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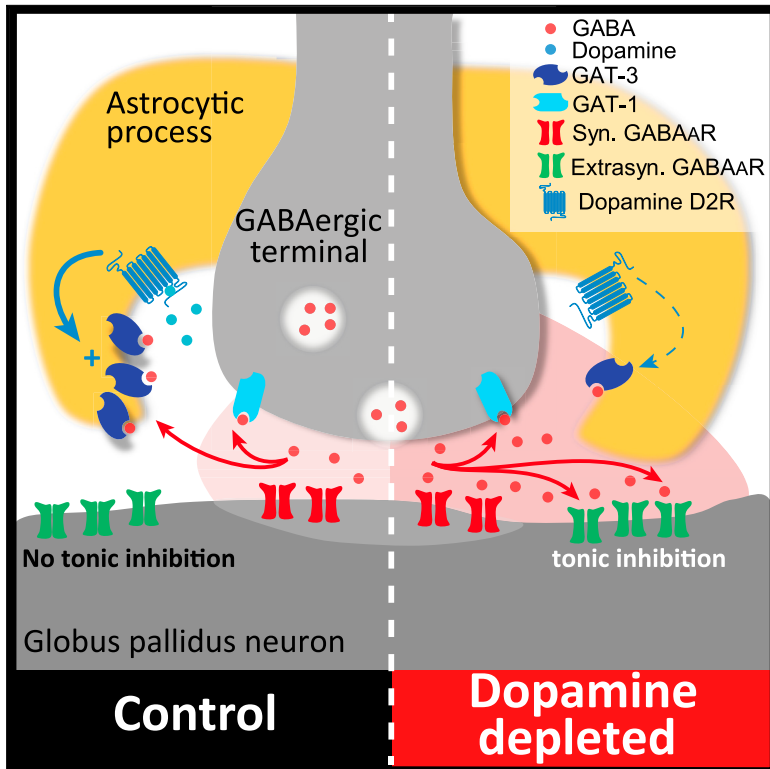
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GAT-3 Dysfunction Generates Tonic Inhibition in External Globus Pallidus Neurons in Parkinsonian Rodents

Graphical Abstract



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In Brief

The globus pallidus (GP) is a key basal ganglia nucleus involved in motor control. In Parkinson's disease, the cellular mechanisms underlying GP neuron hypoactivity are poorly understood. Chazalon et al. find that glial GABA transporters are downregulated in parkinsonian rodents, leading to aberrant GABAergic inhibition in the GP and motor coordination impairment.

Highlights

- Glial transporters (GAT-3) regulate extracellular levels of GABA in the GP
- Astrocytic D2 dopamine receptors regulate GAT-3 activity
- GAT-3 down-expression in GP astrocytes favors tonic GABAergic inhibition in PD
- GAT-3 down-expression may contribute to abnormal motor coordination in PD



GAT-3 Dysfunction Generates Tonic Inhibition in External Globus Pallidus Neurons in Parkinsonian Rodents

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SUMMARY

The external globus pallidus (GP) is a key GABAergic hub in the basal ganglia (BG) circuitry, a neuronal network involved in motor control. In Parkinson's disease (PD), the rate and pattern of activity of GP neurons are profoundly altered and contribute to the motor symptoms of the disease. In rodent models of PD, the striato-pallidal pathway is hyperactive, and extracellular GABA concentrations are abnormally elevated in the GP, supporting the hypothesis of an alteration of neuronal and/or glial clearance of GABA. Here, we discovered the existence of persistent GABAergic tonic inhibition in GP neurons of dopamine-depleted (DD) rodent models. We showed that glial GAT-3 transporters are down-regulated while neuronal GAT-1 function remains normal in DD rodents. Finally, we showed that blocking GAT-3 activity *in vivo* alters the motor coordination of control rodents, suggesting that GABAergic tonic inhibition in the GP contributes to the pathophysiology of PD.

INTRODUCTION

In the CNS, two forms of GABAergic inhibition coexist to control the excitability of neuronal networks (Farrant and Nusser, 2005). Fast, phasic synaptic transmission precisely regulates action potential generation and synchronized activity among neurons (Baufreton et al., 2005, 2009; Cobb et al., 1995), while persistent tonic inhibition sets the global level of excitability of neurons (Semyanov et al., 2004). Both phasic and tonic inhibition transmission are precisely controlled by GABA transporters (GATs), whose main functions are to rapidly remove GABA from the

synaptic cleft, prevent GABA spillover to neighboring synapses (Overstreet and Westbrook, 2003), and limit excessive activation of synaptic and extrasynaptic GABA receptors (Brickley and Mody, 2012). Three subtypes of GATs (GAT1–3) have been identified among the neurotransmitter: sodium symporter family (Scimemi, 2014b), with GAT-1 and GAT-3 expressed widely throughout the brain (Ikegaki et al., 1994). Dysfunctions of GAT-1 or GAT-3 are associated with several neurological disorders, such as absence epilepsy (Cope et al., 2009), Alzheimer's disease (Wu et al., 2014), and strokes (Clarkson et al., 2010), but not yet with Parkinson's disease (PD) pathophysiology.

The basal ganglia (BG) are a collection of subcortical nuclei implicated in motor planning, action selection (Albin et al., 1989), and habit formation (Yin and Knowlton, 2006), and they are also the major site of pathology and dysfunction in PD following the progressive loss of substantia nigra dopamine-synthesizing neurons (Hammond et al., 2007). The external globus pallidus (GP) is a GABAergic nucleus composed of several subtypes of projection neurons (Abdi et al., 2015; Dodson et al., 2015; Hernández et al., 2015; Mastro et al., 2014) that holds a pivotal position in the circuit through its widespread projections to all BG nuclei (Beier et al., 2017; Kita, 2007; Saunders et al., 2016) and the cortex (Saunders et al., 2015). The activity of GP neurons is controlled by extrinsic GABAergic inputs from striatal indirect-pathway medium spiny neurons (ISPNs) and intrinsic pallidal local collaterals (Miguezuez et al., 2012; Rav-Acha et al., 2005; Sims et al., 2008).

GAT-1 and GAT-3 are both expressed in the GP. GAT-1 is predominantly found in axon terminals and glial processes juxtaposed at symmetrical synapses, while GAT-3 is exclusively located on glial processes (Galvan et al., 2005; Jin et al., 2011a; Ng et al., 2000; Wang and Ong, 1999). They exert a powerful regulation of pallidal GABAergic transmission, as pharmacological blockade of GAT-1 and/or GAT-3 prolongs GABA_A-mediated synaptic currents and generates a persistent tonic inhibition in GP neurons *ex vivo* (Jin et al., 2011b) while it reduces



