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Reduction of repetitive behavior by co-administration of adenosine receptor agonists in C58 mice☆

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ABSTRACT

Repetitive behaviors are diagnostic for autism spectrum disorder (ASD) and commonly observed in other neurodevelopmental disorders. Currently, there are no effective pharmacological treatments for repetitive behavior in these clinical conditions. This is due to the lack of information about the specific neural circuitry that mediates the development and expression of repetitive behavior. Our previous work in mouse models has linked repetitive behavior to decreased activation of the subthalamic nucleus, a brain region in the indirect and hyperdirect pathways in the basal ganglia circuitry. The present experiments were designed to further test our hypothesis that pharmacological activation of the indirect pathway would reduce repetitive behavior. We used a combination of adenosine A1 and A2A receptor agonists that have been shown to alter the firing frequency of dorsal striatal neurons within the indirect pathway of the basal ganglia. This drug combination markedly and selectively reduced repetitive behavior in both male and female C58 mice over a six-hour period, an effect that required both A1 and A2A agonists as neither alone reduced repetitive behavior. The adenosine A1 and A2A receptor agonist combination also significantly increased the number of *Fos* transcripts and *Fos* positive cells in dorsal striatum. *Fos* induction was found in both direct and indirect pathway neurons suggesting that the drug combination restored the balance of activation across these complementary basal ganglia pathways. The adenosine A1 and A2A receptor agonist combination also maintained its effectiveness in reducing repetitive behavior over a 7-day period. These findings point to novel potential therapeutic targets for development of drug therapies for repetitive behavior in clinical disorders.

1. Introduction

Repetitive behaviors (e.g., stereotypies, compulsions, rituals) are commonly observed in neurodevelopmental disorders such as Fragile X, Prader-Willi, and non-syndromic intellectual disability including being diagnostic for autism spectrum disorder (ASD; Bodfish et al., 2000; Lewis and Bodfish, 1998; Moss et al., 2009). Repetitive motor behaviors are not unique to neurodevelopmental disorders, as they also manifest in several neurological and psychiatric disorders including Tourette syndrome, obsessive-compulsive disorder, and dementia, particularly the fronto-temporal type (Singer, 2013). Moreover, repetitive behaviors can develop as a consequence of early experiential deprivation including congenital blindness and highly impoverished environments such as orphanages (Fazzi et al., 1999; Rutter et al., 1999). Currently there are very few options, and no FDA-approved medications, for drug treatment of repetitive behavior in neurodevelopmental disorders.

With regard to the clinical neuroscience of repetitive behavior in neurodevelopmental disorders, cortical and basal ganglia regions have received the most attention in neuroimaging investigations of these clinical populations (Wilkes and Lewis, 2018). Unfortunately, due to methodological constraints and clinical heterogeneity, there is no consensus on what brain regions, cell types, or neurochemical systems may lead to repetitive behavior or targeted for novel drug development and treatment.

Animal models provide the opportunity to identify neurobiological mechanisms that mediate the expression of abnormal repetitive behavior. Such work is critical to identifying potential therapeutic targets and developing efficacious medications. Aberrant repetitive behavior can be induced in animals by genetic mutations, drug administration (e.g., amphetamine), and environmental restriction (e.g., zoos, farms). Such behavior can also be observed in specific mouse strains (reviewed in Bechard and Lewis, 2012).

We, and others, have examined the C58 inbred mouse strain (*Mus musculus*), which exhibits a robust repetitive behavior phenotype

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involving high levels of spontaneous jumping and backward somersaulting (Moy et al., 2008; Muehlmann et al., 2012; Ryan et al., 2010). Repetitive jumping has been observed in young C58 mice (postnatal day 20–21) and in the large majority of adolescent and young adult C58 mice observed during behavioral tests (Ryan et al., 2010). In our studies, these repetitive behaviors reach asymptotic levels by about 5 weeks post-weaning when assessed in a test chamber (Muehlmann et al., 2012). The same behaviors occur in the animals' home cage (58–92% of intervals observed; Ryan et al., 2010). The repetitive behavior phenotype of C58 mice is very similar to that of deer mice (*Peromyscus maniculatas*), which exhibit high levels of the same stereotyped motor behavior (vertical jumping, backward somersaulting) as a consequence of being reared in standard laboratory cages (Hadley et al., 2006; Powell et al., 2000; Powell et al., 1999; Presti and Lewis, 2005; Turner and Lewis, 2003; Turner et al., 2003; Turner et al., 2002). Other similarities in the models include the developmental trajectory of repetitive behavior and the efficacy of environmental enrichment to reduce repetitive behavior (Muehlmann et al., 2015; Muehlmann et al., 2012; Powell et al., 2000).

