$GABA_B$ 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. γ-Aminobutyric acid type B receptors ($GABA_BRs$), the metabotropic GABA receptor subtype, are widely expressed in pre- and postsynaptic structures of the SG neurons. Activation of $GABA_BRs$ by exogeneous agonists induces both pre- and postsynaptic inhibitions. However, the actions of endogenous GABA via presynaptic $GABA_BRs$ on glutamatergic synapses, and the postsynaptic $GABA_BRs$ 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 $GABA_BRs$ switched “silent” synapses into active ones and increased the probability of glutamate release onto SG neurons; increasing ambient GABA concentration mimicked $GABA_BRs$ activation on glutamatergic terminals. Next, using holographic photostimulation to uncage glutamate on postsynaptic SG neurons, we found that postsynaptic $GABA_BRs$ modified glutamate-induced postsynaptic potentials. Taken together, our data identify that endogenous GABA heterosynaptically constrains glutamate release via persistently activating presynaptic $GABA_BRs$; and postsynaptically, $GABA_BRs$ modulate glutamate responses. The results give new clues for endogenous GABA in modulating nociception circuit in spinal dorsal horn and shed fresh light on postsynaptic interaction of glutamate and GABA.

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). 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 $GABA_B$ receptors ($GABA_BRs$) are the family of metabotropic G protein-coupled receptors (GPCRs). In the spinal dorsal horn, $GABA_BRs$ are expressed in the presynaptic and postsynaptic structures (Yang et al. 2001b; Yang et al. 2002; Malcangio 2018). Maintaining certain concentrations at the synaptic clefts and extrasynaptic structures, GABA exerts an important role in neuronal homeostasis in the CNS (Bowery 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). $GABA_BRs$ are found at both synaptic cleft and extrasynaptic membrane, making $GABA_BRs$ as 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 the descending pain modulation system (Merighi 2018). Experiments using exogenous agonists revealed that $GABA_B$Rs mediate pre- and postsynaptic inhibitions on glutamate release and action (Ataka et al. 2000; Iyadomi et al. 2000; Yang et al. 2001b). For the action of endogenous GABA, we have previously reported that blocking primary afferents $GABA_B$Rs 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, whether endogenous GABA constrains silent glutamatergic synapses, and whether there is an integration of $GABA_B$Rs and glutamate actions at postsynaptic neurons, are poorly understood. 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 $GABA_B$Rs 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 methods described elsewhere (Yang et al. 2001a; Yang et al. 2021). In brief, male Sprague-Dawley rats (5-7 weeks old) were anesthetized by urethane (1.5 g/kg bodyweight, 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, NaH_2PO_4 1.2, $MgSO_4$ 8, $NaHCO_3$ 26, $CaCl_2$ 0.1, and glucose 11, oxygenated with mixed gas (95% O_2/5% CO_2). 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, NaH_2PO_4 1.2, $NaHCO_3$ 26, $MgSO_4$ 1.5, $CaCl_2$ 2.5, and glucose 11 (pH 7.2, mOsm 300-305) at ~35ºC with mixed gas saturation for 40 min. Slices were then kept in incubation solution at room temperature (23 ± 2ºC) for using up to 8 h.

Visualized whole-cell recordings were obtained by using glass pipettes with resistance 5-8 MΩ when filled with intrapipette solution composed of (in mM) K-gluconate 130, KCl 5, $CaCl_2$ 0.1, $MgCl_2$ 2, EGTA 5, HEPES 5, Na_3-GTP 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 $GABA_B$Rs’ effects (Yang et al. 2001a). Signals were amplified by Axopatch 200B, digitized by Digidata 1550A which was manipulated by software pClamp 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-nitroquinoxaline-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 “presynaptic silent synapses” (Voronin and Cherubini 2003; Safiulina and Cherubini 2009). Monosynaptic synapses 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. 2001a; Yang et al. 2021). To evaluate direct glutamatergic synapses onto SG neurons, only monosynaptic eEPSCs were accepted for further analysis. To evaluate possible postsynaptic $GABA_BRs$-induced K^+ currents, we used a “ramp test” protocol as described before (Liu et al. 2012).

Holographic photostimulation and recordings

For three-dimensional (3-D) holographic stimulation, holographic beam was integrated into the optical axis of 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 to Clampex 10.2 electrophysiology acquisition software (Molecular Devices). The soma and dendrites of recorded neurons were illuminated 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 $GABA_BRs$ with glutamate responses, one dendrite was stimulated by transient glutamate uncaging as “testing”, following a “priming” stimulation on a neighbor dendrite (60 ms interval). 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 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 nulled postsynaptic $GABA_B$Rs by intracellular GDP-β-S dialysis (see 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 then 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 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, 2b). 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 an 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 and induces GABA release and subsequent spillover 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 stimulation (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 (Fig. 3a1); CGP52432 perfusion (1 μM, 5 min) increased the amplitude of the slow current, with a frequency-dependent manner. 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, $n = 9$; Fig. 3b), 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 smaller “net” effects at lower frequency stimuli ($P < 0.05$ or $P < 0.01$, 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 $GABA_B$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 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, one-way ANOVA, n = 8). Intracellular cAMP-dependent protein kinase A (PKA) signaling pathway was indicated in postsynaptic glutamate interaction with $GABA_B$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, one-way ANOVA, n = 8; Fig. 4d).

Taking advantage of holographic stimulation and uncaging glutamate, we compared the effects of postsynaptic $GABA_B$Rs in modulating glutamate responses. The recording pipette did not include GDP-β-S, making postsynaptic $GABA_B$Rs available for investigation (Yang et al. 2001a). In the presence of baclofen (10 μM) which activated $GABA_B$Rs, the average amplitude of uncaged glutamate responses (uEPSPs) decreased to 59.47 ± 11.58% of the control (P = 0.011, paired t-test, n = 10), indicating that postsynaptic $GABA_B$Rs 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 $GABA_B$Rs modulate postsynaptic glutamate responses.

