GABAB receptors constrain glutamate presynaptic release
and postsynaptic actions in substantia gelatinosa of rat spinal cord

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Abstract
The substantia gelatinosa (SG, lamina II of spinal cord gray matter) is pivotal for modulating nociceptive information from the peripheral to the central nervous system. γ-Aminobutyric acid type B receptors (GABABRs), the metabotropic GABA receptor subtype, are widely expressed in pre- and postsynaptic structures of the SG. Activation of GABABRs by exogenous agonists induces both pre- and postsynaptic inhibition. However, the actions of endogenous GABA via presynaptic GABABRs on glutamatergic synapses, and the postsynaptic GABABRs interaction with glutamate, remain elusive. In the present study, first, using in vitro whole-cell recordings and taking minimal stimulation strategies, we found that in rat spinal cord glutamatergic synapses, blockade of presynaptic GABABRs switched “silent” synapses into active ones and increased the probability of glutamate release onto SG neurons; increasing ambient GABA concentration mimicked GABABRs activation on glutamatergic terminals. Next, using holographic photostimulation to uncage glutamate on postsynaptic SG neurons, we found that postsynaptic GABABRs modified glutamate-induced postsynaptic potentials. Taken together, our data identify that endogenous GABA heterosynaptically constrains glutamate release via persistently activating presynaptic GABABRs; and postsynaptically, GABABRs modulate glutamate responses. The results give new clues for endogenous GABA in modulating the nociception circuit of the spinal dorsal horn and shed fresh light on the postsynaptic interaction of glutamate and GABA.

Keywords Spinal cord · GABAB receptors · Minimal stimulation · Holographic photostimulation

Introduction
Spinal dorsal horn plays a pivotal role in receiving and modulating nociception from the peripheral to the central nervous system (CNS) (Finnerup et al. 2021). The neuronal network in the dorsal horn is mainly composed of excitatory and inhibitory neurons where substantia gelatinosa (SG; lamina II of the spinal cord gray matter) is the main origination of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Zeilhofer et al. 2012; Todd 2015). Since the proposal of “gate control theory of pain”, SG has become a target for analyzing nociception modulation (Merighi 2018).

GABA is the primary inhibitory neurotransmitter in the CNS acting on both ionotropic and metabotropic receptors, where GABAB receptors (GABABRs) are the family of metabotropic G protein-coupled receptors (GPCRs). In the spinal dorsal horn, GABABRs are expressed in the presynaptic and postsynaptic structures (Yang et al. 2001c, 2002; Malcangio 2018). Maintaining certain concentrations at the synaptic clefts and extrasynaptic structures, GABA exerts an important role in neuronal homeostasis in the CNS (Bower and Smart 2006). Extrasynaptic GABA (we refer this as “ambient” GABA) is regulated spatially and temporally by GABA transporter activity which is significant in neuronal diseases (Zeilhofer et al. 2012; Smirnova et al. 2020). GABABRs are found at both synaptic cleft and extrasynaptic membrane, making GABABRs to be therapeutic targets for addiction, depression, epilepsy and pain (Goudet et al. 2009; Malcangio 2018).

On the other hand, glutamate is the principal excitatory neurotransmitter in the CNS; glutamatergic synapses onto SG neurons are originated from the primary afferents (Yoshimura and Jessell 1990), intrinsic interneurons
(Kato et al. 2007) and descending pain modulation system (Merighi 2018). Experiments using exogenous agonists revealed that GABA_BRs mediate pre- and postsynaptic inhibitions on glutamate release and action (Ataka et al. 2000; Iyadomi et al. 2000; Yang et al. 2001c). For the action of endogenous GABA, we have previously reported that blocking primary afferents GABA_BRs facilitates action-potential-driven glutamate release, suggesting that endogenous GABA may inhibit the synapses from primary fibers (Yang and Ma 2011). However, the function of endogenous GABA in the spinal dorsal horn circuit remains largely unknown. In specific, it is unclear whether endogenous GABA constrains glutamatergic synapses, and whether there is an integration of GABA_BRs and glutamate actions at postsynaptic neurons. In the present study, employing whole-cell recordings and minimal stimulation strategies at acute rat spinal cord slices, we revealed that endogenous GABA modulates the excitatory glutamate presynaptic release; taking advantage of holographic photostimulation and uncaging glutamate, we found that postsynaptic GABA_BRs blunt glutamate responses.

