GABA-B receptors enhance GABA-A receptor currents by modulation of membrane trafficking in dentate gyrus granule cells

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Activation of postsynaptic GABA-B receptors enhances tonic inhibition mediated by high-affinity extrasynaptic GABA_A receptors in dentate gyrus granule cells (DGGCs), thalamocortical neurons, and cerebellar granule cells. We investigated the mechanism(s) of GABA current modulation by GABA_B receptors in DGGCs using a combination of electrophysiological and biochemical approaches. In acute hippocampal brain slices the GABA_B receptor agonist baclofen increased GABA-evoked currents in ~2/3rds of DGGCs, significantly increasing GABA_A currents by 41% on average. Nonstationary noise analysis was performed to estimate the effects of baclofen on single channel conductance, mean open time, and channel number; these estimates suggest that GABA_B receptor activation increases receptor number but does not modify single channel properties of GABA_A receptors. To directly assess baclofen-induced changes in plasma membrane expression of GABA_A receptors, biotinylated western blots were performed. Treatment of hippocampal slices with baclofen significantly increased the surface expression of GABA_A receptor subunits (both δ and γ2 subunits) and this effect was inhibited by the GABA_B receptor antagonist CGP55845. These data indicate that changes in membrane trafficking and increased number of GABA_A receptors in plasma membrane contribute to the enhancement of GABA currents produced by GABA_B receptor activation in DGGCs.

1. Introduction

High-affinity extrasynaptic GABA_A receptors in many areas of the brain generate a tonic form of inhibition, resulting from activation by ambient GABA levels in extracellular space or spontaneous channel openings [1-3]. Tonic inhibition importantly influences many physiological and pathophysiological processes, including learning and memory, anxiety-related behaviors, and epileptic seizures [4-7]. Deficits in tonic inhibition contribute to neuropsychiatric disease such as post-partum depression and epileptic seizures [6,8], but excessive tonic inhibition can also cause ataxia, seizures, and aggravate recovery following ischemic brain injury [5,9-11]. These findings highlight the importance of regulating extrasynaptic GABA_A receptor function for normal brain function.

There is growing evidence that the strength of tonic inhibition can be regulated over time frames of seconds to minutes by membrane potential, dynamic function of GABA transporters, and neurotransmitter systems acting on G-protein coupled receptors (GPCRs) [12-15]. GABA_B receptors are GPCRs that enhance tonic inhibition in dentate gyrus granule cells, cerebellar granule cells, and thalamocortical neurons [12,14,16]. This effect is mediated by postsynaptic GABA_B receptors, is independent of presynaptic GABA release, and appears to be specific for cells that express GABA_A receptor δ subunits; the effect was absent in cortical neurons, was observed for currents activated by the δ-subunit selective agonist THIP, and was absent in δ - subunit KO mice [12,14].

We investigated mechanisms underlying enhancement of GABA_A receptor currents in DGGCs by the GABA_B receptor agonist baclofen using a combination of electrophysiological, analytical, and biochemical approaches. Our results indicate that enhancement of GABA_A receptor currents by baclofen is primarily due to increased numbers of plasma membrane GABA_A receptors, rather than changes in receptor properties. This effect on membrane trafficking involved δ subunits associated with
extrasynaptic GABA_A receptors in DGGCs, but was also observed for γ2 subunits associated with synaptic GABA_A receptors and an unrelated plasma membrane protein (Na^+/K^+ ATPase). Our results indicate that GABA_A receptor activation alters the equilibrium of trafficking/intercalization of plasma membrane proteins, including GABA_A receptor subunits.

2. Materials and methods

All protocols and animal use were done with approval of local Institutional Animal Care and Use Committee.

2.1. Acute brain slices

Hippocampal brain slices were prepared from 4 to 6 week old male Sprague-Dawley rats. Rats were anesthetized with 4% isoflurane, decapitated, and the brain dissected free. Transverse slices (300 μm) of hippocampus were made using a vibratome (Leica VT1200). Slices were cut in ice-cold solution containing (in mM): 125 NaCl, 3 KCl, 26 NaHCO_3, 1.2 NaH_2PO_4, 0.5 CaCl_2, 4 MgCl_2, 20 dextrose, and 1 kynurenic acid. Solutions were continuously gassed with 95% O_2/5% CO_2 to maintain a pH of 7.4. Slices were stored in cutting solution at room temperature for at least 1 h prior to recording.

