Selective lesions of the cholinergic neurons within the posterior pedunculopontine do not alter operant learning or nicotine sensitization

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Abstract

Cholinergic neurons within the pedunculopontine tegmental nucleus have been implicated in a range of functions, including behavioral state control, attention, and modulation of midbrain and basal ganglia systems. Previous experiments with excitotoxic lesions have found persistent learning impairment and altered response to nicotine following lesion of the posterior component of the PPTg (pPPTg). These effects have been attributed to disrupted input to midbrain dopamine systems, particularly the ventral tegmental area. The pPPTg contains a dense collection of cholinergic neurons, but also large numbers of glutamatergic and GABAergic neurons. Because these interdigitated populations of neurons are all susceptible to excitotoxins, the effects of such lesions cannot be attributed to one neuronal population. We wished to assess whether the learning impairments and altered responses to nicotine in excitotoxic PPTg lesioned rats were due to loss of cholinergic neurons within pPPTg. Selective depletion of cholinergic pPPTg neurons is achievable with the fusion toxin Dtx-UII, which targets UII receptors expressed only by cholinergic neurons in this region. Rats bearing bilateral lesions of cholinergic pPPTg neurons (>90% ChAT+ neuronal loss) displayed no deficits in the learning or performance of fixed and variable ratio schedules of reinforcement for pellet reward. Separate rats with the same lesions had normal a locomotor response to nicotine and furthermore sensitized to repeated administration of nicotine at the same rate as sham controls. Previously seen changes in these behaviors following excitotoxic pPPTg lesion cannot be to be attributable solely to loss of cholinergic neurons. These findings indicate that non-cholinergic and not cholinergic neurons within the pPPTg are responsible for the learning deficits and altered responses to nicotine seen after excitotoxic lesions. The functions of cholinergic neurons may be related to behavioral state control and attention rather than learning.

Introduction

Lesions of the pedunculopontine tegmental nucleus of the upper brainstem disrupt instrumental learning (Alderson *et al.*, 2004) and alter the behavioral responses to several drugs of abuse including nicotine (Alderson *et al.*, 2006; 2008), morphine (Miller *et al.*, 2002) and amphetamine (Inglis *et al.*, 1994). Excitotoxic lesions restricted of the posterior PPTg (pPPTg) impair the ability to learn food rewarded instrumental tasks (Wilson *et al.*, 2009). Such lesions also alter the locomotor response to repeated systemic nicotine – reducing the initial hypolocomotion and increasing subsequent hyperlocomotion (Alderson *et al.*, 2008). Both of these behaviors are dependent on the functional integrity of midbrain dopamine (DA) systems, particularly those of the ventral tegmental area (VTA) and subsequent projections to the nucleus accumbens (NAcc) (Louis & Clarke, 1998; Tsai *et al.*, 2009; Zellner & Ranaldi, 2010). The pPPTg contains a dense population of cholinergic neurons (from which it is often referred to as PPTg pars compacta (Manaye *et al.*, 1999)) and the effects of excitotoxic lesion of PPTg on learning and nicotine might plausibly be explained by loss of cholinergic innervation of dopaminergic systems. Midbrain DA neurons require acetylcholine (ACh) for the switch from tonic to phasic firing, which is essential for normal instrumental learning (Maskos *et al.*, 2005; Maskos, 2008; Zweifel *et al.*, 2009). The PPTg and neighboring LDTg are the sole source of ACh arriving at midbrain DA systems and send innervation in a well-defined topographical manner: the anterior PPTg (aPPTg) principally targets the substantia nigra (SN), the pPPTg the SN and VTA and the LDTg largely innervates the VTA (Oakman *et al.*, 1995; Maskos, 2008). A topographically arraigned cholinergic projection to the striatum and nucleus accumbens has also been identified, with aPPTg preferentially innervating the dorsolateral striatum, pPPTg the medial striatum and NAcc and LDTg the NAcc core and areas of the most medial striatum (Dautan *et al.*, 2014). Up-regulation of nicotinic acetylcholine receptors (nAChRs) within VTA following loss of pPPTg innervation can be presented as an explanation for the enhanced response to systemic nicotine in excitotoxic PPTg lesioned rats (Alderson *et al.*, 2008). However, in addition to containing a dense population of cholinergic neurons,

the pPPTg also contains large numbers of glutamatergic and GABAergic neurons (Wang & Morales, 2009). These neuronal types are topographically arranged within PPTg: cholinergic neurons are densely packed in the posterior portion and sparse in anterior region, the opposite pattern is seen in GABAergic neurons while glutamatergic neurons are relatively equally distributed along the anterior-posterior axis. Studies assessing PPTg function typically create lesions of the region with excitotoxic agents, or temporary inactivation through GABAergic or lidocaine based mechanisms. While valuable, these techniques offer no selectivity for the neuronal sub-population targeted within PPTg, which limits the interpretation of observed effects. Cholinergic neurons within the PPTg selectively express the receptor for the peptide urotensin II (Clark *et al.*, 2001). The genetic fusion of urotensin II (UII) and the ribosome inactivating protein diphtheria toxin (Dtx) creates a recumbent protein toxin (Dtx-UII) which, when directly infused into the PPTg, selectively destroys cholinergic neurons (Clark *et al.*, 2007). Using this toxin, it has recently been found that the deficits in PPI following excitotoxic damage to the PPTg are not present after selective depletion of the cholinergic neuronal sub-population (MacLaren *et al.*, 2014a). Here, we used this toxin to assess specifically the contributions of cholinergic neurons within pPPTg to instrumental learning and the locomotor response to systemic nicotine. In experiment 1, rats were tested in an exact replication of the instrumental learning protocol in which we demonstrated a persistent learning impairment in after excitotoxic lesion of pPPTg (Wilson et al. 2009). In experiment 2, the rate and extent of locomotor sensitization to repeated systemic nicotine was assessed in a replication of the protocol Alderson et al. (2007) which found altered sensitization in excitotoxic pPPTg lesioned rats.

Experiment 1: Instrumental learning after selective depletion of cholinergic pPPTg neurons

Methods

Subjects

Twenty four adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used in these experiments, with a mean weight of 326g at the start of the experiments. Rats were pair housed in temperature and humidity controlled environment and kept on a 12hr light/dark cycle (lights on 7AM, testing carried out in the light phase). Water was always freely available in the homecage. Three days prior to behavioral testing food was restricted to 17-19g/rat/day standard lab chow; rats' body weights were monitored to ensure they did not fall to below 85% free food weight at any point in the experiment. Compliance with the Animals (Scientific Procedures) Act 1986 and European Communities Council Directive of 24/11/86 (86/609/EEC) was maintained throughout these experiments.

Surgery

Rats were anaesthetized with isoflurane (IsoFlo, Abbot Laboratories Ltd, Maidenhead, UK) in an induction box (0% stepped up to 5% isoflurane, 4l/m O_2) before being transferred to a David Kopf stereotaxic frame where anesthesia was maintained through a facemask (2-3% isoflurane, 1.2-1.4l/m O_2). The non-steroidal anti-inflammatory analgesic carprofen (0.05 ml/rat; 5% w/v; Rimadyl Pfizer Ltd, Kent, UK) was administered subcutaneously before the scalp was shaved and a midline incision made. The incisor bar of the stereotaxic frame was adjusted such that the angle between the incisor bar and the interaural line was 8°29′, achieved by multiplying the distance between the IAL and the back of the incisors by the sine of 8°29′, as described by (Whishaw *et al.*, 1977). Two craniotomies were made to allow infusion into the pPPTg at the co-ordinate: -0.8mm from IAL; ±1.9mm from midline; -6.5mm from dura. Dura was cut with the bent tip of a 30 ga needle. In the lesion group rats (n=16) received 300nl of 3% Dtx-UII (toxin kindly gifted from SD Clark, SUNY University at Buffalo, Buffalo, NY, USA). In the sham group (n=8) rats received the vehicle solution (sterile PB). Infusion was made from a hand drawn glass pipette (tip 40-50µ) connected by polythene tubing (containing air) to a 10ml syringe where pressure was applied by hand. The pipette was left *in situ* for 5 min after infusion to allow for diffusion from the tip. Both hemispheres

were infused in the same surgical procedure, with the order of first infusion side counterbalanced (unlike ibotenic acid bilateral lesions of the pPPTg which are normally performed in two separate procedures to reduce post-surgery mortality rate; in our experience recovery complications are not a concern with Dtx-UII infusions). The wound was closed with Michel clips and, once removed from the frame, rats were treated with 1mL Hartmann's solution (Baxter Healthcare Ltd, Norfolk, UK) to aid recovery. Once fully recovered rats were returned to their homecages. Previous studies have shown that the Dtx-UII lesion is not fully complete until 21 days post-surgery (Clark *et al.*, 2007). During this period no behavioral testing was conducted and rats were monitored daily for signs of ill health and bodyweight change.

