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Citation for final published version:

Lindgren, Hanna S., Demirbugen, Merve, Bergqvist, Filip, Lane, Emma L. and Dunnett, Stephen B. 2014. The effect of additional noradrenergic and serotonergic depletion on a lateralised choice reaction time task in rats with nigral 6-OHDA lesions. *Experimental Neurology* 253 , pp. 52-62. 10.1016/j.expneurol.2013.11.015

Publishers page: <http://dx.doi.org/10.1016/j.expneurol.2013.11.015>

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## The effect of additional noradrenergic and serotonergic depletion on a lateralised choice reaction time task in rats with nigral 6-OHDA lesions

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Keywords: 6-OHDA, Parkinson's disease, operant, noradrenaline, serotonin, dopamine

## Abstract

Parkinson's disease (PD) patients often suffer from visuospatial deficits, which may result from a disruption of the representation of external space. The lateralised choice reaction time (CRT) task is an operant task for rodents in which similar visuospatial deficits can be assessed. Specific parameters in this task are disrupted after unilateral nigrostriatal injections of 6-hydroxydopamine (6-OHDA), directly linked to the severe striatal dopamine (DA) depletion that inevitably follows this type of lesion. However, studies have demonstrated that this type of lesion also affects the serotonergic (5HT) and noradrenergic (NA) midbrain pathways. The influence of these systems on visuospatial parameters in the CRT task had not yet been investigated.

To this end, rats were pretrained on the CRT task before receiving selective lesions of the DAergic system, either alone or in combination with bilateral depletion of the NA- or 5HT system. All rats with a 6-OHDA lesion displayed a gradual decline in the selection, initiation and execution of lateralised movements on the side contralateral to the lesion compared to sham-lesion controls. They also displayed a reduced number of useable trials as well as an increased number of procedural errors. Interestingly, the group with an additional noradrenergic lesion was significantly slower in reacting to lateralised stimuli throughout the testing period, as compared to the other two groups with a 6-OHDA lesion. There was however, no pronounced difference between the three different lesion groups in any other parameters assessed in the task.

These data confirm previous findings demonstrating that the majority of the parameters assessed in the lateralised CRT task are strongly dependent on DA. However, an important new finding in this study is the illustration of a putative role for the NAergic system in contributing to the attentive performance influencing the animals' capacity to react to the presented lateralised stimuli.

## Introduction

It is now increasingly recognised that Parkinson's disease is not a pure motor disorder and that patients can experience a variety of additional non-motor symptoms, such as visuospatial deficits, cognitive decline and autonomic dysfunction (Chaudhuri et al., 2006). These non-motor features of PD are a significant burden for the patients and their careers, and strongly contribute to a reduced quality of life (Schrag, 2004). It is also well established that the cardinal motor symptoms of the disorder stem from the progressive degeneration of the dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the subsequent loss of DA in the striatum (Marsden, 1994). However, our knowledge about the pathogenesis underlying the non-motor symptoms is less comprehensive (Chaudhuri et al., 2006). Anti-parkinsonian drugs have a very limited effect, if any, on many of the non-motor symptoms, suggesting that these symptoms cannot solely arise from the nigrostriatal dopaminergic deficits. Indeed, it is well recognised that neither the cell loss nor the classic Lewy body pathology is limited to the DAergic system, and significant effects on the noradrenergic (NA) and serotonergic (5HT) systems have been documented (Halliday et al., 1990; Mann and Yates, 1983; Scatton et al., 1983). It has been postulated that the non-motor symptoms, evident in a majority of PD patients, are linked to this progressive non-dopaminergic pathology (Chaudhuri et al., 2006).

Many patients with PD experience visuospatial deficits (Geldmacher, 2003), which are exemplified as difficulties navigating through confined spaces and interacting with objects in their environment (Lee et al., 2001; Skidmore et al., 2009). Interestingly, unilateral 6-hydroxydopamine (6-OHDA) models of PD show marked impairments in detecting and responding to stimuli on the side contralateral to the dopamine depletion (Brown and Robbins, 1989; Carli et al., 1985; Dowd and Dunnett, 2004; Milton et al., 2004). In an operant lateralised choice reaction time task (CRT; Carli et al., 1985), rats are required to make a sustained nose poke in the central hole of a nine-hole box apparatus (9HB) in response to a stimulus light in the same hole (Robbins et al., 1993). After variable delays, a brief stimulus light is randomly presented on either the left or right side

of the animal's head, and the rats are required to nose-poke into the corresponding hole. Early work utilising this task suggested that the DA depletion disrupts spatial representation of the environment, important for appropriately directing movements in contralateral space (Brown and Robbins, 1989; Carli et al., 1985; Carli et al., 1989).

More recent work has demonstrated that the severity of deficits in the CRT task appears to be dependent upon the site of administration of the dopaminergic toxin. 6-OHDA can be applied either into the striatum to terminal regions of the nigrostriatal fibres, or directly into the medial forebrain bundle (MFB) containing the projecting fibres. Increased severity in the task was associated with the MFB injection as compared to the intrastriatal 6-OHDA injection, and presumed to be related to a greater degree of dopaminergic depletion (Dowd and Dunnett, 2005). However, it has been demonstrated that 6-OHDA applied into the MFB can also have a substantial effect on the other monoaminergic systems, and no attempts were made in these studies to protect non-dopaminergic systems from the toxin (for example by concurrent application of the noradrenaline reuptake blocker, desipramine (Roberts et al., 1975). Even with blockade of noradrenergic reuptake sites (the site of toxin uptake) striatal and cortical levels of NA have been found to be strongly reduced after MFB administration of 6-OHDA (Fulceri et al., 2006). The serotonergic system can also be similarly affected by this approach, with partial damage to the ascending 5HT-projections from the raphe nucleus (Lindgren et al., 2010; Smith et al., 2012). The previous studies utilising the lateralised CRT task in MFB-lesioned rats did not evaluate the extent of damage on the NA- and 5HT-ergic systems, therefore the potential contribution of these to altered parameters in the task remains unknown (Dowd and Dunnett, 2004; Dowd and Dunnett, 2007). We therefore set out to compare the performance of rats with a 'pure' DA lesion versus those with a combined DA/NAergic lesion or DA/5HTergic lesion. To this end, rats were pretrained on the CRT task before being subjected to a specific dopaminergic lesion by applying the 6-OHDA directly into the origin of the nigrostriatal pathway, the SNpc, located ventrally to the ascending noradrenergic and serotonergic fibre bundles at that level of the mesencephalon (Roberts et al., 1975) and then combined with bilateral lesions of either the NA neurons

(targeting the locus coeruleus (LC) with 6-OHDA) or the 5HT neurons (targeted by intraventricular injection of 7-hydroxyserotonin-creatinine-sulphate salt (5,7 DHT)). Once the lesions were established the rats were re-tested in the lateralised CRT task and their performance evaluated and compared between groups and with sham-lesion controls.

## Materials and methods

### Animals

Female Lister hooded rats (220-250g, Charles River, Margate, Kent, UK) were housed in groups of four per cage under a 14:10-h light/dark cycle and controlled temperature (20°C). All rats were allowed *ad libitum* access to water during the study but were food-restricted to 85-90% of their free feeding weight, starting one week prior to each period of testing. All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, with local ethical review and under Home Office personal and project licenses.

### Experimental design

A total of 79 rats were used in this study and the experimental design is shown in Fig. 1. All rats were pre-trained on the lateralised CRT task until a stable baseline was established (approx. six weeks of training) as well as on the staircase task (Montoya et al., 1991), which assess skilled reaching performance. They were divided into two groups based on their baseline performance in the afore-mentioned tasks before receiving either a nigral 6-OHDA lesion (n= 56) or sham lesion (administration of saline; n = 23). Two weeks post-surgery, the extent of dopamine depletion was evaluated by assessing the number of spontaneous rotations elicited during ten minutes, which were then used to divide the 6-OHDA lesioned rats into three balanced groups. One group of 6-OHDA lesion rats received an additional bilateral injection of 6-OHDA in the LC (DA+NA group, n = 16), the second group of rats received an injection of 7-hydroxyserotonin-creatinine-sulphate salt (5,7 DHT) bilaterally into the lateral ventricles (DA+5-HT group, n = 22). The remaining group of nigral 6-OHDA lesion rats did not receive any additional lesions and served as a nigral lesion

only group (DA only, n = 18). One third of the sham-lesion controls (n=6) received additional injections of saline into the LC, while another subgroup (n=6) received saline injections into the lateral ventricles. Three weeks after the second surgery, the rats were retested on the lateralised CRT task for six days and their performance evaluated. The rats were then assessed in the staircase test to evaluate the additional impact of the NA- and 5HT depletion on skilled reaching performance.

