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Increased latencies to initiate cocaine self-administration following laterodorsal tegmental nucleus lesions

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Abstract

Cholinergic input to the ventral tegmental area (VTA), origin of the mesocorticolimbic dopamine system that is critical for cocaine reward, is important for both cocaine seeking and cocaine taking. The laterodorsal tegmental nucleus (LDTg) provides one of the two major sources of excitatory cholinergic input to the VTA, but little is known of the role of the LDTg in cocaine reward. LDTg cholinergic cells express urotensin-II receptors and here we used local microinjections of a conjugate of the endogenous ligand for these receptors with diphtheria toxin (Dtx::UII) to lesion the cholinergic cells of the LDTg in rats previously trained to self-administer cocaine (1 mg/kg/infusion, i.v.). Lesioned rats showed long latencies to initiate cocaine self-administration after treatment with the toxin, which resulted in a reduction in cocaine intake per session. Priming injections reduced latencies to initiate responding for cocaine in lesioned rats, and once they began to respond the rats regulated their moment-to-moment cocaine intake within normal limits. Thus we conclude that while LDTg cholinergic cell loss does not significantly alter the rewarding effects of cocaine, LDTg lesions can reduce the rat's responsiveness to cocaine-predictive stimuli.

Keywords

ACh; acetylcholine; dopamine; pedunculo pontine tegmental nucleus; reward; ventral tegmental area

1. Introduction

While it is widely understood that the mesocorticolimbic dopamine system plays a central role in reward function [1], and that this system is activated by both rewards themselves and by conditioned stimuli that have been associated with rewards [2], little is known about which of the multiple inputs to this system [3, 4] carry reward-relevant information.

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Acetylcholine input to the ventral tegmental area (VTA) is implicated by several lines of study. First, infusions of the cholinergic agonist carbachol directly into the ventral tegmental area (origin of the corticolimbic dopamine system) were found to potentiate the rewarding effects of lateral hypothalamic brain stimulation [5, 6]. Second, such infusions were found to establish conditioned place preferences [5]. Third, rats were found to work for microinjections of carbachol directly into the VTA [7]. That VTA acetylcholine might play a role in cocaine reward is suggested by the finding that extracellular VTA acetylcholine levels are elevated during intravenous cocaine self-administration and during cocaine-seeking under unexpected extinction conditions. An initial rapid elevation in VTA acetylcholine at the beginning of a cocaine self-administration session is seen only in rats previously trained to self-administer cocaine and occurs not only when the rat receives the drug but also when it is expecting the drug but gets saline in its place. A relatively slower increase and subsequently elevated plateau in VTA acetylcholine levels is seen even in cocaine-naïve animals and is thus due to the cocaine itself. The elevation of VTA acetylcholine due to cocaine itself appears to regulate cocaine taking, as blockade of VTA cholinergic receptors in trained rats increases their hourly cocaine intake [8].

There are two sources of cholinergic input to the VTA, the pedunculopontine tegmental nucleus (PPTg) and the laterodorsal tegmental nucleus (LDTg) [9, 10]. Of these, PPTg has been implicated in reward function [11, 12] but lesions of the cholinergic cells of PPTg do not affect intravenous cocaine or heroin self-administration or conditioned place preference [13]. In the present study we used diphtheria toxin conjugated to the ligand for a receptor expressed selectively on cholinergic neurons to assess the effects of loss of cholinergic LDTg neurons on intravenous cocaine self-administration.

2. Materials and Methods

2.1. Animals

Fourteen male Long-Evans rats (Charles River, Raleigh, NC) weighing 275–325 grams at the time of surgery were used. They were individually housed under a reverse light-dark cycle (12/12, lights off at 8 am) with free access to food and water throughout. All experiments were performed in accordance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs and neurotoxin

Ketamine, Xylazine, and (–)-Cocaine hydrochloride were obtained from the NIH. Cocaine was dissolved in 0.9% sterile saline. Diphtheria toxin conjugated to urotensin-II (Dtx::UII) [14] was obtained from One World Biotech (Warminster, PA).

