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Research paper

A novel dopamine D3R agonist SK609 with norepinephrine transporter inhibition promotes improvement in cognitive task performance in rodent and non-human primate models of Parkinson's disease

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ABSTRACT

Mild cognitive impairment is present in a number of neurodegenerative disorders including Parkinson's disease (PD). Mild cognitive impairment in PD (PD-MCI) often manifests as deficits in executive functioning, attention, and spatial and working memory. Clinical studies have suggested that the development of mild cognitive impairment may be an early symptom of PD and may even precede the onset of motor impairment by several years. Dysfunction in several neurotransmitter systems, including dopamine (DA), norepinephrine (NE), may be involved in PD-MCI, making it difficult to treat pharmacologically. In addition, many agents used to treat motor impairment in PD may exacerbate cognitive impairment. Thus, there is a significant unmet need to develop therapeutics that can treat both motor and cognitive impairments in PD. We have recently developed SK609, a selective, G-protein biased signaling agonist of dopamine D3 receptors. SK609 was successfully used to treat motor impairment and reduce levodopa-induced dyskinesia in a rodent model of PD. Further characterization of SK609 suggested that it is a selective norepinephrine transporter (NET) inhibitor with the ability to increase both DA and NE levels in the prefrontal cortex. Pharmacokinetic analysis of SK609 under systemic administration demonstrated 98% oral bioavailability and high brain distribution in striatum, hippocampus and prefrontal cortex. To evaluate the effects of SK609 on cognitive deficits of potential relevance to PD-MCI, we used unilateral 6-hydroxydopamine (6-OHDA) lesioned rats and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated cynomolgus macaques, with deficits in performance in a sustained attention and an object retrieval task, respectively. SK609 dose dependently improved the performance of 6-OHDA-lesioned rats, with peak performance achieved using a 4 mg/kg dose. This improvement was predominantly due to a significant reduction in the number of misses and false alarm errors, contributing to an increase in sustained attention. In MPTP-lesioned monkeys, this same dose also improved performance in an object retrieval task, significantly reducing cognitive errors (barrier reaches) and motor errors (fine motor dexterity problems). These data demonstrate that SK609 with its unique pharmacological effects on modulating both DA and NE can ameliorate cognitive impairment in PD models and may provide a therapeutic option to treat both motor and cognitive impairment in PD patients.

1. Introduction

Parkinson's disease is a progressive neurological disorder that affects more than 6 million patients globally. Although Parkinson's disease (PD) is often considered to be primarily a movement disorder, patients experience numerous non-motor symptoms that affect their quality of

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life. These include sleep disorders, olfactory issues ranging from loss of smell to smell hallucinations, loss of taste, various autonomic disturbances, and a range of cognitive issues that are broadly grouped as mild cognitive impairment and referred to as PD-MCI (Brown and Marsden, 1990; Cooper et al., 1991; Halliday et al., 2014; Hietanen and Teravainen, 1986; Levin et al., 1989; Litvan et al., 2012; Olanow et al., 2009; Owen et al., 1992; Sawamoto et al., 2002; Vale, 2008; Verbaan et al., 2007; Williams-Gray et al., 2007; Masala et al., 2018; Park et al., 2018). PD-MCI is clinically important as it leads to social/ behavioral abnormalities, poor motivation, and reduced "flexibility/ adaptability" in daily life (Vale, 2008; Frank et al., 2006; McNamara et al., 2010) and may be a harbinger of later dementia. Epidemiological studies have shown significantly high rates of 25–50% of PD-MCI at the time of diagnosis suggesting that the development of cognitive impairments may precede motor impairment by several years (Weil et al., 2018). Cognitive impairments in PD patients have proven difficult to treat as effective therapeutic agents used to treat motor impairment can have beneficial or harmful effects on PD-MCI (Brusa et al., 2003; Yarnall et al., 2013).

Although recent studies have highlighted the heterogeneity of the clinical presentation of PD-MCI (Litvan et al., 2012), impairments in attention, executive functioning, and spatial and working memory, are common in many PD-MCI patients (Halliday et al., 2014). The nature of the cognitive impairments suggest involvement of several brain structures including prefrontal cortex (PFC), hippocampus, locus coeruleus (LC), ventral and medial substantia nigra (SN) and striatal regions connected to the PFC (Owen et al., 1992; Owen, 2004; Sawamoto et al., 2007). Similarly, dysfunction in several neurotransmitter systems, including cholinergic (Yarnall et al., 2013; Jellinger, 2012; Kim et al., 2017; Lee et al., 2019; Li et al., 2015; Yarnall et al., 2011) dopaminergic, and noradrenergic systems, have been implicated in PD-MCI. The role of dopaminergic and noradrenergic dysfunction appears to be particularly significant, particularly in the early stages of PD (Halliday et al., 2014; Ko et al., 2013).

The role of the noradrenergic system in various cognitive processes is well established (Bari and Robbins, 2013; Cain et al., 2011; Callahan et al., 2019), with norepinephrine transporters (NET) in the PFC capable of modulating both NE and dopamine (DA) neurotransmission (Carboni and Silvagni, 2004; Moron et al., 2002). Loss of A6 noradrenergic neurons in the LC that project extensively to the forebrain has been observed in PD brain and has been suggested as contributing to PD-MCI. Numerous studies have also shown that the frontocortical dopaminergic system is affected in PD, although the benefits of dopaminergic replacement therapy on frontocortical-based cognitive deficits have been inconsistent (Ko et al., 2013; Mattay et al., 2002; Monchi et al., 2007; Seamans and Yang, 2004). Dopaminergic therapy may provide a therapeutic amount of DA to certain brain areas and improve certain deficits primarily related to that dopaminergic dysfunction, but may also cause excessive DA overflow to other areas with less dopaminergic denervation, resulting in impairments of cognitive functions associated with those regions (Jellinger, 2012; Dymecki et al., 1996; Narayanan et al., 2013; Williams and Goldman-Rakic, 1998). While dopaminergic therapy with levodopa (L-dopa) is the gold standard for providing symptomatic relief of motor symptoms of PD, it has variable effects on cognitive functions related to PD-MCI and may actually exacerbate certain cognitive difficulties in these patients (Cools, 2006; Muller et al., 2001; Pascual-Sedano et al., 2008).