Our work with the C58 mouse model suggests that reduced activation of the indirect and/or hyperdirect pathways of the basal ganglia contributes to the expression of repetitive behavior. We have shown that neuronal metabolic activity in the subthalamic nucleus (STN), a key nucleus within the indirect and hyperdirect pathways of the basal ganglia, was significantly lower in C58 mice compared to C57BL/6J mice that do not show vertical repetitive behaviors (Lewis et al., 2018). Moreover, in the F2 generation of mice that were the products of a C58 by C57BL/6J intercross, high repetitive behavior mice also exhibited significantly reduced neuronal metabolic activity in the STN (Lewis et al., 2018). Furthermore, STN dendritic spine density was lower in C58 mice relative to C57BL/6J mice, which suggests less glutamatergic neurotransmission from the descending cortical hyperdirect pathway. This reduction in dendritic spine density is consistent with lower gene expression of synaptopodin, an actin binding protein found at the neck of dendritic spines, in the STN of C58 mice (Lewis et al., 2018). Reduced neuronal metabolic activity and dendritic spine density in the STN has also been found in deer mice with high rates of repetitive behavior (Bechard et al., 2017; Tanimura et al., 2011).

The relationship between reduced STN function and high rates of repetitive behavior is consistent with the documented role of the STN in mediating behavioral inhibition. Normal function of the STN is thought to be important for inhibiting behaviors (Jahanshahi et al., 2015; Mink, 1996; Yoon et al., 2019) and increasing STN firing rate with optogenetic stimulation can interrupt a bout of self-initiated water licking (Fife et al., 2017). Furthermore, lesioning the STN impairs stopping behavior in a go-trial reaction time task and increases spontaneous locomotion (Eagle et al., 2008). Unfortunately, no selective druggable targets have been identified in the STN.

If high rates of repetitive behavior are due to decreased activation of the STN, then pharmacological activation of the indirect pathway of the basal ganglia that projects to the STN should selectively attenuate repetitive behavior. Indirect pathway neurons of the striatum, which are GABAergic, project to the globus pallidus external segment (GPe). GPe GABAergic neurons then project to the STN. Adenosine A2A receptors are not only highly enriched in striatum, but are selectively expressed on striatal indirect pathway neurons and so become a potentially important target. Moreover, co-administration of agonists for the adenosine A1 and A2A receptors has been shown to increase the firing frequency of indirect pathway neurons in the dorsal striatum (Hernandez-Gonzalez et al., 2014). In deer mice, co-administration of these adenosine A1 and A2A receptor agonists significantly reduced repetitive behavior, though this effect was short-lasting (one-hour duration) and had non-selective effects on motor behavior (Tanimura et al., 2010).

In this study, we evaluated a new preparation of the adenosine A1 and A2A receptor agonist drug combination in peanut oil for its ability to significantly and selectively reduce repetitive behavior in both male and female C58 mice. We also examined the effects of an A1 agonist or A2A agonist in reducing repetitive behavior when administered alone. In addition, we assessed this adenosine A1 and A2A receptor agonist drug combination on *Fos* expression in dorsal striatum. Finally, we tested the effects of sub-chronic administration of the adenosine A1 and A2A receptor agonist drug combination in reducing repetitive behavior in C58 mice.

2. Materials and methods

2.1. Experiment 1: adenosine A1 and A2A receptor agonist combination – acute administration

Thirty-four C58 mice (*Mus musculus*) representing multiple litters were obtained from a breeding colony maintained in our laboratory. Mice sharing the same sex and weaning week were group-caged (3–7 mice/cage) at weaning (postnatal day 21) in standard rodent cages (48 × 27 × 15 cm). The room was maintained at 20–25 °C and 50–70% humidity and under a 12:12 hour light:dark cycle with lights off at 8:00 PM. Food and water were available ad libitum and two Nestlet squares were added for nest construction. All procedures were performed in accordance with the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Florida Institutional Animal Care and Use Committee.

