Development/Plasticity/Repair

Dopaminergic Projections from Midbrain to Primary Motor Cortex Mediate Motor Skill Learning

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The primary motor cortex (M1) of the rat contains dopaminergic terminals. The origin of this dopaminergic projection and its functional role for movement are obscure. Other areas of cortex receive dopaminergic projections from the ventral tegmental area (VTA) of the midbrain, and these projections are involved in learning phenomena. We therefore hypothesized that M1 receives a dopaminergic projection from VTA and that this projection mediates the learning of a motor skill by inducing cellular plasticity events in M1. Retrograde tracing from M1 of Long–Evans rats in conjunction with tyrosine hydroxylase immunohistochemistry identified dopaminergic cell bodies in VTA. Electrical stimulation of VTA induced expression of the immediate-early gene c-*fos* in M1, which was blocked by intracortical injections of D_1 and D_2 antagonists. Destroying VTA dopaminergic neurons prevented the improvements in forelimb reaching seen in controls during daily training. Learning recovered on administration of levodopa into the M1 of VTA-lesioned animals. Lesioning VTA did not affect performance of an already learned skill, hence, left movement execution intact. These findings provide evidence that dopaminergic terminals in M1 originate in VTA, contribute to M1 plasticity, and are necessary for successful motor skill learning. Because VTA dopaminergic neurons are known to signal rewards, the VTA-to-M1 projection is a candidate for relaying reward information that could directly support the encoding of a motor skill within M1.

Introduction

The primary motor cortex (M1) is involved in the acquisition of novel movement sequences and skills (Nudo et al., 1996; Kleim et al., 1998; Luft et al., 2004; Molina-Luna et al., 2008). M1 harbors dopaminergic terminals (for review, see Luft and Schwarz, 2009) that optimize motor skill learning and the ability of M1 synapses to undergo long-term potentiation (LTP)—LTP is a cellular mechanism that is used in M1 during skill acquisition (Rioult-Pedotti et al., 2000, 2007). Blocking dopaminergic transmission in M1 reduces LTP and the effectiveness of skill learning but leaves synaptic transmission and movement execution unaffected (Molina-Luna et al., 2009).

One potential origin of dopaminergic terminals in M1 are midbrain dopaminergic nuclei, which are known to project to prefrontal cortex (Lindvall et al., 1974) (for review, see Luft and Schwarz, 2009). These mesocortical projections play an important role in associative learning paradigms and in the development of addictions by relaying information about reward stimuli to cortex (Schultz, 2006). The mesocortical dopaminergic system is anatomically and functionally distinct from the nigrostriatal system that is critically involved in the control of muscle tone and movement execution (Fuxe et al., 2006).

Here, we test the hypothesis that the dopaminergic terminals in M1 arise in the ventral tegmental area (VTA) of the midbrain and that this projection is necessary for skill learning and cortical plasticity. Using retrograde tracing from M1 and electrical stimulation of VTA, we show that this is indeed the case. Destroying VTA dopaminergic neurons impairs motor skill learning. This impairment partially recovers when dopamine is supplemented by the dopamine precursor levodopa into M1.

Materials and Methods

Animals and surgical procedures. Adult male Long–Evans rats ($n = 67$; 8–10 weeks; 250–350 g; Janvier) were used for all experiments. Animals were housed individually in a 12 h light/dark cycle (light on, 8:00 P.M.; off, 8:00 A.M.). Littermates were distributed equally between experimental groups. All experiments were conducted in accordance with German and Swiss regulations and were approved either by the Animal Commission of the State of Baden-Württemberg or the Committee for Animal Experimentation of the Canton of Zürich. Chemicals and antibodies were purchased from Sigma-Aldrich, unless noted otherwise.

All surgical procedures were performed under ketamine (75 mg/kg, i.p.) and xylazine anesthesia (10 mg/kg, i.p.) with the rats fixated in a stereotactic frame (Stoelting). Additional ketamine doses (30 mg/kg, i.p.) were administered if necessary. Body temperature was controlled using a heating pad. Buprenorphin (0.01 mg/kg, i.p.) was given after surgery for pain relief. All permanent implants were anchored onto the skull by two screws (2 mm diameter) placed in the frontal and occipital skull. Bone flaps were replaced and fixated using bone cement (FlowLine; Heraus Kulzer).

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Injection needles and electrode implantations into VTA were performed using a computer-controlled stereotaxic instrument (Dual Benchmark Angle One; Harvard Apparatus) at coordinates −5.5 mm behind and 0.5 mm lateral of bregma in a depth of 7.8 mm (Paxinos and Watson, 1998) and a microliter injection pump (Nano-injector; Stoelting). After an injection, the needle was left in place for 5 min before being slowly retracted.

M1 infusions of levodopa (5 mg/ml plus 0.2 mg/ml ascorbic acid in saline, 0.9%) was performed using osmotic minipumps (100 μl volume; model 1002; Alzet) that delivered 0.25 μl of solution per hour over a period of 2 weeks. Pump reservoirs were implanted subcutaneously in the neck area and were connected to needles using polyethylene tubing. Needles were placed in the center of the M1 forelimb representation [coordinates, 2 mm lateral and 2 mm anterior to bregma; depth, 800 μm; according to Paxinos and Watson (1998)] contralateral to the limb preferred for reaching. Rats were allowed to recover for 24 h.

For every animal, correct positioning of needles and electrodes was verified histologically by either Nissl staining (M1) or, in the case of VTA, the analysis of anti-tyrosine hydroxylase (TH) immunohistochemistry that allowed for the localization of this region by staining dopaminergic neurons. No animal had to be excluded because of needle or electrode misplacement.