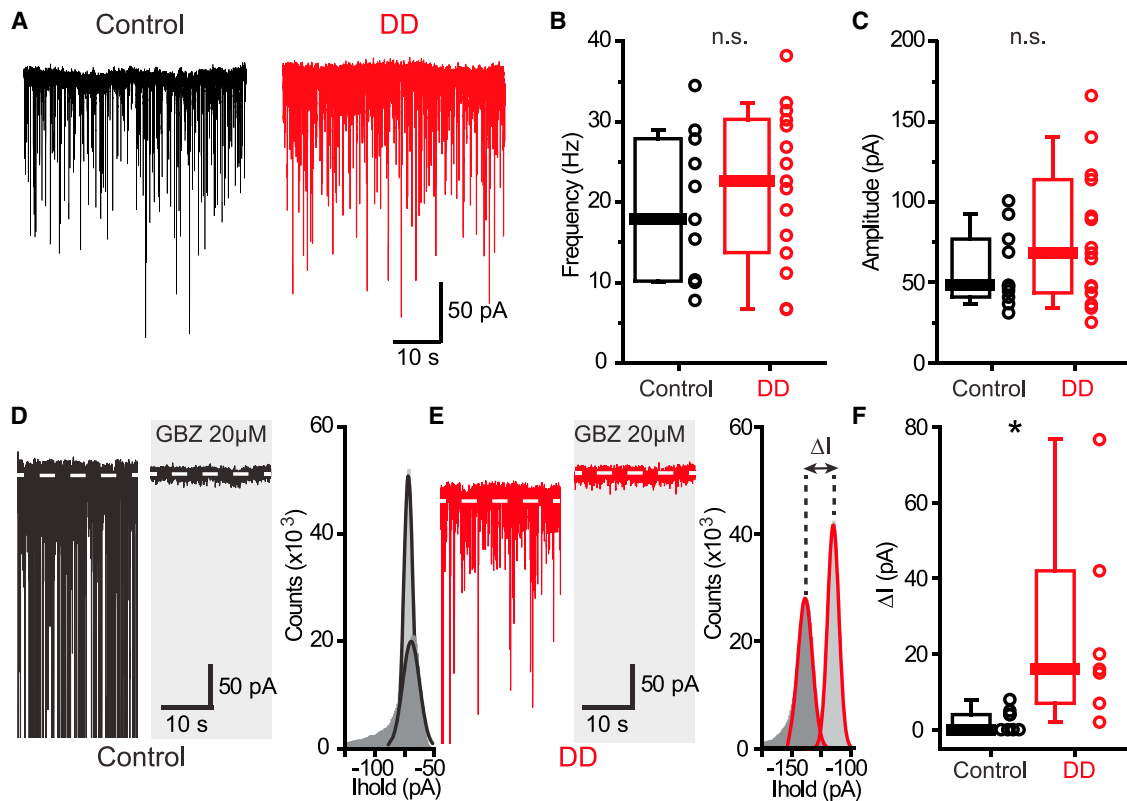


Figure 1. Tonic GABA Inhibition Is Observed in GP Neurons Only in Dopamine-Depleted Rodents

(A) Representative voltage-clamp recording showing spontaneous GABAergic synaptic (sIPSC) transmission in GP neurons recorded in brain slices obtained from a control rat (CTR, black trace) and a dopamine-depleted (DD) rat (red trace).

(B and C) Boxplots showing sIPSC frequency (B) and amplitude (C) for both groups (control, $n = 11$; DD, $n = 15$; non-significant, n.s., $p > 0.05$, MW-U test).

(D and E) Effect of GABAzine (GBZ) on the holding current of a GP neuron under control (D, black traces) and DD conditions (E, red traces). Dashed lines indicate the median currents of all points used to measure shifts in the holding current. The shift in the holding current (ΔI) is also represented on all-point histogram distributions with their corresponding Gaussian fits.

(F) Boxplots depicting the presence of a tonic current only in GP neurons of DD animals (control, $n = 8$; DD, $n = 7$; $*p = 0.0085$; MW-U test).

Whiskers of the boxplots represent 10–90th percentiles.

the firing rate of pallidal neurons in awake monkeys (Galvan et al., 2005). Indeed, the importance of maintaining a controlled discharge of GP neurons is well illustrated in PD, in which GP neurons become hypoactive and synchronized at beta-band frequencies (Bevan et al., 2002; Mallet et al., 2008; Mastro et al., 2017; Raz et al., 2000). This alteration of firing rate and pattern is believed to be the consequence of (1) a reduced cellular excitability, leading to reduced pacemaking in subpopulations of GP neurons (Chan et al., 2011; Hernández et al., 2015); and (2) the hyperactivity of iSPNs (Kita and Kita, 2011; Mallet et al., 2006; Sharott et al., 2017). Dopamine-depleted (DD) rodents show morphological changes at striato-pallidal synaptic terminals (Ingham et al., 1997), increased striato-pallidal synaptic transmission (Cui et al., 2016), and GP astrocytosis (Charron et al., 2014), supporting iSPN hyperactivity as well as local synaptic dysfunctions. While these modifications can account for the elevated extracellular concentrations of GABA observed in the GP of the DD rodents (Galeffi et al., 2003; Ochi et al., 2000), the underlying synaptic mechanism(s) remains unknown. In this study, we used patch-clamp electro-

physiology, biochemistry, and *in vivo* pharmacology in behaving rodents to investigate the alteration of GABA uptake by GATs in the GP of DD rodents.

RESULTS

Persistent Tonic GABAergic Inhibition Appears in GP Neurons after Chronic Dopamine Depletion

According to the classical anatomo-functional model of the BG network, the indirect pathway of the BG is hyperactive after the degeneration of midbrain dopaminergic neurons of the substantia nigra pars compacta (SNc). The primary nucleus of the indirect pathway affected by iSPN hyperactivity is the GP. To characterize the alteration of GABAergic synaptic transmission in GP neurons in DD conditions (Figure 1), induced by unilateral injection of the neurotoxin 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle of rats and mice, only animals with a severe dopamine depletion (>70% reduction in optical density of tyrosine hydroxylase-positive fibers in the striatum) were included in this study (Figure S1).

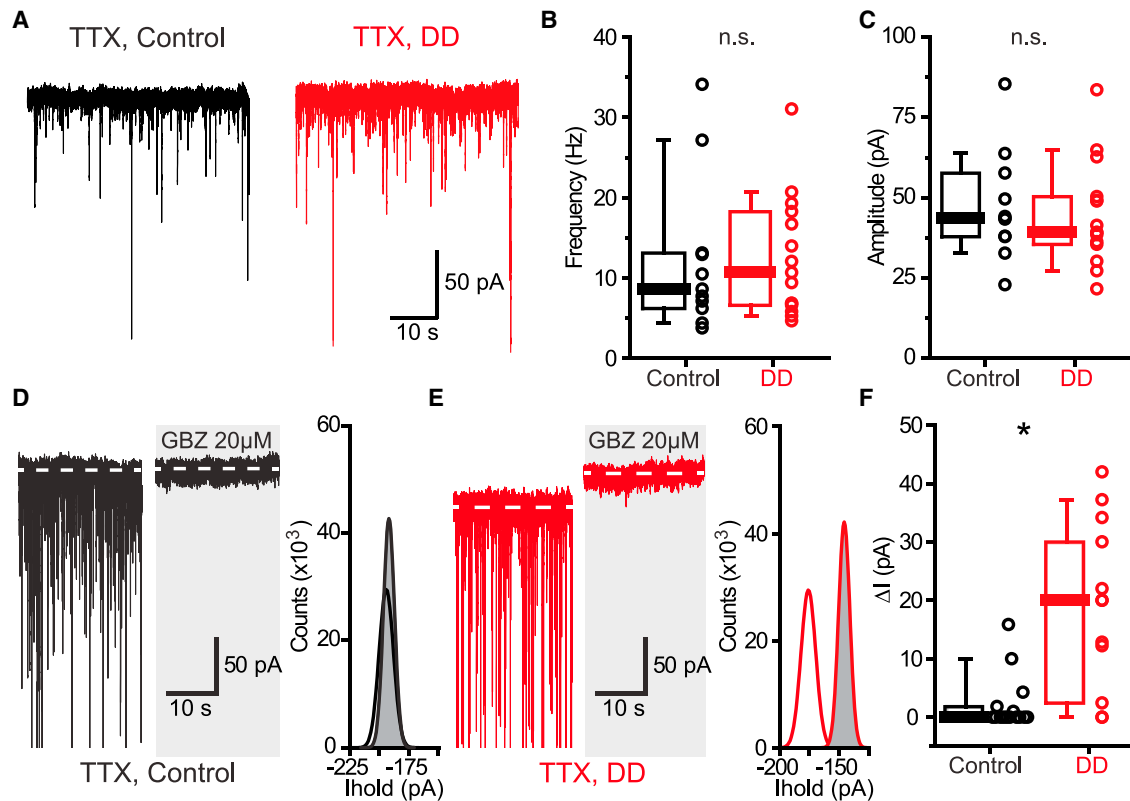


Figure 2. GABA-Mediated Tonic Current Is Independent of Neuronal Activity

(A) Typical example traces of mIPSCs recorded in the presence of 0.5 μM TTX in GP neurons from control (black trace) and DD (red trace) rats.

(B and C) Boxplots depicting the absence of change in the frequency (B) and the amplitude (C) after dopamine depletion (control, $n = 11$; DD, $n = 15$; non-significant, n.s., $p > 0.05$, MW-U test).

(D and E) Effect of GBZ on the holding current of a GP neuron under control (D, black traces) and DD conditions (E, red traces). Dashed lines indicate the median currents of all points used to measure shifts in the holding current. The shift in the holding current (ΔI) under DD conditions can also be observed on Gaussian fits of all-point histograms.

(F) Boxplots depicting that a tonic current is still present in TTX in GP neurons of DD animals (control, $n = 15$; DD, $n = 12$; $*p = 0.001$, MW-U test).

Whiskers of the boxplots represent 10–90th percentiles.

