Control of the Nigrostriatal Dopamine Neuron Activity and Motor Function by the Tail of the Ventral Tegmental Area

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Midbrain dopamine neurons are implicated in various psychiatric and neurological disorders. The GABAergic tail of the ventral tegmental area (tVTA), also named the rostromedial tegmental nucleus (RMTg), displays dense projections to the midbrain and exerts electrophysiological control over dopamine cells of the VTA. However, the influence of the tVTA on the nigrostriatal pathway, from the substantia nigra pars compacta (SNc) to the dorsal striatum, and on related functions remains to be addressed. The present study highlights the role played by the tVTA as a GABA brake for the nigrostriatal system, demonstrating a critical influence over motor functions. Using neuroanatomical approaches with tract tracing and electron microscopy, we reveal the presence of a tVTA–SNc–dorsal striatum pathway. Using *in vivo* electrophysiology, we prove that the tVTA is a major inhibitory control center for SNc dopamine cells. Using behavioral approaches, we demonstrate that the tVTA controls rotation behavior, motor coordination, and motor skill learning. The motor enhancements observed after ablation of the tVTA are in this regard comparable with the performance-enhancing properties of amphetamine, a drug used in doping. These findings demonstrate that the tVTA is a major GABA brake for nigral dopamine systems and nigrostriatal functions, and they raise important questions about how the tVTA is integrated within the basal ganglia circuitry. They also warrant further research on the tVTA's role in motor and dopamine-related pathological contexts such as Parkinson's disease.
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INTRODUCTION

Dopamine regulation of basal ganglia circuitry is critical to fine motor control and to associated pathologies such as Parkinson's disease (Albin *et al*, 1989; Redgrave *et al*, 2010). In this circuit, the nigrostriatal pathway from dopamine neurons of the substantia nigra pars compacta (SNc) to the dorsal striatum is controlled by various inhibitory GABAergic afferents (Tepper and Lee, 2007). The striatum, the external globus pallidus, and the substantia nigra pars reticulata are major contributors to this GABAergic control of SNc dopaminergic activity. Within the midbrain, the tail of the ventral tegmental area (tVTA) (Kaufling *et al*, 2009, 2010a; Perroti *et al*, 2005) also named the rostromedial tegmental (RMTg) nucleus (Jhou *et al*, 2009a, 2009b) appears as a GABAergic inhibitory structure displaying

heavy projections to the VTA and the SNc, suggesting that the tVTA could regulate the activity of dopamine systems (Barrot *et al*, 2012; Bourdy and Barrot, 2012). In the previous years, this inhibitory control has been investigated in the contexts of VTA responses to drugs of abuse (Jalabert *et al*, 2011; Jhou *et al*, 2012, 2013; Lecca *et al*, 2011, 2012; Matsui and Williams, 2011) and of the habenulo–VTA pathway in avoidance behavior (Stamatakis and Stuber, 2012) and in reward prediction error (Hong *et al*, 2011), demonstrating that the tVTA is indeed an important GABA brake for VTA dopamine neurons. However, the influence of the tVTA on the nigrostriatal pathway and on motor functions remains to be explored.

In the present study, we combined neuroanatomical, electrophysiological, and behavioral approaches to demonstrate the functional role played by the tVTA as a GABA brake for the nigrostriatal system and motor function. With pathway tract tracing, electron microscopic analysis of synapses and *in vivo* extracellular single-cell recordings, we demonstrate the inhibitory connectivity between tVTA efferent fibers and SNc dopamine neurons. Moreover, the experimental ablation of tVTA reveals a major influence of this structure on motor skill learning and on motor performance.

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MATERIALS AND METHODS

Subjects

Experiments were performed in adult male Sprague–Dawley rats (Janvier, France). The rats were habituated to the facilities for at least a week before starting the procedures, and the animals were 7–9 weeks old (ie, 250–350 g) at surgery time or at testing time when no surgery was involved. They were housed under standard conditions (22 °C, 12-hour light/dark cycle). The experiments were in accordance with the CNRS and European community's council directives, with animal facilities and procedures approved by regional veterinary office and with scientists having the legal certificate for experimentation on living animals.

Drugs

Ibotenic acid (1% in phosphate-buffered saline (PBS) 0.1 M; 0.3 μl) was injected unilaterally into the VTA and unilaterally or bilaterally into the tVTA using Hamilton syringes with 33-gauge needles. Control animals received 0.3 μl of PBS. The 6-hydroxydopamine hydrobromide (6-OHDA; 0.25% in 0.9% NaCl with 0.01% ascorbic acid) was injected unilaterally into the SNc (4 μl for large lesion or 2 μl for partial lesion) or bilaterally into the dorsolateral striatum (2 μl/hemisphere). D-amphetamine sulfate was injected intraperitoneally (i.p., in 0.9% NaCl) at 3 mg/kg for rotational behavior and 1 mg/kg for rotarod experiments.

Surgery

Rats were anesthetized under ketamine/xylazine (87 mg/kg and 13 mg/kg respectively, i.p.) for tract tracing and 6-OHDA surgeries, under isoflurane for electrophysiology (3% for induction, 2% for surgery) and under sodium pentobarbital (50 mg/kg, i.p.) for ibotenic acid lesions. Stereotaxic coordinates relative to bregma were adjusted to the animal weight, coordinates (in mm) were as follows (Paxinos and Watson, 2007): tVTA, anteroposterior (AP) $= -6.5$, lateral (L) $= \pm 1.5$, ventral (V) $= -7.8$, $\pm 6°$ lateral angle and dorsolateral striatum, AP $= +1$, L $= \pm 4.3$, V $= -4$, $\pm 6°$ lateral angle for tract tracing and behavior; tVTA, AP $= -7$, L $= +0.4$, V $= -7.4$ and SNc, AP $= -5$, L $= \pm 1.5$, V $= -7.5$ for electrophysiology; SNc, AP $= -5.2$, L $= \pm 2.6$, V $= -7.6$, $\pm 6°$ lateral angle and VTA, AP $= -5$, L $= \pm 1.6$, V $= -7.5$, $\pm 6°$ lateral angle for behavior. Verticality was taken from the dura.

Tract Tracing

Biotinylated dextran amine (BDA; 5% in 0.25 M potassium acetate), Phaseolus vulgaris leucoagglutinin (PhaL; 2.5% in 10 mM sodium phosphate buffer) and cholera toxin β-subunit (CTb; 0.25% in 0.1 M Tris) were iontophoretically delivered (+1 to +6 μA, 7–10 s on/off cycles for 10–20 min) with glass micropipettes (10–40 μm tip diameter) as previously described (Balcita-Pedicino et al, 2011; Kaufling et al, 2009; Omelchenko et al, 2009).