We investigated whether ambient GABA induced a tonic postsynaptic inhibition via $GABA_B$Rs to study the role of endogenous GABA. A voltage ramp protocol (from −155 mV to −25 mV with 800 ms ramp) induced a membrane current and 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) as calculated by the Nernst equation (−89.8 mV) under our recording conditions, suggesting that $GABA_B$R-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 (Fig. 5d). Taken together, the results suggest that although endogenous GABA affects postsynaptic membrane K^+ current, it contributes little to glutamate responses; however, activating postsynaptic $GABA_B$Rs by exogeneous agonist baclofen affects postsynaptic glutamate actions.

Postsynaptic $GABA_B$Rs integrate glutamate actions with different arbors

Blocking endogenous $GABA_BRs$ has little effect on postsynaptic glutamate action (see Fig. 5), we next investigated endogenous $GABA_BR$ agonist interaction with glutamate responses. A neuron receives released glutamate at multiple dendrites, but how the inputs at different dendrites are integrated is not clear. 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 Methods). In the presence of baclofen, the integration index significantly decreased (Fig. 6c), indicating that $GABA_BRs$ mediated 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 $GABA_BRs$ integrate glutamate-mediated uEPSPs (Fig. 6c).

Discussion

Technical considerations for presynaptic and postsynaptic $GABA_BRs$ studies

The present study first focused on the presynaptic $GABA_BRs$ in controlling glutamate synapses. Although $GABA_BRs$ are expressed both pre- and postsynaptically, the possible postsynaptic $GABA_BR$ action was ruled out by intracellular GDP-β-S dialysis from the recording electrodes (Yang et al. 2001a). Under these given experimental conditions, the minimal stimulation and the perfusion of CGP52432 only affected presynaptic $GABA_BRs$, making it possible to clarify $GABA_BRs$’ 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; these actions depend upon ambient GABA concentrations.

On the other hand, to study the postsynaptic actions, the presynaptic $GABA_BRs$’ 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 $GABA_BRs$, which revealed that the postsynaptic $GABA_BRs$ modulate glutamate-mediated postsynaptic responses.

Presynaptic $GABA_BRs$’ actions on glutamatergic synapses

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 latter, it is considered as a “presynaptically” silent synapse (Voronin and Cherubini 2003; Safiulina and Cherubini 2009). In the present study, we used paired-pulses 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 (too weak presynaptic stimulation), this notion is in line with a previous report in the spinal dorsal horn (Yasaka et al. 2009). Occlusion of $GABA_B$Rs 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 $GABA_B$Rs by endogenous GABA. 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, suggest that the concentrations of ambient GABA affect $GABA_B$Rs at the presynaptic glutamatergic terminals. The data further support the conclusion that ambient GABA modulates glutamate release.

It should be noted that the present “heterosynaptic hypothesis” is based on assumption that GABA is not co-released 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 $GABA_B$R-mediated inhibition of glutamatergic synapses is due to ambient and/or spillover GABA which binds to $GABA_B$ heteroreceptors at the neighboring glutamatergic terminals (Fig. 7). The common pathway for $GABA_B$Rs modulation on glutamatergic terminals might be through calcium channels (Pfrieger et al. 1994; Yang and Ma 2011).

Postsynaptic $GABA_B$Rs modulation on glutamate-mediated uEPSPs

$GABA_B$Rs mediate calcium channels and potassium channels by intracellular interactions (Kanatamneni 2015; Bettler and Fakler 2017). Since these channels are proteins on the cell surface, it is conceivable that $GABA_B$Rs 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 integration between uEPSPs and $GABA_B$Rs, three possibilities may underlie these results. First, $GABA_B$Rs may modulate the number of excitatory synapses and membrane surface AMPA receptors (Terunuma et al. 2014), Ca^{2+} influx via NMDA receptors may also

be inhibited by $GABA_BRs$ (Terunuma 2018). Second, postsynaptic $GABA_BRs$ 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 $GABA_BRs$ (Chalifoux and Carter 2010). The intracellular PKA signaling pathway plays a certain role (Chalifoux and Carter 2010), however, our current results do not find this modulation pathway in SG neurons (Fig. 4c). And the third, in the present study, baclofen decreased the average amplitude of uEPSPs, these may be resulted by an increasing membrane conductance by $GABA_BRs$ activation, as we reported before (Liu et al. 2012). Nevertheless, $GABA_BRs$' activities do affect the function of the glutamate receptors and vice versa (Kantamneni 2015). A simplest explain is that different structures have different physiological properties and may interact with each other. In the present study, $GABA_BRs$ are GPCRs and have a long extracellular N-terminal domain, a C-terminal intracellular tail and a transmembrane domain, with integration of other receptors, including glutamate receptors (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, the integration is a postsynaptic event, not a presynaptic integration (Zucker and Regehr 2002). Baclofen, presumably activating postsynaptic $GABA_BRs$, impaired the "hold and read" (Santos et al. 2012), suggesting $GABA_BRs$ not only contribute to postsynaptic glutamate response, but also affect information integration. The integration of ionotropic glutamate receptors and metabotropic $GABA_BRs$ is still unsure (Fig. 7), more experiments are required to elucidate how $GABA_BRs$ affect the postsynaptic integration.

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" which is mediated by ionotropic $GABA_ARs$ (Kullmann et al. 2005). Enhancing GABAergic tone alleviates neuropathic and inflammatory pain at the spinal cord level via $GABA_ARs$ (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 $GABA_BRs$, 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). Previous physiological and behavioral reports concerning mechanisms of $GABA_BR$-mediated antinociception were based on exogenous synthetic agonists (see Introduction), our present study focused on endogenous GABA in modulating glutamatergic synapses via $GABA_BRs$. Furthermore, this study also clarified the postsynaptic $GABA_BRs$ 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 $GABA_BRs$ functions depends on the dynamic balance of its actions on excitatory and inhibitory inputs (Malcangio 2018). Given that SG neurons in the superficial dorsal horn play pivotal roles in regulating nociception inflow from primary nociceptors and outflow from projection neurons, the significance of $GABA_BR$ functions manipulated by GABA should be important in the pain circuit. Our data thus reveal an overall view of $GABA_BRs$ on glutamatergic terminals and postsynaptic glutamate actions, paving a way for better understanding the whole scenario of GABA actions in the spinal dorsal horn.