Immunohistochemical procedures. Animals were deeply sedated (pentobarbital, 50 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde (PFA). Brains were removed and kept in 4% PFA for 24 h and transferred to 30% sucrose solution for 3–4 d before rapid freezing in 2-methyl buthan. Coronal sections (50 μm) were prepared using a cryostat (Leica Microsystems). Free-floating sections were rinsed three times in 0.05 M Tris-buffered saline (TBS), treated with 3% H_2O_2 for 30 min, washed three times in 0.05 M TBS, rinsed in 0.1% Triton for 10 min, and blocked for 30 min in 10% fetal cow serum. Sections were incubated with primary antibody (1:200 diluted in 0.05 M TBS and 5% fetal cow serum) for 24 h at 4°C, washed three times in 0.05 M TBS, and incubated with a Cy3-coupled secondary antibody (1:200 diluted in 0.05 M TBS and 2.5% fetal cow serum) at 4°C for 90 min. Sections were mounted with Vectashield (Vector Laboratories) and analyzed using a fluorescent microscope (Axioplan II; Zeiss; equipped with motorized *x–y* stage and 40×/0.75 EC Plan-Neofluar objective).

For anti-TH immunohistochemistry, a monoclonal mouse antibody (Millipore Bioscience Research Reagents) was used as primary, and a goat anti-mouse IgG-Cy3 (Zymed Laboratories) was used as secondary antibody. For c-*fos* immunohistochemistry, monoclonal rabbit anti-c-*fos* antibody (Cell Signaling Technology) and goat anti-rabbit IgG-Cy3 (Jackson ImmunoResearch Laboratories) was used.

Retrograde tracing. Naive rats received 200 nl injections of the retrograde fluorescent tracer Fast Blue (FB) (EMS-Polyloy; 1% suspension in 0.1 M PB and 2% DMSO) at five adjacent injection positions into the M1 forelimb representation of the right hemisphere (depth: 800 μm; coordinates with respect to bregma: first, 1.5 mm lateral, 2.5 mm anterior; second, 2.5 mm lateral, 2.5 mm anterior; third, 2 mm anterior, 2 mm lateral; fourth, 1.5 mm lateral, 1.5 mm anterior; fifth, 2.5 mm lateral, 1.5 mm anterior) over 3 min. Animals were killed 7 d after injection, and TH staining of sections 5.2–7.2 mm posterior to bregma (Paxinos and Watson, 1998) containing midbrain dopaminergic regions was performed. Every fourth section was analyzed quantitatively using StereoInvestigator (MicroBrightField). Based on TH positivity, the contours of VTA, substantia nigra (SN), and the retrorubral field (RRF) were traced on sequential sections. Then, FB-labeled neurons were marked and checked for TH positivity. These neurons are further referred to as double-labeled. All TH-positive cells were quantified by unbiased stereology using the optical fractionator routine of StereoInvestigator (MicroBrightField). Sampling regions were defined using a counting frame of 50 × 50 μm (*x–y* plane) and an optical disector height of 22 μm (*z* plane). The distance between sampling regions was 300 μm in *x*- and 200 μm in *y*-direction. Small-stepwise adjustments of the focus allowed for an assessment of the entire section volume.

*VTA stimulation and c-*fos *expression.* Monopolar tungsten microelectrodes (shank diameter, 125 μm; impedance at 1 kHz, 0.1 MΩ; FHC) were inserted in the right VTA. The reference was connected to a screw implanted in the occipital skull. Constant current pulses (2 ms duration; 300 μA amplitude, cathodal stimulation) were delivered at a frequency of 20 Hz for 5 min. The control group (sham) had the electrode implanted but received no stimulation.

For c-*fos* immunohistochemistry, M1 (4 mm anterior, 1 mm posterior to bregma) ipsilateral to VTA was sectioned at 50 μm, and every 10th section was analyzed. Unbiased stereological quantification of c-*fos*-positive neurons was performed as described above [counting frame: 50 × 50 μm (*x–y* plane); optical disector height, 22 μm (*z* plane); distance between sampling regions, 600 μm in *x*- and 500 μm in *y*-direction].

Dopamine antagonist application. A combination (0.5 μl) of the D_1 antagonist 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol (SCH 23390) (0.6 μg/μl; Tocris Bioscience) and the D_2 antagonist raclopride [10 μg/μl; *S*(−)-raclopride (+)-tartrate salt] was injected into M1 (2 mm lateral, 2 mm anterior to bregma, 800 μm from surface) 30 min before ipsilateral VTA stimulation. The control group received vehicle injections.

Because the spread of DA antagonists was limited as previously reported (Luft et al., 2004; Molina-Luna et al., 2009), every fourth section of a smaller brain volume adjacent to the injection site (right hemisphere, 3 mm anterior to 1 mm anterior to bregma) was analyzed. Unpaired *t* tests were used to compare the number of c-*fos*-positive neurons between the different groups (VTA-stim vs sham; VTA-stim plus $D_{1/2}$ antagonists vs vehicle).