Alternative pharmacological targets and therapies are sorely needed to address cognitive dysfunction in medicated PD patients. Toward this end, we have developed SK609, a novel small molecule agonist of the dopamine D3 receptor. Characterization of the pharmacological properties of SK609 showed it binds to D3R with a EC50 value of ~283 nM to the high affinity sites and has a G-protein to β-arrestin ligand bias of 4.6 with a less than 30% recruitment of β-arrestin (Xu et al., 2017). Further, agonist activity of SK609 at the D3R did not lead to D3R desensitization but resulted in internalization of D3Rs which was independent of β-arrestin recruitment (Xu et al., 2019). SK609 dose dependently improved motor functioning and significantly reduced abnormal involuntary movements associated with L-dopa-induced dyskinesia in a rodent model of PD (Simms et al., 2016). In addition to its D3R agonist activity, SK609 is a preferential inhibitor of NET with an IC50 value of ~570 nM and a substrate of NET but with no psychostimulant activity (Marshall et al., 2019). Given this unique combined pharmacological effect, we recently assessed the effects of SK609 on sustaining attention in normal rats and found a dose dependent improvement in performance. These improvements in performance of the sustained attention task were more prominent among low-performing animals and more significantly, there was no evidence of any psychostimulant like effects (Marshall et al., 2019). Based on these results in normal rats, we hypothesized that SK609 with its DA and NE modulating abilities might improve PD-relevant cognitive impairments. In the present study, we assessed dose-dependent effects of SK609 in a sustained attention task in unilateral 6-hydroxydopamine (6-OHDA)-lesioned rats and in performance of an object retrieval task in cynomolgus monkeys with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism and mild motor impairments.

2. Methods

2.1. Drugs

6-OHDA.HBr, apomorphine, MPTP, saline and ampicillin were purchased from Sigma USA. SK609 was synthesized in house as described previously (Xu et al., 2017). Safety toxicology studies of SK609 in rats have shown the maximum tolerated dose of SK609 is 30 mg/kg and all the doses used in this study are well within the no-observed-adverse-effect level of SK609.

2.2. Animals

All rodent experiments were conducted at Drexel University and care and use of all laboratory animals followed NIH guidelines and all experimental procedures were approved by the Drexel University Institutional Animal Care and Use Committee. Animals were maintained on a 12 h light/dark cycle (lights on at 7 AM) and experimental sessions occurred between the hours of 9 AM and 12 PM. For pharmacokinetics (PK) experiments, seventeen male Sprague Dawley rats (300-400 g, ~4-months old; Charles River) were maintained on food and water ad libitum. For sustained attention testing, a separate group of similar animals were maintained on food ad libitum with water intake limited to 10 min/day (n = 6, 350-450 g, ~4 months old at the beginning of the study and ~ 7 months old at the end of the study; Charles River) and met criteria for apomorphine-induced rotational asymmetry (see below).

2.3. Rodent surgery

Surgeries were conducted as previously described (Simms et al., 2016). Briefly, 6-OHDA (8 μg/2 μl in 0.9% saline and 1% L-ascorbic acid) was injected using a Hamilton syringe unilaterally into the medial forebrain bundle (MFB; coordinates AP: −2.2 mm, ML: ±1.5 mm, DV: −8.0 mm relative to Bregma) at a rate of 1.5 μl/min under isoflurane anesthesia. The syringe was left in place for 5 min to allow the toxin to dissipate into the tissue before slowly removing the syringe and suturing the incision. Post-surgery animals were administered with a subcutaneous injection of bupivacaine (0.05 mg/kg) and a systemic administration of saline (0.9%) and ampicillin (100 mg/kg) and allowed to recover for 2 weeks.

2.3.1. Rodent apomorphine rotation testing

Rats were administered a subcutaneous injection of apomorphine (0.50 mg/kg) and returned to their home cage for 10 min. Animals were placed in a customized plexiglas cylinder (40 cm H x 30 cm D) and each

animal's motor activity within the cylinder was video recorded for 1 h without disturbing the animal. The videos were scored for complete rotations contralateral to the site of the unilateral lesion independently by blind experimenters. Animals were considered to have an effective 6-OHDA lesion if they exhibited ≥60 contralateral rotations with minimal ipsilateral rotations.

