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Reducing falls in Parkinson's disease: Interactions between donepezil and the 5-HT_6 receptor antagonist idalopirdine on falls in a rat model of impaired cognitive control of complex movements

Aaron Kucinski[1], Inge E.M. de Jong[2], and Martin Sarter[1*]

[1]University of Michigan, Department of Psychology, Ann Arbor, MI (USA); [2]H. Lundbeck A/S, Division of Neurodegeneration, Valby (Denmark).

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*Corresponding author: Martin Sarter, University of Michigan, Dept. Of Psychology, 530 Church Street, Ann Arbor, MI 48109; phone: 734-764-6392; email: msarter@umich.edu

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Abstract

Falls are a leading cause of death in the elderly and, in a majority of patients with Parkinson's disease (PD), the leading levodopa-insensitive cause of hospitalization and long-term care. Falling in PD has been attributed to degeneration of forebrain cholinergic neurons that, in interaction with striatal dopamine losses, impairs the cognitive control of balance, gait and movement. We previously established an animal model of these dual cholinergic-dopaminergic losses ("DL rats") and a behavioral test system (MCMCT) to measure falls associated with traversing dynamic surfaces and distractors. Because the combined treatment of the acetylcholinesterase inhibitor donepezil and the 5-HT_6 receptor antagonist idalopirdine (Lu AE58054) was reported to exhibit synergistic pro-cholinergic activity in rats and improved cognition in patients with moderate Alzheimer's disease, here we assessed the effects of this treatment on MCMCT performance and attention in DL rats. Compared with the vehicle-treated group, the combined treatment greatly reduced (Cohen's *d*=0.96) falls in DL rats when traversing dynamic surfaces and when exposed to a passive distractor. However, falls associated with a dual task distractor and sustained attentional performance did not benefit from this treatment. Analyses of the behavior in fall-prone moments suggested that this treatment

improved the efficacy and speed of re-instating forward movement after relatively short stoppages. This treatment may reduce fall propensity in PD patients via maintaining planned movement sequences in working memory and improving the vigor of executing such movements following brief periods of freezing of gait.

Introduction

In addition to the disease-characterizing motor symptoms that reflect primarily striatal dopamine loss, Parkinson's disease (PD) is characterized by widespread neurodegenerative processes in non-dopaminergic brain systems. Degeneration of non-dopaminergic systems has been associated with autonomic, behavioral and cognitive symptoms, including a propensity for falls, which do not benefit from levodopa treatment. The role of non-dopaminergic circuits and how they interact with nigrostriatal circuit loss to cause levodopa-unresponsive symptoms remains poorly understood, posing a critical barrier to the development of therapies for these disabling symptoms (Wood, 2002; Balash *et al.*, 2005; Langston, 2006).

Although multiple cognitive and motor risk factors increase the probability for falls, impairments in the attentional supervision of movement are a major contributing factor for falls, specifically when unfamiliar surfaces and obstacles or secondary tasks challenge gait, balance, and movement control (Brown *et al.*, 1999; Woollacott & Shumway-Cook, 2002; Hausdorff *et al.*, 2006; Yogev-Seligmann *et al.*, 2008; Allcock *et al.*, 2009; LaPointe *et al.*, 2010; Theill *et al.*, 2011). Gait, balance and movement errors normally evoke compensatory attentional control. However, as the disease process also impacts the brain's attention systems, such compensatory deployment of attentional resources is increasingly unavailable for rescuing movement and preventing falls. Consistent with this view, degeneration of a major attention system of the brain, the basal forebrain (BF) cholinergic projections to telencephalic and thalamic regions, but not losses of basal ganglia dopamine, correlate with low gait speed, freezing of gait and falls in PD patients (Bohnen & Albin, 2009; Bohnen *et al.*, 2009, 2013; Yarnall *et al.*, 2011).

The specific cognitive-behavioral consequences of combined BF cholinergic and striatal dopamine losses are not well understood. Accumulating evidence supports the notion that the cholinergic system mediates the detection of cues in attentional contexts and the goal-directed deployment of attention resources (Parikh *et al.*, 2007; Howe *et al.*, 2013; Gritton *et al.*, 2016; Sarter *et al.*, 2014b, 2016). Thus, as a result of cholinergic loss, the cortical processing of cues

is impaired and thus cue-based information, such as those that signal movement errors or imbalance, is not effectively transferred to the striatum. Striatal dopamine loss, in general terms, yields impairments in action selection and sequencing, and decreases in movement vigor and velocity (e.g., Gepshtein *et al.*, 2014; Rueda-Orozco & Robbe, 2015; Yttri & Dudman, 2016). Thus, combined cholinergic-dopaminergic losses have been hypothesized to render striatal circuitry largely incapable of integrating information about ongoing behavior which, in interaction with poor habitual action control, gives rise to major gait and balance errors, ineffective movement recovery after errors, and thus falls (Sarter *et al.*, 2014a; Pelosin *et al.*, 2016).

We recently described a rat model of falls resulting from dual BF cholinergic and striatal dopaminergic losses ("DL rats") and from the attentional taxation of gait, balance and complex movement. DL rats combine bilateral cortical cholinergic and partial striatal dopaminergic deafferentation, produced by intracranial infusions of neurotoxins (192-IgG saporin and 6-hydroxydopamine, respectively). The extent of the cholinergic lesions in these animals has been informed by results from PET imaging studies that indicated moderate decreases in cortical and thalamic cholinergic input in PD patients with a history of falls (Bohnen et al., 2009). Striatal dopamine lesions have targeted the prefrontal projection field in the dorsomedial caudate nucleus, reflecting our goal to determine interactions between dopamine loss and the impact of loss of cholinergic deafferentation of primarily prefrontal regions on striatal function (for more discussion of this model see Sarter *et al.* 2014a). Falls in DL rats have been characterized using a newly designed behavioral test system, the Michigan Complex Motor Control Test (MCMCT) that involves a progressively demanding series of traversal conditions (e.g., stationary planks and rods; rotating beams; distractors). Compared to rats with single lesions and sham-lesioned controls, DL rats exhibit a propensity for falls. As in patients, risk factors for falls in this rat model involves dynamic surfaces, exposure to distractors, relatively slow gait speed, postural instability, and micropauses during forward movement. Furthermore, falls in DL rats correlate with impairments in Sustained Attentional Task (SAT) performance (Kucinski *et al.*, 2013, 2015a; Kucinski & Sarter, 2015; Sarter *et al.*, 2014a), further indicating that this animal model reproduces falls caused by interactions between impairments in attention and movement control.

Here we employed this animal model and the associated behavioral test system (MCMCT) to assess the potential therapeutic efficacy of the combined treatment with the acetylcholinesterase (AChE) inhibitor donepezil (DON) and the 5-HT6 receptor antagonist idalopirdine (IDL). Co-treatment with DON and IDL was found to significantly improve the cognitive abilities of patients with moderate Alzheimer's disease in a phase II trial (Wilkinson *et*

al., 2014). The neuronal mechanisms underlying the interactions between these two drugs remain unclear but may involve synergistic pro-cholinergic effects and associated elevations in telencephalic high frequency oscillations (Herrik *et al.*, 2016).

In the present experiment, DON and IDL were administered separately and together to DL rats performing the MCMCT and the SAT. Compared with control and vehicle-treated DL rats, single drug treatments did not improve performances. In DL rats given both drugs, fall rates were significantly reduced, except for those evoked by a dual task situation. Unexpectedly, decreases in fall rates were not associated with improved SAT performance. Together, the evidence suggests that dual treatment with an acetylcholinesterase inhibitor and a 5-HT_6 receptor antagonist may reduce falls that are associated with relatively brief periods of freezing of gait and with slow and low-vigor re-initiation of movement.

Materials and methods

Subjects. Adult male and female Sprague Dawley rats (Harlan; N=70; 35 males and 35 females) between 3 and 6 months of age were individually housed in opaque single standard cages (27.70 cm × 20.30 cm) in a temperature- and humidity-controlled environment (23℃, 45%). Throughout the experiment male rats weighed more than females ($F(1,69)=460.29$, $P<0.001$; males: 359.86+4.27 g females: 249.24+2.48 g), but weights did not differ across experimental groups (main effect of group and sex x group: both $F<0.29$, both $P>0.88$). The animals were maintained under a 12:12 hour light/dark schedule (lights on at 8:00 AM). Food (Rodent Chow; Harlan Teklad) was available *ad libitum*. Water access was gradually restricted over a 7-day period (12, 8, 5, 3, 1, 0.5, 0.25 hrs of water access per day) in the week before pre- and post-surgery behavioral testing. During testing, water was provided as rewards for correct responses during SAT performance and following beam traversals on the MCMCT (see below). Rats were also provided water *ad libitum* for 15 min following SAT performance each day (*ad libitum* water was not given following the afternoon MCMCT sessions). All procedures were conducted in adherence with protocols approved by the University Committee on Use and Care of Animals at the University of Michigan and in laboratories accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Timeline of experiments. Animals were initially trained on the SAT (~2 months of daily training; ~9:00-11:00 AM). Upon reaching the final stage of training animals remained on this stage for 14 consecutive days. During the final 6 days of training rats were also trained on the MCMCT in the afternoons (~2:00-4:00 PM). Previous experiments showed that performance on either task

was unaffected when rats were trained on both tasks on the same day (Kucinski *et al.*, 2013). Furthermore, because we previously demonstrated that rats' circadian rhythm entrain to daily SAT practice, yielding a robust diurnal phenotype (Paolone *et al.*, 2012; Gritton *et al.*, 2013) all behavioral testing likely occurred during the animals' active phase of the day.

Following pre-surgery training, animals underwent stereotaxic lesion surgeries followed by 4 weeks of recovery. During the final week of recovery animals were gradually water restricted. Drug effects were then assessed over 12 consecutive days of SAT and MCMCT sessions (see Table 1 for the battery of MCMCT test conditions). Drug or vehicle injections were administered 30 minutes prior to SAT sessions (~9:30 injections, SAT testing 10:00-10:45 a.m.) and in the afternoon before MCMCT testing (~3:30-6:00 p.m.). For the MCMCT runs, rats were injected individually so that trials began exactly 30 minutes after injection for each animal.