Materials and methods

Slice preparation and whole-cell recordings

All experiments were approved by the Animal Ethics Committee of the institute. Acute transverse spinal cord slices were obtained following the methods described elsewhere (Yang et al. 2001b, 2021). In brief, male Sprague–Dawley rats (5–7 weeks old) were anesthetized by urethane (1.5 g/kg body weight, i.p.) and a laminectomy was carried out. The spinal cord trunk was quickly transferred to ice-cold “cutting solution” containing (in mM) sucrose 114, KCl 4, NaH₂PO₄ 1.2, MgSO₄ 8, NaHCO₃ 26, glucose 11, oxygenated with mixed gas (95% O₂/5% CO₂). After removing the dura, arachnoid and pia mater, the lumbosacral trunk was mounted on a vibratome (7000smz-2, Campden Instruments Ltd., Leics, UK). The transverse slices (400 µm in thickness) were quickly transferred to an incubation chamber in artificial cerebrospinal fluid (aCSF) containing (in mM) NaCl 124, KCl 4, NaH₂PO₄ 1.2, NaHCO₃ 26, MgSO₄ 1.5, CaCl₂ 2.5, and glucose 11 (pH 7.2, mOsm 300–305) at ~ 35 °C with mixed gas saturation for 40 min. Slices were then kept in incubation solution at room temperature (23 ± 2 °C) for using up to 8 h.

Visualized whole-cell recordings were obtained by using glass pipettes with resistance 5–8 MΩ when filled with an intrapipette solution composed of (in mM) K-gluconate 130, KCl 5, CaCl₂ 0.1, MgCl₂ 2, EGTA 5, HEPES 5, Na₂GTP 0.3, and Mg-ATP 4 (adjusted by KOH to pH 7.3; mOsm 285–290). To exclude GABA_A-R-mediated and glycine receptor-mediated actions, picrotoxin (100 µM) and strychnine (1 µM) were routinely added to the bath unless otherwise stated. In the presynaptic function experiments, 1 mM guanosine-5′-O-(2-thiodiphosphate) (GDP-β-S) was included in the internal solution to block the possible postsynaptic GABA_BRs’ effects (Yang et al. 2001b). Signals were amplified by Axopatch 200B, digitized by Digidata 1550A which was manipulated by software Clampex 10.2 (all from Molecular Devices, Sunnyvale, CA, USA). For presynaptic stimulation, a glass pipette filled with aCSF was preset 100–300 µm away from the recorded neuron. Stimulation intensity was set at the strength that induced detectable evoked excitatory postsynaptic currents (eEPSCs) which were sensitive to 6-cyano-7-nitroquinazoline-2,3-dione (CNQX; 10 µM), an α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonist. All glutamatergic eEPSCs were recorded at a holding potential of ~70 mV. Minimal stimulation protocols were similar to those described elsewhere (Dobrunz and Stevens 1997).

Briefly, a minimal stimulation was accepted when the following criteria were satisfied: (1) eEPSCs latency between stimulation artifact and response onset remained stable (<20% fluctuations); (2) lowering 20% stimulation intensity resulted in total failure of the events; and (3) elevating 20% stimulation intensity yielded no amplitude or shape change. For a subset experiment of synapse evaluation, the stimulation strength was maintained to that about half of all events were detectable. The failures of stimulation responses were estimated by visual discrimination. If the synapses did not respond to the first stimulus but exhibited occasional responses to the second stimulus with 80 ms delay, they were considered as “presynaptically silent synapses” (Voronin and Cherubini 2003; Sautiulina and Cherubini 2009). Synapses with monosynaptic pathway were accepted if the eEPSCs had stable latency from stimulation artifact to the onset of eEPSCs (<20% latency fluctuations) and stable amplitude of eEPSCs (<20% amplitude fluctuations) (Yang et al. 2001b, 2021). To evaluate direct glutamatergic synapses onto SG neurons, only monosynaptic eEPSCs were accepted for further analysis. To evaluate the possible postsynaptic GABA_BRs-induced K⁺ currents, we used a “ramp test” protocol as described before (Liu et al. 2012). Briefly, in voltage-clamp recording mode, a hyperpolarizing current with a slope of 100 mV/s from –155 to –25 mV was injected into the soma to yield voltage-dependent currents.