Behavioural testing

The behavioral testing protocol was an exact replication of our previous study with ibotenic acid (Wilson *et al.*, 2009). Testing was conducted in operant chambers individually housed in sound attenuating boxes (Med-Associates, St Albans, Vermont, USA), monitored and controlled by a computer running Med-PC software (Med-Associates, St Albans, Vermont, USA). Each operant chamber had 2 retractable levers either side of a pellet dispenser. One of the levers had a light above it and there was a houselight on the opposing wall. Three days prior to operant testing rats were food restricted to 17-19g lab chow per day. To allow familiarization with the reward pellet and testing environment, rats were given a single session where 40 pellets (Test Diet purified rodent tablet 5TUL, Sandown Scientific, Middlesex, UK) were freely available in the operant box pellet dispenser. In an attempt to reduce possible latent inhibition, the levers were not extended, the sound attenuating doors were left open and rats were removed once they had consumed all pellets (approx. 20 minutes).

Rats were then tested daily in 40 min testing sessions where pressing one lever the correct number of times led to a pellet being delivered; pressing the other lever had no programmed consequence. At the start of the testing session both levers were extended and the houselight illuminated. Initially rats were

trained on fixed ratio 1 (FR1) where one press on the correct lever (side counterbalanced across rats) triggered pellet delivery and simultaneous illumination of the lever-light. This light remained illuminated for 10 sec and during this time (defined as the inter-trial-interval: ITI) pressing on the correct lever had no consequence. After the 10 sec ITI the lever-light was extinguished and the next trial began. Pressing on the incorrect lever was also monitored. Rats were advanced through a variety of FR and variable ratio (VR) testing schedules (see Table 1) depending on their individual performance. A trial in each session followed the same format, except that correct presses up to the final press in the schedule had no consequence. In the extinction schedules the trials were programed in the format of VR30 but no pellets were delivered.

Throughout all sessions all lever presses and approaches to the food hopper were recorded, allowing the following behavioural measurements to be calculated: *pressing* – the total number of presses on the correct lever during a schedule (not including ITI presses); *incorrect pressing ratio* (the ratio of incorrect : correct presses); *late pressing ratio* (the ratio of [presses on the correct lever between reward delivery and approach to the food hopper] : *pressing*); *reward collection latency* (latency to collect the pellet after delivery); *early pressing ratio* (the ratio of [correct lever presses between reward collection and the start of the next trial] : p*ressing*); *post-reinforcement pause* (latency from the start of the trial to first lever press).

Histology

Rats were administered an intraperitoneal injection of Dolethal (0.6 ml per rat; 200 mg/ml; sodium pentobarbitone; Univet Ltd, Oxford, UK) and once deeply anaesthetized, transcardially perfused with phosphate buffered saline followed by at least 300ml fixative (4% paraformaldehyde in 0.1M phosphate buffer). Brains were removed and stored in 20% w/v sucrose solution in 0.1M PB and once sunk were cut on a freezing microtome. Coronal 30µm sections were taken from the anterior facial nerve through to substantia nigra. A 1:4 series of parallel sections were immunohistochemically processed free floating for

either: (i) neuron specific nuclear protein (NeuN), using mouse derived anti-neuronal nuclear protein monoclonal antibody (Chemicon International Inc, Temecula, CA, USA), a Vector Labs Elite ABC kit (Vector Labs, Peterborough, UK) and Sigma Fast DAB peroxide for final substrate before being mounted onto slides and cresyl violet counterstained; or (ii) choline acetyltransferase (ChAT) using goat derived anti-ChAT polyclonal antibody (Chemicon International Inc, Temecula, CA, USA), Vector Labs Elite ABC kit and DAB peroxide final stain.

Lesion analysis

Sections were viewed under a light microscope (Leica DM LB2) with a high resolution camera (Leica DFC320) connected to a computer for image capture. The pPPTg was defined as the region of PPTg comprised of densely packed ChAT+ neurons posterior to the decussation of the superior cerebellar peduncle, corresponding to the region covering IAL 0.12 mm through to IAL +1.08 mm on the atlas of Paxinos and Watson (2005). The aPPTg was defined as all ChAT+ PPTg neurons anterior to this division. This is the same delineation as used in previous studies (Alderson *et al.*, 2006; 2008; Wilson *et al.*, 2009; Maclaren *et al.*, 2013) and broadly corresponds to the alternative nomenclature PPTg pars compacta (posterior) and PPTg pars dissipata (anterior) (Manaye *et al.*, 1999). On the NeuN/cresyl slides lesion extent was judged by lack of cell bodies and reactive gliosis. On the NeuN/cresyl sections a lesion was judged to be non-selective if there was evidence of areas with no cell bodies. The PPTg is a heterogeneous collection of intermingled glutamatergic, cholinergic and GABAergic neurons (Wang & Morales, 2009) with no region having solely cholinergic neurons. Therefore, even a total loss of cholinergic neurons will leave other populations intact which should be visible throughout all regions of the PPTg on the NeuN stain. The cholinergic lesion was quantified by counting ChAT+ cells within the PPTg. Each section through the anterior-posterior plane was photographed and subsequently loaded into the ImageJ program (ImageJ; US National Institutes of Health, Bethesda, Maryland, USA). Individual ChAT positive cells were

manually tagged using the cell counter plugin. The number of ChAT+ pPPTg neurons in each lesioned rat was then calculated as a percentage of the sham mean. A lesion was considered acceptable if <~80% of ChAT+ pPPTg neurons were destroyed and there was no damage evident on the NeuN/cresyl sections.

Behavioral data analysis

Data were statistically analyzed using SPSS 18 for Windows (SPSS UK, Woking, Surrey, UK). For operant data repeated measures ANOVAs were performed on each behavioral measure across *Day* (schedule day; within subjects factor) and between *Group* (lesion, sham; between groups factor). Latency data were SQRT transformed to correct for positive skew. Significant main effects and interactions were investigated with pairwise comparisons and univariate ANOVAs. Results were considered statistically significant when $p \leq 0.05$.

Results – Experiment 1: instrumental learning and performance

Lesions

All rats recovered well from the surgical procedure. Eight rats which received Dtx-UII had selective bilateral lesions of pPPTg with no indication of non-selective damage on the NeuN sections (Figures 1 and 2). Examination of the NeuN staining at the site of toxin infusion and throughout the posterior-anterior plane of the PPTg showed extensive NeuN+ staining throughout the region with no areas of visibly depleted neurons. Combined with the extensive ChAT+ cell loss and in line with previous studies (Clark *et al.*, 2007; MacLaren *et al.*, 2014b) this indicates that the toxin maintained high selectivity for the UII-R expressing cholinergic PPTg neurons. These lesions destroyed a mean of 93% of ChAT+ pPPTg neurons (range 87.8% to 98.2%). The remaining rats in the lesion group were excluded from all analysis due to having no clear sign of lesion (n = 2); unilateral or partially unilateral lesions (n = 4) or because of non-selective damage (n = 4). There was no indication of lesion in any sham treated rat.

During the 21 day lesion formation period there were no indications of ill health in the lesion group, although lesioned rats did have a transient decrease in bodyweight (Figure 3). A repeated measures ANOVA found a significant effect of day post-surgery ($F_{19,247} = 35.72$ $p < 0.001$), a significant effect of lesion group ($F_{1,13} = 8.17$ $p = 0.013$) and a significant lesion group x day post-surgery interaction ($F_{19,247} = 12.41$ $p < 0.001$). Univariate ANOVAs investigating the interaction found lesioned rats had significantly reduced bodyweight (compared to shams) on days 6 – 15 ($p<0.05$ in all cases).

Behavioral analysis

The primary question addressed was whether selective lesions of cholinergic pPPTg neurons caused impairment in operant learning. This was evaluated in two ways: first by performing an analysis of behavioral measures during initial operant learning of the FR1 schedule (Figure 4) and then by analyzing behavioral changes in response to systematic increase in reinforcement schedules and during extinction (Figure 5, 6, and 7).

Learning on FR1

The number of correct lever presses, reward collection latency and the post reinforcement pause of sham and Dtx-UII lesioned rats are shown in Figure 4. Selective lesions of cholinergic pPPTg neurons had no significant effect on the acquisition of FR1. For correct presses (Figure 4A), repeated measures ANOVA showed a significant effect of session ($F_{2,26} = 100.38$ $p < 0.001$) no significant effect of lesion group ($F_{1,13} = 1.94$ $p = 0.187$) and no significant lesion group x session interaction ($F_{2,26} = 0.45$ $p = 0.643$). Planned pairwise comparisons found that the overall rate of correct pressing in session 2 was higher than session 1 ($p < 0.001$) and higher in session 3 than session 2 ($p = 0.001$). Reward collection latency was unaffected

by lesion: repeated measures ANOVA found a significant effect of session ($F_{2,26} = 181.00$ $p < 0.001$) no significant effect of lesion group ($F_{1,13} = 0.163$ $p = 0.693$) and no significant lesion group x session interaction ($F_{2,26} = 0.088$ $p = 0.916$). Planned pairwise comparisons found that the overall reward collection latency in session 2 was lower than session 1 ($p < 0.01$) and lower in session 3 than session 2 ($p < 0.001$. Post-reinforcement pause was also unaffected by Dtx-UII pPPTg lesion: repeated measures ANOVA found a significant effect of session ($F_{2,26} = 118.04$ $p < 0.001$), no significant effect of lesion group ($F_{1,13} = 1.58$ $p = 0.231$) and no significant lesion group x session interaction ($F_{2,26} = 0.437$ $p = 0.651$). Planned pairwise comparisons found that the overall post reinforcement pause in session 2 was lower than session 1 ($p < 0.001$) and lower in session 3 than session 2 ($p = 0.003$). Taken together, these results show that rats learned to lever press on an FR1 schedule of reinforcement: with no significant main effect of lesion group, or interactions involving group, it can be concluded that lesions of cholinergic pPPTg neurons had no effect on learning using this simple schedule.