2.3. IV catheter implantation

Fourteen rats were each implanted with an intravenous microcatheter (Braintree Scientific, Braintree, MA) inserted into the right external jugular vein. Each rat was first anesthetized with a combination of ketamine and xylazine (57 mg/kg and 9 mg/kg i.p., respectively) followed by isoflurane (2–3% in 1 L/min Oxygen). Catheter tubing was attached to a cannula adaptor backmount assembly at the other end. The backmount

assembly was positioned so that it exited the animals back just caudal to the scapulae. Catheters were flushed daily with heparin (10 USP/ml in sterile saline), containing gentamicin (0.08 mg/ml).

2.4. LDTg Dtx::UII infusion

Following the completion of cocaine self-administration training, each rat was anesthetized and secured in a stereotaxic apparatus and three injections of 200 nl each of either Dtx::UII (42 nanograms protein per infusion, 126 nanograms total per hemisphere) or 0.01M phosphate-buffered saline (vehicle) were made into each LDTg using a syringe controlled by an automated infusion pump (UMP 3; World Precision Instruments; Sarasota, FL) attached to one of the stereotaxic manipulator arms. The injector needle was lowered into each LDTg from the front along an angled trajectory of 30° from vertical. The three injections per hemisphere were aimed at the following coordinates according to the rat brain atlas of Paxinos & Watson [15] relative to Bregma: 1) A-P -8.7, M-L $\pm$ 1.13, D-V -7.0, 2) A-P -8.5, M-L $\pm$ 1.13, D-V -6.8, 3) A-P -7.0, M-L $\pm$ 1.13, D-V -6.4. The needle tip was lowered to the most caudal coordinate and the first injection was made. The needle was then retracted to the second coordinate and a second injection was made. The needle was then retracted to the third coordinate and a third injection was made. Following each infusion, the injector was left in place for five minutes to allow for diffusion of the injected solution from the needle tip. Successive bilateral LDTg ibotenic acid lesions on the same day are associated with high mortality rates [16, 17]. For this reason injections of toxin or vehicle into the left and right LDTg were separated by 48 hours, with the order of injections counter-balanced. No rats died during the post-lesion recovery period.

2.5. Drug self-administration

2.5.1. Drug self-administration apparatus—Operant conditioning chambers, measuring 25 × 27 × 30 cm were used. Each chamber was equipped with a retractable lever and a stationary lever, a red house light, and a white cue light located above the retractable lever and was housed within a sound-attenuating enclosure equipped with a fan that provided both ventilation and a constant source of masking noise.

2.5.2. Drug self-administration procedure—Each rat was housed and trained in a dedicated operant chamber throughout the experiment. Daily 4-hour intravenous drug self-administration training sessions began 7–10 days after i.v. catheter implantation and lasted for 13–15 days. Each rat was placed into its dedicated chamber immediately prior to the first self-administration session. For each rat the backmount catheter was connected to an infusion line attached to a liquid swivel that allowed for free movement of the rat within the operant chamber. The beginning of each daily training session was signaled by illumination of the red house light and insertion of the response lever into the operant chamber. No priming infusions were given during training. Each rat was allowed to self-administer cocaine (1.0 mg/kg/infusion, i.v.) under a fixed ratio 1 schedule of reinforcement with a 20-s time-out period accompanied by illumination of a white cue light located above the retractable lever. Lever presses during the time-out period were recorded but were not reinforced. Presses on the stationary lever were recorded but had no programmed consequence. At the end of the training session the retractable lever was withdrawn from the

chamber and the house light was turned off. During the training phase rats were required to reach a criterion of three consecutive days of stable responding defined as less than 10% variability across days with a minimum of 30 cocaine injections per 4-hour session. Once the rats met the training criteria, they were randomly assigned to either a LDTg toxin lesion or a sham lesion treatment group and underwent the intracranial injection procedure. Self-administration testing was resumed four days after the second LDTg lesion.