Michigan Complex Motor Control Task (MCMCT). Complex movement performance measures including fall propensity were assessed using the MCMCT (for details and an illustration see Kucinski *et al.*, 2013). This beam traversal apparatus was designed to tax the ability of rats to perform attention-demanding movements and correct for stepping errors while crossing a narrow square rod surface (2.54 cm^2). The ends of the beam (2.0 m length) were held in sockets that allowed the rod to be rotated by a gear motor (10 RPM) coupled to one end of the beam element. Traversal of the rod, particularly when rotating, reliably generated falls and other movement impairments in rats with dual lesions of striatal dopaminergic and cortical cholinergic inputs (Kucinski *et al.*, 2013; Kucinski & Sarter, 2015). A flat plank surface (13.3 cm wide) was also used to assess basic motor capacity and for habituation to the apparatus. Two identical platforms (23.0 x 31.5 cm area) situated at the opposite ends of the beam were surrounded by retractable walls (27.0 cm height) to form end-box structures. The walls are raised and lowered manually and secured in position by a friction clamp which rides in a vertical slot in the support structure under the platform. The walls facing the beam had openings (9.0 cm wide) to allow rats to enter and leave the end-boxes. Copper water cups (2.7 cm diameter, 3 cm deep) were embedded on the floors of the end-boxes and rats were provided with ~150 µL of water following each traversal. These rewards were intended as an incentive for self-initiated traversals and thus limited experimenter handling during testing.

At the beginning of a test session, rats were placed on the rod or plank ~10 cm from an end-box and allowed to enter the box and drink the water rewards. Once inside the box on the first trial, and in subsequent trials, rats were given ~45 s to drink the water and explore. Rats were able to leave the end-boxes to traverse to the opposite side of the beam at any time. If after 45 s the

rats did not initiate traversal, the walls were lowered as cues to begin traversal. The majority of rats self-initiated traversal when the walls were lowered, however, if not, the rats were moved to the plank or rod by the experimenter. When falls occurred, animals fell into a safety net (0.7 × 0.2 m) section of a badminton net (generic) placed 20 cm below the beam element. The net frame also served as a mounting point for the various cameras, mirrors, and distractor elements.

Falls, slips, and traversal time were assessed as described previously (Kucinski *et al.*, 2013). A fall was scored in the following instances: when slip/missteps caused a rat to stop forward movement and lose upright walking posture resulting in the underside of the animal hitting the surface of the rod, when the rat fell completely off the rod onto the netting below the rod or hung from the rod by its paws, when a rat ceased forward movement and clung to the rod while it rotated (thus rotating upside down with it). A slip was scored when any of the rats' paws lost contact with the surface of the rod and extended below the lower horizontal border of the rod. Traversal time was defined as the latency to traverse the entire distance of the beam. During trials in which a fall occurred, slips and traversal time were prorated by multiplying the ratio of the distance of a full traversal to the distance where the hind limbs lost contact with the rod during the fall.

Two distractors were presented during traversals. First, a *passive doorframe distractor*, comprised of a 46.0 x 39.4 cm surface with a door-frame shape cutout of 20 cm x 10 cm made of foam core, was incorporated into the MCMCT test sequence. The distractor was placed at the midway point along the beam (100 cm mark) with side jambs 3.5 cm from the rod surface on both sides and the top border of the doorframe cutout 11 cm above the flat rod surface. We previously found that this distractor caused movement disruptions such as freezing of gait and falls (Kucinski *et al.*, 2013, 2015b; Kucinski & Sarter, 2015), therefore modeling the effects of such distractor in PD patients (Cowie *et al.*, 2012). Second, animals were tested with an *active distractor task* in which a water reward (3 drops of water; ~150 µL) was presented on a platform (4.9 cm diameter) during traversals. The platform was also placed at the 100 cm mark, with 2-3 cm separating the rod and the platform.

Rats were first acclimated to the task in 4 shaping trials (test days 8 and 9). In these trials rats were placed directly on the stationary (non-rotating) rod adjacent to the platform (approximately midway across the beam) and allowed to drink the water from the platform. After shaping trials, rats underwent 4 test trials in which the rats performed unassisted traversals of the rotating rod (alternating directions) with presentations of water rewards. In two other test days (10 and 12),

rats performed 10 test runs per day (Table 1). In addition to falls, the number of water rewards earned, defined as licking/drinking water from the platform for ≥1 sec, were counted. All trials were recorded using a system of 4 bullet cameras (KT&C; model KPCS190SH Black/White Bullet Camera with 1/3" SONY Super HAD CCD) with rotatable bases that were fastened to the outer support frame of the outer side of the apparatus by hand clamps. Performance measures were analyzed by video playback by experimenters blind to the lesion status and treatment regimen of the rats.

Insert Table 1 about here

Sustained Attention Task (SAT). *Apparatus.* Training and testing were conducted using 12 operant chambers (MED Associates Inc.) housed within individual sound-attenuating cubicles. Each chamber was equipped with two retractable levers, a central panel white light (2.8 W), and a water dispenser located on the same wall as the panel lights. The water dispenser was capable of administering 45 µL of water per delivery. Signal presentation, lever operation, reinforcement delivery, and data collection were controlled by a Pentium PC and Med-PC for Windows software (version 4.1.3; MED Associates).

Acquisition. Water-deprived rats were initially trained to press a lever for a water reward in accordance with a modified fixed ratio-1 (FR1) schedule for water reinforcement. During this phase of training, any lever press resulted in the delivery of water. Typically, the animals do not exhibit a side bias with regard to which lever is pressed; however, if one lever was pressed 5 times in succession, the FR1 schedule was modified to require the animal to press the opposite lever before the next reward can be obtained. After 3 consecutive days with 120 reinforced lever presses each, the rats began training to discriminate between a signal (1 s illumination of the central panel light) and a non-signal (no illumination) event. Two seconds (s) after a signal or non-signal event, both levers were extended into the operant chamber and remain extended for 4 s or until a lever was pressed. If no press occurred after 4 s, the levers retracted and an omission was scored. Immediately following responses (either correct or incorrect), both levers were retracted and the variable intertrial interval (ITI; 12±3 s) was reset. On signal trials, a press of the left lever was reinforced and termed a "hit," whereas a press of the right lever was not reinforced and termed a "miss." On non-signal trials, a press of the right lever was reinforced and termed a "correct rejection," whereas a press of the left lever was not reinforced and termed

a “false alarm.”

Animals received water rewards only for correct responses (45 µL for each hit and correct rejection), whereas incorrect responses (misses and false alarms) were not rewarded. To eliminate the possibility of a selection bias, half of the animals were trained with the opposite pattern. Signal and non-signal events were presented in pseudorandom order for 81 trials each (total of 162 trials) per session. During this phase of training, incorrect responses were followed by correction trials in which the previous trial was repeated. After three consecutive incorrect responses on correction trials, the animal underwent a forced trial in which the lever was extended for 90 s or until the animal made a response. If the forced choice trial was a signal trial, the signal light remained illuminated for as long as the lever was extended. The house light was not illuminated during this training stage. Animals progressed to the subsequent step of shaping if they responded correctly ≥70% of both signal and non-signal trials for 3 consecutive days.

During the third phase of shaping, multiple signal durations (500, 50, and 25 ms) were introduced and the ITI was reduced to 9±3 s. Correction and forced-choice trials were also eliminated. Trial type and signal duration were pseudorandomly determined for each trial. Session length was set at 40 min. After at least 3 d of stable performance, defined by at least 70% hits to 500 ms signals, 70% correct rejections, and ≤30% omissions, animals began training in the final version of the task. The final version was identical to the previous training stage except that the house light was illuminated throughout the session. The addition of the illuminated house light represents a crucial element of testing sustained attention as it requires the animal to constrain its behavior and focus on the central panel light during task performance. Upon reaching the final stage of training prior to lesion surgeries, animals remained at this stage for 14 consecutive days and scores from the final 5 days of performance were averaged to determine pre-surgery scores for each animal. During post-surgery testing of drug effects, rats were tested only on the final stage for 12 consecutive days and scores from the final 5 days were once again averaged for final analyses.

Measures of SAT performance. The following behavior measures were recorded during each SAT session: hits, misses, false alarms, correct rejections, and omissions. Misses and false alarms are the inverse of hits and correct rejections, respectively. The relative number of hits (hits/hits+misses) for each signal length as well as the relative number of correct rejections (correct rejections/correct rejections+false alarms) were calculated. In addition, an overall measure of attentional aptitude, the SAT score, that integrates both the relative number of hits

(h) and the relative number of false alarms (f), was also determined at each signal duration. The SAT score was calculated using the following formula: $(h-f)/[2(h+f)-(h+f)^2$ (Frey & Colliver, 1973). Thus, SAT scores are not confounded by errors of omission. SAT scores ranged from 1.0 to -1.0, with 1.0 indicating that all responses were hits and correct rejections, 0 indicating an inability to discriminate between signal and non-signal events, and -1.0 indicating that all responses were misses and false alarms. Errors of omission were recorded separately.

Lesions. Of the 70 rats, 56 (28 females and 28 males) received dual striatal-dopaminergic, cortical-cholinergic system lesions (DL) and were randomly assigned to 4 treatment conditions after surgeries (n=14/group, 7/sex). 14 rats (7/sex) received sham lesions. Rats were first placed in vaporization chambers and anesthetized with 4-5% isoflurane (delivered at 0.6 L/min O_2) using a SurgiVet Isotec 4 Anesthesia Vaporizer) until the animals were no longer responsive to a tail pinch and exhibited no hind-limb withdrawal reflex. The animals' heads were shaved using electric clippers and cleaned with a betadine scrub. The animals were then mounted to a stereotaxic instrument (David Kopf Instruments) and isoflurane anesthesia was maintained at 1-3% for the remainder of the surgery. An approximately 2.5 cm incision was made down the midline of the scalp to expose the skull. The animals' body temperature was maintained at 37℃ using Deltaphase isothermal pads (Braintree Scientific). Ophthalmic ointment was provided for lubrication of the eyes. To prevent hypovolemia and hemodynamic instability during prolonged surgeries, 1 mL/100 g 0.9% of NaCl (s.c.) was administered. Animals also received an injection of an analgesic (Carprofen; 5.0 mg/kg; s.c) prior to surgery and once or twice daily as necessary for 48 hours post-operatively.

