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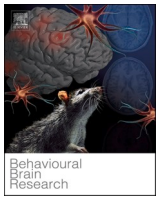

Research report

Striatal adenosine A_{2A} receptor involvement in normal and large nest building deer mice: Perspectives on compulsivity and anxiety

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ABSTRACT

Obsessive-compulsive disorder (OCD) is characterized by recurring obsessive thoughts and repetitive behaviors that are often associated with anxiety and perturbations in cortico-striatal signaling. Given the suboptimal response of OCD to current serotonergic interventions, there is a need to better understand the psychobiological mechanisms that may underlie the disorder. In this regard, investigations into adenosinergic processes might be fruitful. Indeed, adenosine modulates both anxiety- and motor behavioral output. Thus, we aimed to explore the potential associations between compulsive-like large nest building (LNB) behavior in deer mice, anxiety and adenosinergic processes. From an initial pool of 120 adult deer mice, 34 normal nest building (NNB)- and 32 LNB-expressing mice of both sexes were selected and exposed to either a normal water (wCTRL) or vehicle control (vCTRL), lorazepam (LOR) or istradefylline (ISTRA) for 7- (LOR) or 28 days after which nesting assessment was repeated and animals screened for anxiety-like behavior in an anxiogenic open field. Mice were then euthanized, the striatal tissue removed on ice and the adenosine A_{2A} receptor expression quantified. Our findings indicate that NNB and LNB behavior are not distinctly associated with measures of generalized anxiety and that ISTRA-induced changes in nesting expression are dissociated from changes in anxiety scores. Further, data from this investigation show that nesting in deer mice is directly related to striatal adenosine signaling, and that LNB is founded upon a lower degree of adenosinergic A_{2A} stimulation.

1. Introduction

Obsessive-compulsive disorder (OCD) is characterized by recurring intrusive thoughts (obsessions) and rigid repetition of certain mental or behavioral actions (compulsions) that are often associated with anxiety [1,10], even though OCD is no longer classified as an anxiety disorder [2]. In terms of neurobiology, dysfunctional connectivity in distinct cortico-limbic [43,45,49] and cortico-striatal-thalamic circuits has been shown [46,47]. Briefly, the limbic system, via amygdalar, hippocampal, and ventromedial prefrontal-cortical (vmPFC) signaling, is responsible for emotional regulation, fear processing and anxiety sensitivity [53]. Whereas the amygdala and the hippocampus generate and contextualize emotional stimuli, the vmPFC is responsible for its interpretation and exerting top-down control of the associated behavioral output [53]. Two distinct pathways, i.e. direct (striato-nigral) and indirect (striato-pallidal), relay said executive behavioral signals between the prefrontal cortex and the thalamus. Whereas the direct pathway expresses dopamine D_1 receptors, the indirect pathway expresses D_2 receptors [26]. Many theories attempt to explain the role of these pathways in the manifestation and promulgation of repetitive behaviors. For example, it is proposed that the direct pathway facilitates goal-directed behavioral engagement, while the indirect pathway plays a behaviorally inactivating role [30]. In this sense, overactivity in the former is associated with persistent behavioral output [41]. However, such a definite conclusion should be drawn with caution. For example, in a meta-analysis of functional neuroimaging data, Whiteside et al. [50] reported no significant differences between the direct and indirect functional connectivity of OCD patients and healthy controls, while Calzà et al. [7] reported *increased* functional connectivity in the indirect, instead of the direct pathway. In a recent paper, the 'prepare and select' model was proposed [26]. According to this model, D_1 receptor activation is vital for behavioral sequence initiation, whereas the

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D_2-expressing indirect pathway is responsible for the ultimate selection of a context-appropriate behavioral output. Nevertheless, literature agrees that increased dopaminergic signaling at times of behavioral initiation, is needed to facilitate goal-directed behavioral output and that a functional imbalance between these pathways at the time of behavioral engagement underlies repetitive behavioral engagement [30]. In extension, taking into consideration theories of functionally opponent interactions between dopamine and serotonin [12,18,39,4], hyposerotonergic signaling in the cortico-striatal pathways is thought to be related to the manifestation of OCD [27]. Unfortunately, despite convincing evidence that selective serotonin reuptake inhibitors (SSRIs) are modestly successful in the treatment of OCD [32], direct evidence implicating serotonergic dysfunction in the causality of the disorder is lacking [22].

Adenosine, the primary component of the energy-transferring molecule, adenosine triphosphate (ATP) [28] acts as an important neuromodulator via its actions on adenosine A_1 and A_2 receptors. With respect to its effects in the central nervous system, the G_i-coupled A_1 receptor is expressed in the cortex, hippocampus, and cerebellum [42], where it generally causes hyperpolarization of neuronal membranes [48]. The functions of the A_2 receptor are more complex in that striatal (where the A_{2A} receptor subtype is predominantly expressed) and extra-striatal A_{2A} receptors are associated with functionally distinct behavioral profiles [48]. Specifically, scarcely expressed amygdalar and hippocampal A_{2A} receptors modulate anxiety processing and memory consolidation [11,36], although the exact mechanisms underlying these effects remain largely unknown [54]. In turn, striatal A_{2A} receptors modulate behavioral output and regulate motor control [9], and are specifically known to inhibit locomotor activity [5].