Holographic photostimulation and recordings

For three-dimensional (3-D) holographic stimulation, a holographic beam was integrated into the optical axis of an Olympus BX51WI upright microscope (Olympus, Tokyo, Japan) with a 60 × lens (0.9 NA; Olympus), as described before (Tang 2006; Lutz et al. 2008; Yang et al. 2011). The
output beam was expanded to a reflective spatial light modulator (SLM, LCOS Hamamatsu, Hamamatsu, Japan), split and controlled by a custom-made software, integrated into Clampex 10.2 electrophysiology acquisition software. The soma and dendrites of recorded neurons were visualized by Alexa Fluor 594 (10 mM in recording pipette; ThermoFisher Scientific, Waltham, MA, USA) under fluorescence illumination (594 nm wavelength for excitation, 617 nm wavelength for emission). Then 10 mM caged glutamate (DNI-glutamate trifluoroacetate, FEMONICS, Budapest, Hungary) was freshly prepared and added to the superfusion medium. The selected spots as uncaging glutamate targets were aligned and the 3-D photostimulation spots were activated by uncaged glutamate at a resolution of 1 μm. The beam for uncaging glutamate was 405 nm wavelength controlled by a custom-made software (Yang et al. 2011). The uncaging excitatory postsynaptic potentials (uEPSPs) were recorded from neural soma at resting membrane potentials.

To investigate the integration of postsynaptic GABA\(_B\)Rs with glutamate responses, one dendrite was stimulated by transient glutamate uncaging as “testing”, following a “priming” stimulation on a neighbor dendrite. The interval for priming and testing was 60 ms while the distance between spots on two different dendrites was 50–180 μm; the uncaging spots had a distance of 50–220 μm from the soma. A single uncaging (Figs. 4, 5) or a paired-uncaging (Fig. 6) was carried out to test postsynaptic glutamate actions; the repeat tests had a 1 min interval. In our preliminary test, this protocol did not yield synaptic potentiation or depression (Supplementary Fig. S1; see Yang et al. 2011; Santos et al. 2012). The “integration index” was calculated as the ratio of the testing uEPSPs amplitude to the priming uEPSPs amplitude. When applying baclofen, to rule out the possible voltage-dependent membrane response shift, membrane potential was maintained to the baseline value by injecting a direct current through the pipettes via “DC current injection” on the amplifier front panel (Liu et al. 2012).

**Drugs and data analysis**

CNQX, D-2-amino-5-phosphonopentanoic acid (AP-5), (±)-baclofen (baclofen), picrotoxin, strychnine, tiagabine hydrochloride (tiagabine) and GDP-β-S were obtained from Sigma-Aldrich (St. Louis, MO, USA), CGP52432 and H89 dihydrochloride (H89) were obtained from Tocris Bioscience (Bristol, UK). Signals were digitized at 10 kHz and stored in a personal computer. Data were analyzed offline using Clampfit 10.2 (Molecular Devices). Results were expressed as mean ± S.E. The CGP52432 and baclofen actions were measured at the stable response. Statistical comparisons of drug effects were made with Student’s \(t\) test for comparison of two groups and one-way ANOVA followed by a Bonferroni post hoc test for comparison of three or more groups with GraphPad Prism software (version 6.02; San Diego, CA, USA). Linear regression was performed using GraphPad Prism. Significance was determined as \(P < 0.05\).