6-OHDA was delivered bilaterally to dopamine terminals in the dorsal striatum (Sigma-Aldrich; 6.0 µg/2 µL/infusion, bolus; dissolved in 0.9% NaCl with 0.1% ascorbic acid; two infusion sites per hemisphere: AP +1.2 and +0.2 mm; ML ±2.5 and ±3.0 mm relative to bregma; DV -4.8 and -5.0 mm from skull). Desipramine hydrochloride (10 mg/kg; i.p.; Sigma-Aldrich) was administered to rats receiving 6-OHDA infusions 30 min prior to surgeries for protection of noradrenergic neurons (Breese & Traylor, 1971). Basal forebrain cholinergic neurons situated in the nucleus basalis and substantia innominate were targeted with immunotoxin 192 IgG-saporin (Advanced Targeting Systems) in aCSF infused bilaterally (120 ng/µL; 0.5 µL/hemisphere; AP -0.8; ML ± 2.9; DV -7.8). Sham rats received equal volumes of 0.9% NaCl with 0.1% ascorbic acid (striatum) and aCSF (basal forebrain) without neurotoxins. Following infusions the needle was left in position for 5-10 min to foster absorption of the toxins. Non-absorbable sutures were used to close the incisions and a topical antibiotic (Neosporin) was applied to the wounds immediately after surgery.

Drug administration and groups and justification of drug doses. The acetylcholinesterase inhibitor donepezil (DON; 0.3 mg/kg; provided by Lundbeck) and the 5-HT_6 receptor antagonist idalopirdine (IDL; Lu AE58054; 5.0 mg/kg; Lundbeck) were dissolved in 5% 2-hydroxypropyl-beta-cyclodextrin (HP-beta-CD) vehicle solution. Rats were divided into the following drug administration groups (14 per group, 7/sex): sham-lesioned and administered vehicle (SH/VEH), DL and vehicle (DL/VEH), DL and donepezil (DL/DON), DL and idalopirdine (DL/IDL), and DL and idalopirdine plus donepezil (DL/DON+IDL). Solutions were prepared the night before administration and were replaced every 6 days. DON and IDL were dissolved in the same solution for the combination treatment. Rats were injected s.c. (2.0 mL/kg) at four alternating injection sites (left neck, right flank, right neck, left flank; sequence repeated). Rats were injected 30 min prior to performing the SAT in the morning and 30 min prior to performing the MCMCT in the afternoon.

Drug doses were selected based on data from a pilot experiment that assessed, in 4 DL rats each, the effects of DON alone, at 0.1, 0.3 and 1.0 mg/kg, or administered in combination with IDL (5.0 mg/kg). Results suggested that MCMCT performance of DL rats may benefit most clearly from the treatment combination involving the middle dose of DON.

Histology and assessments of lesions. Following the completion of post-surgery drug testing, rats were deeply anesthetized and transcardially perfused at a rate of 50 mL/min with 0.1M phosphate buffer solution (PBS) for 2 minutes followed by perfusion with 4% paraformaldehyde in 0.4M Na-phosphate solution and 15% picric acid (pH 7.4) for 9 minutes. Brains were rapidly removed and postfixed for 2-6 h at 4℃ and then rinsed in 0.1M PBS and stored in 30% sucrose solution and allowed to sink. Coronal sections (40 µM thickness) were sliced using a freezing microtome (CM 2000R; Leica) and stored in antifreeze solution. Tyrosine hydroxylase (TH) and ChAT immunostains were performed as previously described (Kucinski *et al.*, 2013; Kucinski & Sarter, 2015).

TH-immunostained sections were imaged at 4x magnification using a Leica DM400B digital microscope. SPOT 5.1 software (Spot Imaging Solutions) was used to capture images. Two sections (AP +1.2 and +0.2 mm) were used to generate a single score depicting the size and degree of bilateral lesions. 6-OHDA infusions were targeted to the dorsal striatum centered between the medial and lateral boundaries. The lesion size was rated based on the size of the area of TH loss observed in the dorsal striatum, with a score of 10 corresponded to 100% of the dorsal striatum exhibiting TH loss, a score of 5 to 50%, and a score of 1 to 10%. The extent of TH clearance (degree of TH loss) within the lesion size area was also determined based on a

scale of 1-10, with a rating of 10 corresponding to complete depletion of TH within the lesion space, and lower values corresponding to the percent of TH remaining (example: 5 is 50% TH loss, 2 is 20% TH loss). Scores for lesion size and extent were averaged from both sections and hemispheres to yield a single lesion score for each rat.

To assess the extent of cholinergic cell losses, semi-quantitative estimates of the number of cholinergic neurons were generated, as done previously (Kucinski *et al.*, 2013; Kucinski & Sarter, 2015). Photographs of the ChAT-stained basal forebrain of the two hemispheres were taken at 5x magnification using a Leica DM400B digital microscope. Cell count estimates were taken from the area of the nucleus basalis of Meynert (nbM) and substantia innominata (SI) within a 680 µm x 680 µm region, and from the horizontal nucleus of the diagnonal band/preoptic area within a 1000 µm x 1300 µm region. For each rat, one section at about Bregma -0.8 mm (AP) was used to generate a cholinergic cell estimate. The "count" function Photoshop CS6 was used to quantify the number of ACh cells. This feature also tags each neuron already counted to prevent double-counts and allows for review by a second counter. These semi-quantitative estimates from the two hemispheres were averaged to yield a single estimate of residual cholinergic cells per rat. To determine the relationships between the degree of lesions and measures of performance and to verify that all DL lesion groups had comparable lesions, a single composite score, reflecting the severity of the two system lesions, was generated for each rat. For this purpose, cholinergic cell loss of two basal forebrain areas per hemisphere were rated from 5 to 1 (5: >90% cell loss relative to control; 4: >80%; 3: >70%; 2: >60%; 1: >30-50% loss). This score multiplied by 2 was averaged with the TH lesion score described above to generate a single composite score (out of 10) per DL rat.

Statistical analyses. SAT and MCMCT performance measures were compared between the five groups and primarily using within-subjects repeated-measures ANOVAs as well as one or two-way ANOVAs when applicable. Sex was a factor in all analyses. The analysis of SAT scores and hits also included the within-subject factor signal duration (500, 50, and 25 ms). For MCMCT runs on the rod (test days 2-5), traversal time, slips and falls were assessed using condition (stationary, rotating counterclockwise (cc) or clockwise (cw), or rotating in alternating directions) as a within-subjects factor. For each condition (or day), performance measures were averaged over each rats' 6 runs on that day and these averaged values were used for statistical analyses. On doorframe test days (6, 7, and 11), falls evoked by the doorframe distractor were compared between groups and sexes. Falls and rewards obtained during the active distractor condition were compared using two way ANOVAs. Comparisons of doorframe evoked-freeze behavior and other performance measures between DL/VEH and DL/DON+IDL rats were

carried out using two-way ANOVAs. Two-way ANOVAs were also used to compare TH/ChAT composite lesion scores between the drug groups and sexes. Following significant main effects, post hoc multiple comparisons were conducted using the Least Significant Difference (LSD) test. Significant interactions between the effects of group and other factors were followed by one-way ANOVAs on the effects of group and LSD multiple comparison tests. Statistical analyses were performed using SPSS for Windows (version 17.0: SPSS). In cases of violation of the sphericity assumption, Huyhn–Feldt-corrected F- values, along with uncorrected degrees of freedom, are given. Alpha was set at 0.05. Exact *P* values are reported as recommended previously (Greenwald *et al.*, 1996). Variances are reported and illustrated as standard error of the mean (SEM). Effect sizes for selected effects are reported using Cohen's *d* (Cohen, 1988).

Results

Pre-surgery SAT and MCMCT performance

Prior to surgery, rats underwent SAT training until they reached stable criterion performance and they were also familiarized with the MCMCT. Rats required 27-55 (range) practice sessions/days to reach SAT criterion performance and practiced the SAT for at least an additional 14 sessions before being assigned to one of the five experimental groups prior to surgery. Performance data from the final 5 pre-surgery sessions were averaged. There were no sex effects on any measure of performance (SAT score, hits, correct rejections, omissions: all $F<0.38$, all $P>0.51$; SAT scores across signal durations: 0.29±0.16; hits across signal duration: 48.26±1.53%, correct rejections: 79.78±1.22%, omissions: 1.34±0.23%). Rats were then evenly distributed into 5 drug groups based on the SAT scores, with 7 rats per sex in each group. Groups were then randomly assigned to treatment conditions. SAT performance did not differ between these groups prior to surgery (all $F<1.16$, all $P>0.34$) nor were there any interactions between group, sex and signal duration (all $F<1.16$, all $P>0.15$).

Pre-surgery familiarization with the MCMCT involved 3 days of plank traversal training followed by 2 days on the stationary squared rod and, on day 6, the rotating rod (counterclockwise [cc] direction only; Table 1). Performances on the rotating rod trials were analyzed. During this first exposure to the rotating rod, rats on average fell twice across the 6 trials and these falls occurred primarily during the initial runs (1^{st} run: 53% of all rats fell; 6^{th} run: 16%). Sex did not affect traversal time (3.91±0.12 s), the number of slips (1.60±0.09), or the percentage of falls (32.86±2.09%; all $F<1.40$, all $P>0.24$). Comparisons between the groups indicated statistically similar MCMCT performance levels prior to surgery (all $F<1.23$, all $P>0.31$).

DON+IDL reduced fall rate in DL rats

On the first day of MCMCT testing rats performed traversals of the plank surface. This surface rarely causes slips or falls, therefore only traversal time was assessed. Traversal time did not differ between groups and sexes (main effects and interaction: all F<1.46, all *P*>0.23; 4.02±0.16 s per traversal; see Table 2 for an overview of major statistical findings from this study).