2.2. Electrophysiology

Conventional whole-cell patch clamp techniques were used to record membrane currents. Dentate gyrus granule cells in brain slices were visualized with an Axioskop 2 upright microscope with fixed stage using infrared DIC optics (Carl Zeiss Inc., Thornwood, NY). Recordings were made using a Multiclamp 700B amplifier, a Digidata 1200 series A-D converter, and pClamp 10 software (Molecular Devices, Redwood City, CA). Data were acquired at 2–5 kHz and low-pass filtered at 1–2 kHz. Series resistance and whole-cell capacitance were determined by compensating the current transients produced by a ~10 mV voltage step; series resistance was monitored during the experiments and if series resistance exceeded 20 MΩ or changed by more than 30% the experiment was discarded. The recording chamber had a volume of ~2.5 ml and was continuously superfused at a rate of ~1–2 ml/min with bath solution that contained (in mM): 134 NaCl, 3 KCl, 1.4 NaH_2PO_4, 26 NaHCO_3, 10 Dextrose, 2 MgCl_2, 2 CaCl_2, and 1 Kynurenic acid. The pH was 7.35–7.4 when bubbled with 95% O_2/5% CO_2. Osmolarity was measured with a vapor pressure osmometer (Wescor) and adjusted to 300–305 mosm with H_2O. Focal application of drugs was made by pressure ejection from a patch pipette positioned 30–50 μm from DGGCs (Picospritzer II, General Valve Corporation). The picospritzer solution contained (in mM): 150 NaCl, 3 KCl, 2 CaCl_2, 2 MgCl_2, 10 dextrose, and 10 HEPEs with pH adjusted to 7.4 with NaOH. Currents were recorded at ~60 mV.

Patch electrodes were made with borosilicate glass without filament (593400, A-M Systems, Carlsborg, WA) using a micropipette puller (P-97, Sutter Instruments, Novato, CA). Electrodes had resistances of 4–5 MΩ when filled with an intracellular solution containing (in mM): 125 CsCl, 10 QX-314 chloride salt, 10 HEPEs, 1 EGTA (pH corrected to 7.25 with CsOH). Osmolarity was adjusted to 275–285 mosm with H_2O as needed. All chemicals were purchased from Sigma except QX-314 (Alomone Labs). Data acquisition was begun >5 min after establishing a whole-cell recording. Experiments were performed at 32–34 °C.

2.3. Western blots

Biotinylated western blots were performed as previously described (Jung et al., 2011) using EZ link sulfo-NHS-SS Biotin kit (Thermo Scientific). In brief, acute hippocampal brain slices were prepared and stored in same solutions as for electrophysiology experiments. After allowing slices to recover for 1–2 h at room temperature, slices were treated with the GABA_A receptor agonist baclofen (10 μM) for 20 min or pre-treated with the GABA_A antagonist CGP55845 (10 μM) prior to addition of baclofen. Slices were then transferred to ice cold ACSF and hippocampi dissected free. Hippocampi were incubated for 45 min with sulfo-NHS-biotin to label surface proteins, followed by rinsing with ACSF containing lysine (1 μM) to quench the biotinylation reaction. The hippocampus was isolated by dissection in ice-cold solution and then homogenized in a buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitor mixture followed by sonication. This homogenate was centrifuged at 4 °C (15,000 rpm, 5 min) and the supernatant collected for incubation with neutravidin-Avidin beads overnight ( neutravidin-Avidin beads react with biotin-labelled surface proteins, allowing separation of surface proteins). Beads washed three times with homogenization buffer, centrifuged (1000 rpm, 1 min), and the supernatant discarded. The bead pellet, containing biotinylated surface proteins, was suspended in Laemmli buffer and boiled to disrupt the Avidin-Biotin interaction. Total and biotin-labeled (surface) protein were separated by gel electrophoresis (polyacrylamide/SDS-PAGE), transferred to a nitrocellulose membrane, and incubated 12–16 h with antibodies against GABA_A receptor δ subunits (1:200 dilution, Millipore, #AB9752), GABA_A receptor γ2 subunits (1:100, Santa Cruz, #sc-101963), Na/K ATPase (1:200, Abcam, #AB7671), or the intracellular protein GADPH (1:1000, Santa Cruz, #sc-25776). Protein levels in samples were determined with BCA protein assay kit (Thermo Scientific) and each lane of gels was loaded with 50 μg protein. Blots were then incubated with a secondary antibody (anti-rabbit) and proteins visualized by chemiluminescence. Western blots were quantified by densitometry using Image J software, density of bands were normalized to control bands (no baclofen) and reported as percent change from control.