Learning of new fixed and variable schedules of reinforcement

Once rats had learned FR1, we advanced them through various fixed and variable ratio schedules of reinforcement (see Table 1). For clarity, data are presented showing performance of sham and lesioned rats on the first and last day of each schedule. Figure 5 shows the number of correct lever presses on the first and last day of each schedule; Figure 6 the reward collection latency; and Figure 7 the post reinforcement pause. No significant effects involving lesion were found. For correct presses (Figure 5), repeated measures ANOVA found a significant effect of schedule ($F_{6.9,90.4} = 96.92$ $p < 0.001$), no significant effect of lesion group ($F_{1,13} = 0.01$ $p = 0.924$) and no significant lesion group x schedule interaction ($F_{6.9,90.4} = 0.96$ $p = 0.468$). Planned pairwise comparisons investigating the effect of schedule found the number of correct presses increased during FR5, VR5, the switch to VR10 ($p<0.05$ in all cases) and then stayed the same until extinction. Lesions of cholinergic pPPTg neurons had no effect on the

number of correct lever presses across these schedule changes. For reward collection latency (Figure 5), repeated measures ANOVA found a significant effect of schedule ($F_{1.2,15.0} = 10.66$ $p = 0.004$) no significant effect of lesion group ($F_{1,13} = 0.113$ $p = 0.743$) and no significant lesion group x schedule interaction ($F_{1.6,15.0} = 0.17$ $p = 0.725$). Planned pairwise comparisons investigating the effect of schedule, found the reward collection latency changed significantly during extinction, but not during any other point prior to this. For post reinforcement pause, repeated measures ANOVA found a significant effect of schedule ($F_{4.2,54.3} = 7.70$ $p < 0.001$) no significant effect of lesion group ($F_{1,13} = 0.159$ $p = 0.697$) and no significant lesion group x schedule interaction ($F_{4.2,54.3} = 1.44$ $p = 0.232$). Planned pairwise comparisons investigating the effect of schedule found the post reinforcement pause decreased during FR5 and increased during the VR schedules ($p < 0.05$ in all cases). Separate analysis performed on all data (rather than first and last day) produced the same main significant effects as the analysis reported here and no significant effects or interactions involving lesion group (data not shown). Combined, these results show that selective lesions of cholinergic PPTg neurons had no effect on the acquisition or performance of fixed and variable ratio schedules of reinforcement.

Experiment 2: nicotine sensitization

Methods

Subjects

Twenty-four adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used, with a mean weight of 355g (range 331 – 389g) at time of surgery. Rats were pair housed in temperature and humidity controlled environment and kept on a 12hr light/dark cycle (lights on 7AM, testing carried out in the light phase). Food and water was always freely available in the homecage. Compliance with the Animals

(Scientific Procedures) Act 1986 and European Communities Council Directive of 24/11/86 (86/609/EEC) was maintained.

Surgery

Lesion surgery was performed as described in Experiment 1. Sixteen rats received bilateral Dtx-UII infusions into the pPPTg, 8 rats received sham (vehicle only) infusions into pPPTg.

Behavioral testing

Dtx-UII is a protein synthesis inhibitor based toxin which, after entry into the cell, takes up to 21 days for cell death to occur (Clark *et al.*, 2007). To ensure the lesion was formed before testing began, behavioral testing began 21-24 days post-surgery. The behavioral testing protocol is a replication of the protocol previously used to assess nicotine sensitization in excitotoxic pPPTg lesioned rats (Alderson *et al.*, 2008). Locomotor testing was conducted in 6 perspex cages (45.7 cm x 24.1 cm) situated inside "SmartFrame™ Cage Rack Stations" (LED rearing 7x15 High Density, Hamilton Kinder LLC, Poway CA, USA). These contained a 7 x 15 grid of infra-red beams at the height of the rats body. All stations were interfaced with a computer system running "Motor Monitor" software (Hamilton Kinder LLC, Poway CA, USA) which recorded all beam breaks made in the cages. Daily testing sessions were 60 min long, conducted in a dimly illuminated room; each session had a proportionally equal number of sham and lesioned rats. Rats were given 3 habituation sessions where they were placed in the locomotor cages without any injections. This was followed by 7 sessions where rats were injected with 0.9% w/v saline (s.c.; 1mL/kg) immediately prior to testing. After completing this habituation period nicotine testing began. Nicotine sensitization was performed in a day-on day-off routine whereby rats received nicotine (s.c.; 0.4 mg/kg in 0.9% saline; nicotine hydrogen tartrate, Sigma–Aldrich, UK; dose refers to salt) or saline (s.c.; 1mL/kg, 0.9% saline) on alternating days for 14 days. The order of testing was counterbalanced so that on any given day half the rats received nicotine and half saline. All injections were performed in a procedure room opposite the

locomotor testing room: each rat was individually taken to the procedure room, injected, then taken to and placed in the locomotor testing cage, which started recording beam breaks immediately.

Behavioral data analysis

Data were analyzed using SPSS 18 for Windows (SPSS UK, Woking, Surrey, UK). For locomotor data the number of beam breaks per session were SQRT transformed to correct for positive skew in the data (identified by the Shapiro-Wilk test). Separate repeated measures ANOVA were performed across days for the habituation and nicotine testing components of the experiment. Details of particular factors analyzed are reported in the corresponding results section. In the case of significant interactions, these were investigated with planned pairwise comparisons and univariate ANOVAs, where appropriate. Results were considered significant when $p \leq 0.05$.

Lesion analysis

Seven rats had selective bilateral lesions of the cholinergic pPPTg. These lesions destroyed a mean of 89.5% of ChAT+ pPPTg neurons (range 78.8% to 94.8%; see Figure 8) with no evidence of non-selective damage on the NeuN/Cresyl stain. Figure 9 shows photomicrographs from representative lesion and sham rats. The remaining rats in the lesion group were excluded from all analysis because of having unilateral lesions (n = 2); partial ChAT+ lesions (range ˜34-70% cell loss, n = 5), having non-selective damage (n = 2).

Experiment 2: nicotine sensitization – behavioral results

Habituation sessions

The rate of locomotion and rearing during the habituation sessions (where rats had 3 sessions of no injections followed by 7 sessions with saline injections) is shown in Figure 10. Selective lesions of cholinergic pPPTg neurons had no effect on baseline levels of spontaneous locomotion or habituation to the testing environment. For beam breaks during the daily habituation sessions a repeated measures ANOVA found a main effect of session ($F_{6.41,83.40} = 6.46$, $p < 0.001$) but not group ($F_{1,13} = 0.63$, p =

0.44) and no session x group interaction (F6.41,83.40 = 1.27, p = 0.28). Restricted planned pairwise comparisons found that sessions 1, 2 and 3 differed from some, but not all, later sessions (1 from 6 and 8; 2 and 3 from 8) and that from session 4 onwards there were no differences between sessions.

Nicotine sessions

Nicotine testing sessions

Figure 11 shows the mean number of beam breaks (basic movements) during the nicotine and saline testing sessions. Selective lesions of cholinergic pPPTg neurons had no effect on nicotine induced locomotor changes or the rate of nicotine sensitization. Repeated measures ANOVA found a significant effect of session ($F_{6,78} = 27.39$, $p < 0.001$), a significant effect of drug ($F_{1,78} = 4.58$, $p = 0.05$) and a drug x session interaction ($F_{6,78} = 44.52$, $p < 0.001$) and that all effects involving lesion group were non-significant (group ($F_{1,13} = 0.58$, $p = 0.46$); drug x group ($F_{1,78} = 2.76$, $p = 0.121$); group x session ($F_{6,78} = 1.75$, $p = 0.122$); drug x group x session ($F_{6,78} = 0.76$, $p = 0.601$)). Bonferroni corrected paired sample t-tests comparing the effect of nicotine and saline during each session found that during the first session both the lesion and sham groups displayed hypolocomotion (sham $t7 = -4.82$, $p = 0.014$; lesion $t6 = -8.56$, $p < 0.01$) which developed into hyperlocomotion during the later testing sessions (sham session 5: $t7 = -6.33$, $p < 0.01$. Lesion session 6: $t6 = -6.10$, $p = 0.007$). These results show that both groups displayed a sensitized response to repeated systemic nicotine administration and that selective lesions of cholinergic pPPTg neurons had no effect on this.