A critically important trait of adenosine receptors, regardless of subtype, is that they are mostly expressed in clusters with other receptors, either as duplicates of the same receptor (homodimers, e.g. A_{2A}-A_{2A}) or in combination with receptors of other neurotransmitters or other adenosine receptor subtypes (heteromers, e.g. A_{2A}-D_2 or A_{2A}-A_1), or even multimeric complexes involving receptors of three distinct neurotransmitter classes [21]. The implication of such multi-receptor complexes is far reaching, as many unique functional interactions between the constituent receptors may occur, such as changes in affinity of the co-expressed receptor, or interactions between the second messenger systems in question [20]. For example, striatal adenosine and dopamine A_{2A}-D_2 receptor heterodimers are expressed on the neuronal bodies of the indirect pathway [17,16]. Such co-expression facilitates functional opponency, whereby adenosinergic stimulation decreases the activity of dopamine at the D_2 receptor [17]. In this regard, A_{2A} receptor antagonists, e.g. istradefylline (ISTRA), may show promise as a restorative intervention in terms of motor control [11,14,48], since antagonsim of the A_{2A} receptor facilitates the actions of dopamine [17]. Therefore, considering the involvement of both limbic and cortico-striatal processes in OCD as well as the proposed contribution of dysregulated direct and indirect pathway activity in the manifestation of repetitive behavior, the potential of ISTRA as an anti-compulsive pharmacotherapeutic strategy will be explored in this work.

When bred, housed, and reared under standard laboratory conditions, 25–30% of deer mice *(Peromyscus maniculatus bairdii)* express large nest building (LNB) behavior. Although nesting is a common rodent behavior, it shows significant intra-species variation, with some mice expressing excessive and persistent nesting behaviors [51]. Being reminiscent of clinical compulsive-like behavioral engagement, LNB behavior aligns conceptually well with safety/perfectionism OCD [24]. Further, LNB, but not normal nest building (NNB) behavior responds favorably to high-dose, chronic oral exposure to the SSRI, escitalopram [51]. Still, it remains unknown whether LNB behavior is mostly reflective of perturbations in motor control (i.e. striatal processes), or if anxiety-related cognitive impairment (and thus limbic involvement) may play a role in its manifestation. This question, as well as how such associations might be modulated by anti-adenosinergic intervention, have not yet been explored.

2. Materials and methods

2.1. Study layout

The present investigation comprised three phases of study, viz. 1) baseline screening and behavioral categorization, 2) normal water control (wCTRL), vehicle control (vCTRL), or drug exposure and 3) post-exposure behavioral assessment and neurobiological analysis (Fig. 1).

2.2. Mice

Since only 25 – 30% of deer mice express LNB behavior, a total of 120 deer mice (ethics approval no.: **NWU-00422–21-A5**) both sexes; aged 10 – 12 weeks at the onset of investigation), were bred, reared, and housed at the North-West University (NWU) vivarium (SAVC reg: FR15/13458; AAALAC accreditation file: 1717). Experimental subjects were chosen from at least 20 different litters without sex or weight bias. While every effort was made to allocate an equal number of female and male mice to each phenotype and exposure group, the yield of male and female NNB- and LNB-expressing mice differed between litters and hence not all groups were constituted in a 50:50 female:male ratio. At the onset of experimentation, each mouse was allocated to its own home cage (35 cm (l) x 20 cm (w) x 13 cm (h); Techniplast® S.P.A., Varese, Italy). Cages were automatically climate-controlled and kept ambient at 23 °C on a 12-h light/dark cycle (06:00/18:00). Food and water (or drug solutions) were provided ad lib, through study termination. Cages were cleaned and new corncob bedding added, weekly [13]. A piece of white polyvinyl chloride pipe (10 cm (l) x 4 cm (Ø)) and paper towel were provided as environmental enrichment and nesting material (except during periods of nesting assessment), respectively.

2.3. Pre- and post-exposure nest building assessment

To classify mice into the NNB- or LNB-expressing cohorts, we applied the protocol of Wolmarans et al. [51] without modification. Each mouse was screened for nesting behavior over the course of seven consecutive days. On each day between 15:00 and 16:00, an excess of pre-weighed, unscented cosmetic cotton wool was introduced into the roof of each home cage. Every subsequent day, also between 15:00 and 16:00, the remaining cotton wool, i.e. not used for nesting, was weighed, and the built nests removed and discarded. This process was repeated over all seven days of testing. At the end of the assessment period, a grand total nesting score (the sum of the seven daily scores, expressed in grams), was calculated for each mouse. Considering that compulsivity is both excessive and persistent, classification of LNB behavior was based on the extreme end of the total nesting score distribution of all assessed mice [51]. Specifically, LNB behavior was broadly defined as those scores that clustered near to or above the upper 75th percentile of the total nesting score distribution, but that were also associated with coefficients of variance pertaining to the inter-day nesting scores that clustered within the lower 25th percentile of distribution (Fig. 2). Conversely, NNB behavior was characterized by total nesting scores that clustered below the 50th percentile of the distribution. From this data, 34 NNB (18 female; 16 male) and 32 LNB-expressing mice (19 female; 13 male), were selected for further study (Fig. 2). The 34 NNB- and 32 LNB-expressing mice were divided into the different drug-exposure groups (see below) and reassessed for nesting expression as explained before during the last seven days of the 28- or 7-day exposure periods.

