Published in final edited form as:
Eur J Neurosci. 2012 August ; 36(4): 2482–2492. doi:10.1111/j.1460-9568.2012.08147.x.

The GABA Transporters GAT-1 and GAT-3 modulate glutamatergic transmission via activation of presynaptic $GABA_B$ receptors in the rat globus pallidus

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Abstract

Intrapallidal application of GAT-1 or GAT-3 transporter blockers (SKF 89976A or SNAP 5114) reduces the activity of pallidal neurons in monkey. This effect could be mediated through activation of presynaptic $GABA_B$ heteroreceptors in glutamatergic terminals by GABA spillover following GABA transporters (GATs) blockade. To test this hypothesis, we applied the whole-cell recording technique to study the effects of SKF 89976A and SNAP 5114 on evoked excitatory post synaptic currents (eEPSCs) in presence of gabazine, a $GABA_A$ receptor antagonist, in rat GP slice preparations. Under the condition of postsynaptic $GABA_B$ receptor blockade by intracellular application of OX314, bath application of SKF 89976A (10 μM) or SNAP 5114 (10 μM) decreased the amplitude of eEPSCs, without significant effect on its holding current and whole cell input resistance. The inhibitory effect of GATs blockade on eEPSCs was blocked by CGP 58845, a $GABA_B$ receptor antagonist. The paired-pulse ratio (PPR) of evoked EPSCs was increased, while the frequency, but not the amplitude, of miniature excitatory postsynaptic currents (mEPSCs) was reduced in presence of either GAT blockers, demonstrating a presynaptic effect. These results suggest that synaptically released GABA can inhibit glutamatergic transmission through activation of presynaptic $GABA_B$ heteroreceptors following GAT-1 or GAT-3 blockade.

In conclusion, our findings demonstrate that pre-synaptic $GABA_B$ heteroreceptors in putative glutamatergic subthalamic afferents to GP are sensitive to increases in extracellular GABA induced by GATs inactivation, thereby suggesting that GATs blockade represents a potential mechanism by which overactive subthalamopallidal activity may be reduced in parkinsonism.

Keywords

EPSC; patch-clamp; striatum; GATs; GABA receptor

INTRODUCTION

The globus pallidus (GP) (or external globus pallidus, GPe, in primates) plays a central integrative role in the basal ganglia circuitry (Smith *et al.*, 1998; Plenz & Kitai, 1999; Bevan *et al.*, 2002). It receives GABAergic inputs from the striatum (Str) and from axon collaterals of projection neurons, and a major glutamatergic innervation from the subthalamic nucleus (STN). In turn, the GP sends GABAergic projections to the STN and other basal ganglia

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nuclei. The balance between excitatory (glutamatergic) and inhibitory (GABAergic) inputs to GP is crucial for maintaining normal basal ganglia (BG) function.

Several studies have shown $GABA_A$ receptor-mediated effects upon GP neurons (Kita *et al.*, 2004; Galvan *et al.*, 2005; Kita *et al.*, 2006). There is also evidence that metabotropic $GABA_B$ receptors modulate GP synaptic transmission through pre or postsynaptic mechanisms (Chen *et al.*, 2002; Galvan *et al.*, 2005; Kaneda & Kita, 2005). For instance, repetitive intrapallidal stimulation induces postsynaptic $GABA_B$ receptor-mediated slow inhibitory postsynaptic potential (IPSP) (Kaneda & Kita, 2005), while baclofen, a specific $GABA_B$ receptor agonist, reduces the frequency of miniature IPSCs (mIPSCs) without altering their amplitude (Chen *et al.*, 2003; Kaneda & Kita, 2005), in rat GP neurons. Moreover, $GABA_B$ receptors were found to induce high-voltage-activated (HVA) calcium currents in GP neurons (Stefani *et al.*, 1990).

Despite the regulatory functions of $GABA_B$ receptors on GABAergic synaptic transmission, very little is known about their regulatory functions of glutamatergic transmission in basal ganglia (Shen & Johnson, 1997; Chen *et al.*, 2002; Kaneda & Kita, 2005). On the other hand, presynaptic $GABA_B$ receptors activation inhibits glutamatergic transmission in other brain regions (Mitchell & Silver, 2000; Chen & Regehr, 2003; Kolaj *et al.*, 2004). However, because most of these studies were performed using exogenous application of the $GABA_B$ receptor agonist baclofen, they do not provide any evidence that synaptically released γ-aminobutyric acid (GABA) can activate presynaptic $GABA_B$ receptors in glutamatergic terminals. A previous study in the rat GP showed that application of (2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP55845A), a selective $GABA_B$ receptor antagonist, increased the frequency of mEPSCs without any significant effect on its amplitude, suggesting that $GABA_B$ receptors in glutamatergic terminals could be tonically activated by synaptically released GABA (Chen *et al.*, 2002). However, other studies have failed to demonstrate that synaptically released GABA modulates glutamatergic transmission in the rat GP (Hanson & Jaeger, 2002; Kaneda & Kita, 2005). On the other hand, presynaptic $GABA_B$ receptor-mediated inhibition of glutamatergic transmission has been demonstrated in hippocampus (Isaacson *et al.*, 1993) and cerebellar glomerulus (Mitchell & Silver, 2000) under the condition of GAT-1 blockade.

GABA transporter subtype 1 (GAT-1) and GABA transporter subtype 3 (GAT-3) are expressed in unmyelinated axons and glial processes, respectively, in the monkey and rat pallidum (Wang & Ong, 1999; Ng *et al.*, 2000; Galvan *et al.*, 2005; Jin *et al.*, 2011a,b). Although GAT-1-mediated regulation of GABAergic transmission has been studied in various brain regions (Thompson & Gähwiler, 1992; Roepstorff & Lambert, 1992, Isaacson *et al.*, 1993; Roepstorff & Lambert, 1994; Draguhn & Heinemann, 1996; Engel *et al.*, 1998; Overstreet *et al.*, 2000; Overstreet & Wesbrook, 2003; Keros & Hablitz, 2005; Gonzalez-Burgos *et al.*, 2009), most of these studies have focused on the effects of GAT-1 blockade on phasic and tonic $GABA_A$ receptor-mediated inhibition. Much less is known about the roles of GAT-1 and GAT-3 inactivation towards modulation of glutamatergic transmission in the CNS (Mitchell & Silver, 2000). We have demonstrated that *in vivo* application of GAT-1 and GAT-3 blockers inhibits the firing rate of GP neurons in awake monkeys (Galvan *et al.*, 2005). One possible mechanism underlying this effect is that GATs blockade induced increases in extracellular GABA concentration (Fink-Jensen et al., 1992), which then activated presynaptic $GABA_B$ receptors at glutamatergic synapses, and reduced glutamate-mediated excitation upon pallidal neurons. To directly test this hypothesis, we used the whole cell patch-clamp recording technique in brain slices to provide mechanistic insights into the regulatory functions of GATs on glutamatergic transmission in rat GP.