2.3.2. Rodent PK studies

Rats ($n = 17$) were administered with SK609 (4 mg/kg; i.p) dissolved in 0.9% phosphate buffered sterile saline. Fifteen minutes post drug administration, blood was collected from the first group of 12 animals from the saphenous vein into heparinized BD microtainer tubes and plasma was extracted from the blood samples by centrifuging the samples for 6 min at 3000 x g. Post blood collection, the 12 animals were euthanized, and whole brain was rapidly removed on ice and stored. The second group of animals ($n = 5$) was euthanized 15 min post drug administration and prefrontal cortex (PFC), striatum (STR) and hippocampus with amygdala (HIPPO) areas were dissected on ice. All brain samples were homogenized with *w/v* saline and centrifuged at 3000 x*g* and the supernatant was collected and stored at −80 °C. SK609 was quantified by liquid chromatography-high resolution mass spectrometry. All plasma and brain samples were analyzed on an Ultimate 3000 UHPLC (Thermo) with a chilled auto-sampler (6 °C), column heater (60 °C), using a Waters XBridge BEH C18 (2.5 μm, 2.1 mm × 50 mm) column with a water to acetonitrile reversed phase gradient and coupled to a Q Exactive Plus Mass spectrometer in positive ion mode switching between full scan and MS/MS mode. Data were analyzed in Xcalibur (Thermo), Tracefinder v 3.1 (Thermo) and GraphPad v6 (Prism, La Jolla, CA) using the major [M + H] ion at 184.0890 with a 5 ppm mass window. No interfering peaks in analyte or internal standard channels were noted in sample with or without matrix. All solvents were from Thermo Fisher and were Optima LC-MS grade, and analysts were blinded to sample identity until final analysis. For sample processing, 20 μL of the brain homogenate or plasma was added to 10 μL of a 10 ng/μL solution of diclofenac in methanol which was used as an internal standard. The sample was vortexed, and the protein was precipitated with 70 μL ice cold acetonitrile. Brain homogenates were bath sonicated for 5 min to ensure extraction. Samples were clarified by centrifugation at 16,000 x*g* at 4 °C for 10 min. 50 μL of the supernatant was transferred to a HPLC vial, then diluted 1:1 with 50 μL of water, then vortexed. Standard curves, as well three quality control samples at the top, middle and bottom of each range, were generated using dilutions of SK609, diclofenac and 20 μL charcoal stripped serum (Golden West Biologicals) as a surrogate matrix. Since there was no observable noise in the blank samples, conservative limits of detection and quantitation were set at the 1st and 2nd non-zero standard curve point, respectively.

2.3.3. Rodent sustained attention testing

The sustained attention task was performed as described previously, using a sound attenuated operant chamber ((Med Associates Inc.) (Berridge et al., 2006)). 6-OHDA lesioned animals (~3 weeks post lesion surgery) were trained to lever press for water reward using their unaffected paw to avoid any potential motor impairment from affecting task performance. Briefly, animals were trained to sustain attention for a cue light and press the trained lever for signal trials (designated as correct response) and the opposite lever for non-signal trials (designated as correct rejection) to obtain a water reward. Incorrect lever presses were recorded as misses during signal trials or false alarms during non-signal trials and did not result in any reward delivery. Failure to respond to the levers after 5 s in either the signal or signal trials resulted in retraction of the levers and a 5 s timeout period of darkness with no cue or house light and were recorded as omissions. The cue light duration and the intertrial intervals were varied randomly. A visual index (VI) adopted using signal theory was calculated, as described previously (Sahgal, 1988), by taking all of the above parameters into account with 1 being representative of peak performance, 0 being no performance, and 0.35 being considered as minimum cutoff for training (Marshall et al., 2019). To account for the baseline inter-animal variability, performance in the task was measured as difference in VI (DVI) between performance with drug treatment and the previous day vehicle (saline) treatment.

2.3.4. Non-human primates

The study was conducted under an approved Institutional Animal Care and Use Committee protocol by Atuka Inc. at our facility in Jiangsu Province, PRC. Fourteen specific-pathogen-free female cynomolgus (*Macaca fascicularis*, 9.5–12.3 years of age, 3.0–4.5 kg) were used for these studies, eight for PK experiments and six for cognitive testing. All animals were group housed with 2–3 animals per cage. The cage sizes exceeded UK, EU, NIH and CCAC minimum size recommendations, 152 (w) x 136 (d) x 192 (h) cm. The housing room was subject to a 12-h light-dark cycle (lights on 7 AM), temperature 20-28 °C in a room containing only animals of the same sex. Fresh fruit, primate pellets and water were available ad libitum. In preparation for PK studies, animals were acclimatized to the experimental setting and trained to provide blood samples. The animals used for PK and cognition studies were rendered parkinsonian by once daily subcutaneous injection of 0.2 mg/kg MPTP, administered for 8–12 days, until the first appearance of parkinsonism symptoms. After this time, a parkinsonian syndrome progressed over approximately 30 days, and stabilized. Additional administrations of MPTP were given to some animals to titrate to similar degrees of parkinsonism in individuals across the group. If additional MPTP was administered, animals were monitored for a minimum of an additional 30 days to verify that symptoms were stable.

2.3.5. Non-human primate PK studies

In an escalating non-randomized dose design, three oral doses of SK609 (0.5, 2 and 4 mg/kg; $n = 8$ per dose) followed by a single IV dose (2 mg/kg) were administered with a minimum of 3 days washout period between drug administrations. Eleven blood samples for drug level analysis were collected immediately before ($t = 0$ min) and then +5, 10, 15, 30 min and + 1 h, 2 h, 4 h, 6 h, 8 h and 24 h post drug administration for each dose from each animal. Samples of 0.5 ml whole blood were taken from the saphenous or cephalic veins and transferred to blood collection tubes with sodium-heparin additive. The tubes were centrifuged for 10 min at 1500 *g* at 4 °C and the resulting plasma layers removed, split into two equal aliquots and stored at -80 °C. Analytical assays were performed using the same protocol as described for rodent PK studies for analyzing the drug concentration. PK parameters were estimated from curve fitting the data using Graphpad Prism (v8).