2.4. Drugs

Lorazepam (LOR; included as an anxiolytic intervention to discriminate between changes in motor expression and anxiety-related behavioral adaptation; Aspen Pharmacare, Qgeberha, South Africa) and ISTRA

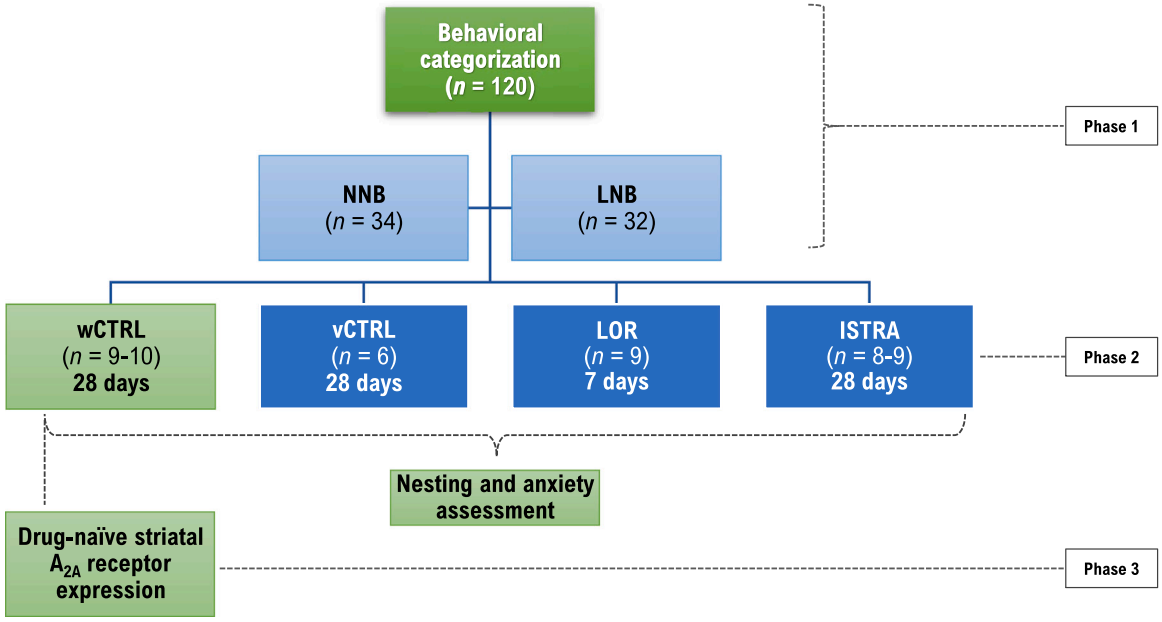

Fig. 1. Schematic representation of the study layout NNB: normal nest building; LNB: large nest building; wCTRL: normal water control; vCTRL: vehicle control; LOR: lorazepam; ISTRA: istradefylline.

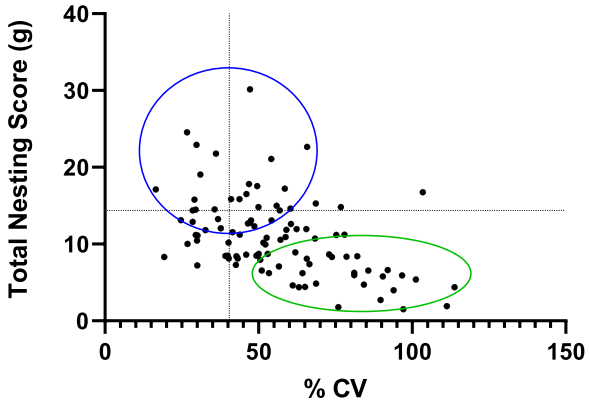

Fig. 2. Association between total nesting scores (g) and the percentage coefficient of variance pertaining to the inter-day nesting scores Vertical line indicate lower 25th percentile for the % CV; Horizontal line indicates the 75th percentile for the total nesting scores; Mice selected for large nest building (LNB) are enclosed in blue, while mice selected for NNB are enclosed in green.

(BLD Pharmatech®, Shanghai, China) were provided ad lib in the normal drinking water (LOR; NNB: $n = 9$, 4 females; LNB: $n = 9$, 5 females) or in a vehicle emulsion (ISTRA; NNB: $n = 9$, 5 females; LNB: $n = 8$, 5 females) as a drinking liquid. The vehicle emulsion consisted of refined soybean oil (10% w/v), purified egg phosphatide (1.2% w/v), glycerol (2.25% w/v), and sterile water (to 100%) [19,40]. LOR [15,52] and ISTRA [14] were constituted as 0.8 mg/100 mL and 8 mg/100 mL solutions or emulsions, respectively. Doses were calculated according to the average liquid intake of deer mice that were previously calculated as 0.25 mL/g/day [38,3]. Liquid intake was recorded daily to confirm drug intake. Normal water (wCTRL; NNB: $n = 10$, 5 females; LNB: $n = 9$, 4 females) or the vehicle emulsion (vCTRL; NNB: $n = 6$, 4 females; LNB: $n = 6$, 5 females) were given as the respective controls to different groups of mice. Drug solutions or emulsions were freshly constituted every other day.

2.5. Mirrored open-field anxiety assessment

On the last day of the post-exposure nesting assessment period (exposure day 7 for the LOR group or 28 for all other mice), all mice were assessed for open-field anxiety between 18:30 and 22:00 under dim red light (40 lux) in a mirrored open field arena [52,8]. Briefly, the arena consisted of a square box (50 cm (l) x 50 cm (w) x 30 cm (h)) constructed from black Plexiglas®. In one corner of the box, an enclosed dark chamber (17 cm (l) x 17 cm (w) x 30 cm (h)) was constructed with a 5 cm × 5 cm opening through which mice could gain access to the larger open field. To evoke anxiety and bolster the aversive character of the open field area, a white Plexiglas® floor and mirrored walls were used [37,8]. All walls were mirrored, except for the area directly adjacent and opposite to the dark compartment. In this assessment, entry into the mirrored open field area was entirely optional, since mice could freely move between the dark 'safe' compartment and the larger open field.