**Results**

**Blockade of presynaptic GABA\(_B\)Rs unsilences glutamatergic synapses**

Because GABA\(_B\)Rs are ubiquitously expressed at both pre- and postsynaptic structures in the dorsal horn, in this subset of experiments, we null postsynaptic GABA\(_B\)Rs by intracellular GDP-β-S dialysis (see “Materials and methods”) to clarify the role of presynaptic GABA\(_B\)Rs (Liu et al. 2013). Silent synapses were found in the spinal dorsal horn (Merrill and Wall 1972) and in other areas of the CNS (Isaac et al. 1995). We here used minimal stimulation technique to investigate the possible roles of presynaptic GABA\(_B\)Rs in keeping glutamatergic synapses silent. In 9 neurons where the stimulation strength virtually yielded no detectable response in the first (1st) stimulus, bath application of CGP52432 (1 μM, 5 min), presumably blocking presynaptic GABA\(_B\)Rs, switched silent synapses into functional ones (Fig. 1a). In specific, CGP52432 perfusion reliably increased the amplitude of the 1st eEPSCs (control: 0.44 ± 0.24 pA, in CGP52432: 5.89 ± 1.18 pA; \(P < 0.01\), paired \(t\) test, \(n = 9\)) and the success rates (control: 1.22 ± 0.81%, in CGP52432: 62.33 ± 6.00%; \(P < 0.01\), paired \(t\) test, \(n = 9\)) (Fig. 1b). In the control tests, the synaptic strength of 4 neurons without CGP52432 treatment showed no significant change in 30 min, excluding a drift possibility under our present recording conditions (data not shown). The results suggest that blocking heterosynaptic GABA\(_B\)Rs on presynaptic glutamatergic terminals facilitates glutamate release, converts some silent synapses into functional ones. The possible mechanisms for GABA\(_B\)Rs keeping glutamatergic synapse silent are shown in Fig. 1c (see figure legends).

**Blockade of presynaptic GABA\(_B\)Rs enhances glutamate release**

Bath application of CGP52432 (1 μM, 5 min) increased the glutamate release probability. As shown in Fig. 2a, in a paired-pulse stimulation where the 1st stimulation yielded ~ 50% events detectable (see “Materials and methods”), CGP52432 perfusion increased the 1st eEPSCs amplitude (control: 6.63 ± 1.21 pA, in CGP52432: 15.00 ± 2.54 pA; \(P < 0.05\), paired \(t\) test, \(n = 8\)), enhanced the events (eEPSCs) success ratio (control: 50.38 ± 2.87%, in CGP52432: 89.63 ± 4.25%; \(P < 0.01\), paired \(t\) test, \(n = 8\)) and altered the paired-pulse ratio (PPR; the ratio of the 2nd eEPSCs amplitude over the
1st eEPSCs amplitude; control: 2.42 ± 0.36, in CGP52432: 1.33 ± 0.27; *P < 0.05, paired t test, n = 8) (Fig. 2a, b). The second stimulation also resulted in higher eEPSCs amplitude than that under control conditions (Fig. 2a).

The decreased PPR indicates that this synaptic strength alteration occurs at presynaptic loci (Zucker and Regehr 2002). As suggested in Fig. 2c, the first stimulation only pushed the synapses to “halfway” (~50% events were successful), while under the condition of GABA_B Rs being blocked, most synapses were activated (see figure legends). Taken together, the results support the idea that presynaptic GABA_B Rs, presumably bound and activated by endogenous GABA, constrain glutamate release.

**High-frequency stimulation facilitates glutamate release revealed by blocking presynaptic GABA_B Rs**

Presynaptic stimulation initiates action potentials, induces GABA release and subsequent spillovers from the synaptic cleft; certain range of stimulation frequency induces more GABA release with frequency increasing (Isaac et al. 1995; Zucker and Regehr 2002). In the present study, a train of presynaptic stimuli (5 pulses, 0.1 ms each shock, 5–100 Hz) induced a slow excitatory membrane current that followed the stimulation volley. This membrane current was mediated by presynaptic glutamate release because it was sensitive to a specific AMPA receptor antagonist, CNQX (10 μM). As shown in a representative neuron, a train of presynaptic stimulation at a certain frequency induced a slow membrane current; CGP52432 perfusion (1 μM, 5 min) increased the amplitude of the slow current, with a frequency-dependent manner (Fig. 3a1). In 9 neurons tested, the average amplitude of 100 Hz stimulation-induced current was increased to 293 ± 51% of that induced by 40 Hz train with CGP52432 perfusion (P < 0.01, unpaired t test, n = 9), indicating that the functional presynaptic GABA_B Rs on glutamatergic terminals were activated by endogenous GABA. Compared to that of 40 Hz test, CGP52432 induced less “net” effects at lower
frequency stimuli \( (P < 0.05 \text{ or } P < 0.01, \text{ one-way ANOVA; Fig. 3a2}) \).