2.3.6. Non-human primate object retrieval testing

Object retrieval (OR) testing was performed as described previously (Schneider and Pope-Coleman, 1995; Schneider and Roeltgen, 1993; Schneider et al., 1998). Briefly, the task required the macaque to reach into a clear Plexiglass box (15 cm by 15 cm by 5 cm) with one open side to retrieve a food reward (raisin or apple piece). The open side of the box can face left, right, or front relative to the animal and the food reward was placed at the front edge, center, or rear edge of the box. The box was mounted on an opaque white tray positioned outside of the testing cage and was centered or placed to the far left or far right relative to the testing cage. A testing session consisted of 30 trials that differed in the combination of placement of the reward within the box, the location of the open side of the box, and the position of the box on the tray, thus affecting the difficulty level of the motor and cognitive demands of each trial. Trials were designated as "easy" or "difficult" based on cognitive requirement of the trial. Easy trials ($n = 15$) were defined as ones in which the opening of the box was facing the animal (requiring only a straight through reach), or when the opening of the box was facing the same way as the previous trial. Difficult trials (n = 15) were defined as ones in which the opening of the box faced to the side (requiring a detour reach) and followed a trial in which the opening either faced the animal or faced the opposite side. Several measures including task completion

time i.e. time between presentation of the box and correct retrieval response; number (percent) of successfully completed trials i.e. those on which reward is retrieved on the first attempt; number (percent) of correctly performed trials i.e. those on which reward is eventually retrieved but not on first attempt, were recorded. In addition, if the reward is not retrieved on the first attempt, the type of errors made were recorded and included: cognitive errors or barrier reach errors (made when an animal reached for and hits a closed side of the box rather than making a detour); motor errors (when an animal reached into the open side of the box but failed to retrieve the reward or dropped the reward before eating it); perseverative reaches (reaches made to a closed side of the box and the reach is to the same location where the opening was on the previous trial). Further, omissions (number of trials that were not attempted within 30 s of trial presentation) and aborted trials (the number of trials that were initiated by the animal, but the animal stopped responding before retrieving the reward) were also recorded.

2.4. Statistics

Data were analyzed using Graphpad Prism v8 and outliers were determined using Grubbs test and removed from further analysis. Significance was tested using repeated measures one-way ANOVA or paired *t*-tests as appropriate and is reported in respective results and $p < 0.05$ was maintained for significance.

3. Results

The distribution of SK609 at 4 mg/kg dose in blood and brain in rats was measured 15 min after injecting the drug intraperitoneally. The blood and brain distribution analysis showed that SK609 was at significantly higher levels in the brain (189 ng/ml of lysate) compared to blood (79 ng/ml) (paired t-test, $t(23) = 8.47$, $p < 0.001$) (Fig. 1A). The distribution of SK609 in brain (PFC, Striatum and hippocampus) showed that levels of SK609 in PFC were significantly less than those in the striatum or hippocampus compared using one-way ANOVA followed by Tukey HSD post hoc test ($F(2,12) = 10.691$, $p = 0.0022$) between regions, with significant differences between PFC and striatum (Qstatic = 5.819, $p = 0.0037$) and PFC and hippocampus (Qstatic = 5.493, $p = 0.0056$) but was not significant between striatum and hippocampus (Qstatic = 0.325, $p = 0.899$)) (Fig. 1B).

The performance of 6-OHDA-lesioned animals ($n = 6$) in the sustained attention task was compared to that of normal animals (n = 6) previously trained to perform the task (Marshall et al., 2019). While, the 6-OHDA animals reached the criteria for performance (VI = 0.35), their performance in the task was significantly lower than that of normal animals (independent t-test; $t(10) = 7.308$, $p < 0.0001$) (Fig. 2A). To assess whether the performance in the sustained attention task was affected by the severity of the lesion, the 6-OHDA-lesioned animals (n = 6) were tested for apomorphine-induced rotational asymmetry. The average VI values from the sustained attention task under baseline conditions for the same group of animals was plotted against the apomorphine rotation scores. Results from the correlation plot (Fig. 2B) shows no significant correlation ($R^2 = 0.20$) between the number of rotations (the index of lesion severity) and their ability to perform the cognitive task. All 6-OHDA-lesioned animals had deficits in sustained attention performance compared to the normal group.

To test if SK609 could rescue the deficits observed in the sustained attention task, animals were treated with saline or various doses of SK609 (2, 4, 6, 8 and 10 mg/kg, i.p.) and tested in the operant task 15 min post dosing. Results from the study was represented as difference in VI (DVI) between the drug induced performance and previous day saline performance. SK609 produced a dose dependent increase in performance with 4 mg/kg and 8 mg/kg producing significant effects when compared to baseline ([$F(4,20) = 4.64$, $p = 0.008$]) (Fig. 3). The inverted-U shaped dose response is very similar to the response observed in normal animals following SK609 administration and other dopaminergic agents such as methylphenidate (Marshall et al., 2019). The performance of 6-OHDA-lesioned animals (4 mg/kg SK609 vs. saline) showed significant improvement in VI values ([$F(1,5) = 12.579$, $p = 0.0038$]) (Fig. 4A). The improved performance after SK609 was not due to the drug effects on either signal hits ([$F(1,5) = 0.236$, $p = 0.647$) or correct rejections ([$F(1,5) = 0.236$, $p = 0.647$) (Fig. 4B) but rather, due to a reduction in the number of misses ([$F(1,5) = 14.36643$, $p = 0.013$) and false alarm hits ([$F(1,5) = 11.2$, $p = 0.020$) (Fig. 4C). There were no changes in the number of omissions between saline or SK609 treatment (data not shown). These results show that SK609 improves performance of the sustained attention task in 6-OHDA-lesioned animals by reducing the number of mistakes made during the task.

In the cynomolgus macaques PK study, all doses of SK609 assessed (0.5, 2 and 4 mg/kg) were well tolerated and no toxicity issues were noted. Oral administration of SK609 (0.5, 2 and 4 mg/kg), was associated with mean Cmax values of 13.7, 69.3 and 218 ng/ml respectively, occurring at (Tmax) 4.1, 6.4 and 5.0 h following administration (Fig. 5) and with corresponding AUC(0-t) values of 171, 904 and 2938 h.ng/ml respectively (supplementary Table 1). Following intravenous administration (2 mg/kg), SK609 had a mean terminal half-life of 7.9 h in plasma. SK609 was highly distributed in macaques (Vdss 8 l/kg) and showed moderate clearance (13.5 ml/kg/min). Comparison of oral and intravenous data suggested an oral bioavailability (F0-t) for SK609 of between 46 and 98%.