### 6-OHDA lesions

Rats were injected with both the NA reuptake inhibitor desipramine (i.p. 20mg/kg dissolved in saline; Sigma Aldrich, Gillingham, Kent, UK) and 5-HT reuptake inhibitor fluvoxamine (i.p. 3mg/kg dissolved in saline; Sigma Aldrich) 20 minutes prior to surgery to protect NA- and 5HT-fibres from any diffused 6-OHDA. The rats were then anaesthetised using 1.5–2.5 % isoflurane with a 2:1 mixture of nitrous oxide and oxygen as carrier gases and mounted into a Kopf stereotaxic frame using atraumatic ear bars with the nose bar set at -3.8 mm below the interaural line (flat skull). A midline incision was made in the skin overlying the skull. A small hole was drilled at the following coordinates: AP = -5.3 and ML = -1.7 from bregma, (Paxinos, 1998). 6-OHDA (4 µg/µl of 0.9 % saline containing 0.05 % ascorbic acid; Sigma Aldrich) or saline was infused at DV = -7.2 from dura via a 30-gauge metal cannula with a flow rate of 0.25µl per minute (total infusion time 8 min), and left to diffuse for a further 2 min before retraction of the cannula. Following surgery, wounds were cleaned and sutured and rats received 30 µl Metacam (5 mg/ml, Boehringer Ingelheim, Germany) subcutaneously for pain relief and glucose-saline to reduce postoperative dehydration. Rats were allowed to recover in a heated cage before being returned to their home cage.

### Serotonergic and noradrenergic lesions

The lesions were performed in a similar manner as for the 6-OHDA lesions using a Kopf stereotaxic frame and atraumatic ear bars with the nose bar set at -3.8 mm below the interaural line (flat skull). Both the 5HT- and NAergic lesions were performed bilaterally to prevent compensatory sprouting from the



intact side and in the knowledge that bilateral lesions of this nature do not compromise the health of the animals.

For the NAergic lesion, rats received an i.p. injection of fluvoxamine (i.p. 3mg/kg dissolved in saline) 20 min prior to surgery to prevent damage of the 5HT fibres. After anaesthesia was induced, two small holes were drilled at: AP = -9.7 and ML =  $\pm 1.3$  from bregma, (Paxinos, 1998) and 6-OHDA (4  $\mu$ g in 2  $\mu$ l of 0.9 % saline containing 0.05 % ascorbic acid) was infused sequentially in the right and left LC at DV = -6.9 from dura with a flow rate of 0.25  $\mu$ l per minute (total infusion time 4 min). The cannula was left to diffuse for a further 2 min before removal. Post-operative care was as described above.

For the 5HT lesions, rats received an i.p. injection of desipramine (i.p. 20mg/kg dissolved in saline) prior to the surgery to prevent any potential damage of the NAergic fibres. After anaesthesia had been induced, two small holes were drilled at: AP = -0.8 and ML =  $\pm 1.4$  from bregma, (Paxinos, 1998) and 5,7-DHT (100  $\mu$ g freebase in 5  $\mu$ l of 0.9 % saline containing 0.05 % ascorbic acid) was infused sequentially in the right and left lateral ventricle at DV = -3.6 from dura with a flow rate of 1  $\mu$ l per minute (total infusion time 5 min). The cannula was left to diffuse for a further 2 min before removal. Post-operative care was as described above.

## **Behavioural tasks**

### **Lateralised choice reaction time task**

The lateralised choice reaction time task (CRT) was first described by Carli et al. (Carli et al., 1985) and requires the rat to make a lateralised response after a sustained centralised nose poke. The task was conducted in a bank of twelve identical 9HB operant chambers, set in sound attenuating wooden cubicles (Cambridge Cognition, UK; (Robbins et al., 1993). Each 9HB box measured 25 cm  $\times$  25 cm  $\times$  25 cm with a grid floor and a door in the front wall for access. A house light was set in the middle of the ceiling. The rear wall of the chamber was curved with a horizontal array of nine equidistant 2 cm  $\times$  2 cm square holes, each 2 cm above floor level. A light-emitting diode in each of the nine holes allowed presentation of a visual stimulus specific to each hole, and vertical infrared beams detected and registered hole entries. A food magazine



covered by a clear Perspex panel was mounted to the front wall providing reward pellets after correct responses. A stimulus light was set at the back of the food magazine to signal delivery of 45 mg sucrose reward pellets (Sandown Scientific, Middlesex, UK). The 9HB chambers were controlled by Cambridge Cognition Control software (Campden Instruments LTD., version 1.23) running on a standard desktop PC using the Windows XP™ operating system.

When using 9HBs for the CRT task, the centre hole, the third hole on the left and the third hole to the right were left open whereas the other holes were covered with metal caps (Dowd and Dunnett, 2004; Dowd and Dunnett, 2005). The training regimen began with accustomising the rats to retrieving reward from the magazine panel by retrieving 30 sucrose pellets at the start of the test session. In a second phase of training, the rats were trained to associate the click of the food pellet dispenser and the magazine light with the delivery of a sugar pellet, i.e. a single sugar pellet was delivered every time the rat poked into the magazine. Thirdly, the rats were trained to poke in the centre hole, which resulted in the delivery of a sucrose pellet in the magazine. Then, the rats were trained to respond to the light stimulus in either the right or the left hole (randomly chosen by the computer), following a central stimulus light. During the training period of six weeks, the time required for the rats to hold their nose in the centre hole gradually increased from 50 to 800 ms, and the duration of the light stimulus was slowly decreased from 10 s to 200 ms, thus progressively making the training more challenging.

During the actual CRT task, each trial began with the presentation of the centre stimulus light. If no centre nose poke was detected within 10s (Limited hold, LH), that trial was terminated and after a 2 s interval (Inter-trial interval, ITI), a new trial began. During a regular and correctly-performed trial the rat had to hold the nose in the centre hole for a randomly chosen delay until a light stimulus (200 ms) was presented either on the left or the right hand side. If poked correctly, the rat was subsequently rewarded with the delivery of one sugar pellet as positive feedback for responding to the light stimulus on the side of stimulus presentation. If the rat failed to perform the lateralised response within the LH (10s), the house light came on and a time-out period started (10s). After the time-out period, the next trial was initiated. The duration of the test

session was 30 min per day with equal number of left and right stimuli presented in a random order. All rats acquired the task using long holds (200, 400, 600, and 800 ms) but shorter holds (50, 100, 150, and 200 ms) were used for the post-lesion testing. This enabled a high enough number of completed trials to permit statistical comparisons, reflecting previous studies showing a pronounced reduction in useable trials when long holds are used following a 6-OHDA lesion (Dowd and Dunnett, 2005; Lindgren et al., 2013).

Accuracy, total usable trials (TTU), movement time (MT), and reaction time (RT) were measured in this task. The accuracy was defined as the percentage of times that a rat made correct lateral nose-poke to the side of stimulus presentation. A usable trial was defined as a trial on which the rats completed the required hold into the central stimulus hole. The RT was the time from onset of the lateral stimulus to withdrawal from centre hole after the lateralised stimulus was presented, and MT the time taken to execute the lateralised nose-poke after withdrawing from centre hole. The nature of errors was also evaluated: the number of premature withdrawals from the centre hole during the holds, nose-poking into the incorrect hole, panel presses during time-outs and the number of time-outs (failure to respond to the stimulus within the LH).

### Staircase test

The staircase test was first described by Montoya and co-workers (Montoya et al., 1990) and is a measure of skilled forelimb use and grasping performance. Rats were first habituated to the apparatus, a Plexiglas box with a removable baited staircase (seven graded stages), for 15 minutes for two to three days with the staircase baited with sugar pellets in excess. Thereafter, the rats were trained for 15 min each day until a stable baseline performance was achieved (approx. 6-7 days of training) with the staircase baited with four sugar pellets on each stage, giving a total of 28 pellets per side/paw. The number of pellets eaten was calculated for both paws (left and right). All rats were trained prior to the initial dopaminergic lesion and retested on the staircase task for four days immediately after finishing the final re-test in the lateralised CRT task. The testing was performed in a similar manner as during the baseline training.

## Histological analysis

### Tissue preparation

Rats were terminally anaesthetized with sodium pentobarbital (150 mg / kg i.p., Euthatal™ 1.0 mL / rat, 200 mg / ml: Merial, Harlow, UK) and sacrificed by transcardial perfusion with 50 ml of 0.01 M phosphate buffer followed by 250ml of 1.5 %, buffered (pH 7.4) paraformaldehyde (Sigma Aldrich). Brains were post-fixed for 24 hours in paraformaldehyde, transferred to 20 % sucrose overnight and sectioned coronally on a freezing microtome at 40 µm thickness. Free-floating sections were stored in cryoprotective solution at -20 °C until further processing.

### Immunohistochemistry

Immunohistochemistry was performed using a peroxidase-based detection method and 3'3'-diaminobenzidine (DAB; Sigma Aldrich) as the chromogen. Sections were washed in TRIS-buffered saline (TBS) and thereafter pre-treated with 10 % methanol and 3 % hydrogen peroxide to quench endogenous peroxidase activity. After washing with TBS, the sections were incubated with 5 % horse serum in TBS containing 0.25 % Triton X-100 (TXTBS) for 1 h and thereafter left overnight with the primary antibody in TXTBS with 5 % horse serum at room temperature. The following primary antibodies were used: mouse anti-Tyrosine hydroxylase (TH) (1:2000; Millipore, Watford, UK), mouse anti-Dopamine-β-Hydroxylase (DβH) (1: 750; Millipore), mouse-anti serotonin transporter (SERT)(1:750; Millipore). After washing in TBS, sections were incubated with the secondary biotinylated horse anti-mouse antibody (1:200, Vector Laboratories, Peterborough, UK). The secondary antibody was visualised using a standard peroxidase-based method system (Vectastain Elite ABC, Vector Laboratories) with DAB as chromogen. Sections were rinsed in TBS to stop the DAB reaction, mounted on chromalum-coated slides and coverslipped using DPX mounting medium (Sigma Aldrich).

