to parallel fibers in the cerebellum or generalizable to other synapses in the central nervous system. Future work will be required to determine if other synapses expressing presynaptic GABA_BRs (or other classes of receptors) also display activity-dependent plasticity, and if so, what patterns of activity are required.

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FIGURE 1.

Heterogeneity of presynaptic GABA_BR-mediated inhibition of glutamate release. (a) Diagram showing experimental protocol used to measure presynaptic GABA_BR-mediated inhibition of PF-SC synapses. Stimulation electrode was placed in the molecular layer to activate parallel fiber synapses while evoked EPSC were recorded in stellate cells. On alternating sweeps, release of GABA was induced by photolytic uncaging of RuBi-GABA to activate GABA_BRs. (b) Current traces of evoked EPSCs from three stellate cells in control (black) or after photolytic uncaging of RuBi-GABA (red) showing low (left), intermediate (middle), or high (right) GABA_BR-mediated inhibition. (c) Frequency histogram of GABA_BR-mediated inhibition for all cells tested ($n = 133$) showing the heterogeneity of inhibition across cells

FIGURE 2.

4 Hz stimulation reduced presynaptic GABA_B-mediated inhibition. (a) Example current traces (average of 10 sweeps) of EPSCs from interleaved control sweeps (black) and RuBi-GABA uncaging sweeps (red). EPSCs were recorded before (–10, –5 min) or after (+5, +10, +15 min) 4 Hz stimulation. (b) Plot of the mean GABA_B-mediated inhibition across the time-course of the experiment showing a transient reduction in inhibition after 4 Hz stimulation ($n=14$). (c) Example paired-pulse stimulation (20 ms ISI) current traces in control (black) or following uncaging of RuBi-GABA (red) either before (top) or after 4 Hz stimulation. Traces have been normalized to the amplitude of the first EPSC to show the change in PPR. (d) Mean PPR of EPSCs in control sweeps (black) and GABA uncaging sweeps (red). Results from individual cells plotted as connected gray circles ($n=11$). (e) Mean change in variance of EPSC amplitudes ($1/CV^2$) by GABA uncaging before (base), immediately after (4 Hz) or 15–20 min after (recov) 4 Hz stimulation. Results from individual cells plotted as connected gray circles ($n=11$). (f) Mean GABA_B-mediated inhibition before (baseline) or after 1, 4, 8, 20, 50 and 100 Hz stimulation of parallel fibers. Only 4 Hz stimulation significantly altered GABA_B inhibition

FIGURE 3.

Forskolin decreased GABA_B2R-mediated inhibition and expression. (a) Example current traces (average of 10 sweeps) of EPSCs from control sweeps (black) or Rubi-GABA uncaging sweeps (red). GABA_B2R-mediated inhibition is reduced following bath application of forskolin (right) compared with baseline (left) (b) Mean PPR of EPSCs in control sweeps (black) and GABA uncaging sweeps (red) ($n=14$). (c) Plot of the mean GABA_B2R-mediated inhibition across the time-course of the experiment showing a transient reduction in inhibition after bath application of forskolin. (d1) Mean GABA_B2R-mediated inhibition in control conditions and in the presence of KT5720 (PKA inhibitor) ($n=11$). (d2) Mean PPR before and after forskolin application in the presence of KT5720. (e1) Example images of cerebellar slices immunolabeled using an antibody against the extracellular domain of the GABA_{B2} subunit. Slices were briefly (6 min) incubated in control aCSF (left) or forskolin (right) prior to fixation. PL, Purkinje cell layer, ML, molecular layer, GL, granule cell layer. (e2) Fluorescence intensity of extracellular GABA_{B2} subunit labeling in control or after forskolin treatment. Connected gray circles represent pairs of samples processed in parallel ($n=4$)

FIGURE 4.

Forskolin reduces GABA_BR-mediated inhibition of presynaptic calcium influx. (a) Image of a granule cell loaded with Alexa 594 (left) showing the soma, ascending axon, and parallel fibers. Inset, expanded image of a parallel fiber varicosity and location of the line scan. Right, Calcium-dependent fluorescence ($\Delta G/R$, top) during a single action potential evoked at the soma (bottom). Traces are the average of 10 sweeps. (b) Traces of action potential-evoked calcium transients recorded from a parallel fiber varicosity in control (black) or during pressure application of baclofen (red). Action potential-evoked calcium transients were recorded before (baseline, top) or after bath application of forskolin (bottom). Each trace is the average of 5 sweeps. (c) Mean inhibition of the calcium transient induced by baclofen during the baseline or after application of forskolin ($n=11$) or 4 Hz stimulation ($n=3$). Results from individual cells plotted as connected gray circles