From 18:00 on each given assessment day, mice were moved to the assessment room (located on the same floor of the vivarium) in their home cages and allowed 30 min to habituate. Mice were then transferred to the dark compartment of the open-field arena and left to freely explore the entire arena for 6 min. Digital video cameras were mounted above all arenas and assessment sessions were recorded. Ambulatory activity was assessed by means of automated digital tracking (Ethovision XT® 15 software, Noldus Information Technology®, Wageningen, The Netherlands). To establish whether NNB- and LNB-expressing mice differ in terms of general anxiety-like behavior, **(i)** time spent, and **(ii)** distance travelled in the open arena, **(iii)** number of arena entries, **(iv)** duration per arena entry, and **(v)** border-to-center entry ratio, were measured. After each assessment, mice were returned to their home cages and the arenas cleaned with 90% ethanol, rinsed with normal water, and allowed to dry.

2.6. Study endpoint and brain sampling

On the day following the last nest building and open field assessment, mice were euthanized by means of cervical dislocation. Since the neurobiological objective of this work was to explore the striatal A_{2A} receptor expression profiles of NNB- and LNB-expressing mice, the striata of wCTRL-exposed mice were removed on ice, snap frozen in

liquid nitrogen and stored at − 80 °C until further analysis.

2.7. Radioligand binding assay for the profiling of A_{2A} receptors

Striatal A_{2A} expression was determined by means of a saturation binding assay adapted from Bruns et al. [6] and Janse van Rensburg et al. [25]. Three assays were performed per behavioral phenotype. Briefly, striatal tissue from three same-phenotype mice were pooled to perform one assay. This pooling was necessary to ensure that enough protein was included in the final assay mixtures. As such, a total of nine mice were used per phenotype to complete three binding assays per phenotype (Table 1). Pooled tissues were added to ice cold buffer (50 mM Tris-HCl, containing 10 mM $MgCl_2$; pH adjusted to 7.7; 25 °C) to constitute a 100 volumes dilution ($MgCl_2$ was included to increase radioligand binding and decrease non-specific binding). Subsequently, the pooled tissues were homogenized (Polytron®, setting 6, 5 s), centrifuged at 50 000 x *g* for 10 min, and the supernatant discarded. Pellets were resuspended in the same quantity of fresh buffer, transferred to glass tubes, and homogenized using a Teflon® drill bit. Homogenates were centrifuged again at 50 000 x *g* for 10 min. The supernatant was again discarded, and the last two steps repeated. The correct amount of fresh buffer to result in a 100 volumes dilution (e.g. 8.4 mL buffer added to 84 mg of tissue) was added to the resulting pellets and finally homogenized. All procedures were performed on ice or in a cold room (3 – 4 °C).

For the purpose of the current assay, a concentration range of eight points was used, i.e. between 0.5 and 20 nM of the radioactive ligand, ^{3}H-5'-ethylcarboxamido adenosine (^{3}H-NECA) (specific activity: 29.6 Ci/mmol; 25 μL added per concentration). Binding of ^{3}H-NECA to A_1 receptors was blocked by the addition of 20 μL N^6-cyclopentyladenosine (CPA; 100 μM). Specific binding was determined by subtraction of the non-specific binding which was measured in the presence of 10 μL of unlabeled NECA (30 μM). Adenosine deaminase was added to the homogenate at a concentration of 0.2 units/mL to catabolize any remaining adenosine. Subsequently, 445 μL of the tissue homogenates were added to each tube. The reaction mixtures (final volume of 500 μL per mixture) were vortexed and allowed to incubate for 1 h at 25 °C in a shaking water bath.

Following the 1-h incubation period, binding reactions were terminated by rapid vacuum filtration through Whatman® GFB glass microfiber filters that were pre-soaked in ice-cold buffer. Thereafter, filters were again washed twice with 4 mL of ice-cold buffer under vacuum, added to scintillation vials, and immersed in 4 mL Filter-Count® scintillation liquid. The series of radioactive standards were constituted by adding 25 μL of each radioactive concentration (0.5–20 nM) to 4 mL Filter-Count® (without filters). Prepared vials were left for 2 h after which it was subjected to scintillation counting (Tri-Carb® 2100 TR Liquid Scintillation Counter, PerkinElmer®). The binding data were analyzed using GraphPad® Prism® 9 to calculate values for the maximal number of binding sites (B_{max}) and the binding affinity (K_D).

Table 1
Total nesting scores and sexes of mice of which striatal tissues were pooled for the respective binding replicates.

