



**Anxiety-like behaviour and adenosinergic processes in compulsive-like deer mice
(*Peromyscus maniculatus bairdii*)**

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This dissertation is dedicated to our **heavenly Father**, as this was only possible through His **GRACE**.

* * *

John 15:5

“I am the vine; you are the branches. If you remain in me and I in you, you will bear **much** fruit; apart from me you can do nothing”

* * *

Stefan Saaiman (1 September 1971 – 28 January 2021)

Aan my liefste pappa.

Nooit vergeet, veraltd met ons.

Baie lief vir pappa.

* * *

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Congress proceedings

The results from this investigation were presented at the annual meeting of the South African Society for Neuroscience which was hosted at the Biological Psychiatry meeting in 2022. The full reference for the presentation was:

Saaiman D., Brand L., Krahe T., Wolmarans D. “Distinct anxiety-like behaviours in normal and large nesting deer mice (*Peromyscus maniculatus bairdii*): towards an understanding of compulsive-like behavioural persistence”, September 2022, Century City, Cape Town.

Abstract

Obsessive-compulsive disorder (OCD)¹ is characterized by recurring thoughts (obsessions) and rigid repetition of certain mental or behavioral actions (compulsions) that are often associated with anxiety. In terms of neurobiology, dysfunctional connectivity in distinct cortico-limbic and cortico-striatal-thalamic circuits has been shown. Two pathways, i.e. direct and indirect, relay executive signals between the prefrontal cortex and the thalamus. Whereas the direct pathway expresses dopamine D₁ receptors, the indirect pathway expresses D₂ receptors. Dysregulation between the activities of these pathways is proposed to underlie obsessive-compulsive symptom manifestation. Further, literature agrees that a functional imbalance between these pathways at the time of behavioral engagement, underlies repetitive behavioral output. Unfortunately, despite convincing evidence that selective serotonin reuptake inhibitors (SSRIs)² are modestly successful in the treatment of OCD, treatment resistance remains a fundamental dilemma.

Adenosine is an important neuromodulator that exerts its actions via A₁ and A₂ receptors. Of especial importance in this work is the A_{2A} receptor which is located mainly in the striatum. Here, they modulate behavioral output and regulate motor control. Importantly, adenosine and dopamine receptors co-occur as A_{2A}-D₂ receptor dimers on the neuronal bodies of the indirect, behaviorally inactivating pathway. Such co-expression facilitates functional opponency, whereby adenosinergic stimulation decreases the activity of dopamine at the D₂ receptor. In this regard, A_{2A} receptor antagonists, e.g. istradefylline (ISTRA)³, may show promise as an anxiolytic and motor restorative intervention. 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 anxiolytic and anti-compulsive pharmacotherapeutic strategy was explored.

When bred, housed, and reared under standard laboratory conditions, 25 - 30 % of deer mice (*Peromyscus maniculatus bairdii*) express large nest building (LNB)⁴ behavior. Being reminiscent of clinical compulsivity, LNB behavior is excessive and purposeless. Still, it remains unknown whether LNB behavior is mostly reflective of perturbations in motor control (i.e. striatal processes), or if anxiety-related cognitive processes (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, has not yet been explored and thus formed the focus of this investigation.

¹ obsessive-compulsive disorder

² selective serotonin reuptake inhibitors

³ istradefylline

⁴ large nest building

Abstract

* * *

An initial pool of 120 deer mice of both sexes underwent initial nest building screening to select 34 NNB¹- and 32 LNB²-expressing deer mice that were used in further study (ethical approval number: **NWU-0042-21-A5**). The selected mice were exposed to either a normal water (wCTRL)³ or vehicle control (vCTRL)⁴ for 28 days, lorazepam (LOR)⁵ for seven days (as an anxiolytic control) or ISTRA⁶ for 28 days. Mice were subsequently reassessed for nesting expression, this time in combination with open field anxiety testing. On the morning following the last nesting and anxiety assessment, mice were decapitated, the striatal tissue harvested on ice, and analyzed for adenosine A_{2A} receptor expression.

* * *

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

* * *

The first main finding of this investigation was that NNB- and LNB-expressing mice showed similar behaviors in an open field, indicating that NNB- and LNB-expressing mice present with similar anxiety-like behavioral responses. This finding was somewhat unexpected, given our earlier results that LNB-expressing mice, assessed over the course of a full dark cycle, engage in increased risk-taking behaviors, albeit to obtain cotton wool. Nevertheless, from the present data, it can be concluded that when open field behavior is measured over the short term in a novel open field environment, NNB- and LNB-expressing mice both exhibit similar levels of exploratory behavior. Thus, it can be concluded that LNB expression is not associated with generalized underlying anxiety.

* * *

The second and third main findings of this study were highly informative. Not only was it observed that ISTRA, but not control or LOR, bolstered NNB behavior, but also that LNB behavior associated with a noteworthy increase in striatal A_{2A} receptor density (B_{max}), compared to NNB behavior. While we

¹ normal nest building

² large nest building

³ normal water control

⁴ vehicle control

⁵ lorazepam

⁶ istradefylline

Abstract

could not report these differences on a statistically significant level, the pharmacological and neurobiological data from this work show that naturalistic LNB¹ behavior is associated with distinct indirect pathway mechanisms that are most likely founded upon lower adenosinergic, and by implication higher dopaminergic, stimulation. Therefore, it can be concluded that the act of nesting itself is intrinsically dependent on the executive balance between the direct and indirect pathways of the CSTC² circuits. This finding paves the way for continued investigations of adenosinergic agonists in the model system as a potential ameliorative strategy.

* * *

In conclusion, our findings show that NNB³ and LNB behavior are not distinctly associated with measures of anxiety and that ISTR⁴-induced changes in nesting expression are dissociated from changes in anxiety scores. Further, nesting in deer mice is directly related to striatal adenosine signaling, and 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.

Keywords: deer mouse; obsessive-compulsive; anxiety; nest building; adenosine; istradefylline; lorazepam

Solemn declaration: I, Devan Saaiman (28801393) herewith declare that this dissertation is my own work and that no part thereof has been copied from any other sources.

¹ large nest building

² cortico-striatal-thalamo-cortical

³ normal nest building

⁴ istradefylline

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1 Introduction

1.1 Dissertation approach and layout

This dissertation has been prepared in article format, as specified by the North-West University (NWU)¹, South Africa. The main experimental body of research will be presented in the form of a journal article, i.e. **Chapter 3**. This chapter will be submitted for publication in an internationally accredited, peer-reviewed journal, i.e., '*Journal of Neuroscience Research*'.

The complete dissertation will comprise four chapters. **Chapter 1** provides a concise summary of the investigation, including the problem statement, working hypothesis, study aims and objectives, experimental layout, and ethical considerations. In **Chapter 2**, a detailed but focused literature review lays the theoretical foundations for the body of research presented here. Last, **Chapter 4** contains a broad summary of the complete dissertation, potential study shortcomings, and future considerations.

Three addenda are included. **Addendum A** contains supplementary details regarding the behavioral methods and results that are not provided in **Chapter 3**. **Addendum B** contains supplementary methodological detail regarding the receptor binding experiments of which the main results are included in **Chapter 3**. Last, **Addendum C** contains the letters of permission from the co-authors of **Chapter 3** that were not formally involved as functionaries in this project, stating that **Chapter 3** may be included in this dissertation for assessment purposes.

Chapter 3 was prepared according to the "Instructions to Authors" as specified by '*Journal of Neuroscience Research*'. As such, this dissertation is written in US English and referenced in the style of said journal.

* * *

¹ North-West University

1.2 Problem statement

Obsessive-compulsive disorder (OCD)¹ is a neuropsychiatric disorder that is characterized by recurring and intrusive thoughts (obsessions) and rigid repetition of specific actions (compulsions) that often associate with increased levels of anxiety (APA 2013).

On a neurobiological level, OCD mostly associates with irregular activity in the cortico-striatal-thalamo-cortical (CSTC)² loops (Ting and Feng 2011). A direct, behaviorally activating pathway and an indirect, behaviorally inactivating pathway comprise the applicable CSTC circuits (Evans et al. 2004). Dysregulation between the dopamine D₁ receptor-expressing direct pathway and the indirect, D₂ receptor-expressing pathway serves as one proposed explanation for obsessive-compulsive symptomology (Denys 2004; Kravitz et al. 2012). Furthermore, given the known behaviorally opponent interactions between dopamine and serotonin (Daw 2002), hyposerotonergic signaling in the CSTC circuits is postulated to underlie symptom manifestation (Lissemore 2018). Nevertheless, despite convincing evidence that selective serotonin reuptake inhibitors (SSRIs)³ are successful for the treatment of OCD, direct evidence implicating serotonergic dysfunction is restricted. Still, SSRIs remain the mainstay of anti-compulsive pharmacological therapy, with 40 – 60 % of patients showing symptom improvement (Albert et al. 2018). Thus, an improved understanding of the condition and how various psychobiological processes may contribute to its phenomenology, will be fruitful.

To this end, investigations into the potential role of anxiety-processing in OCD might be informative. Although no longer classified as an anxiety disorder (APA 2013), obsessive-compulsive symptoms often co-occur with anxiety (Abramovitch and Cooperman 2015). Also, it is hypothesized that functional impairment in OCD is positively associated with anxiety severity (Citkowska-Kisieleska et al. 2020; Welkowitz et al. 2000), a finding that might inform suboptimal treatment outcomes in OCD. To explain, while a causal relationship between OCD and anxiety has not yet been shown, traditional anxiolytics, i.e. the benzodiazepines, are also mostly ineffective for the treatment of the condition (Starcevic et al. 2016). However, different obsessive-compulsive phenotypes may present with unique underlying anxiety-related profiles (Ferreira et al. 2017; Torres et al. 2016), a factor that should arguably be considered when treatment regimens are conceptualized.

* * *

¹ obsessive compulsive disorder

² cortico-striatal-thalamo-cortical

³ selective serotonin reuptake inhibitors

Adenosine is the primary component of the energy-transferring molecule, adenosine triphosphate (ATP)¹ (López-Cruz et al. 2017). Adenosine also acts as an important neuromodulator via its actions on adenosine A₁ and A₂ receptors. A₁ receptors generally suppress neuronal signaling by causing hyperpolarization and minimized excitability of neuronal membranes (van Calker et al. 2019). The functions of the A_{2A} receptor, which is predominantly expressed in the striatum, are more complex (van Calker et al. 2019). Specifically, A_{2A} receptor activation modulates motor control on the levels of both reward- and goal-directed feedback processing (Li et al. 2018) and directly influences striatal signaling (Chen and Cunha 2020; Tanimura et al. 2010). A_{2A} receptor antagonists, e.g. istradefylline (ISTRA)², shows demonstrable pro-cognitive and motor improving effects in patients suffering from Parkinson's disease (Chen and Cunha 2020) and also improves anxiety in patients diagnosed with mood and anxiety disorders (Coelho et al. 2014; van Calker et al. 2019). In terms of the neurobiology of OCD³, the A₂ and D₂ receptor subtypes are co-expressed, whereby activation of the A₂ receptor decreases the affinity of dopamine for and its activity at the D₂ receptor (Ferré et al. 1996). Although clinical data remain scant, ISTRA improved compulsive-like behaviors induced by quinpirole, a dopamine D_{2/3} receptor agonist (Asaoka et al. 2019) and also attenuated the expression of high motor stereotypy (HS)⁴ in the deer mouse model of OCD (de Brouwer et al. 2021). Thus, the present investigation will seek to expand on the current literature base and extend earlier work on HS, by exploring the potential effects of ISTRA on anxiety- and compulsive-like behavior in deer mice showing large nest building (LNB)⁵ behavior.

* * *

When bred, housed, and reared under standard laboratory conditions, 25 - 30 % of deer mice (*Peromyscus maniculatus bairdii*) express spontaneous LNB behavior. Although nest building is a common rodent behavior, it shows significant intra-species variance, with some mice expressing exacerbated, inflated, and persistent nesting behaviors. Being reminiscent of clinical compulsivity, LNB behavior aligns conceptually well with safety/perfectionism OCD (Hoffman and Rueda Morales 2009), while LNB, but not normal nest building (NNB)⁶ behavior also responds favorably, though not fully, to high-dose, chronic oral exposure to the SSRI⁷, escitalopram (Wolmarans et al. 2016). Still, associations between LNB behavior, anxiety-related processes, and striatal dysregulation remain unexplored. This, as well as how such associations might be modulated by anti-adenosinergic

¹ adenosine triphosphate

² istradefylline

³ obsessive compulsive disorder

⁴ high motor stereotypy

⁵ large nest building

⁶ normal nest building

⁷ selective serotonin reuptake inhibitors

intervention, will be explored in this work. Indeed, considering that anti-adenosinergic modulation has shown promise as both an anxiolytic and a motor restorative intervention, and that OCD¹ involves with both anxiety and excessive behavioral engagement, the adenosinergic system is a realistic target for investigation in a naturalistic model system of compulsive-like persistence.

* * *

1.3 Research hypothesis

Following from the above, the overarching hypothesis of this work is that NNB² and LNB³-behavior will show distinct psychobiological signatures so that LNB-expressing mice will present with an inflated degree of general open field anxiety and striatal adenosine A_{2A} receptor dysregulation, compared to NNB-expressing mice. In extension, based on earlier clinical (Yamada et al. 2014) and pre-clinical findings (Asaoka et al. 2019; de Brouwer et al. 2021; Horita et al. 2013), we hypothesize that LNB behavior will be improved by ISTRA⁴, a selective adenosine A_{2A} receptor antagonist. Specifically, we postulate that ISTRA will attenuate both the anxiety (if shown) and nesting scores of LNB-expressing mice without affecting the behavioral responses of NNB-expressing mice. Last, in line with the clinical treatment response of OCD, we hypothesize that lorazepam (LOR)⁵, a benzodiazepine with strong anxiolytic action, will attenuate the anxiety-like behavior of LNB-expressing mice, without affecting nesting scores, thereby separating the neurobiological actions of ISTRA from that of LOR.

1.4 Research questions

- i) Will NNB- and LNB-expressing mice differ with respect to their general anxiety-related behavioral output?
- ii) Will ISTRA, a selective adenosine A_{2A} receptor antagonist, decrease the expression of LNB behavior and will such decrease correspond with parallel improvements in anxiety-like behavioral expression?
- iii) Will LNB, compared to NNB behavior, associate with striatal pathway dysregulation as shown by altered adenosine A_{2A} receptor expression?

¹ obsessive-compulsive disorder

² normal nest building

³ large nest building

⁴ istradefylline

⁵ lorazepam

1.5 Study aims and objectives

1.5.1 Aims

The current research aims to:

- i) Investigate the potential association between compulsive- and anxiety-like behavioral expression in NNB¹- and LNB²-expressing deer mice;
- ii) Determine whether ISTR³A will abrogate the expression of LNB behavior with or without affecting any potential and parallel anxiety-like behaviors; and
- iii) Characterize baseline differences in the striatal A_{2A} receptor expression profiles in NNB and LNB mice.