We first recorded spontaneous GABAergic inhibitory postsynaptic currents (IPSCs) received by GP neurons in voltage-clamp mode in acute brain slices from control and DD rats. GABA_A IPSCs were recorded in the presence of 50 μM D(-)-2-Amino-5-phosphonopentanoic acid (APV), 20 μM 6,7-Dinitroquinoxaline-2,3-dione (DNQX), and 1 μM (2S)-3-[[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP55845) to selectively block NMDA, AMPA, and GABA_B receptors, respectively. The frequency and amplitude of spontaneous inhibitory post-synaptic currents (sIPSCs) were not affected by dopaminergic degeneration (Figures 1A–1C) as previously reported (Miguez et al., 2012). Unexpectedly, we discovered that blocking GABA_A extrasynaptic receptors with 20 μM of the selective GABA_A competitive antagonist SR95531 (GABAzine [GBZ]) induced a shift in the holding current attributable to tonically activated GABA_A receptors only in GP neurons recorded from DD rat slices, suggesting an increased extracellular concentration of GABA (control, $\Delta I = 2$ [0;4] pA, $n = 8$; DD, $\Delta I = 16$ [7;42] pA, $n = 7$; $p = 0.0085$, Mann-Whitney [MW]-U test; Figures 1D–1F). Similar results were obtained from DD

mice (control, $\Delta I = 0$ [–2;6] pA, $n = 7$; DD, $\Delta I = 13$ [6;40] pA, $n = 10$; $p = 0.0446$, MW-U test). This form of inhibition, known as tonic inhibition, has only been seen in GP neurons of control rats when researchers have pharmacologically blocked GABA transporters using selective antagonists (Jin et al., 2011b). Thus, our results suggest that GABAergic transmission is altered in the GP following the chronic loss of dopamine.

Several reports in the literature support the hypothesis that iSPNs are hyperactive in PD (Mallet et al., 2006; Sharott et al., 2017), and this GABAergic input to the GP is believed to be responsible for the aberrant rate and pattern of activity of GP neurons (Abdi et al., 2015; Kita and Kita, 2011; Mallet et al., 2006). To test if tonic inhibition results from an increase in GABA release from putatively hyperactive striato-pallidal synapses, we recorded quantal synaptic GABAergic transmission in the presence of 0.5 μM tetrodotoxin (TTX) to block activity-dependent GABAergic transmission.

Miniature IPSC (mIPSC) amplitude and frequency medians were not statistically different between control and DD slices (Figures 2A–2C). While action potential-independent synaptic

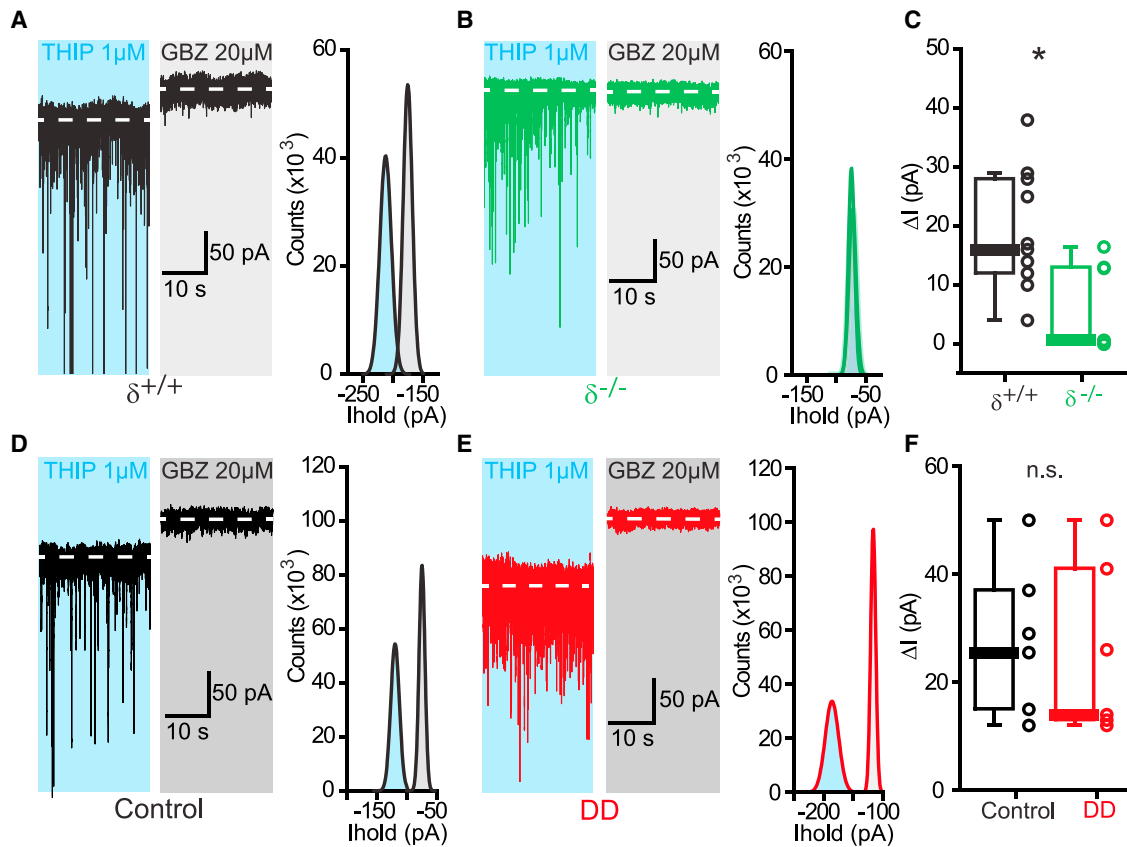


Figure 3. Pallidal Tonic Inhibition Is Mediated by δ -Containing Extrasynaptic GABA_A Receptors that Are Activated by Elevated Levels of Extracellular GABA

(A and B) Examples of the effect of 1 μ M THIP (an agonist of δ -containing GABA_A receptors) on the holding current of GP neurons recorded from wild-type mice ($\delta^{+/+}$) (A) and knockout mice for the GABA_A receptors δ subunit ($\delta^{-/-}$) (B). Shifts in holding currents are represented by dashed lines in electrophysiological recordings and by Gaussian fits in all-point histograms.

(C) Boxplots showing the lack of effect of THIP in $\delta^{-/-}$ mice ($\delta^{+/+}$, n = 10; $\delta^{-/-}$, n = 7; p = 0.0217, MW-U test).

(D and E) Examples of the effect of 1 μ M THIP on the holding current of GP neurons recorded from control (D) and DD (E) rat slices.

(F) Boxplots indicating that THIP causes a tonic current of similar magnitude in control and DD conditions (control, n = 6; DD, n = 6; p = 0.809, MW-U test), suggesting that tonic inhibition is not due to an upregulation of δ -containing GABA_A receptors.

Whiskers of the boxplots represent 10–90th percentiles.

transmission remained similar, a tonic current was still observed in the presence of TTX in DD slices when GBZ was bath applied to block GABA_A receptors. In identical conditions, no current was detectable in slices obtained from control rats (Figures 2D–2F; control, $\Delta I = 0$ [0;1.83] pA, n = 15; DD, $\Delta I = 20$ [2.44;30] pA, n = 12; p = 0.001, MW-U test). These results support the conclusion that tonic inhibition in DD animals is independent of action potential-dependent synaptic release of GABA, and, therefore, it is not directly related to ISPN hyperactivity.

Tonic GABA Inhibition Is Mediated by Extrasynaptic δ Subunits Containing GABA_A Receptors

In the CNS, tonic inhibition is mediated by GABA_A receptors composed of specific subunits, such as δ , $\alpha 4$, $\alpha 5$, or $\alpha 6$ (Brickley and Mody, 2012), which give these receptors a high affinity for GABA, slow desensitization, and fast deactivation kinetics (Bianchi et al., 2001; Haas and Macdonald, 1999). In the GP, δ , $\alpha 4$, and

$\alpha 5$ subunits have been detected at the protein level (Pirker et al., 2000; Schwarzer et al., 2001; Kultas-Ilnisky et al., 1998; Waldvogel and Faull, 2015). As δ and $\alpha 4$ are often associated in extrasynaptic GABA_A receptors, we investigated the presence of δ subunits in GP neurons using the δ -selective superagonist 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) in wild-type mice ($\delta^{+/+}$) and δ subunit knockout mice ($\delta^{-/-}$). THIP was applied at a concentration of 1 μ M, which has been reported to selectively activate δ -containing GABA_A receptors (Drasbek et al., 2007; Meera et al., 2011), and GBZ was used to measure the magnitude of the tonic current. THIP induced a tonic current only in wild-type mice, but not in $\delta^{-/-}$ mice (Figures 3A–3C; $\delta^{+/+}$, $\Delta I = 16.5$ [12;28] pA, n = 10; $\delta^{-/-}$, $\Delta I = 0.6$ [0;13] pA, n = 7; p = 0.0217, MW-U test), suggesting that extrasynaptic GABA_A receptors have δ subunits in GP neurons. We also analyzed the properties of spontaneous IPSCs in slices from both $\delta^{+/+}$ and $\delta^{-/-}$ mice, and we did not find any differences in the amplitude, frequency, rise time, and decay time (Table S1), suggesting

that δ subunits do not participate in the composition of synaptic GABA_A receptors.