2.4. Analysis

Data analysis was performed with Clampfit (pClamp 10, Molecular Devices) and Origin (Microcal Software) software. Tonic current amplitudes were measured as the difference in mean holding current in the absence and presence of the GABA_A receptor agonist bicuculline methiodide (30 μM). The mean holding current values were recorded as the center of Gaussian fits to all-point current amplitude histograms before and during bicuculline application [12].

Nonstationary noise analysis was performed on GABA-evoked currents to estimate changes in single-channel conductance and receptor number in response to GABA_A receptor activation with baclofen [17]. Mean current and variance were calculated from 100 ms epochs of current responses to focal GABA application, variance was calculated from slope-subtracted current data and corrected for baseline variance. Mean current and variance data from 3 to 4 GABA applications was averaged into 50–100 pA bins for curve-fitting with a parabolic form of the function:

\[ \sigma^2 = i^2 - I^2/N \]

where \(\sigma^2\) is variance, \(I\) is mean current, \(i\) is the estimate of single-channel current, and \(N\) is the estimate of channel number. Single channel conductance, \(\gamma\), was estimated as \(i/\sigma^2\) mV (\(I\) is ~ 0 mV). Estimates of peak channel open probability (Po) were made by dividing peak current (I) by derived parameters for channel number and single channel current [Po = 1/(N+z)].

2.5. Power spectral analysis

Power spectral analysis of GABA-evoked currents was performed to estimate mean channel open time before and during baclofen application [17]. Power spectra of GABA-evoked currents was computed by fast Fourier transform (after subtracting the corresponding average spectral of baseline noise) and the average of 3–4 GABA applications was fit with
a single Lorentzian equation:

\[
S(f) = \frac{S(0)}{1 + (f/f_c)^2}
\]

where \( S(f) \) is the spectral density at frequency \( f \), \( S(0) \) is the spectral density at zero frequency, and \( f_c \) is the cut-off frequency at which the spectral density is half of \( S(0) \). The corresponding time constants (\( \tau \)) are calculated from the fit-derived, cut-off frequency (\( f_c \)):

\[
\tau = 1/(2\pi f_c)
\]

At low open probability (\( P_o \)), the forward rate constant is negligible and \( \tau \) is simply the reciprocal of the closing rate or the mean channel open time. Thus, this analysis allows the effect of baclofen on channel kinetics (i.e. mean open time) to be estimated.

Data values are presented as mean ± standard error of mean (SEM). Statistical analyses were performed using Microsoft Excel and MicroCal Origin. A two-tailed, paired or unpaired Student’s t-test was used with a p-value of 0.05 considered as significant. Western blot data was analyzed by two-way ANOVA to assess effect of treatment and effect of control baclofen treatment across measured proteins. Where appropriate the actual p-values are reported.

3. Results

3.1. \( \text{GABA}_A \) receptor currents were enhanced by baclofen

Membrane currents were recorded at –60 mV in response to focal application of GABA (10 \( \mu \)M); these currents were blocked by bath application of the \( \text{GABA}_A \) receptor antagonist bicuculline indicating they were due to \( \text{GABA}_A \) receptors (data not shown). As previously reported [12,14], the \( \text{GABA}_B \) receptor agonist R-baclofen (10 \( \mu \)M) increased currents evoked with exogenous GABA, an effect that over 6–8 min during baclofen application and was reversible in some cells (solution exchange was complete within 3 min) (Fig. 1A, B). This enhancement was absent in 6/19 cells tested, but ensemble analysis of all experiments showed a significant increase of \( \text{GABA}_A \) receptor currents from –863 ± 88 pA under control conditions to –1220 ± 139 pA during baclofen application (n = 19 cells from 14 animals, \( p < 0.01 \)) (Fig. 1C-E). The distribution of the current potentiation produced by baclofen is illustrated in Fig. 1F, current increased by \( \geq 25\% \) in 10/19 cells. There was a weak correlation between baseline GABA current amplitude and relative potentiation with baclofen (Fig. 1G, \( r = 0.30 \)).