1.5.2 Objectives

To realize the aims of this work, the following objectives were addressed:

- i. Identifying 34 NNB- and 32 LNB-expressing deer mice of both sexes from an initial pool of 120 mice;
- ii. Subdividing each behavioral cohort, into the following exposure groups (*n* = 6 - 10 per cohort per exposure group):
 - a. Normal water control (wCTRL)⁴: normal water administered for 28 days;
 - b. Vehicle control (vCTRL)⁵: vehicle emulsion administered for 28 days;
 - c. LOR⁶ (2 mg/kg/day; Chesley et al. (1991); Fahey et al. (2006)): administered in normal water sub-acute for seven days; and
 - d. ISTR (20 mg/kg/day; de Brouwer et al. (2021); Orr et al. (2018)): administered in the vehicle emulsion for 28 days.

Continue next page.../...

¹ normal nest building

² large nest building

³ istradefylline

⁴ normal water control

⁵ vehicle control

⁶ lorazepam

- iii. Assessing the nest building and open-field behavioral expression of all mice after 21 days of uninterrupted control, vehicle, or drug exposure and while such drug exposure is continued for a further seven days of behavioral testing (wCTRL¹, vCTRL², and ISTRA³) or during the seven days of behavioral testing (LOR)⁴; and
- iv. Quantifying striatal adenosine A_{2A} receptor expression in the wCTRL-exposed NNB⁵- and LNB⁶-expressing mice, with and without ISTRA.

* * *

1.6 Study Layout

The present investigation comprised three phases of study, viz. 1) baseline screening and behavioral categorization, 2) wCTRL, vCTRL, and drug exposure and 3) post-exposure behavioral assessment and neurochemical analysis (**Fig. 1-1**).

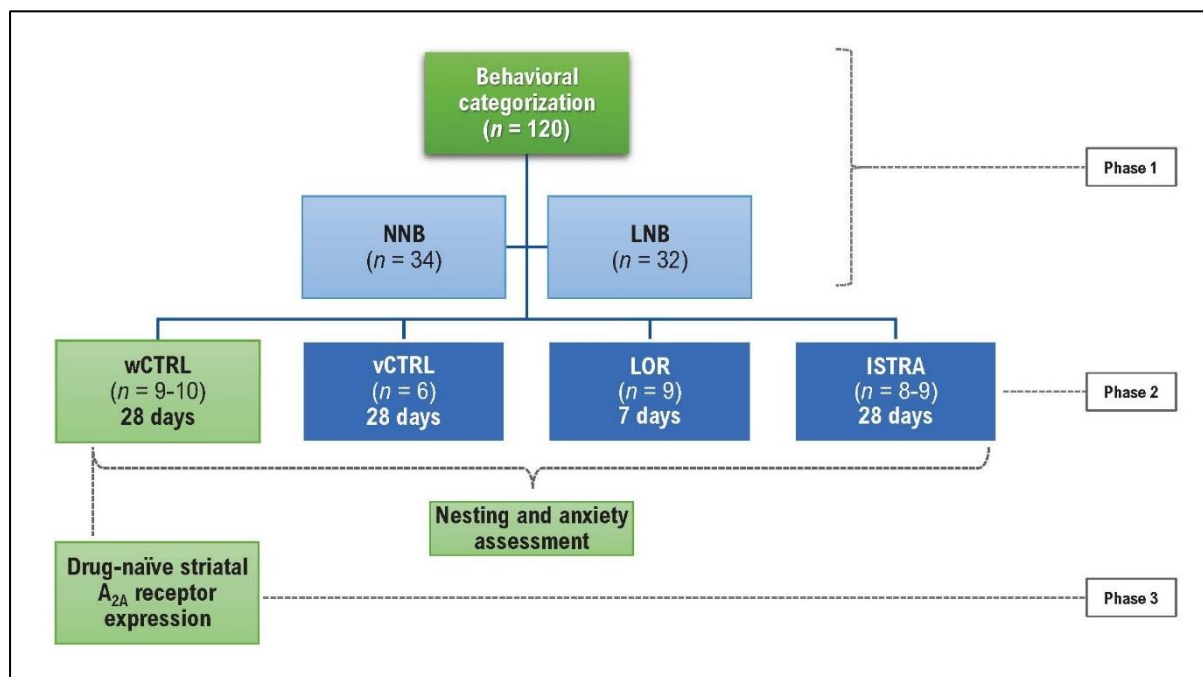


FIGURE 1-1: SCHEMATIC REPRESENTATION OF THE STUDY LAYOUT

NNB: NORMAL NEST BUILDING; LNB: LARGE NEST BUILDING; wCTRL: NORMAL WATER CONTROL; vCTRL: VEHICLE CONTROL; ESC: ESCITALOPRAM; LOR: LORAZEPAM; ISTRA: ISTRADefylline

¹ normal water control

² vehicle control

³ istradefylline

⁴ lorazepam

⁵ normal nest building

⁶ large nest building

1.7 Ethical approval

Prior to the onset of experimentation, this investigation was approved by the scientific committee of '*Translational Neuroscience and Neurotherapeutics*' (a subprogram of Pharmacén, NWU¹) as well as by the NWU AnimCare Research Ethics Committee (approval number: **NWU-00422-21-A5**). Throughout the investigation, the four R's (4R) as they pertain to mice experimentation, were adhered to (Arora et al. 2011; Doke and Dhawale 2015; Ferdowsian and Beck 2011; Hooijmans et al. 2010). All procedures were performed in accordance with the code of ethics stipulated in the relevant national legislation (South African National Standard for the Care and Use of Animals for Scientific Purposes; SANS² 10386:2008).

¹ North-West University

² South African National Standards

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2 Literature review

2.1 Obsessive-compulsive disorder – a clinical background

Obsessive-compulsive disorder (OCD)¹ is a neuropsychiatric disorder with a one-year prevalence rate of 1.8 % (APA 2013). OCD is characterized by recurring obsessive thoughts (obsessions) and rigid repetition of certain overt or covert actions (compulsions) that are often associated with increased levels of symptom-related anxiety (APA 2013). OCD shows a high degree of heritability with at least 6 % to 55 % of cases diagnosed in individuals that had at least one first-degree relative suffering from the condition (Browne et al. 2014). That said, environmental triggers, both in childhood and adulthood also increase the risk for OCD. These include life experiences that place a significant psychobiological strain on individuals, e.g. social segregation, abandonment, and physical, emotional, and sexual abuse (Grisham et al. 2011).

From a symptomological perspective, OCD is phenotypically heterogeneous, and symptoms are idiosyncratic, i.e. they present in various contexts about different themes in different individuals (Kuehne et al. 2020). Irrespective of symptom theme, obsessions are characterized by four fundamental traits, i.e. 1) they are constant and frequent intrusive thoughts that are 2) ego-dystonic, repugnant or inconsistent with the individual's values (Veale and Roberts 2014), 3) cause a high degree of anxiety, and 4) are perceived by the suffering individual as a product of their own mind; in other words, OCD patients demonstrate insight into the irrationality of their symptoms. Typical examples of undesirable obsessive-compulsive thoughts include images of hurting loved ones, constant doubting if doors are locked or electrical appliances are switched off, thoughts of being contaminated, as well as repugnant thoughts of a sexual or religious nature. Importantly, while these themes are reminiscent of the everyday worries expressed by most persons, worry and obsessions differ from one other on the basis of content, frequency, and its ascribed level of importance (Burns et al. 1996; Calleo et al. 2010).

Currently, four symptom categories are described (Abramowitz and Reuman 2020), i.e.:

- Safety obsessions associated with checking compulsions and reassurance-seeking behaviors;
- Symmetry obsessions associated with ordering and counting routines;
- Contamination obsessions associated with washing and cleaning routines; and
- Intrusive thoughts related to aggression, sexual, and religious themes, which are mostly associated with covert mental routines, e.g., excessive praying.

¹ obsessive compulsive disorder

For said symptoms and behaviors to satisfy a diagnosis of OCD¹, they must 1) cause severe discomfort, 2) be notably time-consuming (taking up more than 1 hour per day), 3) significantly impair the normal day-to-day functioning of the patient, 4) not be attributable to drug use (APA 2013), and 5) not be directly related to another mental disorder (APA 2013). In terms of its presentation in men and women (Lochner et al. 2004; Mathis et al. 2011), OCD shows a younger age of onset in men compared to women (Stein et al. 2019; Veale and Roberts 2014), while men tend to present with more severe symptoms which often associate with a worse prognosis (Lochner et al. 2004; Mathis et al. 2011). Women more typically present with cleaning and washing compulsions (Benatti et al. 2020), while men more commonly suffer from intrusive thoughts of a sexual or religious nature (Torresan et al. 2013).

2.2 The neurobiology of obsessive-compulsive disorder

2.2.1 Neuroanatomy

In the last few decades, substantial advancements in the pharmacological, psychological, and neuroimaging fields have led to a better understanding of the pathophysiology of OCD. A growing body of evidence indicates that OCD associates with dysfunctional processing in various brain regions (Yuste 2015), notably so the cortico-striatal-thalamo-cortical (CSTC)² circuits (Ting and Feng 2011) (**Fig. 2-1**).

¹ obsessive-compulsive disorder

² cortico-striatal-thalamo-cortical

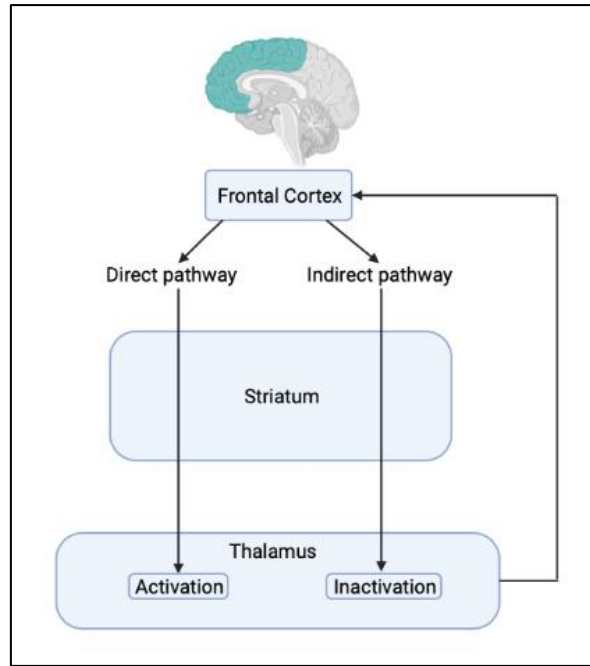


FIGURE 2-1: THE DIRECT, BEHAVIORALLY ACTIVATING PATHWAY AND INDIRECT, BEHAVIORALLY INACTIVATING PATHWAY OF THE CSTC CIRCUITRY RELATED TO OBSESSIVE-COMPULSIVE DISORDER (OWN FIGURE)

Briefly, the CSTC¹ circuit comprises two distinct pathways, i.e. a direct and an indirect pathway (Bartz and Hollander 2006; Evans et al. 2004). In concert, these two pathways regulate the planning and execution of voluntary behaviors (Simmler and Ozawa 2019). Both pathways originate in the prefrontal cortex (PFC)² and project to the thalamus via striato-thalamic projections (Bartz and Hollander 2006). Specifically, the direct pathway projects from the PFC, through the striatum via the globus pallidus interna and the substantia nigra pars reticulata, activating the thalamus before again terminating in the PFC (Bartz and Hollander 2006). In contrast, the indirect pathway projects from the PFC via the globus pallidus externa, subthalamic nuclei, then only passing through the substantia nigra pars reticulata, ultimately inhibiting the thalamus before closing the loop in the PFC (Bartz and Hollander 2006). In healthy, non-clinical individuals, activity in the direct and indirect pathways is constantly balanced so that executive behaviors are only executed when planned (Bartz and Hollander 2006; Pauls et al. 2014). Thus, under resting state conditions, the indirect pathway dominates. However, upon planning and executing voluntary motor behavior, the executive balance switches in favor of the direct pathway (Ting and Feng 2011). In patients suffering from OCD³, dysregulated activity in the direct and indirect pathways has been shown (Robbins et al. 2019; Saxena and Rauch 2000; Shephard et al. 2021). While earlier research in clinical OCD cohorts pointed to a relative neurophysiological bias in favor of the direct pathway in OCD (Saxena et al. 1998), more recent models point to overactivity in both pathways whereby the planning of various potential situational

¹ cortico-striatal-thalamo-cortical

² prefrontal cortex

³ obsessive-compulsive disorder

outcomes are ascribed to the activation of the direct pathway and the ultimate selection of the appropriate response to activation of the indirect pathway (Keeler et al. 2014). Irrespective, persistent behavioral output, mostly dissociated from a realistic outcome, is observed in OCD. The neurophysiological dynamics that govern the executive output of these pathways, are predominantly regulated by the neurotransmitter, dopamine. To this, more attention will be afforded later in this chapter. For the purposes of this review, a brief synopsis of the relevant regions of the PFC, basal ganglia and thalamus will be provided against the background of OCD.

2.2.1.1 Prefrontal and cingulate cortices

In the mammalian brain, the PFC forms part of the anterior cortical region. It receives projections from, among others, the thalamus (Fuster 2015), which serves as a pivotal subcortical relay for executive and cognitive output (Ferguson and Gao 2015). The PFC, which is organized into distinct neuroanatomical areas, i.e. the orbitofrontal cortex (OFC)¹, and the medial and ventral aspects of the dorsal and ventral regions, is responsible for, among others, the planning and execution of goal-directed behavior (Fuster 2015). With respect to OCD², the OFC is especially important, since it plays a pivotal role in goal-directed behavioral flexibility (Simmler and Ozawa 2019), and orbitofrontal hyperactivity is often correlated with obsessive-compulsive symptom severity (Beucke et al. 2013; Ursu and Carter 2009). The dorsolateral PFC³ is central to the initiation of voluntary behavior (Hutcherson et al. 2012), and overactivity in this region is associated with the repetitive generation of executive output (Robbins et al. 2012), notably also in patients diagnosed with OCD (Friedlander and Desrocher 2006; Hazari et al. 2019). Considering that most patients suffering from OCD also present with high levels of anxiety, the anterior cingulate cortex (ACC⁴; a cortical area primarily involved in limbic processes) has also been implicated in the neurobiology of the condition (Kühn et al. 2013; Shim et al. 2009). Indeed, perturbations in anterior cingulate processing is believed to impair decision making in patients showing heightened trait anxiety (Paulus et al. 2004), which is also occasionally in those with OCD (McGovern and Sheth 2017; Raines et al. 2014).