Declarations

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Author contributions

Mingwei Zhao, Jiaxue Dong, Caifeng Shao, Qian Chen: Investigation; Formal analysis. Rui Ma, Ping Jiang, Wei-Ning Zhang: Formal analysis. Kun Yang: Conceptualization; Methodology; Investigation; Supervision; Project administration; Resources; Writing-original draft; Writing-review & editing.

Competing interests

The authors declare no competing interests.

Additional information

Data Availability Statement: The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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Figures

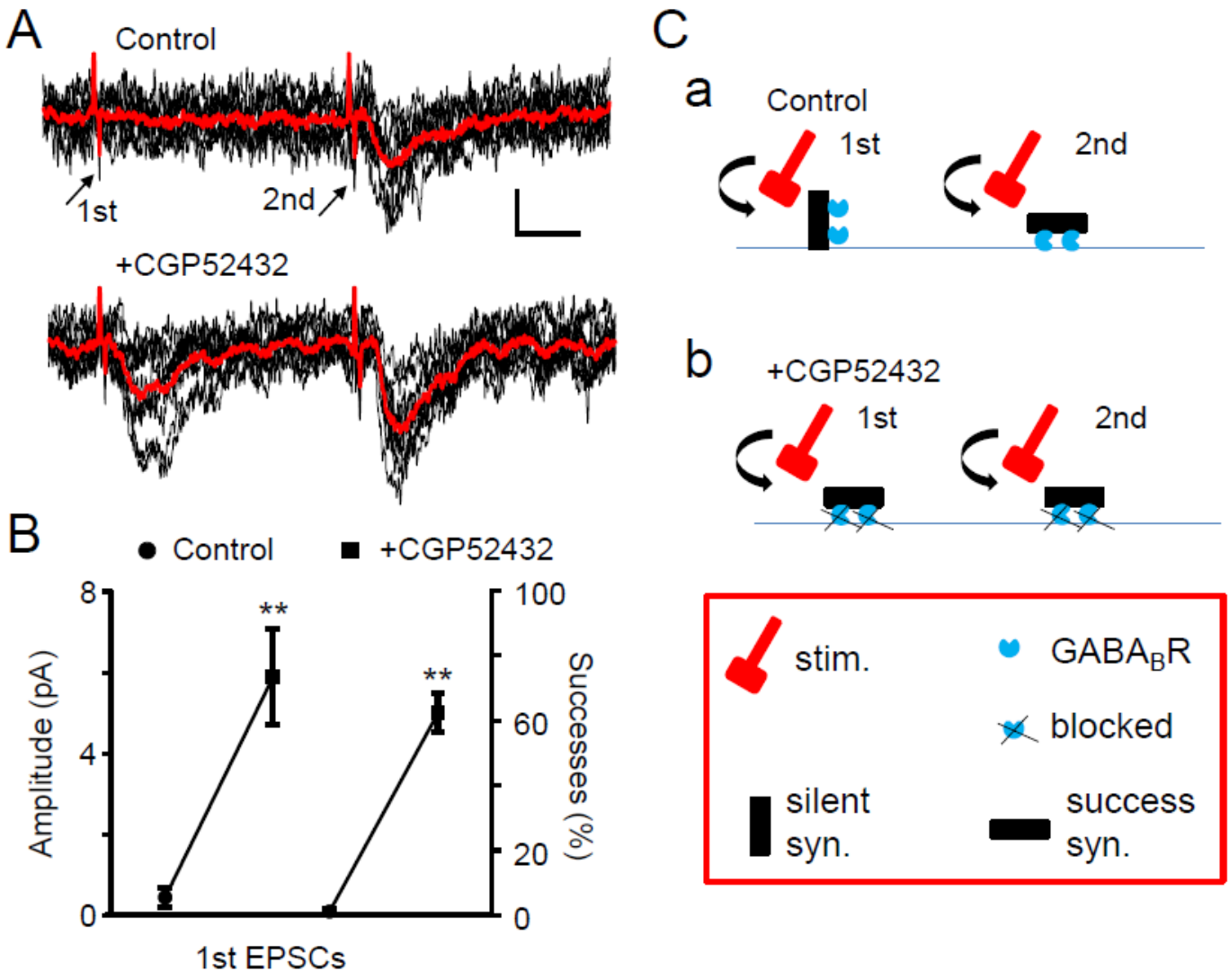

Figure 1

Blockade of GABABRs with CGP52432 switches “silent” glutamatergic synapses to functional ones onto SG neurons via a presynaptic mechanism. a A representative neuron shows silent synapses to the first (1st) stimulus while it responds to the second (2nd) stimulus in a paired-pulses paradigm (80 ms interstimulus interval; Control). Perfusion of CGP52432 (1 μM, 5 min) reveals some detectable evoked excitatory postsynaptic currents (eEPSCs) to the 1st stimulus (+CGP52432). Black and red lines indicate 8 consecutive traces at 0.033 Hz and the averaged traces, respectively. Scale bars, 5 pA, 20 ms. Arrows indicate the 1st and the 2nd stimulus artifacts, respectively. b Group data show the 1st eEPSCs from all 9 neurons tested with increased amplitude (Amplitude) and success ratio (Successes) to CGP52432 treatment. Results are shown as mean ± S.E. **, P < 0.01. c cartoon shows the possible mechanism for unsilencing synapse by blocking GABABRs. (a) In paired stimuli (red hammers and curved arrows), the 1st shock yields no detectable eEPSCs because GABABRs constrain the release, while the 2nd shock induces some successful synapse events, indicating “presynaptic silence”. (b) Perfusion of CGP52432, presumably blocking GABABRs (× symbols), the 1st stimulus switches silent synapse (silent syn.) to successful one (success syn.).