Dopamine depletion can induce an alteration in GABA_A receptor level of expression that could be responsible for the appearance of tonic inhibition in the GP. To test if δ subunits are overexpressed in the GP after 6-OHDA-induced destruction of midbrain dopaminergic neurons, we measured the magnitude of the THIP-induced currents in control and DD rats. No differences in the magnitude of THIP-induced currents were found between the two groups (Figures 3D–3F; control, $\Delta I = 27.25 [15;37]$ pA, $n = 6$; DD, $\Delta I = 20 [13;41]$ pA, $n = 6$; $p = 0.809$, MW-U test), suggesting that pallidal tonic inhibition is not due to δ -containing GABA_A receptor overexpression in DD rats. THIP did not modify the kinetics of sIPSCs (Table S2), excluding the presence of GABA_A δ subunits in the composition of synaptic GABA_A receptors in GP neurons.

Finally, we tested the contribution of GABA_A $\alpha 5$ subunits in pallidal tonic inhibition using the $\alpha 5$ inverse agonist L655,708. Perfusion of 1 μ M L655,708 did not modify the holding current in DD slices, suggesting that $\alpha 5$ subunits do not contribute to tonic inhibition under dopamine-deprived conditions in GP neurons (Figures S2A and S2B). Furthermore, the magnitude of tonic current was also similar after the application of GBZ in the presence of L655,708 compared to GBZ alone in DD slices. L655,708 did not modify the kinetics of sIPSCs (Figures S2C–S2F), excluding the presence of GABA_A $\alpha 5$ subunits in the composition of synaptic GABA_A receptors in GP neurons.

Altogether, these results suggest that tonic inhibition in the GP is mediated by δ -containing extrasynaptic GABA_A receptors but does not rely on an overexpression of these receptors in pathological conditions.

Tonic Inhibition Is Present in All Subtypes of GP Neurons

Several recent reports have shown that the GP is populated by two main neuronal subtypes: prototypic and arky pallidal GP neurons (Abdi et al., 2015; Dodson et al., 2015). Prototypic GP neurons are characterized by the expression of PV and or Nkx2.1, while arky pallidal GP neurons are devoid of these two markers but selectively express FoxP2. We used PV::Ai9T and Nkx2.1::Ai9T reporter mice to selectively record prototypic (PV- and Nkx2.1-expressing neurons are labeled by the fluorescent protein dTomato in these mouse lines) and arky pallidal (FoxP2-expressing neurons identified by post hoc immunohistochemistry) GP neurons and test if tonic inhibition was present in all these cell types. We artificially augmented extracellular GABA concentrations by blocking GAT-1 transporters, and we tested the effect of a saturating concentration of GBZ on the holding current of prototypic PV-expressing and Nkx2.1-expressing GP neurons. In these two subtypes of prototypic GP neurons, a tonic current was detected (Figures S3A–S3H; PV, $\Delta I = 12.81 [10.98;27]$ pA, $n = 5$; Nkx2.1, $\Delta I = 22.58 [8.54;37.84]$ pA, $n = 9$). The same experiment was performed on arky pallidal neurons and also revealed tonic inhibition in these neurons (Figures S3I–S3L; FoxP2, $\Delta I = 27.46 [0;43.94]$ pA, $n = 5$). These results indicate that both GP neuron populations express extrasynaptic GABA_A receptors responsible for the tonic inhibition triggered by elevated extracellular GABA concentrations.

Reduced Expression of Glial GAT-3 Transporters Is Responsible for Tonic GABA Inhibition in DD Rats

Extracellular GABA concentrations are tightly regulated by neuronal and glial GABA transporters GAT-1 and GAT-3, respectively. We first investigated GAT-1 function in control and DD conditions in rats. In control slices, bath application of 10 μ M NNC711, a selective inhibitor of GAT-1, generated a tonic current compared to the application of GBZ alone (Figures S4A–S4C; GBZ, $\Delta I = 0 [0;9.8]$ pA, $n = 9$; NNC711 + GBZ, $\Delta I = 25 [14;40]$ pA, $n = 12$; $p = 0.0027$, MW-U test). In DD slices, the application of NNC711 produced a significantly greater current compared to the application of GBZ alone (Figures S4D–S4F; GBZ, $\Delta I = 15 [7;31]$ pA, $n = 7$; NNC711 + GBZ, $\Delta I = 36 [22;60]$ pA, $n = 11$; $p = 0.0459$; MW-U test), suggesting that blocking these neuronal transporters can exacerbate the tonic current observed in basal conditions in DD rats. Nevertheless, the amount of tonic inhibition due to GAT-1 in control and DD conditions was of similar magnitude, which suggests that GAT-1 transport is not altered in DD rats. GAT-1 blockade did not affect IPSC kinetics (Table S3), which suggests that, under basal synaptic transmission, GAT-1 contributes little to the regulation of synaptic GABA_A receptor currents in GP neurons. In addition, GABA uptake experiments performed from punches of the GP revealed that GAT-1 transporters tended to uptake GABA more efficiently in DD conditions (Figures S4G–S4I). This better efficiency is attributable to a greater maximal speed (V_{max}) of GABA uptake (control, $V_{max} = 1.67 \times 10^6 [1.56 \times 10^6; 2.32 \times 10^6]$ CPM/mg, $n = 3$; DD, $V_{max} = 3.88 \times 10^6 [2.85 \times 10^6; 4.48 \times 10^6]$ CPM/mg, $n = 4$; $p = 0.057$).

GAT-3 transporters contribute to GABA clearance from GABAergic synapses, but they can also be a source of GABA release when they function in a reverse transport mode (Sci-memi, 2014a). To determine the mode of transport of GAT-3 in the GP, we pharmacologically blocked GAT-3 in control and DD slices. In control slices, GAT-3 limits extrasynaptic GABA_A receptor activation, as the GAT-3 selective antagonist SNAP-5114 (20 μ M) induced a tonic current in GP neurons; this result is in agreement with a recent report (Jin et al., 2011b). This tonic current was significantly greater than in control conditions (Figures 4A–4C; GBZ, $\Delta I = 0 [0;4]$ pA, $n = 14$; SNAP-5114 + GBZ, $\Delta I = 16 [3;27]$ pA, $n = 14$; $p = 0.0022$, MW-U test). In contrast, the same pharmacological treatment failed to increase tonic current in GP neurons recorded from DD animals (Figures 4D–4F; GBZ, $\Delta I = 20 [15;30.5]$ pA, $n = 7$; SNAP-5114 + GBZ, $\Delta I = 12.8 [11.6;20]$ pA, $n = 7$; $p = 0.816$, MW-U test), suggesting that GAT-3 function was altered by chronic dopamine depletion. As with GAT-1, GAT-3 blockade did not affect spontaneous IPSC kinetics (Table S4), suggesting that, under basal synaptic transmission, GAT-3 also contributes little to shape synaptic GABA_A receptor currents in GP neurons. Western blot experiments with anti-GAT-3 antibodies revealed that GAT-3 expression was significantly reduced by approximately 30% in GP punches obtained from DD animals compared to control GP punches (Figures 4G and 4H; control, GAT-3/Actin = 1.02 [0.857;1.077], $n = 11$; DD, GAT-3/Actin = 0.70 [0.633;0.911], $n = 7$; $p = 0.0289$, MW-U test). These results support the conclusion that tonic inhibition is due to a down-expression of GAT-3, presumably located on astrocytes, in the GP of DD rodents.