EXPERIMENTAL PROCEDURES

Slice preparation

All animal procedures were performed in accordance with the *NIH Guide for the Care & Use of Laboratory Animals*, and were approved by the Emory University's Animal Care and Use Committee. The electrophysiological experiments were performed on slices from 14- to 17-d old Sprague Dawley rats (Charles River Laboratories, Wilmington, MA). The animals were decapitated, and the brains removed and submerged in the ice-cold oxygenated sucrose buffer (containing, in mM 233.4 sucrose, 20 glucose, 47.3 $NaHCO_3$, 3 KCl, 1.9 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$). Parasagittal (300μm thickness) were made on a Vibratome 3000 (The Vibratome Company, St. Louis, MO) in ice-cold oxygenated sucrose buffer. The slices were stored at room temperature in a chamber containing artificial cerebrospinal fluid (ACSF) [containing, in mM: 124 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 2.0 $CaCl_2$, 20 glucose, 26 $NaHCO_3$], pH 7.3–7.4 with 95% O_2, 5% CO_2 bubbling through it. The osmolarity of the ACSF was ~310 mOsm.

Drug application

All drugs were purchased from Toris Cookson (Ellisville, MO). All compounds were prepared as a concentrated solution (1000 x), stored at −20 °C, and diluted into ACSF to the appropriate concentrations immediately before bath application.

Whole-Cell Patch Clamp Recording

Whole cell patch-clamp recordings were performed as described previously (Jin et al., 2006; Jin and Smith, 2007; Jin et al., 2011a). During the recording, the slice was maintained fully submerged in a recording chamber which was perfused with oxygenated ACSF (~3ml/min). The ACSF was heated to 32~35C° by an in-line heater (Warner Instruments, Hamden, CT), and the temperature was monitored by a thermistor placed in the recording chamber. GP neurons were visualized with a IR-differential interference contrast microscopy (BX51Wl) using a 40X water immersion objective (Olympus, Pittsburgh, PA). Electrodes were pulled from borosilicate glass on a vertical patch pipette puller (Narishige, Tokyo, Japan) to have resistance in the range of 3.5–5 MΩ when filled with the internal solution containing (in mM): 140 CsCl, 2 $MgCl_2$, 1 CaCl, 10 HEPES, 10 EGTA, 2 Mg_2ATP, 0.3 GTP (pH 7.4; 300–310 (mOsm). QX314 was included in the pipette solution to block action potential generation and postsynaptic $GABA_B$ responses (Nathan *et al.*, 1990; Andrade 1991; McLean *et al.*, 1996).

Tight-seal (>1GΩ) whole-cell recording was obtained from the cell bodies of GP neurons. The series resistances (20~30 MΩ) were regularly monitored during recording, and cells were rejected if the resistance changed by 20% or more. Neurons were voltage-clamped at a holding potential of −60 mV and whole-cell membrane currents were recorded with a Patch-Clamp PC-501A (Warner Instruments, Hamden, CT). In some experiments, the input resistance of GP neurons was determined during the SKF 89976A or SNAP 5114 application, which was indicated by changing the holding current during a −10 mV step from the command voltage −60 mV (Jin *et al.*, 2006). To isolate ionotropic glutamate receptor-mediated EPSCs, 10 μM gabazine was added to the ACSF. The mEPSCs were recorded in the presence of gabazine and 1 μM of tetrodotoxin (TTX). Data were collected continuously for 10–20 min.

Electrical stimulation

A bipolar matrix stimulating electrode (FHC, Bowdoinham, ME) was placed in the GP. Excitatory postsynaptic currents (EPSCs) in GP neurons were synaptically evoked by stimulation in the GP with single pulses (150–200 μs) that ranged from 7–10 volts delivered

once every 20 sec. The baseline holding current was determined from the averaged holding current of three 200-ms epochs free of EPSCs. The paired-pulse ratio (PPR) of evoked EPSCs was performed as follows: two stimuli of the GP were paired with an interstimulus interval of 40–50 ms. The ratio of peak 2/peak 1 was calculated.

Data analysis

The signals were low-pass filtered at 5 kHz, digitized with a Digidata 1322A and analyzed off-line using pClamp 9 (Molecular Devices, Unioon City, CA). Three or six EPSCs were averaged, and their peak amplitude was measured before, during and after SKF 89976A or SNAP 5114. The mEPSCs were detected and analyzed using the Mini Analysis software (Synaptosoft, Fort Lee, NJ). Cumulative probability distributions were compared with Kolmogorov-Smirnov tests. All group data were expressed as means ± SEM. The significance of differences between groups was assessed with Student's *t*-test.

RESULTS

Activation of presynaptic $GABA_B$ receptors reduces glutamatergic transmission in rat GP

Bath application of baclofen (10 μM) reversibly reduced the EPSC amplitude (61 ± 7% of control; $P < 0.01$) without any significant effect upon the input resistance (95% ± 7 of control; $P > 0.05$) and base line holding currents (111 ± 6% of control; $P > 0.05$) in a total of 7 GP cells tested when perfused with 10 μM baclofen (Fig. 1A,B). The baclofen-induced inhibition of EPSCs was blocked in the presence of CGP55845 (3 μM), a $GABA_B$ receptor antagonist (102 ± 11% of control, $P > 0.05$) (Fig. 1A,B).

We then conducted two sets of additional experiments to determine if the effect of baclofen on eEPSC amplitude was due to presynaptic $GABA_B$ activation. First, we studied the effect of baclofen on PPR of eEPSCs. To record paired EPSCs, two local GP stimuli were paired with an interstimulus interval of 40 ms (Fig. 1C). The ratio of peak 2/peak 1 in the presence or absence of baclofen was then calculated, and found to be significantly increased in the presence of baclofen compared with control (1.45 ± 0.13 and 1.05 ± 0.1, respectively, $P < 0.01$, $n = 7$) (Fig. 1D). Next, we tested the effect of baclofen on mEPSCs in the presence of TTX (Fig. 1E). The mEPSCs frequency was significantly reduced (Fig. 1F and H), but the amplitude was not significantly affected in the presence of baclofen (59 ± 8%, $P < 0.01$ and 92 ± 7%, $P > 0.05$ of control, respectively, $n = 6$) (Fig. 1G and H). Together, these results further demonstrate that activation of presynaptic $GABA_B$ receptors in glutamatergic terminals reduce glutamatergic synaptic transmission in the rat GP.

Blockade of GAT-1 or GAT-3 inhibits eEPSCs

Presynaptic $GABA_B$ receptor activation in glutamatergic terminals can be induced following GAT-1 blockade in the cerebellum (Mitchell & Silver, 2000) and hippocampus (Isaacson & Nicoll, 1993). A previous in vivo study from our laboratory suggested pre-synaptic $GABA_B$ heteroreceptor-mediated inhibition of pallidal neurons in monkeys (Galvan *et al.*, 2005). To further address the potential synaptic mechanisms underlying this physiological response, we examined the effects of SKF 89976A or SNAP 5114 on the amplitude of eEPSCs in GP neurons. To evoke glutamate and GABA release in GP, a bipolar stimulating electrode was placed into the GP ~200–300 μm away from the recorded reurons. EPSCs were evoked every 20 sec in GP neurons at a holding pontential of −60 mV in presence of gabazine (10 μM). As shown in Fig. 2A, bath application of SKF 89976A (10 μM) reversibly decreased EPSC amplitude. On average, the EPSC amplitude was 64.8 ± 7.8% ($n = 8$, $P < 0.001$) of control (Fig. 2B). We did not detect any significant effect of SKF 89976A on input resistance and baseline holding currents (Fig. 2B) suggesting that SKF 89976A-induced inhibition of EPSC amplitude was not due to changes in postsynaptic membrane properties.