### Optical density measurement

Striatal TH-positive fibre density was measured by optical density (OD) analysis with the assessments performed at four equidistant rostrocaudal levels

(one section per level), spanning from the rostral pole of the striatum (AP: +2.20) to the rostral pole of the globus pallidus (AP: -0.92). Photomicrographs of TH-stained striatal sections were captured using a Leica DFC420 camera (Leica, Milton Keynes, UK) mounted on a freestanding microscope (Wild-Heerburgg M420), so that the whole section could be visualised in the photomicrograph. The ODs was then measured using the NIH-free software Image J (<http://rsbweb.nih.gov/ij/>). For every rat, the ODs from both the intact and the lesioned side were determined and the final reading was calculated by subtracting the value from the corpus callosum from each section from the striatal OD values, to account for non-specific staining. OD values are expressed as percentages of the intact side.

### **Stereological counts**

The number of TH-positive cells in the SNpc was estimated using the Olympus CASTgrid stereology system (Visiopharm, Hoersholm, Denmark) on 5 sections in a series of 1:6. The fractionator method was used, which samples a fraction of the total cell number, i.e. the fraction of the area sampled, the fraction of sections stained and the ratio between the section thickness and the dissector from which they were counted (West, 1999). The CASTgrid software was used to delineate the borders of SNpc at 4x magnification and generate counting areas of 150x150µm. The counting of cells was made at 100x magnification and the counting frame (3568 µm<sup>2</sup>) was placed randomly on the first counting area and systematically moved through all counting areas until the entire delineated area was sampled. The sampling frequency was chosen so that approximately 110 TH-positive cells from the intact side was sampled. Since this method was not suitable for the lesioned side in the 6-OHDA rats where very few TH-positive cells remained, the number of cells on this side was counted manually and the values corrected for split cell counts (Abercrombie, 1946).

### **Fibre density counts**

The number of NA and 5HT fibres was estimated in a 800 x 800 µm area of the striatum and medial prefrontal cortex using the Olympus CASTgrid stereology system. The region of interest was delineated at 4x magnification and counting areas of 200x200µm was generated using the CASTgrid software. The

counting of fibres within these areas was made at 100x magnification by randomly placing the counting frame ( $3568\ \mu\text{m}^2$ ) on the first counting area and then systematically moved until the entire delineated square of  $800 \times 800\ \mu\text{m}$  was sampled. The sampling frequency resulted in approximately 16 sampling areas and the counting was performed on one section per rat and from both the right and the left side of the brain. The data are expressed as the total sum of fibres from both hemispheres in the area of  $800 \times 800\ \mu\text{m}$ .

## HPLC analysis

### Tissue sample preparation

Rats were terminally anaesthetized with sodium pentobarbital and the striatum and prefrontal cortex dissected out rapidly on a cold glass plate and kept frozen at  $-80^\circ\text{C}$  until analysis.

### Biochemical analysis

The monoamine transmitters (DA, 5HT and NA) were quantified in the prefrontal and striatal tissue samples by high performance liquid chromatography (HPLC) followed by electrochemical detection. After ultrasound homogenization (Branson Analog Sonifier 450; Branson Ultrasonic Co.) in 0.1 M perchloric acid with 2.5 mM EDTA, 0.65 mM reduced glutathione and an internal standard ( $\alpha$ -methyldopamine, 418 nM) and subsequent centrifugation, the supernatant was injected into a sample splitting HPLC system. A cation-exchange chromatography system was used for the separation of NA, DA, and 5HT. A fixed loop of  $26\ \mu\text{l}$  was used to inject a portion of the sample on a  $0.2 \times 15\ \text{cm}$  Nucleosil SA  $5\ \mu\text{m}$  column (Macherey-Nagel, Düren, Germany) with a mobile phase consisting of citric acid 0.049 M, NaOH 0.114 M, methanol 20%,  $\text{Na}_2\text{-EDTA}$  0.012 mM. The analytes were detected with an amperometric detector (Waters 460) operated at 0.55 V versus an Ag/AgCl reference. The analysis of HVA, DOPAC and 5-HIAA were performed with a reverse phase chromatography system, into which  $10\ \mu\text{l}$  were injected from a fixed loop. The reverse phase system consisted of a Nucleosil RP-18  $3\ \mu\text{m}$  column ( $0.2 \times 5\ \text{cm}$ ), a mobile phase (0.010 M  $\text{K}_2\text{HPO}_4$ , 0.040 M citric acid, 0.012 mM  $\text{Na}_2\text{-EDTA}$ , 6% methanol) and an identical Waters amperometric detector operated at 0.75 V. The currents

from the detectors were recorded using the Chromeleon Chromatography software (Thermo Scientific Dionex, Sunnyvale, CA).

### Statistical analysis

All data are presented as group means  $\pm$  S.E.M. The statistics for the histology, biochemistry and staircase test were analysed using a two-factor ANOVA where side and group were added as variables. Time course data from the lateralised CRT task was analysed a three-factor ANOVA where side, group and day was added as variables. Errors during the operant task was analysed using one-factor ANOVA for the different groups. Post-hoc analysis was performed using Student-Newman Keul's test or Bonferroni's when appropriate. The threshold for statistical significance was set at  $\alpha$  level of 0.05 and all analyses were made using Genstat v.10.

## Results

### Immunohistochemical evaluation of the lesion extent

The selectivity of the lesions was evaluated using both immunohistochemistry and biochemical analysis in two subset of rats (see Fig. 1).

The unilateral injection of 6-OHDA in the SNpc resulted in pronounced reduction of TH-positive neurons in the SNpc on the side contralateral to the lesion compared to sham-lesion controls (Fig. 2A, C; main effect of group  $F_{3,46} = 17.94$   $p < 0.001$ , main effect of side  $F_{1,46} = 105.30$   $p < 0.001$ ). As expected, there was no difference in the number of TH-positive neurons between the three different lesion groups (DA only, DA + NA, DA + 5HT; Fig. 2A, C). The striatal TH-innervation was also evaluated using optical density of TH immunoreactivity. The injection of 6-OHDA resulted in an almost complete depletion of TH-positive fibres in the striatum compared to sham-lesion controls and to the contralateral side (Fig. 2B, D; main effect of group  $F_{3,46} = 12.40$   $p < 0.001$ , main effect of side  $F_{3,46} = 163.40$   $p < 0.001$ ) but there was no difference in lesion extent between the three lesion groups.

Bilateral injection of 5,7-DHT in the lateral ventricles resulted in a reduction of striatal SERT-positive fibre innervation with approximately 70% in both the left and the right hemisphere (Fig. 3A, A'; main effect of group  $F_{3,46} = 227.00$   $p < 0.001$ ) compared to both the sham-lesion controls and the other two lesion groups (DA only, DA + NA). There was however no difference in SERT-innervation between the left and the right side of the brain (Fig. 3A; main effect of side, n.s.).

Since the NA innervation of the striatum is sparse, histological assessment of D $\beta$ H fibre density was made in the medial prefrontal cortex to which the LC extensively projects (Cenci et al., 1992). Bilateral injection of 6-OHDA in the LC caused an 85% reduction in D $\beta$ H- positive fibres in the medial prefrontal cortex (Fig. 3B, B'; main effect of group  $F_{3,46} = 95.04$   $p < 0.001$ ) compared to both sham-lesion controls and the other two lesion groups (DA only, DA + 5HT). There was however no difference between the left and the right side of the brain (Fig. 3A; main effect of side, n.s.).

### Biochemical evaluation of lesion extent

Another subset of rats was prepared for biochemical analysis of the lesion extent in both the striatum and prefrontal cortex. The unilateral injection of 6-OHDA into the SNpc had no effect on the tissue levels of DA in the prefrontal cortex with no difference found between lesion groups and sham lesion controls (Fig. 4A; main effect of group  $p = \text{ns}$ ). In contrast, the striatal levels of DA were strongly reduced in all three groups that had received the unilateral 6-OHDA injection into the SNpc compared to both the contralateral side and the sham-lesion group (Fig. 4B; main effect of group  $F_{3,94} = 23.43$   $p < 0.001$ , main effect of side  $F_{3,94} = 146.40$   $p < 0.001$ ). There was however no difference in striatal DA levels between the three different lesion groups.

Bilateral injections of 5,7 DHT into the lateral ventricles resulted in a reduction of the 5HT levels in the prefrontal cortex compared to both sham-lesion controls and the other two lesion groups on the left side (Fig. 4C, D; main effect of group  $F_{3,98} = 15.32$   $p < 0.001$ ), and towards sham-lesion controls and the DA + 5HT group in the right hemisphere (Fig. 4C, right panel). The striatal levels of 5HT were also reduced after the additional 5HT lesion compared to the sham-



lesion controls and the two other lesion groups (Fig. 4C, D; main effect of group  $F_{3,94} = 78.71$   $p < 0.001$ ). In addition, the DA only lesion group had significantly lower levels of 5HT on the side ipsilateral to the lesion compared to sham lesion controls in this region (Fig. 4D, second panel).

Depletion of the noradrenergic innervation by 6-OHDA injection in the LC resulted in a 50% reduction of NA levels in the prefrontal cortex compared to both the sham-lesion group and the other two lesion groups (Fig. 4E; main effect of group  $F_{3,116} = 15.34$   $p < 0.001$ ). In the striatum, the additional NAergic lesion reduced the levels of NA compared to the other groups but this did not reach significance (Fig. 4F; n.s.).