Mice were weighed and then individually placed in testing cages (22 × 28 × 25 cm) made of Plexiglas and habituated for at least 1 h prior to the beginning of the dark cycle. Up to six mice were tested on any given testing day. Food and water were provided. Rates of spontaneous repetitive behavior (hindlimb vertical jumping and backward somersaulting) were assessed using a modified automated photocell detection apparatus (Columbus Instruments; Muehlmann et al., 2012; Tanimura et al., 2011; Tanimura et al., 2010). Photocells were placed around test chambers such that vertical activity (jumping and somersaulting) resulted in beam breaks with photobeams located high enough to avoid recording counts for rearing, drinking, eating, or any other behavior that did not include all four paws leaving the ground. Each occurrence of a detected photocell interruption, be it jumping or somersaulting, was registered with a real time value and these counts were termed “repetitive behavior counts.” The test session consisted of the 12 h of the dark cycle (8:00 PM–8:00 AM). All sessions were digitally video-recorded for accuracy of the automated counters and to measure the occurrence of non-stereotyped behavior.

Duration of grooming, digging, eating, drinking, and inactivity was scored by human observers, who were blind to treatment condition, using BORIS (Friard and Gamba, 2016) for a randomly selected subgroup of mice. Grooming was defined as the licking of the paws or body and paw strokes over the head and face. Digging was scored when the mouse moved the bedding with their paws or their face. Eating was scored when the mouse held a food pellet with the paws and was actively gnawing at it. Drinking was scored when the mouse approached and kept in contact with the spout of the water bottle. Inactivity was scored when the mouse was still for longer than 3 s. The repetitive behavior phenotype, either jumping or backward somersaulting, was also documented for each mouse.

The selective adenosine A1 receptor agonist N6-cyclopentyladenosine (CPA, PubChem CID: 657378, Sigma) was suspended in peanut oil in a volume of 10 ml/kg. The selective adenosine A2A receptor agonist 2-p-(2-carboxyethyl)phenethylamino-50-N-ethylcarboxamidoadenosine (CGS21680, PubChem CID: 3086599, NIMH Drug Supply Program) was also suspended in peanut oil in a volume of 10 ml/kg. Each drug suspension was made at least 1 h before injection and was left stirring up until the solution was drawn in the syringe. When drugs were given together as a double combination, a single solution was made and administered in a single injection. All injections were administered subcutaneously.

Seven female and nine male C58 mice were used for our assessment of 0.1 mg/kg CPA + 0.1 mg/kg CGS21680 drug combination. These mice were tested between five and eight weeks post-weaning, in a random order crossover design. Peanut oil vehicle and the 0.1 mg/kg dose of the adenosine A1 and A2A receptor agonist drug combination were administered to each mouse, with at least seven days separating the two tests. Drug or vehicle injections were given as the lights turned off at 8:00 PM.

Eight female and 10 male C58 mice were used for our assessment of 0.3 mg/kg CPA + 0.3 mg/kg CGS21680 drug combination. These mice were also 5–8 weeks post-weaning and also received peanut oil vehicle or the 0.3 mg/kg dose of the adenosine A1 and A2A receptor agonist combination in a random order crossover design with at least seven days between tests. Drug or vehicle injections were given as the lights turned off at 8:00 PM.

For assessment of non-stereotyped motor behavior, five females and two males in the high dose group (0.3 mg/kg of each drug) were scored on both their vehicle and adenosine A1 and A2A receptor agonist drug combination testing day.

After inspecting the repetitive behavior time course data (using 15 minute time bins) for all mice, we opted to use the repetitive behavior counts in the six hours following injection. It was clear from the data that drug effects had markedly attenuated by this time as behavior counts for drug and vehicle animals were quite similar (Supplementary Fig. 1). We totaled the number of repetitive behavior counts for the six hours following injection for each mouse and each testing day and analyzed the data with a two-way repeated measures analysis of variance (RM-ANOVA; GraphPad Prism, v5.03), using drug and sex as factors. When there was no significant main effect of sex, we collapsed the two sex groups and graphed the data accordingly. For the non-repetitive behaviors (grooming, digging, eating/drinking, and inactivity), we used paired *t*-tests to compare the total duration of each of the individual behaviors for the 25 min of scored video.

2.2. Experiment 2: individual adenosine A1 and A2A receptor agonists – acute administration

Fifteen C58 mice (7 females, 8 males) were weaned and housed as described in Experiment 1. Repetitive behavior was quantified as described in Experiment 1. For the A1 agonist (CPA) and the A2A agonist (CGS21680), each was suspended in peanut oil, and injected as described in Experiment 1. All mice received injections of peanut oil vehicle, 0.3 mg/kg CPA, and 0.3 mg/kg CGS21680 in a random order crossover design with at least seven days separating the three test sessions.