A similar result was also found following bath application of SNAP 5114 (Fig. 2C). In that case, the EPSC amplitude was 70 ± 7.3% (n = 6, P < 0.01) of control (Fig. 2D), while neither the input resistance (95.3 ± 7.8% of control; P > 0.05) and baseline holding current (90 ± 6.4% of control; P > 0.05) were significantly affected (Fig. 2D).

As expected, the EPSC amplitude was further reduced when both SKF 89976A and SNAP 5114 were applied together (Fig. 3A,B). In six neurons, the EPSC amplitude was reduced to 45 ± 6.2% (n = 6, P < 0.01) of control following the combined application of both GAT blockers, which was significantly more pronounced than the effects induced by the application of individual GAT-1 or GAT-3 blocker (64.8 ± 7.8% and 70 ± 7.3%, respectively). Together, these results provide evidence that GAT-1 and GAT-3 blockade synergistically regulates glutamatergic transmission in the rat GP.

GAT-1- or GAT-3-mediated depression of glutamatergic transmission in GP involves presynaptic mechanisms

The effect of SKF 89976A and SNAP 5114 on the amplitude of eEPSCs was examined under the condition that $GABA_A$ and postsynaptic $GABA_B$ receptors were blocked by bath application of gabazine and QX314 in the recording electrode (Nathan *et al.*, 1990; Andrade, 1991; McLean *et al.*, 1996; Kaneda & Kita, 2005). The lack of significant effects of both GAT blockers on cell's input resistance and baseline holding current under these conditions suggests a presynaptic GABAB receptor-mediated mode of action. To test this hypothesis, we examined the effect of SKF 89976A and SNAP 5114 on PPR of evoked EPSCs, and found that the ratio of peak 2/peak 1 was significantly increased in the presence of SKF 89976A or SNAP 5114 compared with controls (Fig. 4A–C), thereby suggesting a presynaptic effect.

To further address this issue, we also examined the effect of GAT-1 or GAT-3 blockade on the frequency and amplitude of mEPSCs recorded from GP neurons in the presence of 1 μM TTX and 10 μM gabazine. As shown in Fig. 4D, bath application of 10 μM SKF 89976A reduced the frequency, but had no significant effect on the amplitude of mEPSCs. The inter-mEPSC intervals were significantly increased following 10 μM SKF 89976A application (P < 0.01, Kolmogorov-Smirnov test) (Fig. 4E). On the other hand, SKF 89976A had no significant effect on the mEPSC amplitude (Fig. 4F). In seven cells tested, the mEPSC frequency was reduced to 62 ± 8% (P < 0.01) of control, while the mEPSC amplitude was not significantly affected (94 ± 8% of control; P > 0.05) (Fig. 4G) by SKF 89976A application. Similarly, blockade of GAT-3 with SNAP 5114 also decreased mEPSC frequency (66 ± 7% of control, P < 0.01; n = 5), without any significant effect upon the amplitude of mEPSC (102 ± 6% of control, P > 0.05; n = 5) (Fig. 4H–K). We confirmed that mEPSCs were α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPA)/*N*-methyl-D-aspartate (NMDA) receptor-mediated events, because they were completely blocked by 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) plus D-(−)-2-amino-5-phosphonopentanoic acid (DAP-5) (data not shown). Taken together, these data strongly support the hypothesis that SKF 89976A and SNAP 5114-induced inhibition of glutamatergic transmission in GP is mediated presynaptically.

GAT-1 or GAT-3 blockade mediates presynaptic depression of glutamatergic transmission in GP through activation of presynaptic $GABA_B$ receptors

To demonstrate that the presynaptic inhibitory effects of SKF 89976A or SNAP 5114 on glutamatergic transmission are mediated through $GABA_B$ receptor activation, we repeated the experiments described above in the absence or presence of CGP 55845 (3 μM), a selective $GABA_B$ receptor antagonist. As shown in Fig. 5A and B, we found no significant

effect of SKF 89976A plus SNAP 5114 on eEPSC amplitude in the presence of CGP 55845 (90.7 ± 6.8 of control, n = 7, P > 0.05).

Furthermore, there was also no significant effect of SKF 89976A or SNAP 5114 on the PPR of evoked EPSCs and on the frequency of mEPSCs in the presence of CGP 55845. The ratio of peak 2/peak 1 was 1.04 ± 0.14 in control and 1.01 ± 0.16 in the presence of CGP 55645 together with SKF 89976A and SNAP 5114 (P > 0.05, n = 7) (Fig. 6A,B), while the mEPSC frequency was 93 ± 9% (P > 0.05, n = 6) and 94 ± 8% of control (P > 0.05, n = 6) in the presence of SKF 89976A/CGP 55845 or SNAP 5114/CGP 5114, recpectively (Fig. 6G, H). Together, these results provide evidence that GAT-1 and GAT-3 blockade-induced inhibition of glutamatergic transmission in the rat GP is mediated through presynaptic $GABA_B$ receptor activation.

DISCUSSION

The findings presented in this study provide evidence that GAT-1- and GAT-3-mediated regulation of extracellular GABA can modulate glutamatergic transmission at the subthalamopallidal synapse in the rat GP, presumably through presynaptic $GABA_B$ heteroreceptors activation in STN terminals (Fig. 7). Together with our recent data showing that GAT-1 and GAT-3 also regulate GABAergic transmission in the rat GP (Jin *et al.*, 2011), these findings suggest that GATs modulation could play a critical role in maintaining homeostasis of GABAergic and glutamatergic transmission in the GP. Our data also raise the possibility that GATs activity regulation may have some therapeutic benefits in disorders such as Parkinson's disease, which relie on an imbalanced transmission at key GABAergic and glutamatergic synapses along the basal ganglia network.