Phenotype	Tissue Pool	Total nesting score (g)	Sex
NNB	1	6.6	F
		5.4	M
		8.7	F
	2	4.9	F
		7.2	M
		8.5	M
	3	5.9	F
		8.1	M
		6.6	F
LNB	1	30.1	F
		21.1	M
		19.0	M
	2	22.7	F
		21.8	M
		17.6	M
	3	22.9	F
		15.8	F
		17.8	M

2.8. Statistical analysis

All statistical analyses were performed, and graphics drawn with GraphPad® Prism® 9. Spearman's rank-order correlation was applied to assess the relationship between the individual total nesting scores and the coefficients of variance (CV) calculated with respect to the daily nesting scores. Descriptive statistics were applied to broadly determine the 75th percentile of the individual total nesting score distribution and the lower 25th percentile of the CV distribution. Two-way analysis of variance (ANOVA), regarded as sufficiently robust for the analysis of smaller data sets, was applied to analyze changes in nesting scores over time as well as the various measures of anxiety-like behavior. Behavioral parameters were set as dependent variables, while phenotype and drug exposure were set as independent variables. Where applicable, Bonferroni post-hoc analysis was applied for pairwise comparisons of group means. Striatal A_{2A} receptor expression, represented as B_{max}, and K_D values were determined by means of non-linear regression as applied for saturation binding analyses. Differences between the A_{2A} receptor expression and K_D values of NNB- and LNB-expressing mice were analyzed by means of one-tailed, unpaired *t*-tests. Statistical significance was set at $p < 0.05$ for all analyses.

3. Results

3.1. Anxiety-like behavior of NNB- and LNB-expressing mice

No significant exposure-phenotype interactions nor main effects of exposure or phenotype were shown for any of the measured parameters

Table 2
Interaction statistics and main effects for behavioral outcomes in the mirrored open field.

Parameter measured in the mirrored open field	Interaction / main effect	Statistical descriptor
Total time spent	Exposure-phenotype	$F(3, 58) = 1.15$, $p = 0.23$
	Exposure	$F(3, 58) = 2.23$, $p = 0.14$
	Phenotype	$F(1, 58) = 0.84$, $p = 0.48$
Distance travelled	Exposure-phenotype	$F(3, 58) = 0.83$, $p = 0.49$
	Exposure	$F(3, 58) = 0.32$, $p = 0.81$
	Phenotype	$F(1, 58) = 4.93$, $p = 0.03$
Number of entries	Exposure-phenotype	$F(3, 58) = 1.64$, $p = 0.19$
	Exposure	$F(3, 58) = 2.14$, $p = 0.11$
	Phenotype	$F(1, 58) = 1.57$, $p = 0.24$
Duration per entry	Exposure-phenotype	$F(3, 58) = 1.27$, $p = 0.29$
	Exposure	$F(3, 58) = 0.50$, $p = 0.69$
	Phenotype	$F(1, 58) = 0.003$, $p = 0.95$
Border to center entry ratio	Exposure-phenotype	$F(3, 58) = 1.69$ $p = 0.18$
	Exposure	$F(3, 58) = 0.73$, $p = 0.54$
	Phenotype	$F(1, 58) = 1.00$, $p = 0.32$

(Table 2), except for the total distance traveled, where phenotype had a significant main effect on the total distance traveled in the open field arena [Fig. 3**A**; $F(1, 58) = 4.93$, $p = 0.03$], although no significant pairwise differences were observed between any of the exposure groups. Thus, data were pooled per phenotype across exposure groups, which revealed a significantly decreased total distance traveled in the open field by LNB- compared to NNB-expressing mice (Fig. 3**B**; $U = 388$, $p = 0.04$, $d = 0.5$).

3.2. Pre- and post-exposure nesting expression

Differences between the total post-drug-exposure and baseline nesting scores, expressed as a percentage change for each mouse, are reflected in Fig. 4. A significant exposure-phenotype interaction impacted the result [$F(3, 57) = 4.06$, $p = 0.01$], with exposure being the main driver [$F(3, 57) = 4.90$, $p = 0.004$] of the result. Post-hoc analyses revealed a significant increase in the post-exposure nesting scores of ISTRA-exposed (77.3 ± 76.3%) compared to wCTRL- (−10.8 ± 22.8%, $p = 0.0004$) and LOR-exposed (−17.9 ± 42.6%, $p = 0.0002$) NNB-expressing mice. Further, the change in average baseline nesting scores of ISTRA-exposed NNB-expressing mice, also differed significantly from that of ISTRA-exposed LNB-expressing mice (77.3 ± 76.3% vs. 1.2 ± 27.8%, $p = 0.003$).

3.3. Striatal A_{2A} receptor expression

The individually measured (i.e. replicate) B_{max} values with respect to the striatal A_{2A} receptor in NNB- and LNB-expressing mice are represented in Fig. 5A. The measured K_D values for ^{3}H-NECA are represented in Fig. 5B. Crosses resemble the software generated averages when all three replicate results are analyzed as a whole (i.e. analyzed as the average of three measurements at each concentration point). While the difference between the mean B_{max} values obtained from NNB- and LNB-expressing mice failed to reach statistical significance despite having a large effect size (23.97 vs. 45.32 fmol/mg protein, $p = 0.200$, $d = 1.12$), a significant difference between the two cohorts with respect to the measured K_D values (25.13 vs. 91.02 nM, $p = 0.02$, $d = 3.00$), was shown.