GABA in the extrasynaptic structures is regulated partially by GABA transporters (GATs) (Isaacson et al. 1993; Sem’yanov 2005). In all 10 neurons tested, tiagabine (30 µM), a GABA transporter 1 (GAT-1) inhibitor, decreased the amplitude of eEPSCs. As shown in a representative neuron in Fig. 3b, further perfusing CGP52432 (1 µM) rescued the eEPSCs; CNQX (10 µM) completely blocked the amplitude, indicating that eEPSCs were mediated by glutamate AMPA receptors.

**Postsynaptic GABA\(_{\text{B}}\)Rs alter glutamate responses**

Holographic photostimulation and whole-cell recordings are shown in Fig. 4a. We first verified the methodology of holographic photostimulation. Recording pipette with 10 mM Alexa Fluor 594 revealed the soma and dendrites morphology (Fig. 4b, insert picture). Under the high magnification of the microscope, the uncaging spots were firstly aligned as described before (Tang 2006; Lutz et al. 2008; Yang et al. 2011). Increasing the photostimulation light “flashing” pulse width which uncaged more glutamate increased the amplitude of uEPSPs and finally triggered action potentials (Fig. 4b). The subthreshold uEPSPs showed a linear relationship between uncaging light beam duration and amplitude \( (R^2 = 0.9967; P < 0.001) \). uEPSPs were reversibly inhibited by CNQX (10 µM) and AP-5 (100 µM) to 19.25 ± 4.53% of the control, revealing their ionotropic glutamate receptor-mediated nature (Fig. 4c; control: 14.29 ± 1.23 mV, in the presence of CNQX/AP-5: 2.75 ± 0.65 mV; \( P < 0.01, \text{ one-way ANOVA, } n = 8 \)). Intracellular cAMP-dependent protein kinase A (PKA) signaling pathway was indicated in postsynaptic glutamate interaction with GABA\(_{\text{B}}\)Rs (Chalifoux and Carter 2010), we thus tested the PKA roles in the present study. The uEPSPs were not significantly changed in the presence of H89 (10 µM, perfused to the slices with superfusion medium), a specific PKA blocker, indicating that the postsynaptic PKA pathway did not mediate the interaction (90.13 ± 6.80% of the control; \( P > 0.05, \text{ one-way ANOVA, } n = 8 \); Fig. 4d).
Taking advantage of holographic stimulation and uncaging glutamate, we compared the effects of postsynaptic GABABRs in modulating glutamate responses. The recording pipette did not include GDP-β-S, making postsynaptic GABABRs available for investigation (Yang et al. 2001b). In the presence of baclofen (10 µM) which activated GABABRs, the average amplitude of uncaged glutamate responses (i.e., uEPSPs) decreased to 59.47 ± 11.58% of the control (P = 0.011, paired t test, n = 10), indicating that postsynaptic GABABRs activation blunted glutamate responses (Fig. 5a). Uncaged glutamate-evoked action potentials were depressed by baclofen, in a reversible manner after washout (Fig. 5b). These results suggest that postsynaptic GABABRs modulate postsynaptic glutamate responses.

We investigated whether ambient GABA induced a tonic postsynaptic inhibition via GABABRs to study the role of endogenous GABA. A voltage ramp protocol (from −155 mV to −25 mV ramp, see “Materials and methods”) induced a membrane current and the perfusion of CGP52432 (1 µM) shifted the curve with a reversal potential of −85.3 ± 2.5 mV (n = 8; Fig. 5c), which was close to the K⁺ equilibrium potential (E_K) calculated by the Nernst equation (−89.8 mV) under our recording conditions, suggesting that GABABR-mediated actions via potassium channels. We also tested possible endogenous GABA effects on uEPSPs. However, perfusing CGP52432 (1 µM, 5 min) did not change uEPSPs amplitude (control: 13.55 ± 0.71 mV, in CGP52432: 12.28 ± 0.78 mV; P = 0.18, paired t test, n = 6), suggesting that endogenous GABA had little effect on postsynaptic glutamate actions under the basic conditions (Fig. 5d). The tiagabine pretreatment (30 µM, 3 min) which presumably increased ambient GABA concentration, significantly reduced uEPSPs amplitude (control: 11.90 ± 1.01 mV, in tiagabine: 8.42 ± 0.57 mV; P = 0.015, paired t test, n = 5;
Fig. 5d). Taken together, the results suggest that endogenous GABA affects postsynaptic membrane K⁺ current; although under the basic condition GABA contributes little to glutamate responses, activating postsynaptic GABABRs by baclofen, or elevated GABA concentration, affects postsynaptic glutamate responses.