### Lateralised CRT task

All rats were pretrained on the lateralised CRT task for approximately six weeks until a stable base line was established. All rats acquired the task readily and there was no difference between the different lesion groups in any of the parameters assessed (baseline data not shown). The rats were retested in the operant boxes three weeks after the second lesion (and approx. five weeks from the baseline recordings) and their performance on the total trials usable (TTU), accuracy, reaction time (RT) and movement time (MT) was assessed for six consecutive days.

Each trial started with the illumination of the centre stimulus light. If no nose poke was detected within 10s (the limited hold), an inter-trial-interval of 2s was started and a new trial initiated. Thus, a useable trial was defined as a trial in which the rat completed the sustained nose-poke in the central hole. On the first day of testing, rats in the DA + NA group performed less TTU's than the sham-lesion controls (Fig. 5A; main effect of group  $F_{3,76} = 18.71$ ,  $p < 0.01$ , day \* group  $F_{15,380} = 2.36$ ,  $p < 0.01$ ; *post hoc*  $t = 4.72$ ,  $p < 0.01$ ) but there was no difference between the controls and the other two lesion groups at this time point ( $t < 3.38$  n.s.). From day two and onwards, a deficits in the rats with a 6-OHDA manifested and they performed fewer TTU's than the sham-lesion controls rats for the rest of the testing period (Fig. 5A; *post hoc*  $t > 5.62$ ,  $p < 0.01$ ). There was no difference in number of TTU's between the three different types of

lesion at any time point examined (DA only, DA + NA, DA + 5HT; *post hoc* all  $t < 1.84$ , n.s.).

The response accuracy was recorded for both left and right stimuli, and was defined as the percentage of times a rat made a correct nose-poke, i.e. on the side of stimulus presentation. The response accuracy on the right side (i.e. the side ipsilateral to the 6-OHDA lesion) was close to the performance of sham-lesion controls throughout the six days of testing with the exception of the DA + NA group on the first day of testing (Fig. 5B left panel; side  $\times$  day  $\times$  group  $F_{15, 380} = 3.85$ ,  $p < 0.001$ ; *post hoc* vs. sham  $t = 3.74$ ,  $p < 0.05$ ). On the side contralateral to the lesion, the rats with a 6-OHDA lesion had similar response accuracy as sham-lesion controls on the first day of testing (Fig. 5B right panel; *post hoc*  $t < 3.04$  n.s.) but thereafter a deficit gradually developed. From day two of testing and onwards, all rats with a 6-OHDA-lesion gradually worsened in their response accuracy on the side contralateral to the lesion, to around 20% accuracy on the 6<sup>th</sup> day of testing (*post hoc* vs. sham  $t > 9.90$   $p < 0.01$ ). There was however no difference between the group of rats with a 'pure' DA lesion compared to those with a combined DA + 5HT or DA + NA lesion in the response accuracy on any of the testing days (*post hoc* vs. all  $t < 3.05$ , n.s.).

The RT, i.e. the time to initiate a response on presentation of the stimulus was also gradually affected on the contralateral side by the injection of 6-OHDA (Fig. 5C; group  $\times$  side  $\times$  day  $F_{15, 380} = 3.24$ ,  $p < 0.001$ ). Indeed, on the first and second day of testing there was no difference in the RT between the 6-OHDA lesion groups and the sham lesion controls on the side contralateral to the lesion (Fig. 5C right panel; *post hoc* all  $t < 1.49$ , n.s.). However, on the third day a deficit emerged but only in the rats with a combined DA+NA lesion (Fig. 5C right panel; *post hoc* vs. all other groups  $t < 3.08$ ,  $p < 0.05$ ), which sustained for the duration of testing. By day four, also the rats in the DA had a slower reaction to stimuli contralateral to the lesion than the DA + 5HT and sham lesion controls (Fig. 5C right panel; *post hoc* vs. both groups  $t < 4.51$   $p < 0.01$ ) which persisted to day six. On the same testing day the DA + 5HT group also showed a longer RT relative to the sham-lesion controls (Fig. 5C right panel; *post hoc*  $t = 5.52$   $p < 0.001$ ) as well as towards the DA only group ( $t = 3.61$   $p > 0.05$ ), which in turn was no longer different from sham-lesion controls ( $t < 1.90$ , n.s.). On the side

ipsilateral to the lesion, there was no effect of the 6-OHDA lesion on the time to react to a lateralised stimuli (Fig. 5C left panel *post hoc*  $t < 3.71$ ,  $p < 0.05$ ) with the exception of day six where the DA + NA group was significantly slower than all other groups in reacting to lateralised stimulus (*post hoc*  $t > 3.46$ ,  $p < 0.05$ ).

A similar phenomenon was evident when assessing the MT, i.e. the time taken to execute the lateralised movement (Fig. 5D; group  $\times$  side  $\times$  day  $F_{15, 380} = 3.82$ ,  $p < 0.01$ ). Although there was no difference in MT on the first day of testing between the sham-lesion controls and the 6-OHDA lesion rats on the side contralateral to the lesion, a movement deficit developed gradually, with the difference from the sham-lesion controls reaching significance on day two for the DA only group (Fig. 5D right panel *post hoc* vs. sham  $t = 4.00$ ,  $p < 0.05$ ) and from day three for both the DA + NA group and the DA + 5HT group (Fig. 5D right panel *post hoc* vs. sham  $t > 8.79$ ,  $p < 0.01$ ). On day four, the lesion deficit had really manifested and all groups with a 6-OHDA lesion had a significantly slower MT than the sham-lesion controls for the rest of the testing period (Fig. 5D right panel *post hoc* vs. sham  $t > 6.10$ ,  $p < 0.01$ ). In addition, on the same day (day four), the DA + NA group was slower in executing lateralised movements than the other two lesion groups (*post hoc* vs. DA only, DA + 5HT  $t > 3.84$ ,  $p < 0.01$ ). There was however no difference between the three lesion groups on day five or six of testing. A movement deficit was not apparent on the side ipsilateral to the lesion in the rats with a 6-OHDA lesion, but for day five where the DA+NA and DA+5HT group was slower than the sham-lesion controls (Fig. 5D left panel *post hoc* vs. sham  $t < 3.73$ ,  $p < 0.05$ ).

The types of procedural errors made by the rats in the lateralised CRT were also evaluated between the four different groups. The type of errors included (1) premature withdrawal from the centre hole; (2) incorrect nose-pokes after a successful sustained central nose-poke; (3) incorrect panel responses and (4) time-outs, i.e. when the rat did not respond to a lateralised stimulus. The number of each type of error was averaged over the six days of testing and compared between the groups. The number of premature withdrawals from the centre hole was on average 8% for the sham-lesion controls of the total number of trials started (Fig. 6A). Only rats with a pure DA-lesion had a higher rate of premature withdrawals from the centre hole than

sham-lesion controls (rate approx. 14%; Fig. 6A;  $F_{3,79} = 4.28$   $p < 0.01$ ), whereas the other two lesion groups (DA + NA and DA + 5HT) displayed similar premature withdrawal rates as sham-lesion controls. In contrast, the number of incorrect nose-pokes after a successful central nose-poke, i.e. responding to the unlit response hole instead of the lit hole was higher in all three groups with a 6-OHDA lesion compared to the sham-lesion controls (Fig. 6B;  $F_{3,79} = 17.14$ ,  $p < 0.0001$ ). The number of incorrect panel responses per session, i.e., repeated pressing of the panel to the reward magazine without any reward was delivered, did not differ between the rats with a lesion and the sham-lesion controls (Fig. 6C). In contrast, the number of time-outs was increased in both the DA only and the DA + NA group compared to the DA+5HT and sham lesion controls (Fig. 6D  $F_{3,79} = 8.84$ ,  $p < 0.0001$ ).

### Staircase test

All rats were also evaluated on a task of skilled reaching performance to further evaluate the impact of an additional depletion of NA and 5HT on motor skill performance. On the side contralateral to the lesion, the rats with a 6-OHDA lesion were impaired in their performance to reach and eat the sugar pellets compared to sham-lesion controls but there was no difference between the three different lesion groups (Fig. 7; main effect of group  $F_{3,150} = 9.73$   $p < 0.001$ , main effect of side  $F_{1,150} = 45.35$   $p < 0.001$ , group x side  $F_{3,150} = 3.81$   $p < 0.011$ ).

There was no difference between the 6-OHDA lesion rats and the sham-lesion controls on the side ipsilateral to the lesion.

### Discussion

By selectively targeting the midbrain DA, NA and 5HT systems, this study has been able to dissect out the contribution each monoaminergic pathway makes towards the different parameters evaluated in the operant CRT task. This study has demonstrated that DA is critical for complete accurate performance in the task whereas NA appears to play an important role in the time it takes to react to lateralised stimuli. The effect of the NA depletion was in fact global and not isolated to responses made to stimuli contralateral to dopamine depletion but also to the dopaminergically intact side. These findings confirm that many

aspects of deficits in the lateralised choice reaction time task after an injection of 6-OHDA are indeed dependent on DA but also that the NA system may play a role in attentive performance influencing the capacity to react to lateralised stimuli.