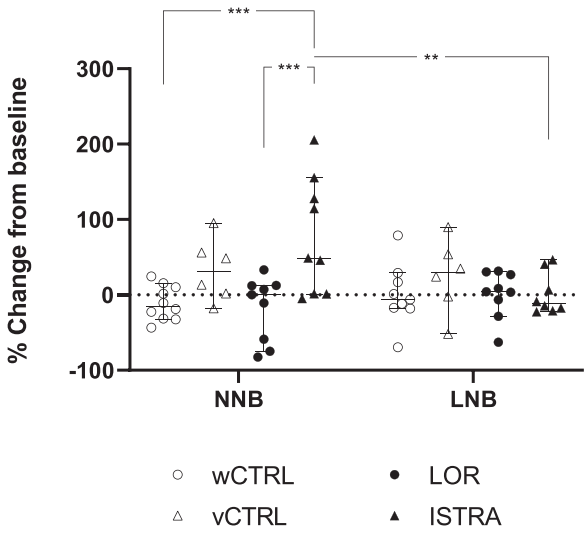

Fig. 4. Change in nesting expression over time expressed as a percentage change from the baseline scores NNB: normal nest building; LNB: large nest building; wCTRL: normal water control; vCTRL: vehicle control; LOR: lorazepam; ISTRA: istradefylline. * * $p < 0.01$; * ** $p < 0.001$, Two-Way ANOVA followed by Bonferroni Post-Hoc. Data represent median ± 95CI.

4. Discussion

The main findings of this investigation are that 1) NNB- and LNB-expressing mice show similar behavior in an anxiogenic open field, 2) ISTRA increased the nesting expression of NNB mice, without affecting LNB behavior or the open field behavior of either phenotype, and 3) LNB, compared to NNB behavior, is associated with a noteworthy increase in the expression of lower affinity striatal A_{2A} receptors.

Excessive nest building in rodents is proposed to be a behavioral phenotype suitable for the study of compulsive-like processes in animal model systems [23,24,29,51]. Considering that clinical compulsivity is varyingly associated with limbic and cortico-striatal involvement [43], it remains mostly unknown whether naturalistic LNB as opposed to NNB behavior in deer mice, uniquely associates with anxiety or perturbations in striatal processing. To this end, investigations into adenosinergic

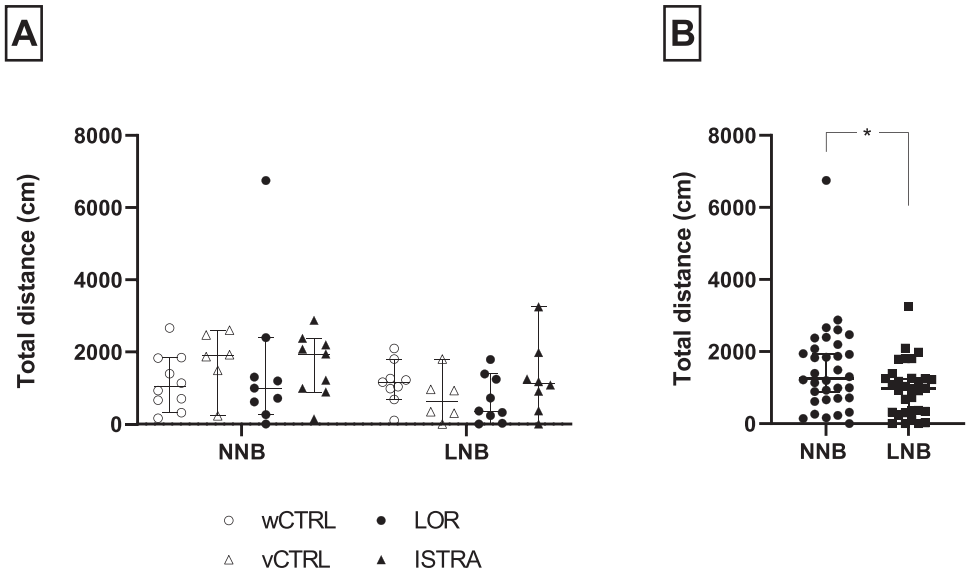

Fig. 3. Total distance traveled in the open field by mice of the NNB- and LNB-expressing phenotypes per exposure group (A) and pooled across exposure group (B) NNB: normal nest building; LNB: large nest building; wCTRL: normal water control; vCTRL: vehicle control; LOR: lorazepam; ISTRA: istradefylline. * $p = 0.045$, Two-Way ANOVA followed by Bonferroni Post-Hoc. Data represent median ± 95CI.

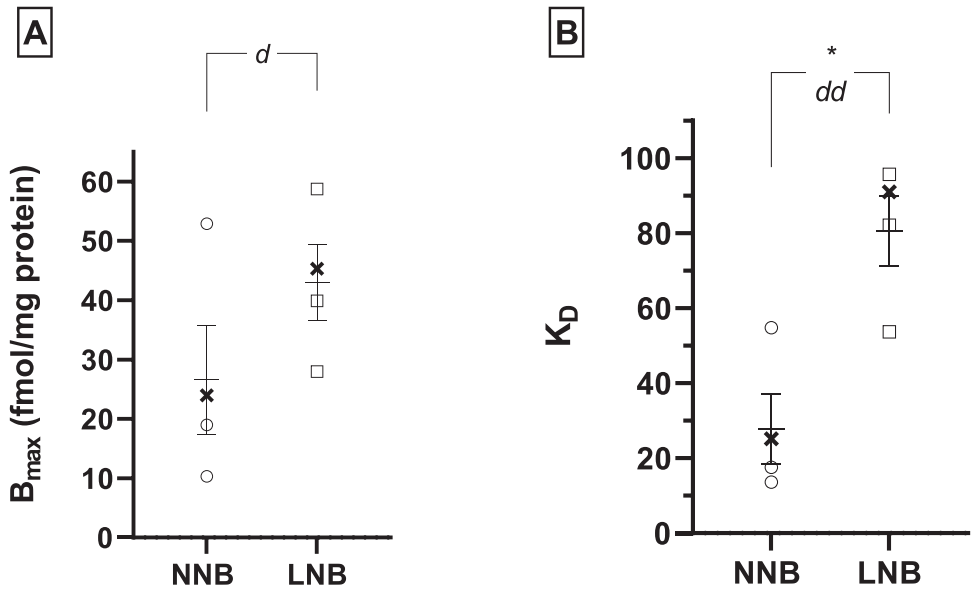

Fig. 5. Comparisons of striatal A_{2A} receptor B_{max} and K_D values reported in NNB- and LNB-expressing mice NNB: normal nest building; LNB: large nest building. * $p = 0.02$, Unpaired, one-tailed student's t-test; $0.8 < d < 1.3 < dd$. Data represent mean ± SEM.