2.2.1.2 Basal ganglia

Receiving executive input from the PFC, the basal ganglia comprise various subcortical nuclei that each serves a distinct function (Macpherson and Hikida 2019). Over and above its regulation of ‘go-no-go’ signals via the direct (go) and indirect (no-go) pathways as explained above, the CSTC⁵ loops

¹ orbitofrontal cortex

² obsessive-compulsive disorder

³ prefrontal cortex

⁴ anterior cingulate cortex

⁵ cortico-striatal-thalamo-cortical

that relay signals from the PFC to the thalamus, can be organized into three main circuits, i.e. the sensorimotor, associative, and limbic circuits (Haber 2003; Macpherson and Hikida 2019). Briefly, the sensorimotor circuit recruits both sensory and motor processes that control executive movement that is associated with a specific environmental context, and which may develop into habitual behavior over time (Gremel and Lovinger 2017; Parent 1990). In contrast, the associative circuit is responsible for goal-directed planning and action-outcome feedback valuation (DeLong and Wichmann 2010; Gremel and Lovinger 2017). In turn, the limbic circuit is responsible for the way organisms perceive and respond to favorable (rewarding) and aversive (punishing) stimuli, evaluate fear and experience anxiety (Gremel and Lovinger 2017). It stands to reason that dysfunction within any of these circuits may contribute to or associate with the symptomological manifestation of OCD (Macpherson and Hikida 2019). For example, dysfunction in the limbic circuits may underlie obsessions that are mostly borne from fear, i.e. safety/checking OCD, while sensorimotor and associative perturbations may contribute to the expression of habit-like compulsions, i.e. counting, ordering, and behaviors aimed at ensuring symmetry (Macpherson and Hikida 2019). Irrespective, dysregulation in any of these circuits may arguably involve dysregulation of direct or indirect cortico-striatal signaling, thereby contributing to OCD¹ (Macpherson and Hikida 2019; Saxena et al. 2001; Saxena et al. 1998).

2.2.1.3 *Thalamus*

The thalamus, being the primary behavioral striato-cortical output relay (Li et al. 2019), is responsible for processing all sensory information, including that which is used to plan voluntary motor output, prior to such being interpreted by the cerebral cortex (Kurata 2005; Rasmussen et al. 2013). Indeed, distinct thalamic projections can, in addition to those that relay striato-cortical executive output, also relay sensory information directly to the PFC² and ACC³, thus bypassing striatal processing to instigate reflexive behaviors in anxiogenic, traumatic or fearful circumstances (Miller and Cohen 2001; Wimmer et al. 2015). With respect to OCD and given the inflated degree of striato-cortical output, thalamic hyperactivity also sometimes ensues (Hallett 1993).

2.2.2 The neurotransmission of OCD

2.2.2.1 *Glutamate and GABA*

Glutamate, an amino acid neurotransmitter, is the most abundant excitatory neurotransmitter in the mammalian brain (Kvamme 2018; Pittenger et al. 2011; Trevor 2014). It is also the precursor of the

¹ obsessive-compulsive disorder

² prefrontal cortex

³ anterior cingulate cortex

main inhibitory neurotransmitter gamma-aminobutyric acid (GABA)¹ (Karthik et al. 2020; Zhou and Danbolt 2014). Glutamate receptors can be described as either metabotropic or ionotropic, where metabotropic receptors (mGluRs)² are G-protein coupled (Karthik et al. 2020; Niswender and Conn 2010; Purves et al. 2001). In contrast, ionotropic receptors such as, N-methyl-D-aspartate (NMDA)³, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)⁴, and kainate receptors are responsible for most of the rapid excitatory signals within the central nervous system (Karthik et al. 2020; Pittenger et al. 2011; Zhou and Danbolt 2014). They are also especially important for maintaining synaptic plasticity (Collingridge et al. 2009). Glutamate may have neurotoxic properties via the overstimulation of NMDA receptors (Karthik et al. 2020; Pittenger et al. 2011) and its concentrations are regulated carefully. Glutamate influences a variety of processes within specific neuroanatomical regions of the brain. With respect to OCD, these processes include cognition, memory, and motor output (Karthik et al. 2020; Muller and Roberts 2005). During the neurodevelopmental window, cortical neurons mature to project to the striatum to form glutamatergic synapses with GABA⁵ergic interneurons (Karthik et al. 2020). These connections eventually form the direct- and indirect cortico-striatal pathways, alluded to earlier. Against this background, it is proposed that glutamatergic hyperactivity is associated with cortico-striatal overactivation (Rosenberg et al. 2004), thus leading to persistent thalamo-cortical go-signaling and thereby contributing to the pathophysiological picture of OCD⁶ (Karthik et al. 2020; Wu et al. 2012). However, despite this knowledge, drugs targeting glutamate signaling are not overly effective for the majority of OCD patients, with selective serotonin reuptake inhibitors (SSRIs)⁷ still being regarded as the first line pharmacotherapeutic intervention (Kellner (2022); see **paragraph 2.3.1** also). This is likely because the pathophysiology of OCD is complex, involving several biological and cognitive processes that interact to yield the typical picture of OCD; these may each be uniquely modulated by glutamate (Pittenger 2015). In contrast with the excitatory nature of glutamate, GABA is the primary inhibitory neurotransmitter in the brain (McCormick 1989). The inhibitory effect of GABA stands in opposition to the excitatory effects of glutamate (Karthik et al. 2020), a balance that must be maintained to prevent neuronal hyperexcitability, which is in turn associated with, among others, anxiety, impulsivity, hyperactivity, and psychosis (Bauer et al. 2018; Lydiard 2003; Nasir et al. 2020). With respect to OCD, significant reductions in orbito-frontal cortical GABA concentrations have been shown in OCD patients,

¹ gamma-aminobutyric acid

² metabotropic glutamate receptors

³ N-methyl-D-aspartate

⁴ α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

⁵ gamma-aminobutyric acid

⁶ obsessive-compulsive disorder

⁷ selective serotonin reuptake inhibitors

compared to healthy individuals (Zhang et al. 2016). Such findings arguably support theories of heightened cortico-striatal executive signaling as a potential neurobiological construct underlying OCD. That said, considering also the known relationship between anxiety and the expression of compulsive symptoms, it is worth mentioning that, as is true for glutamatergic modulation, GABAergic potentiation does not hold promise as a pharmacotherapeutic intervention (Nasir et al. 2020). To this, we will allude later in this chapter.

2.2.2.2 Dopamine

As explained earlier, dysregulation in the activity of the direct and indirect cortico-striatal pathways is often shown in OCD (Bartz and Hollander 2006; Keeler et al. 2014; Saxena and Rauch 2000). Since the planning and selection of goal-directed behavior simultaneously activate both pathways, executive conflict between thalamic activation and inactivation, is triggered. This is resolved by dopamine, which is released in parallel upon anticipating an expected goal-directed outcome. To explain, dopamine D₁ receptors are expressed on the direct pathway, while D₂ receptors are expressed on the striato-pallidal neurons of the indirect pathway (Berke 2018; Daubner et al. 2011; Denys 2004). When stimulated, D₁ receptors amplify signal propagation along the direct route which leads to thalamic activation (Denys 2004; Macpherson et al. 2014; Ting and Feng 2011). In comparison, activation of the D₂ receptors expressed on the indirect pathway, *inhibits* firing of these neurons, which resultantly inhibits the otherwise thalamus-inhibiting pathway (Freeze et al. 2013; Ting and Feng 2011). Thus, the anticipation and planning of goal-directed behavior in parallel with an increase in dopamine, shifts the executive balance in favor of behavioral activation (Ting and Feng 2011), thereby solving the apparent conflict between direct and indirect pathway activation.

These effects of dopamine on CSTC¹ functioning, and thus a potential role for its involvement in the expression of obsessive-compulsive behaviors, are reflected by the successful use of low-dose anti-dopaminergic drugs, e.g. haloperidol or risperidone (Grassi et al. 2020; Tost et al. 2006), in combination with SSRIs² to manage the condition (Grassi et al. 2020). However, since OCD³ is a multifaceted condition that is likely borne from heterogenous psychobiological mechanisms, such involvement is not consistent among patients, a frustrating fact highlighted by the inadequate treatment response of patients to currently available pharmacotherapeutic interventions (see **paragraph 2.3.1** also; Thamby and Jaisoorya (2019)).

¹ cortico-striatal-thalamo-cortical

² selective serotonin reuptake inhibitors

³ obsessive-compulsive disorder

2.2.2.3 Serotonin

Dysregulation of the serotonergic system is variably linked with most psychiatric disorders including OCD (Grove et al. 1996; Stein and Hollander 1997), anxiety disorders (Ressler and Nemeroff 2000), schizophrenia (Selvaraj et al. 2014), and major depression (Nautiyal and Hen 2017). In the brain, serotonin originates in the raphe nuclei which primarily comprise serotonergic neurons (Mohammad-Zadeh et al. 2008; Stein et al. 2019). From here, projections are directed to several other brain regions including the frontal cortex, hippocampus, amygdala, thalamus, and putamen (Kong et al. 2020; Mohammad-Zadeh et al. 2008). Serotonin acts on fourteen receptor subtypes; however, with respect to OCD, the serotonin 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors are the most studied (Sinopoli et al. 2017).

5-HT_{1A} receptors, which are expressed both pre- and post-synaptically in the raphe nuclei and limbic system (Katzung and Trevor 2012; Newman-Tancredi 2011), are mostly known to modulate anxiety (Garcia-Garcia et al. 2014; Niedzielak et al. 2020). Interestingly, 5-HT_{1A} receptor stimulation has been shown to exacerbate obsessive-compulsive like behavior (Goddard et al. 2008). Specifically, post-synaptic 5-HT_{1A} receptor activation increases dopamine release in the cortico-striatal projections (Blier et al. 1996; Haleem 2015), while presynaptic 5-HT_{1A} receptors in the raphe nuclei reduces serotonergic output (Altieri et al. 2013; Barnes and Sharp 1999). In concert, these effects result in the propagation of excessive CSTC¹ signaling (Ahmari and Dougherty 2015; Ahmari et al. 2013). Such a theory also agrees with our current understanding of how SSRIs² may attenuate obsessive-compulsive symptoms, that is, by increasing synaptic serotonin concentrations, which then down-regulate 5-HT_{1A} receptor expression (Blier and Ward 2003). 5-HT_{2A} receptors, which are abundantly expressed in the cortical and striatal regions, modulate dopaminergic neurotransmission (Kantrowitz 2020). Specifically, stimulation of the 5-HT_{2A} receptors increases cortico-striatal dopamine release which, in this way, also contributes to CSTC hyperactivation (Aznar and Hervig 2016). Last, 5-HT_{2C} receptors are expressed in the striatum, although to a lesser extent than the 5-HT_{2A} receptor subtype (Mengod Los Arcos 2011). However, in contrast to the effects of 5-HT_{1A} and 5-HT_{2A} receptors, 5-HT_{2C} receptor activation decreases dopamine release (Mengod Los Arcos 2011), highlighting a potential role for 5-HT_{2C} receptor agonists in the management of OCD³. In fact, evidence for the potential symptom-ameliorating role of 5-HT_{2C} receptor activation on compulsive symptom expression has been gained from both pre-clinical (Chou-Green et al. 2003) and clinical (Delgado and Moreno 1998; Gross-Isseroff et al. 2004; Moreno and Delgado 1997) data. Sadly, these findings have not translated into 5-HT_{2C}

¹ cortico-striatal-thalamo-cortical

² selective serotonin reuptake inhibitors

³ obsessive-compulsive disorder

receptor targeting pharmacotherapies for OCD, except in cases of augmentation strategies (Goddard et al. 2008), as will be highlighted later in this chapter.

Taken together and considering that SSRIs still are the mainstay of current anti-compulsive pharmacotherapeutic strategies, it is widely accepted that this neurotransmitter is a fundamental neurobiological role player in the condition. In fact, OCD is often described as a condition of hyposerotonergic functioning, since the chronic elevation of central serotonin concentrations results in symptom attenuation in most, though not all, patients (Wolmarans et al. 2013; Wood et al. 2018).

2.3 The treatment of OCD

2.3.1 Pharmacotherapy

As alluded to earlier in this literature overview, SSRIs are considered the first line pharmacotherapeutic agents for the treatment of OCD. These agents, e.g. fluoxetine, escitalopram, paroxetine and sertraline (Albert et al. 2018; Stein et al. 2019), act by blocking the serotonin transporter, thereby preventing the reuptake of serotonin from the synaptic cleft (Goddard et al. 2008). Their use is not exclusive to OCD though; drugs in this class are also used for the treatment of among others, major depressive disorder (MDD)¹ (Kupfer et al. 2012) and anxiety (Wehry et al. 2015). However, when used for OCD² and anxiety, higher doses and a longer duration of administration (i.e. 8 – 12 weeks) are typically used (Pittenger and Bloch 2014). Interestingly, there is some evidence that higher doses are associated with a greater initial clinical response, while moderate to lower doses seem more successful to prevent symptom relapse (Fineberg et al. 2007). Typically, higher doses are used if patients do not show signs of improvement after four weeks of SSRI³ treatment. This is achieved by incrementally increasing the dosage to a tolerable and effective dose (Lambert 2008). Notably, when anxiety is a comorbid diagnosis, the anxi-selective effect of SSRI treatment tends to be slower compared to when anxiety is diagnosed as a primary disorder, i.e., six to eight weeks (sometimes up to 24 weeks). This may point to a unique role for serotonergic processes under conditions of complex limbic and striatal interplay that are yet to be elucidated. Still, that SSRIs administered in this manner are effective for the treatment of both OCD and anxiety disorders, clouds an understanding of the relationship between these disorders in general, but also of their association with serotonergic perturbations (Stein and Stahl 2000).

In terms of treatment response, clinical evidence points to some phenotypes of the disorder responding better to SSRI intervention than others. For example, SSRIs are more effective for

¹ major depressive disorder

² obsessive-compulsive disorder

³ selective serotonin reuptake inhibitors

phenotypes such as symmetry/ordering and obsessive thoughts (Hazari et al. 2019; Mataix-Cols et al. 2005), while contamination and washing compulsions mostly respond better to exposure and response prevention (ERP¹; see below) (Buchanan et al. 1996; De Araujo et al. 1995). Interestingly, safety/checking OCD seems equally responsive to both pharmaco- and psychotherapy (Landeros-Weisenberger et al. 2010). Although not undisputed, such differences in treatment response arguably point to distinct psychobiological underpinnings of various obsessive-compulsive phenotypes, thereby highlighting areas of uncertainty that may elucidate key areas for future research.

Nevertheless, only 40 - 60 % of patients experience noticeable symptomatic relief when treated with SSRIs (Goddard et al. 2008; Kellner 2022; Stein et al. 2019). In SSRI refractory cases, several strategies can be followed. These include increasing the dose of the current SSRI being used, switching to another SSRI with a unique receptor binding profile (e.g. switching from sertraline to escitalopram), or augmenting the existing SSRI regimen with low-dose anti-dopaminergic agents (Lambert 2008). Indeed, the aforementioned explanation of how dopamine promotes goal-directed striato-cortical signaling, explains why anti-dopaminergic treatments are effective in some patients (Albert et al. 2018). In this regard, most currently available D_{1/2} or D₂ receptor antagonists, e.g. haloperidol, risperidone, or quetiapine are used, with the choice of drug being influenced mostly by clinician preference and adverse-effect profile (Fineberg et al. 2015). Unfortunately, this approach also yields inconsistent therapeutic results (Kellner 2022).