Histochemistry

One week following tracer injection or immediately after behavioral testing, rats were anesthetized with sodium pentobarbital and perfused with 100 ml phosphate buffer (0.1 M, pH 7.4) followed by 500 ml of a paraformaldehyde solution (4% in phosphate buffer). Brains were collected, postfixed overnight, and coronal sections (40 μm) were cut on a vibratome. Histochemistry for BDA and immunohistochemistry was done as previously described (Kaufling et al, 2009, 2010a; Omelchencko et al, 2009). Primary antibodies were directed against: CTb (Sigma C3062, 1/20000), tyrosine hydroxylase (TH) (Millipore-Chemicon MAB318, 1/2500), or NeuN (Millipore MAB377, 1/5000). Visualization was performed with biotinylated secondary antibodies and peroxidase/DAB reaction after avidin–biotin–peroxidase complex amplification (ABC Elite; Vector Laboratories, Burlingame, CA, USA).

Electron Microscopy

Rats were a subset of those used for the prior study of the tVTA/RMTg projection to the VTA (Balcita-Pedicino et al, 2011). For the present analysis, four animals were chosen as also having good transport of PhaL to the SNc. Ultrathin sections (60 nm) were cut from the core of the SNc (with slight inclusion of the immediately adjacent pars reticulata), stained with heavy metals, and examined by electron microscopy as previously described (Balcita-Pedicino et al, 2011). Primary antibodies were specific for PhaL (Vector AS-2300, 1/1000) and TH (MAB318, 1/5000). PhaL visualization was performed with biotinylated secondary antibodies and peroxidase/DAB reaction after avidin–biotin–peroxidase complex amplification. Then, the sections were processed for TH immunogold–silver staining, using gold-conjugated secondary antibody goat anti-rabbit (Aurion, Electron Microscopy Science, 1/50, Hatfield, PA, USA) and IntenSE M silver kit reagent (GE Healthcare, Chalfont St Giles, UK) for the silver intensification of gold label. The semiquantitative ultrastructural analysis was done as previously described (Omelchenko et al, 2009; Balcita-Pedicino et al, 2011).

Electrophysiology

A glass micropipette (2–3 μm tip diameter; 4–6 MΩ) filled with 2% pontamine sky blue in 0.5 M sodium acetate was lowered into the SNc. Dopamine neurons were identified according to their electrophysiological features (Ungless et al, 2004). The extracellular potential was recorded with an Axoclamp2B (Axon Instrument) or Neurodata IR283 (Cygnys Technology; experiment Figure 4b) amplifier in the bridge mode vs a reference electrode in contact with the skull ($\times 10$ gain). The signal was further amplified ($\times 100$ gain) and filtered (low-pass 300 Hz, high-pass 0.5 kHz) via a differential amplifier (AC amplifier 1700, A-M Systems; or Brownlee Precision 440, Instrumentation Amplifier). Single neuron spikes were discriminated and digital pulses were collected online using a laboratory interface and software (CED1401, Spike2; Cambridge Electronic Design; or Datapac 2K2, Run Technologies). The chemical manipulation of the tVTA was done on one cell only per animal. The electrical stimulation of the tVTA and the lesion study allowed multiple recordings from the same animal in which case recording tracks were done 100 μm apart along the mediolateral and/or AP axes. At the end of the experi-

ments, the recording electrode placement was marked by iontophoretic deposit of pontamine sky blue ($-20\,\mu A$, continuous current, 12–15 min). Electrical stimulation sites were marked by electrolytic lesion ($+20\,\mu A$, continuous current, 2 min). Rats were deeply anesthetized with isoflurane (5%) and decapitated. Brains were removed, snap-frozen in isopentane at $-80\,^{\circ}C$, sectioned (30 μm coronal sections), mounted, and stained with neutral red to visualize recording, stimulation, and injection sites.

Electrical stimulation of the tVTA was conducted with a concentric bipolar electrode (250 μm diameter overall, 100 μm diameter inner electrode that extended 100 μm beyond the outer electrode; Phymep). Stimulations (100, 0.2–1.0 mA, 0.5 Hz, 0.5 ms duration pulses) were administered using a square pulse stimulator and stimulus isolator. In one set of experiments, we delivered single pulses of stimulation and in an independent experiment we delivered trains of three consecutive pulses of stimulations, separated by 50 ms, at 1 mA. Responses to electrical stimulation of the tVTA were evaluated and a peristimulus time histogram (PSTH) was generated online (Spike2 software) for each neuron.

An injection pipette (30 μm tip diameter) was used for chemical manipulation of tVTA activity, either stimulation by injecting 60 nl of L-glutamate (100 mM in PBS) or inactivation by injecting 120 nl of muscimol-BODIPY (0.8 mM in PBS).

Rotometer

Each animal was introduced into a cylindrical plastic chamber (40 cm diameter, 46 cm high) placed under a camera. The number of contralateral and ipsilateral rotations was counted. Rats were first tested for baseline rotation, which allowed the exclusion of rats showing spontaneous rotation bias. The chosen time window (30 min) for maximal locomotor activity was between 25 and 55 min post injection. In a first set of experiments, rats underwent stereotaxic surgery for unilateral lesion of the tVTA, of the SNc or of the VTA. After 2 weeks of recovery, rats were tested in the rotometer following an acute injection of saline or amphetamine (3 mg/kg, i.p.). In a second set of experiments, we compared animals with unilateral partial lesion of the SNc to animals with concomitant ipsilateral lesion of the tVTA, the test was done 1 week after the surgeries. The lesions were visualized by using immunohistochemistry directed against TH or NeuN. The extent of the partial unilateral SNc lesion was evaluated by counting the number of SNc TH-positive cells on both sides of the brain, analyzing one section every 160 μm over the full AP extent of the SNc.

Rotarod

Experiment 1. At least 2 weeks after bilateral lesion of the tVTA or the dorsolateral striatum, we assessed the overall rotarod performance (ORP) (Roto-Rod Series 8), a motor parameter shown to be affected in a rat model of Parkinsonism (Rozas et al, 1997). Rats were first trained at low speed (5 rotations per minute (r.p.m.)) to remain on the rod for 5 min. Each fall was recorded and the animal immediately put back on the rotarod until the end of the 5 min training. On day 2, animals were tested at successively

incremented rotation speeds with a 5 min cut off. ORP was calculated as the area under the curve for time against speed plot. On day 3, animals were tested with an accelerating speed ramp (0–45 r.p.m., 120 s). The locomotor activity was also measured during 2 h through a photocell system.

Experiment 2. A separate set of animals was used to assess the effect of bilateral tVTA lesion on motor skill learning. They were tested repeatedly on the rotarod under an accelerating speed ramp, allowing the measurement of increased performance over days (0–45 r.p.m., 5 min cut off, 3 consecutive times per day, over 4 days). The daily values correspond to the average of the three consecutive daily exposures to the rotarod.