Nonstationary noise analysis was performed to estimate single-channel conductance and receptor number before and during baclofen exposure to assess whether current enhancement was due to a change in single channel conductance or an increase in receptor number [17,18]. Cells that showed \( \geq 25\% \) enhancement during baclofen application were selected for analysis; cells with frequent spontaneous synaptic currents that confounded calculation of current variance, or with a linear variance vs. mean current curve (i.e. data could not be fit with a non-constrained parabolic function to estimate receptor number), were excluded from this analysis. Fits were performed on averaged variance and mean current data from 3 to 4 GABA applications at baseline and during baclofen application for each cell. Parameters of a parabolic function fit to the variance-mean current data indicated that single
channel conductance, $\gamma$, was unaffected by baclofen but receptor number, $N$, was increased (Fig. 2A-B). On average, estimates of $\gamma$ were 9.9 ± 1.8 pS at baseline and 10.6 ± 1.3 pS in the presence of baclofen ($n = 7$ cells, $p = 0.67$) (Fig. 2B, E). In contrast, estimates of $N$ indicated a significant increase in receptor number during baclofen application (3093 ± 969 vs. 5086 ± 1194, $n = 7$, $p < 0.01$) (Fig. 2E). As an additional measure of single channel conductance we performed linear fits of current variance data at low values of mean current; estimates of single channel conductance from linear fit of data was in good agreement with values obtained from parabolic fit of data and did not show any significant effect of baclofen (Fig. 2C-D, F). Maximum channel open probability (Po) was also estimated using derived parameters (single channel current and $N$) and measured peak current; estimates of maximum Po were unaffected by baclofen treatment (Po of 0.52 ± 0.3 and 0.53 ± 0.06 for baseline and baclofen, respectively; $n = 7$, $p = 0.79$) (Fig. 2E). Power spectra analysis was also performed on GABA-evoked currents before and during baclofen application; this analysis did not show any significant changes in estimates of mean channel open time (i.e. channel kinetics) after GABA$_{A}$ receptor activation ($\tau$ was 7.0 ± 1 ms vs. 8.2 ± 2 ms for control and baclofen, respectively, $n = 7$, $p = 0.34$)

Fig. 2. Nonstationary noise analysis of GABA-evoked currents indicates that baclofen increases surface expression of GABA$_{A}$ receptors. A: GABA-evoked currents before (black) and during baclofen (red) application; black and red traces are average of 3 GABA applications at baseline and during baclofen application (light gray traces). B: Current variance as a function of mean current for data in (A). Data represents mean ± SEM of three GABA applications before and during baclofen application. Solid lines are a parabolic function fit to the data. Estimated single channel conductance ($\gamma$) and channel number ($N$) were derived from fit to data. C-D: Current variance vs. mean current plot with linear fit to data at low mean current amplitude (i.e. low Po) before (C) and during baclofen (D) application. Slope of these fits used as an additional estimate of single channel conductance. E: Summary data (mean ± SEM) for parabolic fit parameters estimating single channel conductance ($\gamma$), channel number ($N$), and open probability (Po). Baclofen increased estimates of $N$ but did not significantly affect estimates of $\gamma$ or Po ($n = 7$). Connected data points represent paired measurements from individual cells. F: Summary data for estimates of $\gamma$ from linear fits of current variance – mean current data. G: Spectral density of GABA currents at baseline and during baclofen application. Red lines are a single Lorentzian function fit to data to estimate corner frequency (Fc) and mean channel open time ($\tau$). H: Summary data for estimates of $\tau$ as in (G). ** - $p < 0.01$, n.s. – nonsignificant.
(Fig. 2G-H). These analyses suggest that GABA$_A$ receptor activation increases the number of functional GABA$_A$ receptors but does not significantly affect single-channel properties.