Similar to Experiment 1, we totaled the repetitive behavior counts for the first six hours post-injection for each mouse for each of their test sessions. These data were analyzed using a two-way RM-ANOVA with drug and sex as factors.

2.3. Experiment 3: Fos mRNA induction following adenosine A1 and A2A receptor agonist combination – acute administration

Thirteen C58 mice were weaned and housed as described in Experiment 1. The A1 agonist (CPA) and the A2A agonist (CGS21680) were suspended in peanut oil, and injected as described in Experiment 1. Seven C58 mice (4 females, 3 males) received peanut oil injections and six C58 mice (three females, three males) received injections of the 0.3 mg/kg dose of the adenosine A1 and A2A receptor agonist combination. Repetitive behavior was not assessed in these mice. Mice were removed from their home cages at approximately 8:15 PM (15 min after lights off), weighed, randomly assigned to group, and injected with either vehicle or the adenosine A1 and A2A receptor agonist combination. After injection, each mouse was returned to its home cage. After 30 min, each mouse was killed by cervical dislocation followed by decapitation and its brain was immediately removed, frozen in chilled 2-methylbutane, and stored at −80 °C. The 30 min time point was chosen because the abundance of Fos transcript was measured, instead of translated protein (which peaks at 90–120 min), and to assess the direct effects of the adenosine agonists on the dorsal striatal neurons, without potential effects on subsequent cortico-basal ganglia circuitry that may feedback onto the dorsal striatum.

Starting at approximately bregma +1.18, the brains were sliced at 12 μm on a sterilized cryostat and thaw mounted onto Superfrost Plus microscope slides (Fisherbrand) and stored at −80 °C. RNAscope Fluorescent Multiplex in situ hybridization was completed according to the manufacturer's instructions (Advanced Cell Diagnostics, Inc.). Slides were fixed, rinsed, dehydrated, and pretreated with protease IV. Positive and negative control probes for each of the three color channels were included in the assay. The three RNA probes used were: Drd1a probe (GenBank accession number NM_010076.3; target region, 444-1358), Drd2-C2 probe (GenBank accession number NM_010077.2; target region, 69-1175), and Fos-C3 probe (GenBank accession number NM_010234.2; target nt region, 407-1427). Amplification and labeling were completed with the provided reagents (Amp 4 Alt A-FL). Amp 4 Alt A-FL contains green (Alexa 488), orange (Atto 550), and far red (Atto 647) fluorphores and was used to detect *Drd1*, *Drd2*, and *Fos*, respectively. Slides were coverslipped with ProLong Gold antifade reagent with DAPI mounting medium and stored at −80 °C until imaging.

A Nikon Ti-E Motorized Inverted Fluorescent Microscope was used to image the stained sections. Images were captured at 20× with filters for four channels: DAPI, TRITC, FITC, and Cy5. Cropped images of the dorsal striatum (962 × 1236 pixels) were analyzed in two ways. The number of *Fos* transcripts per image was analyzed by counting the number of fluorescent dots in the Cy5 channel using SpotsInNucleiBot developed in Matlab (Cicconet et al., 2017). The same sigma and threshold values were used for all samples. To determine the cell type (i.e., dopamine D1 receptor positive or dopamine D2 receptor positive), a blinded observer manually counted the number of Fos positive neurons (designated as having > 10 transcript dots in or around a single DAPI-labeled nucleus) in each image and noted whether that cell was D1 positive, D2 positive, or neither D1 or D2 positive. An unpaired *t*-test was used to compare the number of *Fos* transcripts per image and the number of Fos positive cells per image per mouse between the peanut oil vehicle and the adenosine A1 and A2A receptor agonist combination groups. To evaluate any differential Fos activation across striatal cell types, a two-way ANOVA was used with drug and cell type as factors.

2.4. Experiment 4: adenosine A1 and A2A receptor agonist combination – sub-chronic administration

Thirty C58 mice were weaned and housed as described in Experiment 1. Repetitive behavior was quantified as described in Experiment 1. Each mouse was injected each day for seven days, although mice were only tested for repetitive behavior on days 1, 4, and 7. The A1 agonist (CPA at 0.3 mg/kg) and the A2A agonist (CGS21680 at 0.3 mg/kg) were suspended in peanut oil and injected as described in Experiment 1.