Presynaptic $GABA_B$ receptor activation modulates glutamatergic transmission in GP

Presynaptic $GABA_B$ receptors activation inhibits glutamatergic transmission in the hippocampus (Isaacson *et al.*, 1993; Guetg *et al.*, 2009), cerebral cortex (Porter & Nieves, 2004), lateral geniculate nucleus (LGN) (Chen & Regehr, 2003), and cerebellar glomerulus (Mitchell & Silver, 2000). Similar results were also reported in various basal ganglia nuclei including the striatum (Nisenbaum *et al.*, 1992; 1993), the globus pallidus (Chen *et al.*, 2002), the subthalamic nucleus (STN) (Shen & Johnson, 2001), and the substantia nigra pars reticulata (SNR) (Shen & Johnson, 1997). In line with these observations, our data show that baclofen-induced inhibition of glutamatergic transmission in the rat GP is associated with a significant increase in PPR of evoked EPSC, and a decrease in the frequency, but not the amplitude, of mEPSCs. Our results are also consistent with previous electron micoscopic immunocytochemical data showing that $GABA_B$ receptors are expressed in glutamatergic axon terminals in the rat GP (Chen *et al.*, 2004). Because $GABA_B$ receptors immunoreactivity is also expressed at postsynaptic sites in GP neurons (Chen *et al.*, 2004), it was important for us to use pipettes filled with QX 314 to block these postsynaptic $GABA_B$ receptors (Nathan *et al.*, 1990; Andrade, 1990; McLean *et al.*, 1996; Kaneda & Kita, 2005), to rule out the possibility that any of the $GABA_B$ receptor-mediated effects on glutamatergic transmission could have been mediated through postsynaptic mechanisms. The stability of the baseline holding currents and input resistance of GP neurons in the presence of baclofen are additional evidence for the lack of postsynaptic mechanisms in mediating these effects.

Most previous studies used the $GABA_B$ receptor agonist baclofen to study presynaptic $GABA_B$ receptor-mediated effects on glutamatergic transmission in the CNS (Shen & Johnson, 1997; Shen & Johnson, 2001; Chen & Regehr, 2003; Porter & Nieves, 2004; Guetg *et al.*, 2009). Only a few studies have demonstrated that synaptically released GABA can activate $GABA_B$ receptors in glutamatergic terminals. For instance, repetitive stimulation of the stratum radiatum and dentate gyrus can induce presynaptic $GABA_B$ receptor-mediated

inhibition of glutamatergic transmission at the CA1 and CA3 pyramidal cell synapses, respectively (Isaacson *et al.*, 1993; Vogt & Nicoll, 1999), while presynaptic $GABA_B$ receptors in glutamatergic terminals can be activated by tonically released GABA in the cerebellar glomerulus (Mitchell & Silver, 2000). However, the physiology and sources of activation of presynaptic $GABA_B$ heteroreceptors in the GP remain poorly characterized. One study in GP slices showed that bath application of $GABA_B$ receptor antagonist increased the frequency of mEPSCs, suggesting that $GABA_B$ receptors in glutamatergic terminals can be activated by tonically released GABA (Chen *et al.*, 2002). However, other slice preparation studies failed to confirm this result (Hanson & Jaeger, 2002; Kaneda & Kita, 2005).

GAT-1 blockade-induced depression of glutamate release has been demonstrated in the cerebellar glomerulus (Mitchell & Silver, 2000) and hippocampus (Isaacson *et al.*, 1993). In these experiments, bath application of the GAT-1 blockers, NO711 or SKF89976A significantly reduced the amplitude of eEPSCs or field EPSPs, and this effect was blocked by $GABA_B$ receptor antagonist. Consistent with these results, our data demonstrated that bath application of SKF 89976A induced a $GABA_B$ receptor-mediated reduction of eEPSCs amplitude in the rat GP. We further demonstrated that this effect was mediated through presynaptic $GABA_B$ heteroreceptors, thereby indicating that the spillover of GABA induced by GAT-1 blockade is sufficient to activate presynaptic $GABA_B$ receptors on the surface of glutamatergic terminals in the GP. Thus, GAT-1-induced inhibition of glutamatergic transmission in the GP is mediated through presynaptic $GABA_B$ receptor activation.

In contrast to GAT-1, the role of GAT-3 in the presynaptic regulation of GABAergic and glutamatergic synaptic transmission is not clear, although the importance of GAT-3 in GABA reuptake, most particularly during intense neuronal activity, is well documented in various CNS regions (Keros *et al.*, 2005; Kirmse *et al.*, 2009). One study has reported that the sole application of the GAT-3 blocker, SNAP 5114, increases GABAergic transmission in the rat neocortex (Kinney, 2005). In primates, *in vivo* data from our laboratory have demonstrated that application of SNAP 5114 or SKF 89976A have similar stimulatory effects on GABA levels, and comparable inhibitory effects on the firing rate of pallidal neurons (Galvan *et al.*, 2005). Knowing that GAT-1 and GAT-3 are largely expressed in different neuronal and glial compartments in the rat and monkey pallidum, respectively (Wang & Ong, 1999; Ng *et al.*, 2000; Galvan *et al.*, 2005; Jin et *al.*, 2011a), combined with evidence from our study that the cocktail of GAT-1/GAT-3 blockers has a significantly stronger inhibitory effect upon eEPSCs than individual transporter blockade alone, suggest that GAT-1 and GAT-3 partly function through different mechanisms to regulate extracellular GABA levels, and modulate GP neuronal activity. The extracellular level of GABA can be increased by up to threefold in the rat GP after GAT-1 blockade (Fink-Jensen et al., 1992). Although diffusion and spillover of synaptically released GABA is likley to be the main source of the GABA overflow, non-vesicular glial GABA release mechanisms may also involved, as shown in other brain regions (for reviews, see Semyanov et al., 2004; Koch & Magnusson, 2009) (Fig. 7B). Taking into consideration the fact that presynaptic $GABA_B$ receptors are highly sensitive to GABA (Jones *et al.*, 1998; for reviews, see Bowery, 2002; Pinard *et al.*, 2010), they become a priviledged target for raises in extracellular GABA concentrations. Thus, our findings demonstrate that the reuptake of GABA through both SNAP 5114 and SKF 89976A plays a powerful control over $GABA_B$ receptor-mediated presynaptic inhibitory effects upon glutamatergic synaptic transmission in the rat GP.

Functional implications

Overactivity of the glutamatergic subthalamopallidal system is a cardinal pathophysiological feature of Parkinson's disease (Albin *et al.*, 1989; Bergman *et al.*, 1990). Thus, the development of pharmacological or surgical procedures aimed at reducing STN activity

have been at the forefront of Parkinson's disease therapeutics for the past two decades (for recent review, see Smith *et al.*, 2012). The results presented in our study provide strong evidence that the modulation of GABA reuptake through blockade of GAT-1 and GAT-3 can presynaptically reduce glutamatergic synaptic transmission from the STN via $GABA_B$ receptor activation. In addition, our data provide additional evidence for the physiological, and possibly therapeutic, relevance of GABA reuptake regulation in the GP to activate presynaptic $GABA_B$ heteroreceptors. Taking into consideration that $GABA_B$ receptors activation has some therapeutic relevance in various CNS disorders, combined with the fact that they play a crical role in regulating the intrinsic pattern of neuronal activity in the basal ganglia circuitry (Kaneda & Kita, 2005; Bevan *et al.*, 2007), our findings suggest that the fine tuned modulation of $GABA_B$ receptors activation through the regulation of GAT-mediated GABA spillover offers some therapeutic relevance as potential antiparkinsonian therapy. This promising strategy is further supported by the recent evidence for indirect modulation of GATs activity by A2A adenosine and CB1 cannabinoid receptors, two basal ganglia-enriched G-protein-coupled receptors with known antiparkinsonian effects (Kanda et al., 2000; Romero et al; 2002; Brotchie, 2003; Chase et al., 2003; Venderova *et al.*, 2005; Gonzalez *et al.*, 2006; Jin *et al.*, 2011a,b).