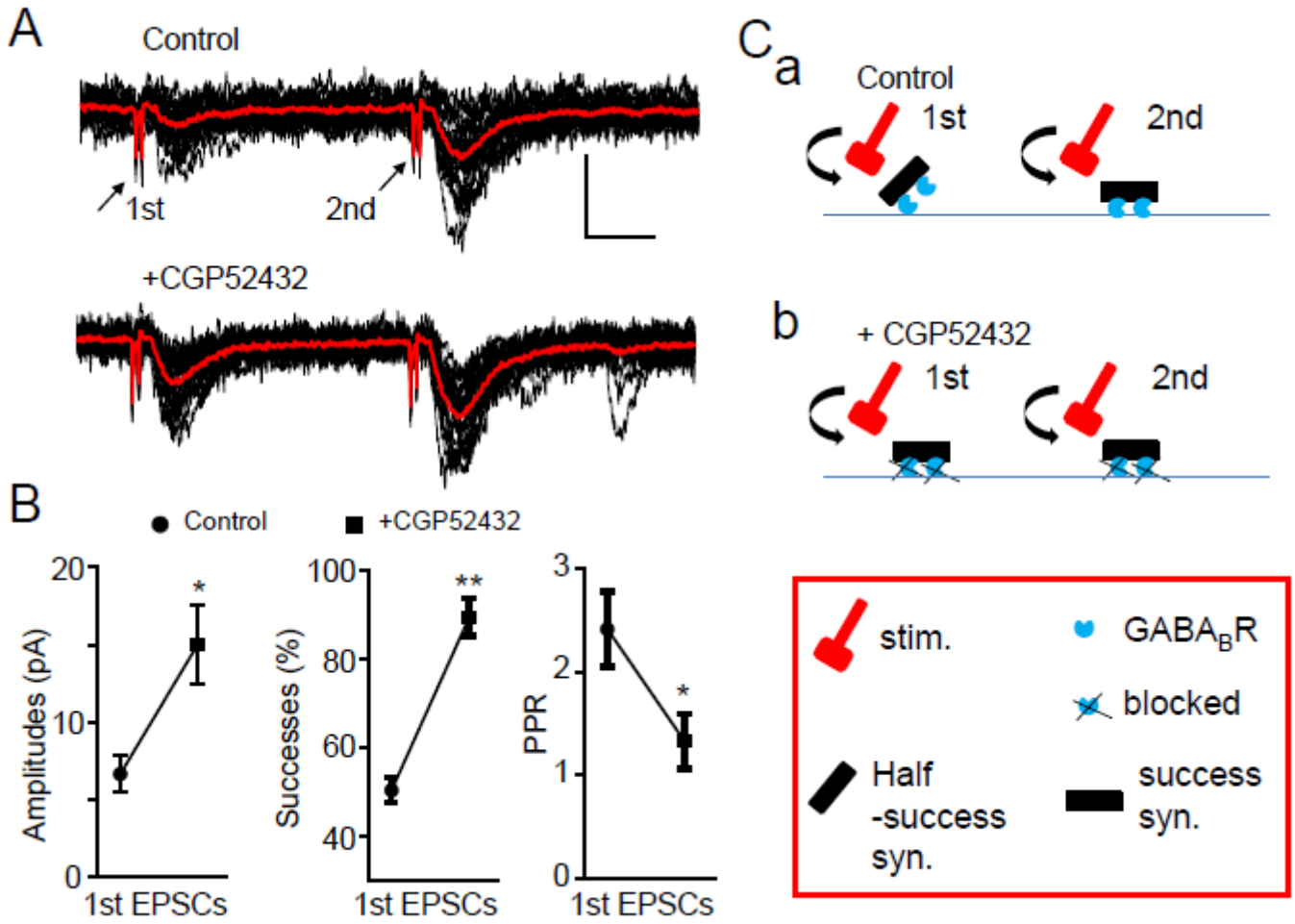

Figure 2

Presynaptic GABABRs constrain glutamate release onto SG neurons by endogenous GABA via a presynaptic mechanism. a A paired presynaptic stimulation (80 ms interval) with a strength that induces

~50% of detectable eEPSCs events in the 1st stimulus (Control); perfusion of CGP52432 (1 μM, 5 min) increases amplitude and success ratio of eEPSCs at the 1st stimulus, as well as the amplitude of eEPSCs at the 2nd stimulus (+CGP52432). The raw data (black) show 8 consecutive traces at 0.033 Hz, the red lines show the average of these traces. Scale bars, 20 pA, 20 ms. b Perfusion of CGP52432 significantly increases the average amplitude (Amplitudes), success ratio (Successes) of the 1st EPSCs, and changes paired-pulse ratio (PPR) eEPSCs. All data are expressed as mean ± S.E. *, P < 0.05, **, P < 0.01. c cartoon shows the mechanism for increasing synapse by blocking GABABRs. (a) In paired stimuli (red hammers and curved arrows), the 1st shock yields about half events of eEPSCs detectable (half-success syn.) because GABABRs constrain the release, while the 2nd shock induces more successful events. (b) Perfusion of CGP52432 which blocks GABABRs (× symbols) increases more successful events (success syn.) and eEPSCs amplitude by the 1st stimulus.