Experiment 3. To assess the effect of amphetamine on motor skill learning, rats were tested repeatedly for performance on an accelerating speed ramp (0–45 r.p.m., 180 s) over two days (3 sessions/day, 45 min between sessions). On the first day, a single injection of amphetamine was delivered immediately after the second session.

Experiment 4. The effect of amphetamine on motor performance was assessed in a separate group of animals. Rats were first overtrained with an accelerating speed ramp (0–45 r.p.m., 120 s) during 4 days, which allowed stable performances. On day 5, the animals were tested 30 min after saline or amphetamine injection.

Statistical Analysis

Results are expressed as mean ± SEM. Statistics are given in figure legends. We used Student's t-test or ANOVA to analyze the electrophysiological data. We performed LSD *post hoc* for the analysis of the SNc neuron evoked activity after electrical stimulation and Dunnet *post hoc* for the analysis of neuron activity after chemical manipulation. For the behavioral experiments, we used Student's t-test, test of comparison between the mean and a standard value (rotation experiments and tVTA *vs* DLS comparison), and ANOVAs followed by Duncan *post hoc* when applicable.

RESULTS

The tVTA Neurons Project to the Nigrostriatal System

Using tract tracing, a projection from the posterior end of the VTA to the SNc had been previously described (Ferreira et al, 2008). This SNc projecting region extends beyond the VTA *per se*, encompassing the newly described GABAergic tVTA (Jhou et al, 2009a; Kaufling et al, 2009, 2010a). Consistent with this view, we injected the anterograde tracers BDA (Supplementary Figure S1) or PhaL (Figure 1d–g) into the tVTA, and highlighted a plexus of fibers in the SNc with appositions onto dopamine neurons and processes. To identify the specific link with the nigrostriatal pathway, we then delivered the retrograde tracer CTb into the dorsolateral striatum together with BDA into the tVTA. tVTA fibers displayed appositions onto striatum-projecting SNc neurons (Figure 1a–c). In order to evaluate whether the bouton contacts between tVTA fibers and dopamine neurons were actual synapses, we analyzed the
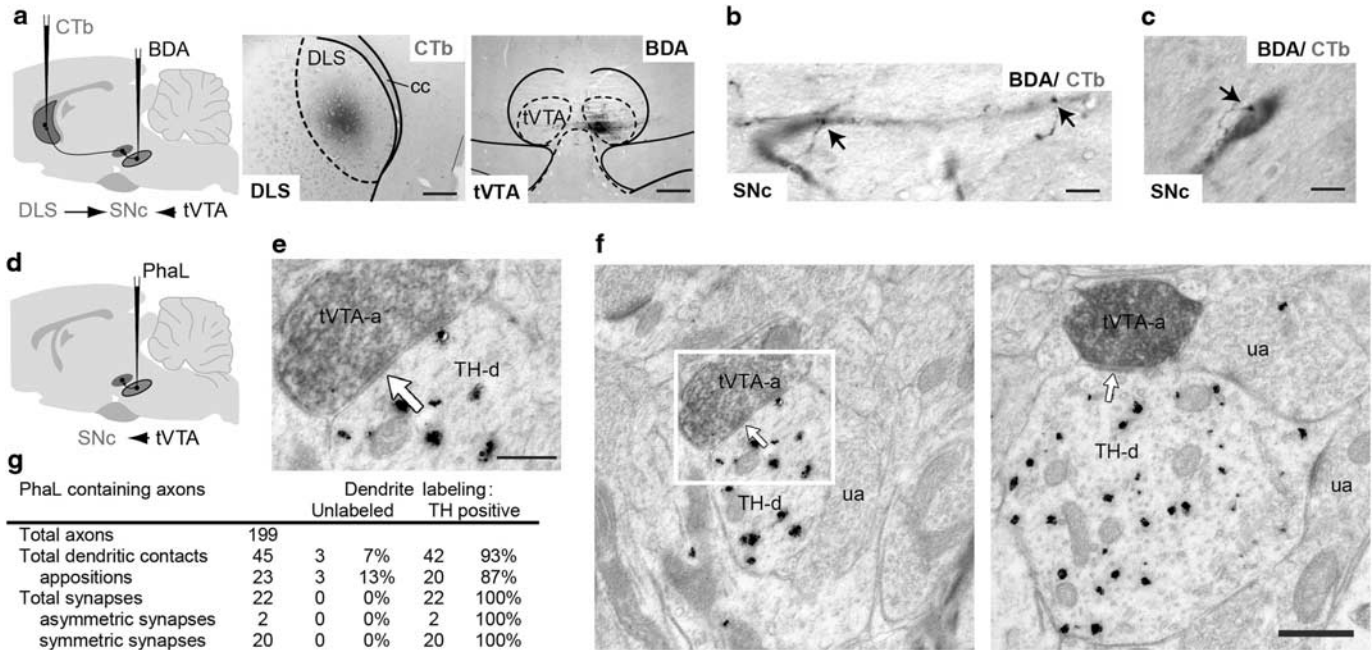
Figure I The tVTA neurons project to the nigrostriatal system. (a) Injection of the anterograde tracer BDA into the tVTA and of the retrograde tracer CTb into the dorsolateral striatum ($n = 5$). (b and c) tVTA terminals (arrows) contact dendrites and cell soma of striatum-projecting SNc neurons. (d) Injection of the anterograde tracer PhaL into the tVTA ($n = 4$). (e and f) Electron micrographs showing tVTA axon terminals (tVTA-a) forming synapses (arrow) onto SNc dendrites labeled for tyrosine hydroxylase (TH-d) and contacted by additional unlabeled axons (ua) (f), larger view; (e), details of boxed area. (g) Quantitative analysis of the electron microscopy experiment. Most of the tVTA terminals synapse onto dendrites in the SNc that are immunoreactive for TH. BDA, biotinylated dextran amine; cc, corpus callosum; CTb, cholera toxin β-subunit; DLS, dorsolateral striatum; PhaL, Phaseolus vulgaris leucoagglutinin. Scale bars, 500 μm (a), 20 μm (b, c), 0.25 μm (e), 0.5 μm (f).

SNc by electron microscopy after PhaL delivery into the tVTA. Most tVTA axons formed symmetric synapses with SNc dendrites immunoreactive for the dopamine synthetic enzyme TH (Figure 1d–g), showing that the tVTA–SNc projection is organized similarly to the tVTA–VTA projection (Balcita-Pedicino et al, 2011).