On days 2, 3, 4 and 5 (Table 1) rats performed traversals with the rod surface and were assessed for traversal time as well as slips and falls (6 traversals per day, 24 total runs). On day 2 the rod remained stationary (non-rotating) followed by rotation of the rod (10 RPM) in the familiar counterclockwise (cc) direction on day 3. On day 4 the rod's direction was reversed to the unfamiliar clockwise (cw) direction and on day 5 the direction of rotation was alternated between successive trials (cc-cw-cc-cw-cc-cw). Performance measures were analyzed for the effects of group, sex, and testing condition (stationary rod, cc, cw, alternating) as the within-subjects factor.

Insert Figure 1 and Table 2 about here

Falls. Rotation of the rod generally increased the number of falls across all animals (main effect of testing condition: F(3,180)=6.48, *P*<0.001; falls from stationary rod: 21.67±1.90%, cc: 32.62±1.90%, cw: 30.00±2.34%, alternating: 30.00±2.00%; less falls on stationary rod than all other conditions (all *P*<0.001). Across these trials, a main effect of group on fall frequency (F(4,60)=2.62, *P*=0.04) reflected that compared with the frequency of falls in DL/VEH rats, SH/VEH and DL/DON+IDL rats fell significantly less frequently (Cohen's *d*=0.96; for multiple comparisons see Fig. 1*A*). Furthermore, and in contrast to DL/DON+IDL rats, with a fall frequency that was statistically similar to that seen in SH-VEH rats, rats treated only with IDL fell as frequently as untreated DL rats (DL/VEH). The frequency of falls in DL/DON rats tended to be lower than in DL/VEH rats but the effect did not reach significance (*P*=0.17). The effects of group and testing condition did not interact (F(12,180)=1.00, *P*=0.45) indicating that all performance across all rod conditions contributed to the demonstration of the large (as indicated by *d*) treatment effect by DON+IDL. There was no sex effect and no interactions involving sex (all F<1.69, all *P*>0.17).

Although the effects of group and testing conditions on fall frequency did not interact, inspection of falls for individual testing conditions (Figs. 1*B-E*) may assist the interpretation of the main finding. Notably, falls elicited by the unfamiliar cw-rotating condition (Fig. 1*D*) and, subsequently,

by alternating the rotation direction, appeared to have contributed to the DON+IDL treatment effect found in the overall analysis of these data. In contrast, treatment with DON alone did not reduce falls during cw-rotating rod as traversals (Fig. 1*D*) and therefore did not significantly ameliorate the overall fall rate of DL rats.

Traversal speed. Similar to the effects on falls, traversal speed differed between groups ($F(4,60)=3.31$, $P=0.02$), with the treatment group that fell as often as vehicle-treated DL rats, DL/IDL, exhibiting the slowest traversals (SH/VEH: 3.96±0.19 s; DL/VEH: 4.62±0.25 s; DL/DON: 4.48±0.36 s; DL/IDL: 5.48±0.45 s, DL/DON+IDL: 4.58±0.32 s; DL/IDL significantly slower than all other groups). Moreover, males were generally slower than females $F(1,60)=13.54$, $P=0.001$; males: 5.12±0.23 s, females: 4.13±0.17 s; see Methods for greater body weights of males). In either sex, body weights were not correlated with traversal speeds (both $R^2<0.06$). Traversal speed was not affected by testing condition and no 2- or 3-way interactions between the 3 factors were found (all $F<0.91$, $p>0.33$).

Slips. Traversing rotating rods generally caused more slips compared with the stationary rod ($F(3,180)=37.53$, $P<0.001$; stationary: 1.02±0.67 slips; cc: 2.31±0.16; cw: 2.39±0.15; alternating: 2.35±0.13). The number of slips did not differ among groups ($F(4,60)=2.37$, $P=0.06$), with the trend reflecting that DL/IDL rats appeared to slip more frequently than all other DL rats (SH/VEH: 1.60+0.11; DL/VEH: 2.04+0.16; DL/DON: 2.04+0.14; DL/IDL: 2.51+0.38, DL/DON+IDL: 1.92+0.16). Males slipped more often than females ($F(1,60)=5.64$, $P=0.02$; males: 2.24±0.17; females: 1.79±0.10) but once again there were no interactions between the three factors (all $F<1.01$, $p>0.44$). Slips in males, but not females were positively correlated with their body weights (males: $R^2=0.14$, $P=0.03$; females: $R^2=0.08$, $P=0.10$).

DON+IDL-treated DL rats are protected against doorframe-associated falls

In PD patients who already have a propensity for freezing of gait, tight doorways are highly effective in evoking freezing and thus increasing the risk for falls (Cowie *et al.*, 2012). This effect is hypothesized to reflect a shift of limited attentional resources away from supporting forward movement to the processing of this distractor. The doorframe distractor (see illustration in Fig. 2*A*) was placed along the rod on test days 6, 7 and 11. On days 6 and 7 the doorframe was presented 3 times in 6 trials and six times in 10 trials on day 11. In total, there were 12 presentations of the doorframe and 10 non-doorframe trials. The direction of rotating rod was alternated between cc and cw directions for these trials.

Insert Figure 2 about here

Overall, the door distractor more than tripled the percentage of trials in which falls occurred ($F(1,60)=117.10$, $P<0.001$; door falls: 38.34±2.89%, non-door falls: 11.30±1.34%). The main effect of group ($F(4,60)=3.24$, $P=0.018$) and a significant door x group interaction ($F(4,60)=3.15$, $P=0.02$) reflected that doorframe-evoked falls accounted for the group differences (Fig. 2*B*). DL rats treated either with VEH, DON or IDL experienced more doorframe-associated falls than SH/VEH rats. In contrast, in the presence of the doorframe, fall rates in DL/DON+IDL rats did not differ from those in SH/VEH rats and were significantly lower than fall rates in DL/IDL rats (Cohen's $d=0.74$; Fig. 2*B*). In all rats, the rate of doorframe-associated falls decreased across the three testing days (main effect of day: $F(1,120)=48.31$, $P<0.001$; day 1: 64.76±4.42% falls, 2: 38.10±4.14%, 3: 12.14±2.14; all interactions with other factors: $F<1.34$, $P>0.23$).

The doorframe caused almost twice as many falls in males than females, however males did not fall more than females in the absence of the doorframe (sex x condition: $F(1,60)=34.24$, $P<0.001$; percentage of door falls over trials; females: 25.69±3.76%, males: 50.99±3.22%, $t(69)=26.09$, $P<0.001$; non-door falls females: 13.27±1.81%, males: 9.32±1.95%, $t(69)=2.20$, $P=0.14$). Doorframe-associated falls in males did not correlate with their body weights or traversal speeds (both $R^2<0.03$). However, slower females had more falls in this condition ($R^2=0.21$, $P=0.005$). There were no significant interactions between the 3 factors (all $F<1.31$, all $P>0.25$).

Microbehavioral correlates of doorframe falls and treatment effects. To explore potential behavioral correlates of the DON+IDL treatment effect on doorframe-associated falls, a video-based analysis of the animals' microbehavior during doorframe traversal was conducted. To optimize the potential insights gained from this analysis we selected doorframe runs from DL/VEH and DL/DON+IDL male and female rats from days when their fall rates reflected their group means (days 6 and 7 for females, and 11 for males). In general, we observed that the rats of both groups stopped forward movement when approaching the doorframe (effects of groups on freezing duration: $F(1,27)=1.22$, $P=0.28$; DL/VEH: 1.25±0.22 s/freeze; DL/DON+IDL: 0.93±0.21 s). Furthermore, the individual rats' duration of freezing periods correlated with their fall rates (Fig. 2*C*). Because we observed that longer freezes nearly consistently were associated with falls in all rats, doorframe-evoked freezes were grouped into long (≥2 s) versus short freezes (<2s). The percentage of trials in which a long freeze occurred did not differ between the two groups (6 trials per animal; $F(1,27)=2.59$, $P=0.12$; 26.79±4.95% of trials involved long freezes). In addition, rates of falls associated with long freezes - in animals that

displayed at least one such freeze (10 DL/VEH and 9 DL/DON+IDL) - did not differ between these two groups ($F(1,18)=0.004$, $P=0.95$; DL/VEH: 62.67±12.62% falls in runs involving long doorframe-associated freezes; DL/DON+IDL: 63.89±13.89%). However, fall rates associated with short freezes were higher in DL/VEH than the DL/DON+IDL rats (14 rats per group; $F(1,27)=5.27$, $P=0.03$, Figure 2*D*). Thus, these observations indicated that treating DL rats with DON+IDL did not increase the proportion of doorframe runs that were associated with relatively short freezes, but DON+IDL-treated rats were more capable of resuming and continuing the traversal after short freezes, perhaps by better maintaining or recovering posture and balance, and re-initiating forward movement than was the case for DL/VEH rats.

To further detail the potential effects of the combined treatment with DON+IDL, we selected representative runs (first two trials on doorframe day 2 in females and on day 3 for males; 2 trials per rat) and counted the rate of the following behaviors for a 1-s period beginning shortly after and during short freezes (0.5-1.5 s of freezing periods): (1) sudden increases of traversal speed after a freeze; (2) high and firm tail position while passing under the doorframe (associated with controlled, upright posture and forward focus in contrast to a low, dragging tail and slouched posture and downward focus typical of DL rats; Kucinski *et al.*, 2013); (3) swinging of the tail to maintain balance following doorframe-associated slips; (4) the use of forelimbs to 'push' the upper trunk of the body back onto the rod after slips; (5) active hind limb movements ('walking in place') during freezes to maintain balance on the rod; and (6) small 'hops' after freezes to reestablish forward momentum through the doorframe. Results indicate that, first, a composite score collapsing counts of all 6 behaviors negatively correlated with fall rates in both groups (both $R^2>0.42$, both $P<0.02$), indicating that active recovery movements following short freezes are a strong predictors of successful doorframe runs. Second, DL/DON+IDL rats exhibited more instances of category #2 behavior (above) than DL/VEH rats ($X^2=7.22$, $P=0.03$, DL/VEH: 0.18±0.10 counts/trial, DL/DON+IDL: 0.54±0.11; all other categories of behavior: $P>0.30$; Figs. 2*E*). Together, these observations suggest that DON+IDL treatment fosters the maintenance and, after relatively short stoppages, facilitates the recovery of posture, balance, and forward movement.