Western blot analysis. M1 dopaminergic terminals were quantified after lesioning VTA by anti-TH Western blots. The right VTA was injected with 6-OHDA after rats had received desipramine, 20 mg/kg, i.p.) to protect noradrenergic neurons. Controls received vehicle (0.5 ml of 0.1% ascorbic acid in 0.9% NaCl) and desipramine. After 3 d, animals were deeply sedated (pentobarbital; 50 mg/kg, i.p.) and transcardially perfused with PBS. Brains were quickly dissected over ice to isolate the ipsilateral M1 before rapid freezing in 2-methyl buthan. Tissue samples were pestled in liquid nitrogen and subsequently brought into lysis buffer containing protease inhibitors. Lysates were centrifuged at 6000 rpm for 5 min and the supernatant extracted for gel electrophoresis. Equal amounts of lysates were transferred to SDS-PAGE gels and then to nitrocellulose membranes. After blocking with 5% Blotting Grade Blocker (Bio-Rad Laboratories), blots were incubated with primary monoclonal mouse anti-TH antibody (1:1000; Millipore Bioscience Research Reagents). Antibody binding was detected using a horseradish-conjugated secondary goat anti-mouse antibody (1:1000; Santa Cruz Biotechnology) and enhanced chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific). Membranes were washed three times for 10 min in 0.05% PBS-Tween and sequentially reprobed with a monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:1000; Jackson ImmunoResearch) to ensure equal protein loading across samples. Exposed films were scanned (G:Box; Syngene) and analyzed using band densitometry (GeneTools; Syngene). Unpaired *t* tests were used to compare densitometry results between groups. Additionally, the brains of two VTA-lesioned and two control rats were evaluated using anti-TH immunohistochemistry.

VTA lesion. Rats were injected either with 0.5 μl of 6-OHDA (6 μg/μl in saline containing 0.1% ascorbic acid) to selectively eliminate dopaminergic neurons in VTA or vehicle (sham). To protect noradrenergic neurons, desipramine was administered (20 mg/kg, i.p.) 1 h before 6-OHDA. After 72 h of recovery, reach training was performed for eight sessions before an osmotic minipump containing either levodopa (5 mg/ml plus 0.2 mg/ml ascorbic acid in saline, 0.9%) or vehicle was implanted. The sham group also received levodopa-containing minipumps. Twenty-four hours were allowed for recovery before reach training was continued for 8 d. Then, pumps and infusion needles were explanted and training was continued for 8 additional days.

Motor skill training and analysis. To investigate the behavioral role of dopaminergic projections from VTA to M1, rats were trained in a precision forelimb reaching task. Training sessions were performed at the beginning of the dark phase. Animals were food-restricted for 24 h before the first session of pretraining. During subsequent training, animals were kept slightly over their initial weight (336.7 ± 31.2 g) by providing 50

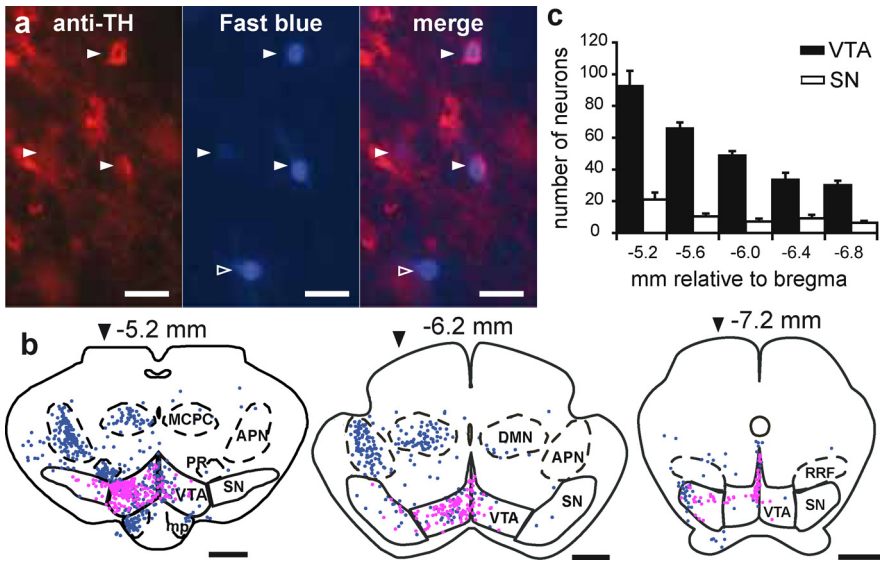

Figure 1. Retrograde tracing from M1 identifies dopaminergic neurons in the VTA and SN. ***a***, Representative images from VTA (magnification, 20×). The white arrowheads indicate dopaminergic neurons (left, anti-TH), neurons projecting to M1 (middle, retrograde Fast Blue labeling), double-labeled neurons (right, anti-TH and Fast Blue). The open arrowheads indicate a non-dopaminergic neuron (anti-TH negative) projecting to M1 (Fast Blue-positive). Scale bar, 30 μm. ***b***, Superposition of double-labeled neurons (magenta) and Fast Blue-labeled neurons (blue) in three representative sections (positions relative to bregma) derived from six animals. ▼, Tracer-injected hemisphere; APN, anterior pretectal nucleus; DMN, deep mesencephalic nucleus; MCPC, magnocellular nucleus of posterior commissure; mp, mamillary peduncle; PR, prerubral field. Scale bars, 1 mm. ***c***, Average number of double-labeled neurons in VTA and SN for selected sections (position relative to bregma; error bars indicate SEM).

mg/kg standard laboratory diet after each training session. Water was given *ad libitum*.