The tVTA Controls the Activity of SNc Dopamine Neurons

To examine the functional connectivity between the tVTA and the SNc, we then recorded *in vivo* the extracellular activity from single SNc dopamine neurons in anesthetized rats. Ninety-five percent of the recorded neurons were inhibited by a single pulse of electrical stimulation of the tVTA (Figure 2a–e and Supplementary Figure S2), with an onset latency for inhibition of 3.2 ± 1.3 ms and a mean duration of 60.5 ± 11.4 ms. We also observed that the duration of inhibition of SNc dopamine neurons was inversely correlated with the basal firing rate, while the magnitude of the response was uncorrelated (Supplementary Figure S2). Using a train of three pulses of stimulation instead of one, we observed a more prolonged inhibition of SNc cells (206.1 ± 25.0 ms; Supplementary Figure S2). The tVTA is, however, partially embedded within the fiber bundles of the superior cerebellar peduncle (Kaufling et al, 2009) and the dense passing fibers surrounding the tVTA could mediate part of the consequences of the electrical stimulation. Hence, microinjection of L-glutamate or a

labeled GABA$_A$ receptor agonist, muscimol-BODIPY, was used to chemically stimulate or inhibit tVTA neurons, respectively, without affecting passing fibers. These mirror manipulations decreased (L-glutamate) or increased (muscimol-BODIPY) SNc dopamine neuron firing rate ($-61 \pm 6\%$ and $+113 \pm 43\%$ of basal activity, respectively) and bursting rate ($-72 \pm 7\%$ and $+132 \pm 42\%$ of basal bursting rate, respectively) (Figure 2 f–i), thus revealing that the tVTA exerts both phasic and tonic inhibitory controls on the activity of these cells.

The tVTA Controls Motor Behavior

The neuroanatomical and electrophysiological data were supportive of a main inhibitory control exerted by the tVTA onto nigrostriatal dopamine neurons, which raised the question of the behavioral significance of this influence. The rotation model following SNc unilateral lesion has been widely used in Parkinson's disease research, with lesions inducing ipsilateral rotations after amphetamine administration (Figure 3c and d). This rotation bias is specific to the nigrostriatal pathway, as it is not present with VTA-restricted lesions (Kelly and Moore, 1976) (Figure 3e and f). Unilateral excitotoxic lesion of the tVTA alone increased contralateral rotational behavior ($+25.4\%$) (Figure 3a and b), opposite to what was observed following unilateral SNc lesions (Figure 3d) and consistent with a dysregulation of the inhibitory control of SNc dopamine neurons. We then tested whether a lesion of the tVTA might compensate the
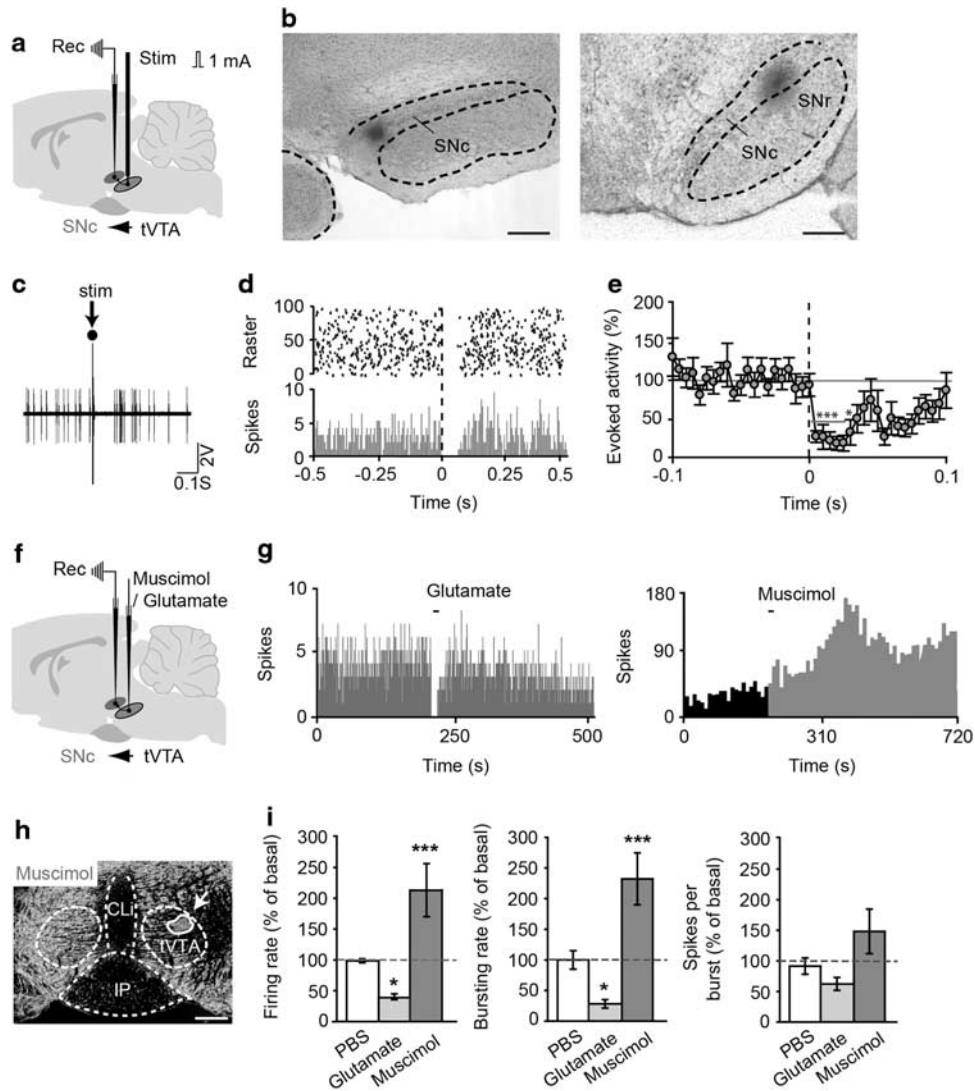
Figure 2 The tVTA controls SNc dopamine neuron activity. (a) Experimental protocol used for electrical stimulation. (b) Microphotographs of coronal sections through the SNc showing blue spots done at the end of recordings (left, anteromedial spot; right, posterolateral spot). (c) Example of a spike trace of a SNc dopamine neuron with a single pulse of tVTA stimulation at 1 mA. (d and e) Consequence of tVTA stimulation on a single SNc dopamine neuron (d) and on the mean of 19 neurons (e), $n = 19$, $F_{40,720} = 4.2$, $*p < 0.05$, $***p < 0.001$). (f) Experimental protocol used for chemical stimulation and inhibition of the tVTA. (g) Examples of unitary activities from single SNc dopamine neurons after tVTA chemical manipulation. (h) Injection site for muscimol-BODIPY in the tVTA. (i) Consequences of tVTA inhibition (muscimol, $n = 9$) or excitation (glutamate, $n = 16$) on SNc dopamine neuron activity (vs PBS vehicle, $n = 15$) (Firing rate $F_{2,37} = 22.3$, $p < 0.001$; bursting rate $F_{2,37} = 23.6$, $p < 0.001$; spikes/burst $F_{2,37} = 4.9$, $p < 0.02$; $*p < 0.05$, $***p < 0.001$). Graphs represent mean ± SEM. CLi, caudal linear nucleus of the raphe; IP, interpeduncular nucleus. Scale bar, 500 μm (b and h).

rotation bias induced by a partial ipsilateral lesion of the SNc. The extent of the SNc lesion (Figure 3g) was similar in both groups ($53.9 \pm 7.1\%$ vs $57.0 \pm 7.5\%$ of SNc dopamine lesion for the SNc and the SNc–tVTA groups, respectively). While a strong ipsilateral amphetamine-induced rotation was observed after partial SNc lesion, this bias was absent in animals with concomitant lesion of the tVTA (Figure 3h).