As previously reported, tonic GABA currents due to endogenous GABA, measured as the shift in holding current produced by the GABA$_A$ receptor antagonist bicuculline, were also increased by baclofen in 5/8 cells (Fig. 3) \[12,14\]. On average, tonic current increased from $-14.2 \pm 3$ pA at baseline to $-20.3 \pm 4$ pA during baclofen application ($p = 0.03$, $n = 8$ cells from 6 animals).

### 3.2. GABA$_B$ receptor activation increases plasma membrane expression of GABA$_A$ receptor subunits

To directly assess whether GABA$_B$ receptor activation affects the number of GABA$_A$ receptors in plasma membrane, we performed biotinylated western blots on hippocampus, a procedure that allows isolation of plasma membrane proteins for separation by gel electrophoresis and immunoblot detection. Antibodies against three plasma membrane/surface proteins (Na$^+$/K$^+$ ATPase and GABA$_A$ receptor $\delta$- and $\gamma$-2-subunits) and one strictly intracellular protein (GAPDH) were used. Data are from samples obtained from 5 animals for both control and baclofen-treated conditions, 3 animals for CGP + baclofen. Representative western blots for total protein and surface protein from hippocampal slices under control conditions, after 20 min treatment with R-baclofen (10 µM), and after baclofen treatment of slices pre-treated with the GABA$_B$ antagonist CGP55845 (10 µM) (Fig. 4A). The GAPDH bands are absent from the surface protein fractions, indicating that the biotinylation procedure effectively isolated plasma membrane protein. Treating acute hippocampal brain slices with R-baclofen for 20 min significantly increased the surface expression (quantified by densitometric analysis) of GABA$_A$ receptor $\delta$-subunits and $\gamma$-2-subunits compared to control by $80 \pm 24\%$ and $169 \pm 87\%$, respectively ($p < 0.05$) (Fig. 4A-B). Surface expression of Na$^+$/K$^+$ ATPase was also significantly increased by $34 \pm 6\%$ ($p < 0.05$). Analysis of these data by one-way ANOVA did not indicate significant differences of baclofen-induced changes in surface expression between the three proteins. Baclofen-induced increases in surface expression were sensitive to pre-incubation with the GABA$_B$ receptor antagonist CGP55845 (10 µM), confirming that the effect was due to GABA$_B$ receptor activation (Fig. 4A-B). Treatment of slices with both baclofen and CGP did not change band density compared to control for all three proteins; there was a trend towards lower surface expression of $\gamma$-2-subunits after combined treatment but this was not significant ($n = 3$ blots for tissue from 3 separate animals, $p = 0.29$). There were also CGP-sensitive increases in total protein seen with baclofen treatment for both Na/K ATPase and GABA$_A$ receptor $\delta$-subunits, but not GABA$_A$ receptor $\gamma$-2-subunits. On average, baclofen treatment increased total protein levels of Na/K ATPase and GABA$_A$ receptor $\delta$-subunits increased by $29 \pm 12\%$ and $28 \pm 7\%$, respectively ($p < 0.05$).

### 4. Discussion

We investigated the cellular mechanisms underlying the enhancement of GABA$_A$ receptor currents by the GABA$_B$ receptor agonist baclofen, an effect that is mediated by postsynaptic GABA$_A$ receptors and present in DGGCs, cerebellar granule cells, and thalamocortical neurons \[12,14,16\]. Specifically, we sought to distinguish between a change in receptor properties versus a change in receptor number as the cause of current enhancement during GABA$_B$ receptor activation. Our results, obtained using a combination of analytical approaches (nonstationary noise analysis) and biotinylated western blots, support the conclusion that GABA$_B$ receptor activation increases the number of GABA$_A$ receptors in plasma membrane. These data indicate that alterations in membrane trafficking contribute (at least in part) to the increased GABA$_A$ currents produced by GABA$_B$ receptor activation.