Acknowledgments

This research was supported by NIH grants RO1 NS 043293, the Emory UDALL Center of Excellence for Parkinson's Disease (P50 NS071669), and the Yerkes National Primate Research Center NIH/NCCR base grant (RR-00165).

ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
BG	basal ganglia
D-AP5	D-(−)-2-amino-5-phosphonopentanoic acid
eEPSCs	evoked excitatory post synaptic currents
GABA	γ-aminobutyric acid
GATs	GABA transporters
GAT-1	GABA transporter subtype 1
GAT-3	GABA transporter subtype 3
GP	globus pallidus
IPSP	inhibitory postsynaptic potential
mEPSCs	miniature excitatory postsynaptic currents
NMDA	*N*-methyl-D-aspartate
PD	Parkinson's disease
PPR	paired-pulse ratio
QX314	N-(2,6-Dimethylphenylcarbamoylmethyl)-triethylammonium bromide
SNc	substantia nigra parts compacta
SKF 89976A	1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride

SNAP 5114	1-[2-[tris(4-methoxyphenyl)methoxy]ethyl]-(S)-3-piperidinecarboxylic acid
SNR	substantia nigra pars reticulata
STN	subthalamic nucleus
Str	striatum
TTX	tetrodotoxin

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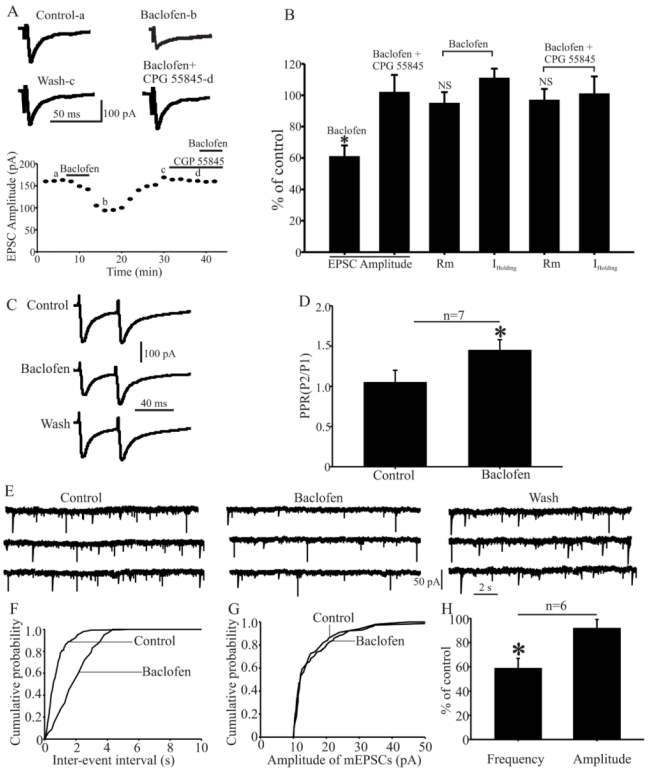

FIG. 1. Presynaptic effects of baclofen on evoked glutamatergic synaptic transmission in the rat GP

(A) Time course of the effect of baclofen and baclofen plus CGP 55845 on AMPA/KA and NMDA-mediated excitatory postsynaptic current (EPSC) amplitude (pA) in the presence of 10 μM gabazine. Six EPSCs are averaged in each trace at the time indicated by the corresponding letters in the graph. (B) Summary bar praph showing the effect of baclofen alone and baclofen together with CGP 55845 on the EPSC amplitude, cell's input resistance, and base line holding current, as percent of control ± SEM. *: indicates significance, $P <$ 0.01. NS: not significant. (C) Paired EPSCs were recorded in control condition (top trace), in the presence of baclofen (middle trace), and after washout of baclofen (lower trace). (D) Bar graph summarizing the PPR expressed as a mean ratio of P2/P1 ± SEM, in the absence or presence of balofen. (E) Sample traces showing mEPSCs recorded in GP neurons under control condition (left), during bath application of baclofen (10 μM) (middle) and after the wash out of baclofen (right). These mEPSCs were recorded in the presence of 10 μM gabazine and 1 μM TTX. (F–G) The cumulative distributions of the amplitude, inter-event interval of mEPSCs obtained from the same neuron as in panel E. Baclofen has significant effect ($p < 0.01$) on the interevent interval (left), but not the amplitude (middle), distribution curves of mEPSCs. (H) A summary bar graph shows that baclofen significantly reduced the frequency, but not the amplitude, of mEPSCs. * $P < 0.01$. In this and following figures, ns indicates non-significant difference; 'n' indicates the number of cells recorded.