To further explore nigrostriatal functions, we then studied motor performance. After bilateral tVTA lesion, we observed that the basal activity of SNc dopamine neurons was chronically enhanced (control, 3.5 ± 0.3 Hz; lesion, 5.2 ± 0.5 Hz) (Figure 4b), even though no significant locomotor hyperactivity was observed per se (Jhou et al, 2009b) (Figure 4c). Animals with ablation of the tVTA,

however, displayed a major increase in motor performance in rotarod tasks. During habituation to the apparatus at low speed, tVTA-lesioned rats displayed fewer falls than control rats (control, 3.3 ± 0.5; lesion, 0.6 ± 0.2) (Figure 4d). After the habituation day, control rats had good performance at this low speed (Figure 4e), but a major difference with tVTA-lesioned rats was present at a more challenging fixed speed, with an improvement of ORP in bilateral tVTA-lesioned rats compared with non-lesioned animals (ORP: control, 2262 ± 149; lesion, 4098 ± 450) (Figure 4e and f). The improvement also resulted in the capacity for animals without the tVTA to remain on the rotarod at higher speed than controls, as shown by the fall thresholds under a ramp acceleration procedure (r.p.m: control, 10.0 ± 1.4; lesion,
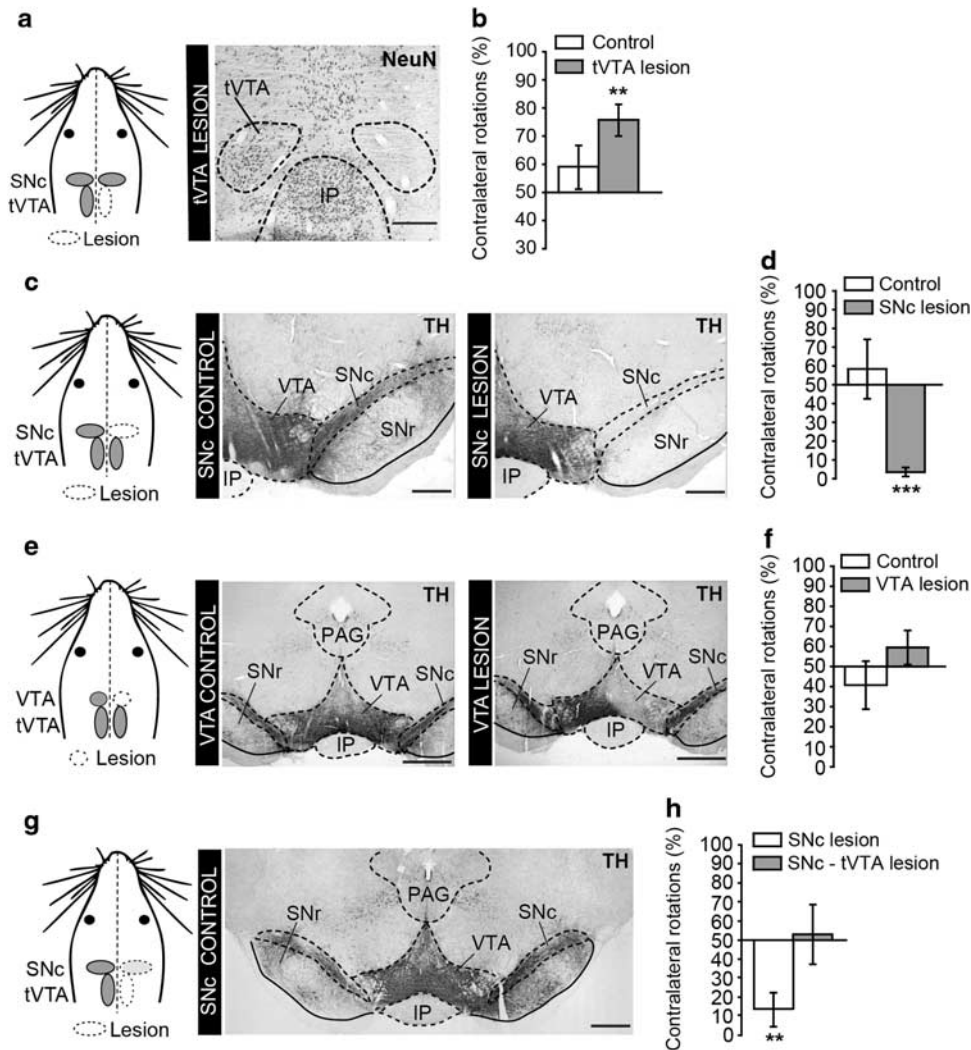
Figure 3 The tVTA controls rotation bias behavior. (a) Experimental protocol and histological evidence for unilateral tVTA lesion. (b) Unilateral tVTA lesion induces a contralateral rotation bias after amphetamine 3 mg/kg (control, $n = 17$; lesion, $n = 12$; **$p < 0.01$). (c) Experimental protocol and histological evidence for 6-OHDA unilateral SNc lesion. (d) Unilateral SNc lesion induces an ipsilateral rotation bias after amphetamine administration (control, $n = 12$; lesion, $n = 14$; ***$p < 0.0001$ referred to 50%). (e) Experimental protocol and histological evidence for unilateral VTA lesion with ibotenic acid. (f) Unilateral VTA lesion does not induce a rotation bias after amphetamine administration (control, $n = 9$; lesion, $n = 7$; referred to 50%). (g) Experimental protocol for unilateral SNc–tVTA lesions. The microphotograph illustrates a left side partial SNc lesion. (h) A tVTA lesion prevents the amphetamine-induced ipsilateral rotation bias observed after partial unilateral SNc lesion (SNc lesion, $n = 8$; SNc–tVTA lesion, $n = 7$; **$p < 0.01$ referred to 50%). Graphs represent mean ± SEM. 6-OHDA, 6-hydroxydopamine; IP, interpeduncular nucleus; PAG, periaqueductal gray; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; TH, tyrosine hydroxylase; VTA, ventral tegmental area. Scale bars, 400 µm (a), 500 µm (c, e, g).