There has recently been a shift in the conceptualization of PD, from having previously been considered a pure motor disorder where the cardinal motor symptoms arises from the progressive degeneration of DAergic neurons in SNpc. Current thinking now recognises the heterogeneity in disease pathology and that this mirrors the complexity of the non-motor symptoms also associated with the disorder (Chaudhuri et al., 2006). Indeed, beyond causing pronounced dopaminergic disturbances, the degeneration also involves the serotonergic, noradrenergic and cholinergic systems as evidenced by neuronal losses and inclusion pathology in the locus coeruleus (Cash et al., 1987; Zarow et al., 2003), dorsal raphé nuclei (Scatton et al., 1983) and the basal nucleus of Meynert (Mann and Yates, 1983) respectively. The disorder is associated with a large number of intrinsic non-motor symptoms such as visuospatial deficits, autonomic dysfunction, cognitive impairment and neuropsychiatric problems. These deficits, as well as the other non-motor symptoms of PD, have been shown to have a great impact on the quality of life for the individuals with PD and their careers (Schrag, 2004) and effective treatment options are currently lacking. One type of visuospatial deficit is disruption of the conceptual representation of external space, which is often exemplified as difficulties in navigating through doorways, narrow corridors and other confined spaces (Azulay et al., 2006).

Unilateral dopamine-depletion models of PD show marked impairments in detecting and responding to stimuli on the side contralateral to the lesion (Brown and Robbins, 1989; Carli et al., 1985; Carli et al., 1989; Dowd and Dunnett, 2004; Dowd and Dunnett, 2005; Milton et al., 2004). In the lateralised CRT task, it has been demonstrated that unilateral nigrostriatal dopamine depletion in both rats and mice disrupts the response accuracy on the side contralateral to the lesion. Furthermore, the latency to react to a lateralised stimulus is markedly increased as well as the time taken to execute the lateralised response (Dowd and Dunnett, 2004; Dowd and Dunnett, 2005; Heuer et al., 2013). Interestingly, the rats are able to accurately select, respond and execute a lateralised response regardless of direction relative to the dopamine

depletion when first reintroduced to the testing chambers. However, while the performance directed towards the side ipsilateral to the dopaminergic lesion remains stable, the response to the side contralateral to the lesion gradually deteriorates with further testing (Dowd and Dunnett, 2005). This waning of the response strongly suggests that the deficit is not due to a primary motor impairment. Critically, Brown and Robbins demonstrated that if rats first had to make a centralised nose poke and thereafter respond to a proximal or distal hole on the contralateral side, the rat would demonstrate a remarkable bias to the proximal hole (Brown and Robbins, 1989). Interestingly, when this hole was covered, the rats were able to re-direct their responses to the distal hole demonstrating that the deficit in response accuracy could not be explained as a primary sensory deficit. In a conceptually similar experiment, Heuer et al. demonstrated that the contralateral accuracy was only impaired in the distal response option whereas the accuracy at the proximal hole was similar to the performance exhibited by the controls (Heuer et al., 2012). Taken together, these findings suggest that the impaired accuracy cannot be explained as a primary sensory deficit or by a simple motor impairment *per se*.

Although the pattern of impairment is similar if the dopamine depletion is mediated through 6-OHDA administered into the fibre bundle or into the terminal areas of the striatum, there is a remarkable difference in the degree of impairment (Brown and Robbins, 1989; Carli et al., 1985; Carli et al., 1989; Dowd and Dunnett, 2005). Impairments are milder if the striatum is the target for the dopamine depletion, which may be attributed to a lesser degree of striatal DA depletion and/or the fact that a bundle lesion will deplete dopamine from the nucleus accumbens and the prefrontal cortex, in addition to the striatum (Dowd and Dunnett, 2005). Intra-striatal administration of 6-OHDA usually results in approximately 75-85% depletion of TH positive fibres in the striatum and leaves the nucleus accumbens relatively spared, whereas injections into the MFB give rise to a near complete depletion of striatal TH fibres as well as some degree of depletion in the nucleus accumbens (Dowd and Dunnett, 2005). However, no additive impairing effect was evident after a combined striatal and accumbens lesions in the same task (Carli et al., 1989), which may suggest the level of DA in the other extra-striatal region affected by the MFB-lesion, i.e. prefrontal cortex,

may be of more importance (Dowd and Dunnett, 2005). What has been omitted to date, is consideration of the other monoamine systems which are significantly altered when 6-OHDA is targeted to the MFB (Fulceri et al., 2006; Lindgren et al., 2010).

Locus Coeruleus, although being a relatively small nucleus, projects extensively to many brain regions such as cerebral cortex, hippocampus, thalamus, midbrain, brain stem and spinal cord in a highly regional and laminar specificity (Foote et al., 1983). The relative contribution of the NA system to cognitive function has received considerable interest, especially along the dimensions of attention, arousal and behavioural flexibility (Aston-Jones et al., 1999). Indeed, recent studies have highlighted the importance of an optimal level of noradrenergic tone in these functions (Aston-Jones et al., 1999). It has been demonstrated that depletion of NA after 6-OHDA-injections into the dorsal noradrenergic bundle results in impaired attention, as assessed by the five-choice serial reaction time task (Carli et al., 1983) particularly under certain testing conditions associated with greater “effortful processing” (Carli et al., 1983; Cole and Robbins, 1992). In addition, Milstein and co-workers recently demonstrated that selective depletion of medial prefrontal levels of NA using the immunotoxin anti-D $\alpha$ H-saporin, resulted in increased latencies to react correctly when responding under high event rate conditions (Milstein et al., 2007). In line with these findings, the current investigation demonstrated that depletion of forebrain NA, resulted in increased latencies to react to stimuli presented both to ipsi- and contralateral sides relative to nigrostriatal dopamine depletion. The deficit was however more pronounced on the side contralateral to the DAergic lesion, suggesting an additive effect of the two catecholamine systems on reaction time performance. While occasional deficits were observed in accuracy and movement time, neither was as significantly or consistently affected as reaction time. Both the biochemical and histological analysis confirmed that a high selective although only partial bilateral lesion of pre-frontal cortex NA was achieved. There was also a non-significant but apparent reduction in NA levels in the striatum after the additional NA-depletion. However, baseline NA is very low in the striatum (Cenci et al., 1992), indeed very close to the limit of detection,



which might explain the lack of significant reduction towards the other groups in this region.

5HT has not been implicated as consistently as NA in attentional processes but more so in different aspects of impulsivity (Boulougouris and Tsaltas, 2008). Indeed, several studies have shown that global depletion of 5-HT using 5,7-DHT has no effect on attentional performance and latencies in the five-choice serial reaction time task but significantly increased the impulsivity, assessed as increased premature responding (Harrison et al., 1997; Koskinen et al., 2000; Winstanley et al., 2004). These findings are both in line and incongruent with the findings in the current study. The forebrain depletion of 5HT in addition to the unilateral DA depletion had no effect on the response accuracy or on the time to react to lateralised stimuli in the lateralised CRT task, which is in agreement with previous studies. We did however not see an increased rate of premature withdrawal from the centre hole after the 5HT depletion, which could be considered as a measure of impulsivity. A possible explanation for this discrepancy is the different tasks used in the studies, i.e. the five choice serial reaction time task vs. the lateralised CRT. Another reason could be the suggested delicate interaction between the DA and the 5HT-system in regulating impulsive choices (Boulougouris and Tsaltas, 2008). Indeed, it has been shown that the increased impulsivity caused by either a 5HT<sub>2</sub> receptor agonist or by global depletion of 5HT using 5,7-DHT, was completely blocked by selective D1- and D2 receptor antagonist treatment. (Harrison et al., 1997; Koskinen and Sirvio, 2001).

The rats were also tested in a task of skilled reaching as an additional measure of motor function. The reduction in the number of pellets eaten on the side contralateral to the lesion in the rats with a 6-OHDA lesion is in line with a massive body of studies utilising this task as a measure of skilled reaching after striatal DA depletion (Barneoud et al., 2000; Jeyasingham et al., 2001; Klein et al., 2007; Samoudi et al., 2012; Whishaw et al., 1997). There was however no difference between the rats with a pure DA depletion and those with the additional depletion of 5HT and NA, which readily matches the findings from the movement time in the lateralised CRT task.

In summary, this study has demonstrated that the majority of parameters in the lateralised CRT are strongly dependent on DA, which is in line the body of literature utilising the same type of operant task (Brown and Robbins, 1989; Carli et al., 1985; Carli et al., 1989; Dowd and Dunnett, 2004; Dowd and Dunnett, 2005). Importantly, this study has also demonstrated that the NA system contributes to the attentive performance in the CRT task by influencing the capacity to react to the presented lateralised stimuli. These findings confirms the importance of NA in attentive function and considering the loss of NAergic innervation in individuals with PD (Halliday et al., 1990), this loss may contribute to different aspects of the visual dysfunction evident in the disease symptomology.

## Acknowledgements

H.S.L. was funded by a postdoctoral fellowship from the Swedish Research Council. S.B.D was supported by EU FP7 REPLACES (EC contract number 222918 FP7, Thematic priority HEALTH). The authors would also like to acknowledge the UK Medical Research Council and Parkinson's UK for their generous funding. Special thanks to Andreas Heuer for his help with statistical analysis and to Jane Heath for her excellent technical support.