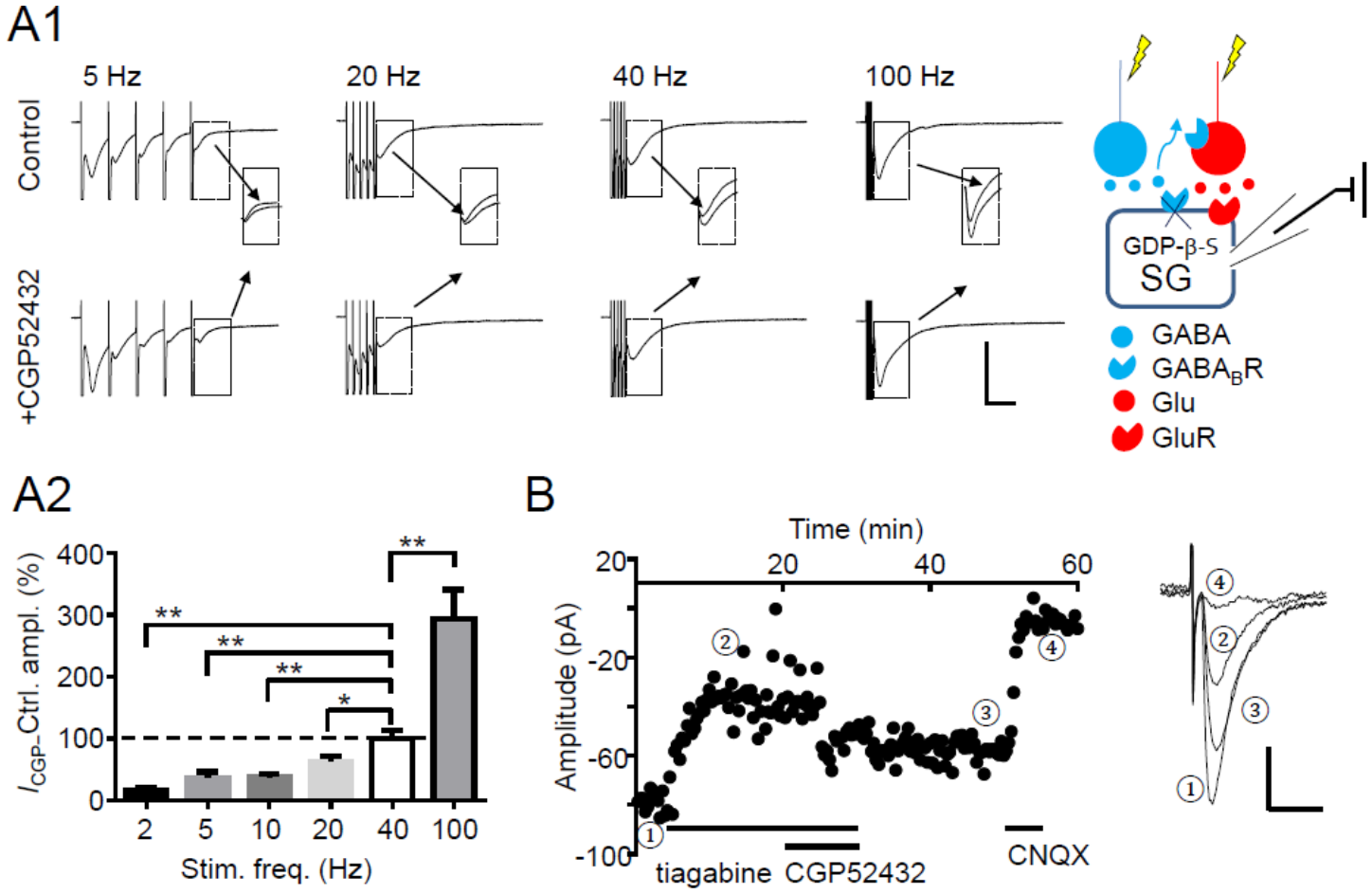

Figure 3

Elevating ambient GABA level decreases presynaptic glutamate release by activating presynaptic GABABRs with a frequency-dependent manner or GABA transporter inhibition. a1 Representative recordings show EPSCs of the control (Control; upper) and in the presence of 1 μM CGP52432 (+CGP52432; bottom). All traces are averages of 8 consecutive trails at 0.033 Hz. Broken line rectangle boxes indicate the peak of averaged post-train EPSCs at the control and in the presence of CGP52432, overlaps for comparing (indicated by arrows). Scale bars, 200 pA, 200 ms. Cartoon in the right indicates

that in a recorded SG neuron (SG), the presynaptic electric stimulation induces both GABA and glutamate (Glu) release where EPSCs amplitude is affected by GABA escaped from neighbor GABAergic terminal. Note the postsynaptic GABABR-mediated action is nulled by intracellular GDP-β-S dialysis. a2 correlation of the normalized amplitude of the "net" CGP52432 action (ICGP–Ctrl. ampl.) at different stimulation frequency (stim. freq.; n = 9 for each group). All data are expressed as mean ± S.E. and the average amplitude at 40 Hz is set as 100%, indicated by the broken line. *, P < 0.05, **, P < 0.01. b Left panel: a representative neuron shows that tiagabine (a GAT-1 inhibitor; 30 μM) decreases eEPSCs, and this action is partially attenuated by adding 1 μM CGP52432. CNQX (10 μM) abolishes EPSCs, suggesting their glutamate AMPA receptor-mediated nature. Each drug was perfused to the slice in the bath solution at times indicated by the horizontal time. Right panel: raw current traces are shown at the time indicated by circled numbers in the left panel. Scale bars, 40 pA, 20 ms.