Given the complex neurobiological footprint of the disorder alluded to throughout this chapter, other pharmacotherapeutic approaches have also been explored. These include other serotonin-targeting antidepressants, e.g. the tricyclic antidepressants (TCAs)² and serotonin-noradrenalin reuptake inhibitors (SNRIs)³ (Pittenger and Bloch 2014). In the case of TCAs, clomipramine is the preferred choice, but due its potential for causing anticholinergic adverse effects, it is not used as a first-line intervention (Pittenger and Bloch 2014). With respect to the SNRIs, a response rate of 76 % have been shown in a small open-label investigation (29 patients) using venlafaxine (Hollander et al. 2003). However, due to the lack of more current double-blind, placebo-controlled trials, SNRIs are not recommended as monotherapy for OCD⁴ (Pittenger and Bloch 2014).

Considering the complex interplay between anxiety and compulsivity, which will also be afforded some attention in this work, attention must also be paid to anxiolytic interventions for the treatment of OCD. Benzodiazepines (e.g. lorazepam; LOR⁵) are mostly ineffective to treat patients with OCD (Robbins et

¹ exposure and response prevention

² tricyclic antidepressants

³ serotonin-norepinephrine reuptake inhibitors

⁴ obsessive-compulsive disorder

⁵ lorazepam

al. 2019). However, benzodiazepines are still frequently used in the early stages of treatment with other interventions to alleviate the anxiogenic effects of acute-onset SSRI¹ treatment and to facilitate adherence to ERP², which is known to be highly anxiogenic and associated with high patient drop-out rates (Hirschtritt et al. 2017). While the relationship between OCD and anxiety is well-known, our understanding of how anxiety and obsessive-compulsive processes interact, clearly needs further investigation.

2.3.2 Psychotherapy

As alluded to earlier in this work, and as will be referred to throughout this chapter, OCD is founded upon complex interactions between cognitive and neurobiological perturbations. As such, patients also respond favorably to cognitive and behavioral interventions, e.g. cognitive behavioral therapy (CBT)³ and ERP (O'Neill and Feusner 2015; Pittenger and Bloch 2014; Stein et al. 2019; Veale and Roberts 2014). Briefly, CBT is aimed at redefining the realistic context of a patient's obsessive-compulsive experiences and thoughts during therapist-guided discussions, whereas ERP involves exposure of individuals to the triggers of their obsessive-compulsive symptoms, while they are prevented from engaging in compulsive neutralizing behaviors (Pittenger and Bloch 2014; Veale and Roberts 2014). Given the nature of ERP⁴ intervention, patients experience a significant degree of distress during these sessions and patient compliance remains an obstacle (Abramowitz 2006). Further, its efficacy largely depends on the symptom subtype treated (Rufer et al. 2006), with especially patients suffering from intrusive thoughts associated with covert compulsions, e.g. excessive praying (Magee et al. 2012), showing poor therapeutic outcomes. In such scenarios, the so-called "rebound effect" might prevent optimal response. The "rebound" effect refers to a scenario when the accessibility of and importance ascribed to an unwanted thought or idea increases when a clinician attempts to suppress such a thought (Purdon 2004). Considering the above, psychotherapeutic interventions, like drug treatment, are also associated with suboptimal treatment outcomes in roughly 30 % of patients (Kellner 2022; Pittenger and Bloch 2014). Further, up to 50 % of patients present with lingering symptoms after intensive treatment (Kellner 2022; Pittenger and Bloch 2014).

¹ selective serotonin reuptake inhibitors

² exposure and response prevention

³ cognitive behavioral therapy

⁴ exposure and response prevention

2.4 A cognitive perspective: the role of anxiety and fear in OCD

As explained before, anxiety has always been and still is, regarded as a core component of OCD¹ (Nutt and Malizia 2006; Stein et al. 2010). However, since the publication of the DSM-V², OCD is no longer described as an anxiety disorder, rather as the archetype disorder in the recently added ‘Obsessive-Compulsive and Related Disorders’ (OCDs)³ chapter (APA 2013). The reasons for this decision were multifactorial, but mainly related to the fact that repetitive behaviors without the presence of a significant degree of anxiety, can be the main clinical feature of the condition (Nutt and Malizia 2006). However, the discussion is complex. We have explained earlier in this chapter that while OCD generally does not respond to classic anxiolytics, e.g. the benzodiazepines (Starcevic et al. 2016), the condition is responsive to high-dose SSRIs⁴, the same intervention which is also used to treat anxiety disorders (Wehry et al. 2015). Moreover, that compulsions generally arise in response to an inflated sense of anxiety which are borne from specific fears, worries or doubts, undoubtedly links OCD with anxiety and thus, attempts at improving our understanding of the condition should remain sensitive to the potential relationships between innate anxiety and compulsive symptom expression (Samuels et al. 2017; van Rijsoort et al. 2001).

Anxiety is primarily linked to abnormal processing of fear- and/or stress-provoking stimuli by the limbic system (de Brouwer et al. 2019; Nutt and Malizia 2006). In contrast, the repetitive motor and cognitive symptoms are primarily associated with dysfunctional cortico-striatal processes (Ting and Feng 2011). Interestingly, the cortico-striatal pathways implicated in the expression of obsessions and compulsions show a high degree of anatomical and functional overlap with parts of the limbic system (Nutt and Malizia 2006); this, we explained earlier. Thus, it stands to reason that patients suffering from OCD⁵ might also present with distinct psychobiological perturbations in the limbic system. While most persons experience distressing thoughts related to obsessive themes, e.g. fears of self-harm, OCD patients ascribe an inflated sense of importance to these thoughts, believing that the feared outcome is more likely to transpire and that engagement in compulsive neutralizing rituals would prevent such outcomes (Abramowitz and Reuman 2020; Cervin et al. 2021; Moulding and Kyrios 2006). Such inflation of everyday fears likely interacts with anxiety-related processes in a bidirectional manner. On the one hand, inflated anxiety can trigger compulsive engagement, while the expression of compulsive behavior, knowing that such actions are irrational and futile (Lewin et al. 2010; Matsunaga et al. 2002), can also *cause* and exacerbate anxiety (Logan 2007). These theories form the

¹ obsessive-compulsive disorder

² Diagnostic and Statistical Manual of Mental Disorders, 5th edition

³ obsessive-compulsive and related disorders

⁴ selective serotonin reuptake inhibitors

⁵ obsessive-compulsive disorder

basis of CBT¹, but more specifically ERP², in which exposure to a specific object of obsession is purposefully aimed at causing a high degree of distress in the patient undergoing therapy. As such, cognitive neuropsychological theory dictates that either implicit (i.e. borne from the 'self') or explicit (sensory) activation of the limbic system is at least in part responsible for triggering compulsive (read, cortico-striatal) processes (Campbell et al. 1999; Catani et al. 2013; Joseph 1992).

It should then be considered whether OCD patients, or at least some individuals suffering from the condition, present with a lower anxiogenic threshold towards everyday stimuli. To explain, OCD patients are not excessively anxious in general. Rather, they often experience distress due to specific concerns. In other words, when a patient is confronted with everyday stimuli that are thematically related to their specific symptom phenotype, they tend to inflate the fear associated with the stimuli and overestimate the potential consequences of not responding to such stimuli (Wheaton et al. 2012). For example, patients experiencing repugnant thoughts (e.g. ideation of harming loved ones), fear that they might act on these intrusive thoughts (Wheaton et al. 2012), while such fears are absent in those suffering from, for example, symmetry/ordering OCD. Thus, it remains unknown whether anxiety in patients suffering from OCD is implicitly generated in response to certain external stimuli, or whether a lower threshold of anxiety sensitivity triggers obsessive-compulsive symptoms.

2.5 The adenosinergic system – a potentially valuable target for investigation in OCD

Considering the overall suboptimal response of OCD³ to current treatments and highlighting the growing body of evidence pointing to distinct psychobiological processes underlying various obsessive-compulsive phenotypes, a dire need exists for an improved understanding of the condition. Towards this goal, investigations of the adenosinergic system in animal model systems of compulsive-like behavioral repetition are fruitful.

2.5.1 General background

Adenosine is a naturally occurring nucleoside that functions as a metabolic intermediate throughout the mammalian body (Chen et al. 2014). It is the primary component of the energy-transferring molecule, adenosine triphosphate (ATP)⁴ (López-Cruz et al. 2017). However, apart from being essential for many energy transfer processes, adenosine also acts as an important neuromodulator (van Calker et al. 2019). Concentrations of adenosine in the central nervous system are primarily

¹ cognitive behavioral therapy

² exposure and response prevention

³ obsessive-compulsive disorder

⁴ adenosine triphosphate

regulated based on the balance between the rate of energy expenditure and supply (Fredholm et al. 2005). Notably, increased neural activity and reduced oxygen supply increase adenosine concentrations (Chen et al. 2014; Fredholm et al. 2005). Thus, given its ability to restore energy imbalances, adenosine is regarded as a retaliatory metabolite (Fredholm et al. 2005). Adenosine differs from other classic neurotransmitters in that it is not stored in synaptic vesicles, is not released in response to an action potential, and does not predominantly act in the synapses to transfer information unidirectionally from the pre- to the postsynaptic membranes (Chen et al. 2014). In fact, its distribution is rather ubiquitous, while much of its neuromodulatory actions are not yet elucidated.

Apart from its known functions in the energy transport chain, adenosine controls neuronal excitability, the release of different neurotransmitters such as glutamate, GABA¹, acetylcholine, and dopamine, and modulates synaptic development and function (Sebastiao and Ribeiro 1996). To this end, it exerts its actions via binding to four receptor subtypes, i.e. A₁, A_{2A}, A_{2B} and A₃. Of these, the most relevant receptor subtype with respect to obsessive-compulsive symptom manifestation is the A_{2A} receptor, known for its ability to influence both the motor and cognitive domains in patients suffering from Parkinson's disease (Cieślak et al. 2008; Schwarzschild et al. 2006). Further, considering that istradefylline (ISTRA)², a selective A_{2A} receptor antagonist, has shown promise as both an anxiolytic motor restorative intervention (Torti et al. 2018; Vorovenci and Antonini 2015), and that OCD involves dysfunctions across both of these domains, the adenosinergic system may be a realistic target for investigation in a naturalistic model system of compulsive-like persistence.

2.5.2 Adenosinergic neurotransmission

Since it became known that adenosine receptors affect a variety of signal-transducing systems, the approach to describe its receptor subtypes based on their ability to modify adenylyl cyclase activity was replaced by a classification system based on the actions of various agonists and antagonists at the respective proteins (van Calker et al. 2019). Classically viewed, the A₁ and A₂ receptor subtypes are distinguished by their ability to inhibit and stimulate adenylyl cyclase, respectively (Chen et al. 2014; Fredholm et al. 2005; van Calker et al. 2019). Both the A_{2A} and A_{2B} receptors are G_s-coupled in the periphery; the A_{2A} receptor are also G_{olf} coupled in the striatum (Jacobson 2013; Jacobson and Gao 2006; van Calker et al. 2019), maybe even more so than its coupling to G_s (Corvol et al. 2001; Svenningsson et al. 1999). In contrast, A₁ receptors are G_{i/o} coupled, thus mediating the inhibition of adenylyl cyclase. In broad terms, the neuromodulatory actions of adenosine are realized by

¹ gamma-aminobutyric acid

² istradefylline

influencing the release of neurotransmitters upon A_{2A} receptor stimulation (Chen et al. 2014; Fredholm et al. 2003), and the inhibition thereof by A_1 receptor activation (Fredholm et al. 2003).

2.5.3 With respect to the present body of work: insights into A_{2A} receptor function

Significant progress has been made to clarify the role of A_{2A} receptor signaling (Chen et al. 2014) in the central nervous system. While A_{2A} receptors are widely distributed throughout the brain, its expression in the dorsal and ventral striatum is highest (Chen et al. 2001; Domenici et al. 2019; Rosin et al. 2003). Extrastrially, the A_{2A} receptor is scarcely expressed in certain structures of the limbic system. Adenosine is a modulator for dopaminergic neurotransmission (Chen 2014; Tanimura et al. 2010). Co-localized adenosine and dopamine receptor dimers, i.e. A_{2A}/D_2 -complexes on the indirect pathway, display functional antagonistic interactions (Fredholm et al. 2005). In other words, the activation of A_{2A} receptors decreases the binding affinity and intrinsic activity of dopamine at the D_2 receptor (Ferre et al. 1991; Stockwell et al. 2017). Therefore, a dynamic state of functional opponency, whereby adenosinergic stimulation decreases the activity of dopamine at its respective receptors (Ferré et al. 1996), is maintained. Given the focus of the present investigation, i.e., exploring the effects of adenosine A_{2A} receptor antagonism in a model of compulsive-like behavioral persistence, a brief explanation of adenosinergic A_{2A} signaling in the potential neurocognitive processes that might underlie the expression of OCD¹, is necessary.

Goal-directed behaviors are borne from the evaluation of certain expected outcomes which guides our ability to flexibly adjust our behavioral output in response to contextual and environmental change (Gillan et al. 2011; Li et al. 2018). In contrast, habits evolve after repeated exposures to the same contextual stimulus that necessitates a uniform behavioral output. This produces an automatic response evoked by external and internal triggers while performing routine procedures at a lower necessitated cognitive resource (Li et al. 2016; Li et al. 2018). For the optimal execution of behavioral tasks, a balance and seamless transition between flexible goal-directed behaviors and habit-like behaviors should be established. Indeed, dysregulation in this relationship gives rise to impaired goal-directed decision making and increased stereotypical behavior (Furlong et al. 2017; Li et al. 2018; Li et al. 2020). Importantly, A_{2A} receptor expression within the dorsomedial and dorsolateral striatum is associated with the control of balance between goal-directed and habit-like behavioral output (Li et al. 2016) and in this regard A_{2A} receptor antagonists, e.g. ISTR², may show promise as motor restorative interventions (Coelho et al. 2014; de Brouwer et al. 2021; van Calker et al. 2019).

¹ obsessive-compulsive disorder

² istradefylline

It follows that the search for novel adenosine-modulating agents, notably A_{2A} receptor antagonists, has gained significant interest over the past three decades (Armentero et al. 2011; Müller and Jacobson 2011; Preti et al. 2015). In addition, our understanding of the functions and effects of A_{2A} antagonism has also improved (Stone et al. 2009). Therefore, considering the involvement of both limbic and cortico-striatal processes in OCD¹ 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. In fact, within a framework where an imbalance between goal-directed and habit-like behavioral control is thought to be a possible neurocognitive construct underlying the rigid motor behaviors associated with OCD (Burguiere et al. 2015; Robbins et al. 2012), findings that antagonism of striatal A_{2A} receptors attenuates the expression of rigid habit-like behaviors and improve goal-directed behavioral output (Li et al. 2018), are highly informing. Furthermore, because anxiety is a frequent trigger for obsessions and compulsions, the anxiolytic effects of A_{2A} receptor antagonism may to some extent contribute to symptomatic relief. Therefore, understanding how A_{2A} receptor antagonists may function, could be fruitful to clarify the involvement of the adenosinergic system within the context of OCD.