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## Figure legends

**Fig. 1:** Schematic illustration of the experimental design applied in this study.

CRT: choice reaction time task

**Fig. 2:** TH-positive cells in the SNpc were assessed using stereology whereas striatal TH-positive fibre density was measured by optical density (OD) analysis **(A)** Unilateral injection of 6-OHDA into the SNpc resulted in a pronounced reduction in nigral TH-positive cells on the side ipsilateral to the lesion but there was no difference between the three different lesion groups. **(B)** Striatal TH-innervation was reduced by 90-95% after the 6-OHDA injection on the side ipsilateral to the lesion. Representative photomicrographs illustrating the loss of TH-positive cells in the SNpc **(C)** and TH-fibre innervation in the striatum **(D)** ( $p < 0.05$  \* vs. sham-lesion controls). Scalebar: 5mm

**Fig. 3:** The specificity of the selective lesions was evaluated using fibre counts. **(A, A')** The number of striatal serotonin transporter (SERT) positive fibres was only reduced in the group of rats that had received the additional depletion of

5HT. **(B, B')** The number of prefrontal dopamine  $\beta$  hydroxylase (D $\beta$ H) positive fibres was only reduced in the group of rats that had received the additional depletion of NA ( $p < 0.05$  \* vs. sham-lesion controls, § vs. DA only, # vs. DA + NA, ¶ vs. DA + 5HT). Scalebar: 100 $\mu$ m

**Fig. 4:** The specificity of the selective lesions was also evaluated by assessing the levels of DA, NA and 5HT in the prefrontal cortex and striatum using HPLC. **(A)** The levels of DA in the prefrontal cortex were not affected by the 6-OHDA lesion whereas **(B)** the striatal levels were strongly reduced on the side ipsilateral to the lesion compared to the intact side and to sham-lesion controls. **(C)** The levels of 5HT in the prefrontal cortex were only reduced in the DA + 5HT group and there was no difference between the left and the right side. **(D)** The striatal levels of 5HT were also reduced bilaterally in this group and in this region the DA only group had also lower striatal levels of 5HT compared to sham-lesion controls. **(E)** The NA levels in the prefrontal cortex were only reduced in the DA + NA group whereas **(F)** there was no difference in NA levels in the striatum ( $p < 0.05$  \* vs. sham-lesion controls, § vs. DA only, # vs. DA + NA, ¶ vs. DA + 5HT).

**Fig. 5:** Postlesion performance in the lateralised choice reaction time task was assessed for six days. **(A)** The number of useable trials remained stable in the sham-lesion controls over the six days of testing whereas it gradually declined in the DA only, DA + 5HT and DA + NA groups **(B) right panel;** The response accuracy on the side ipsilateral to the lesion was stable and similar in all groups over the treatment period but for the first day in the DA + NA group. *left panel;* On the side contralateral to the lesion, the deficit gradually developed during the testing period in the DA only, DA + 5HT and DA + NA groups. **(C) right panel;** The latency to react to lateralised stimuli was similar and stable over the testing period in all groups on the side ipsilateral to the lesion, but for the last day in the DA + NA group. *left panel;* On the side contralateral to the lesion, the DA + NA group displayed a slower reaction time than all other groups from day three and onwards. **(D) right panel;** The latency to execute the lateralised movement towards the side ipsilateral to the lesion was similar and stable among the different groups but for on day five where the DA + NA and DA + 5HT group was

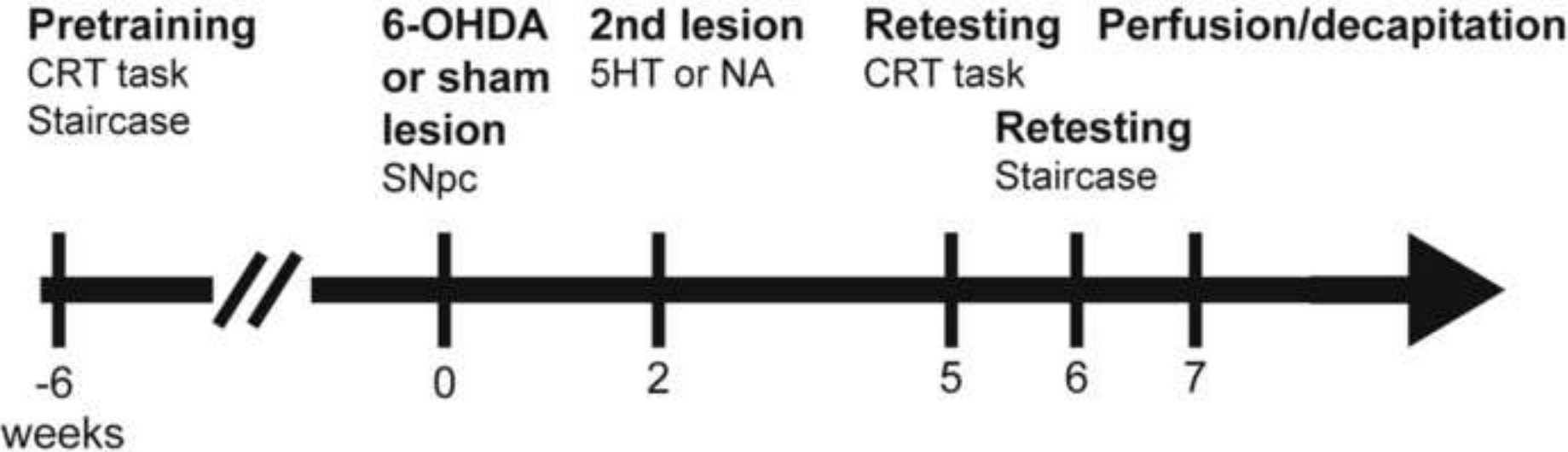
slower than controls. *left panel*; On the side contralateral to the lesion, a deficit first emerged in the DA only group subsequently in the DA+ NA and DA + 5HT group. ( $p < 0.05$  \* vs. sham-lesion controls, § vs. DA only, # vs. DA + NA, ¶ vs. DA + 5HT).

**Fig. 6:** Errors performed during the lateralised CRT task. **(A)** The number of premature withdrawals from the centre hole was increased in the DA only group compared to sham-lesion controls and the DA + NA group. **(B)** The number of incorrect response was increased in all groups with a 6-OHDA lesion compared to sham-lesion controls. **(C)** There was no difference in the number of incorrect panel presses between the different groups whereas **(D)** the number of time outs was increased in the DA only and in the DA + NA group compared to sham-lesion controls and the DA + 5HT group ( $p < 0.05$  \* vs. sham-lesion controls, § vs. DA only, # vs. DA + NA, ¶ vs. DA + 5HT).

**Fig 7:** Performance in the staircase task, *right panel*; there was no difference in the number of sugar pellets eaten on the side ipsilateral to the lesion. *Left panel*; in contrast, the number of pellets was significantly reduced compared to sham-lesion controls on the side contralateral to the lesion. ( $p < 0.05$  \* vs. sham-lesion controls).