DON+IDL-treated DL rats are not protected against active distractor associated falls

Falls in aged humans and PD patients are correlated with poor dual task performance (Shumway-Cook *et al.*, 1997; Marchese *et al.*, 2003; Amboni *et al.*, 2013). To model the impact of the reallocation of attentional resources to a secondary task on complex movement control, water-deprived rats were offered water for retrieval while traversing the rotating rod. In total,

there were 28 runs with water being offered (see Methods and Table 1).

Insert Figure 3 about here

Overall, 54 of the 70 (77.14%) animals retrieved water (defined as licking water for at least 1 s, regardless of falls) in at least one trial (13 SH/VEH, 9 DL/VEH, 10 DL/DON, 11 DL/IDL, and 11 DL/DON+IDL; 26 males and 28 females). A main effect of group indicated that all DL rats retrieved water less frequently than SH/VEH rats ($F(4,60)=3.32$, $P=0.016$) although multiple comparisons indicated that DL/DON rats' retrieval count was not significantly lower than DL/VEH (Fig. 3*A*). Females retrieved water more frequently than males ($F(1,60)=10.56$, $P=0.002$; females: 49.78±6.32%, males: 26.69±4.36%], however the effects of sex did not interact with group ($F(4,60)=1.50$, $P=0.22$). In this test, water retrievals and falls are confounded measures as stopping forward movement to retrieve water is a high risk for falls. SD/VEH rats succeeded more frequently in retrieving water without falling than DL rats ($F(4,60)=5.84$, $P<0.001$; Fig. 3*B*). Furthermore, females successfully retrieved water without falling more frequently than males ($F(1,60)=10.81$, $P=0.002$; females 33.98+5.02% of trials; males: 16.73+3.36%) but this effect did not interact with group ($F(1,69)=1.56$, $P=0.19$). In neither sex did this measure correlate with body weights, traversal speed or doorframe-associated falls all $R^2<0.1$). Thus, DL lesions reduced the engagement with the active distractor and elevated associated falls and the drug treatments did not benefit rod traversal performance in the presence of this active distractor.

Lesion-induced impairment in SAT performance and lack of treatment effects

As detailed in Methods, following the completion of the postsurgery recovery period of four weeks, animals were returned to morning SAT and afternoon MCMCT testing, and drug treatments were administered 30 min prior to the morning as well as the afternoon test session. The analysis of post-surgery SAT performance was based on the data from the final five SAT sessions (averaged for individual rats). Overall SAT performance, as indicated by SAT scores, was impaired in all lesioned groups when compared with SH/VEH rats (main effect of group: $F(4,60)=3.38$, $P=0.015$; SH/VEH: 0.33±0.02; DL/VEH: 0.18±0.04; DL/DON: 0.23±0.04; DL/IDL: 0.19±0.03; DL/DON+IDL: 0.19±0.03; SH/VEH had higher score than all other groups, all $P<0.05$, no other group differences).

Insert Figure 4 about here

In addition to the main effect of drug group on SAT scores, there was a significant interaction between the effects of group and signal duration (main effect of signal duration: $F(2,138)=272.98$, $P<0.001$; group x signal: $F(8,120)=4.59$, $P<0.001$; Fig. 4*A*). To identify the locus of this interaction, one-way ANOVAs on the effects of group were computed for the SAT scores for individual signal durations and followed up, if applicable, by multiple comparisons (see Fig. 4*A*). SH/VEH rats had higher scores than all other groups with respect to longest signals ($F(4,60)=5.36$, $P=0.001$) and higher scores than DL/VEH and DL/IDL rats for medium duration signals. SAT scores calculated over hits to shortest signals were relatively low across groups (Fig. 4*A*), thus probably preventing the manifestation of the effects of lesions on these measures. Similar to pre-surgery SAT performance, there were no sex effects and no interactions involving sex, group or signal duration (all $F<1.51$, all $P>0.21$).

The results of the analysis of hits partly mirrored the findings on SAT scores, indicating that SAT score group differences primarily reflected the effects of lesions on hits (main effect of group: $F(4,60)=0.61$, $P=0.66$; main effect of signal duration: $F(2,138)=197.90$, $P<0.001$; group x signal: $F(8,120)=4.44$, $P<0.001$; Fig 4*B*). Three out of the four DL groups scored less hits to longest signals than SH/VEH (one-way ANOVA on effects of group on hits to 500 ms signals: $F(4,60)=2.93$, $P=0.028$). Hit rates to shorter signals did not differ between groups (both $F<0.42$, both $P>0.79$). Finally, neither correct rejections (Fig. 4*C*) nor errors of omission differed between groups (both $F<2.23$, both $P>0.08$; Fig. 4*D*), and there were no effects of sex (all main effects and interactions: all $F<1.29$, all $P>0.14$). Together, these findings reproduce the effects of DL lesions on SAT performance (Kucinski *et al.*, 2013), with the loss of cholinergic neurons likely being primarily responsible for the decrease in hits (McGaughy *et al.*, 1996). However, none of the DL rats treated with DON, IDL or the two drugs combined showed improved performance compared with DL/VEH rats.

Histological analyses

Semi-quantitative cholinergic cell density estimates in the basal forebrain indicated that the lesions removed about 60-70% of the cholinergic cell bodies in the rostral nucleus basalis of Meynert (nbM) and about 50% of the cholinergic neurons in the more ventral horizontal nucleus of the diagonal band (HDM; Fig. 5). Such a partial loss was intended to model the limited cholinergic losses in non-demented PD fallers (Bohnen *et al.*, 2009) and it is also reflected in the limited severity of the impairments in SAT performance (Fig. 4). In the analysis of cholinergic

losses in both BF subregions, main effects of group (both F(4,59)>4.34, both *P*<0.005) indicated a lower number of cholinergic cells in all DL rat groups, with no differences among DL groups (Fig. 5). Cholinergic losses did not differ by sex and there were no sex x group interactions (all F<2.08. all *P*>0.10).

Insert Figure 5 about here

Striatal dopamine depletions were rated based on size (mean score: 4.13±0.28) and the intensity of TH-immunoreactivity losses (5.33±0.21; see Methods). Scores did not differ between the groups or sexes (main effects and interactions: all F<0.81; all *P*>0.37*).* We further investigated whether cholinergic or dopaminergic lesion scores correlated with the propensity for falls in DL/VEH or DL/DON+IDL rats and found only one relationship that indicates that in treated DL rats, falls were lower in rats with larger striatal dopamine lesions (Fig. 5*F*; all other groups: all R^2<0.20, all *P*>0.17).

Discussion

We tested the effects of donepezil (DON) and idalopirdine (IDL) in rats with cortical cholinergic and striatal dopaminergic losses (DL rats) traversing rotating rods and performing the Sustained Attention Task (SAT). (1) Co-treatment with DON+IDL reduced falls on the rotating rod as well as falls evoked by the doorframe distractor. (2) DON+IDL prevented falls primarily by enhancing the efficacy and vigor of the re-initiation of rotating rod traversal following relatively brief movement stoppages. (3) Falls evoked by the active distractor/dual task were not affected by the treatment. (4) SAT performance did not benefit from DON+IDL treatment. (5) Neither DON nor IDL significantly improved SAT or MCMCT performance when administered alone. (6) DON+IDL was more effective in rats with relatively larger dopamine depletions.

Previous evidence rejects the possibility that the present effects of DON+IDL were based on pharmacokinetic interactions between the two drugs (Herrik *et al*., 2016). Furthermore, it seems unlikely that the contrast between effects of DON+IDL on MCMCT performance, assessed following the afternoon administration of the compounds, and the absence of effects on SAT performance, assessed following the morning administration of the compounds, was due to the relatively shorter delay between the morning and the afternoon injections (6 hrs). DON+IDL-induced increases in basal extracellular acetylcholine (ACh) levels, measured by microdialysis,

return to baseline within 2-3 hrs (Herrik *et al.* 2016) and thus were unlikely to have carried over from the morning to the afternoon session.

DL rats treated with DON+IDL exhibited less falls while traversing the rotating rod than other DL groups, and they specifically fell less often in association with relatively short doorframe-evoked stoppages. In human fallers, as in DL rats, slow gait speed and movement stoppages, evoked by distractors or occurring without obvious causes, destabilize forward movement and increases the risk for falls (Springer *et al.*, 2006; Plotnik *et al.*, 2011). Stoppages or freezing of gait involve both instabilities in posture and gait control as well as disruption of movement selection and planning, and thus such stoppages may reflect the breakdown of cortico-striatal communication, rather than solely losses of striatal dopamine (Lewis & Barker, 2009; Vercruysse *et al.*, 2012; Heremans *et al.*, 2013; Bohnen *et al.*, 2014; Kucinski *et al.*, 2015a).

In humans, as in DL rats, freezing episodes may occur for a couple of seconds or longer (Nutt *et al.*, 2011). However, while traversing dynamic surfaces, shorter stoppages allow for planned movements to stay "online" in working memory so that motor plans can be updated to account for surface changes and adjustments of limb placements and balance, and to allow for corrections after stepping errors (Seidler *et al.*, 2012). Such cognitive-motor interactions favor successful movement re-initiation after relatively short stoppages. Indeed, traversing the rotating rod at 10 RPM and stopping for 2 s (the upper limit of "short" stoppages in our analyses) requires coping with 120° of rod rotation, including rebalancing and posture adjustments and re-placing limbs as forward movement resumes. DL rats treated with DON+IDL were significantly more effective in mastering this situation. After having resumed forward movement, DL rats treated with DON+IDL often rapidly displayed effective posture and balance control and displayed a level of vigor of movement less frequently observed in vehicle-treated DL rats.

Maintaining motor plans in working memory and resuming movement with relatively high vigor are confounded variables and thus it is difficult to further isolate the cognitive-behavioral mechanisms that mediated the effects of DON+IDL. However, if the treatment had primarily improved the vigor of movement, thought to reflect the status of striatal dopamine systems (Mazzoni *et al.*, 2007; Niv *et al.*, 2007; Wang *et al.*, 2013; Gepshtein *et al.*, 2014), we would have expected that this treatment also reduced the portion of relatively long stoppages. As this was not the case, enhanced vigor alone is unlikely to account for the treatment effect. Rather, following doorframe-evoked freezes or slips, DL rats treated with DON+IDL may have been superior in integrating information about the rod status, balance and limb positioning with ongoing movement programs, while also benefiting from greater vigor of corrective movements.