The reaching task was performed as previously described (Buitrago et al., 2004b). The training cage was a 15 × 40 cm chamber (height, 30 cm) with a vertical window (1 cm wide, 5 cm high, lower edge 2 cm above ground) in the front wall and a small light sensor in the rear wall (7 cm above ground). Animals were first pretrained for 5 d learning to open the motorized sliding door that covered the front window by nose poking the sensor in the rear. Opening the window gave access to one food pellet (45 mg; Bioserve) located on a horizontal board so close to the window (0.5 cm relative to the outside window edge) to allow retrieval by tongue. Pellets were automatically replaced by a pellet dispenser (Lafayette Instrument). At the end of the last pretraining session, forelimb preference was determined by moving the pellet further away from the window and observing the rats' forelimb use. Forelimb reaching was then trained for 12 or 24 d. The pellet board was replaced by a small vertical post 1.5 cm away from the window, a position in which pellets were only retrievable by forelimb. Because the diameter of the post was approximately that of the pellet, the pellet was in a position from which it was easily kicked off the post. The post was shifted to the side opposite of the preferred forelimb to allow for reaching with the preferred limb only. To retrieve the pellet rats had to extend the forelimb toward the target, pronate, open the paw, grasp, and pull the forelimb back while supinating to bring the pellet toward the mouth (Whishaw and Pellis, 1990). Each reaching trial was scored as "successful" (reach, grasp, and retrieve) or "unsuccessful" (pellet pushed off pedestal or dropped during retraction). One session consisted of 100 trials. The average number of forelimb movements per session was 141 ± 5.7 (mean ± SEM, recorded by a sensor between cage wall and pellet pedestal). Sessions lasted 22 ± 0.5 min (mean ± SEM). Success rates were analyzed using JMP (version 7; SAS Institute). First and second 8 d training phases were analyzed separately. General linear models were used to test for effects of training day and group (including their interaction) on success rate. Whether data met the sphericity condition was tested using Mauchly's criterion, and if not met, Geisser and Greenhouse correction was used. *Post hoc* tests were performed using Bonferroni's correction for multiple comparisons.

For within-session analysis, the individual performance-over-time curves were approximated by an exponential function [$y = \text{plateau}/(1 + e^{k*(a - x)})$] and the estimated plateau values were compared between groups using Wilcoxon's signed-rank tests.

To construct between-session learning curves, reaching performance per session was quantified by calculating the success rate [number of successful trials/total number of trials per session (i.e., 100)]. Within-session performance curves were constructed using a two-step procedure: (1) step–time curves were computed using Matlab (version 2009a; The MathWorks). Each step represented the result of one reaching trial, a step of +1 if the pellet was grasped but dropped while pulling the forelimb back and a step of +3 if the pellet was successfully retrieved—"+3" was assigned to successful retrieval to emphasize the value of this performance result over a mere grasp ("+1"). If the pellet was kicked of without grasping it, step size was zero. Step–time curves were then converted to splines and smoothed (Matlab function "spaps"). (2) Because step–time curves represented cumulative performance over time, the first derivative had to be computed to obtain performance over time curves (Matlab function "fnder"). Derivation curves were then averaged across animals.

The latency between pellet removal and subsequent door opening was used as an index of motivation.

To screen for motor deficits related to surgery or injection, an accelerated rotarod test was performed at baseline, 24 h after VTA lesioning, after implantation of the osmotic pump and after the first intraperitoneal injection. The rod was 7 cm in diameter and accelerated at 1 cm/s^2. Maximum velocity at the time the rat fell off the rod was used to index motor function. Twenty runs were performed per session with a 15 s rest period between runs. Because rotarod performance improves with practice (Buitrago et al., 2004a), two training sessions were performed on 2 consecutive days before surgery. For the analysis, data from the second session were considered as the presurgical baseline.

Results

Dopaminergic neurons projecting to M1 are located in VTA

For the hemisphere analyzed, 48% of TH-positive midbrain neurons were located in the VTA (11,623 ± 249 neurons; $n = 6$), 47% were found in the SN (10,895 ± 292 neurons; $n = 6$), and 5% were detected in the RRF (1246 ± 95 neurons; $n = 6$).

Intracortical injection of the retrograde tracer Fast Blue into the M1 forelimb area combined with anti-TH immunostaining revealed the VTA as the main source of dopaminergic projections to M1. Double-labeled neurons (Fig. 1*a*, arrowheads) were found in the ipsilateral VTA (630 ± 106 neurons, equates to 5.4% of TH-positive cells in that region; $n = 6$). Fewer cells were identified in the contralateral VTA (104 ± 14 neurons, equates to 0.9% of TH-positive cells in that region; paired t test, $p = 0.006$; $n = 6$). The number of double-labeled neurons decreased in rostrocaudal direction (Fig. 1*b*). Additionally, a lateromedial gradient of decreasing density was observed in the rostral part of VTA, whereas the caudal part showed a mediolateral gradient (Fig. 1*c*). Some double-labeled neurons were also identified in the SN (ipsilateral, 125 ± 22, equates to 1.2% of TH-positive cells in that region; contralateral, 5 ± 3, equates to 0.05% of TH-positive cells in that region; paired t test, $p = 0.007$), but those were signifi-

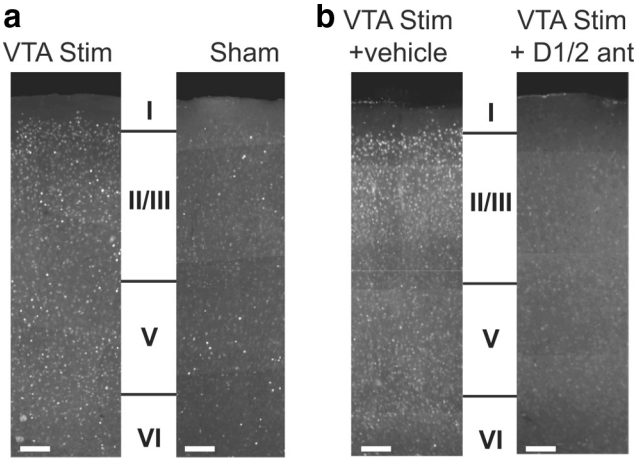

Figure 2. VTA stimulation induces c-*fos* expression in M1 via release of dopamine. ***a***, Representative image of M1 demonstrating c-*fos* expression in response to ipsilateral VTA stimulation (VTA Stim) and unstimulated control (Sham). Images were taken from the right hemisphere, 2.5 mm anterior to bregma. Scale bars, 250 μm. ***b***, Representative images of c-*fos* expression patterns in M1 after VTA stimulation combined with either intracortical vehicle (VTA Stim + vehicle) or D_1 plus D_2 antagonist injections (VTA Stim + D1/2 ant). Images were taken from the right hemisphere at 2.5 mm anterior to bregma. Scale bars, 250 μm.