2.6. Cocaine priming

Priming injections of cocaine (10 mg/kg, i.p.) or saline (1 ml/kg, i.p.) were introduced five minutes prior to the beginning of the 12th and 13th post-lesion sessions in lesioned rats; the order of treatments was counterbalanced across rats. Each rat was allowed to earn cocaine infusions normally for the duration of the test sessions. Normal testing—testing without priming injections—resumed on post-lesion test day 14.

2.7. Cocaine dose-response testing

Because lesioned rats did not initiate cocaine self-administration reliably in response to lever-insertion, cocaine priming injections (10 mg/kg, i.p.) were given five minutes prior to the beginning of each of the next 9 daily testing sessions. The dose of cocaine was set to 0.5, 1, or 2 mg/kg/infusion for each session. Each rat was tested three times at each dose with the order of doses randomized across rats. Dose response data were also collected from four LDTg vehicle-treated rats. However, priming injections were not required for the control rats, which started promptly with lever insertion.

2.8. Histology

Following completion of behavioral testing, each rat was deeply anesthetized with a mixture of pentobarbital (30 mg/kg, i.p.) and chloral hydrate (140 mg/kg, i.p.) and was then transcardially perfused with 0.1 M phosphate buffer (PB) followed by 4% (W/V) paraformaldehyde in 0.1 M PB, pH 7.3. The brains were removed and left in 4% paraformaldehyde for 2 hours at 4 °C and then transferred to an 18% sucrose solution in PB. Coronal sections (20 µm) were prepared for each rat; some were processed for choline acetyltransferase (ChAT) immunocytochemistry and others were stained with cresyl violet.

2.8.1. Immunocytochemistry—Sections processed for ChAT immunocytochemistry were rinsed with 3× 10 min PB, followed by 15 minutes incubation in 0.3% H₂O₂, followed by 1 hour incubation in PB supplemented with 4% bovine serum albumin (BSA) and 0.3% Triton X-100. The sections were then incubated overnight at 4°C with anti-ChAT goat polyclonal antibody (Chemicon, Temecula, CA, AB144P; dilution 1:100). After rinsing with 3×10 min PB, the sections were incubated for 1 hour at room temperature in a 1:200 dilution of biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), rinsed with PB, and incubated with avidin-biotinylated horseradish peroxidase for 1 hour. The sections were then rinsed and the peroxidase reaction was developed with 0.05% 3,3-diaminobenzidine-4 HCl (DAB) and 0.03% hydrogen peroxide (H₂O₂). Finally, the sections were mounted on gelatin-coated slides and air-dried for inspection. Processed sections were viewed, analyzed, and photographed with bright field microscopy using Surveyor with Turboscan software (Objective Imaging, Cambridge, UK) at 20×.

2.8.2. Cell counting—One representative coronal section was chosen from each sham-lesioned rat showing each of the rostral (A/P –8.52), medial (A/P –8.76) or caudal (A/P –9.12) portions of the LDTg. The boundaries of the LDTg were determined in sham-lesioned rats based on the extent of ChAT staining. The outlines were traced accordingly and the numbers of ChAT-positive cells were counted within these boundaries at each of the three rostro-caudal levels. Outlines of several adjacent brain nuclei were traced as well. The outlines created from the sham-lesioned rats were superimposed onto sections from lesioned rats to identify comparable sections. For each lesioned rat the numbers of ChAT-positive cells were counted within the previously identified boundaries at each rostro-caudal level. The numbers of ChAT-positive cells within the LDTg boundaries at each of the three rostro-caudal levels were then added and analyzed in each of the lesioned and control rats.