Mice were randomly assigned to either the peanut oil vehicle group (n = 15, 6 females, 9 males) or the adenosine A1 and A2A receptor agonist combination group (n = 15, 7 females, 8 males). On days when repetitive behavior was not tested, each mouse was removed from the home cage, weighed, injected with either vehicle or the adenosine A1 and A2A receptor agonist combination (0.3 mg/kg of each drug) at 8:00 PM and returned back to its home cage.

The total repetitive behavior counts in the six hours following injection were calculated for each mouse on each testing day (day 1, 4, and 7). Data were analyzed for main effects and interactions with a generalized linear model (SPSS Statistics 25) with sex, drug, and time as factors. We did not assess the effects of sub-chronic administration of the A1 and A2A receptor agonist combination on non-repetitive behaviors.

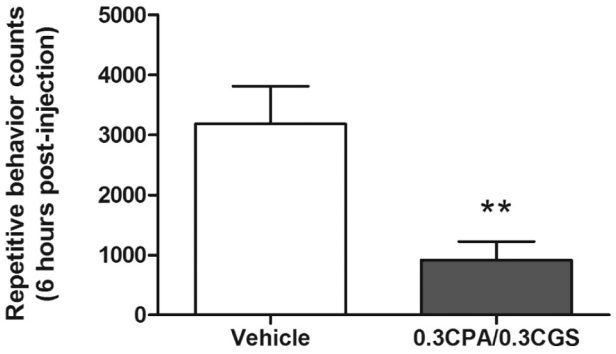

Fig. 1. Repetitive behavior is reduced following co-administration of the adenosine A1 agonist (CPA at 0.3 mg/kg) and the adenosine A2A agonist (CGS21680 at 0.3 mg/kg). Eighteen mice received both vehicle and adenosine agonists in a random order crossover design. Data are expressed as mean + standard error of the mean (S.E.M.; ** represents the significant main effect of drug ($p < 0.01$) revealed by two-way RM-ANOVA).

3. Results

3.1. Experiment 1

We evaluated the efficacy of two dose combinations of the adenosine A1 and A2A receptor agonist combination in both male and female C58 mice. With the lower dose of the A1 and A2A receptor agonist combination (0.1 mg/kg CPA + 0.1 mg/kg CGS21680), we found no significant main effects of drug ($F(1,14) = 0.25$, $p = 0.63$) or sex ($F(1,14) = 1.05$, $p = 0.32$), nor a significant drug x sex interaction ($F(1,14) = 0.62$, $p = 0.44$), when we compared the total number of repetitive behavior counts in the six hours following injection. The higher dose of the A1 and A2A receptor agonist combination (0.3 mg/kg CPA + 0.3 mg/kg CGS21680), however, significantly reduced repetitive behavior in both male and female C58 mice in this same six hour window (Fig. 1). We found a significant main effect of drug ($F(1,16) = 12.7$, $p < 0.01$) and no significant main effect of sex ($F(1,16) = 1.29$, $p = 0.27$) or drug x sex interaction ($F(1,16) = 0.1$, $p = 0.75$). In a subset of these C58 mice, we quantified the total duration of several behaviors in sampled videos taken at 60, 90, 120, 150, and 180 minutes following drug and vehicle administration. We found no significant effects of the adenosine A1 and A2A receptor agonist combination (0.3 mg/kg of each drug) on time spent grooming ($t(6) = 1.66$, $p = 0.15$), digging ($t(6) = 0.45$, $p = 0.67$), eating/drinking ($t(6) = 2.4$, $p = 0.06$), or inactive ($t(6) = 1.45$, $p = 0.2$). The lack of significant effects of the high dose of the drug combination on these non-repetitive behaviors suggests that non-selective effects of the drugs were not responsible for the significant reduction in repetitive behavior. We also found no demonstration that the repetitive behavior phenotype (jumping or backward somersaulting) changed in any mouse following drug administration.

3.2. Experiment 2

The successful reduction of repetitive behavior using the high dose A1 and A2A receptor agonist combination supported our hypothesis that activation of both receptor subtypes was required to change basal ganglia pathway output and alter behavior. This hypothesis was further tested with single drug challenges of either 0.3 mg/kg CPA or 0.3 mg/kg CGS21680 compared to peanut oil vehicle. A two-way, RM-ANOVA found no significant main effects of drug ($F(2,26) = 2.12$, $p = 0.14$) or sex ($F(1,26) = 3.41$, $p = 0.09$) or a drug x sex interaction ($F(2,26) = 0.29$, $p = 0.76$) for our analysis of repetitive behavior counts in the six hours following injection.