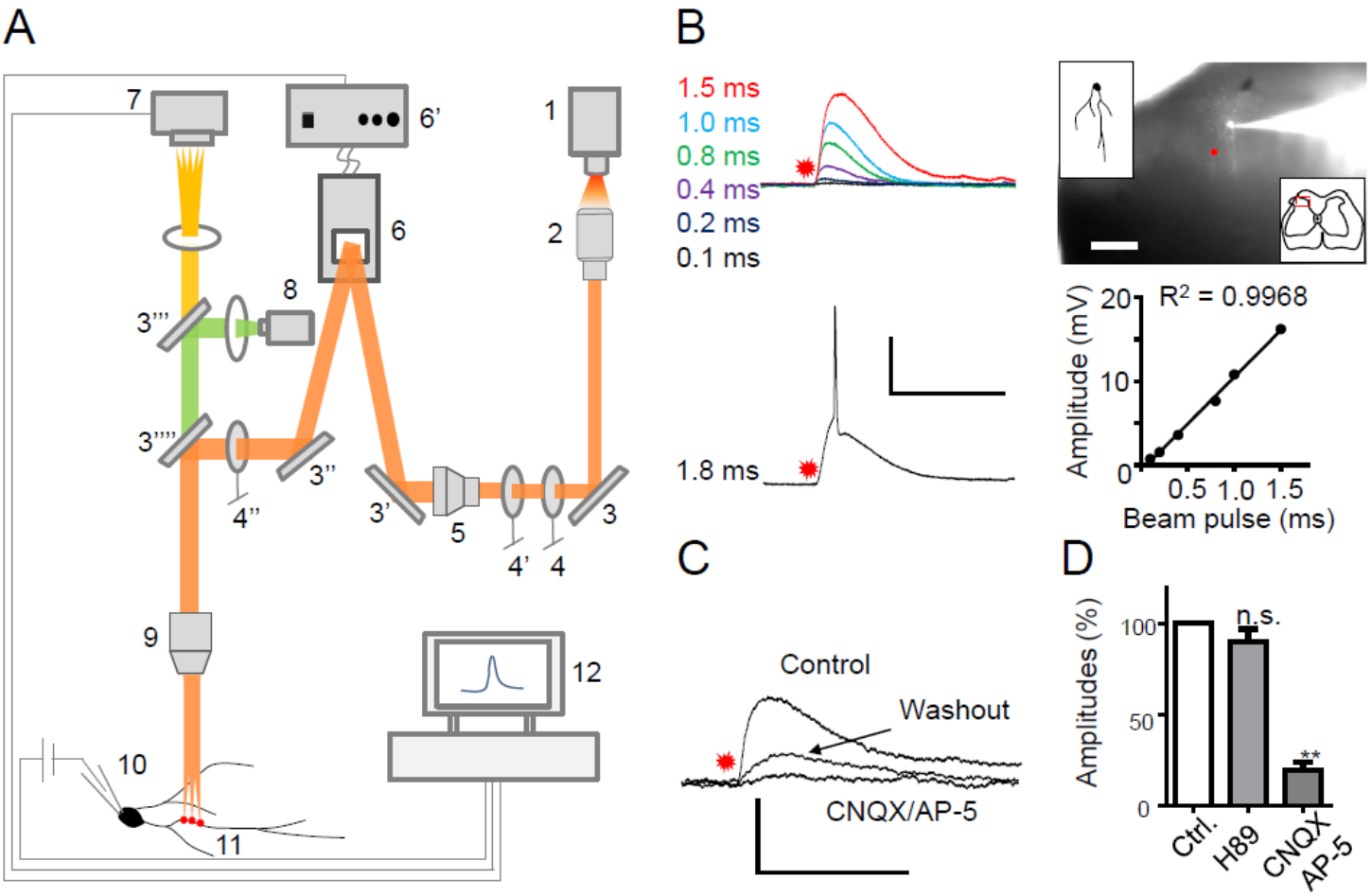

Figure 4

Uncaging glutamate induces postsynaptic excitatory responses by activating glutamate receptors. a Setup of holographic stimulation and recordings (drawn not to scale for clarity). 1, laser generator (405 nm, 150 mW; Optoelectronics Tech, Changchun, China); 2, spatial filter collimator (Melles Griot 09Lsf011, SPW Industrial, Laguna Hills, CA, USA); 3-3"", broadband dielectric mirrors (BB2-E02; Thorlabs, NJ, USA); 4-4", neutral density filters (Thorlabs); 5, BE-05 5× galilean beam expander (Thorlabs); 6 and 6', Hamamatsu

LEOS-SLM head and controller (Hamamatsu, Japan); 7, CCD camera (iXon Ultra; Andor, Belfast, UK); 8, arc lamp; 9, water immersion objective (N.A. 0.9; Olympus, Tokyo, Japan); 10, whole-cell recording (amplifier and signal converter not shown); 11, target neuron and uncaging spots (red dots) on a dendrite; 12, PC integration with recording and stimulation synchronization (Molecular Devices). b Uncaging excitatory postsynaptic potentials (uEPSPs) evoked by uncaged glutamate to different degrees by varying light beam “flashing” time (upper, left). The explosion symbol represents uncaged glutamate at the initial time of uEPSPs. Uncaging glutamate yields an action potential (bottom, left). The times on the left of charts indicate the different flashing widths of uncaging light beam. Vertical bars, 10 mV for upper and 20 mV for bottom, respectively; horizontal bars, 400 ms. Upper right: the recorded neuron viewed by intracellularly dialyzing Alexa Fluor 594 (10 mM) from recording pipette. The red explosion symbols indicate the uncaging spots on a dendrite. The inserts in the photo indicate the area of the photo taken from a transverse spinal cord slice (right) and a reconstruction of the recorded neuron (left). Scale bar, 140 μm. Bottom right: linear regression fits to the relationship between beam duration and subthreshold uEPSPs amplitude in this neuron. c In a different neuron, uncaging glutamate to a dendrite induces uEPSP (Control) which is mostly abolished by CNQX 10 μM and AP-5 100 μM (CNQX/AP-5), with a reversible manner after washout (Washout). Scale bars, 10 mV, 400 ms. d Pooled data summarize uncaging responses under different conditions. Responses to uncaging glutamate are sensitive to the perfusion of CNQX 10 μM + AP-5 100 μM, but not a specific PKA inhibitor H89 (30 μM). Data are shown as mean ± S.E., n = 8 for each group. n.s., not significant; **, P < 0.01.