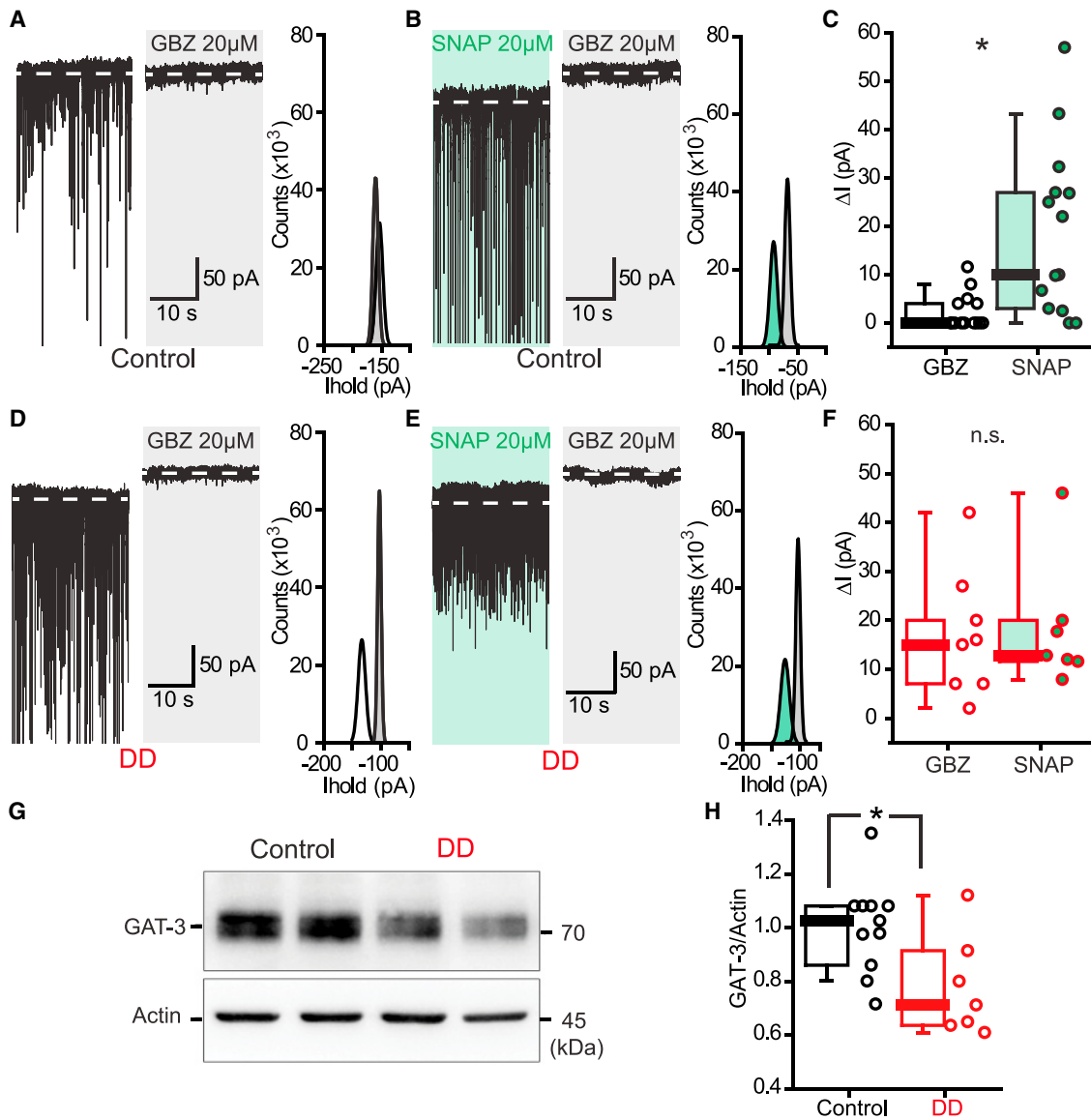


Figure 4. Pallidal Tonic Inhibition Results from a Reduced Expression of GAT-3 in DD Rats

(A and B) Representative voltage-clamp traces with their corresponding Gaussian fits of all-point histograms recorded in the presence of 20 μM GBZ alone (A) and in the presence of GBZ in combination with 20 μM SNAP-5114, a selective blocker of the GABA transporter GAT-3 (B).

(C) Boxplots showing that the blockade of GAT-3 significantly increases the magnitude of tonic inhibition in control animals (control, $n = 14$; DD, $n = 14$; $*p = 0.0022$, MW-U test).

(D and E) (D) is the same as (A) and (E) is the same as (B) in DD slices.

(F) Boxplots indicating that the blockade of GAT-3 does not significantly increase the tonic inhibition in DD conditions (control, $n = 8$; DD, $n = 7$; $p = 0.405$, MW-U test).

(G) Western blot showing the significant reduction in GAT-3 expression in DD conditions.

(H) Summary graph highlighting the significant reduction of GAT-3 protein expression in DD rats (control, $n = 11$; DD, $n = 7$; $*p = 0.0289$, MW-U test).

Whiskers of the boxplots represent 10–90th percentiles.

To confirm the loss of function of GAT-3 in GP astrocytes, we directly measured GAT-3 activity by recording the current generated by GABA uptake. GP astrocytes were initially identified on the basis of their size and morphology (Figure 5A), followed by their electrophysiological properties, which included linear I-V relationship (Figure 5B), low input resistance (Figure 5C; control,

$R_m = 33.49$ [28.04; 41.48] $\text{M}\Omega$, $n = 18$), and hyperpolarized resting membrane potential (Figure 5D; control, $V_m = -71.94$ [–75.29; –70.01] mV, $n = 17$). We did not find any modification in the I/V curve (Figure 5B), membrane resistance (DD, $R_m = 38.85$ [34.35; 73.06] $\text{M}\Omega$, $n = 15$; $p = 0.143$, MW-U test), and resting membrane potential (DD, $V_m = -75$ [–77.6; –68.72] mV, $n = 15$;