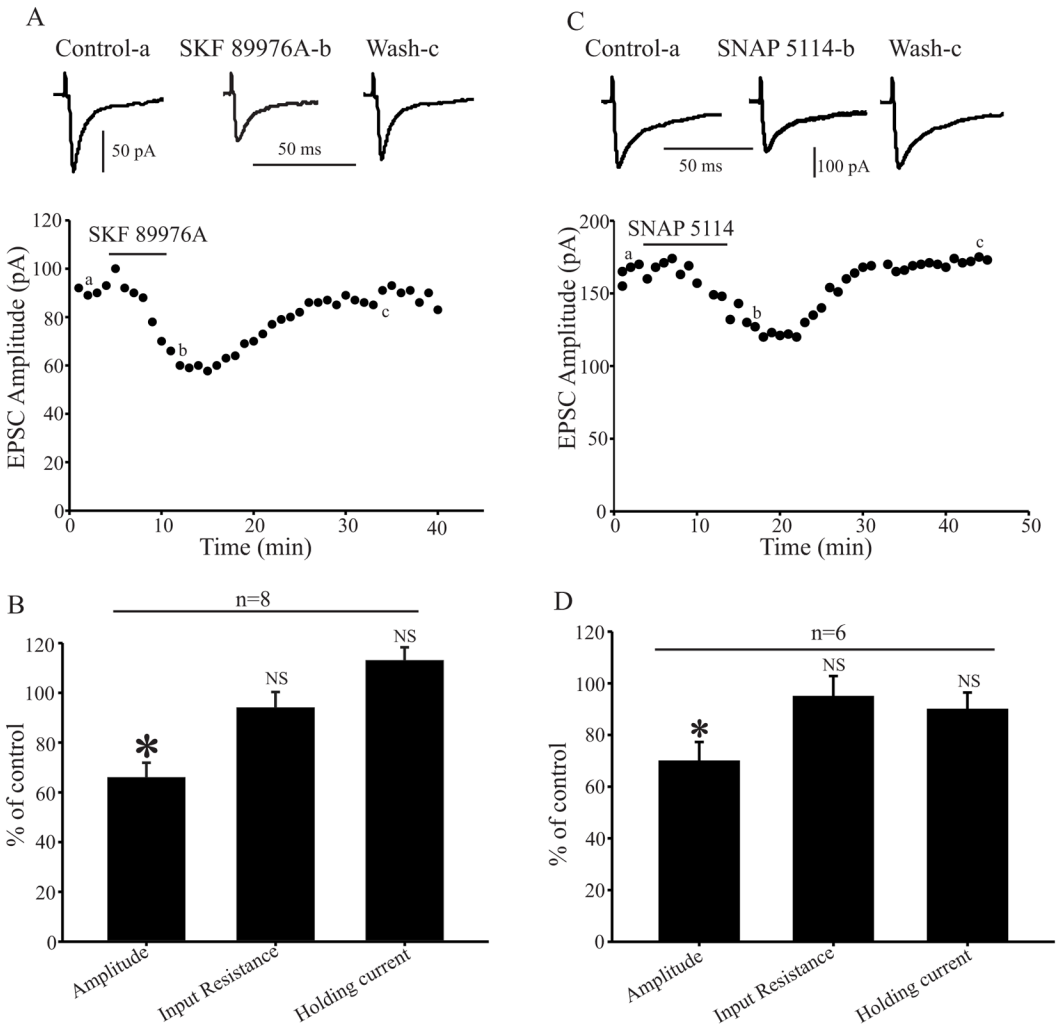

FIG. 2. Effects of GAT-1 or GAT-3 blocker on eEPSCs in rat GP neurons

(A) Time course of the effect of SKF 89976A on eEPSC amplitude in the presence of 10 μM gabazine. Three EPSCs are averaged in each trace at the time indicated by the corresponding letters in the graph. (B) A summary bar graph shows that SKF 89976A significantly reduced the amplitude without affecting on cells' input resistance and base line holding current. * $P < 0.001$. (C) Time course of the effect of SNAP 5114 on eEPSC amplitude in the presence of 10 μM gabazine. Three EPSCs are averaged in each trace at the time indicated by the corresponding letters in the graph. (D) A summary bar graph shows that SNAP 5114 significantly reduced the eEPSC amplitude. * $P < 0.01$.

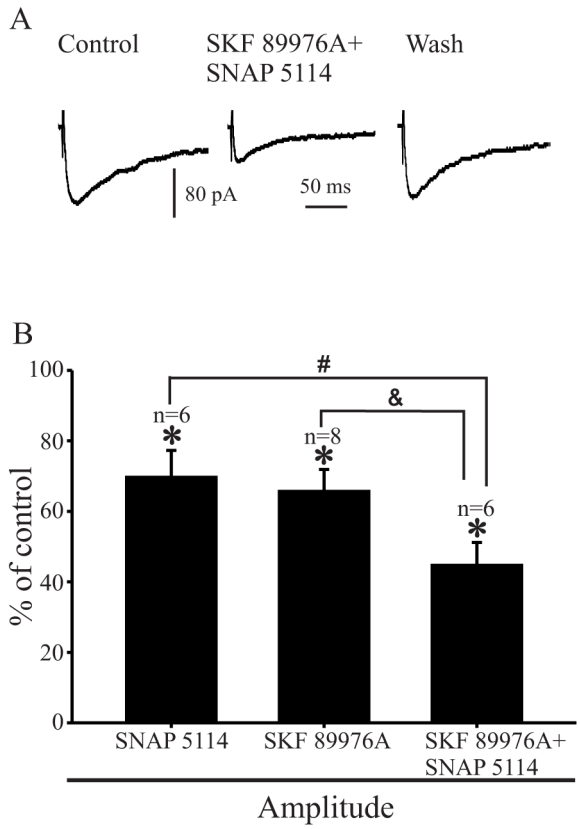

FIG. 3.

Effects of GAT-1 and GAT-3 blockade on eEPSCs in rat GP neurons. (A) Sample traces showing eEPSCs recorded in GP neurons under control condition (left), or during bath application of SKF 89976A together with SNAP 5114 (middle). (B) A summary bar graph shows that SKF 89976A together with SNAP 5114 significantly reduced the eEPSC amplitude expressed as percentage of control ± SEM (* $P < 0.01$). For comparison, the effect of SNAP 5114 (from Fig. 2D) and SKF 89976A from (Fig. 2B) on eEPSCs is shown. Application of SNAP 5114 together with SKF 89976A had a significantly more pronounced effect than application of SNAP 5114 (# $P < 0.01$) or SKF 89976A alone (& $P < 0.01$) on eEPSC amplitude.