Mean plasma exposure profiles and summary PK parameter calculations are shown in Fig. 5. Key individual animal pharmacokinetic parameters for SK609 in macaque plasma following oral and IV administration are shown in Supplementary Table 1. The PK profile of SK609 showed significant distribution in the plasma with a long half life of ~9.6 h at 4 mg/kg dose.

Based on the PK properties, the object retrieval (OR) task was tested in six macaques with mild to moderate MPTP-induced Parkinsonism and

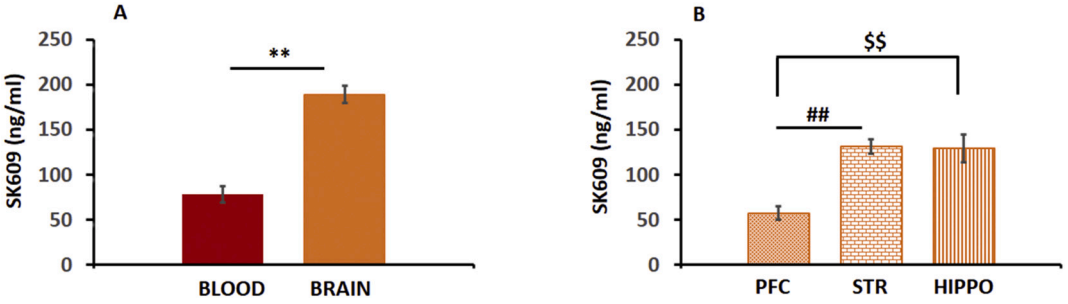

Fig. 1. Distribution of SK609 in rodent blood and brain after systemic administration. A) Rats ($n = 12$) were administered 4 mg/kg of SK609 intraperitoneally and blood and brain levels were estimated 15 min post drug administration. SK609 achieved significantly high levels in the brain compared to blood (**$p < 0.001$) B) Measurement of SK609 levels in PFC, striatum and hippocampus showed significantly less accumulation of SK609 in PFC compared to striatum or hippocampus (## $p = 0.003$, $$ $p = 0.005$).

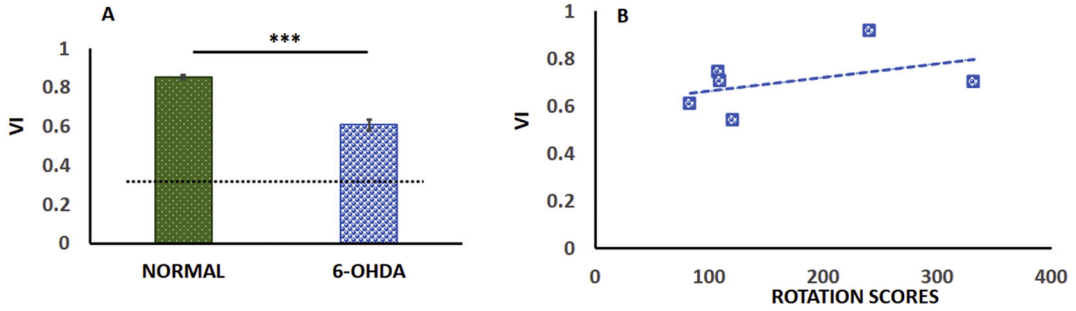

Fig. 2. Sustained attention performance in normal and 6-OHDA-lesioned rats. A) Average baseline vigilance index (VI) scores from the sustained attention task were plotted for normal ($n = 6$) and 6-OHDA-lesioned (n = 6) rats. While both groups of rats were successful in performing the task, indicated by a VI cutoff of 0.35 (black dotted line), 6-OHDA-lesioned rats had significant deficits in performance, compared to normal rats (***$p = 0.0001$) B) Average VI values from 6-OHDA-lesioned rats were plotted against apomorphine induced contralateral rotation scores for individual rats. Results showed no significant correlation ($R^2 = 0.20$) between performance in the sustained attention task to that of severity of the lesion.