Discussion

Summary

These experiments examined the effects of lesions of cholinergic neurons within the posterior pedunculopontine tegmental nucleus (pPPTg – also described as PPTg pars compacta) on instrumental learning and nicotine sensitization. Selective depletion of cholinergic PPTg neurons was achieved using the fusion toxin Dtx-UII (Clark *et al.*, 2007). Behavioral testing in the instrumental learning and performance experiments consisted of assessing the ability to learn under fixed and variable ratio schedules of reinforcement for food pellet reward. This experiment was a replication of our previous study which showed that rats bearing excitotoxic (ibotenic acid) lesions of pPPTg were persistently impaired in learning every schedule of reinforcement tested and, once having learned the schedules, displayed behavioral changes during performance of them (Wilson *et al.*, 2009). In contrast to this, selective depletion of cholinergic pPPTg neurons caused no measurable effect on any aspect of learning or performance across all schedules tested. The second experiment examined (in separate rats) the rate of locomotor sensitization to repeated systemic nicotine following selective depletion of cholinergic pPPTg neurons. The experimental protocol was a replication of previous work in our laboratory showing enhanced sensitization to systemic nicotine following excitotoxic lesion of the pPPTg. Mirroring the excitotoxic lesion, Dtx-UII lesions had no effect on baseline levels of spontaneous locomotion. However, in contrast to the excitotoxic lesion, Dtx-UII lesions had no effect on the rate of sensitization to repeated systemic nicotine.

Neuronal sub-types affected by lesion techniques

The key difference between this study and the previous reports is that the previous reports used techniques which non-selectively target all neuronal types within PPTg, whereas in this current work Dtx-UII caused selective and extensive depletion of the cholinergic neuronal sub-population. The PPTg is a

heterogeneous collection of interdigitated cholinergic, glutamatergic and GABAergic neurons (Wang & Morales, 2009). These neuronal types are not equally distributed: In the posterior PPTg, there are more glutamatergic than cholinergic neurons, with the GABAergic population being the smallest population. In the anterior PPTg, the GABAergic population is the largest, glutamatergic second largest and cholinergic population the smallest. Previously, experimental work in laboratory animals assessing PPTg function has typically used excitotoxic agents to create lesions, or GABA agonists such as muscimol to induce transient inactivation. Excitotoxic agents bind to NMDA channels and lock them open, causing unregulated calcium influx which rapidly becomes neurotoxic (Berdichevsky *et al.*, 1983). While these agents are selective for neurons (unlike electrolytic lesions which also destroy fibers of passage) they are not selective for the type of neuron they target. The mechanism of action of muscimol (activation of inhibitory GABA receptors) also, in this region of brain, has no selectivity for the neuronal subtype targeted. In contrast to this, Dtx-UII targets cells which express the receptor for the peptide urotensin II, which, within the mesopontine tegmentum, is selectively expressed by cholinergic neurons (Clark *et al.*, 2001). Furthermore, not only does Dtx-UII selectively target the cholinergic neuronal sub-population, but it results in more extensive cell loss within this population than generally experienced with the use of excitotoxic agents. For example in the instrumental learning experiment here, cholinergic cell loss was 93% but in our previous study using the same paradigm with ibotenate lesions, cholinergic cell loss in the pPPTg was only 64% (Wilson *et al.*, 2009).

Relation to previous work – instrumental learning and performance

There is an extensive body of literature showing deficits in learning and performance of goal directed tasks following excitotoxic lesion of PPTg. Rats bearing bilateral lesions of the whole PPTg (anterior and posterior) are impaired at learning to navigate a maze for food reward (Dellu *et al.*, 1991) and at the delayed spatial win shift radial maze task (Keating & Winn, 2002). Likewise, excitotoxic PPTg lesioned rats are impaired at acquiring lever pressing for intravenous self-administration of d-amphetamine (Alderson

et al., 2004) and heroin (Olmstead *et al.*, 1998) and do not form conditioned place preference to morphine or amphetamine (Bechara & Vanderkooy, 1989; Olmstead & Franklin, 1994) (although, interestingly, the same lesions do not block cocaine place preference (Parker & van der Kooy, 1995)). These learning deficits are not the result of altered reward perception or reduced motivation – performance of lesioned rats increases when reward value in increased (Taylor *et al.*, 2004; Ainge *et al.*, 2006) and if the task is learned prior to surgery, PPTg lesioned rats have identical levels of responding as sham controls (Alderson *et al.*, 2004). More recently, the learning impairment has been shown to be a result of loss of posterior but not anterior PPTg (Wilson *et al.*, 2009) and furthermore to be specifically in the updating of goal directed action-outcome associations (Maclaren *et al.*, 2013). These studies implicate disrupted input to midbrain DA systems as being the core reason for learning impairment after PPTg manipulation. Midbrain DA neurons switch from a tonic to a phasic firing pattern in response to unexpected reward, or stimuli that predict expected reward (Schultz, 1998; Schultz, 2010). This firing pattern (described as reward prediction error signal, or alternatively sensory prediction error signal) is crucial for normal instrumental learning (Redgrave *et al.*, 2008; Zweifel *et al.*, 2009). The switch in firing patterns is critically dependent on cholinergic input – it does not happen in the absence of functioning cholinergic receptors (Maskos *et al.*, 2005; Maskos, 2007; Maskos, 2008; Steidl *et al.*, 2011a). The PPTg sends extensive excitatory glutamatergic and cholinergic innervation to midbrain DA neurons (Mena-Segovia *et al.*, 2008b) and is able to switch the firing pattern of these neurons from tonic to phasic (Lodge & Grace, 2006; Chen & Lodge, 2013). PPTg neurons are known to encode non-physical aspects of sensory stimuli such as salience and reward prediction and, crucially, to do this at a shorter latency than midbrain DA (Okada *et al.*, 2009; Thompson & Felsen, 2013). This has led to the hypothesis that PPTg may provide crucial information required for generating the reward prediction error signal (Kobayashi & Okada, 2007; Okada & Kobayashi, 2013). This interpretation is entirely consistent with the finding that in trained rats, inactivation of PPTg has no effect on baseline firing of midbrain DA neurons, but selectively silences the phasic firing in

response to reward predicting stimuli (Pan & Hyland, 2005). Previously it has been shown that selective loss of cholinergic PPTg neurons has no effect on the continued performance of a drug self-administration task learnt prior to lesion surgery (Steidl *et al.*, 2011b). Our current results show that normal instrumental learning can occur in the absence of cholinergic input from pPPTg to midbrain DA neurons. Two interpretations emerge from this. The first is that a functioning cholinergic pPPTg has no role in normal instrumental learning and therefore absence of functioning cholinergic PPTg has no impact on this behavior. The second interpretation is that a functioning cholinergic pPPTg may contribute to instrumental learning, but in its absence, compensatory mechanisms allow this to continue despite a reduction in input. While cholinergic innervation of midbrain DA is essential for normal firing patterns, the loss of pPPTg will not lead to a total loss of cholinergic input to any midbrain sub region. In the case of the VTA (particularly involved in instrumental learning) the loss of pPPTg will reduce numbers of cholinergic neurons projecting to VTA by only around 26% (numbers used for calculation taken from: Wang and Morales, 2009). Whichever of these explanations is correct, the key finding is that instrumental learning can occur in the absence of a functioning cholinergic pPPTg, but *cannot* occur normally after lesioning or inactivation targeting all neuronal types within PPTg. The most parsimonious interpretation of this difference is that the non-cholinergic pPPTg is critically involved in normal instrumental learning – without it, this process is severely disrupted.

Relation to previous work – nicotine sensitization

Repeated systemic administration of nicotine causes reliable, dose dependent, locomotor sensitization. First administration induces locomotor depression which, over a period of re-administration (the speed of which depends on dose) develops into hyperlocomotion (Benwell & Balfour, 1992). This effect is believed in part to be mediated via activation and subsequent up-regulation of nAChRs on VTA DA neurons (Reavill & Stolerman, 1990; Vezina *et al.*, 2007; Govind *et al.*, 2009). However, nicotine also activates nAChRs on VTA glutamatergic (Grillner & Svensson, 2000) and GABAergic neurons (Mansvelder *et al.*,

2002). It has been proposed that nicotine has a prolonged action on glutamatergic VTA which in turn increases glutamate driven VTA DA activation, while the action on GABAergic (inhibitory) VTA neurons has been shown to be short transient activation followed by prolonged desensitization leading to depression of inhibition (Mansvelder *et al.*, 2002). Therefore, the action of nicotine in the VTA is more complex than simply acting directly upon DA neurons and driving DA output, but instead may involve several parallel events: immediate excitatory action on DA neurons, prolonged activation of DA neurons mediated by glutamatergic activity and additional persistent depression of inhibitory GABAergic input, with the net result being rapid and sustained increase in mesoaccumbens DA levels. Excitotoxic lesions of the pPPTg alter the locomotor response to nicotine (Alderson *et al.*, 2008). The initial hypolocomotion seen in sham animals was absent and the rate of subsequent hyperlocomotion accelerated. One interpretation of this effect is that the pPPTg lesioned rats had an enhanced rate of sensitization, a consequence of up-regulation of VTA nAChRs in response to the reduction in innervation arriving from the pPPTg, leading to a greater response when systemic nicotine acts on this system. A second interpretation is that nicotine has a direct action on the pPPTg, and loss of the pPPTg therefore changes the nicotinic response in a manner independent from direct alterations within VTA. There is some evidence to support the hypothesis that nicotine has an effect within PPTg. PPTg neurons expresses various nAChRs and systemic nicotine induces *c-fos* activation within the PPTg (Lanca *et al.*, 2000). Interestingly, this activation appears to be almost exclusively within the non-cholinergic neuronal sub-populations. While there is less behavioral evidence to support the view that nicotine has a direct effect on PPTg neurons, one study reports that nicotine micro-infused directly into PPTg induces a significant conditioned place preference for nicotine rather than vehicle infusion (Iwamoto, 1990). The results from our current study are not compatible with the hypothesis that the altered sensitization to nicotine following excitotoxic pPPTg lesion are a direct consequence of up regulation of nAChRs within VTA. The cholinergic neuronal loss within PPTg in our study was higher than following excitotoxic lesion, yet there were no indications of

altered sensitization to nicotine. Our results are compatible with the view that nicotine may have a direct effect on non-cholinergic PPTg, or that the altered sensitization following excitotoxic lesion is a result of disrupted signaling within VTA following combined loss of cholinergic and non-cholinergic PPTg.