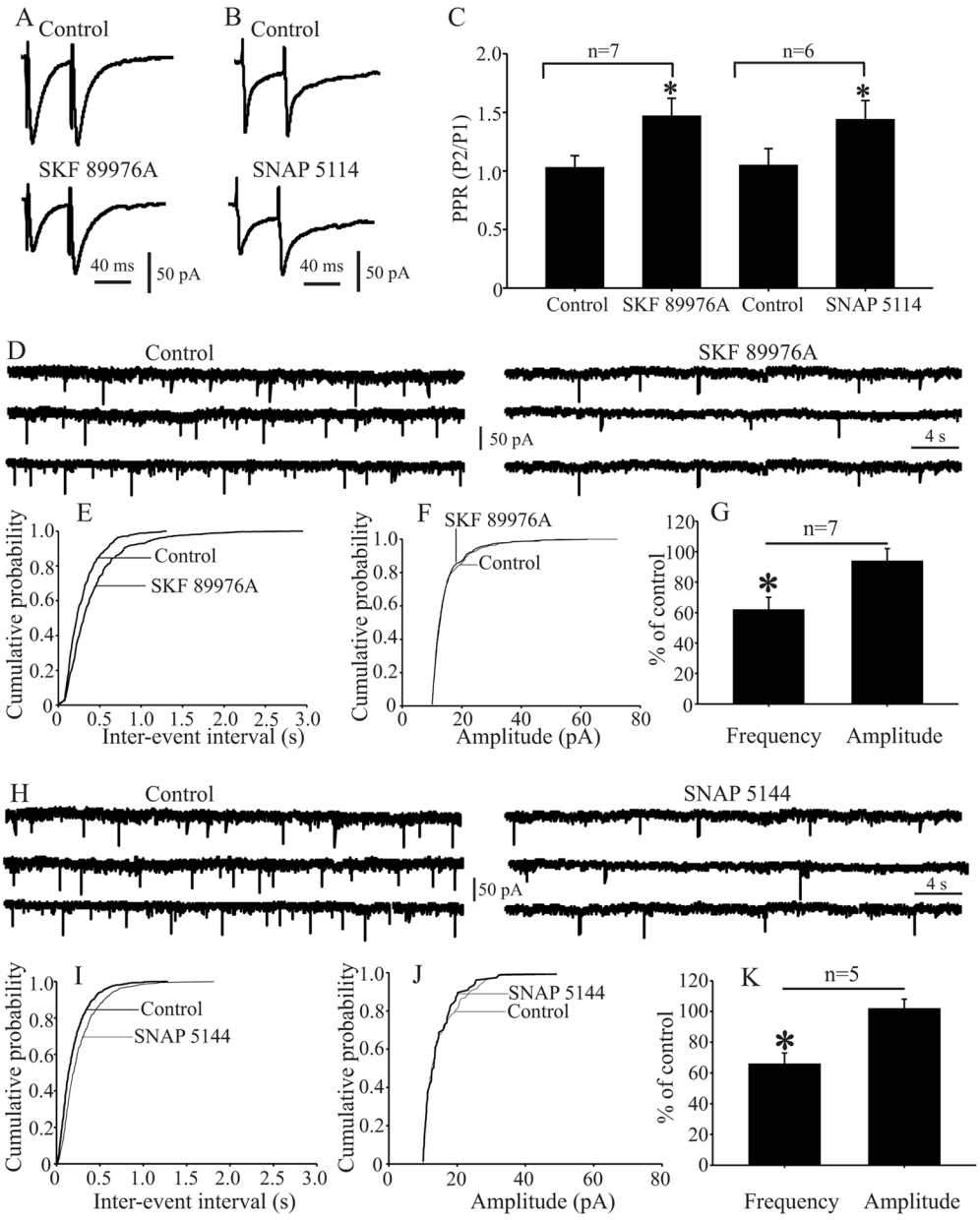

FIG. 4. Presynaptic effects of GAT-1 or GAT-3 blockade on glutamatergic synaptic transmission in the GP

(A, B) Paired EPSCs were recorded before (top) and during applications of SKF 89976A (bottom left) or SNAP 5114 (bottom right). (C) Bar graph summarizing the PPR expressed as a mean ratio of P2/P1 ± SEM, in the absence or presence of SKF 89976A or SNAP 5114. * $P < 0.01$. (D) Sample traces show mEPSCs recorded before and during 10 μM SKF 89976A application. These mEPSCs were recorded in the presence of 10 μM gabazine and 1 μM TTX. (E, F) The cumulative distribution of the inter-mEPSC interval and amplitude of mEPSCs obtained from the same neuron as in panel A. SKF 89976A significantly shifted the inter-event interval distribution curve to the right (left, $P < 0.01$), but had no significant effect on the distribution of mEPSCs amplitude (middle, $P > 0.05$). (G) A summary bar graph shows that SKF 89976A significantly reduces the frequency, but not the amplitude of

mEPSCs. * $P < 0.01$. (H) Sample traces show mEPSCs before and during 10 μM SNAP 5114 application. (I, J) The cumulative distributions of the inter-mEPSC interval and amplitude of mEPSCs obtained from the same neuron as in panel H. SNAP 5114 significantly shifted the inter-event interval distribution curve to the right, but had no significant effect on the distribution of mEPSCs amplitude. (K) A summary bar graph shows that SNAP 5114 significantly reduces the frequency, but not the amplitude, of mEPSCs. * $P < 0.01$.

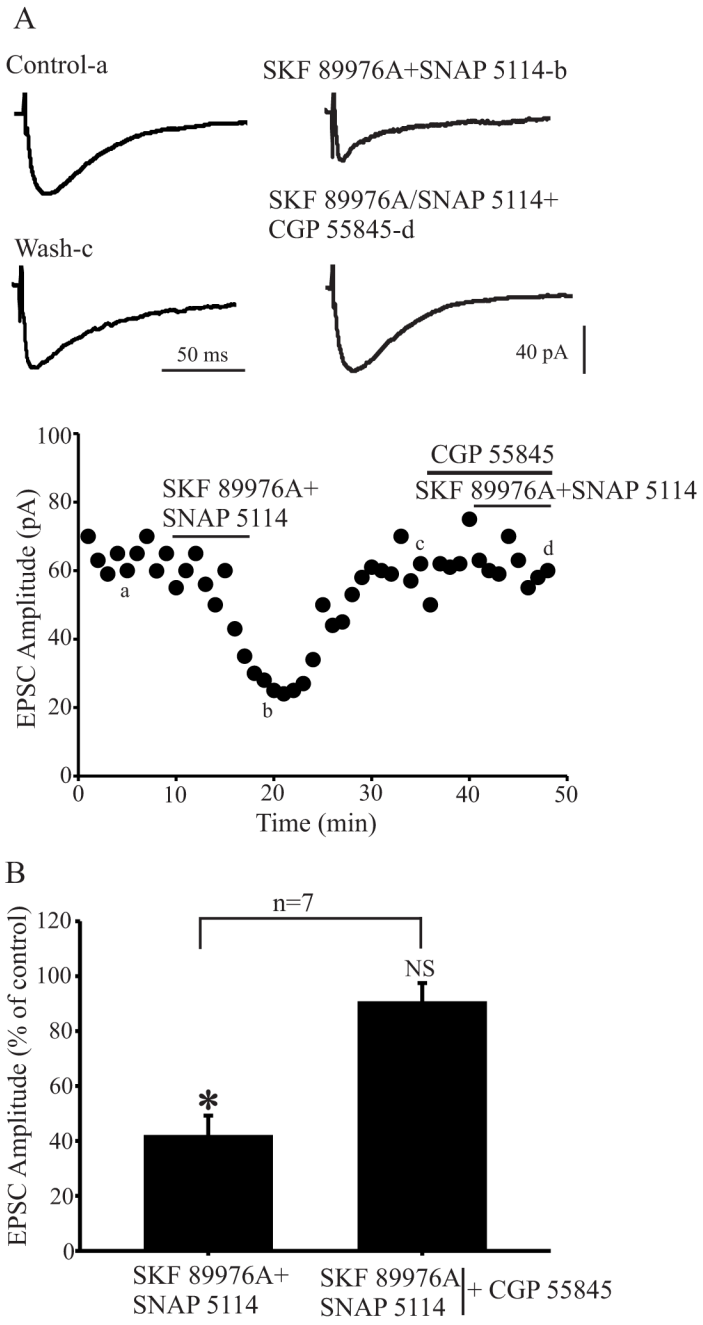

FIG. 5. GAT-1 and GAT-3 blockade-induced inhibition of eEPSCs is abolished by CGP 55845

(A) Time course of the effect of SKF 89976A/SNAP 5114 on eEPSCs in absence or presence of 3 μM CGP 55845. Three EPSCs are averaged in each trace at the time indicated by the corresponding letters in the graph. (B) Summary bar praph showing the effect of SKF 89976A/SNAP 5114 alone, or together with CGP 55845 on eEPSC amplitude as precent of control ± SEM. There was a significant difference from control: * $P < 0.01$. NS, not significant from control, $P > 0.05$.

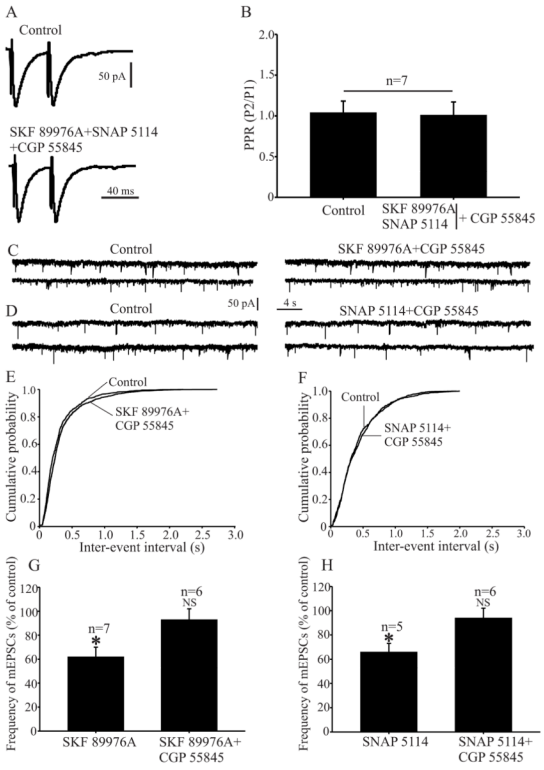

FIG. 6.