**Postsynaptic GABAʙRs integrate glutamate actions with different arbors**

Blocking GABAʙRs has little effect on postsynaptic glutamate actions (see Fig. 5), we next investigated GABAʙRs interaction with glutamate responses. A neuron receives released glutamate at multiple dendrites, but how the inputs from different dendrites are integrated is unclear. In the present study, we took advantage of the holographic photostimulation and whole-cell recordings to study the postsynaptic glutamate actions from different arbors. Activating different dendrites from the same soma (Fig. 6a) resulted in uEPSPs integration. In specific, activating a neighbor dendrite facilitated another dendrite uEPSP to the photostimulation (Fig. 6b). We termed this phenomenon as “integration index” (the amplitude ratio of the “testing” uEPSP to the “priming” uEPSP; see “Materials and methods”). In the presence of baclofen, the integration index significantly decreased (Fig. 6c), indicating that GABAʙRs mediate integration of glutamate responses between two different arbors (control: 8.83 ± 1.14, in the presence of baclofen: 4.39 ± 0.90; P = 0.02, paired t test, n = 10), further supporting the idea that postsynaptic GABAʙRs integrate glutamate-mediated uEPSPs.
Technical considerations for presynaptic and postsynaptic GABABRs studies in the spinal dorsal horn

The present study first focused on the presynaptic GABABRs in controlling glutamate synapses. Although GABABRs are expressed both pre- and postsynaptically, the possible postsynaptic GABABR action was ruled out by intracellular GDP-β-S dialysis from the recording electrodes (Yang et al. 2001b). Under these given experimental conditions, the minimal stimulation and the perfusion of CGP52432 only affected the presynaptic GABABRs, making it possible to clarify the GABABRs’ presynaptic roles. Our study shows that ambient GABA is able to maintain the silence of some glutamatergic synapses and that endogenous GABA constrains glutamate release probability onto SG neurons.

On the other hand, to study the postsynaptic actions, the presynaptic GABABRs’ role should be ruled out. We here used uncaging glutamate with a defined time and volume which approximated the transmitter release with a couple of micrometer diameter (Dodt et al. 1999; Yang et al. 2011; Yang and Yuste 2018). The present holographic photostimulation combined with whole-cell recordings made it possible to precisely give repeated stimulation (Yang et al. 2011), allowing us to bypass the presynaptic terminals and directly activate the postsynaptic glutamate receptors. Our sophisticated holographic photostimulation resulting in glutamate uncaging allowed us to selectively study postsynaptic GABABRs, which revealed that the postsynaptic GABABRs modulate glutamate-mediated postsynaptic responses, as previously observed (e.g., Kangrga et al. 1991). Although previous studies have suggested postsynaptic GABABR-mediated depression (for review, see Bardoni et al. 2013; Malcangio 2018; Merighi 2018), our photostimulation methods excluded a possible presynaptic GABABR-mediated contamination. It is worth noting that the current single spot stimulation protocol or “priming-testing” protocol did not yield synaptic plasticity (Fig. S1; Yang et al. 2011; Santos et al. 2012).

Presynaptic GABABRs’ actions on glutamatergic synapses

It is well known that GABABRs pre- and postsynaptically modulate spinal dorsal horn synapses and neurons (for review, see Bardoni et al. 2013; Merighi 2018), but the in-depth functions remain unclear. Intensive studies have demonstrated that presynaptic GABABRs modulate (mainly depress) either excitatory glutamatergic synapses (Dickenson et al. 1985; Malcangio and Bowery 1993; Ataka et al. 2000; Yang et al. 2001c; Fukuhara et al. 2013) or inhibitory GABAergic/glycinergic synapses (Yang et al. 2001a). Most of these used exogeneous GABABRs agonists. Our previous study investigated endogenous GABA actions on depressing synaptic transmission in the dorsal horn (Yang and Ma 2011). However, little is known about GABABRs modulation on silent synapses. In the present study, we employed minimal stimulation to separately investigate GABABRs.
To our knowledge, the present study is the first report suggesting that GABABRs silence heterogeneous glutamatergic synapses in the adult spinal dorsal horn. A synapse that connects two neurons can be “silent”. Conventional notion suggests that the postsynaptic silencing is attributed to the lack of AMPA receptors on the subsynaptic membrane, and the presynaptic silencing is thought of lacking or insufficient presynaptic neurotransmitter release (Voronin and Cherubini 2003; Kerchner and Nicoll 2008). If a synapse does not respond to the first stimulus but exhibits occasional responses to the second one 30–100 ms later, it is considered as a “presynaptically” silent synapse (Voronin and Cherubini 2003; Safiulina and Cherubini 2009). In the present study, we used paired-pulse test and clarified that the postsynaptic AMPA receptors were in the presence and the failure of synaptic events was due to too few glutamate release (i.e., weak presynaptic stimulation), this notion is in line with a previous report in the spinal dorsal horn (Yasaka et al. 2009). Occlusion of presynaptic GABABRs switched silent glutamatergic synapses to functional ones, our present results suggest that ambient GABA silences glutamatergic synapses onto SG neurons (Fig. 7).