2.9. Data Analysis

One rat developed a leaking i.v. catheter during the training period and one toxin-treated rat showed no cholinergic cell loss within the LDTg. Data from each of these rats were excluded from the statistical analyses, and statistical analyses were conducted on data from 6 lesioned and 6 sham-lesioned control rats. Data were analyzed using 2-way between-within subjects Analysis of Variance (ANOVA) tests. Significant 2-way interactions were followed by analysis of simple main effect, and significant main effects were followed by Fisher's LSD post-hoc test.

3. Results

LDTg injections of the neurotoxin Dtx::UII had no effect on latency to initiate responding (Figs 1A–B), numbers of lever-presses per session (Fig 2B), or number of cocaine infusions earned (Fig 2A) on the first post-lesion test day. On the second and subsequent post-lesion test days 6 of the 7 rats that were given Dtx::UII injections failed to initiate responding with normal latency (the animal that responded normally was subsequently found not to be lesioned and was excluded from subsequent analysis.) Mean latency for the six effectively lesioned animals shifted from under five minutes on the first post-lesion test day to over two hours on the second post-lesion test day (Fig 1B). The effects on response initiation latency were reflected in a significant interaction between group (lesion vs. sham) and time (pre- vs. post-lesion); there were no significant differences in initiation between the lesioned and sham-lesioned groups before the lesion, and only response initiation latency of the lesioned group was significantly increased after the toxin injections (Figure 1B; LESION X TIME interaction [$F_{13,130} = 3.13$, $P < 0.001$] and simple main effects analysis comparing latencies in lesioned and sham-lesioned groups on individual pre- and post-lesion test days: P 's between $< .05$ and $< .00001$ on post-lesion test day 2 and post-lesion test days 4–11, and P 's $> .1$ on pre-lesion test days and post-lesion test days 1 and 3).

As a result of delayed response initiation, the lesioned animals made significantly fewer responses and earned significantly fewer injections in the second and subsequent sessions (Fig. 2). The effects on drug intake were reflected in a significant interaction between group (lesion vs. sham) and time (pre- vs. post-lesion); there were no significant differences in intake between the lesioned and sham-lesioned groups before the lesion, and only the intake

of the lesioned group was significantly depressed after the toxin injections (LESION $\times$ TIME interaction [$F_{13, 130} = 6.32, P < .00001$] and simple main effects analysis comparing intake in lesioned and sham-lesioned groups on individual pre- and post-lesion test days: P 's between $< .05$ and $< .0001$ on post-lesion test days 2–11, and P 's $> .1$ on pre-lesion test days and post-lesion test day 1).

The decreased drug intake after the first post-lesion day of testing reflected only the long latencies to respond and not decreased responding once responding was initiated. Response rates were normal following response initiation on post-lesion days 1–11 (Fig 1A shows representative event records; independent samples t-test comparing mean inter-infusion intervals: $t_{85} = 1.66$, NS). Priming injections of cocaine, but not saline, significantly reduced the latencies in lesioned rats (Figure 1C; repeated-measures ANOVA main effect of DAY [$F_{3,15} = 4.07, P < .05$; Fisher's LSD post-hoc tests show shorter latencies on the cocaine priming day relative to the other test days [P 's $< .01$]). On post-lesion days 15–23 when the animals were given a single priming injection of cocaine 5min prior to the start of each session and tested under each of three different doses per injection, the lesioned animals showed normal compensation for changes in dose, responding less for the higher and more for the lower dose per injection (Fig. 3 repeated-measures ANOVA on the number of cocaine infusions: main effect of LESION [$F_{1,8} = 0.19$, NS] and repeated-measures ANOVA on the mean inter-infusion intervals: main effect of LESION [$F_{1,8} = 0.02$, NS]).