Treatment with IDL was previously demonstrated to potentiate the effect of DON on basal cortical and hippocampal extracellular ACh levels, with no effects of IDL on ACh levels alone (Foraster *et al.*, 2014; Herrik *et al.*, 2016). Relatively high levels of cortical cholinergic neuromodulation may benefit SAT performance when challenged by distractors (St Peters *et al.*, 2011) and, in DL rats, about half of the cholinergic systems remained intact and thus might have supported treatment-induced increases in extracellular ACh levels. However, neither DON alone nor in combination with IDL improved SAT performance. Although the effects of these treatments on cortical ACh levels in this model remain unknown, the absence of treatment effects on SAT performance suggests that cortical pro-cholinergic/pro-attentional mechanisms do not sufficiently explain the reduction in falls in DON+IDL-treated DL rats.

Impairments in SAT performance in DL rats largely reflect their cholinergic losses. In contrast, rats with lesions of only the BF or only the dopaminergic striatum, with one minor exception, were not significantly impaired in the MCMCT (Kucinski *et al.*, 2013). Thus, in contrast to SAT impairments, impaired MCMCT performance requires interactions between cholinergic-attentional deficits and striatal-motor deficits. Consequently, the effects of DON+IDL treatment may have acted at the intersection between these two systems. BF cholinergic lesions or optogenetic silencing of cholinergic neurons disrupt a complex cognitive process that allows a cue to control behavior (Gritton *et al.*, 2016; Sarter *et al.*, 2014b, 2016) and thus impair the cortico-striatal, glutamatergic transfer of information about external and proprioceptive cues that normally assists balance, gait and movement control (see Fig. 2 in Sarter *et al.*, 2014a). Such cue-related information may be transferred specifically to synapses on dendritic spines of medium spiny neurons (Dubé *et al.*, 1988) of direct-pathway projections (Wall *et al.*, 2013). Cortico-striatal signaling is modulated by D1 receptors expressed on the neck of the spines that are also contacted by cortico-striatal projections (Pickel *et al.*, 1981) and thus, in DL rats, both cortico-striatal signaling and the dopaminergic modulation of such signaling may be disrupted. The predominant expression of 5-HT_6 receptors on pyramidal neurons throughout the cortex and in basal ganglia output neurons, and their co-localization with striatal dopamine receptors (Helboe *et al.*, 2015), are consistent with the speculation that the treatment restored, to a degree, such cortico-striatal, glutamatergic-dopaminergic interactions. The neuropharmacological interactions between DON and IDL on striatal output neurons is not clear and may involve diverse signaling pathways converging onto striatal output neurons (e.g., Yan & Surmeier, 1996; Threlfell *et al.*, 2012; Nelson *et al.*, 2014).

In functional terms, elevation of striatal-cholinergic interneuronal activity may have interacted with 5-HT_6 receptor blockade to maintain movement plans during short stoppages and thus

maintained goal-directed behavior (e.g., Bradfield *et al.*, 2013; Aoki *et al.*, 2015). The absence of treatment effects on SAT performance and on falls evoked by the active water distractor is also consistent with the possibility that DON+IDL reduced fall propensity by partly restoring cognitive-motor interactions that, in cognitive terms, may be downstream from effects on the attentional control of movement. Such downstream effects may remain mediated via synergistic effects of DON+IDL on telencephalic circuitry (Foraster *et al.*, 2014; Herrik *et al.*, 2016). However, in addition to potential effects on DON+IDL on telencephalic-striatal circuitry, this treatment may enhance the functions of brainstem ascending cholinergic systems also thought to be involved in movement control (e.g., Kucisnki & Sarter, 2015). The simultaneous measurement of striatal ACh, amino acids and monoamines in DL rats traversing the rotating rod and treated with DON+IDL may be a first future step toward identifying relevant neuropharmacological interactions between the two drugs.

Finally, the current experiment indicated that males generally performed poorer across the more demanding MCMCT conditions than females, and that, with one exception, the greater body weight of males did not seem to account for this sex effect. Similar sex effects have been observed in humans (Pereira *et al.*, 2013) and, as in the current DL rats, their neuronal or behavioral origins are unclear. Importantly, however, in the present study, treatment effects did not differ by sex. As SAT performance did not differ between the sexes (see also McGaughy & Sarter, 1999) but as females retrieved water without falling more frequently than males, greater dual task capacity in females may be a primary mediator of their relatively better MCMCT performance (see also Hollman *et al.*, 2011).

Conclusions

The present evidence indicates the efficacy of treatment with DON+IDL in reducing falls in DL rats and thus may form the basis for predictions reagrding the clinical efficacy of this combination treatment. However, it is important to first acknowledge that DL rats do not constitute a model of PD; rather, DL rats are a hypothesis-driven animal model of impaired cortico-striatal interactions hypothesized to underlie falls. As we previously demonstrated (Kucinski *et al.*, 2013), DL rats lack the primary motor and sensor-motor deficits that characterize rats with relatively larger uni- or bilateral striatal dopamine depletions (for review see Deumens *et al.*, 2002). Indeed, the presence of such deficits would severely confound interpretations in terms of cognitive-motor interactions underlying falls in this model.

Our results in DL rats suggest that the co- treatment with an acetylcholinesterase inhibitor and a 5-HT_6 receptor antagonist may reduce the fall propensity in PD patients who also exhibit a

propensity for relatively brief movement stoppages evoked by distractors or occurring spontaneously. This treatment may be less effective in more severely impaired PD fallers who exhibit longer periods of freezing of gait (Lewis & Barker, 2009; Vercruysse *et al.*, 2012). Finally, considering that in the present experiment rats continued daily traversal practice while undergoing pharmacological treatment, the clinical efficacy of the present pharmacological combination treatment may be enhanced based on interactions with parallel behavioral programs aimed at reducing falls.

Figure Legends

Figure 1. Falls during rod traversal (N=70, n=14 per group and 7/sex). Across all testing conditions, DL rats fell more frequently than SH/VEH rats. Furthermore, compared with DL/VEH rats, treatment with DON+IDL significantly lowered fall rates (*A*). Inspection of individual testing conditions (*B-E*) indicated that reduction of falls associated with traversing the rod rotating in the unfamiliar clockwise (cw) direction and alternatingly rotating in the cw and counterclockwise (cc) directions contributed primarily to the overall effect of DON+ILA in DL rats. This and the following figures indicate the results of *post hoc* multiple comparisons that were based on significant results from ANOVAs that are described in Results (*,**,***, $P<0.05$, 0.01, 0.001; abbreviations used in this and other figures: SH, sham-operated; DL, dual basal forebrain cholinergic and striatal dopaminergic lesions; DON, donepezil, IDL, idalopirdine).

Figure 2. Effects of the doorframe distractor (N=70, n=14 per group and 7/sex). All DL rats, except for DL/DON+IDL rats, fell more frequently than SH/VEH rats when exposed to this passive distractor (*A,B*). To gain insight into potential behavioral mechanisms mediating the effects of DON+IDL, the doorframe-associated behavior of DL/VEH and DL/DON+IDL rats was further analyzed. Doorframe-associated falls were associated with stoppage of movement or freezing of gait, as rats approached or reached the frame. Generally, longer freezes were associated with more falls (*C*) in both DL/VEH and DL/DON+IDL rats. Indeed, falls associated with longer freezes did not differ between the groups (*D*). However, DON+IDL treated rats fell significantly less frequently when freezes remained relatively short (<2 s). The proportion of short freezes itself did not differ between the groups. As illustrated in *E* (see Results for quantification), following short freezes, and even if they did not fall, DL/VEH rats resumed forward movement relatively slowly, generally with the tail positioned relatively low and with a slouched posture (note that this rat slips after passing through the door). In contrast, when

DL/DON+IDL rats resumed forward movement, sometimes starting with a hop (as shown here), they quickly regaining regular traversal speed and fluid forward movement, with high and firm tail position and upright posture.

Figure 3. Performance on active distractor task (N=70, n=14 per group and 7/sex). To model the impact of a secondary task, water was offered as rats traversed the rotating rod (see top photograph; rats were water-deprived because of the parallel, daily SAT testing). DL rats were generally less likely to engage in this competing activity as indicated by lower number of attempts to retrieve the water (*A*). DL rats were also less likely to retrieve water without incurring a fall (*B*). The drug treatments did not significantly improve the performance of DL rats in the presence of this active distractor.

Figure 4. SAT performance (N=70, n=14 per group and 7/sex). Overall SAT performance, indicated by SAT scores, was lower in all rats with DL lesions (main effect). Moreover, a significant interaction between the effects of group and signal duration (*A*) reflected that "floor effects" limited the impact of the DL lesions on SAT scores calculated over hits to shorter signals. The effects of the lesions on overall performance were largely reproduced in the analyses of hits (*B*) while, similar to prior studies on the effects of DL lesions or solely lesions of the cortical cholinergic inputs system, correct rejections (*C*) and errors of omission (*D*) remained unaffected. The drug treatments did not improve SAT performance of DL rats (in *A,* DL/VEH versus DL/DON: P=0.40).

Figure 5. Histological analyses and relationship with treatment effects. Semi-quantitative counts of cholinergic neurons in the nucleus basalis of Meynert (nbM) and the horizontal nucleus of the diagonal band (HDB; schematically illustrated in *A*) indicated lesion-induced losses ranging from 60-70% in the nbM (B) to about 50% in the HDB (*C*; M; SEM; see Results for statistical analyses). *D* (AP from bregma: -0.8mm) and *E* (AP: -0.10 mm) are coronal sections showing ChAT-immunoreactive neurons in the BF of a sham-operated rat (*D*) and an immunotoxin-infused rat (*E*) (bars: 0.5 mm; m depicts the midline of the brain). *F*: Correlations between dopaminergic lesion scores and falls by DL/DON+IDL rats (*F*; R^2=0.34, P=0.03; note that the correlation remains significant following the removal of data from the animal with the highest fall rate; R^2=0.38, P=0.022). This correlation suggests that DON+IDL was more effective at reducing falls in rats with larger striatal dopamine depletions. *G* and *H* show examples of a smaller and a large striatal dopamine lesion, respectively, and their positions in *F* are indicated by the green and blue, respectively, halo around their data points.