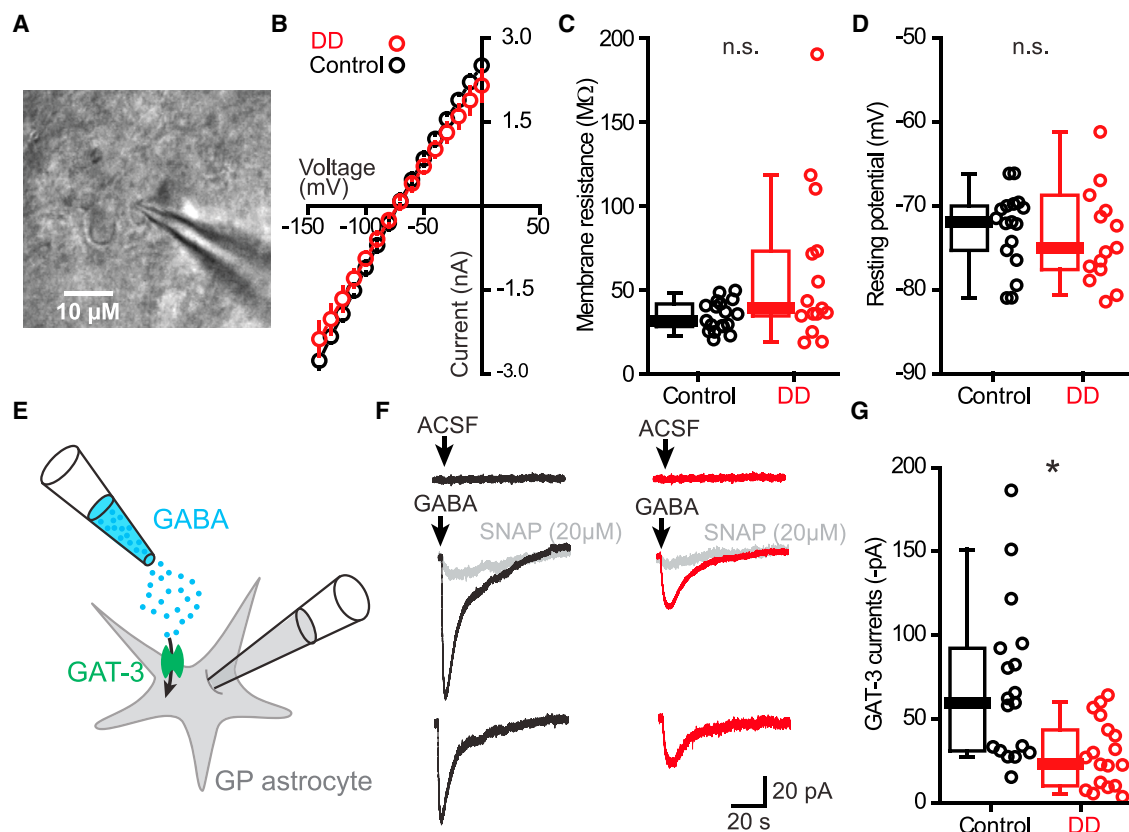


Figure 5. GAT-3 Currents Are Reduced in GP Astrocytes of DD Rats

(A) Differential interference contrast (DIC) image of a GP astrocyte with a visible recording patch pipette.
 (B) Current-voltage relationship of control (black) and DD (red) GP astrocytes obtained in response to 10-mV voltage steps.
 (C) Boxplot representing GP astrocyte membrane resistance (control, $n = 18$; DD, $n = 15$; $p = 0.1431$, MW-U test).
 (D) Boxplot depicting GP astrocyte membrane resting potential (control, $n = 17$; DD, $n = 15$; $p = 0.597$, MW-U test).
 (E) Schematic diagram illustrating the experimental design for astrocyte GAT-3 current measurements.
 (F) Typical responses of GP astrocytes to focal application of ACSF (top traces) and 10 mM GABA (middle traces) in the absence (black/red) and presence of GAT-3 blocker (SNAP-5114, 20 μM ; gray traces). The bottom traces represent the GAT-3 current obtained by subtracting the trace obtained by GABA puff alone from the one in the presence of SNAP-5114.
 (G) Boxplots showing a significant reduction in the amplitude of GAT-3 currents in GP astrocytes from DD rats (control, $n = 18$; DD, $n = 18$; $*p = 0.0022$, MW-U test). Whiskers of the boxplots represent 10–90th percentiles.

$p = 0.597$, MW-U test) of GP astrocytes recorded from DD compared to control animals, which is in agreement with a recent report (Cui et al., 2016).

To isolate GAT-3-mediated currents, we added the GABA_A and GABA_B receptor blockers GBZ (20 μM) and CGP55845 (1 μM), respectively, and the GAT-1 transporter blocker NNC711 (10 μM) and TTX (0.5 μM) to the perfusion solution. Under these experimental conditions, pressure puffs of GABA (150 ms; 10 mM) evoked GAT-3-mediated currents in GP astrocyte plasma membranes during GABA uptake (Figure 5E). GABA, but not artificial cerebro-spinal fluid (ACSF) puffs, generated inward currents that were abolished by 20 μM SNAP-5114, indicating that these currents were mediated during GABA uptake by GAT-3 (Figure 5F). Consistent with the decreased expression of GAT-3 in the GP of DD rats, as measured by western blot analysis, the GAT-3 current amplitude was reduced by ~60% in DD rats (Figure 5G; control, $i\text{GAT-3} = 60.97$ [31;92.2]

pA, $n = 18$; DD, $i\text{GAT-3} = 25$ [10.2;43.5] pA, $n = 18$; $p = 0.0013$, MW-U test), which strongly supports the conclusion that tonic inhibition observed in GP neurons from DD rats is due to reduced expression of GAT-3 transporters in GP astrocytes.

It has been recently reported that GP astrocytes express D2 dopamine receptors (Cui et al., 2016). Therefore, it is possible that dopamine regulates ambient GABA levels by controlling GAT-3 activity or addressing at the plasma membrane. To test this hypothesis, we artificially increased extracellular GABA concentration by applying 2 μM GABA in the superfusion to generate tonic inhibition in GP neurons. To avoid the contribution of GAT-1 in GABA uptake, the recordings were done in the presence of 10 μM NNC711. Addition of the D2 receptor agonist quinpirole (2 μM) induced a significant positive shift of the holding current, suggesting that D2 receptor activation boosts GABA uptake by GAT-3 in GP astrocytes (Figures 6A and 6C; $n = 6$). The effect of quinpirole was completely occluded when it was applied in

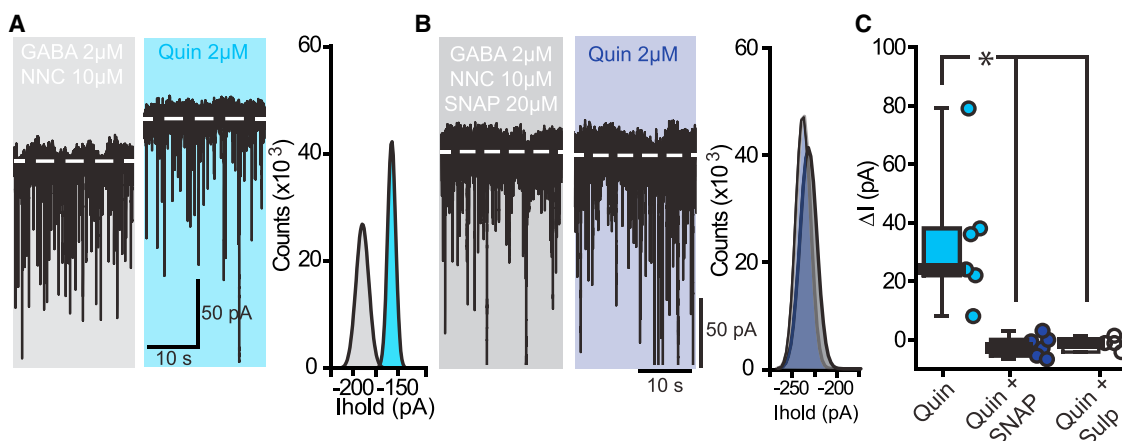


Figure 6. GAT-3 Activity Is Regulated by D2 Dopamine Receptors

(A) Representative voltage-clamp traces with their corresponding Gaussian fits of all-point histograms recorded in the presence of 2 μM GABA and 10 μM NNC711 to artificially elevate extracellular GABA concentrations and block GAT-1 activity, respectively. The addition of 2 μM quinpirole (Quin), a selective D2 dopamine receptor agonist, induced a shift in holding current.

(B) Same as (A) but in combination with 20 μM SNAP-5114, a selective blocker of the GABA transporter GAT-3. Note the absence of change in holding current. (C) Boxplots indicating that quinpirole action of holding current is dependent on GAT-3 activity and is selective of D2 dopamine receptors, as its action is occluded by the blockade of GAT-3 or by sulpiride (a D2 receptor selective antagonist), respectively (Quin, $n = 6$; Quin + SNAP, $n = 6$; Quin + Sulp, $n = 4$; $p = 0.0049$, Kruskal-Wallis test). These experiments were performed in the presence of 50 μM APV, 20 μM DNQX, and 1 μM CGP55845 to block NMDA, AMPA, and GABA_B receptors, respectively.

Whiskers of the boxplots represent 10–90th percentiles.

either the presence of the GAT-3 antagonist SNAP-5114 (20 μM) or the D2 dopamine receptor antagonist sulpiride (2 μM), supporting the conclusion that the action of quinpirole on GP neuron's holding current is selective of D2 dopamine receptors and dependent on GAT-3 activity (Figures 6B and 6C). Further experiments will be required to determine if D2 dopamine receptors control the membrane expression or the transport efficacy of GAT-3 in the GP.

Intrapallidal GAT-3 Blockage Worsens Motor Coordination in Control Animals

To address the functional role of GP GAT-3 transporters *in vivo*, we administered SNAP-5114 intrapallidally (Figures 7A and 7B), and we evaluated motor behavior in control and DD rats. We performed two types of behavioral tests: rotarod and cylinder tests. The former evaluates coordination and balance during motor execution and the latter measures forelimb asymmetry during volunteer exploration.

To determine the dose of SNAP-5114 to use in the following experiments, we first unilaterally injected five rats with vehicle and seven animals with 2 μM , 2 mM, and 20 mM SNAP-5114 in the GP, and we tested their motor performance using the rotarod. The rotarod was performed in the same animals, which were tested with different doses every 4 days. From all doses tested, the largest one, 20 mM, produced a significant worsening in motor coordination compared to those animals injected with the vehicle ($p < 0.001$, Fisher's least significant difference [LSD] post hoc test).