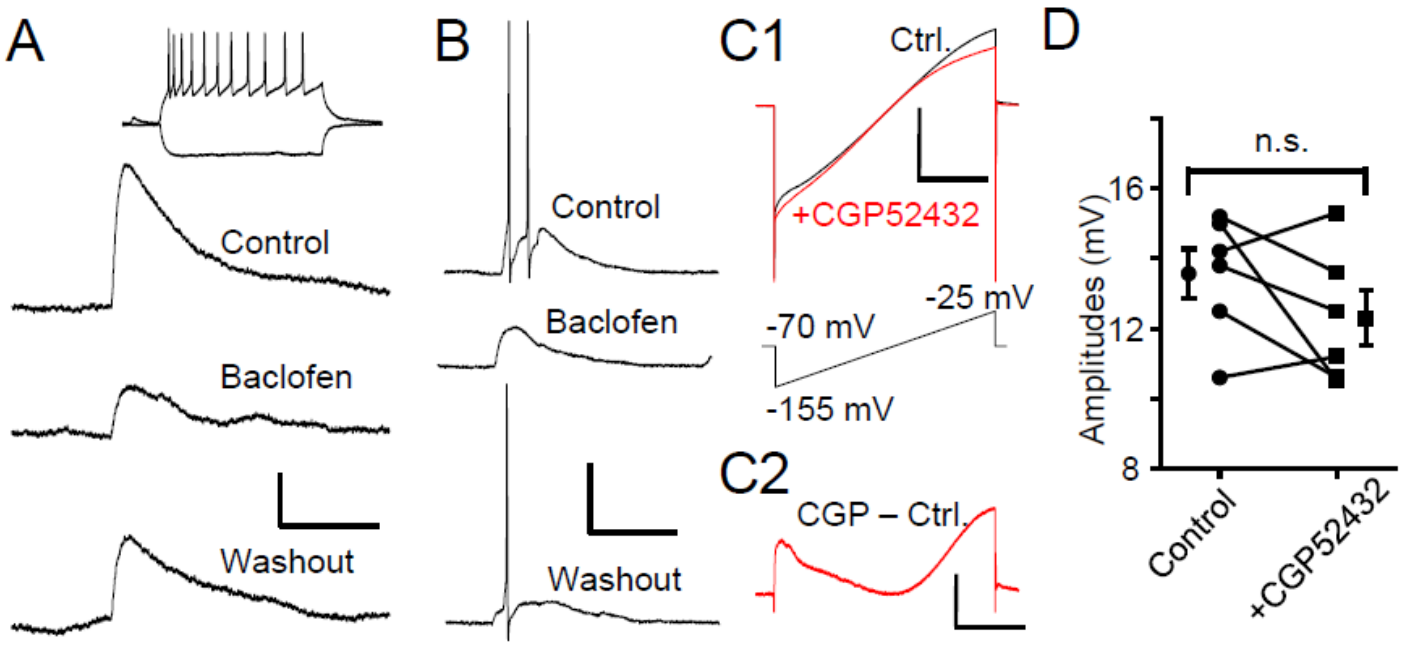

Figure 5

Postsynaptic GABABRs modulate glutamate-induced uEPSPs. a Bath application of baclofen (10 μM) reduces the amplitude of uEPSPs from the control (Control) to in the presence of baclofen (Baclofen); the depression recovers after washout (Washout). Insert: response to depolarizing and hyperpolarizing current injections (± 20 pA, 0.8 s; resting membrane potential −63 mV) displaying a stereotypic SG

neuron. Scale bars, 5 mV, 0.3 s. b In a different neuron, baclofen (10 μM) depresses action potentials induced by postsynaptic uncaging glutamate, with a reversible manner after washout. Scale bars, 40 mV, 0.2 s. c Postsynaptic GABABRs suppress the excitability of SG neurons through postsynaptic mechanism. c1 Typical results from blocking GABABRs by CGP52432 (1 μM) reveal a voltage-dependent membrane current. Black trace indicates the control (Ctrl.) while red trace shows in the presence of CGP52432 (+CGP52432). Insert: the holding voltage ramp protocol. Scale bars, 1 nA, 0.4 s. c2 The “net” postsynaptic membrane current of GABABR-mediated component was calculated by subtracting control from in the presence of CGP52432 (CGP−Ctrl.). Scale bars, 200 pA, 400 ms. d Perfusion of 1 μM CGP52432 for 5 min (+CGP52432; black squares) does not change uEPSPs amplitude from the control (Control; round dots). n.s., not significant.