cantly fewer than in VTA (paired *t* test, $p = 0.004$), suggesting the VTA as the main source of dopaminergic projections to M1. No double-labeled neurons were detected in the RRF.

VTA stimulation induced DA-dependent c-*fos* expression in M1

DA is known to induce the expression of the immediate-early gene (IEG) c-*fos* in striatal (Mao and Wang, 2003) and in cortical neurons (Wang et al., 1995). To examine whether the dopaminergic projections from the VTA to M1 are functional, we measured c-*fos* expression in M1 after electrical stimulation of VTA. Stimulation increased c-*fos* expression in the ipsilateral M1 to 141.2% ($n = 6$) compared with sham control ($n = 6$, 390,051 ± 35,281 vs 229,555 ± 16,894 neurons, respectively; $p = 0.002$) (Fig. 2*a*). Whether c-*fos* expression was indeed caused by stimulation of dopaminergic VTA–M1 neurons was examined after blocking dopaminergic transmission in M1. Intracortical injection of D_1 and D_2 antagonists (SCH 23390 and raclopride; $n = 6$) reduced c-*fos* expression after VTA stimulation to 43.2% compared with vehicle-injected controls ($n = 5$; 594,120 ± 51,330 vs 337,211 ± 41,831 neurons, respectively; $p = 0.004$) (Fig. 2*b*). These results provide clear evidence for a functional dopaminergic projection from VTA to M1.

Destroying dopaminergic neurons in VTA depletes M1 of dopaminergic terminals and prevents motor skill learning

Considering the above findings, destruction of dopaminergic neurons in the VTA by 6-OHDA is expected to deplete most dopaminergic terminals in M1. VTA dopaminergic neurons were destroyed by injection of 6-OHDA directly into the VTA. Figure 3*a* shows representative anti-TH immunofluorescence of M1 after sham (vehicle injected; left) and 6-OHDA injections into VTA (right) that removed most dopaminergic VTA neurons (examples in Fig. 3*b*). Qualitatively, dopaminergic terminals were clearly reduced in VTA-lesioned versus sham rats throughout all cortical layers. Quantitatively, Western blot analysis confirmed significantly lower TH expression in the ipsilateral M1 compared with sham (densitometry: 0.018 ± 0.001, $n = 4$ lesioned, vs 0.062 ± 0.018, $n = 4$ sham; $p = 0.048$) (Fig. 3*c*), whereas there was no difference in the expression of the unspecific protein GADPH.

Rats that received 6-OHDA to eliminate dopaminergic neurons in VTA were not able to successfully learn the reach-and-grasp task. Improvement in reaching skill during the first training phase (days 1–8) was significantly lower in 6-OHDA-treated (two groups, lesion_1, $n = 7$, and lesion_2, $n = 9$, that were later treated differently) compared with sham-injected controls ($n = 8$; group by time effect, $p = 0.0005$; *post hoc* comparisons sham–lesion_1, $p = 0.0014$; sham–lesion_2, $p = 0.0004$) (Fig. 4*a*). Short-term performance gains evaluated during the first training session (day 1) were not significantly different between lesioned and sham rats ($p = 0.87$) (Fig. 4*b*).

Lesioning the VTA could have specifically affected learning or it could have merely worsened performance, thereby causing spurious learning impairments. To investigate whether VTA lesions affect motor performance, an additional group of rats ($n = 6$) was trained for 8 d before the VTA was lesioned. After 8 d of motor skill training, these animals had reached a performance plateau; hence, they did not learn any further. Lesioning the VTA at that time point neither affected reaching performance nor latencies between pellet removal and subsequent door opening (Fig. 4*c*). In fact, reaching performance even improved slightly (paired *t* test, day 8 vs day 9: $p = 0.049$). These results suggest that lesioning dopaminergic neurons in the VTA has no effect on motor performance but specifically impairs skill learning.

Supplementing dopamine in form of its precursor levodopa continuously into the M1 of VTA-lesioned rats (lesion_1) during training sessions 9–16 restored the ability to learn the reaching skill (group by time effect, $p = 0.015$; *post hoc* comparison lesion_1 vs lesion_2, $p = 0.035$) (Fig. 4*a*, gray-shaded part). Infusing vehicle instead of levodopa (lesion_2) had no effect. The performance of levodopa-supplemented VTA-lesioned rats, however, did not reach the performance of sham-lesioned controls.

This partial recovery of the learning ability could have been the result of levodopa compensating for the lack of DA that was induced by lesioning VTA. Alternatively, it could have reflected the combination of two unrelated mechanisms (i.e., levodopa boosting skill learning by a mechanism unrelated to the VTA-to-M1 projection and VTA lesioning depressing it). The summation of the two mechanisms could have resulted in the observed finding. We tested these alternate possibilities by infusing levodopa into the M1 of sham-treated rats. These rats did not show any additional improvement in performance (Fig. 4*a*, gray-shaded part), suggesting that levodopa was responsible for recovering the learning ability in VTA-lesioned rats.