In the following set of experiments, the intrapallidal administration of 20 mM SNAP-5114 was tested in consecutive days. As shown in Figure 7C, 20 mM SNAP-5114 resulted in significantly

worse motor performance in the rotarod from the first day of drug administration (interaction factor: $F_{(2,36)} = 3.2$, $p = 0.05$; treatment factor: $F_{(2,36)} = 4.7$, $p = 0.04$; repeated-measure [RM] two-way ANOVA). SNAP-5114 did not produce forelimb asymmetry in the animals evaluated in the cylinder test (Figure 7D) (interaction factor: $F_{(2,36)} = 0.07$, $p = 0.92$; treatment factor: $F_{(2,36)} = 0.32$, $p = 0.72$; RM two-way ANOVA). These behavioral data support the conclusion that GP GAT-3 is essential for normal motor coordination.

We also tested the effect of GAT-3 blockade in DD rats. In this case, SNAP 5114 had no negative effect on rotarod performance (Figure 7E; interaction factor: $F_{(2,32)} = 0.34$, $p = 0.714$; treatment factor: $F_{(2,32)} = 2.70$, $p = 0.08$; RM two-way ANOVA) or in forelimb asymmetry, evaluated with the cylinder test (Figure 7F; interaction factor: $F_{(2,32)} = 1.84$, $p = 0.17$; treatment factor: $F_{(2,32)} = 1.64$, $p = 0.21$; RM two-way ANOVA). These results agree with our findings that GAT-3 is impaired in DD conditions and, therefore, blockage of the glial GABA transporter does not worsen motor coordination.

One limitation of the present study is the high concentration of SNAP-5114 used in the behavioral experiments, which could lead to the blockage of GAT-1 in addition to GAT-3. However, the lack of effect of SNAP-5114 in DD rats confirms the specific GAT-3 effect mediated by the drug in these experiments.

DISCUSSION

Using a combination of electrophysiological and biochemical approaches, we show that chronic dopamine depletion in rodents triggers the development of a tonic form of GABAergic inhibition in the GP. Our data demonstrate that the synaptic

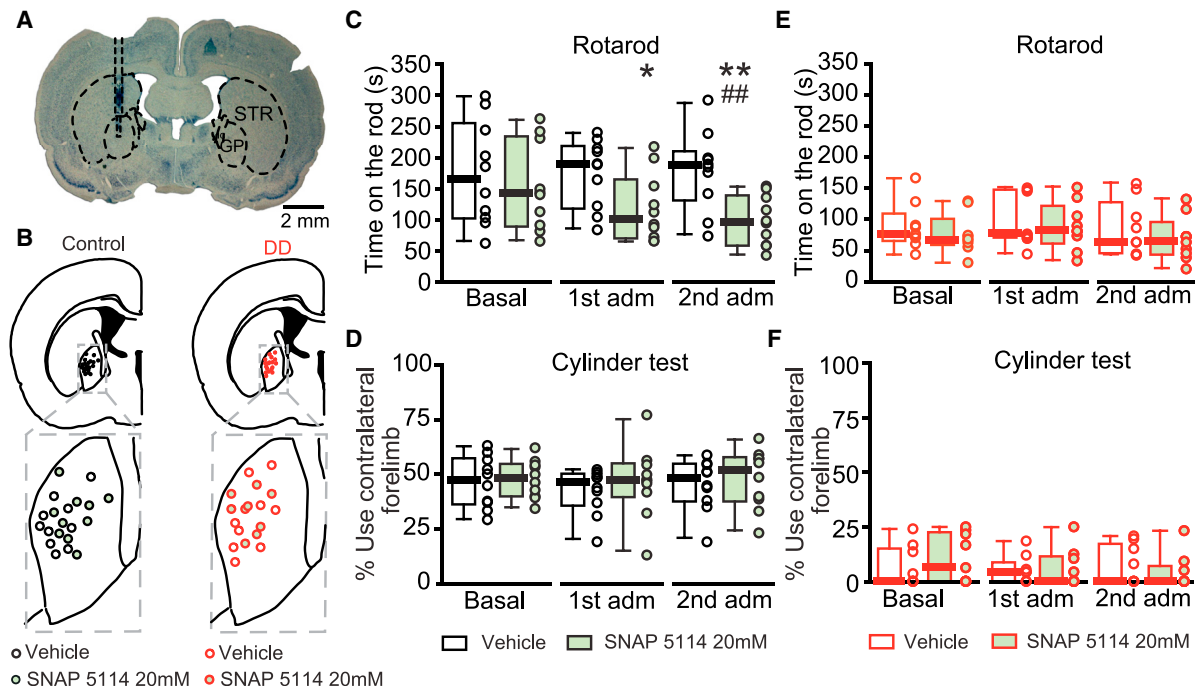


Figure 7. Intrapallidal GAT-3 Blockage Impairs Motor Coordination in Control Rats

(A) Micrograph showing cannula emplacement in the GP taken for a control rat. (B) Cannula placements in the GP for the control (n = 20) and DD (n = 18) groups. Each circle represents the position of one cannula for a given animal. (C) Motor coordination and balance were evaluated in the rotarod for 3 consecutive days. The first day, the animals did not receive any drug; the second and third days, animals received either 20 mM SNAP-5114 or vehicle. Animals treated with 20 mM SNAP showed significant impairment after drug administration. (D) The same animals were also tested in the cylinder test but the drug produced no forelimb asymmetry. n = 10 rats in each group. *p < 0.05 and **p < 0.01 versus respective vehicle group; ##p < 0.01 versus respective basal group, Fisher's LSD post hoc test. (E and F) (E) is the same as (C) and (F) is the same as (D) in DD rats. n = 9 DD rats in each group. Whiskers of the boxplots represent 10–90th percentiles.

mechanism responsible for this aberrant tonic inhibition is a downregulation of GAT-3 in GP astrocytes. Finally, we show that GP GAT-3 blockade impairs motor coordination in control rats, highlighting the instrumental role played by this GABA transporter in GP motor functions. Altogether, our study suggests that GAT-3 dysfunction is associated with and may contribute to the pathophysiology of PD.

The Nature of Extrasynaptic GABA_A Receptors Involved in Tonic Inhibition in GP Neurons

In the CNS, tonic inhibition is mediated by extrasynaptic GABA_A receptors composed of particular subunits that confer high affinity for GABA and slow deactivation and desensitization kinetics. These receptors most often contain $\alpha 4$, $\alpha 5$, $\alpha 6$, or δ GABA_A subunits. In the GP, the presence of $\alpha 5$ and δ subunits is controversial due to the low level of protein expression compared to the striatum (Pirker et al., 2000; Schwarzer et al., 2001; Waldvogel and Faull, 2015). We combined pharmacological experiments with $\delta^{-/-}$ mice to test for the involvement of this subunit in the extrasynaptic receptors present in GP neurons. Our results suggest that δ -containing GABA_A receptors are expressed in control animals but are not activated under basal conditions when GABA transporters are functional. Furthermore, no tonic currents were detectable in $\delta^{-/-}$ mice, suggesting that the presence of this

subunit is mandatory to have functional extrasynaptic GABA_A receptors in GP neurons. Our results also show that the δ -selective drug THIP induced tonic currents of similar magnitude in both control and DD animals, which excludes an increased expression of δ subunits following chronic dopamine depletion. We also tested the contribution of $\alpha 5$ subunits, which significantly contribute to tonic inhibition in the striatum (Ade et al., 2008), cortex (Clarkson et al., 2010), and hippocampus (Wu et al., 2014). In contrast with previous results in those brain areas, the inverse agonist L655,708 failed to modify the holding current in GP neurons of DD rats, which excludes *de novo* expression of this subunit in the GP following dopamine depletion.

The GP is composed of several neuronal populations with embryonic, molecular, and functional specifications (Abdi et al., 2015; Dodson et al., 2015; Hernández et al., 2015; Mastro et al., 2014; Nóbrega-Pereira et al., 2010). Two main cell populations, prototypic and arkyallidial neurons, have been identified (Abdi et al., 2015; Dodson et al., 2015; Hegeman et al., 2016; Mallet et al., 2012). All prototypic neurons express the transcription factor (TF) Nkx2.1, and a great majority (~70%) co-expresses the calcium-binding protein parvalbumin, while arkyallidial neurons selectively express the TF FoxP2. While we observed tonic inhibition in most of our recordings of unidentified GP neurons in DD rodents, we also demonstrated that both

prototypic (Nkx2.1 and PV positive) and arky pallidal neurons (FoxP2 positive) were equipped with extrasynaptic receptors, which suggests that DD-induced tonic inhibition is present in all pallidal neuronal populations and, thus, affects the entire GP.