Functionally dissecting the PPTg: cholinergic v non-cholinergic PPTg systems

Previously, functionally dissecting the PPTg has focused on investigating the behavioral roles of the anterior and posterior PPTg components (Alderson *et al.*, 2006; 2008; Wilson *et al.*, 2009; Martinez-Gonzalez *et al.*, 2011). In these studies we have undertaken a different approach and attempted to examine the functions of one neuronal population within PPTg. While the cholinergic and non-cholinergic neurons appear to innervate very similar structures (Hallanger & Wainer, 1988; Semba & Fibiger, 1992; Mena-Segovia *et al.*, 2008a; Kita & Kita, 2011) the profile of these projections appears very different. Single cholinergic PPTg neurons are known to send massively bifurcated projections, whereby one single neuron targets multiple efferent structures (Jourdain *et al.*, 1989; Semba *et al.*, 1990; Losier & Semba, 1993; Dautan *et al.*, 2014). In contrast to this, the non-cholinergic projections, despite innervating the same regions, appear to form far simpler projections whereby one neuron targets only one or two efferent regions (Mena-Segovia *et al.*, 2008a). Furthermore, while there are relatively few studies analyzing the relative densities of cholinergic versus non-cholinergic projections to target regions, where these have been studied (VTA and STN) it appears that in terms of number of projecting neurons, there are more non-cholinergic than cholinergic neurons innervating target regions (Wang *et al.*, 2010; Kita & Kita, 2011). Given the apparent different projection patterns in the presence of similar projection regions, it is interesting to speculate what the different functions of the cholinergic and non-cholinergic projections may be. Cholinergic PPTg neurons have a long association with involvement in behavioral state control. While the PPTg is not essential for normal sleep (Deurveilher & Hennevin, 2001), cholinergic PPTg neurons do change their activity across sleep-wake transitions (Ros *et al.*, 2010), are most active during wake and REM states, and are linked to changes in cortical EEG (Mena-Segovia & Bolam, 2011). The cholinergic PPTg

neurons, sending relatively sparse yet diverse innervation to numerous efferent regions, seem ideally suited to be coordinating synchrony across multiple brain regions and/or to be involved in integrating information across regions. In contrast, the non-cholinergic PPTg, particularly the glutamatergic component, is ideally suited to send rapid excitatory input to a subset of target regions. This interpretation is also consistent with the results from our current studies. The instrumental learning task we used is relatively straightforward: despite having high and variable schedules of reinforcement, it involves little complexity beyond association formation and adjusting levels of lever pressing. Learning of goal directed operant tasks is critically dependent on basal ganglia and midbrain DA systems, but can be acquired normally despite large lesions of, for example, hippocampal and entorhinal circuitry (Corbit & Balleine, 2000; Reichelt *et al.*, 2011). This supports the view that a disruption in integration of information across circuitry outside basal ganglia could have little impact on standard operant learning. Operant learning, however, is critically dependent on accurate rapid processing of sensory input and the ability to attribute non-physical properties (such as salience) to these stimuli. This process is dependent on a functioning non-cholinergic PPTg (Alderson *et al.*, 2004; Wilson *et al.*, 2009). It would be of interest to test this working hypothesis of the function of cholinergic PPTg by assessing the effects of loss of cholinergic PPTg neurons in behavioral tasks with a considerably stronger reliance on multi-modal integration of information – for example context dependent instrumental learning (Corbit & Balleine, 2000; Reichelt *et al.*, 2011), occasion setting (Reichelt *et al.*, 2011) or cue driven behavioral changes, which are known to be highly susceptible to cholinergic manipulation (Palmatier *et al.*, 2006; Farquhar *et al.*, 2011).

Conclusions

We assessed the involvement of cholinergic neurons within the pPPTg in operant learning and nicotine sensitization. Lesions of cholinergic pPPTg neurons, created with Dtx-UII, were highly destructive to this neuronal population (over 90% cell loss). These lesions had no effect on instrumental learning or the rate of nicotine sensitization – two behaviors which are severely and persistently affected by lesions of all

neuronal types within pPPTg. Our results strongly implicate the role of the non-cholinergic PPTg in these behaviors and highlight the importance not attributing the deficits observed after excitotoxic manipulation of this region solely to loss of cholinergic neurons.

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Tables

Table 1

Schedule	Number of correct presses required	Criteria to be met before advancing to the next schedule
FR1	1	2 consecutive sessions of >80 trials completed
FR5	5	2 consecutive sessions of >60 trials completed, or 5 sessions.
VR5	1-9 (mean 5)	5 sessions completed
VR10	1-19 (mean 10)	2 sessions completed
VR15	1-29 (mean 15)	2 sessions completed
VR30	1-59 (mean 30)	7 sessions completed
Extinction	No reward delivered	7 sessions completed

Table 1: Reinforcement schedules and criteria used to advance rats through the schedules. Rats were advanced through all schedules from top to bottom. The number of correct presses required refers to the number of correct presses required on a given trial to earn food reinforcement. In VR schedules, this number was randomly picked from the range on a trial-to-trial basis.

Table 2

	Correct lever presses		Reward collection latency		Post reinforcement pause	
Schedule	**Dtx-UII**	**Ibotenic**	**Dtx-UII**	**Ibotenic**	**Dtx-UII**	**Ibotenic**
FR1	-	↓	-	↑	-	↑
FR5	-	↓	-	↑	-	↑
VR5	-	↓	-	-	-	-
VR10	-	↓	-	-	-	-
VR15	-	↓	-	-	-	-
VR30	-	↓	-	↑	-	-
Extinction	-	-	-	-	-	-

Table 2: *Comparison of effects of selective lesions of cholinergic pPPTg neurons (Dtx-UII) in this study and excitotoxic (ibotenic acid) lesions performed in our earlier study (Wilson et al. 2009). Arrows indicate significant difference and direction of difference (compared to sham controls). "-" indicates no significant difference.*