In 6 of 7 treated animals, the toxin treatment caused major loss of cholinergic cells within the LDTg but did not appear to encroach significantly on those of the adjacent PPT (Figures 4 A–D). In the one animal that continued to respond with normal latency and intake after treatment, no evidence was found for cholinergic cell loss; this animal's data were excluded from the behavioral analyses. Cresyl violet staining of representative LDTg coronal sections from the successfully lesioned animals revealed no gross abnormalities in LDTg tissue from lesioned rats (Figures 4A'' and 4B''). Counts of ChAT-positive cells in the LDTg of toxin-injected animals were 78.62 % ($\pm 5.78\%$) lower than those from vehicle-injected animals (Figures 4E; main effect of LESION [$F_{1,10} = 148.07, P < .00001$]). Lesioned left and right LDTg showed comparable losses of cholinergic cells (main effect of HEMISPHERE [$F_{1,10} = 4.06$, NS]).

4. Discussion

LDTg cell loss had no effect on cocaine self-administration on the first day of testing following the recovery period after neurotoxin administration. The rats initiated self-administration promptly when the response lever was inserted into the cage, and they responded at pre-lesion rates and earned pre-lesion amounts of cocaine in the session. This was an unexpected finding, given the facts that LDTg is the strongest source of ACh into the VTA [10], that cholinergic blockers in the VTA attenuate the rewarding effects of medial forebrain bundle electrical stimulation [5, 6], that ACh is elevated in the VTA by cocaine-predictive stimuli [8], and that cholinergic blockade in the VTA causes increased cocaine intake presumably by blocking the contribution of cocaine-predictive stimuli to the rewarding effect of the drug for conditioned animals [8].

While ACh cell loss did not affect cocaine-self-administration on the first day of post-lesion testing, it resulted in delayed initiation of responding on the second and all subsequent days. This did not reflect a change in either the response-maintaining or the response-initiating effects of cocaine; once initiated, earned cocaine maintained normal response patterns and rates and when given as a priming injection, cocaine triggered normal response initiation. Thus the effect of the lesion appeared to be to weaken the control of the environmental stimuli—the sound and sight of insertion of the retractable lever—that normally triggered the initiation of binge cocaine self-administration.

Cholinergic cell loss following LDTg Dtx::UII treatment was extensive but not complete: lesioned rats had $78.62\% \pm 5.78\%$ fewer ChAT-positive cells than did control rats in the selected LDTg sections. PPTg Dtx::UII treatment results in preferential loss of cholinergic cells and can cause some non-specific damage to neighboring glutamatergic and GABAergic cell populations [13]. While cresyl violet staining of LDTg tissue in lesioned rats did not show obvious non-specific damage we cannot rule out the possibility that some degree of loss of glutamatergic or GABAergic cell populations contributed to the increases in latency to initiate cocaine self-administration in our lesioned rats.

Our data clearly confirm, however, that loss of over half the cholinergic cells of LDTg does not alter the rewarding and response-initiating (priming) effect of cocaine. Inasmuch as loss of 95% of the cholinergic cells of PPTg does not affect cocaine self-administration, this leaves unanswered the question of the origin of the cholinergic input that is implicated in the contribution of conditioned reinforcement to cocaine reward [8]. One possibility is that our lesions were simply not complete enough to eliminate the reward-associated release of ACh in VTA. Some support for this notion comes from the finding that patients can have 70% loss of dopaminergic cells in the substantia nigra and show only minimal symptoms of Parkinson's Disease [18]. Alternatively, it may be necessary to lesion both LDTg and PPTg in order to effectively deprive the VTA of all cholinergic input, in order alter cocaine self-administration as is seen following local VTA pharmacological manipulations [8].