2.6 The present model system: naturalistic compulsive-like behavioral expression in deer mice (*Peromyscus maniculatus bairdii*)

2.6.1 General background and validation

When considering the development and application of animal model systems, it is important to consider their main purpose. Animal models have been described by McKinney (2012) as “*experimental preparations developed in one species for the purpose of studying phenomena occurring in another.*” However, while the overall purpose of animal models is to provide suitable translational frameworks for the study of among others, human-specific psychobiological processes, progress especially in the field of neuropsychiatric research is slow (Stanford 2020). It is therefore vital that preclinical research as a collective, should strive to conceptualize, apply, and explore experimentation on animals appropriately within the context of the translational questions asked.

With respect to compulsivity, such a foundation will arguably be built on the demonstration of specific behavioral expressions (e.g. repetitiveness, excessiveness), neurobiological traits (e.g. dopaminergic or serotonergic involvement), or evidence for relevant responses to drug or other therapeutic interventions (e.g. showing attenuation following SSRI² intervention). Collectively, these three

¹ obsessive-compulsive disorder

² selective serotonin reuptake inhibitor

principles can be defined as face, construct, and predictive validation criteria (Alonso et al. 2015; Willner 1986) and which animal models need to meet to be relevant frameworks for investigation.

Specific subpopulations of deer mice (*Peromyscus maniculatus bairdii*) spontaneously develop persistent behaviors, which have collectively been validated as a naturalistic model system for the study of compulsive-like processes. Specifically, deer mice present with three main compulsive-like phenotypes, i.e. high motor stereotypy (HS¹; i.e. vertical jumping and pattern running) (Korff et al. 2008), large nest building (LNB)², and high marble-burying (HMB)³ (Wolmarans et al. 2013; Wolmarans et al. 2018; Wolmarans et al. 2016). Such behaviors develop without any triggering intervention, other than mice being bred, reared, and housed under standard laboratory conditions (Korff et al. 2008).

In terms of face validity, up to 45 % of laboratory-housed deer mice exhibit these persistent behaviors. Not only do they emerge in certain animals exclusively, but also occur in animals of both sexes between the ages of 10 and 12 weeks. Irrespective of phenotype, said behaviors are purposeless, persistent, and repetitive, in this regard resembling clinical compulsivity. In terms of its representation of compulsive-like processes on a neurobiological level, deer mice presenting with HS behavior also show hyposerotonergic signaling as evinced by a significantly lower level of striatal serotonin transporter expression, compared to non-stereotypical controls (Wolmarans et al. 2013). Further, more recent data implicated distinct patterns of immune system involvement in LNB⁴-expressing mice (Scheepers et al. 2020), as well as compositional changes in the gut-microbiota composition of LNB-, compared to normal nesting (NNB)⁵ mice. Also, perturbations in the metabolism of the essential amino acid, tryptophan (which is required to produce serotonin (Jenkins et al. 2016)) has also been described (Karsten 2022). Such findings align well with clinical compulsivity since OCD⁶ is thought to be a condition founded upon dysfunctional serotonergic processes (Goddard et al. 2008; Karsten 2022). Last, compulsive-like behavioral expression has also been validated against its response to clinically used anti-compulsive interventions. As such, LNB and HS, but not HMB, respond well to chronic (28 days), high dose (50 mg/kg/day), oral escitalopram treatment (Wolmarans et al. 2013). This suggests that different neurobiological mechanisms might underly these behaviors within the model species (Wolmarans et al. 2018).

¹ high motor stereotypy

² large nest building

³ high marble-burying

⁴ large nest building

⁵ normal nest building

⁶ obsessive-compulsive disorder

2.6.2 A closer focus on large nest building behavior

In this work, closer attention will be afforded to the potential involvement of adenosinergic signaling in the expression of nesting, and notably so LNB expression. From an evolutionary perspective, nest building is an intrinsic component of the normal behavioral repertoire of many animal species, including small mammals (Smithers 1983). To build adequate and well-constructed nests is necessary for protection against adverse weather conditions, safety from predators (Smithers 1983), and to provide a suitable environment for breeding and nursing. Competition between different species or between different animals of the same species also plays a significant role in the architecture, distribution, and overall success of nests as breeding habitats. Furthermore, environmental and seasonal fluctuation causes animals to adapt their nesting behavior, since different climates are associated with temporal changes in the availability of various nesting materials or because nesting behavior itself may be responsive to changes in temperature (Beintema and Muskens 1987; Lynch 1980; Wolton 1985). However, what remains indisputable is that rather than not building nests, most nesting animals, including rodents, present with an innate motivational drive to nest, irrespective of the circumstances. In other words, nesting behavior can be understood as a necessary form of coping behavior, which although innate, is modifiable by circumstantial change.

Against this background, it could be expected that all laboratory-housed animals of the same species uniformly express nest building behavior which could at most differ between sexes (Deacon 2012; Jirkof 2014). This would be a valid conclusion, since all subjects of a laboratory-housed rodent species are bred, reared, and maintained under identical conditions. Interestingly, this is not the case. A robust body of research has demonstrated over time that vast inter-individual variance characterizes the nest building expression of different animals of the same species (Greene-Schloesser et al. 2011; Wolmarans et al. 2016). Further, larger nesting scores do not cluster based on sex, pointing to the likelihood that instead of being modulated by the factors referred to above as well as other evolutionary influences, nesting behavior as expressed in the laboratory is subject to psychobiological intervention (de Brouwer et al. 2020). However, an important question should be asked here: What is ‘normal’? This should be considered carefully, since ‘abnormal’ behavior in laboratory-housed animals is a subjective concept. One can argue that against the background of OCD¹, persistence and repetition should be key. For example, if animals engage in a single bout of ‘large’ nesting behavior over the course of several repeated assessments, such an animal will likely not be considered to express ‘compulsive-like’ behavior. Also, the behavior should be perceived as being excessive and time-consuming, in that, when measured against the nesting score distribution of the larger population, compulsive-like behavior will be characterized by nesting scores that are consistently

¹ obsessive-compulsive disorder

higher than the average scores generated by the majority of subjects in the study population, especially since there is no apparent function for or advantage of larger than ‘normal’ nests in the laboratory.

In this investigation, we will explore the association between LNB¹ expression and general anxiety in a modified version of the open field, i.e. an ‘unsafe’ and anxiogenic mirrored and white-floored open space (Carola et al. 2002; Fuss et al. 2013). Further, considering the aforementioned overview of adenosinergic involvement in executive processes, we will investigate the possibility that an adenosine A_{2A} receptor antagonist, i.e. ISTRA², could engender a certain degree of control over the expression of LNB, either by influencing an associated anxiety-like construct, or merely by influencing the motor behavioral output of LNB- as opposed to NNB³-expressing animals. Indeed, since we regard LNB as an excessive behavioral phenotype that is dissociated from a normal goal-directed control mechanism, we believe that modifying the balance between the direct and indirect striatal pathway activity, might be fruitful in LNB-expressing animals. While such a modification in said balance can only be inferred indirectly from the behavioral data observed, our findings will be lent strength by a parallel neurobiological investigation of the striatal A_{2A} receptor expression levels in LNB- compared to NNB-expressing animals.

This investigation will be useful for three primary reasons. First, it will be valuable to determine the extent to which LNB⁴ is related to generalized anxiety. Second, by differentiating LNB from NNB⁵ behavior on a neurobiological level in terms of adenosinergic involvement, important insights can be gained with respect to nesting behavior in the model organism. The most important of these would be to confirm whether NNB and LNB behavior represent distinct ‘normal’ and ‘abnormal’ phenotypes, respectively and whether targeting adenosine signaling might prove to be a useful avenue for continued study into other related treatment alternatives. Last, given the evolutionary function of nesting behavior, investigations into NNB and LNB behavior as expressed by deer mice, will bring us closer to a translationally significant model, since it would be possible to test several hypotheses pertaining to the manner in which innate behaviors evolve to become ‘abnormal’ (Fineberg et al., 2010; Robbins et al., 2012; Robbins et al., 2019).

¹ large nest building

² istradefylline

³ normal nest building

⁴ large nest building

⁵ normal nest building

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3 Journal article

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)¹ 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)² behavior in deer mice, anxiety and adenosinergic processes. From an initial pool of 120 adult deer mice, 34 NNB³- and 32 LNB-expressing mice of both sexes were selected and exposed to either a normal water (wCTRL)⁴ or vehicle control (vCTRL)⁵, lorazepam (LOR)⁶ or istradefylline (ISTRA)⁷ 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 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 obsessive-compulsive disorder

² large nest building

³ normal nest building

⁴ normal water control

⁵ vehicle control

⁶ lorazepam

⁷ istradefylline

3.1 Introduction

Obsessive-compulsive disorder (OCD)¹ is characterized by recurring intrusive thoughts (obsessions) and rigid repetition of certain mental or behavioral actions (compulsions) that are often associated with anxiety (Abramovitch and Cooperman 2015; Citkowska-Kisielewska et al. 2020), even though OCD is no longer classified as an anxiety disorder (APA 2013). In terms of neurobiology, dysfunctional connectivity in distinct cortico-limbic (Shephard et al. 2021; Thorsen et al. 2019; Via et al. 2014) and cortico-striatal-thalamic circuits has been shown (Ting and Feng 2011; Vaghi et al. 2017). Briefly, the limbic system, via amygdalar, hippocampal, and ventromedial prefrontal-cortical (vmPFC)² signaling, is responsible for emotional regulation, fear processing and anxiety sensitivity. 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 (Wood and Ahmari 2015). Two 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 D₁ receptors, the indirect pathway expresses D₂ receptors (Keeler et al. 2014). 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 (Nakanishi et al. 2014) and that overactivity in the former is associated with persistent behavioral output (Saxena and Rauch 2000). However, such a definite conclusion should be drawn with caution. For example, in a meta-analysis of functional neuroimaging data, Whiteside et al. (2004) reported no significant differences between the direct and indirect functional connectivity of OCD patients and healthy controls, while Calzà et al. (2019) reported *increased* functional connectivity in the indirect, instead of the direct pathway. In a recently proposed model, i.e. 'prepare and select' (Keeler et al. 2014), it was proposed that D₁ receptor activation is vital for behavioral sequence initiation, whereas the D₂-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. In extension, taking into consideration theories of functionally opponent interactions between dopamine and serotonin (Balasubramani et al. 2015; Daw et al. 2002; Fischer and Ullsperger 2017; Rogers 2011), hyposerotonergic signaling in the cortico-striatal pathways is thought

¹ obsessive-compulsive disorder

² ventromedial prefrontal-cortical

to be related to the manifestation of OCD¹ (Lissemore 2018). Unfortunately, despite convincing evidence that selective serotonin reuptake inhibitors (SSRIs)² are modestly successful in the treatment of OCD (Pittenger and Bloch 2014), direct evidence implicating serotonergic dysfunction in the causality of the disorder is lacking (Goodman et al. 2021).

Adenosine, the primary component of the energy-transferring molecule, adenosine triphosphate (ATP)³ (López-Cruz et al. 2017) acts as an important neuromodulator via its actions on adenosine A₁ and A₂ receptors. With respect to its effects in the central nervous system, A₁ receptors are expressed in the cortex, hippocampus and cerebellum (Schindler et al. 2001) where it generally causes hyperpolarization of neuronal membranes (van Calker et al. 2019). The functions of the A₂ 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 (van Calker et al. 2019). Specifically, scarcely expressed amygdalar and hippocampal A_{2A} receptors modulate anxiety processing and memory consolidation (Coelho et al. 2014; Rau et al. 2015), although the exact mechanisms underlying these effects remain largely unknown (Yamada et al. 2014). In turn, striatal A_{2A} receptors modulate behavioral output and regulate motor control (Chen and Cunha 2020). In the striatum, adenosine and dopamine A_{2A}-D₂ receptor dimers are expressed on the neuronal bodies of the indirect pathway (Ferré et al. 1996; Ferre et al. 1991). Such co-expression facilitates functional opponency, whereby adenosinergic stimulation decreases the activity of dopamine at the D₂ receptor (Ferré et al. 1996). In this regard, A_{2A} receptor antagonists, e.g. istradefylline (ISTRA)⁴, may show promise as a motor restorative intervention (Coelho et al. 2014; de Brouwer et al. 2021; van Calker et al. 2019). 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)⁵ behavior. Although nesting is a common rodent behavior, it shows significant intra-species variance, with some mice expressing excessive and persistent nesting behaviors. Being reminiscent of clinical compulsive-like behavioral engagement, LNB behavior aligns conceptually well with safety/perfectionism OCD (Hoffman and

¹ obsessive-compulsive disorder

² selective serotonin reuptake inhibitors

³ adenosine triphosphate

⁴ istradefylline

⁵ large nest building

Rueda Morales 2009). Further, LNB, but not normal nest building (NNB)¹ behavior responds favorably to high-dose, chronic oral exposure to the SSRI², escitalopram (Wolmarans et al. 2016). Still, it remains unknown whether LNB³ 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.

3.2 Materials and methods

3.2.1 Study layout

The present investigation comprised three phases of study, viz. 1) baseline screening and behavioral categorization, 2) normal water control (wCTRL)⁴, vehicle control (vCTRL)⁵, or drug exposure and 3) post-exposure behavioral assessment and neurobiological analysis (**Fig. 3-1**).

3.2.2 Mice

Since only 25 – 30 % of deer mice express LNB behavior, a total of 120 deer mice (both sexes; aged 10 – 12 weeks at the onset of investigation), were bred, reared, and housed at the North-West University (NWU)⁶ 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 NNB⁷- 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 (NWU Animcare Ethics Approval No.: **NWU-00422-21-A5**), 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 (de Brouwer et al. 2020). A piece of white polyvinyl chloride pipe (10 cm (l) x 4 cm (Ø)) and paper towel were provided as a form of environmental enrichment and nesting material (except during periods of nesting assessment), respectively.

¹ normal nest building

² selective serotonin reuptake inhibitor

³ large nest building

⁴ normal water control

⁵ vehicle control

⁶ North-West University

⁷ normal nest building

3.2.3 Pre- and post-exposure nest building assessment

To classify mice into either the NNB¹- or LNB²-expressing cohort, we applied the protocol of Wolmarans et al. (2016) 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 (Wolmarans et al. 2016). 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. 3-2**). 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. 3-2**). The 34 NNB- and 32 LNB-expressing mice were divided into the different drug-exposure groups (see below) and retested during the last seven days of the 28- or 7-day exposure periods (**Fig. 3-1**).

3.2.4 Drugs

Lorazepam (LOR³; Aspen Pharmacare, Qgeberha, South Africa) and ISTR⁴A⁴ (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 %) (Fresenius-Kabi 2016; Rotenberg et al. 1991). LOR (Fahey et al. 2006; Wolmarans et al. 2022) and ISTRA (de Brouwer et al. 2021) 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 (Aschhoff et al. 2000; de Ridder et al. 2022). Liquid intake was recorded

¹ normal nest building

² large nest building

³ lorazepam

⁴ istradefylline

daily to confirm drug intake (data not shown but represented in **Addendum A**). Normal water (wCTRL¹; NNB²: $n = 10$, 5 females; LNB³: $n = 9$, 4 females) or the vehicle emulsion (vCTRL⁴; 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.