3.3. Experiment 3

We assessed Fos mRNA expression in the dorsal striatum of male and female mice injected with either peanut oil vehicle or 0.3 mg/kg CPA + 0.3 mg/kg CGS21680. We looked at Fos mRNA expression 30 min after injection and categorized each Fos positive cell as either dopamine D1 receptor positive, dopamine D2 receptor positive, or D1 and D2 receptor negative. There were only three Fos positive neurons that were not classified as either D1- or D2-positive and these were not included in the analysis. For our initial examination of Fos mRNA induction in vehicle- or adenosine A1 and A2A receptor agonist combination-treated C58 mice, we found a significant increase in the number of Fos transcripts in the dorsal striatum samples of mice given the adenosine A1 and A2A receptor agonist combination ($t(11) = 3.4$, $p < 0.01$). The number of Fos positive cells in the dorsal striatum samples of the drug-treated group were also significantly higher ($t(11) = 4.25$, $p < 0.01$; Fig. 2A). A subsequent analysis revealed *Fos* induction occurred in both D1- and D2-positive cells. A two-way ANOVA revealed a significant main effect of drug ($F(1,11) = 16.19$, $p < 0.01$) and no significant main effect of cell type or drug x cell type interaction (Fig. 2B).

3.4. Experiment 4

We extended our examination of the adenosine A1 and A2A receptor agonist combination by administering either peanut oil vehicle or the drug combination (0.3 mg/kg CPA + 0.3 mg/kg CGS21680) each day for seven days in both male and female C58 mice. A generalized linear model was used to test the main effects of the two between-subjects factors, sex and drug, and the one within-subjects factor, time. We also

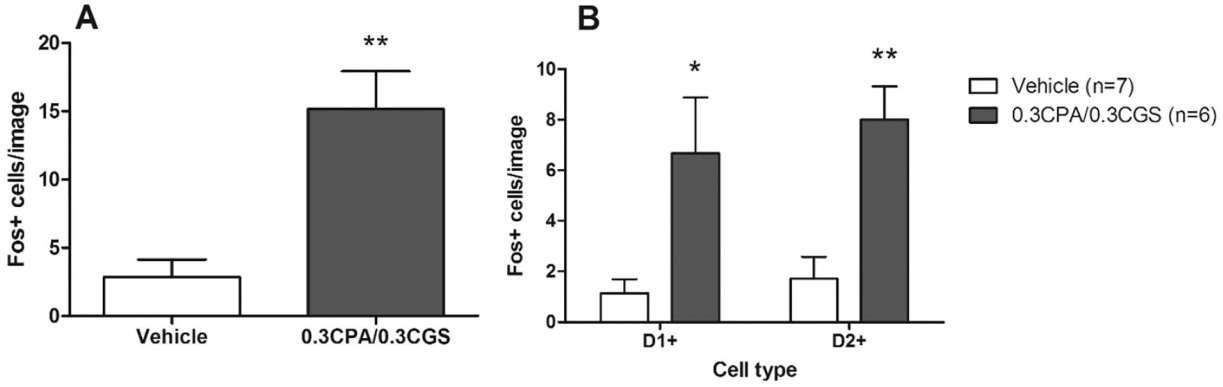

Fig. 2. A) Co-administration of the adenosine A1 agonist (CPA at 0.3 mg/kg) and the adenosine A2A agonist (CGS21680 at 0.3 mg/kg) significantly increased the number of Fos positive neurons in the dorsal striatum (** $p < 0.01$). B) This increase in Fos positive neurons occurred in both dopamine D1 receptor expressing neurons and in dopamine D2 receptor expressing neurons (* $p < 0.05$, ** $p < 0.01$, for Bonferroni post-tests). Data are expressed as mean + S.E.M.