Discontinuation of intracortical levodopa infusions (day 17, lesion_1) had no effect on performance (Fig. 4*a*, gray-striped part) indicating that levodopa supplementation is not necessary for skill execution without additional learning.

To explore whether the learning deficits were spuriously caused by reduced motivation, the latencies between pellet removal and subsequent door opening were measured. Latencies continued to decrease over the entire training period without differences between the groups (time effect, $p < 0.0001$; group effect, $p = 0.30$) (Fig. 4*d*). To screen for a confounding influence of a general motor function deficit, rotarod performance was recorded at baseline, after every surgical procedure and at the end of the experiment. Rotarod performance declined equally in all groups after the VTA injection of 6-OHDA or vehicle and remained stable thereafter regardless of group assignment ($p = 0.13$) (Fig. 4*e*).

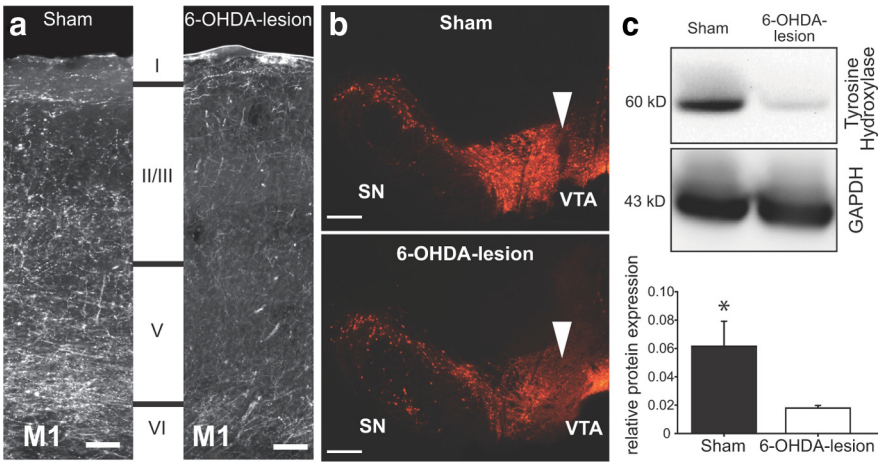

Figure 3. Destroying dopaminergic neurons in VTA depletes dopaminergic terminals in M1. ***a***, Representative TH immunofluorescence staining of M1 shows a clear reduction of TH-positive dopaminergic terminals throughout all cortical layers in 6-OHDA-lesioned animals. Scale bar, 250 μm. ***b***, Example of TH immunohistochemistry shows effective destruction of dopaminergic neurons in the VTA after 6-OHDA injection in conjunction with desipramine intraperitoneally to protect noradrenergic neurons (right) compared with the vehicle-injected VTA (left). The arrow indicates injection site. Scale bar, 50 μm. ***c***, Western blot analysis of M1 TH immunoreactivity demonstrates quantitatively the loss of dopaminergic neurons after 6-OHDA lesions in VTA compared with vehicle-injected controls ($n = 8$; $^*p = 0.048$). GAPDH was used as a positive control protein and remained unchanged after 6-OHDA lesions. Error bars indicate SEM.

Discussion

These findings prove the existence of an ipsilateral dopaminergic pathway from the VTA of the midbrain to the M1 of rats. This pathway is necessary for acquiring a novel motor skill through repeated training. Dopaminergic signals to M1 not only optimize motor skill learning as previously suggested (Molina-Luna et al., 2009) but are a requirement. Destroying VTA dopaminergic neurons prevented rats from learning a novel reaching skill. Stimulation of the VTA-to-M1 dopaminergic pathway induced IEG expression in M1. IEG expression represents a first step in the cellular cascade of events leading to synaptic plasticity (Guzowski, 2002), a cortical mechanism of motor skill learning (Kleim et al., 1996; Rioult-Pedotti et al., 2000).

VTA dopaminergic neurons project to many areas of cortex (Lindvall et al., 1974; Albanese and Bentivoglio, 1982; Swanson, 1982; Descarries et al., 1987; Pirot et al., 1992; Ikemoto, 2007). Although some of these studies also report dopaminergic terminals in M1 (for review, see Luft and Schwarz, 2009), a direct demonstration of a dopaminergic VTA-to-M1 projection in rodents and a characterization of its functional role were lacking.

In rodents and in primates, dopaminergic neurons are located in the VTA, SN, RRF, and adjacent nuclei (regions A8, A9, and A10) of the midbrain. There are substantial differences between species. Compared with rodents, primates have three to seven times more dopaminergic neurons in the midbrain, which are inhomogeneously distributed across nuclei (German and Manaye, 1993). The topography of dopaminergic projections originating in the midbrain differs between species (Williams and Goldman-Rakic, 1998). In owls and rhesus monkeys, M1 receives dopaminergic input from the ipsilateral SN (~40%), the VTA (~45%), and the RRF (~15%) (Gaspar et al., 1992; Williams and Goldman-Rakic, 1998). Our study shows that in rats the ipsilateral VTA is the major source of dopaminergic input to M1 (~73%), whereas the contralateral VTA and ipsilateral SN contribute little (12 and 15%, respectively). Interestingly, we did not find neurons in RRF that projected to M1. This is congruent with previous reports suggesting that rodents lack a retrorubral–neocortex projection (Loughlin and Fallon, 1984; Deutch et al., 1988). This projection can be considered a consequence of the phylogenetic evolution of the primate neocortex (for more information, see Williams and Rakic, 1998).