3.2.5 Mirrored open-field anxiety assessment

On the last day of the post-exposure nesting assessment period (exposure day 7 or 28), 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 (Carola et al. 2002; Wolmarans et al. 2022). 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 x 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 (Carola et al. 2002; Reddy and Kulkarni 1997). 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 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 arenas 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 (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.

3.2.6 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}

¹ normal water control

² normal nest building

³ large nest building

⁴ vehicle control

receptor expression profiles of NNB¹- and LNB²-expressing mice, the striata of wCTRL³-exposed mice were removed on ice, snap frozen in liquid nitrogen and stored at -80 °C until further analysis.

3.2.7 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. (1986) and Janse van Rensburg et al. (2019). Three assays were performed per behavioral phenotype. Briefly, striatal tissue from three same-phenotype mice were pooled to perform one assay. As such, a total of nine mice were used per phenotype to complete three binding assays per phenotype (**Table 3-1**). Pooled tissues were added to ice cold buffer (50 mM Tris-HCl, containing 10 mM MgCl₂; pH adjusted to 7.7) to constitute a 100 volumes dilution (MgCl₂ was included to increase radioligand binding and decrease non-specific binding). Subsequently, the pooled tissues were homogenized (Polytron®, setting 6, 5 sec), 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 was added to the resulting pellets and finally homogenized. All procedures were performed on ice or in a cold room (3 – 4 °C).

Continue next page .../...

¹ normal nest building

² large nest building

³ normal water control

TABLE 3-1: 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

For the purpose of the current assay, a concentration range of eight points were used, i.e. between 0.5 and 20 nM of the radioactive ligand ^3H -5'-ethylcarboxamido adenosine (^3H -NECA¹) (specific activity: 29.6 Ci/mmol; 25 μL added per concentration). Binding of ^3H -NECA to A_1 receptors were blocked by the addition of 20 μL N^6 -cyclopentyladenosine (CPA²; 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 stopped 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, 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). The vials were left for 2 h after which it was subjected to scintillation counting. 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).

¹ 5'-ethylcarboxamido adenosine

² N^6 -cyclopentyladenosine

3.2.8 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)¹ 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 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 variable, while phenotype and drug exposure were set as independent variables. Where applicable, Bonferroni post-hoc analysis was applied for pairwise comparisons of group means. Where data were pooled across exposure or phenotype, Mann-Whitney U-tests were applied to compare the mean ranks of the different groups. 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 NNB²- and LNB³-expressing mice were analyzed by means of one-tailed, unpaired *t*-tests. Statistical significance was set at $p < 0.05$ for all analyses.

3.3 Results

3.3.1 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, except for the total distance traveled, where phenotype had a significant main effect on the total distance traveled in the open field arena [Fig. 3-3A; $F(1, 58) = 4.93$, $p = 0.03$], 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. 3-3B; $U = 388$, $p = 0.045$, $d = 0.5$).

3.3.2 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. 3-4. A significant exposure-phenotype interaction impacted the result [$F(3, 57) = 4.06$, $p = 0.011$], 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

¹ coefficients of variance

² normal nest building

³ large nest building

post-expression nesting scores of ISTRA¹-exposed (77.3 ± 76.3 %) compared to wCTRL²- (-10.8 ± 22.8 %, $p = 0.0004$) and LOR³-exposed (-17.9 ± 42.6 %, $p = 0.0002$) NNB⁴-expressing mice. Further, the change in average baseline nesting scores of ISTRA-exposed NNB-expressing mice, also differed significantly from that of ISTRA-exposed LNB⁵-expressing mice (77.3 ± 76.3 % vs. 1.2 ± 27.8 %, $p = 0.0033$).

3.3.3 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. 3-5A**. The measured K_D values for ³H-NECA⁶ are represented in **Fig. 3-5B**. 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 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, $p = 0.2$, $d = 1.12$), 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.

3.4 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 bolstered the expression of NNB, 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 (Greene-Schloesser et al. 2011; Hoffman and Rueda Morales 2009; Lustberg et al. 2020; Wolmarans et al. 2016). Considering that clinical compulsivity is varyingly associated with limbic and cortico-striatal involvement (Shephard et al. 2021), 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 mechanisms might be fruitful. Adenosine, via its actions on A_{2A} receptors, modulates the activity of associated, dimerized dopamine receptors in the indirect, D₂-expressing striatal pathway (Fuxe et al. 2010). Of specific relevance for this investigation, the binding of adenosine or similar

¹ istradefylline

² normal water control

³ lorazepam

⁴ normal nest building

⁵ large nest building

⁶ 5'-ethylcarboxamido adenosine

agonists to adenosine A_{2A} receptors *decreases* the affinity of the associated dimerized dopamine D₂ receptor for its endogenous ligand, thus blunting subsequent dopaminergic signaling in the indirect pathway (Fuxe et al. 2010). Conversely, antagonists of adenosine receptors appear to institute the opposite effect, i.e. increasing the affinity of the associated dopamine receptors (Prasad et al. 2021). Since dysregulated activity in the direct and indirect cortico-striatal pathways is believed to be associated with repetitive behavioral expression (Nakanishi et al. 2014; Saxena and Rauch 2000), including compulsions (Keeler et al. 2014; Shephard et al. 2021), modulation of adenosinergic signaling might provide additional insights into the neurobiological architecture of LNB¹-expressing mice.

Concerning the first main finding, which broadly shows that NNB²- and LNB-expressing mice show similar behaviors 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 (Carola et al. 2002). Of all the assessed parameters, only the total distance travelled differed between the phenotypes, irrespective of exposure group (**Fig. 3-3**). However, whereas LNB-expressing mice traveled shorter distances in the open field on average, the reported effect size is small (0.5). Further, if one outlier was to be removed from the NNB cohort (NNB-LOR³; **Fig. 3-3**), 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 (Wolmarans et al. 2022). 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.

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 ISTRA⁴, but not control or LOR, bolstered the nest building behavior of NNB mice (**Fig. 3-4**). Strikingly, LNB behavior remained unresponsive to ISTRA and all other interventions, while neither NNB-, nor LNB-expressing mice showed any change in anxiety-like behavior as a function of drug exposure. These

¹ large nest building

² normal nest building

³ lorazepam

⁴ istradefylline

data are highly informative, especially given the overwhelming, though statistically insignificant, increase in the striatal A_{2A} receptor density (B_{max}) of mice in the LNB¹ cohort, relative to the NNB² cohort (**Fig. 3.5A**; $d = 1.12$). Further, the A_{2A} receptors of LNB-expressing mice showed a significantly lower affinity (K_b) for ^3H -NECA³ (**Fig. 3-5B**; $p = 0.02$, $d = 3.0$). 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 (Fuxe et al. 2010). 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 ^3H -NECA, is bolstered by ISTRA⁴, in the absence of parallel changes in anxiety scores. This is important since the inflation in the nesting scores of NNB-expressing mice can likely not be ascribed to the modulation of underlying anxiety. In fact, 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)⁵ circuit. That LNB-expressing mice, despite their level of A_{2A} receptor expression did not respond in a similar manner to NNB-expressing mice, would support a conclusion that LNB behavior is linked with lower adenosinergic sensitivity in the indirect pathway of the CSTC circuitry, 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 (Powell et al. 1999). 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 (Presti and Lewis 2005). Further, when assessed over the short-term, high stereotypical behavior responded favorably to acute adenosinergic agonist administration (Tanimura et al. 2010). In contrast, we have shown that high stereotypy attenuates after chronic exposure to ISTRA (de Brouwer et al. 2021), 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.

¹ large nest building

² normal nest building

³ ^3H -5'-ethylcarboxamido adenosine

⁴ istradefylline

⁵ cortico-striatal-thalamo-cortical

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 NNB¹- and LNB²-expressing mice is not always possible, as shown in **Fig. 3-2**. 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. Further, 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 an anxiogenic space, was striking. Mice of this species seem to be relatively risk-engaging compared to other strains, e.g. DBA/2J mice, that have shown significant anxiety-like behavior in the mirrored open field (Paterson et al. 2010). In contrast, mice of the *Peromyscus* genus are likely less risk-averse, 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.

Continue next page.../...

¹ normal nest building

² large nest building

3.5 Conclusion

In this work, we explored the potential associations between compulsive-like LNB¹ 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, ISTR², would abrogate the expression of LNB. In summary, our findings indicate that NNB³ and LNB behavior are not distinctly associated with measures of generalized anxiety and that ISTR-induced changes 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.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Author Contributions

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. 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. and D.W.W.

¹ large nest building

² istradefylline

³ normal nest building

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Figures

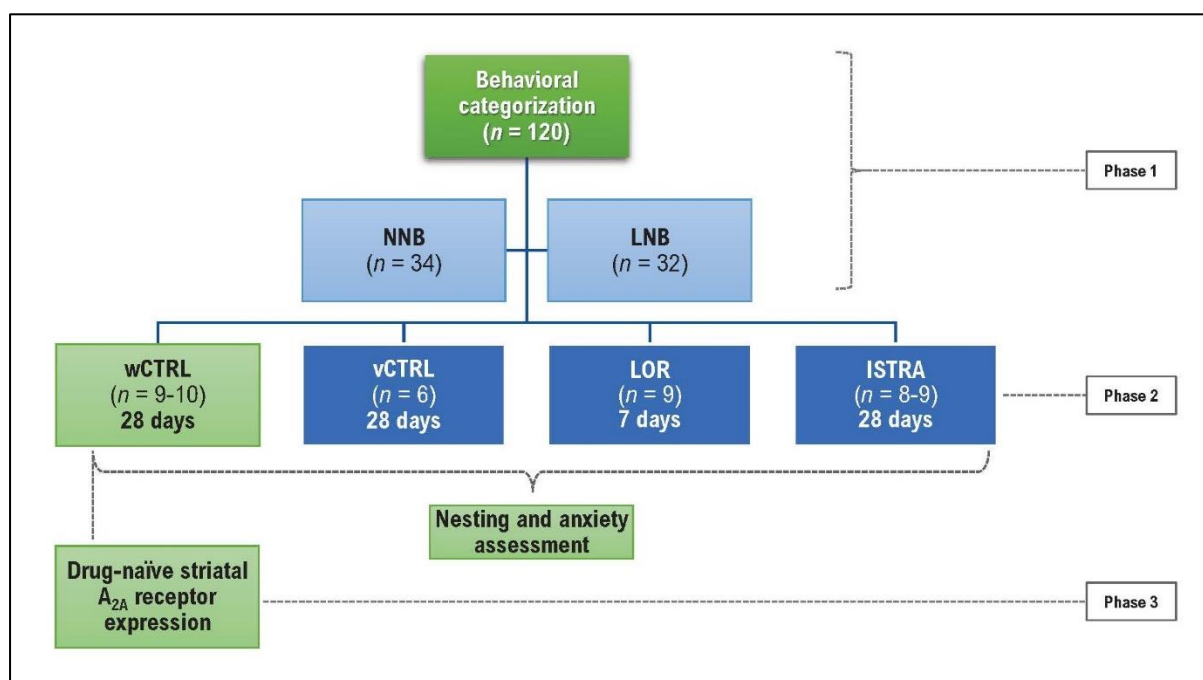


FIGURE 3-1: SCHEMATIC REPRESENTATION OF THE STUDY LAYOUT

NNB: NORMAL NEST BUILDING; LNB: LARGE NEST BUILDING; wCTRL: NORMAL WATER CONTROL; vCTRL: VEHICLE CONTROL; LOR: LORAZEPAM; ISTRA: ISTRADefylline

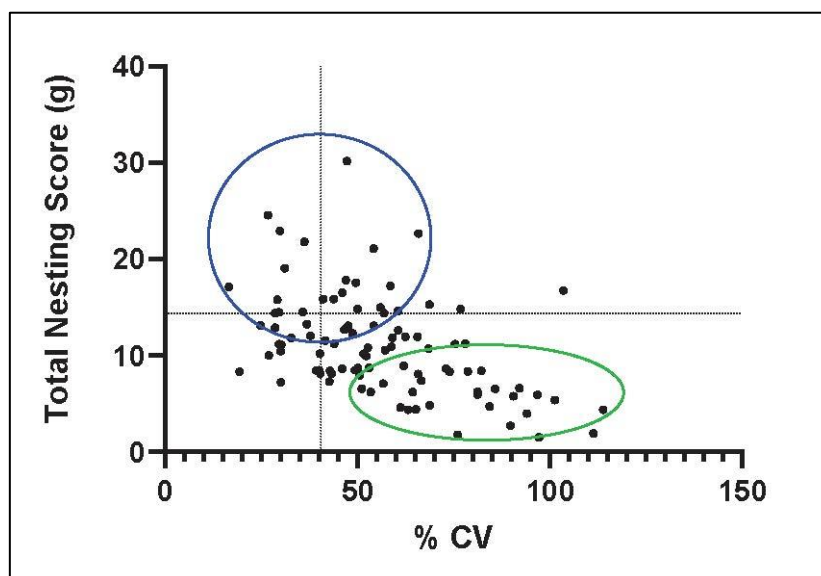


FIGURE 3-2: ASSOCIATION BETWEEN TOTAL NESTING SCORES (g) AND THE PERCENTAGE COEFFICIENT OF VARIANCE PERTAINING TO THE INTER-DAY NESTING SCORES

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.

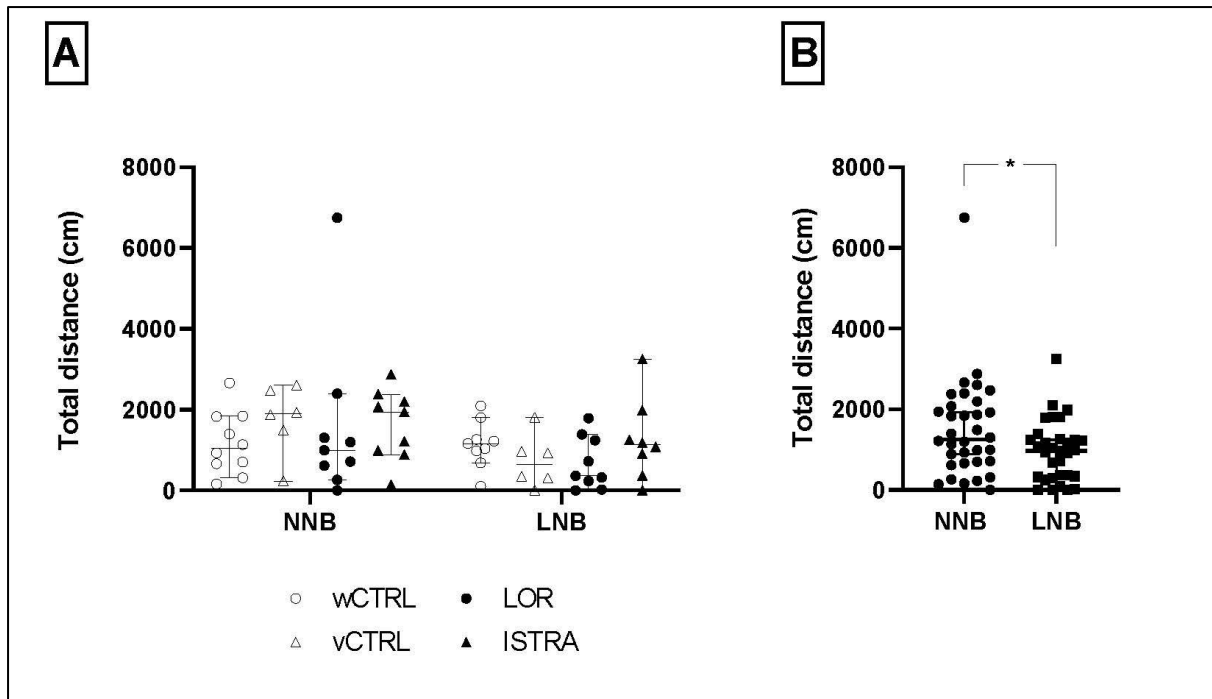


FIGURE 3-3: TOTAL DISTANCE TRAVELED IN THE OPEN FIELD BY MICE OF THE NNB- AND LNB-EXPRESSING PHENOTYPES PER EXPOSURE GROUP (A) AND POOLED ACROSS EXPOSURE GROUP (B)

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.