mechanisms might be fruitful. As alluded to in the introduction, adenosine, via its actions on A_{2A} receptors, modulates the activity of associated, heterodimerized dopamine receptors in the indirect, D_2-expressing striatal pathway [20]. Of specific relevance for this investigation, the binding of adenosine or similar agonists to adenosine A_{2A} receptors *decreases* the affinity of the associated dimerized dopamine D_2 receptor for its endogenous ligand, thus blunting subsequent dopaminergic signaling in the indirect pathway [20]. Conversely, antagonists of these adenosine receptors appear to induce the opposite effect, i.e. increasing the affinity of the associated dopamine receptors [34]. Since dysregulated activity in the direct and indirect cortico-striatal pathways is believed to be associated with repetitive behavioral expression [30,41], including compulsions [26,43], modulation of adenosinergic signaling might provide additional insights into the neurobiological architecture of LNB-expressing mice.

Concerning the first main finding, which broadly shows that NNB- and LNB-expressing mice show similar behavior in an anxiogenic open field, subjects were screened for anxiety in a modified version of the standard open field, which allowed mice to move freely between an enclosed, but open-doored dark compartment, and an anxiogenic white-floored and mirrored open space [8]. Of all the assessed parameters, only the total distance travelled differed between the phenotypes, irrespective of exposure group. However, whereas LNB-expressing mice traveled shorter distances in the open field on average, the reported effect size is small ($d = 0.5$). Further, if one outlier was to be removed from the NNB cohort, said difference would no longer be significant. As such, it can be concluded that NNB- and LNB-expressing mice present with similar behavioral responses in a novel, anxiogenic open field, at least when assessed in the absence of any goal-triggering object, i.e. cotton wool, as was used before [52]. Indeed, in earlier work, we performed the same test, albeit over 12 h and while cotton wool was supplied in the large open space. At the time, we showed that LNB-expressing mice engaged in active risk-engaging behavior that was solely aimed at obtaining cotton wool. In contrast, the present data indicate that when open field behavior is measured over the short term in a novel, anxiogenic space, NNB- and LNB-expressing mice exhibit similar levels of exploratory behavior and that NNB- and LNB-behavior is not differentially associated with measures of generalized anxiety. Such conclusion is also elegantly supported by the clear lack of any anxiolytic effect of lorazepam, which was specifically included here to discriminate between behavioral adaptations related to motor control and limbic mechanisms.

The second and third main findings of this study broadly speak to the effects of adenosinergic processes in the manifestation of deer mouse nesting behavior. Firstly, it was observed that ISTRA, but neither control nor LOR, bolstered the nest building behavior of NNB mice. Importantly, since the vehicle emulsion also trended towards increasing the nesting sizes of both cohorts (NNB: $d = 1.3$; LNB: $d = 0.6$), we considered the possibility that the effect of ISTRA on the behavior of NNB-expressing mice, might be an artefact of an underlying, though insignificant, vehicle effect. However, this is unlikely since the effect of ISTRA in NNB animals was strikingly different from its effect on LNB expression. In fact, LNB behavior remained wholly unresponsive to ISTRA and all other interventions, despite the vehicle-exposed LNB-expressing animals showing a similar degree of behavioral adaptation compared to that seen in NNB-expressing mice. These data are highly informative, especially given the robust, though statistically insignificant, increase in the striatal A_{2A} receptor density (B_{max}) of mice in the LNB cohort, relative to the NNB cohort ($d = 1.12$). Further, the A_{2A} receptors of LNB-expressing mice showed a significantly lower affinity (K_D) for ^{3}H-NECA ($p = 0.02$, $d = 3.0$). When viewed against the background of our current understanding of the interactions between A_{2A} and D_2 receptors, the pharmacological and neurobiological data from this work are striking in showing that naturalistic LNB behavior is associated with distinct indirect pathway mechanisms that are most likely founded upon lower adenosinergic, and by implication higher dopaminergic, stimulation [20]. This notion is supported by the finding that NNB behavior, being associated with a lower expression of A_{2A} receptors that have a greater affinity for ^{3}H-NECA, is bolstered by ISTRA, in the absence of parallel changes in anxiety scores. This is important, since the inflation in the nesting scores of NNB-expressing is not attributed to the modulation of underlying anxiety. We show here that the act of nesting itself is intrinsically related to adenosinergic signaling in the indirect pathway of the cortico-striatal-thalamo-cortical (CSTC) circuit. Our data further suggest that control over the expression of motor behavior on the level of striatal adenosine-dopamine interactions is, given the high affinity of A_{2A} receptors in NNB mice, an important mechanism that regulates behavioral output in this cohort. In contrast, LNB-expressing mice, despite their level of A_{2A} receptor expression, did not respond in a similar manner, indicating a potential naturalistic ceiling effect with respect to adenosinergic behavioral output regulation. Importantly, it is entirely possible that the low affinity of striatal A_{2A} receptors expressed

by LNB mice, renders this mechanism of control over D_2 receptor activity mostly ineffective, thus explaining why ISTRA did not have any effect on the nesting expression of LNB mice. Collectively, the data presented here support a conclusion that LNB behavior is linked with lower adenosinergic sensitivity in the indirect pathway of the CSTC circuitry at baseline, thus promulgating excessive behavioral output. This finding paves the way forward for continued investigations of adenosinergic agonists in the model system as a potential ameliorative strategy.