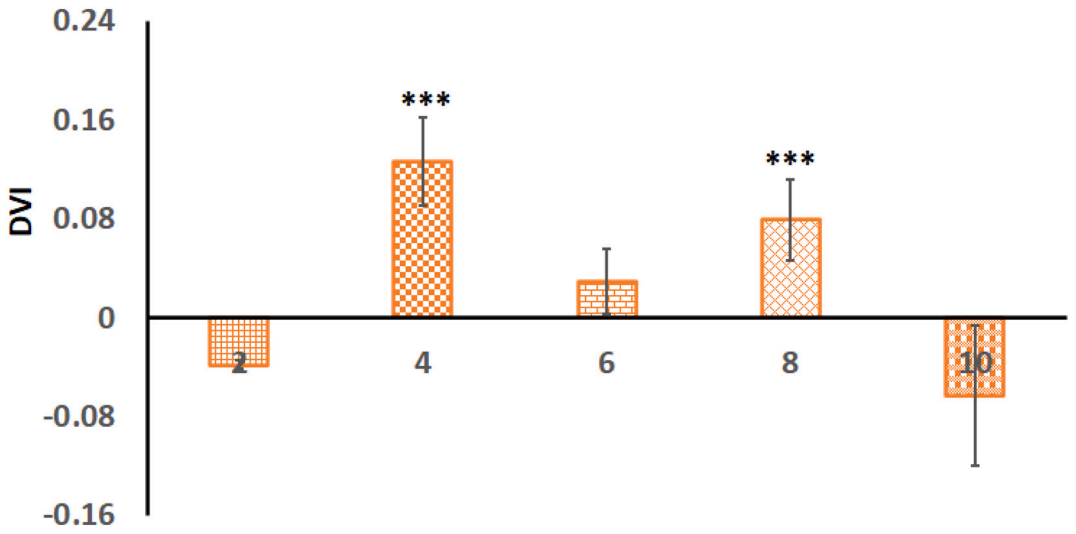

Fig. 3. SK609 had a broad inverted U dose-response effect in the sustained attention task, as measured by DVI and plotted against the doses administered (2-10 mg/kg). Improvement in performance as defined by DVI scores >0 were observed following administration of 4 and 8 mg/kg SK609 when compared to vehicle (represented as 0) (***$p = 0.008$). Post-hoc analyses revealed the peak dose of SK609 (4 mg/kg) resulted in an improvement in sustained attention significantly greater than 10 mg/kg ($p = 0.03$). Data presented are means ± SEMs.

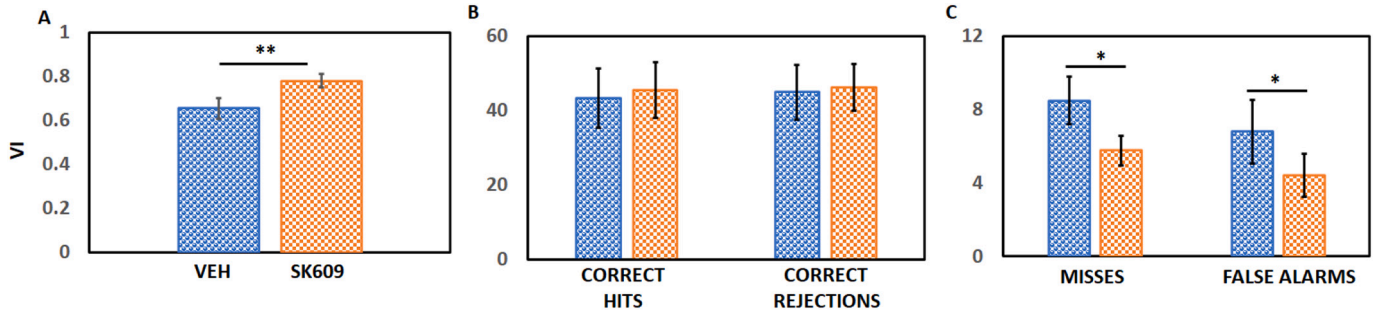

Fig. 4. SK609 improves performance in sustained attention task A) Sustained attention performance of 6-OHDA lesioned rats (n = 6) significantly improved after treatment with SK609 (4 mg/kg), compared to vehicle treatment. Data presented are means ± SEMs. **$p = 0.0038$. (B) Lesioned rats showed no significant change in either signal hits or correct rejections following SK609 administration. (C) SK609 decreased misses (*$p = 0.013$) and reduced false alarm in signal trials (*$p = 0.020$) during the sustained attention task. Data presented are means ± SEMs.

that that were known to have deficits in performing the OR task. SK609 (4 mg/kg) or saline (vehicle) was administered by intravenous injection and tested occurred 30 min after administration. There were at least 3 days between subsequent drug or vehicle injections. Macaques had significant deficits in object retrieval task performance (on both easy and difficult trials) under the vehicle condition, and SK609 administration improved performance by reducing the number of cognitive errors (barrier reaches), particularly on difficult trials (Fig. 6A). SK609 treatment (in comparison to saline treatment) also decreased the number of motor errors made during both easy and hard trials (Fig. 6B). Although there was a trend toward improvement in the percentage of successful first responses and the percent of correct

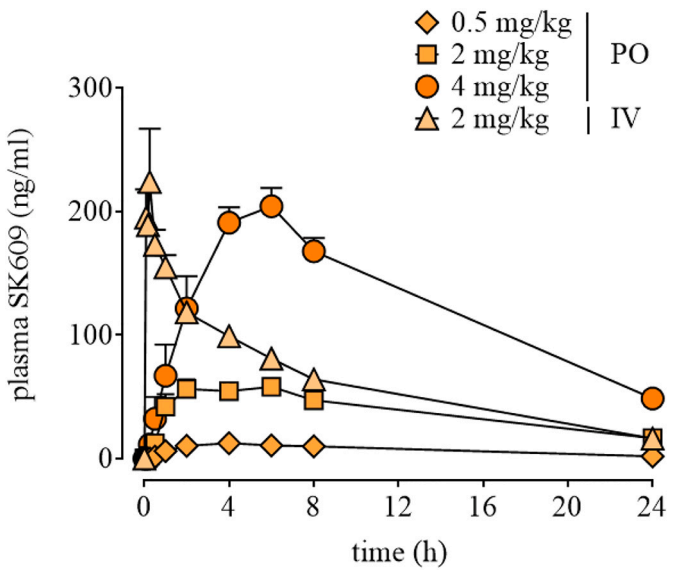

Fig. 5. Plasma exposure profiles of SK609 following oral and IV administration in the MPTP-lesioned macaque. In an escalating non-randomized dose design, three oral doses of SK609 (0.5, 2 and 4 mg/kg; $n = 8$ per dose) followed by a single IV dose (2 mg/kg) were administered and SK609 levels in plasma was evaluated. Results show a dose dependent increase in PK parameters including half life and maximum concentration and 98% oral bioavailability at 4 mg/kg dose. Data presented are means ± SEMs.

responses in both easy and difficult trials following SK609 treatment, these did not reach statistical significance (Fig. 7). There was no difference in time taken to complete the task nor in the number of omissions or perseverative errors in either easy or difficult trials between saline and SK609 treatments (data not shown).