Figure  
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**Figure 2**  
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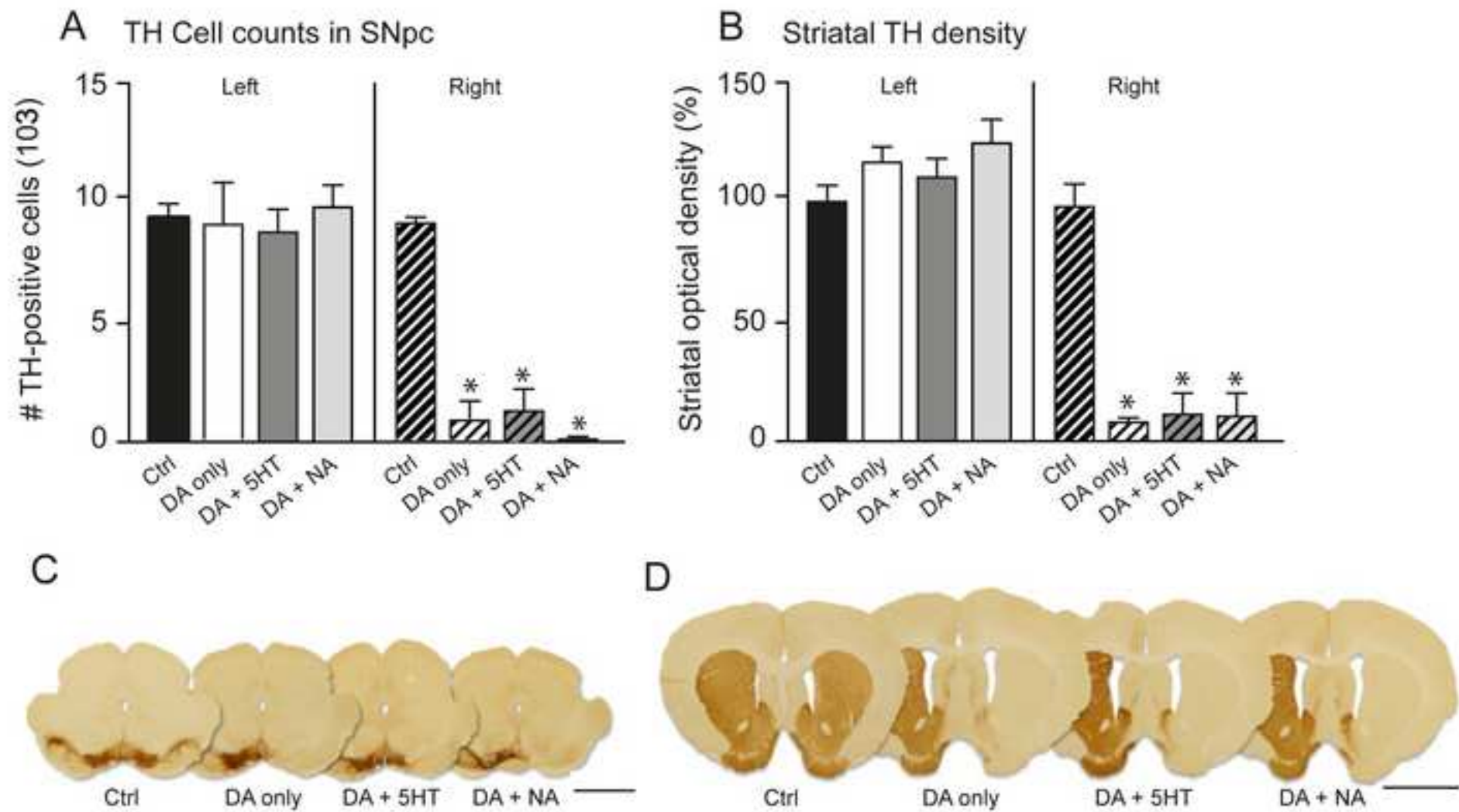
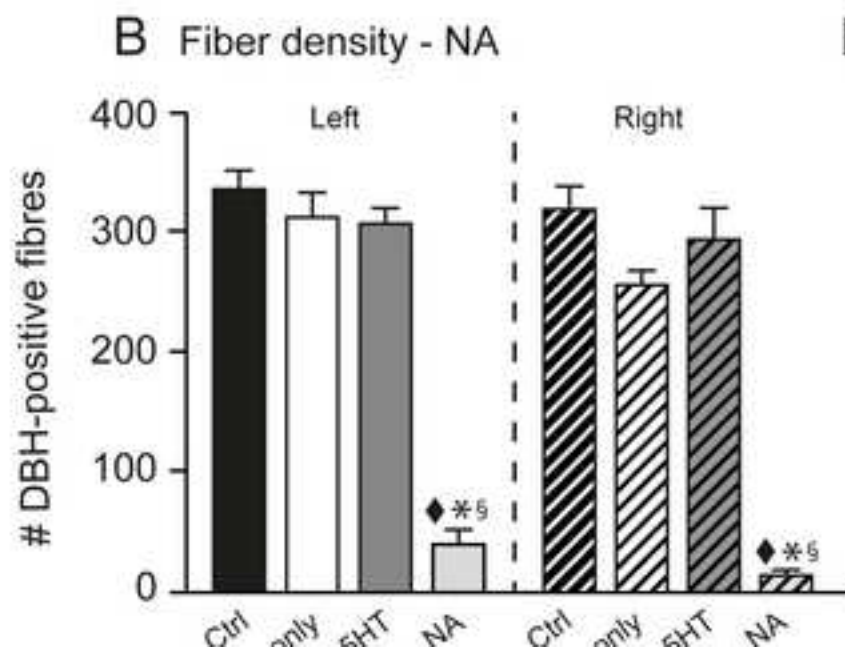
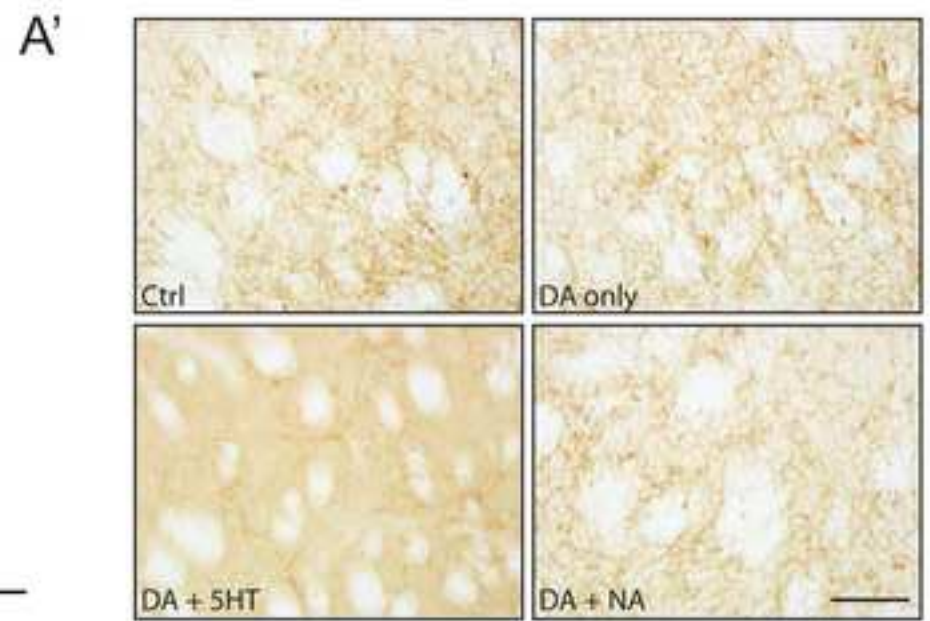
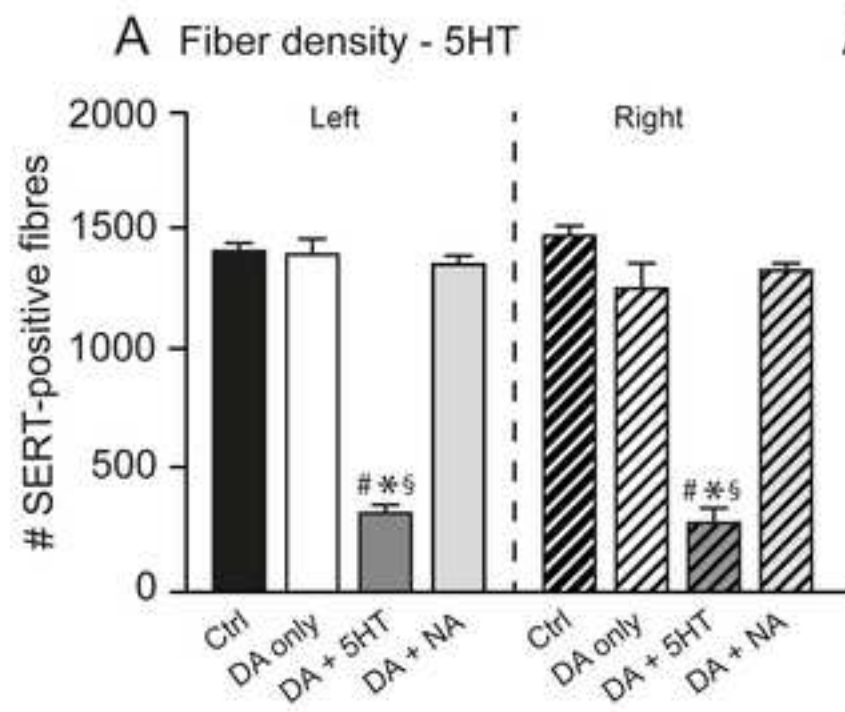
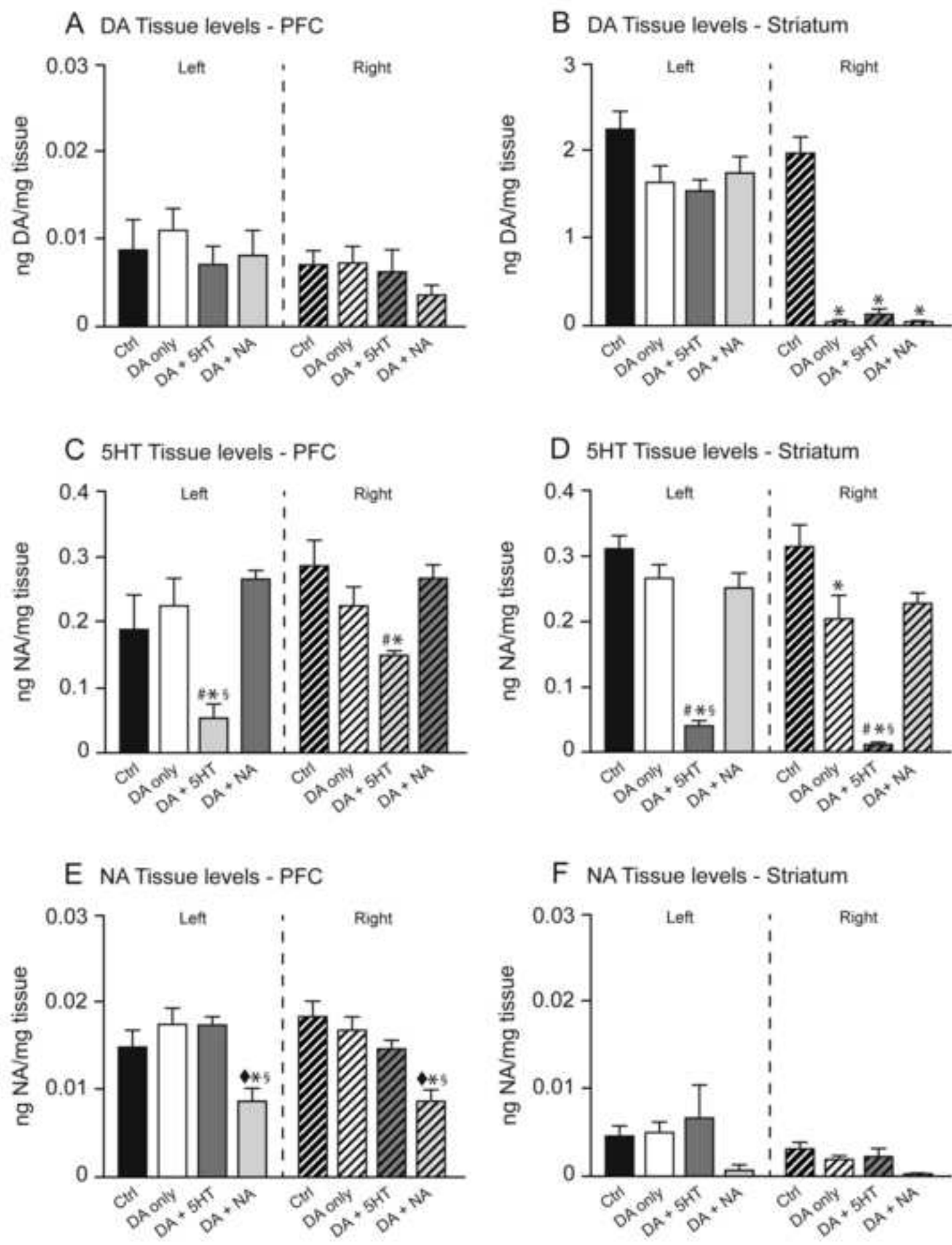