Tonic Inhibition in GP Neurons Is Independent of iSPN Overactivity

According to the classical BG model (Albin et al., 1989) and experimental data (Kita and Kita, 2011; Mallet et al., 2006; Sharott et al., 2017), iSPNs become hyperactive following dopamine deprivation. It has been shown that striato-pallidal synapses are enlarged (Ingham et al., 1997) and striato-pallidal transmission is overactive (Cui et al., 2016) in DD rodents. This hyperactivity could result in GABA spillover from striato-pallidal synapses and the activation of extrasynaptic GABA_A receptors, meaning that pallidal tonic inhibition could simply be the consequence of the hyperactivity of the striato-pallidal connection. Our data do not support this hypothesis, as tonic inhibition was still present in the GP neurons of DD slices when recordings were performed in the presence of TTX, which abolishes activity-dependent synaptic release and significantly reduces synaptically released GABA. This finding implies that pallidal tonic inhibition is independent of iSPN hyperactivity and instead involves local modifications at the level of pallidal GABAergic synapses.

Astrocytic GAT-3 Dysfunction Is Responsible for Tonic Inhibition in GP Neurons

GATs represent the main buffering system to control extracellular levels of GABA and to shape GABAergic transmission (Sciemmi, 2014b). In the GP, extracellular GABA concentrations are abnormally elevated in the DD condition (Galeffi et al., 2003; Ochi et al., 2000), suggesting that GABA uptake is deficient. Electron microscopy analysis has shown that GAT-1 is present in unmyelinated axons as well as in GP glial processes (Galvan et al., 2005, 2010). Contrary to the thalamus, where down-expression and reduced activity of GAT-1 are responsible for excessive tonic inhibition in absence epilepsy (Cope et al., 2009; Errington et al., 2011; Pirttimaki et al., 2013), in the GP we found that GAT-1-mediated GABA uptake was not affected by DD. This result is in agreement with anatomical data showing that GAT-1 expression and subcellular location are not affected in rodent and primate models of PD (Galvan et al., 2010).

GAT-3 is found exclusively in glial processes that ensheath dendrites and terminals in the GP (Galvan et al., 2005; Ng et al., 2000), suggesting that pallidal astrocytes actively contribute to the regulation of GABA levels in the GP. GAT-3 provides an additional source of GABA through reverse transport in the striatum and the hippocampus under several physiological and pathological conditions (Allen et al., 2004; Wójtowicz et al., 2013; Wu et al., 2007, 2014). In agreement with another study (Jin et al., 2011b), we found that pharmacological blockade of GAT-3 induced a significant GABA_A-mediated tonic current in dopamine (DA)-intact rodents, indicating that, under physiological conditions, GAT-3 works in the uptake mode and removes GABA from the synaptic cleft. Under the DD condition, the level of tonic inhibition was not significantly modified by GAT-3 inhibition, which suggests that GAT-3 does not work in reverse mode in PD, and that its functioning is altered in the GP. Using western

blot analysis, we demonstrated that GAT-3 expression was indeed reduced by ~30% in the GP of DD animals. Furthermore, direct recording of the currents generated during GABA uptake by GAT-3 in GP astrocytes conclusively proved a reduced activity indicative of a loss of function. GAT-1 has been suggested to prevent GABA escape from the synaptic cleft during phasic synaptic transmission, while GAT-3 could be responsible for controlling the ambient GABA concentration that mediates tonic inhibition (Beenhakker and Huguenard, 2009). If this is the case in the GP, it could explain why down-expression of GAT-3 is not compensated by the apparent increase uptake activity of GAT-1 we observed in DD conditions.

Other factors can contribute to the deficit of GABA uptake by GAT-3, such as maladaptive structural plasticity or the alteration of GAT-3 trafficking to astrocyte plasma membranes. Structural plasticity has been observed in GP astrocytes during running activity (Tatsumi et al., 2016), which suggests that they actively contribute to motor functions. We recently showed that astrogliosis takes place in the GP in response to chronic dopamine loss (Charron et al., 2014), which suggests that maladaptive structural plasticity of the tri-partite synapse, such as astrocyte processes retraction, could also contribute to GAT-3 deficiency by removing the transporters from GABAergic terminals. Only a few signaling pathways regulating GAT-3 have been described in the literature. Among them, metabotropic ATP and adenosine receptors as well as TRPA1 channels are known to modulate GABA transport by GAT-3 into the astrocytes (Cristóvão-Ferreira et al., 2013; Jacob et al., 2014; Shigetomi et al., 2011). In our study, we report a direct modulation of GAT-3 activity by D2 dopamine receptors. It will be interesting to investigate in more detail the signaling pathway involved in this modulation of GAT-3 activity.

Functional Considerations

Balanced GABAergic inhibition in the GP is essential for proper motor control. Artificial elevation of GABAergic transmission by the pharmacological inhibition of GATs induces a reduction of GP neuron firing discharge in awake DA-intact monkeys (Galvan et al., 2005, 2010), which somehow mimics the hypoactivity observed in PD. On the other hand, blocking GABA_A receptors in DD rodents increases GP neuron activity and produces marked antiparkinsonian effects (Maneuf et al., 1994; Xue et al., 2010). As no specific agonist or positive modulator of GAT-3 is currently available, we inhibited GAT-3 using microinjections of the selective antagonist SNAP-5114 in the GP of DA-intact rats, and we found that, while forelimb akinesia was intact, motor coordination was significantly worse compared to vehicle-injected rats. The same experiments in DD rats had no impact on motor coordination, which is in line with the impairment of GAT-3 confirmed by electrophysiological and molecular approaches. These results unambiguously demonstrate the importance of GAT-3 in preventing excessive GABAergic inhibition and maintaining the role in motor function played by the GP. As overactivity of GABAergic signaling in the GP is one of the cardinal hallmarks of PD (DeLong, 1990), the development of novel agonists or positive modulators of GAT-3 appears to be a promising therapeutic avenue to alleviate motor symptoms in PD.

In conclusion, GAT-3 downregulation/malfunction in the GP is responsible for the persistent tonic inhibition observed in this

nucleus in models of PD. This modification could contribute to the appearance of some of the motor symptoms that characterize rodent PD models, and it also may have clinical consequences in PD patients.

EXPERIMENTAL PROCEDURES

All experiments were performed in agreement with the Bordeaux University ethical committee (agreement A5012075) and are in compliance with European Economic Community guidelines (86/6091EEC). Sprague-Dawley rats and C57BL6/J mice received a unilateral injection of 6-OHDA into the medial forebrain bundle to lesion the nigrostriatal dopaminergic system.

Rat and mice of either sex were used for *ex vivo* experiments. Whole-cell voltage- and current-clamp recordings of GP neurons and astrocytes were performed on acute brain slices on a Nikon Eclipse workstation equipped with a 60× water-immersion objective. Tonic inhibition was measured as a shift in holding current induced by bath application of GBZ, a selective GABA_A receptor antagonist. Standard procedures were used to perform immunohistochemistry and GAT-3 western blot experiments. Motor asymmetry and motor coordination in 6-OHDA rats were assessed using rotarod and cylinder tests.

Data analyses were done using ClampFit 9.2, Mini Detection 8.0 (in-house software), Origin 7, and ImageJ. Data in the text are presented as median and interquartile range. Boxplots with individual data points were used for graphic representation. Wilcoxon signed-rank (WSR), MW-U, and Kruskal-Wallis tests were used for statistical comparisons in *ex vivo* experiments. For behavior experiments, repeated-measure two-way ANOVA followed by Fisher's LSD post hoc test were used. Data were considered significant at $p < 0.05$.

Mouse lines, reagents, and pharmacological tool details are provided in Table S5.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and five tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.04.014>.

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AUTHOR CONTRIBUTIONS

M.C. and C.M. designed the experiments, conducted electrophysiology and immunohistochemistry experiments, analyzed the data, and wrote the manuscript. M.C., C.M., and S.M. performed stereotaxic surgery. M.C. and A.P. performed astrocyte recordings. A.M. and E.B.-G. performed western blot experiments. S.C.-F., S.V., and A.S. designed and performed GABA uptake experiments. E.P.-R. and C.M. designed and performed the behavioral experiments. J.B. designed the experiments, conducted electrophysiology experiments, wrote the manuscript, and directed the project. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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