18.5 ± 1.9) (Figure 4g). These performance enhancements following the tVTA ablation are opposite to the impairments observed following dopaminergic lesion of the dorsolateral striatum (Deumens et al, 2002) (Figure 5).

The motor improvement after bilateral tVTA lesion affected not only motor performance but also motor skill learning, as revealed in a specific experiment with repeated training over days (Figure 4h). In these conditions, tVTA-lesioned animals increased their performance over days while a ceiling effect was present in controls (control, 135 ± 22 s on day 3; lesion, 252 ± 40 s on day 4). The increased nigrostriatal activity and associated motor performance following tVTA lesion somewhat parallel those of the 'performance enhancing' drug, amphetamine. Indeed,

amphetamine enhances extracellular dopamine levels (Rebec, 2006; Sulzer, 2011), and in agreement with previous reports (Pryor et al, 1978), we observed that amphetamine increased motor performance in animals that had already learned the motor task (r.p.m: control, 13.9 ± 1.6; amphetamine, 21.2 ± 2.5) (Figure 6a). Interestingly, we also found that amphetamine can improve motor skill learning (Figure 6b). This action can be seen while under the acute influence of the drug (day 1 in Figure 6b), and the resulting improvement is long-lasting, persisting even though the animal is no more under the influence of the drug, as shown by the increased performance still present 24 h after the training done under amphetamine (day 2 in Figure 6b).
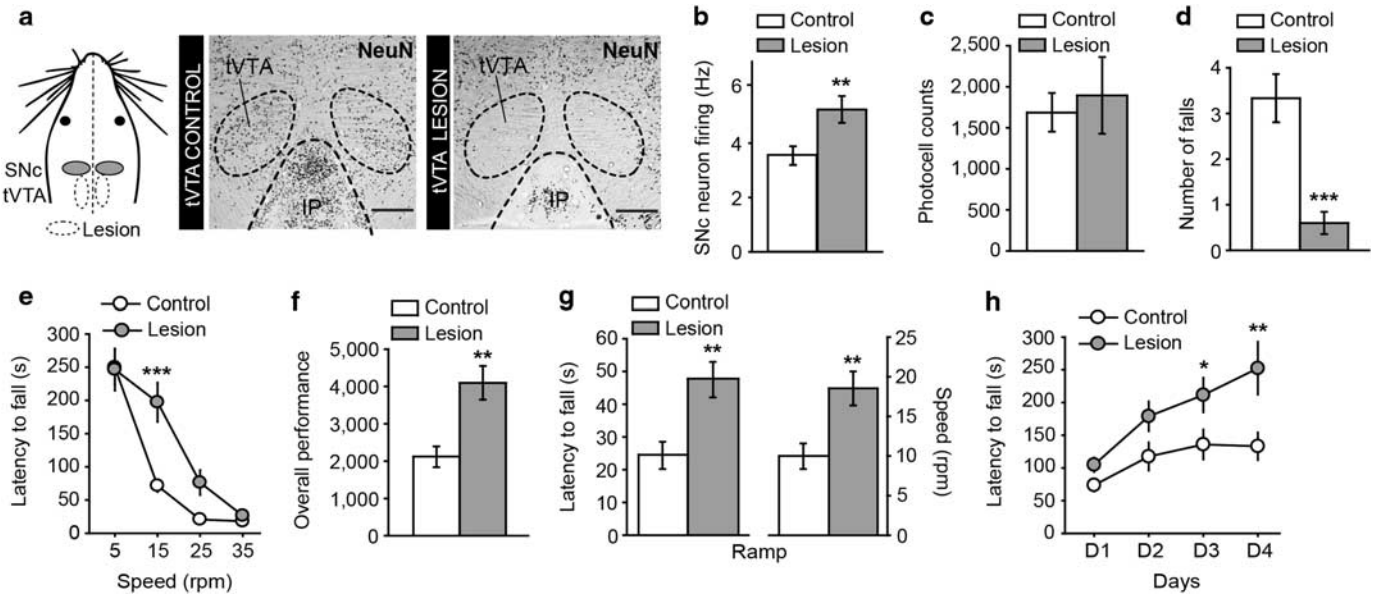
Figure 4 The tVTA controls motor behavior. (a) Experimental protocol and histological evidence for bilateral tVTA lesion. (b) Bilateral tVTA lesion increases SNc neuron basal firing (control, $n = 22$; lesion, $n = 23$; **$p < 0.01$). (c) Locomotor activity of the bilateral tVTA-lesioned animals after subcutaneous saline injection (over 2 h) (control, $n = 9$; lesion, $n = 10$). (d) Number of falls in bilateral tVTA-lesioned and non-lesioned animals during their first rotarod training at low speed (5 r.p.m., 300 s) (control, $n = 9$; lesion, $n = 10$; ***$p < 0.001$). (e) Motor performance of the bilateral tVTA-lesioned animals in a rotarod task at successively increasing fixed speeds (control, $n = 9$; lesion, $n = 10$; $F_{3,51} = 5.1$, $p < 0.01$, ***$p < 0.001$). (f) Overall performance comparison between bilateral tVTA-lesioned and non-lesioned animals, corresponding to e (**$p < 0.01$). (g) Latency to fall and speed of bilateral tVTA-lesioned animals in a ramp rotarod task (0–45 r.p.m. in 120 s) (control, $n = 9$; lesion, $n = 10$; **$p < 0.01$). (h) Motor skill learning of the bilateral tVTA-lesioned animals in a rotarod task measured over successive days (0–45 r.p.m., in 300 s) (control, $n = 10$; lesion = 9; $F_{3,51} = 3.5$, $p < 0.05$; *$p < 0.05$, **$p < 0.01$). Graphs represent mean ± SEM. r.p.m., rotations per minute; IP, interpeduncular nucleus. Scale bars, 500 μm (a).
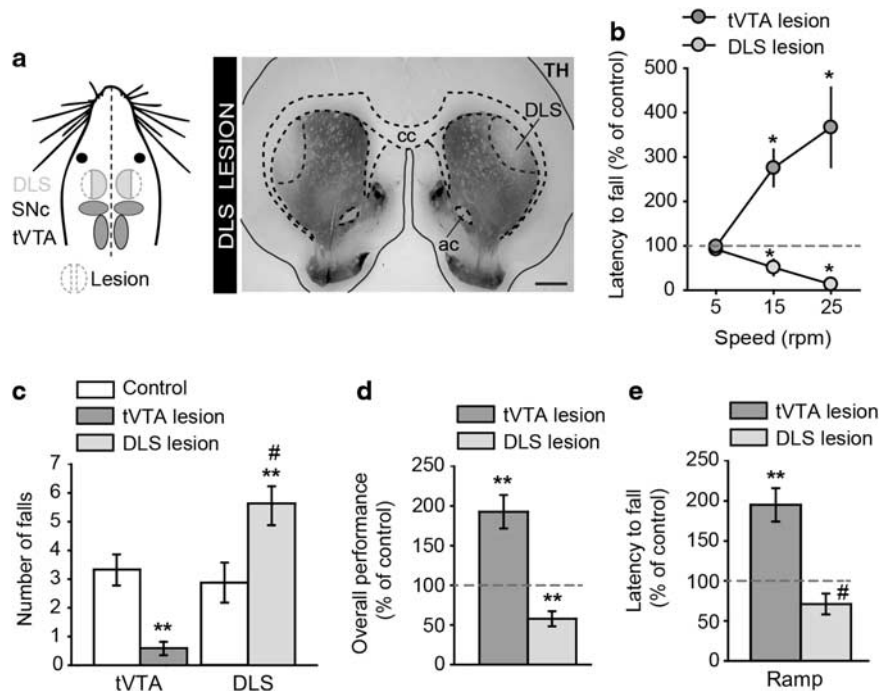