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Table 1. MCMCT testing sequence

Pre-Surgery Sequence

Day	Trial type	Rotating (10 rpm)	Distractor	Number of trials
1	plank			6
2	plank			6
3	plank			6
4	rod			6
5	rod			6
6	rod	cc		6

Post-Surgery Sequence

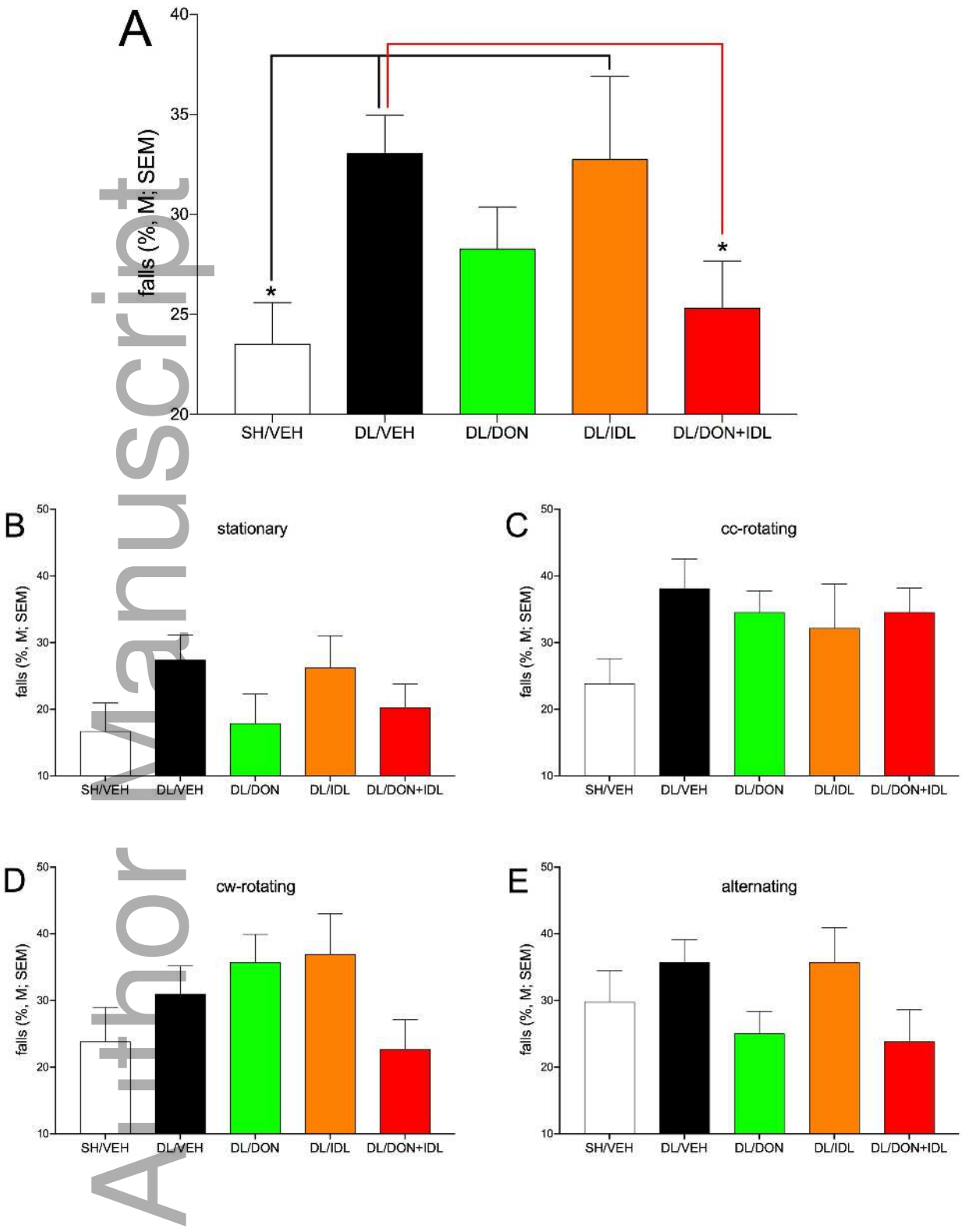
1	plank			6
2	rod	stationary		6
3	rod	cc		6
4	rod	cw		6
5	rod	cc-cw-cc-cw-cc-cw		6
6	rod	cc-cw-cc-cw-cc-cw	doorframe distractor	6 (3 with doorframe)
7	rod	cc-cw-cc-cw-cc-cw	doorframe distractor	6 (3 with doorframe)
8	rod	cc-cw-cc-cw	active distractor	8 (4 shaping; 4 test trials)
9	rod	cc-cw-cc-cw	active distractor	8 (4 shaping; 4 test trials)
10	rod	cc-cw-cc-cw-cc-cw-cc-cw-cc-cw	active distractor	10
11	rod	cc-cw-cc-cw-cc-cw-cc-cw-cc-cw	doorframe distractor	10 (6 with doorframe)
12	rod	cc-cw-cc-cw-cc-cw-cc-cw-cc-cw	active distractor	10

cc, cw: counterclockwise, clockwise

Table 2: Summary of main treatment effects

	test days	# of trials	main effects of treatment group or significant group x test condition	multiple comparisons
falls (percentage of trials with falls)	2, 3, 4, 5	24	group: $F(4,60)=2.62$, $P=0.04$	SH/VEH less falls than DL/VEH and DL/IDL* DL/DON+IDL less falls than DL/VEH*
traversal time	2, 3, 4, 5	24	group: $F(4,60)=3.31$, $P=0.02$	DL/IDL slower than all other groups (** vs SH/VEH; * vs all other groups)
slips	2, 3, 4, 5	24	group: $F(4,60)=2.37$, $P=0.06$ (n.s.)	N/A
falls with doorframe distractor	6, 7, 11	12	group x condition: $F(4,60)=3.15$, $P=0.02$	SH/VEH less door falls than DL/VEH*, DL/DON*, & DL/IDL*. DL/DON+IDL less falls than DL/IDL*
active distractor task: retrievals without falling	8, 9, 10, 12	28	group: $F(4,60)=5.84$, $P<0.001$	SH/VEH more retrievals without falling than all other groups**

* $p < 0.05$; ** $p < 0.01$



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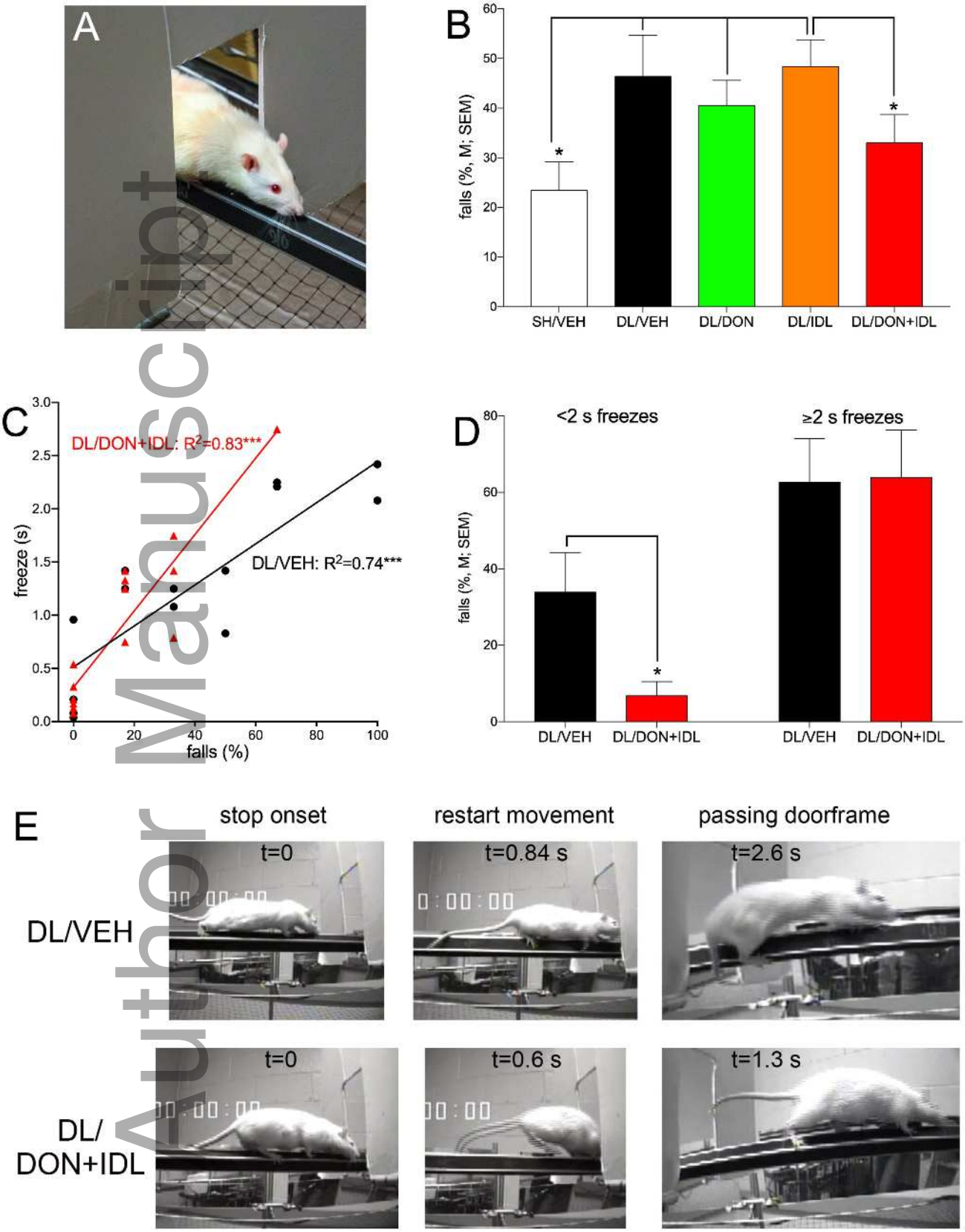