Figures

Figure 1

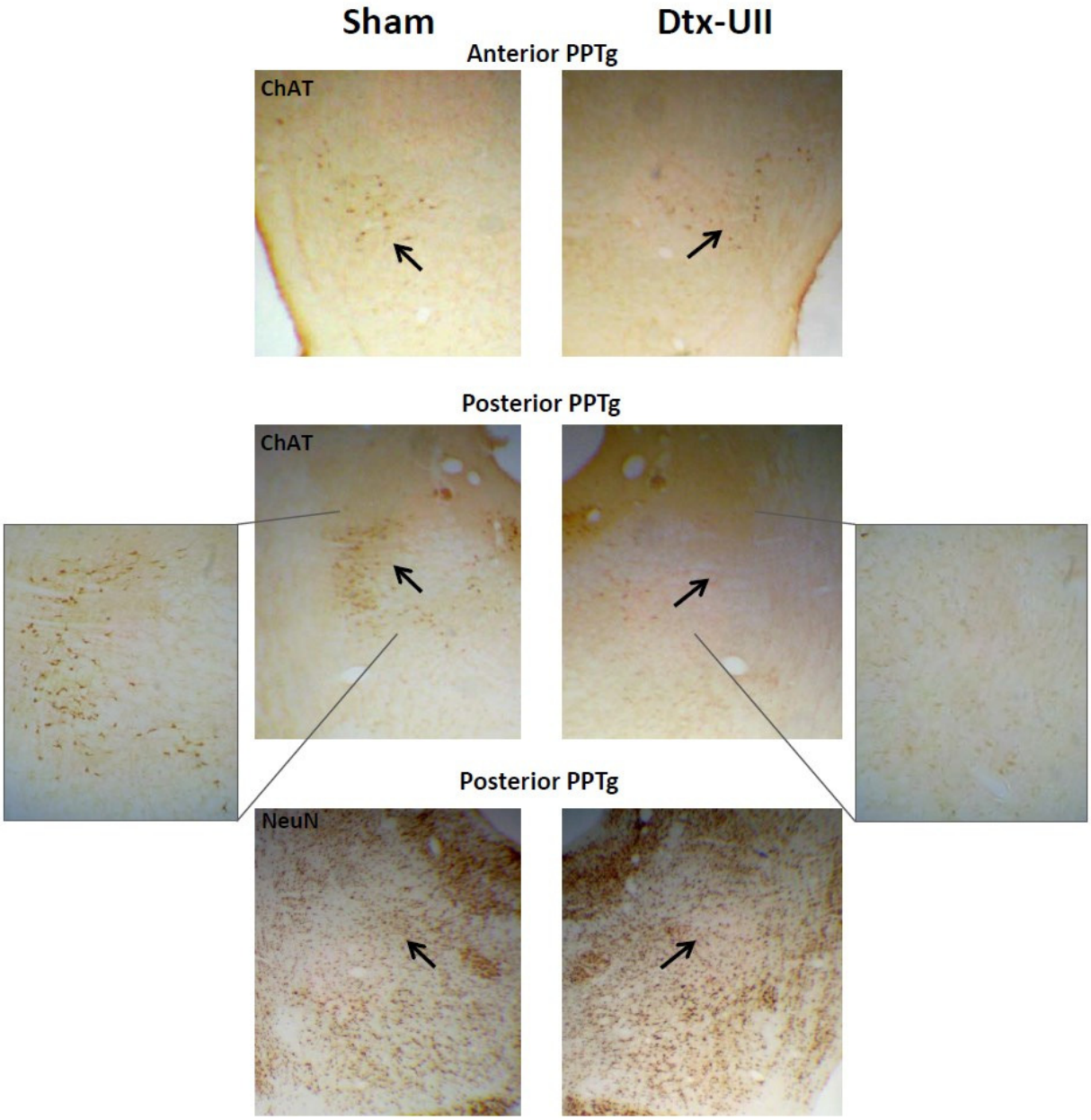

Figure 1: Photomicrographs from a sham lesioned rat (left panels) and Dtx-UII lesioned rat (right panels). The top row shows cholinergic neurons within anterior PPTg, the middle rows shows cholinergic neurons within the posterior PPTg, the cut out shows a high magnification image of the same posterior PPTg section. The bottom row shows a NeuN / Cresyl double stained section immediately parallel the ChAT section above it, which is through the region of the greatest ChAT cell loss. The black arrows indicate the location of the PPTg.

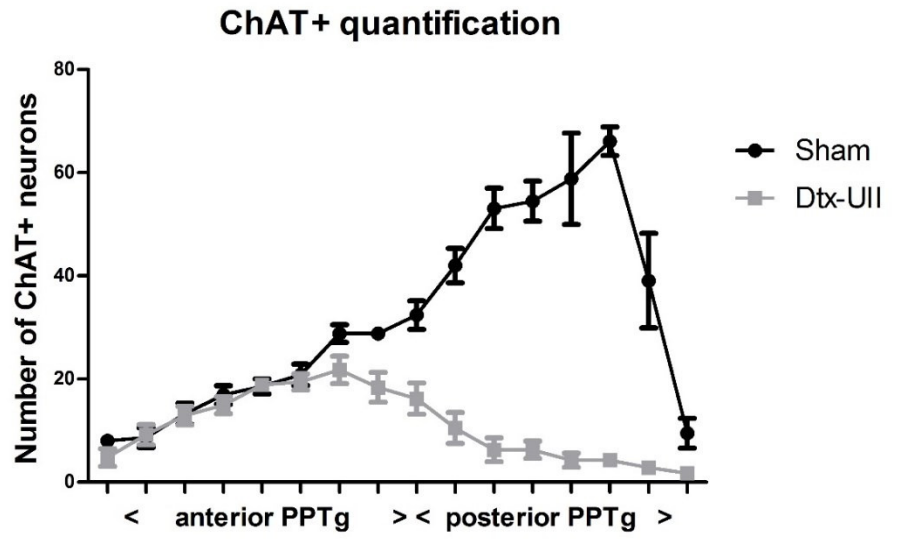

Figure 2: Quantification of number of ChAT+ neurons present along the anterior-posterior axis of the PPTg.

Graph shows group means ± SEM

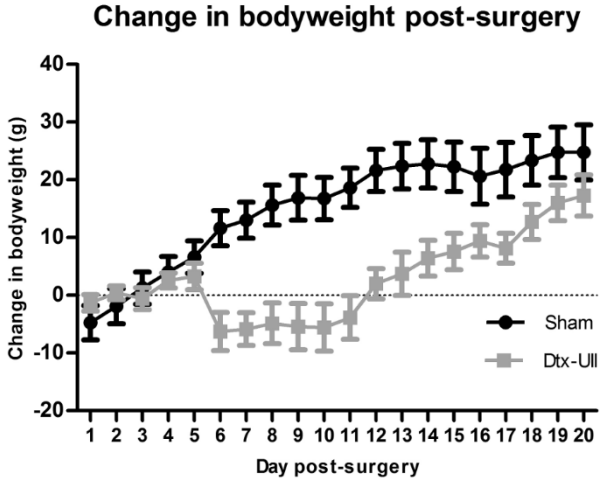

Figure 3: Post-surgery bodyweight of sham control and successful selective cholinergic pPPTg lesioned rats.

Graph shows group means ± SEM.

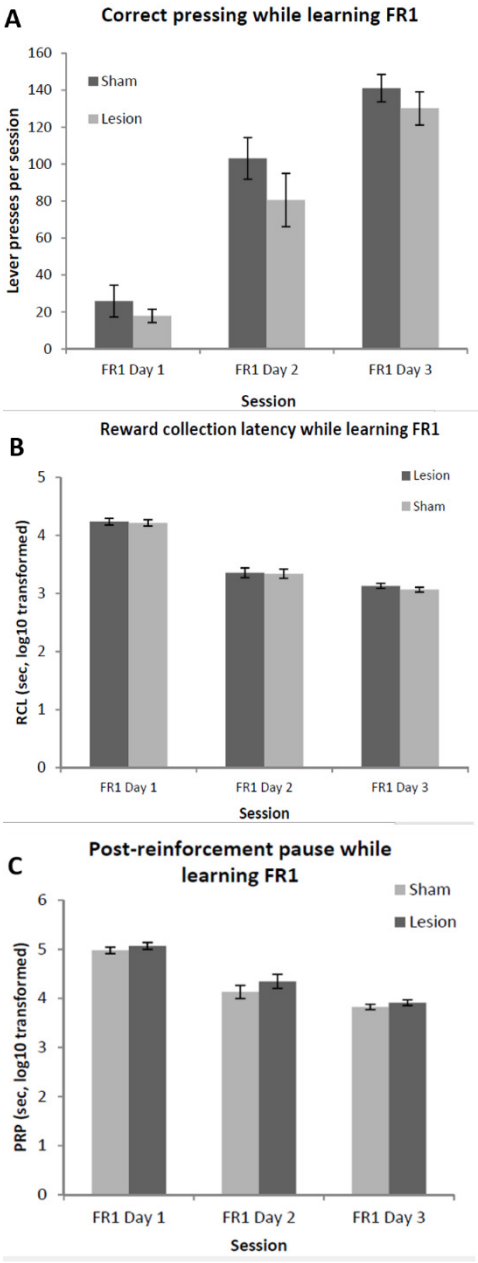

Figure 4: Lesions of cholinergic pPPTg neurons had no significant effect on acquisition of a novel instrumental action (FR 1). Panel A shows the number of correct lever presses; panel B shows the reward collection latency; and panel C shows post-reinforcement pause. All graphs, group means ±SEM.

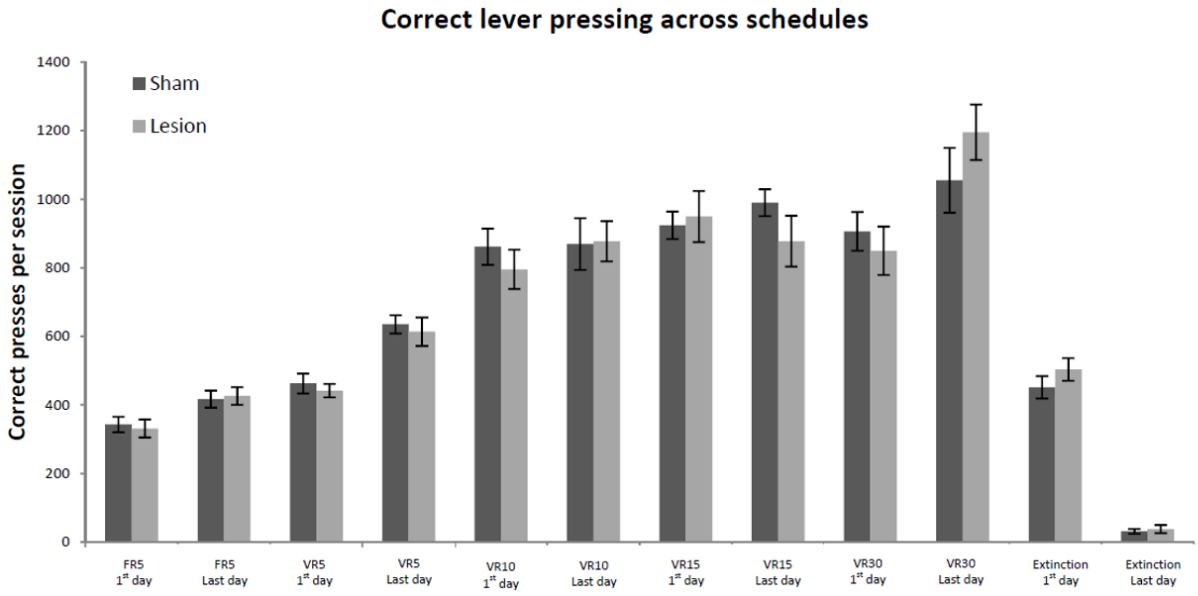

Figure 5: Number of correct lever presses performed on the first and last day of each schedule after FR1.