Figure 5 Comparison of motor performances between tVTA and dorsolateral striatum lesions. (a) Experimental protocol and histological evidence for bilateral DLS lesion with 6-OHDA. (b) Latency to fall of bilateral tVTA and DLS-lesioned animals in a rotarod task at fixed speed (tVTA, $n = 10$, DLS, $n = 11$; referred to 100%, *$p < 0.05$. (c) Number of falls during the first day of rotarod training at low speed (control, $n = 8$–9; tVTA lesion, $n = 10$; DLS lesion, $n = 11$; **$p < 0.01$ compared with controls; tVTA–DLS comparison, #$p < 0.001$). (d) Overall performance comparison corresponding to Figure 5b (**$p < 0.01$ referred to 100%). (e) Latency to fall in a ramp rotarod task (0–45 r.p.m. in 120 s) (tVTA, $n = 10$; DLS, $n = 11$; **$p < 0.01$, #$p = 0.0507$, referred to 100%). Graphs represent mean ± SEM. ac, anterior commissure; cc, corpus callosum; DLS, dorsolateral striatum; r.p.m., rotations per minute; TH, tyrosine hydroxylase. Scale bar, 1 mm (a).
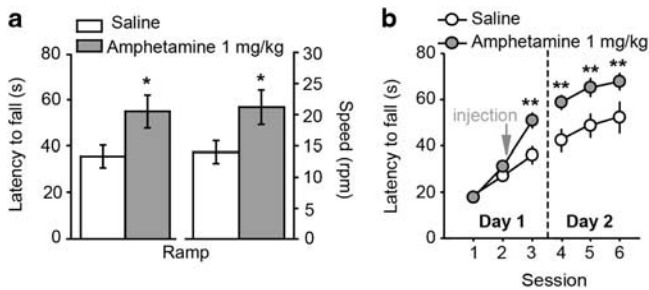
Figure 6 Amphetamine increases motor performance. (a) Amphetamine increases motor performance in a learned rotarod task (0–45 r.p.m., 120 s) (saline, $n = 10$; amphetamine, $n = 10$; *$p < 0.05$). (b) Amphetamine increases motor skill learning in a rotarod task assessed over successive days (0–45 r.p.m., 180 s) (saline, $n = 12$; amphetamine, $n = 13$; $F_{5,115} = 3.6$, $p < 0.01$, **$p < 0.01$). Graphs represent mean ± SEM.

DISCUSSION

In this study, tract tracing and electron microscopy approaches allowed the visualization of a tVTA–nigrostriatal pathway. We then revealed the inhibitory influence of tVTA on the activity of SNc dopamine neurons by conducting *in vivo* electrophysiological studies. Together, these findings were supportive of a main role of the tVTA as a GABA brake for the nigrostriatal pathway. We then confirmed it at a behavioral level, first by observing an amphetamine-induced contralateral rotation bias following unilateral tVTA ablation, then by studying motor coordination and motor skill learning performances after bilateral ablation of the tVTA. The behavioral consequences of tVTA lesion are similar to the performance-enhancing action of a low dose of amphetamine, which also recruits dopamine systems. These findings highlight the major influence that the tVTA exerts over basal ganglia functions.

The anatomical data reveal a heavy projection from the tVTA to the nigrostriatal system. While studying the possible interconnections between the mesencephalic dopaminergic areas, a discrete subregion in the caudal pole of the VTA was previously shown to display efferents to the VTA–SNc complex (Ferreira *et al*, 2008). This subregion is now identified as the rostral part of the tVTA, a long GABA structure whose first tier is bilaterally inserted within the VTA and that spans rostrocaudally over 1 mm in rats, laterally to the median raphe nucleus and partially embedded with the superior cerebellar peduncle (Jhou *et al*, 2009a, 2009b; Kaufling *et al*, 2009, 2010a). Study of the tVTA–VTA efferents revealed that more than 80% of the synapses formed by tVTA axons in the VTA contacted the dendrites of dopamine cells (Balcita-Pedicino *et al*, 2011). The present data confirm the presence of a tVTA–SNc projection, and show appositions from tVTA fibers onto nigrostriatal neurons and the presence of symmetric synaptic connections between tVTA axons and TH-containing dendrites in the SNc. This structural organization is thus similar to the one that was observed in the VTA (Balcita-Pedicino *et al*, 2011), suggesting that the GABAergic tVTA should exert a main inhibitory control on both populations of dopamine cells.

Dopamine cells are under the control of excitatory and inhibitory inputs whose balance finely tunes cell activity

(Morikawa and Paladini, 2011). The tVTA is now recognized among the important inhibitory control regions for VTA cells (Barrot *et al*, 2012; Bourdy and Barrot, 2012; Jalabert *et al*, 2011; Lecca *et al*, 2011, 2012; Matsui and Williams, 2011), and the present data demonstrate that the tVTA is also critical for the regulation of SNc dopamine cell activity. Indeed, we show that the tVTA influences both the firing rate and bursting rate of SNc dopamine cells and that this control is both phasic and tonic. Single-pulse electrical stimulation of GABAergic inputs originating in the striatum, the globus pallidus or the substantia nigra pars reticulata produces an inhibition lasting around 30 ms (Paladini *et al*, 1999), whereas a similar stimulation in the tVTA produces an inhibition lasting around 60 ms (Figure 2c–e). These two independent sets of data collected in very similar experimental conditions highly suggest that the tVTA exerts a greater inhibition of the SNc dopamine neurons than the classical inhibitory inputs arising from the basal ganglia itself.