4. Discussion

Dopamine D3 receptors (D3Rs) are uniquely located in corticostriatal regions of the brain that control both motor and cognitive functions [51–54]. In the past decade, several postmortem studies on human PD patients, and in rodent and non-human primate PD models, have provided evidence for the involvement of D3Rs in the etiology of motor and cognitive symptoms in PD (Sokoloff et al., 2006; Joyce and Millan, 2005; Loiseau and Millan, 2009; Bezard et al., 2003; Guillin et al., 2003; Leriche et al., 2003; Sokoloff and Le Foll, 2017, Narendran et al., 2006). As the expression and distribution of D3Rs differs in primates compared to rodents (Sokoloff et al., 2006; Nakajima et al., 2013), we evaluated the effects of SK609, a selective D3R agonist and NET inhibitor, on cognitive task performance in in both rodent and nonhuman primate PD models.

Our previous studies on SK609 in cognition in normal rats as well as in improving motor impairment and dyskinesia in parkinsonian animals, suggested that SK609 exhibits a classical inverted U dose-response, with peak effects seen at a 4 mg/kg dose in these behavioral assays (Simms et al., 2016). In the present study, we evaluated the PK distribution of SK609 in plasma and brain in normal rats as well as in parkinsonian monkeys. In rats, SK609, at a 4 mg/kg dose, had significantly higher distribution in brain compared to plasma and was highly distributed in striatum, hippocampus and moderately in PFC. This distribution pattern of SK609 correlates with the D3R distribution in the rat brain (Clarkson et al., 2017; Sokoloff et al., 1990). The PK profile of SK609 in non-human primate plasma suggested a dose dependent increase in oral bioavailability as well as peak distribution at the 4 mg/kg dose. Dose (4 mg/kg) and time of testing relative to administration (30 min. Post administration) for use in the sustained attention task and the OR task were based on these PK results.

6-OHDA-lesioned rats had significant deficits in performance of a sustained attention task, compared to normal animals, with a level of performance comparable to that previously observed in low performing normal animals and that could be significantly improved by SK609 administration (Marshall et al., 2019). Analysis of the individual components of this assay revealed that the improvement in performance produced by SK609 at a peak dose of 4 mg/kg was due to a reduction in the number of errors committed during both signal (misses) and non-signal trials (false alarms). These results are similar to those from previous studies using other D3R agonists which were found to reduce errors and perseverative behavior in a stop signal task in rodents, suggesting that there could be a common mechanism of action of D3R agonists in attention specific tasks (Bari and Robbins, 2013).

The D3R distribution in the brains of non-human primates are different from that in the rat brain and more is more similar to that of human brain. D3Rs in the monkey brain are distributed more in associative and limbic regions of the striatum (caudate-putamen), as compared to the sensorimotor striatum (Morissette et al., 1998). Thus, in primates, D3Rs may be more specifically involved in cognitive and motivational aspects of striatal functions, which are elaborated in prefrontal, temporal, parietal, cingulate and limbic cortices (Morissette et al., 1998). MPTP administration also significantly decreases levels of D3Rs in the primate striatum (Morissette et al., 1998). In the present study, monkeys with MPTP-induced parkinsonism had deficits in performing an OR task and administration of SK609 improved the performance in these monkeys, with a significant reduction in the number of cognitive errors (i.e., barrier reaches) made particularly during difficult trials. SK609 treatment also reduced the number of motor errors made during task performance.

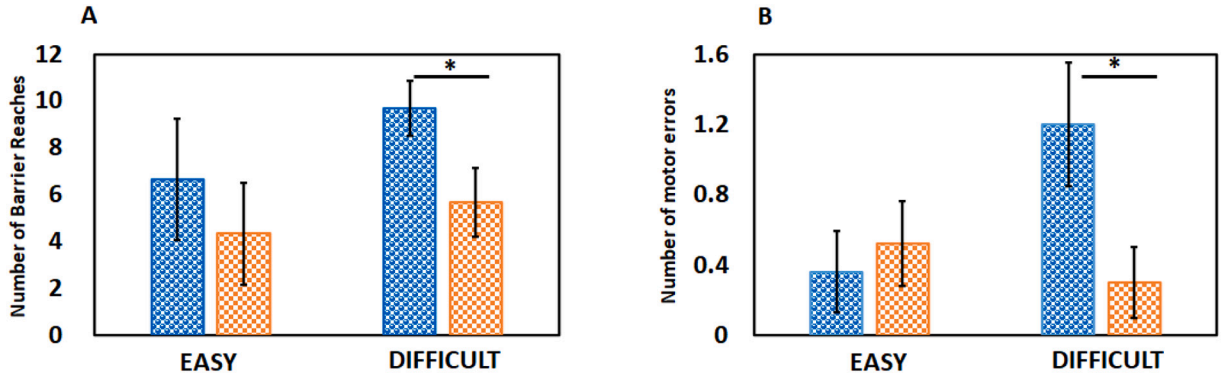

Fig. 6. Object retrieval performance on easy and difficult trials after i.v. saline (blue bars) or SK609 (4 mg/kg) (orange bars) in administration in MPTP-lesioned macaques. A significant decrease in number of A) barrier reach errors (*$p = 0.0457$) and B) motor errors (*$p = 0.0422$) was observed 30 min following SK609 administration. Data presented are means ± SEMs.