We also revealed that endogenous GABA constrained heterogeneous glutamatergic terminals by decreasing the release probability, and this process was under the control of presynaptic GABABRs by endogenous GABA. This conclusion is in line with our previous report (Yang and Ma 2011). Taken together, we conclude that GABA...
heterogeneously constrains presynaptic glutamatergic synapses, as suggested in Fig. 7.

Certain frequency of train stimulation increases presynaptic neurotransmitter release (Zucker and Regehr 2002). The present data showed a frequency-dependent manner of eEPSCs revealed by CGP52432, suggesting that the concentration of ambient GABA affects GABABRs at the presynaptic glutamatergic terminals. The data further support the conclusion that ambient GABA modulates glutamate synapses.

It should be noted that the present “heterosynaptic hypothesis” is based on assumption that GABA is not coreleased from glutamatergic terminals. It has been proposed that glutamate is released at GABAergic synapses together with GABA in the auditory system (Noh et al. 2010) and the lateral habenula (Shabel et al. 2014); this co-release idea, however, was not the case in many brain areas (Uchigashima et al. 2007). In the spinal cord, to our knowledge, there is no evidence showing that inhibitory GABA and excitatory glutamate co-release from the same terminal (Jonas et al. 1998; Jo and Schlichter 1999). Therefore, a logical explanation for the present results is that GABAergic R-mediated inhibition of glutamatergic synapses is due to ambient and/or spillover GABA which binds to GABA₉ heteroreceptors at the neighboring glutamatergic terminals (Fig. 7). The common pathway for GABA₉Rs modulation on glutamatergic terminals might be through calcium channels (Pfrieger et al. 1994; Yang and Ma 2011).

**Postsynaptic GABA₉Rs modulation on glutamate-mediated uEPSPs**

GABA₉Rs mediate calcium channels and potassium channels by intracellular interactions (Kantamneni 2015; Bettlér and Fakler 2017). Since these channels are proteins on the cell surface, it is conceivable that GABA₉Rs may integrate glutamate receptors which are virtually membrane proteins. However, there is no complex formation or physical contact has been found between these two kinds of receptors (Bettler and Fakler 2017). In the present study, we found the integration between uEPSPs and GABA₉Rs, three possibilities may underlie these results. First, Ca²⁺ influx via NMDA receptors may also be inhibited by GABA₉Rs (Terunuma 2018). Second, postsynaptic GABA₉Rs suppress NMDA receptor responses via an intracellular signaling pathway (Lur and Higley 2015). It should be noted that some reports do not support this notion by showing that neither AMPA nor NMDA receptors are modulated by postsynaptic GABA₉Rs (Chalifoux and Carter 2010). The intracellular PKA signaling pathway plays a certain role (Chalifoux and Carter 2010), however, our current investigations do not find this modulation pathway in SG neurons (Fig. 4c). And the third, in the present study, baclofen decreased the amplitude of uEPSPs, these may be resulted from an increasing membrane conductance by GABA₉Rs activation, as we reported before (Liu et al. 2012). Nevertheless, GABA₉Rs’ activities do affect the function of the glutamate receptors and vice versa (Kantamneni 2015).