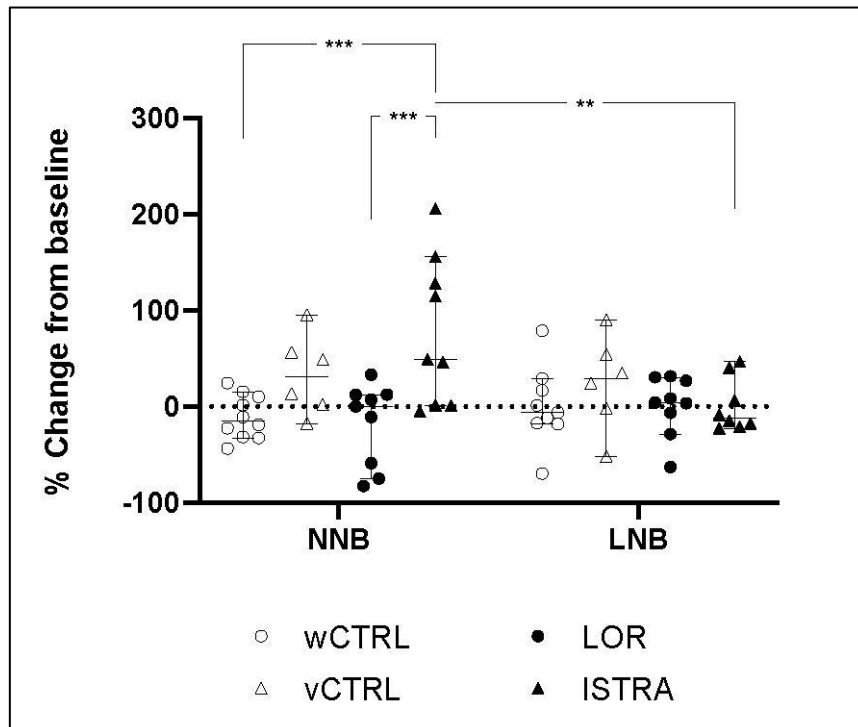


FIGURE 3-4: CHANGE IN NESTING EXPRESSION OVER TIME EXPRESSED AS A PERCENTAGE CHANGE FROM THE BASELINE SCORES

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.

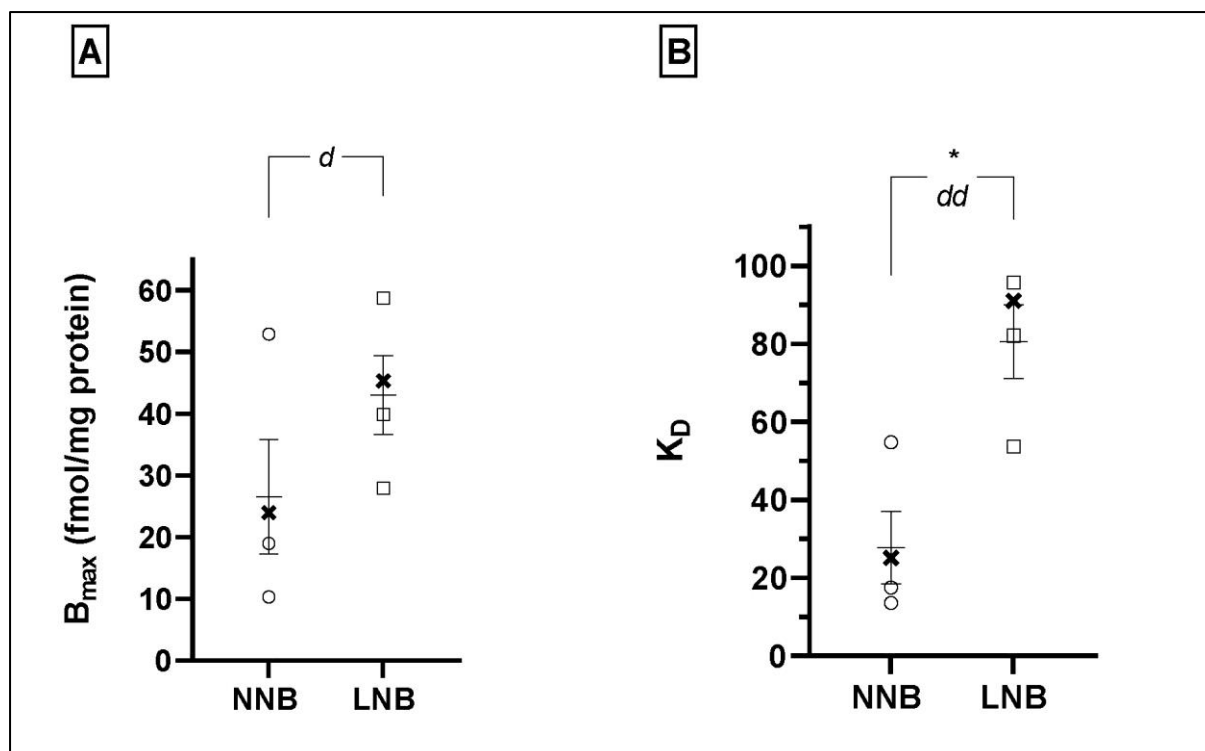


FIGURE 3-5: COMPARISONS OF STRIATAL A_{2A} RECEPTOR B_{MAX} AND K_D VALUES REPORTED IN NNB- AND LNB-EXPRESSING MICE
 NNB: NORMAL NEST BUILDING; LNB: LARGE NEST BUILDING. **P* = 0.02, UNPAIRED, ONE-TAILED STUDENT'S T-TEST;
 0.8 < *D* < 1.3 < *DD*

4 Conclusion

The present research explored the potential associations between compulsive-like large nest building (LNB)¹ behavior in deer mice (*Peromyscus maniculatus bairdii*), anxiety and striatal adenosine involvement. Obsessive-compulsive disorder (OCD)² is variably associated with anxiety. However, the psychobiological impact of anxiety on obsessive-compulsive symptom manifestation remains unclear, especially since patients may either experience anxiety that is only related to the expression of their OCD symptoms (e.g. experiencing safety-related anxiety that manifests as checking compulsions), or present with anxiety as a unique co-morbid diagnosis (Abramovitch et al. 2013). Neurobiologically, OCD mostly associates with irregular patterns of activity in the cortico-striatal-thalamo-cortical (CSTC)³ loops (Ting and Feng 2011). A direct, behaviorally activating and an indirect, behaviorally inactivating pathway comprise the applicable CSTC circuits that are involved in OCD (Evans et al. 2004). Dysregulation between the dopamine D₁ receptor-expressing direct pathway and the indirect, D₂ receptor-expressing pathway serves as one proposed explanation for obsessive-compulsive symptomology (Denys 2004; Kravitz et al. 2012), although their exact contributions remain unclear. Thus, and considering the suboptimal response of OCD to currently used pharmacotherapies, e.g. the selective serotonin reuptake inhibitors (SSRIs)⁴, an improved understanding of the condition will be fruitful.

Adenosine acts as an important neuromodulator via its actions on adenosine A₁ and A₂ receptors. Specifically, A_{2A} receptor activation modulates motor control via its effects on striatal signaling (Chen and Cunha 2020; Tanimura et al. 2010). A_{2A} receptor antagonists, e.g. istradefylline (ISTRA)⁵, have shown demonstrable pro-cognitive and motor improving effects and improves anxiety in patients diagnosed with mood and anxiety disorders (Coelho et al. 2014; van Calker et al. 2019). With respect to this investigation, an important theoretical point of departure was the fact that adenosine A₂ and dopamine D₂ receptor subtypes are co-expressed, whereby activation of the A₂ receptor (notably the A_{2A} subtype) decreases the affinity of dopamine for and its activity at the D₂ receptor (Ferré et al. 1996). In line with theories of dysregulation between the direct and indirect pathway activity, ISTRA has been shown to improve compulsive-like behaviors induced by quinpirole, a dopamine D_{2/3} receptor agonist (Asaoka et al. 2019) in a rat model. Further, ISTRA also attenuated the expression of high motor stereotypy (HS)⁶ in the present model system (de Brouwer et al. 2021). Thus, we sought to

¹ large nest building

² obsessive-compulsive disorder

³ cortico-striatal-thalamo-cortical

⁴ selective serotonin reuptake inhibitors

⁵ istradefylline

⁶ high motor stereotypy

Conclusion

expand prior work and understand the role of anxiety and adenosinergic processing in compulsive-like LNB¹-expressing mice.

* * *

Briefly, 34 NNB²- and 32 LNB-expressing deer mice were selected from an initial pool of 120 mice and exposed to either a normal water (wCTRL)³ or vehicle control (vCTRL)⁴, lorazepam (LOR)⁵ or ISTRA⁶ for 7- (LOR) or 28 days after which nesting assessment was repeated and mice screened for anxiety-like behavior in an anxiogenic open field. Mice were subsequently euthanized, and the striatal tissue removed and analyzed for A_{2A} receptor expression.

* * *

The first main finding of this investigation was that NNB- and LNB-expressing mice showed similar behaviors in an open field, indicating that NNB- and LNB-expressing mice present with similar generalized anxiety-like behavioral responses. This finding was somewhat unexpected, given our earlier result showing that LNB-expressing mice, assessed over the course of a full dark cycle, engage in increased risk-taking behaviors, albeit to obtain cotton wool. Nevertheless, from the present data, it can be concluded that when open field behavior is measured over the short term in a novel open field environment, NNB- and LNB-expressing mice both exhibit similar levels of exploratory behavior and thus, it can be concluded that NNB and LNB behavior are not differentially associated with measures of generalized anxiety.

The second and third main findings of this study were highly informing. Not only was it observed that ISTRA, but not control or LOR, bolstered normal nest building behavior, but also that such behavior associated with a robust decrease in the expression of high-affinity striatal A_{2A} receptor density (B_{max}) in NNB- compared to LNB-expressing mice. While we could not report the difference between the B_{max} values on a statistically significant level, the pharmacological and neurobiological data from this work show 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 (Fuxe et al. 2010). Therefore, it can be concluded that the act of nesting itself is intrinsically related to adenosinergic signaling in the indirect pathway of the CSTC⁷ circuit. This finding paves the way for

¹ large nest building

² normal nest building

³ normal water control

⁴ vehicle control

⁵ lorazepam

⁶ istradefylline

⁷ cortico-striatal-thalamo-cortical

Conclusion

continued investigations of adenosinergic agonists in the model system as a potential ameliorative strategy.

* * *

In conclusion, our findings indicate that NNB¹ and LNB² behavior are not distinctly associated with measures of anxiety and that ISTRA³-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. Future studies should explore the effects of adenosine receptor agonists as potential ameliorative strategies.

* * *

Continue next page.../...

¹ normal nest building

² large nest building

³ istradefylline

4.1 Final summary of study aims and outcomes

In **Table 4-1**, a final summary of the study questions posed in this work and the associated answers that were gained, is provided.

TABLE 4-1: SUMMARY OF STUDY QUESTIONS AND OUTCOMES

Expected Outcome	Actual Outcome
Will NNB- and LNB-expressing mice differ with respect to their general anxiety-related behavioral output?	 No differences were observed between the general open field behavior of drug-naïve NNB- and LNB-expressing mice. However, our data cannot exclude the potential association between LNB expression and anxiety-like processes that might be more related to the potential underlying triggers of LNB, e.g. safety- or perfectionism-related obsessions. Future study is needed to explore this possibility.
Will ISTRA, a selective adenosine A_{2A} receptor antagonist, decrease the expression of LNB behavior and will such decrease correspond with parallel improvements of anxiety-like behavioral expression?	  The answer to this question is complex. Although ISTRA had no influence on either the expression of LNB or the anxiety-like behavior of deer mice in either cohort, it inflated the expression of NNB behavior. Thus, adenosine influences the expression of nesting behavior, although this result largely points to an intrinsic relationship between the goal-directed expression of nesting itself. Further, it can be concluded that LNB expression might be related to a lower degree of adenosinergic innervation in the indirect pathway of LNB-, as opposed to NNB-expressing mice.
Will LNB, compared to NNB behavior, associate with striatal pathway dysregulation as shown by altered adenosine A_{2A} receptor expression?	 Yes. Data from this work overwhelmingly point to dysregulation between direct and indirect pathway activity in LNB-expressing mice. Specifically, it is likely that decreased activity of adenosine in the indirect pathway of the LNB-expressing mice underlie the expression of excessive nesting behavior.
NNB: normal nest building; LNB: large nest building; ISTRA: istradefylline.	

* * *

4.2 Study shortcomings and future directions

The findings of this work are important for our understanding of LNB¹-expression in deer mice and its resemblance of compulsive-like and persistent behaviors in preclinical model systems. However, some shortcomings need mentioning and elaboration.

* * *

An important aspect of the current work, though intrinsic to the manner of investigation in naturalistic animal models, may contribute to a lack of a robust separation in neurobiological data. This difficulty speaks to the subjective nature of cohort selection. Indeed, from **Chapter 3**, it is evident that a clear separation between NNB²- and LNB-expressing mice, as investigated here, is not always possible with the sometimes-marginal differences showed between the nesting expression of mice included in the NNB and LNB cohorts, respectively. Further contributing to the potential confounding effect of such method of selection, is the fact that tissues from different mice were pooled to complete a single binding assay. As such, tissue from mice that expressed differing intensities of nesting behaviors, were pooled—this was intentional, since we aimed not to bias the binding data within a specific cohort. In order to overcome this, two possible solutions can be suggested. First, larger groups of mice will have to be screened to ensure a better separation between cohorts, bearing in mind the ethical constraints that such an approach might be bound to. Secondly, more sensitive methods of quantifying receptor expression, e.g. immunohistochemistry might be fruitful. This will arguably result in tissues from a single mouse used to obtain a single data point, thereby increasing the number of data points that can be analyzed.