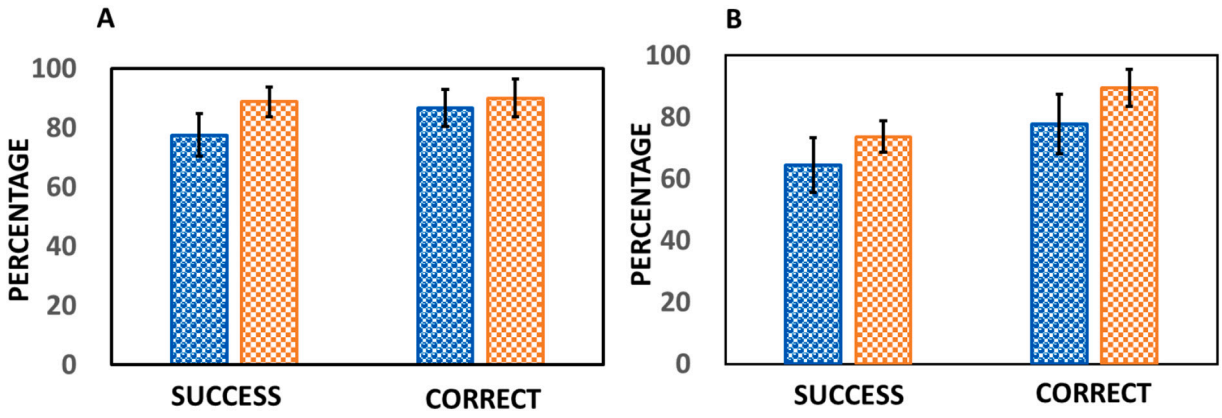

Fig. 7. No significant differences in either the percent of trials performed successfully (reward retrieved on first attempt) or the percent of trials performed correctly (reward eventually retrieved) on either easy (A) or difficult (B) trials were observed after SK609 administration (orange bars), compared with performance following saline administration (blue bars). Data presented are means ± SEMs.

A recent study in mice has highlighted that in the PFC, D3Rs have a unique distribution and reside on the layer V glutamatergic cells and inhibit high frequency action potentials via low-voltage-activated CaV3.2 calcium channels localized to the axon initial segment (Clarkson et al., 2017). These glutamatergic neurons containing D3Rs project to nucleus accumbens, basolateral amygdala and contralateral cortex and likely contribute to the feedback loop mechanisms (Clarkson et al., 2017; Sokoloff et al., 2013). In animal models of ADHD such as the spontaneously hypertensive rats, aberrant glutamate signaling in the PFC has been implicated in impaired performance in a sustained attention task (Sagvolden, 2006; Dimatelis et al., 2015; Miller et al., 2014). Further, in ADHD patients with deficits in sustaining attention, magnetic resonance spectroscopy studies have revealed increases in glutamate levels in PFC suggesting that aberrant glutamatergic activity in the PFC may be causally related to an impairment in sustaining attention (Courvoisie et al., 2004; Avisar and Shalev, 2011). Agonist activity of SK609 at D3Rs on these glutamate neurons of the PFC may help dampen this aberrant activity thereby improving performance in sustained attention tasks.

In addition to the D3R agonist activity, SK609 is a selective NET inhibitor and systemic treatment with SK609 increases DA levels by 150% and NE levels by ~300% of the baseline values in PFC (Marshall et al., 2019). The noradrenergic projections from LC and mesolimbic DA neurons that converge in PFC have a high expression of NET (Berger et al., 1974; Lindvall and Bjorklund, 1974; Thierry et al., 1973), which also participates in DA reuptake in the PFC (Carboni and Silvagni, 2004; Moron et al., 2002; Tanda et al., 1997). It is possible that changes in noradrenergic signaling induced by SK609 may have a higher impact on performance of PFC-mediated tasks than direct effects on D3Rs. In this regard, atomoxetine, a selective NET inhibitor, has been shown to preferentially improve PFC- mediated cognitive deficits similar to those observed in our study (Cain et al., 2011; Callahan et al., 2019; Bradshaw et al., 2016; Redding et al., 2019).

The results from the present study are qualitatively different from several published results on the role of D3R in PD-MCI that have suggested that antagonistic activity at D3Rs are beneficial in improving PD-MCI while agonistic activity may further impair cognition (Bari and Robbins, 2013; Nakajima et al., 2013; Millan et al., 2010). The differences in effects reported following the manipulation of D3R signaling may be related to differences in: a) involvement of different brain regions in addition to PFC that may influence the level of D3R involvement in different tasks; b) inherent pharmacological properties of the molecules used that could influence other neurotransmitter systems (SK609 affects both DA and NE levels); c) dose dependent effects of the molecules – an inverted U dose response for most dopaminergic agents requires dose-response testing to establish optimal dosing; and d) in vivo selectivity of the molecules tested for a given target and the ability to modulate downstream signaling pathways. SK609 is a G-protein biased agonist with significantly differentiated mechanism of action (Xu et al., 2017; Xu et al., 2019) that may effectively influence cognitive functioning in comparison to unbiased agonists. However, given that SK609 is both a D3R agonist and a NET inhibitor, it is impossible to delineate what aspects of the behaviors described here would be mediated specifically by D3R agonism versus NET inhibition. Future studies will be designed to answer these questions by choosing a selective D3R agonist (eg: Pramipexole at doses less than 0.25 mg/kg) and a selective inhibitor of NET (eg: Atomoxetine) to further delineate the D3R effects from those of NET inhibition in sustained attention and object retrieval tasks, by dosing them individually as well as in combination and using physiological and biochemical methods to validate the mechanism. In summary, the studies presented here demonstrate that SK609, a novel D3R agonist and a selective NET inhibitor, may provide a unique opportunity to treat cognitive dysfunction related to PC-MCI as well as potentially improve motor functioning. This provides justification for a shift away from dopaminergic agents in the design and development of therapeutics for treating cognitive and motor dysfunction associated with PD.

5. Invention disclosure

SK609 and its analogs are new chemical entities and covered under patent applications PCT/US2014/062644 and PCT/US2011/47263 and are licensed to Polycore Therapeutics LLC.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2020.113514.

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