Of note, deer mice also present with varying degrees of motor stereotypy, i.e. vertical jumping and pattern running [33]. In earlier work, it was shown that high stereotypical, as opposed to low stereotypical behavior is associated with a bias in favor of activity in the direct pathway [35]. Further, when assessed over the short-term, high stereotypical behavior responded favorably to acute adenosinergic agonist administration [44]. In contrast, we have shown that high stereotypy attenuates after chronic exposure to ISTRA [14], without any effect on low stereotypical behavior, a finding that contrasts with the present result. Collectively, historical, and current data from the deer mouse model highlight an intriguing role for adenosinergic processing in the manifestation of normal and repetitive behaviors that should be afforded more attention in future study.

Despite the insights gained, the present body of research was not without some shortcomings. An important aspect of work of this nature, speaks to the relatively subjective nature of cohort selection. Indeed, clear separation between NNB- and LNB-expressing mice is not always possible, as shown in Fig. 2. Further contributing to the potential confounding effect of such method of selection on the resulting data output, is the fact that tissues from different mice were pooled to complete a single binding assay. To overcome this, larger groups of mice are needed to ensure a better separation between cohorts, bearing in mind the ethical constraints that such an approach might be bound to. In addition, it would have been fruitful to explore the anxiety-like behavior of NNB- and LNB expressing deer mice in anxiety-tests that make use of actual safety- and perfectionistic-related triggers. While the present work did employ an anxiogenic version of the open field test, the fact that most deer mice presented with an innate drive to explore such and anxiogenic space, was striking. Mice of this species seem to be relatively risk-engaging compared to other strains, e.g. DBA/2 J mice, that have shown significant anxiety-like behavior in the mirrored open field [31]. In contrast, mice of the *Peromyscus* genus are likely less risk-aversive, a trait that could have clouded the interpretation of the present data. Indeed, it is possible that LNB- but not NNB-expressing mice, might show inflated anxiety when the contextual triggers of their expression, are manipulated or modified. In other words, if LNB is associated with safety-related anxiety, exposing such mice to an aggressor for example, might yield a different result in tests of anxiety, as opposed to what was reported here. Therefore, a clear conclusion cannot be drawn with respect to the potential innate associations between compulsive-like behavioral expression and an inflated degree of underlying, contextually-related anxiety.

5. Conclusion

In this work, we explored the potential associations between compulsive-like LNB expression and anxiety-like behavior in deer mice. We further sought to investigate the relationship between LNB behavior and striatal A_{2A} receptor expression and determine whether chronic intervention with the A_{2A} receptor antagonist, ISTRA, would abrogate the expression of LNB. In summary, our findings indicate that NNB and LNB behavior are not distinctly associated with measures of generalized anxiety and that an ISTRA-induced increase in the nesting expression of NNB-expressing mice are dissociated from changes in anxiety scores. Further, data from this investigation show that nesting in deer mice is directly related to striatal adenosine signaling, and that LNB is founded upon a lower degree of adenosinergic A_{2A} stimulation. Future studies should explore the effects of adenosine receptor agonists as potential ameliorative strategies.

Ethics approval

This study was approved by the Animcare Research Ethics Committee of the NWU (approval number: NWU-00526–20-A5). All procedures were in accordance with the rules and guidelines stipulated by the South African National Standard 10386:2021 *"The care and use of animals for scientific purposes"*.

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CRediT authorship contribution statement

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, D.S., L.B., T.K., and D.W.W.; *Methodology*, D.S., L.B., G.d.B., H.j.V., G.T., and D.W.W.; *Investigation*, D.S., L.B., G.d.B., H.j.V., and D.W.W.; *Formal Analysis*, D.S., L.B., G.d.B., and D.W.W.; *Resources*, L.B., L.L., and D.W.W.; *Writing - Original Draft*, D.S. and D.W.W.; *Writing - Review & Editing*, D.S., L.B., T.K., and D.W.W.; *Visualization*, D.S. and D.W.W.; *Supervision*, L.B., T.K., and D.W.W.; *Funding Acquisition*, L.B., G.T., L.L., and D.W.W.

Data Availability

Data will be made available on request.

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Significance statement

Our understanding of repetitive and persistent behaviors, similar to that which is seen in patients suffering from obsessive-compulsive disorder (OCD), remains inadequate. Modulation of adenosinergic processes in patients suffering from neuropsychiatric illness has gained increasing interest. Adenosine, an endogenous nucleoside, plays an important role in numerous physiological processes, including energy transfer. However, it is also an important neuromodulator via its actions on adenosine receptors in the central nervous system. Here, we show that adenosine is also vital to regulate goal-directed behavioral output and that manipulation of adenosinergic processes in conditions characterized by both cognitive and motor dysfunction, might prove useful.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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