We have previously shown that dopaminergic signaling within M1 optimizes motor skill learning and modulates the synaptic plasticity of horizontal connections in superficial M1 layers (Molina-Luna et al., 2009). After removing M1 dopaminergic terminals, learning was reduced. Here, we show that these terminals come from VTA (and to a lesser degree from SN).

Retrograde tracing identified dopaminergic neurons in VTA that projected to M1. A small number of these neurons was also found in SN, but these seem to be functionally irrelevant since SN neurons were not able to compensate for the loss of VTA neurons during skill learning in animals with VTA 6-OHDA lesions. Lesioning might also have damaged SN neurons. Immunohistochemistry, however, confirmed that 6-OHDA injections were restricted to VTA (Fig. 3*b*). If dopaminergic projections from SN were crucial for skill learning, their destruction should have had more profound behavioral consequences. Because most SN neurons project to striatum and this nigrostriatal dopaminergic system controls muscle tone and movement initiation, SN lesioning should have caused parkinsonian symptoms such as freezing behavior. We then should have observed a marked increase in intertrial latencies and reduced rotarod performance, which we did not. Thus, SN-to-M1 projections were not able to compensate for the loss of VTA-to-M1 neurons. The small number of neurons in the SN projecting to M1 does not seem to have functional relevance at least in the context of motor skill learning.

6-OHDA lesioning of VTA eliminated at least 70% of dopaminergic terminals in M1. Thirty percent were left intact because selective destruction of dopaminergic neurons in the VTA spared the DA neurons located in the ipsilateral SN and in the nuclei in the contralateral hemisphere. In conjunction with the complete elimination of skill learning after VTA lesioning, this finding indicates that even a partial compromise of dopaminergic projections to M1 has dramatic behavioral consequences for motor skill learning.

The increase in M1 c-*fos* expression in response to VTA stimulation depended on DA release. This increase was absent when the DA action was blocked by injecting DA receptor antagonists into M1 before VTA stimulation. Therefore, c-*fos* upregulation is not the consequence of the electrical stimulus spreading beyond VTA (e.g., activating sensory afferent pathways to M1 would lead to glutamate release not DA release). From other brain regions and cell types, we know that DA receptors are linked to c-*fos* expression (Wang et al., 1995). Hence, the M1 c-*fos* expression observed here is likely the result of a direct activation of the VTA-to-M1 pathway.

Lesioning dopaminergic neurons in VTA suppressed skill learning. Because motor skill learning relies (among other brain regions) on M1, these findings are considered indirect proof of the VTA-to-M1 dopaminergic pathway.

Our results clearly demonstrate a behavioral relevance of the VTA-to-M1 pathway. Remarkably, VTA lesioning completely abol-

ished training-related improvements in reaching performance, which is in contrast to our previous findings that indicated learning impairment when 6-OHDA was directly injected into MI (Molina-Luna et al., 2009). This difference is most likely a result of technical origin. Direct injections reduced M1 dopaminergic neurons by 45%, whereas a 70% reduction was observed after VTA lesions. 6-OHDA injected in the cortex spreads in a perimeter of ~1 mm, which is smaller than the entire forelimb representation (Neafsey et al., 1986). Therefore, many dopaminergic neurons were left intact. Our present results highly suggest that dopaminergic signaling in M1 is a requirement for motor skill learning, not merely a necessity.

Lesions of the VTA impair avoidance learning and operant conditioning (Hefco et al., 2003). The deficits mainly affected retention, not acquisition, which is in accordance with our findings of unaffected within-session gains in reaching success. Instead, the between-session (i.e., longer term) learning processes were affected by VTA lesioning.

Substitution of DA in M1 only partially reversed the effect of VTA lesioning that might result from the limited spread of levodopa infused into M1. The drug might not have reached all sites where dopaminergic innervation was depleted. Alternatively, the unphysiological continuous administration of DA might have been an insufficient replacement of the physiological dopaminergic drive provided by the VTA.

Considering the widespread projections from the VTA to various cortical areas, one would expect that a VTA lesion produces also other deficits in addition to impaired motor skill learning. We can rule out deficits in motivation, spatial memory, and conceptual learning (i.e., learning the concept of obtaining a food pellet by opening the door and using the forelimb to reach) because intertrial latencies were unaffected. Movement deficits can also be excluded because (1) lesioning VTA in overtrained animals did not affect skill performance, (2) discontinuation of levodopa substitution in VTA-lesioned animals did not affect skill performance, and (3) rotarod performance between lesioned and sham-lesioned animals was not different. Nonmotor behavioral consequences of lesioning VTA neurons that project to different brain regions would certainly have been detected, if the appropriate tests were used. It is important that these theoretical deficits did not introduce confounds for the interpretation of the skill learning paradigm. The fact that skill learning deficits recovered after supplementing levodopa to M1 proves that the deficit was caused by the elimination of VTA-to-M1 projections.