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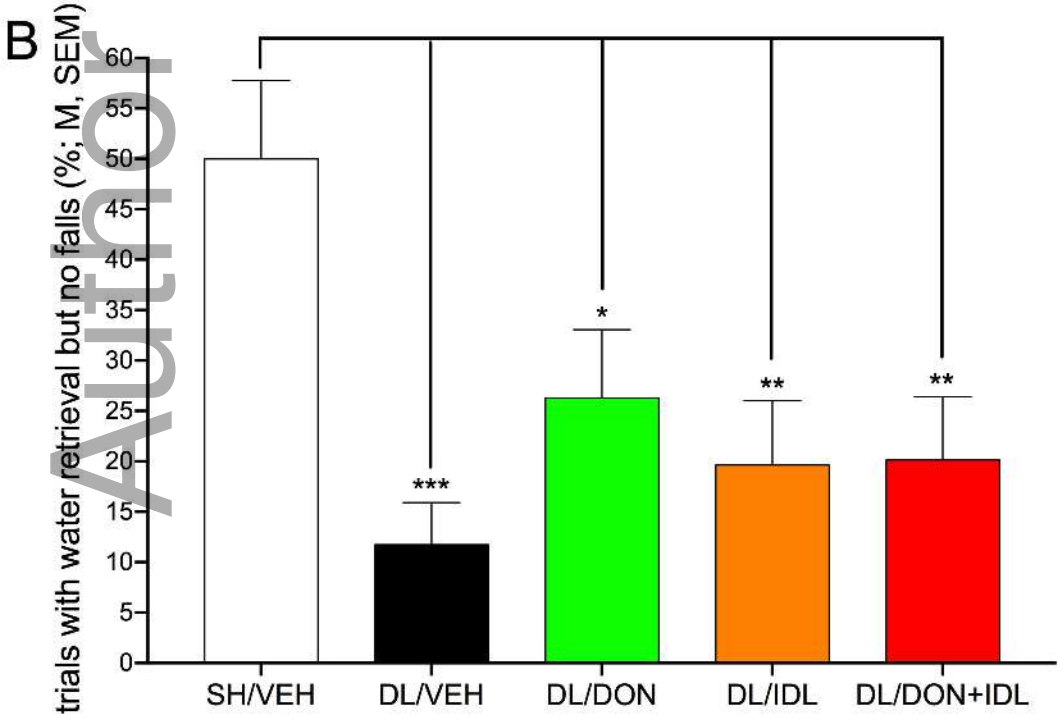

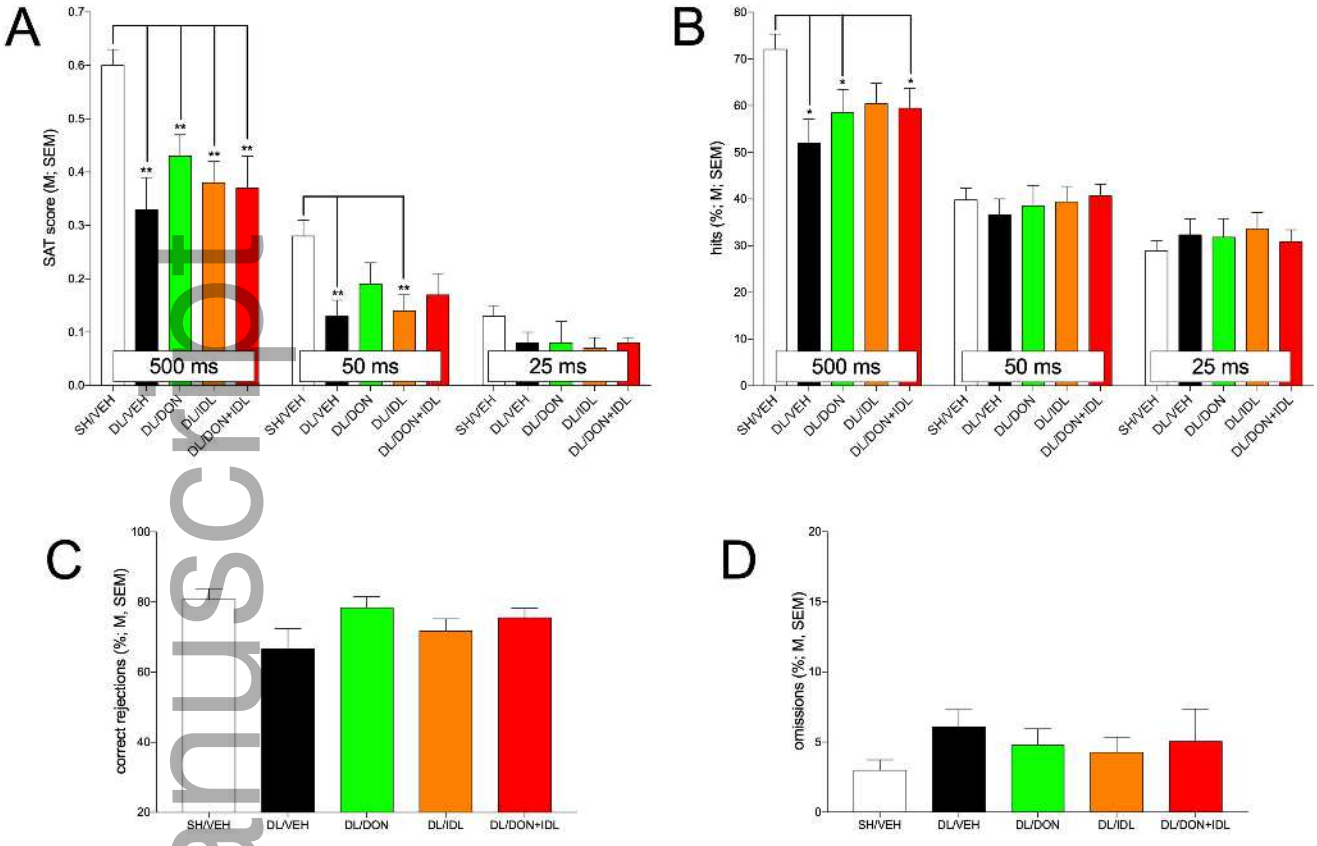

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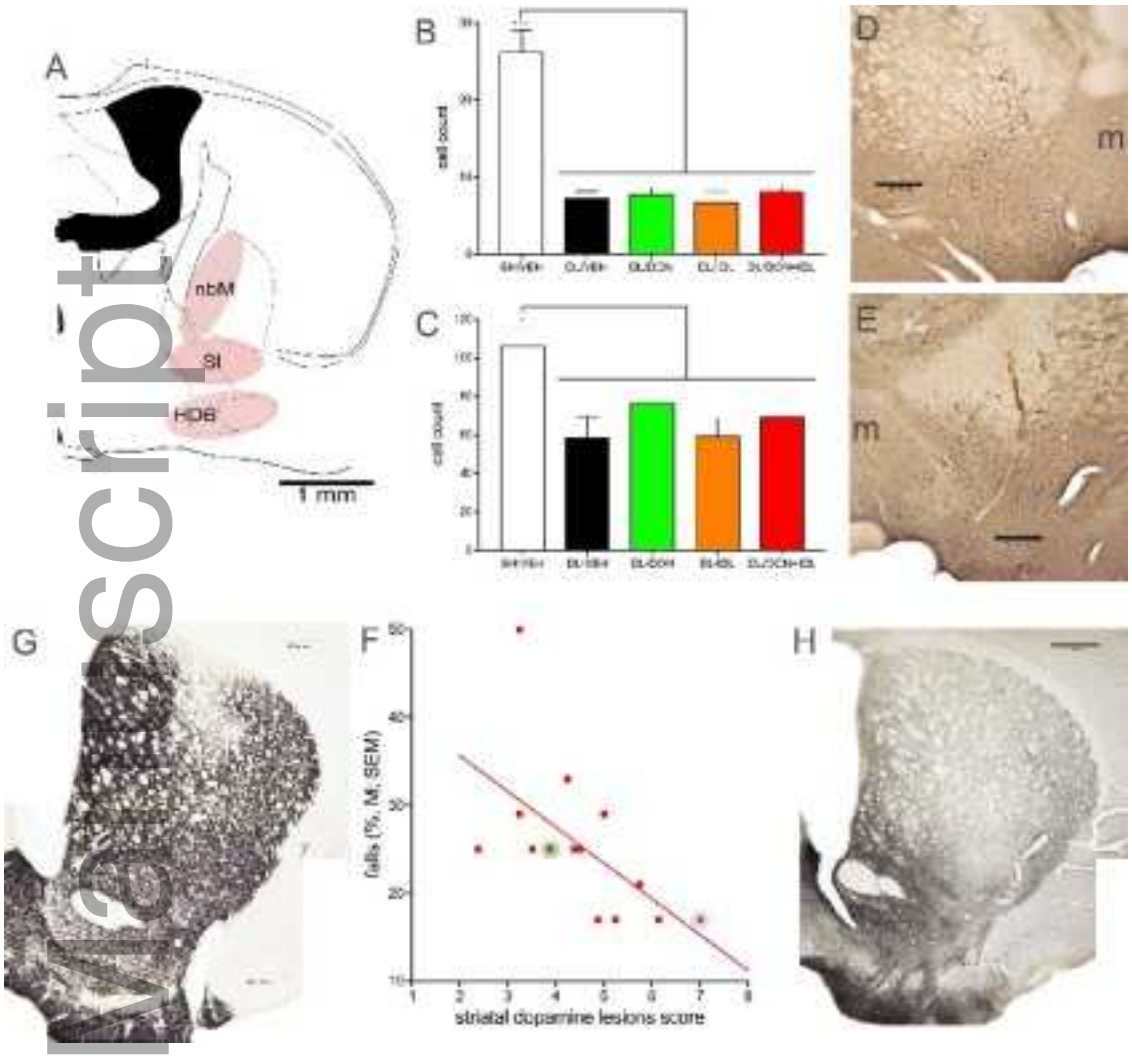

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