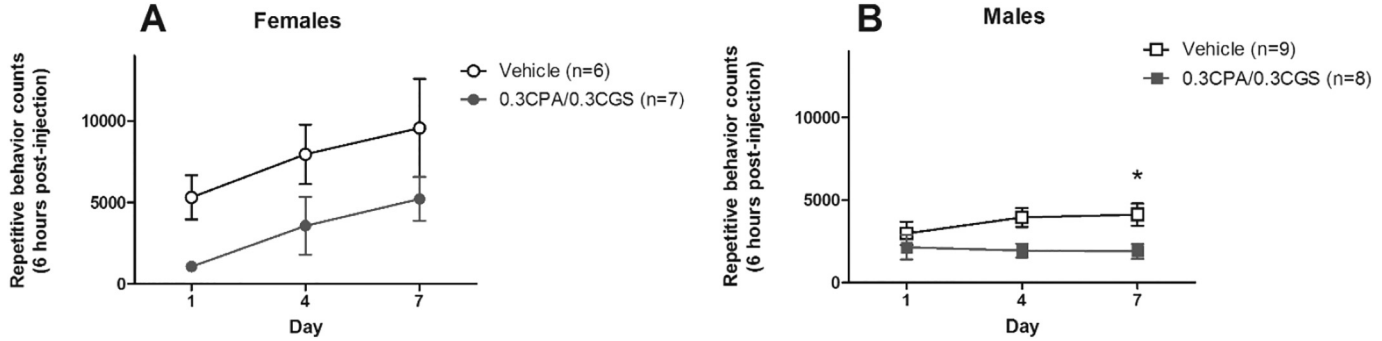

Fig. 3. Female (A) and male (B) C58 mice exhibited fewer repetitive behaviors across the three testing days when the adenosine A1 agonist (CPA at 0.3 mg/kg) and adenosine A2A agonist (CGS21680 at 0.3 mg/kg) were co-administered each day for seven days. Data are expressed as mean ± S.E.M. (*$p < 0.05$ for Bonferroni post-test following two-way RM-ANOVA on sex segregated data).

tested the time x sex, time x drug, and time x sex x drug interactions. We found significant main effects of all three factors. Repetitive behavior changed across the three test days ($F(2,52) = 10.15$, $p < 0.0001$) and females exhibited more repetitive behavior than the males ($F(1,26) = 6.84$, $p < 0.05$). We also found a significant reduction in repetitive behavior following administration of the adenosine A1 and A2A receptor agonist combination ($F(1,26) = 8.89$, $p < 0.01$). The only significant interaction was time x sex ($F(2,52) = 6.19$, $p < 0.01$) where female C58 mice showed increased repetitive behavior across days (Fig. 3A), while the repetitive behavior of the male C58 mice remained relatively constant across time (Fig. 3B).

4. Discussion

Our previous findings using two mouse models, deer mice and C58 mice, have implicated reduced STN activation in the mediation of repetitive behavior (Lewis et al., 2018; Lewis et al., 2019; Tanimura et al., 2011; Tanimura et al., 2010). In Tanimura et al. (2010) and Lewis et al. (2019), we provided evidence for pharmacological agents selected to increase indirect basal ganglia pathway activity to reduce repetitive behavior in deer mice. The present findings provide important generalizability to our findings by showing that the adenosine A1 and A2A receptor agonist combination substantially reduced repetitive behavior in C58 mice. This drug combination, when administered in a peanut oil vehicle, had a significant, selective, and long lasting effect on repetitive behavior. Similar to our previous study, we also showed that, at the same dose used in the two drug combination, the adenosine A2A agonist alone had no effect. This is in contrast to the demonstration that the adenosine A2A agonist alone significantly reduces grooming behavior in BTBR mice (Amodeo et al., 2018; Ansari et al., 2017). Grooming is the repetitive behavior phenotype expressed in this preclinical model of ASD. The adenosine A1 agonist alone also had no effect in the C58 mice, an outcome we did not assess in our prior work. Finally, we showed that our adenosine A1 and A2A receptor agonist combination could maintain its effectiveness in reducing repetitive behavior over a 7-day period. Interestingly, we found a significant sex effect in only this subchronic administration experiment. There were no significant sex effects in any of the three other cohorts used in the acute administration single or double drug experiments. This highlights the need for a more thorough analysis of sex effects on repetitive behavior in C58 mice and the potential role of the estrous cycle.