Regardless of whether lesioned rats initiated responding spontaneously or as a result of priming injections, once initiated, responding continued with normal rates and reliability for the rest of the session. Following priming in lesioned rats the inter-infusion intervals measured with each cocaine dose tested with a between-session dose-response paradigm were very similar to the inter-infusion intervals that have been reported previously with a variable dose paradigm [19, 20]. In the variable dose paradigm, a random sequence of low, medium, and high doses of cocaine (0.5, 1.0, or 2.0 mg/kg) are self-administered; decreases in nucleus accumbens dopamine below a particular threshold level, following dopamine elevations induced by the previous cocaine injection, reliably precede the next lever press for cocaine. Larger elevations in nucleus accumbens dopamine induced by higher intravenous cocaine doses take longer to return to the threshold level, consequently increasing the subsequent inter-infusion interval. The similarity in inter-infusion intervals between the non-lesioned rats in earlier studies and the lesioned rats tested in the present study confirms that the regulation of cocaine intake in lesioned rats is under similar pharmacological control as in non-lesioned rats.

It is not known what role LDTg cells might play in cue-driven initiation of responding. While LDTg recording studies are not available, PPTg cells respond to environmental stimuli though they are not selectively sensitive to those with conditioned significance [21]. To the extent that LDTg and PPTg are part of the same functional unit [9, 10] it seems unlikely that LDTg cells selectively respond to environmental stimuli with conditioned significance. It seems more likely that LDTg lesions resulted in more general attentional, arousal or sensory processing deficits. In this regard lesions of PPTg cells can result in general attentional deficits [22]. Cholinergic input to the thalamus, which partly arises from the LDTg [9, 10, 23], modulates thalamocortical processing [24] consistent with an arousal function of the LDTg [25]. LDTg inputs to ventral tegmental area dopamine neurons may similarly modulate their ability to respond to inputs from other brain areas that relay sensory information with conditioned significance. In support of this, reversible chemical inactivation of the LDTg in anesthetized rats blocks burst firing of dopamine neurons induced by direct glutamate application [26]. In the present experiments, the rats were continuously housed in the operant chambers with tethers and i.v. infusion lines always attached. With the exception of the first training day, the rats were not transported or handled prior to the beginning of the cocaine self-administration sessions. Insertion of the retractable lever into the operant chamber, which provides visual and auditory cues, and illumination of the house light, another visual cue, were the only events that reliably marked the beginning of the self-administration sessions and thus the availability of cocaine. These cues—both in the control group and in the test days prior to lesioning in the experimental animals—normally caused immediate initiation of self-administration. Cues that signal the availability of rewards are known to gain the ability to activate glutamatergic [27, 28] and cholinergic [8] inputs to the VTA, increase burst firing activity of ventral midbrain dopamine neurons [2, 21, 29], and induce dopamine transients in the core regions of the nucleus accumbens [31–32].

In conclusion, LDTg lesions did not significantly alter the rewarding effects of cocaine and appear to have reduced the rat's responsiveness to cocaine-predictive stimuli. Cocaine intake was reduced but only as result of delayed initiation in daily sessions. The lesions did not impair locomotion or normal function significantly; responding was normal once it was initiated. Overall our data are not inconsistent with previous findings suggesting a role for an as yet unidentified LDTg cell population in behavioral responding for drugs of abuse [16,17, 33, 34].

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Highlights

- We used a neurotoxin designed to lesion LDTg cholinergic cells.
- LDTg-lesioned rats failed to regularly initiate cocaine self-administration.
- Cocaine priming reduced response initiation latencies in LDTg-lesioned rats.
- Lesions did not significantly alter the rewarding effects of cocaine.
- Lesions likely altered the rats' responsiveness to cocaine-predictive stimuli.

Figure 1. LDTg lesions result in significantly increased latencies to initiate cocaine self-administration

Panel A: Representative event records from two sham-lesioned control rats (Sham 1 and Sham 6) and two lesioned rats (Lesion 3 and Lesion 8) on post-lesion testing days 1 (Post 1), 5 (Post 5) and 8 (Post 8). Each event record shows the distribution of cocaine infusions during the course of a 4-hour cocaine self-administration session, with each vertical tick mark showing the time of a single infusion of cocaine. Sham-lesioned rats took the first cocaine infusion very soon after the beginning of the session (0 minutes) and continued

taking cocaine at regular intervals until the end of the session (240 minutes) on all post-lesion testing days shown. Lesioned rats were similar to controls on the first post-lesion test day, but latencies to initiate cocaine self-administration were significantly increased on post-lesion test days 5 and 8.