GAT-1 and GAT-3 blockade-induced presynaptic inhibition of glutamatergic transmission is abolished by CGP 55845. (A) Sample traces show paired-eEPSCs recorded in control and during 10 μM SKF 89976A/SNAP 5114 together with CGP 55845. (B) Bar graph summarizing the PPR expressed as a mean ratio of P2/P1 ± SEM, in the absence or presence of SKF 89976A/SNAP 5114 combined with CGP 55845 application. (C, D) Sample traces show mEPSCs recorded before and during SKF 89976A/CGP 55845 and SNAP 5114/CGP 55845 application, respectively. (E, F) The cumulative distribution of the inter-mEPSC interval of mEPSCs obtained from the neuron shown in panels C and D, repectively. SKF 89976A/CGP 55845 and SNAP 5114/CGP 55845 did not significantly alter the inter-event interval distribution curves. (G, H) Summary bar graphs show that SKF 89976A and SNAP 5114-induced inhibition of mEPSCs frequency was blocked by CGP 55845. For comparison, the effects of 10 μM SKF 89976A or SNAP 5114 in absence of CGP 55845 on mEPSCs (from Fig. 4G, K) are shown. There were no significant difference of GATs blockade on mEPSCs frequency in the presence of CGP 55845 as compared with control. ns, not significant.

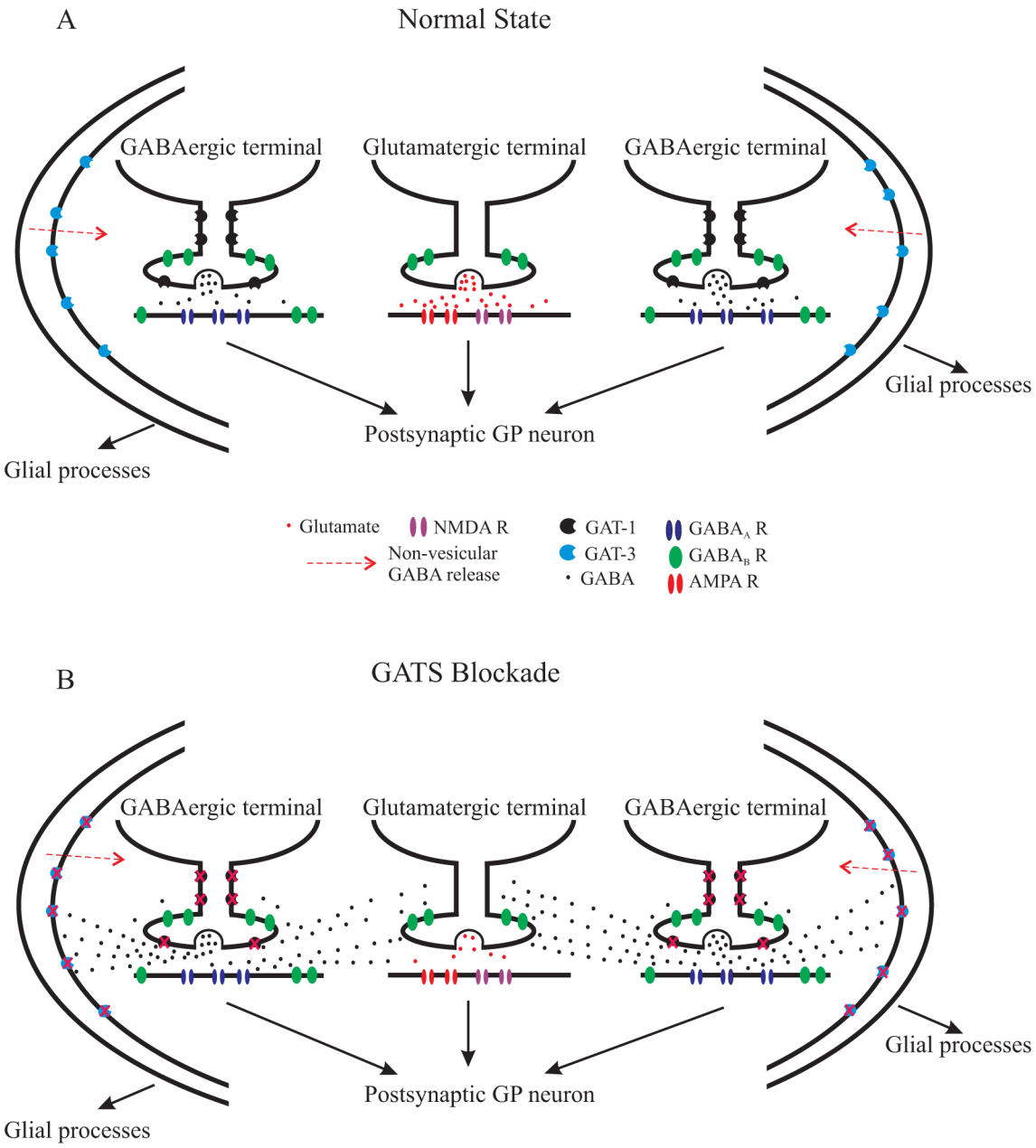

FIG. 7.

Summary diagram showing the location of GAT-1, GAT-3 and $GABA_B$ receptors, and their relative functions at GABAergic and glutamatergic synapses in the GP under normal (A) and GATs blockade (B) conditions. GAT-1 is mainly expressed in axons and GABAergic terminals, whereas GAT-3 is almost exclusively found in glial processes. GAT-3-labeled glial elements are often wrapped around axo-dendritic synaptic complexes in the GP. $GABA_B$ receptors are expressed presynaptically in both GABAergic and glutamatergic terminals, and at postsynaptic sites on the surface of GP neurons. (A) Under normal condition, synaptically released GABA does not diffuse or spill over much oustide the synaptic cleft due to the active GATs-mediated GABA reuptake, allowing for actication of postsynaptic GABA-A receptors in the active zone, but limited extrasynaptic GABAB-mediated effects. (B) Following GAT-1 or GAT-3 blockade, synaptically released GABA

can diffuse away from GABAergic synapses, to activate distant presynaptic GABAB heteroreceptors and reduce glutamate release in the GP. When both GAT-1 and GAT-3 are blocked, a larger number of presynaptic GABAB heteroreceptors are activated, which leads to further inhibition of glutamate release in the GP. The possibility for non-vesicular glial release of GABA is also indicated (red arrows), although its contribution to changes in extracellular GABA in the GP is unknown.