In a previous report of this adenosine A1 and A2A receptor agonist combination, Karcz-Kubicha et al. (2006) demonstrated a selective increase in Fos protein in enkephalin positive neurons in the nucleus accumbens and no increase in Fos protein in dynorphin positive neurons. This group also reported increased Fos protein in the dorsal striatum following the adenosine agonist co-administration, though cell type selectivity was not investigated (Karcz-Kubicha et al., 2006; Karcz-Kubicha et al., 2003). These reports informed our hypothesis that the adenosine A1 and A2A receptor agonist combination would reduce repetitive behavior by selectively increasing activation of indirect pathway neurons (which also selectively express enkephalin) in the dorsal striatum – an effect we measured by using Fos transcription as a proxy of neuronal activity. We found no such cell type selectivity for transcriptional activation of Fos with our dose selections, in the peanut oil formulation, and at the 30 minute post-injection time point. These factors were all different from Karcz-Kubicha et al. (2003, 2006). Measuring Fos expression is a common technique in neuroscience, but perhaps quantification of Fos transcriptional targets in the direct and indirect pathway neurons would have been more informative (Chandra and Lobo, 2017).

Alterations in indirect basal ganglia pathway function have been associated with repetitive behavior in other animal models as well as movement disorders in clinical conditions. For example, stereotyped digit licking and biting was induced in monkeys by infusion of the GABA antagonist bicuculline into the GPe (Grabli et al., 2004) and blocked by deep brain stimulation (DBS) applied to STN (Baup et al., 2008). Ibotenic acid lesions to STN resulted in increased compulsive lever pressing in a rat model of obsessive-compulsive disorder (Winter et al., 2008b). Bilateral high frequency stimulation of STN as well as pharmacological inactivation of STN reduced quinpirole-induced compulsive checking in rats (Klavir et al., 2009; Winter et al., 2008a; Winter et al., 2008c). Moreover, excessive grooming behavior expressed by the Shank3B mutant mouse was rescued by selective activation of indirect basal ganglia pathway neurons in the striatum (Wang et al., 2017). In addition, high frequency stimulation of the STN reduced excessive self-grooming in two mouse models relevant to ASD (Chang et al., 2016). Clinically, degeneration of indirect pathway striatal neurons has been linked to Huntington's disease, which involves the expression of uncontrollable movements (Deng et al., 2004; Starr et al., 2008). DBS of STN reduced the severity of symptoms in treatment refractory OCD patients (Burdick et al., 2009). Finally, DBS of STN and GPe has been found to be effective in ameliorating L-DOPA induced dyskinesias in Parkinson's patients (e.g., Kleiner-Fisman et al., 2006) whereas increased A2A receptor density has been linked to the expression of such dyskinesias (Calon et al., 2004; Zeng et al., 2000). These findings highlight that degeneration or inactivation of indirect pathway neurons produces repetitive behavior.

Our Fos data indicate that the effectiveness of the adenosine A1 and A2A receptor agonist combination may be due to normalization of the balance of direct and indirect pathway activation. This balance is important for the adaptive expression of behavior (Cazorla et al., 2015). We have shown an imbalance of these pathways in deer mice with high levels of repetitive behavior using the direct and indirect pathway-specific endogenous opioids. There is significantly decreased leu-enkephalin content and significantly increased dynorphin/enkephalin content ratios in high repetitive behavior mice relative to low repetitive behavior mice. Moreover, we found a significant negative correlation

between striatal enkephalin content and frequency of repetitive behavior as well as a significant positive correlation between the dynorphin/enkephalin content ratio and frequency of repetitive behavior in these mice (Presti and Lewis, 2005). Met-enkephalin levels in the striata of C58 mice are also lower relative to controls (Blum et al., 1987). Adenosine A1 and A2A receptors form isoreceptor complexes in both neurons and astrocytes (Cristovao-Ferreira et al., 2013). In the dorsal striatum, these A1-A2A receptor complexes reside on the terminals of glutamatergic cortical neurons (Ciruela et al., 2006). Here activation of A2A receptors inhibits A1 receptor binding and increases glutamate release (Ciruela et al., 2006). This glutamatergic signaling stimulates both direct and indirect pathway striatal neurons, as no pathway selectivity has been found (Borroto-Escuela and Fuxe, 2019). Glutamatergic stimulation of both direct and indirect pathway neurons may be responsible for Fos expression across the two cell types. There is also evidence that sustained activation of the direct pathway increases activation of the STN (Lee et al., 2016).

Another feature of this work is identifying novel potential therapeutic targets for development of drug therapies. There are currently no effective pharmacological treatments to ameliorate repetitive behavior in neurodevelopmental disorders such as ASD (Carrasco et al., 2012; King et al., 2009). Thus, there is a pressing need for the development of effective drug treatments, based on an understanding of the specific pathophysiological mechanisms of repetitive behaviors. The present findings point to striatal adenosine receptors as potential targets as well as other targets capable of altering basal ganglia circuitry activity.

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