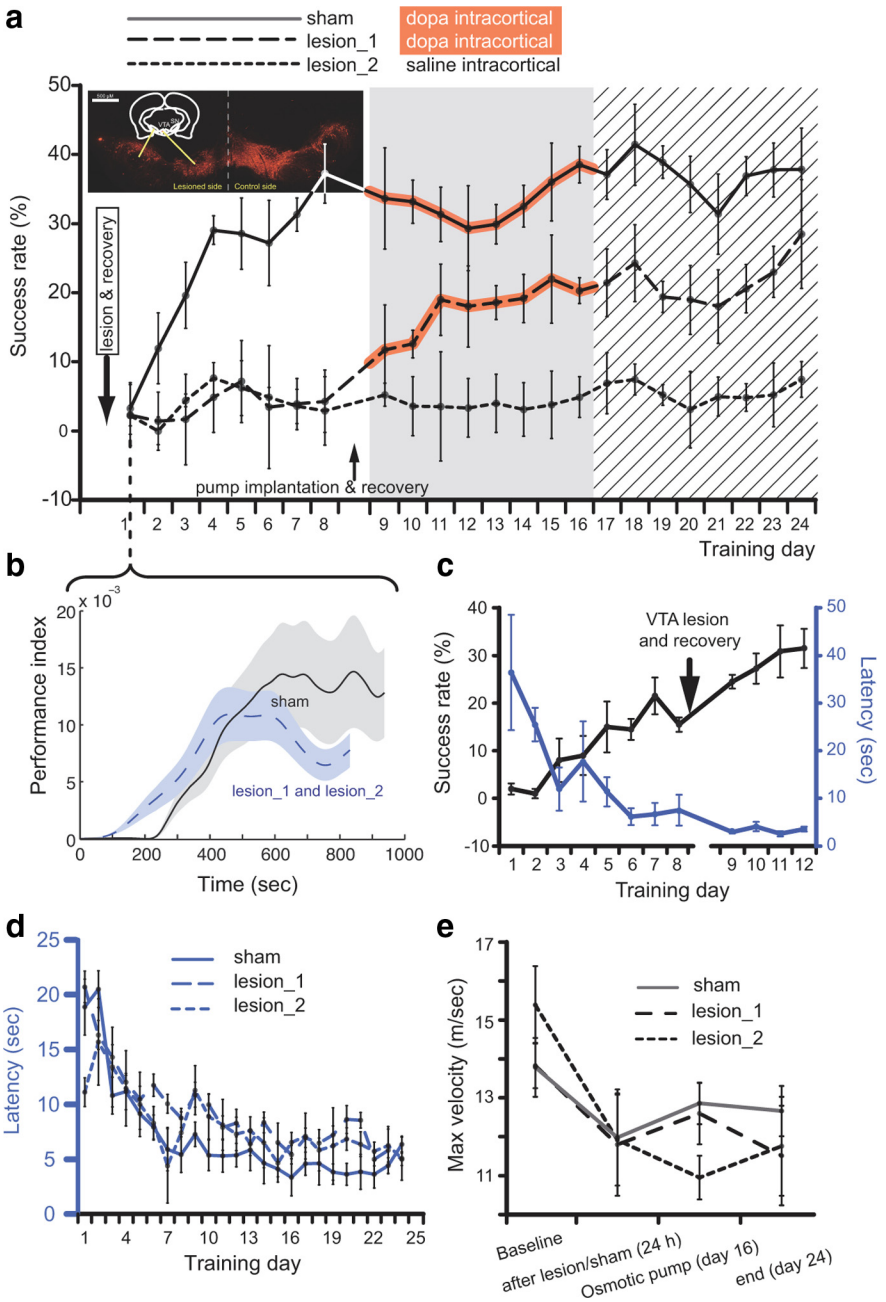

Figure 4. Intact dopaminergic projection from VTA to M1 is required for motor skill learning. ***a***, Lesioning VTA prevented training-induced improvements in reaching performance (lesion_1 and lesion_2) compared with sham-lesioned controls (days 1–8; white background). Infusing levodopa directly into M1 (days 9–16; gray background) rescued the VTA lesion-induced deficit (lesion_1; $n = 7$), whereas there was no such restoration on vehicle injection (lesion_2; $n = 9$) and no additional improvement by levodopa injections in rats that had already acquired the task (sham; $n = 8$). Lesion_1 animals maintained performance after discontinuation of levodopa substitution (days 17–24; gray-striped background). ***b***, Short-term improvement in session 1 is maintained after lesioning VTA (blue) compared with sham (black). ***c***, Destroying dopaminergic neurons in VTA after 8 d of training (plateau phase without additional learning; $n = 6$) does neither affect performance indicating unaffected movement execution, nor latency. ***d***, Latencies between pellet removal and subsequent door opening, an index of motivation, were not affected by VTA lesions. ***e***, Rotarod performance, an index of general motor function, decreased because of surgical instrumentation but not because of 6-OHDA injections into VTA. Error bars indicate SEM.

Our findings have interesting implications. Dopaminergic neurons in VTA encode reward, especially unexpected rewards (Schultz, 2007). Routing reward signals from VTA to M1 very likely initiates synaptic plasticity in M1, which is one mechanism of motor skill learning (Rioult-Pedotti et al., 2000). Therefore, via the VTA-to-M1 projection rewards may directly influence motor cortical learning processes.

Patients with Parkinson's disease (PD) have deficits in skill learning (Frith et al., 1986; Verschueren et al., 1997). PD is characterized by degradation of the nigrostriatal dopaminergic system. Mesocortical dopaminergic neurons also degenerate (McRitchie et al., 1997). Consequently, PD patients show reduced ^{18}F-dopa uptake in positron emission tomography in motor cortex as a marker for the degradation of cortical dopaminergic terminals (Moore et al., 2008). Degeneration of the VTA-to-M1 projection may explain the skill learning deficits in PD.

In conclusion, dopaminergic terminals in M1 originate in the VTA and are specifically required for learning a novel motor skill, but not for movement execution. Dopaminergic signals from VTA induce immediate-early gene expression in M1 that may enable subsequent plastic modifications. These modifications may account for DA-dependent synaptic plasticity in M1 (Molina-Luna et al., 2009), a key mechanism for skill learning (Rioult-Pedotti et al., 2000).

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