The presence of excitatory glutamate signaling can also be important to increase dopamine cell activity following the removal of the GABA brake on these neurons (Jalabert *et al*, 2011). In the case of SNc dopamine cells, such excitatory inputs are provided by structures within the basal ganglia circuitry but also from the somatosensory and from the primary and secondary motor cortices (Watabe-Uchida *et al*, 2012). This connectivity suggests that removing the tVTA braking influence could amplify the impact of these sensorimotor-related excitatory afferents on the nigrostriatal system whose stimulated state could then favor specific striatal functions.

Previous functional data on the tVTA concerned its influence on the mesolimbic system, more particularly in relation to aversive and avoidance behaviors (Jhou *et al*, 2009b; Stamatakis and Stuber, 2012) and to drugs of abuse (Jalabert *et al*, 2011; Jhou *et al*, 2012, 2013; Lecca *et al*, 2011, 2012; Matsui and Williams, 2011). Thus, GABA cells of the tVTA are targets for psychostimulants, including amphetamines, as shown by the expression of Fos proteins (Cornish *et al*, 2012; Jhou *et al*, 2009a, 2009b; Kaufling *et al*, 2009, 2010a, 2010b; Geisler *et al*, 2008; Lavezzi *et al*, 2011; Lecca *et al*, 2011; Matsui and Williams, 2011; Perroti *et al*, 2005; Rotllant *et al*, 2010; Scammel *et al*, 2000; Zahm *et al*, 2010). In rats, this expression is classically used to visualize the tVTA. Electrophysiologically, the tVTA neurons are also targets for cannabinoids and opiates (Jalabert *et al*, 2011; Lecca *et al*, 2011, 2012; Matsui and Williams, 2011). In the latter case, investigations of the tVTA recently led to an updated disinhibition model for opiate recruitment of VTA dopamine cells (Bourdy and Barrot, 2012; Jalabert *et al*, 2011; Johnson and North, 1992; Matsui and Williams, 2011) and behavioral evidence further supports the role of the tVTA in the rewarding and reinforcing properties of opiates (Jhou *et al*, 2012). Behaviorally, the tVTA has also been shown to be important for freezing in a fear conditioning paradigm or to a predator odor (Jhou *et al*, 2009b), and to promote active, passive, and conditioned avoidance behaviors when its inputs from the lateral habenula are stimulated (Stamatakis and Stuber, 2012). In monkeys, tVTA neurons can detect reward prediction errors (Hong *et al*, 2011). While progress has been made concerning the physiology of this brain region, behavioral studies have

focused on reward/avoidance aspects. The neuroanatomical and electrophysiological data nevertheless suggest that the tVTA should also regulate nigrostriatal functions such as motor control.

Rotation bias and motor coordination performance are supportive of a main influence of the tVTA on nigrostriatal functions. The amphetamine-induced rotation bias following unilateral lesions is selective for the nigrostriatal pathway, as shown by the lack of such bias following unilateral VTA lesion (Kelly and Moore, 1976; present data); and the opposite bias observed with SNc and tVTA lesions is in agreement with an inhibitory influence of tVTA on the nigrostriatal pathway. The tVTA-lesion rotation bias is, however, milder than the SNc lesion one, which may be related to the connectivity of these regions and/or to amphetamine's properties. Indeed, the tVTA–SNc pathway also includes contralaterally directed fibers, although it is principally ipsilateral (Jhou et al, 2009a; Kaufling et al, 2010a), whereas the nigrostriatal pathway is more strictly lateralized. Moreover, amphetamine is able to directly release dopamine (Sulzer, 2011), an action that would be less affected by changes in dopamine neuron activity. Despite this mild effect of tVTA lesion alone, such a lesion can compensate for the strong rotation bias induced by a partial ipsilateral lesion of the SNc, which further highlights the control exerted by the tVTA on the nigrostriatal pathway.

Under mildly increased dopamine cell activity, as obtained by removing the tVTA GABA brake on SNc neurons, or after mildly enhanced aminergic transmission, as provided by a low dose of amphetamine, we observed an improvement of both motor performance and motor-related learning. The execution of challenging coordinated movements requires a concerted action of motor and sensory systems, for which the cerebral cortex, the cerebellum, and the basal ganglia are crucial components (Groenewegen, 2003). The fine-tuning of these brain regions participates not only in the execution of movement but also in associated motor learning (Groenewegen, 2003; Penhune and Steele, 2012). Indeed, complex movements have to be learned and practiced repeatedly to improve their execution. Nigrostriatal dopamine is an important modulator of these mechanisms, which are impaired following the partial loss or disruption of this pathway (Akita et al, 2006; Beeler et al, 2010; Ogura et al, 2005; Willuhn and Steiner, 2008). In this context, plasticity at glutamatergic corticostriatal synapses within the dorsal striatum has been implicated in motor learning (Dang et al, 2006; Pisani et al, 2005; Yin et al, 2009) and is regulated by dopamine (Calabresi et al, 2007). Both D_1 receptor-expressing striatonigral and D_2 expressing striatopallidal medium-sized spiny neurons appear to be critical for optimal performance during early motor learning (Durieux et al, 2012). However, striatonigral neurons seem to be more crucial than striatopallidal neurons for longer-term gradual motor skill acquisition or for the execution of a previously learned motor sequence (Durieux et al, 2012), even though synaptic potentiation is preferentially expressed in striatopallidal neurons after extensive motor training (Yin et al, 2009). The tVTA, by setting the level of activity of dopamine systems, appears to be a major controller for basal ganglia functions. The fact that the lesion of the tVTA or a low dose

of amphetamine lead to motor improvement suggests that these manipulations may operate within the physiological range and could thus favor a harmonious recruitment of the basal ganglia circuits and of the above-mentioned plasticity. In this respect, much higher doses of amphetamine are known to impair motor performance (Gerald and Gupta, 1977). Further studies will, however, be required to detail the mechanism leading to motor improvement after tVTA ablation, and in particular, its impact on the direct and indirect pathways and on striatal plasticity.

The neuroanatomical, electrophysiological and behavioral evidence supports a main role of the tVTA as a GABA brake for dopamine systems, and they raise the question of tVTA integration within basal ganglia circuitry (Albin et al, 1989; Redgrave et al, 2010; Tepper and Lee, 2007). Increasing nigrostriatal transmission in this circuit, either through tVTA suppression or an amphetamine-like performance enhancer, not only improves motor coordination but also motor skill learning, highlighting the learning influence of the dopamine system (Wise, 2004). These data warrant further functional research on the tVTA influence over dopamine-related functions in both physiological and dopamine-related pathological contexts.

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