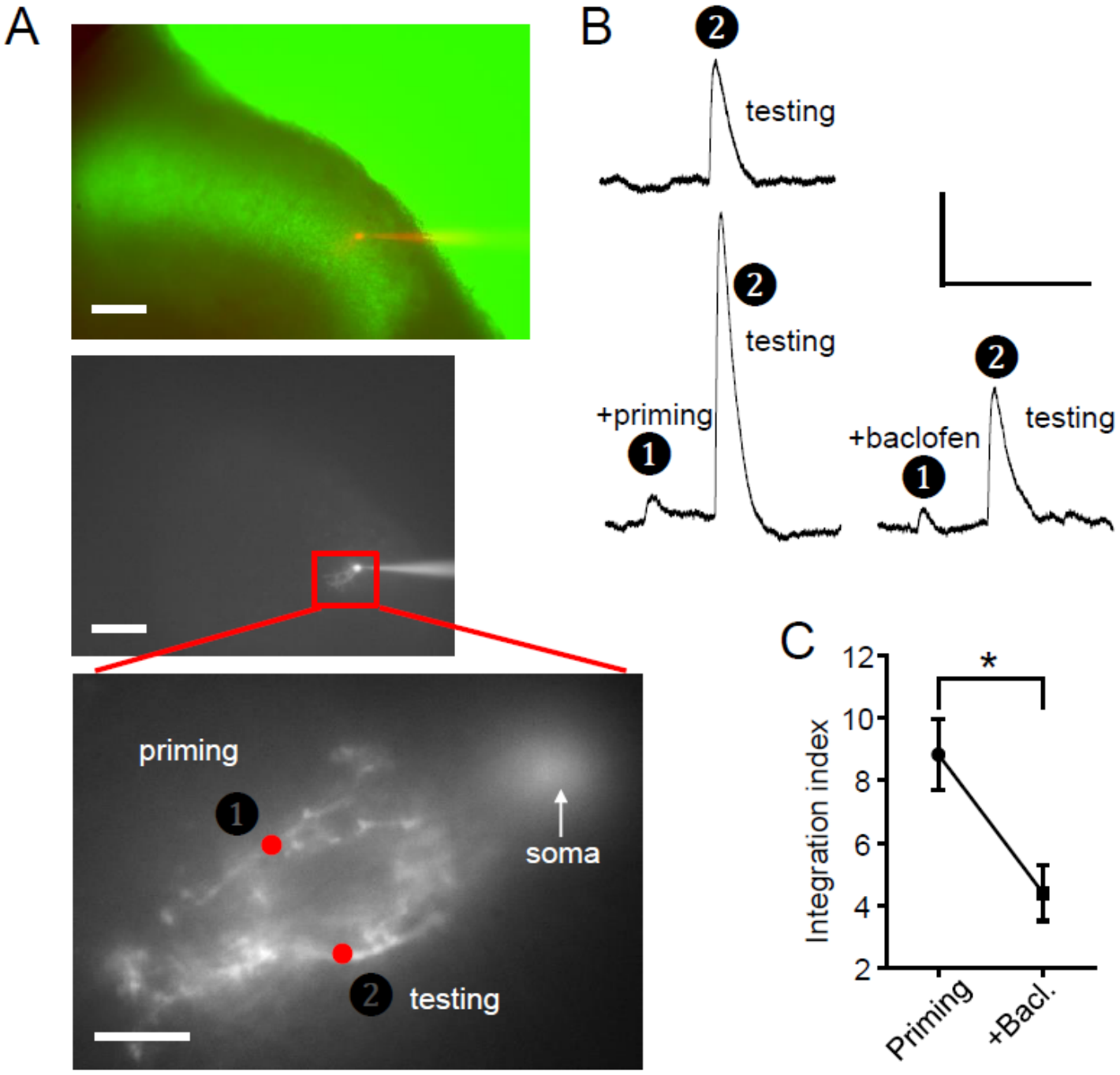

Figure 6

Postsynaptic GABABRs integrate glutamate-induced uEPSPs from different arbors. a Image of the whole-cell recordings from a SG neuron filled by Alexa Fluor 594 from the recording pipette (top and middle; scale bars, 150 μm), SG under dissection scope illuminated by transparent light shown in a transversal slice. The higher magnification (bottom) indicates dendritic spots for uncaging glutamate. Scale bar, 30 μm. Note at higher magnification, the soma (indicated by an arrow) is obscure because of the plane difference between the cell body and the dendrites. The red dots indicate sites for activated by uncaged glutamate as priming (□) and testing (□), respectively. b Uncaging glutamate from different arbors shows integration. Testing caged glutamate induces eEPSP (top, testing), which is enhanced by a priming eEPSP from another dendrite (+priming), resulting in an integration. Further perfusing baclofen (+baclofen) yields an integration index change. Scale bars, 5 mV, 120 ms. c The integration index changes in the presence of baclofen (+Bacl.), which activated GABABRs. *, P < 0.05; unpaired t-test.

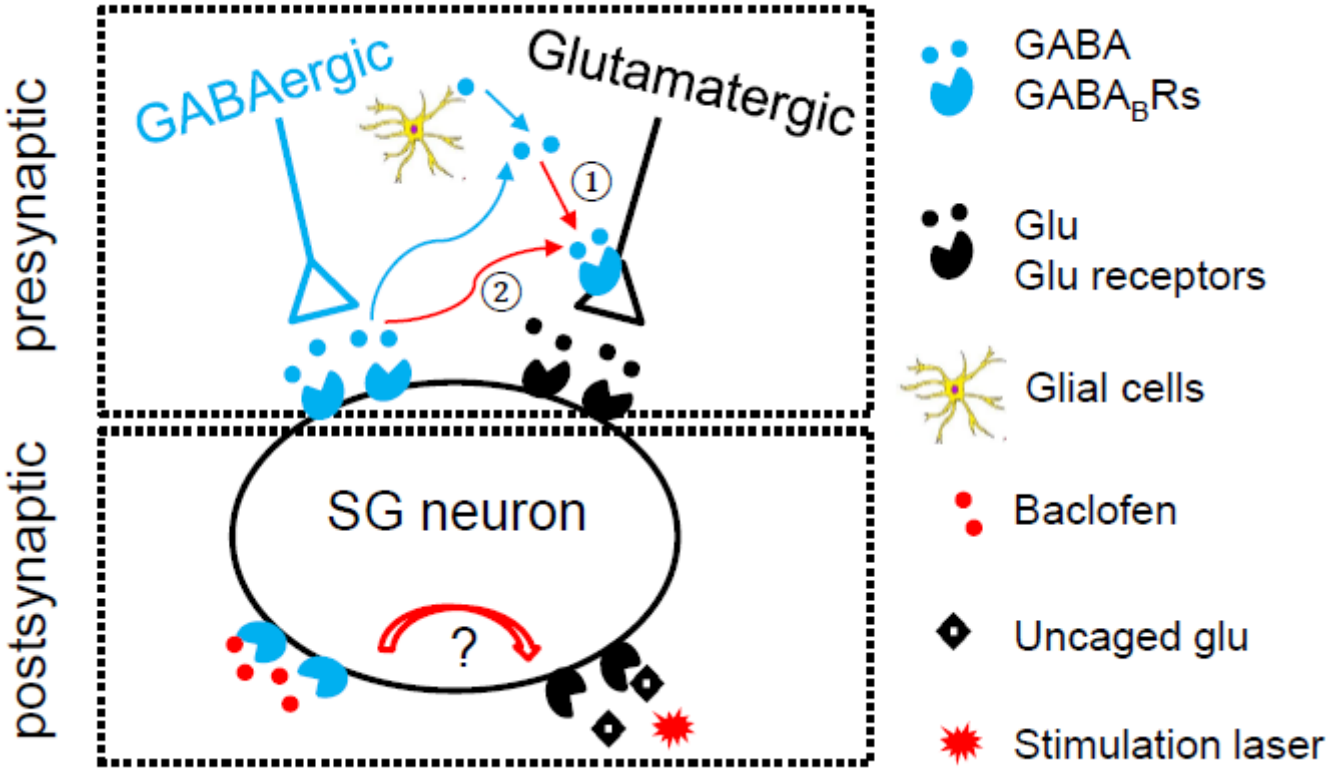

Figure 7

Schematic diagram illustrating the modulation of glutamate presynaptic releases and postsynaptic responses by GABABRs onto SG neurons in the dorsal horn. At the presynaptic (upper broken line rectangle), ambient GABA binds glutamatergic terminals (indicated by □) and/or spillover GABA from GABAergic terminals (indicated by □), 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), GABABRs,

activated by baclofen, impair postsynaptic glutamate response (see Results), with an uncertain mechanism (indicated by a question mark).