#### 4.1. Enhancement of GABA$_A$ currents by baclofen and noise analysis estimates

Our results indicate that the GABA$_B$ receptor agonist baclofen enhances GABA$_A$ receptor currents in the majority of DGGCs, including currents evoked with exogenous GABA and tonic currents due to endogenous GABA. In the subset of cells that responded to baclofen (i.e. > 25% increase in current amplitude), potentiation of GABA$_A$ currents was substantial and similar to prior reports (77% increase on average) \[12\]. A minority of cells (5/19) showed small current reduction (~20% on average) during baclofen application; we do not believe this is a technical issue (series resistance was monitored throughout experiments) and may relate to well-described rundown of GABA currents due to receptor internalization \[19,20\]. GABA$_B$ receptors are G-protein coupled receptors (Gi/o) that reduce cAMP levels and PKA activity in both pre- and post-synaptic neurons, and also activate inwardly-rectifying K$^+$ channels \[21\]. Bath application of baclofen could activate all of these mechanisms. However, presynaptic GABA$_A$ receptors or GABA$_B$ receptor activation of K$^+$ channels are unlikely to have contributed to our results; the use of exogenous GABA to activate GABA$_A$ receptors would minimize any contribution of presynaptic mechanisms to the measured currents and the use of CsCl-based pipette solutions with QX-314 would block inwardly-rectifying K$^+$ channels \[22\]. Additionally, prior work has shown that baclofen modulation of GABA$_A$ receptors is blocked by intracellular GDP-β-S, a GTP analogue that inhibits G-protein signaling, and is unaffected by inhibition of presynaptic GABA

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**Fig. 3.** Baclofen increased endogenous tonic currents. A: Membrane currents at baseline and during baclofen application in response to focal application of the GABA$_B$ bicuculline (Bic, 30 µM); tonic current amplitude is defined as shift in holding current produced by GABA$_A$ receptor antagonist. Black traces are mean of 3 Bic applications (light gray traces). Inset to right of current traces are Gaussian fit to all-point histograms from average currents at baseline and during Bic application used to define holding current amplitude. B: Time course of current change for experiment in (A). C: Summary data (mean ± SEM) for tonic currents at baseline and during baclofen application. Connected data points are paired measurements from individual cells ($n = 8$). * $p < 0.05$. 

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**Fig. 4.** Baclofen and GABA$_B$ receptor activation increases plasma membrane expression of GABA$_A$ receptor subunits.
release [12]. The observed effects of baclofen are therefore due to effects on intracellular signaling pathways that cause an increase in GABA<sub>A</sub> receptor currents.

Our noise analysis estimates of single channel conductance yielded a value of ~10 pS on average (range of 5.7 pS–16 pS). These estimates fall within the range of conductances for GABA<sub>A</sub> receptor currents. GABA<sub>A</sub> receptor activation also increased surface expression of δ-subunits of GABA<sub>A</sub> receptors and this effect was blocked by CGP55845.

4.2. Membrane trafficking of GABA<sub>A</sub> receptors

Biotinylated western blots directly confirmed that GABA<sub>A</sub> receptor activation increases the number of GABA<sub>A</sub> receptors in plasma membrane, consistent with our noise analysis estimates. These data, demonstrating increased surface expression of both δ- and γ-2-subunits of GABA<sub>A</sub> receptors, indicate that GABA<sub>A</sub> receptor activation enhances GABA<sub>A</sub> receptor currents via effects on signaling pathways that affect membrane trafficking proteins, thereby enhancing GABA<sub>A</sub> receptor currents. GABA<sub>A</sub> receptor activation also increased surface expression of Na/K-ATPase, suggesting that the effect of GABA<sub>A</sub> receptors on trafficking of proteins to plasma membrane is not specific to GABA<sub>A</sub> receptor subunits. Our use of exogenous GABA (10 µM) does not distinguish between currents due to δ- or γ-2-subunit-containing GABA<sub>A</sub> receptors, nor their relative contribution to measured currents, but data showing enhancement of endogenous tonic currents indicates that high-affinity, extrasynaptic receptors are involved in this modulation. We suspect that increased surface expression of γ-2-subunits reflects an extrasynaptic pool of these receptors, because baclofen reduces frequency of sIPSCs (a presynaptic effect) but does not modify the characteristics of sIPSCs (suggesting GABA<sub>A</sub> receptors activated at synapses.
are unaffected by GABA$_B$ activation) [12,25,26]. Another limitation of our data is that membrane currents were recorded from DGGCs but western blots were performed on entire hippocampus; baclofen could have different effects on membrane trafficking in different cell types but our data cannot distinguish where proteins in our western blots originated and the possibility of cell type-specific effects of GABA$_B$ receptors.