It is intriguing that uncaging glutamate activating from two separate dendrites has integration (Mueller and Egger 2020; see Fig. 6 in the present study), although the underlying mechanism is still elusive, a “hold and read” theory may explain this. In specific, when one dendrite is activated, this dendrite “holds” a short-term information storage by a small depolarization in the soma, the subsequent depolarization from another dendrite “reads out” this information and yields a stronger response (Santos et al. 2012). Single dendrite, and even single spine can serve as basic functional units of neuronal integration by individually detecting the temporal coincidence of postsynaptic activity (Yuste and Denk 1995; Mueller and Egger 2020). It is worth noting that in the present study, first, the integration is a postsynaptic event, rather than a presynaptic integration (Zucker and Regehr 2002). Baclofen, presumably activating postsynaptic GABA₉Rs, impaired the “hold and read” (Santos et al. 2012), suggesting GABA₉Rs not only contribute to postsynaptic glutamate response but also affect information integration. Second, this integration is not due to plasticity induced by repeat photostimulation (Santos et al. 2012). The integration of ionotrophic glutamate receptors and metabotropic GABA₉Rs is still unsure (Fig. 7), more experiments

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Fig. 7 Schematic diagram illustrating the modulation of glutamate presynaptic releases and postsynaptic responses by GABA₉Rs onto SG neurons in the dorsal horn. At the presynaptic (upper broken line rectangle), ambient GABA binds glutamatergic terminals (indicated by ③) and/or spillover GABA from GABAergic terminals (indicated by ②), subsequently modulates the glutamatergic synapse. Note ambient GABA comes from escapee of GABAergic synaptic release, GABA reuptake (not shown in this figure) and/or glial cells (indicated by blue arrows). At the postsynaptic (lower broken line rectangle), GABA₉Rs, activated by baclofen, impair postsynaptic glutamate response (see “Results”), with an uncertain mechanism (indicated by a question mark)
Significance of the findings

GABAergic neurons constitute as many as one-third of neural cells in the brain (Bowery and Smart 2006). Studies on endogenous GABA inhibition are mainly focused on “tonic inhibition” and “phasic inhibition” which are mediated by ionotropic GABA\(\text{\(A\)}_2\text{\(R\)}s (Kullmann et al. 2005). Enhancing GABAergic tone alleviates neuropathic and inflammatory pain at the spinal cord level via GABA\(\text{\(A\)}_2\text{\(R\)}s (Eccles et al. 1963; Witschi et al. 2011; Hanack et al. 2015; Petitjean et al. 2015; Neumann et al. 2021). Relatively, little attention has been paid to the action of GABA\(\text{\(B\)}_1\text{\(R\)}s, although they are the first targets for the released GABA in the spinal dorsal horn and mediate the majority of prolonged inhibitory signaling (Chéry and De Koninck 2000). While many previous physiological and behavioral reports concerning mechanisms of GABA\(\text{\(B\)}_1\text{\(R\)}-mediated antinociception were based on exogenous synthetic agonists (see “Introduction”), our previous study reported that endogenous GABA depressed both glutamate and GABA release (Yang and Ma 2011). Our present study focused on endogenous GABA in silencing glutamatergic synapses via GABA\(\text{\(B\)}_1\text{\(R\)}s. Furthermore, this study also clarified the postsynaptic GABA\(\text{\(B\)}_1\text{\(R\)} interaction with glutamate.

Clinical use of GABA analogues is limited largely due to our insufficient understanding of its actions in the “pain circuit”. Ambient GABA modulates glutamatergic synapses, while ambient glutamate may also modulate GABA release from heterosynaptic GABAergic terminals (Mitchell and Silver 2000; Drew et al. 2008; Yang et al. 2015; Bonalume et al. 2021). The “net effect” of GABA\(\text{\(B\)}_1\text{\(R\)}s functions depends on the dynamic balance of its actions on excitatory and inhibitory inputs (Malcangio 2018). Given that SN neurons in the superficial dorsal horn play a pivotal role in regulating nociception inflow from primary nociceptors and outflow from projection neurons, the significance of GABA\(\text{\(B\)}_1\text{\(R\)} functions manipulated by GABA should be important in the pain circuit. Our data thus reveal an overall view of GABA\(\text{\(B\)}_1\text{\(R\)}s on glutamatergic terminals and postsynaptic glutamate actions, paving a way for better understanding the whole scenario of GABA actions in the spinal dorsal horn.

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Data availability statement The data that supports the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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