Panel B: Mean latency to the first infusion of cocaine in lesioned (black bars, $n = 6$) and sham-lesioned rats (white bars, $n = 6$) on the last three days of the training period and throughout the post-lesion testing period. Starting on post-lesion test day 2 and continuing through post-lesion test day 11, latencies to initiate cocaine self-administration were significantly increased in lesioned rats. Error bars represent $\pm$ SEM; * $P < .05$ LDTg lesion vs. sham control.

Panel C: Lesioned rats ($n = 6$) were given either cocaine priming (10 mg/kg, i.p.) or saline priming (1 ml/kg, i.p.) 5 minutes prior to the beginning of a cocaine self-administration session on post-lesion test days 12 and 13, with the order of priming conditions counterbalanced across rats. On post-lesion test day 14, lesioned rats were not given cocaine priming injections and again showed long latencies to initiate cocaine self-administration. Post-lesion test day 11 replicates part of the data shown in Figure 1B. Error bars represent $\pm$ SEM; * $P < .05$ cocaine-priming vs. saline-priming, and cocaine-priming vs. test day 11 or test day 14.

Figure 2. Intravenous cocaine self-administration is reduced following LDTg lesions
 Starting on the second post-lesion test day lesioned rats (solid circles, $n = 6$) took significantly fewer cocaine infusions during daily 4-hour training sessions (top panel) and pressed the cocaine lever significantly less often (bottom panel) relative to control rats (open squares, $n = 6$). Rats' ability to distinguish between the retractable cocaine and stationary (bottom panel) levers was comparable in lesioned (solid circles vs. solid triangles) and control (open squares and open triangles) rats. Error bars represent $\pm$ SEM.

Figure 3. Cocaine self-administration is under the control of reinforcement magnitude in LDTg lesioned rats following priming injection of cocaine

Lesioned rats (solid circles, $n = 6$) were given priming injections of cocaine (10 mg/kg, i.p.) 5 minutes prior to the beginning of nine consecutive daily 4-hour cocaine self-administration sessions. The dose of intravenous cocaine available per session was either 0.5, 1, or 2 mg/kg/infusion, with the order of doses randomized across sessions and responding for each dose tested three times. A sub-set of control rats (open squares, $n = 4$) was also tested, but these rats were never given priming injections of cocaine. Similar to control rats, lesioned rats

took fewer infusions of cocaine (top panel) and waited longer between infusions (bottom panel) with higher doses of cocaine. Error bars represent $\pm$ SEM.

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Figure 4. LDTg cholinergic cell loss following toxin treatment

Panels A–B: Representative coronal LDTg sections showing choline acetyltransferase immunoreactivity (ChAT-IR; brown cells) following bilateral LDTg toxin lesions (B) or sham lesions (A). Panels A' and B' highlight staining of ChAT-IR within the areas outlined in A and B, respectively. Panels A'' and B'' show surrounding cresyl violet-stained LDTg tissue following bilateral toxin lesions (B'') or sham lesions (A''). **Panels C and D:** Representative coronal sections showing choline acetyltransferase immunoreactivity (ChAT-IR; brown cells) in the PPTg following bilateral LDTg toxin lesions (D) or sham

lesions (C). **Panel E:** Mean numbers of ChAT-IR cells counted in each of two LDTg hemispheres across three representative A-P levels of the LDTg following LDTg toxin lesions (black bars, $n = 6$) or sham lesions (white bars, $n = 6$). In all cases tissue was processed for ChAT immunoreactivity approximately 4 weeks after LDTg toxin or vehicle infusions. Error bars represent $\pm$ SEM; * $P < .00001$.