Figure 3  
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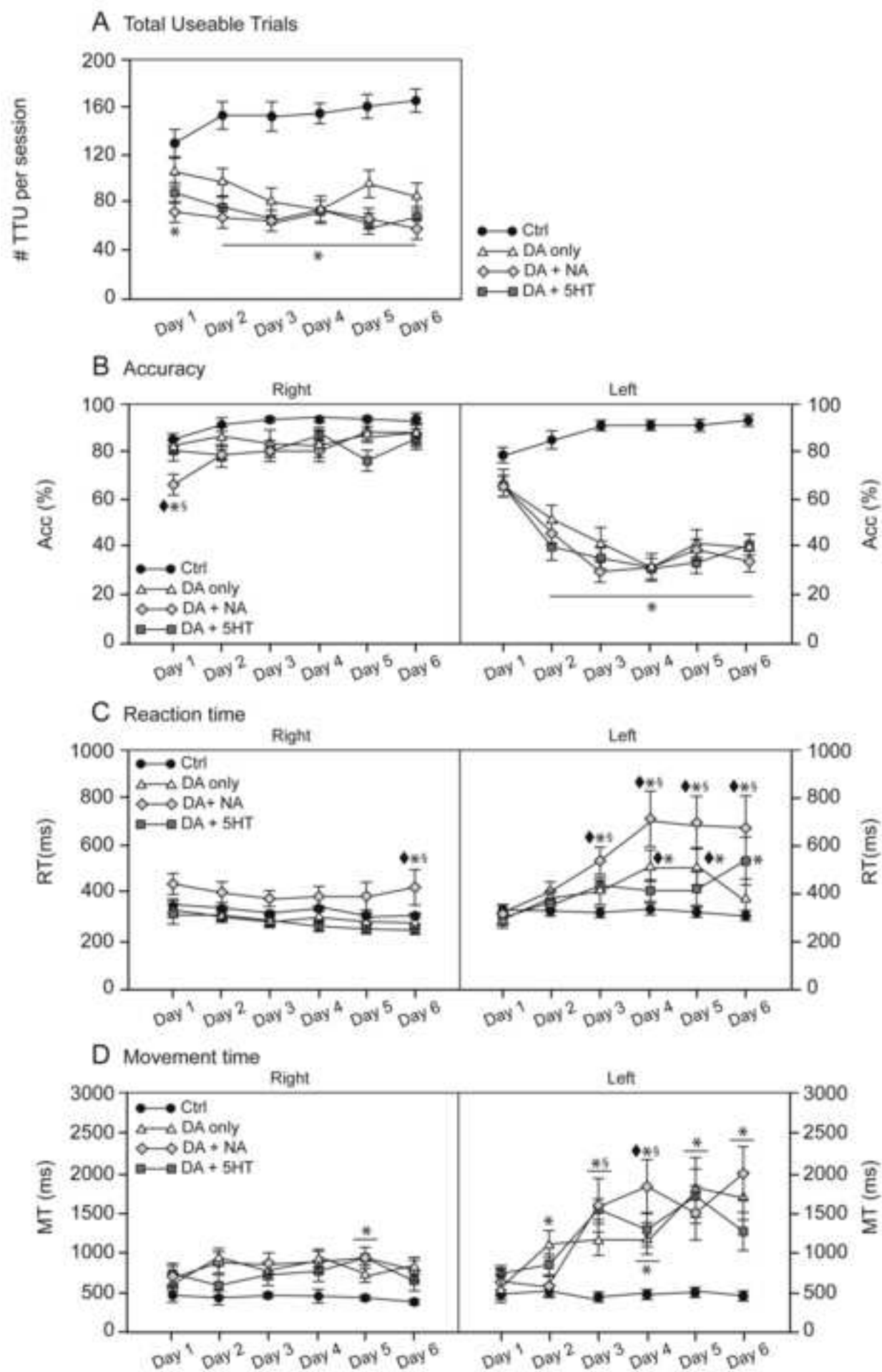


**Figure 4**  
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**Figure 5**  
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**Figure 6**  
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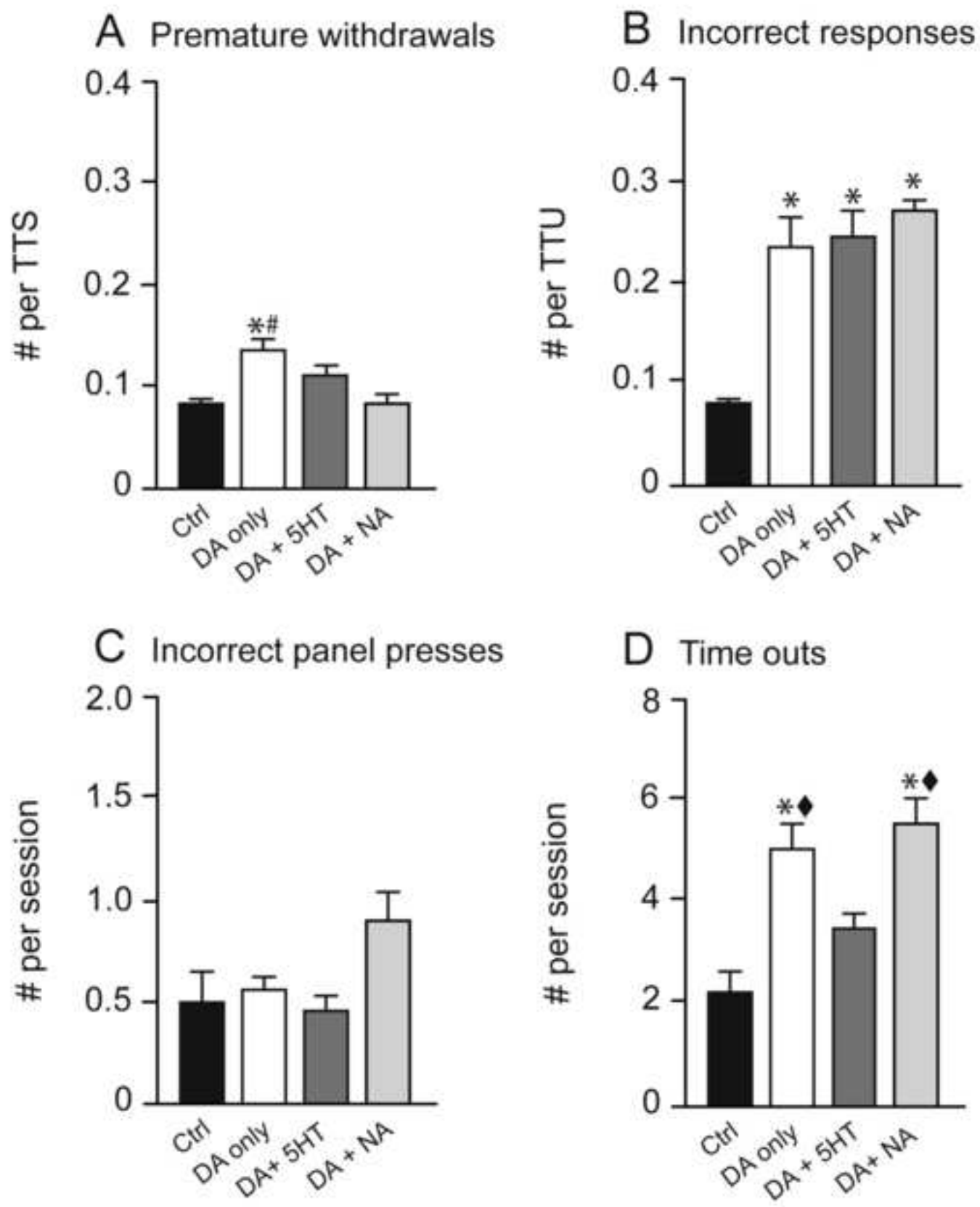


Figure 7  
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