Activation of GABA$_A$ receptors, like other GPCRs, produces changes in intracellular signaling pathways that modulate many cellular processes. This includes membrane trafficking and alteration in the balance of insertion and retrieval of plasma membrane proteins. Our data showing increase in surface expression of GABA$_A$ receptors after stimulation of GABA$_A$ receptors does not distinguish between a primary effect of accelerating insertion of protein-containing vesicles into plasma membrane or a slowing of endocytosis. GABA$_B$ receptors are linked to the Gi/o signaling pathway (inhibition of adenylate cyclase with decreased cAMP/PKA activity) and manipulation of this pathway has predictable effects on tonic GABA currents; inhibition of adenylate cyclase mimics the effect of baclofen and increases GABA currents, the catalytic subunit of PKA decreases tonic currents and occludes the effect of baclofen, indicating that PKA activity favors reduced plasma membrane expression of GABA$_A$ receptors [14]. In addition to canonical Gi/o signaling, GABA$_B$ receptors can also lead to activation of PKC signaling which can affect membrane trafficking of GABA$_A$ receptor subunits, including those involved in tonic inhibition [27-29]. PKC activation, however, causes GABA$_A$ receptor internalization and reduced GABA currents [30-32], and is therefore unlikely to have contributed to the effects of GABA$_B$ receptor activation in our experiments. In addition to Intracellular signaling changes that affect trafficking of GABA$_A$ receptors, GABA$_B$ receptor activation also produced small but significant increases in total δ subunit protein levels (and Na$^+$/K$^-$/ATPase), suggesting additional effects on translation and/or gene expression.

4.3. Significance of results

Dynamic trafficking of neurotransmitter receptors and ion channels is a fundamental mechanism underlying forms of activity-dependent plasticity, neuromodulation, and disease processes [33-36]. Alterations in trafficking of GABA$_B$ receptors is particularly relevant to seizures and epilepsy. Models of chronic epilepsy have been associated with increased surface expression of synaptic GABA$_A$ receptors (kindling) or subunit-specific changes (status epilepticus models, genetic models) [18,37,38]. Acute seizures also trigger GABA$_A$ receptor internalization, an effect believed to importantly contribute to the pathophysiology of status epilepticus and benzodiazepine resistance [39-41]. In addition to direct phosphorylation of GABA$_A$ receptors, kinase/phosphatase activity can rapidly modulate the equilibrium of membrane trafficking to affect receptor number and thereby alter cellular and network excitability. Our data do not identify the specific molecules involved in GABA$_B$ receptor trafficking that are affected by GABA$_B$ receptor signaling (see reviews [42-44]), but this effect is dependent on reductions of cAMP/PKA signaling [14,16]. Our data suggest that activation of GABA$_B$ receptors on DGCCs can provide feed-forward enhancement of GABA$_A$ receptor number and function. Because of the extrasynaptic location of GABA$_B$ receptors, this proposed mechanism is most likely manifest during periods of intense neural activity associated with GABA spillover required for GABA$_B$ activation [22]. However, GABA$_B$ receptor antagonists reduce GABA$_A$ currents in thalamocortical cells and cerebellar granule cells, indicating that this mechanism is also operant under basal conditions in some neurons [14,16]. Activity-dependent effects on GABA$_A$ receptor trafficking likely exist on a continuum; repetitive/high-frequency presynaptic activity that promotes GABA spillover and GABA$_B$ receptor activation will lead to increased surface expression of GABA$_A$ receptors, while more intense activity/prolonged seizures that cause widespread depolarization, NMDA-R activation, and increased intracellular Ca$^{2+}$ (and other signaling pathways) will favor receptor internalization [40,45].

CRediT authorship contribution statement

Ning Li: Investigation, Project administration, Formal analysis, Data curation. Wucheng Tao: Investigation, Formal analysis. Liu Yang: Methodology, Formal analysis. William Spain: Methodology, Supervision, Writing – review & editing. Christopher Ransom: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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