No significant differences were found between sham and lesioned rats. Graph shows group means ±SEM

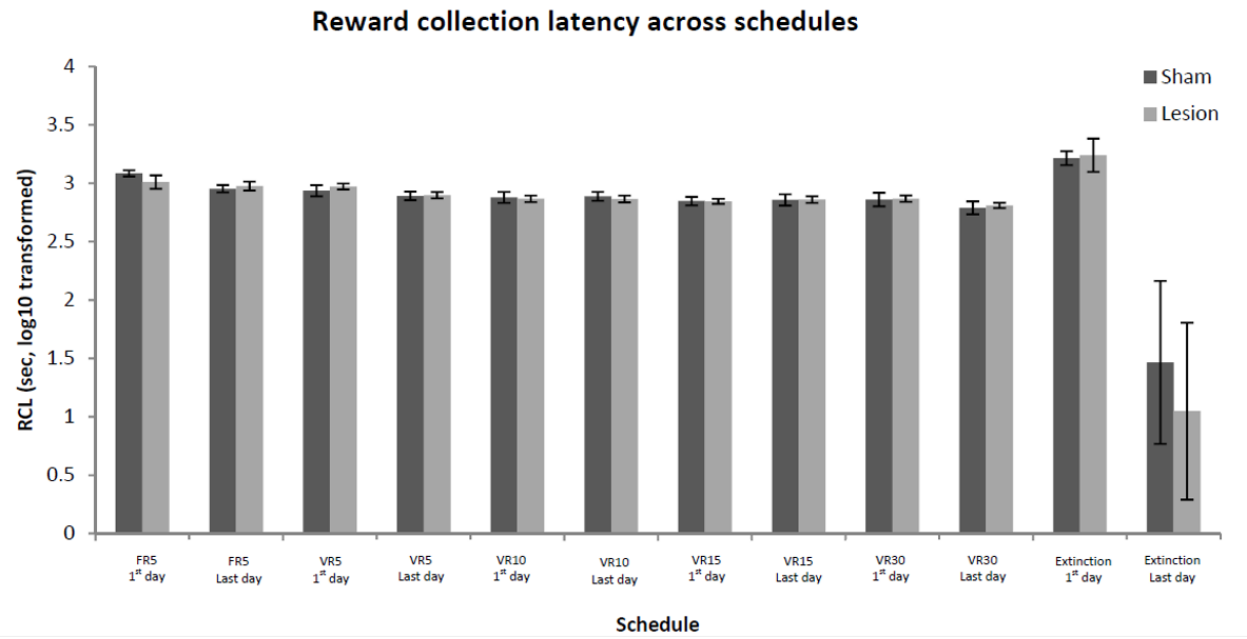

Figure 6: Reward collection latency on the first and last day of each schedule after FR1. No significant differences were found between sham and lesioned rats. Graph shows group means ±SEM

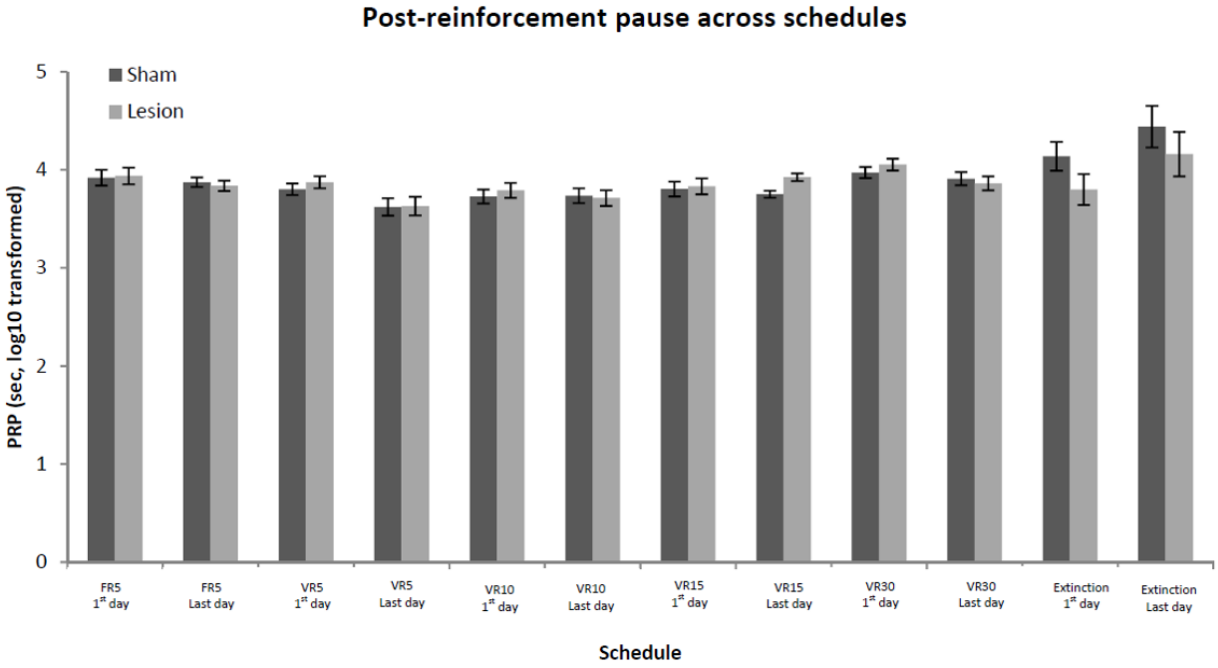

Figure 7: Post reinforcement pause on the first and last day of each schedule after FR1. No significant differences were found between sham and lesioned rats. Graph shows group means ±SEM

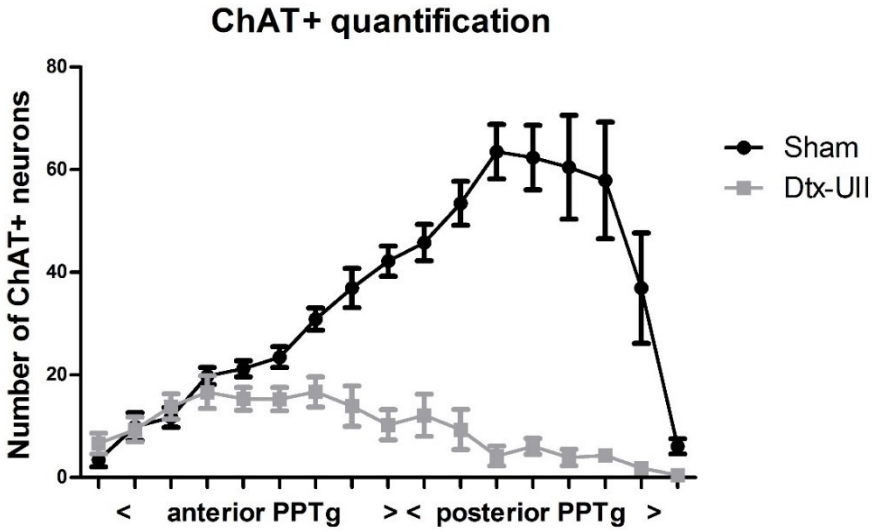

Figure 8: Quantification of number of ChAT+ neurons present along the anterior-posterior axis of the PPTg.

Graph shows group means ± SEM

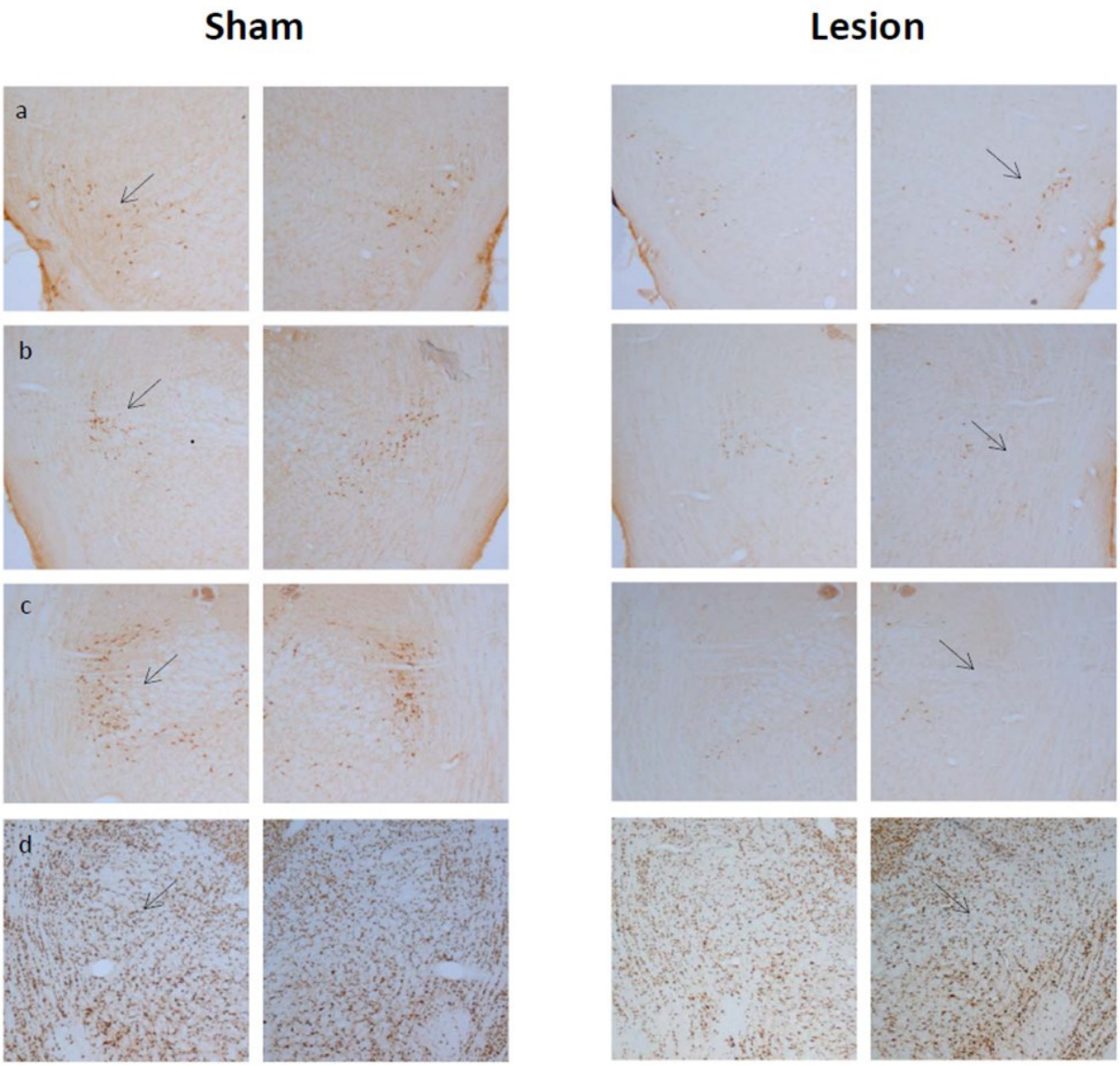

Figure 9: Photomicrographs from a sham (left panels) and a Dtx-UII lesioned (right panels) rat. Rows a – c show ChAT stained sections of anterior PPTg (row a), division between anterior and posterior PPTg (row b) and posterior PPTg (row c). Row d shows a NeuN / Cresyl double stained section immediately parallel to row c, at the level of the posterior PPTg and greatest ChAT cell loss. Dotted arrow indicates the location of the PPTg

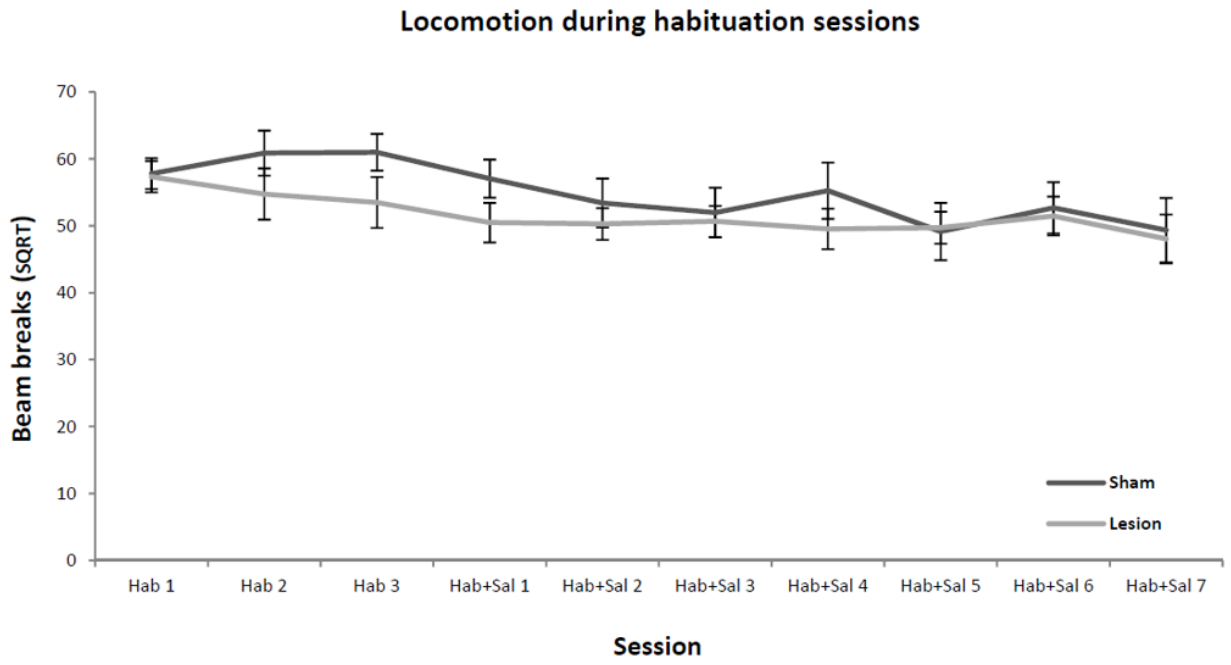

Figure 10: Beam breaks made by sham and Dtx-UII pPPTg lesioned rats during the habituation sessions. Hab = habituation session; Hab+sal = saline injection and habituation session. Graph shows group means ± SEM.

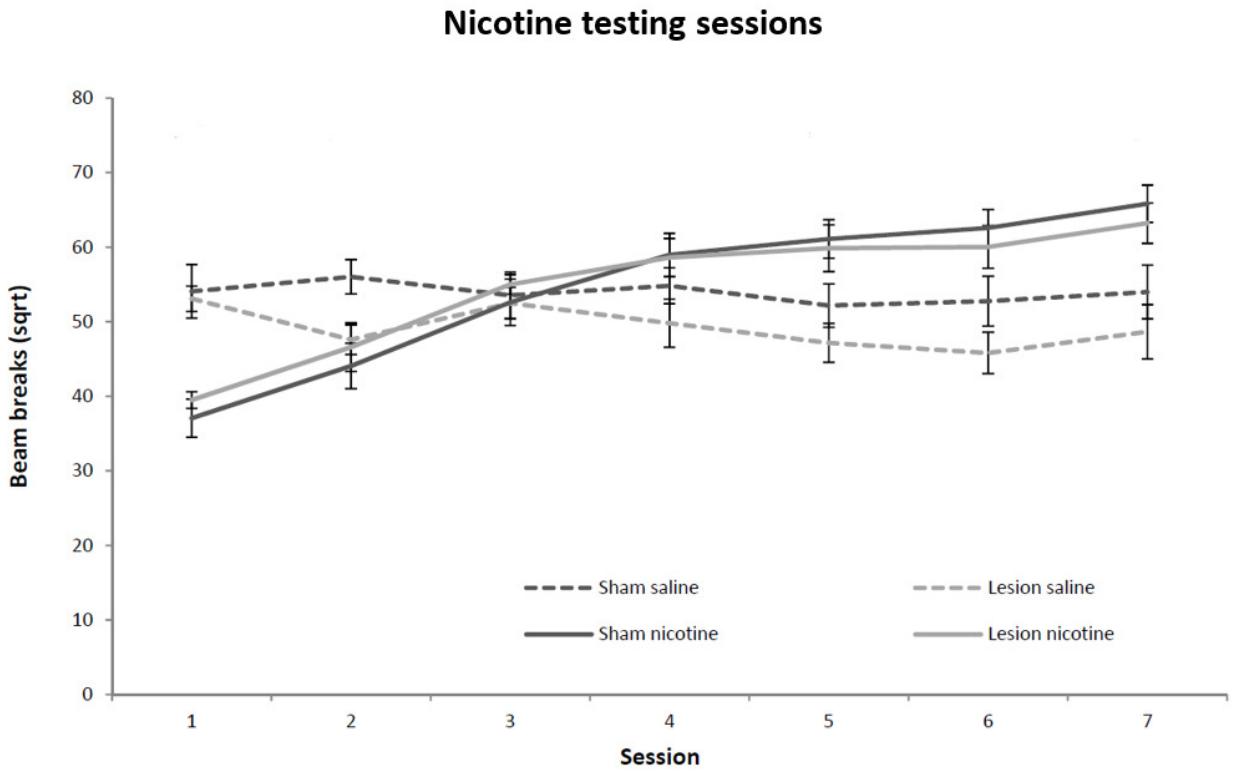

Figure 11: 6.8: Basic movements made during the nicotine testing sessions by sham and Dtx-UII lesioned rats. Graph shows group means ± SEM.

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