A second shortcoming of this work relates to the use of only one dose of ISTR³. ISTR is known to have differential effects on the expression of anxiety-like behavior and, while we based our present approach on prior data in the model system, we have not explored the effects of ISTR on anxiety in deer mice before. Still, the present project was hampered by significant time constraints that were brought about by a slower breeding rate of mice during a critical window of the study and the inclusion of another, lower dose ISTR-exposed group, was not possible. Future studies should bear this in mind and, in parallel with proposed changes in anxiety testing (see below), explore the effects of both adenosinergic agonists and ISTR at different concentrations, to clearly identify any potential effects of adenosine modulation on both anxiety and compulsive-like behavior in deer mice.

¹ large nest building

² normal nest building

³ istradefylline

Conclusion

Last, it would have been fruitful to explore the anxiety-like behavior of NNB¹- and LNB² 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. C57/bl mice, that have shown significant anxiety-like behavior in the mirrored open field. In contrast, mice of the *Peromyscus* genus are likely less risk-averse, 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.

* * *

THE END

¹ normal nest building

² large nest building

4.3 References

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Addendum A

This Addendum contains supplementary methodological detail that was not relevant for inclusion in the main body of work, i.e. **Chapter 3**. As such, the information contained in this Addendum should be read with **Chapter 3**.

General details about mouse husbandry, housing, and care prior to the onset of investigation

- Prior to onset of the study, mice were bred in the North-West University (NWU)¹ vivarium, Potchefstroom, South Africa (SAVC² registration number: FR15/13458; SANAS3 GLP compliance number: G0019; AAALAC accreditation file: 1717).
- Mice were weaned at the age of 21 days, and group housed (4 - 6 same sex mice per cage; 35 cm (l) x 20 cm (w) x 13 cm (h); Tecniplast® S.P.A., Varese, Italy), until selection for further study.
- Every day, the welfare monitoring procedure included the checking and recording of the following parameters: 1) the humidity of the housing room (40 – 55 %), 2) temperatures of the housing room (22 ± 1 °C), and 3) food and water or drug availability. If discrepancies were noted, the relevant stakeholders, i.e. staff of the vivarium, were informed, the problem identified and resolved.
- Once a week, all cages were cleaned by replacing the corncob bedding (in addition to the daily change of water or drug solutions, where applicable). Cages were cleaned with F10® veterinary disinfectant cleaning solution (Reg No Act 29, GNR 529/29990/040/150; DAFF Registration Number: G3073). No paper towel or polyvinyl chloride pipes (100 mm x 40 mm Ø) was provided during the experimental (i.e. nesting assessment) window.
- Prior to the onset of the baseline investigation, mice (10 - 12 weeks old) were single housed in identical cages, remaining so for the duration of study.
- All food (mouse pellets; Labchef; Nutritionhub®, Reg nr: 2012/141960/07) were autoclaved at 90°C for 15 min before use.

Behavioral experimentation

General points

- On each morning between 08:00 and 10:00 throughout this investigation, water bottles were weighed to monitor liquid intake. Water (or drug solutions/emulsions, where applicable) were replaced every day.
- Each day the bottles were weighed and subtracted from the previous day's value to determine the liquid intake over the 24-hour cycle (see **Fig. A-1**)

¹ North-West University

² South African Veterinary Council

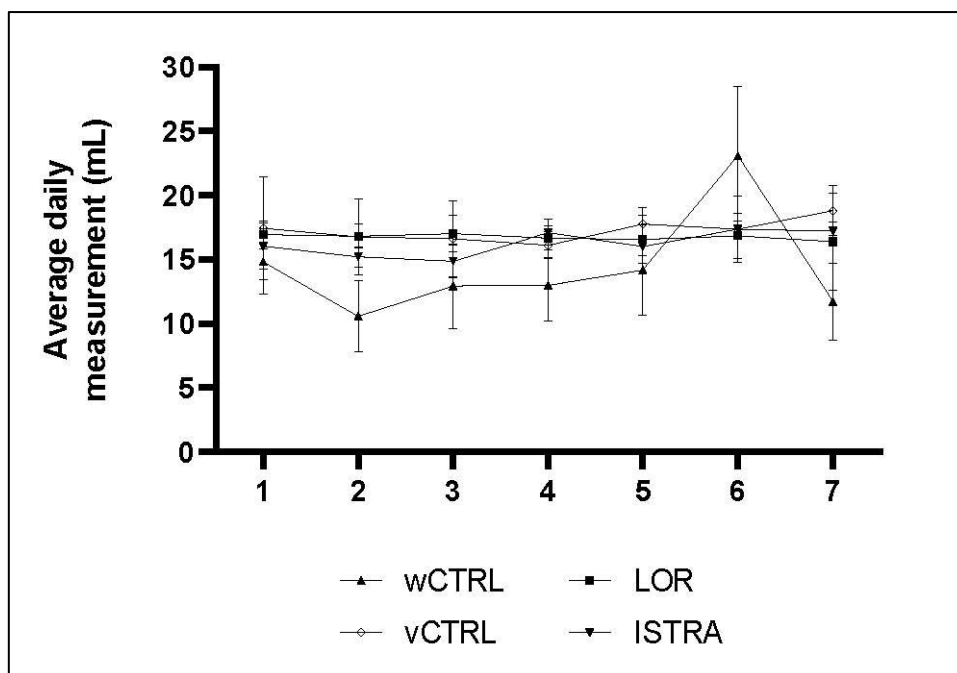


FIGURE A-1: LIQUID INTAKE DATA REPRESENTATIVE OF 7 DAYS OF ADMINISTRATION TO MICE IN EACH RESPECTIVE EXPOSURE GROUP
wCTRL: NORMAL WATER CONTROL, vCTRL: VEHICLE CONTROL, LOR: LORAZEPAM, ISTRA: ISTRADefylline. NO SIGNIFICANT DIFFERENCES OBSERVED BETWEEN ANY OF THE GROUPS ON ANY TESTING DAY. TWO-WAY REPEATED MEASURES ANOVA.

Nest building

- Two nest building assessments were performed for each mouse as described in detail in **Chapter 3**. The first assessment marked the onset of investigation. Data from the first baseline assessment were used to classify mice as either large (LNB)¹ or normal nest building (NNB)². Following the end of the first assessment, a one- or four-week drug exposure period began (group dependent; see **Table A-1**), after which the second nesting assessment was performed.

TABLE A-1: DURATION OF EXPOSURE PER EXPOSURE GROUP (REFER ALSO TO CHAPTER 3)

Exposure group	Duration of exposure
Water control	4 weeks
Vehicle control	4 weeks
Lorazepam	1 week (sub-acute exposure)
Istradefylline	4 weeks

¹ large nest building

² normal nest building

- The experimental procedure, data reporting and statistical analyses were carried out exactly as explained in **Chapter 3**.
- Mice not selected for inclusion in the experimental group, were euthanized according to standard Vivarium operating procedure (i.e. by means of cervical dislocation) without being used for sample collection or breeding.
- During periods of nest building analysis, food and water were available as normal, although mice were not provided with any additional form of nesting material.

Mirrored open field test

- On days on which anxiety assessment took place, mice were moved in groups of four (since only four mice could be assessed at a time) from the housing room to the experimental rooms at 19:00. Since the light cycle of the vivarium was set at 06:00/18:00, mice were allowed 60 min to wake prior to the onset of testing. Mice were habituated in black boxes constructed from Plexiglas® (50 cm x 50 cm) for 5 min, prior to the onset of mirrored open field assessment (**Chapter 3**).
- The experimental procedure then followed according to the detailed method alluded to in **Chapter 3**.
- Ethovision® 16 (Noldus Information Technology®, Wageningen, The Netherlands) was used to track the ambulatory activity of each mouse and the data were analyzed according to the method set out in **Chapter 3**.

Additional information pertaining to drug administration

This study involved the administration of two drugs, each with its own unique dosing and practical considerations. Lorazepam (LOR¹; Ativan® 4 mg/mL, Aspen Pharmacare®, Qheberha) is freely water soluble and effective over a sub-acute (7 day) exposure period (Wolmarans et al. 2022). It was thus added to the normal drinking water of mice as per the concentration specified in **Chapter 3**. Istradefylline (ISTRA²; BLD Pharma®, Shanghai, China) is poorly water soluble and was thus prepared in an emulsion (**Table A-2**) for chronic, 28-day administration (de Brouwer et al. 2021).

¹ lorazepam

² istradefylline

TABLE A-2: INGREDIENTS USED FOR EMULSION

Ingredient	Function	Per kg of emulsion	g/mL
Soybean oil	Internal Phase	100 g	0.1
Egg phosphatide	Surfactant	12 g	0.012
Istradefylline	Active	0.04 g	0.00004
RO water or WFI	Continuous phase	860 g	0.86
Glycerol	Osmotic agent	22.5 g	0.023
NaOH (q.s.)	pH	0.07 g	0.00007

Method for the preparation of the emulsion

Preparation of the aqueous and oil phases

Aqueous phase

1. Heat water on a stirring hotplate to 65 °C in a heat-resistant glass beaker.
2. Add glycerol and dissolve.
3. Adjust pH (pH = 7.5 - 8.5) with NaOH.
4. Cover the aqueous phase to prevent evaporation and keep at 65 °C.

Oil phase

1. Add the soybean oil to a heat-resistant glass beaker. Heat to 30 °C.
2. Add the active (ISTRA)¹ and dissolve.
3. Further heat the oil phase to 65 – 70 °C.
4. While heating, add the egg phosphatide and dissolve using vigorous magnetic stirring. Maceration of the egg phosphatide before adding can speed up the dissolution process. Egg phosphatide will only sufficiently start dissolving after the phase reaches 60 °C. From this point onwards full dissolution could take 30 – 60 min.
5. The temperature of the oil phase should be kept at 68 °C to prevent degradation and hydrolysis of the oil phase.

¹ istradefylline

Preparation of the primary emulsion

1. Place the aqueous phase on the magnetic stirring plate. Vigorous mixing is needed, but mixing should be initiated slowly, gradually increasing speed.
2. Slowly add the oil phase to the aqueous phase without adding too much oil to the water phase at once, while continuously stirring.
3. Once all of the oil phase has been added, stir for another 3 min.
4. Homogenize the primary emulsion for 2 min by moving the tip of a homogenizer from the top to the bottom of the beaker slowly for the duration of this step.
5. Transfer the finished emulsion into a clean Schott glass bottle.
6. Let the emulsion cool to room temperature before administration.

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Supplementary images

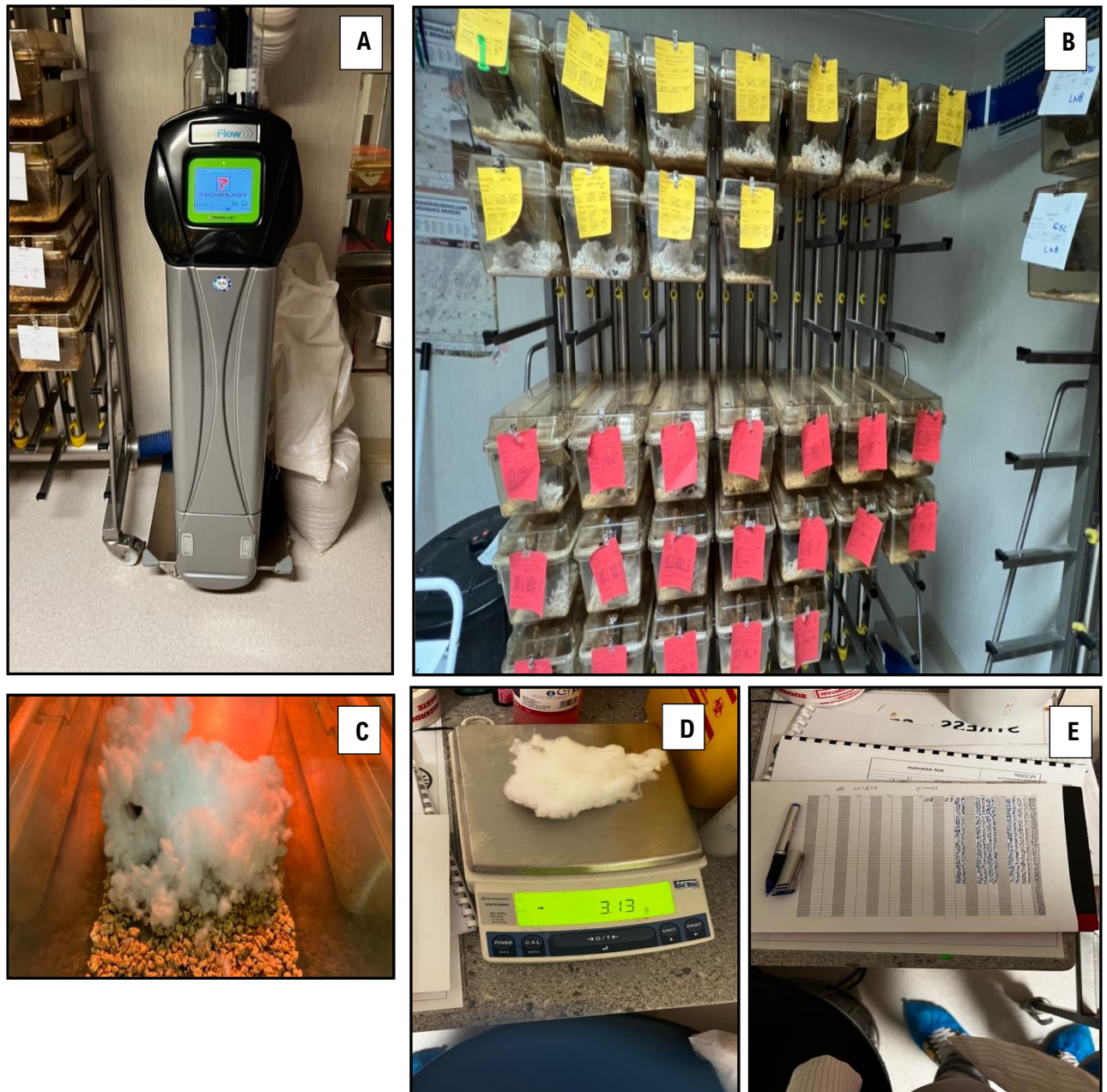


FIGURE A-2: (A): TECHNIPLAST® SMART FLOW FOR THE MONITORING OF VENTILATION, HUMIDITY, AND TEMPERATURE IN THE CAGES; (B): DEER MICE HOUSING SYSTEM (CAGES: 35 CM (L) x 20 CM (W) x 13 CM (H); TECHNIPLAST® S.P.A., VARESE, ITALY). EACH CAGE WAS MARKED ACCORDING TO THE DIFFERENT GROUPS FOR THE IDENTIFICATION OF MICE; (C-E): EXCESSIVE NEST BUILDING SCREENING AND RECORDING

Addendum A

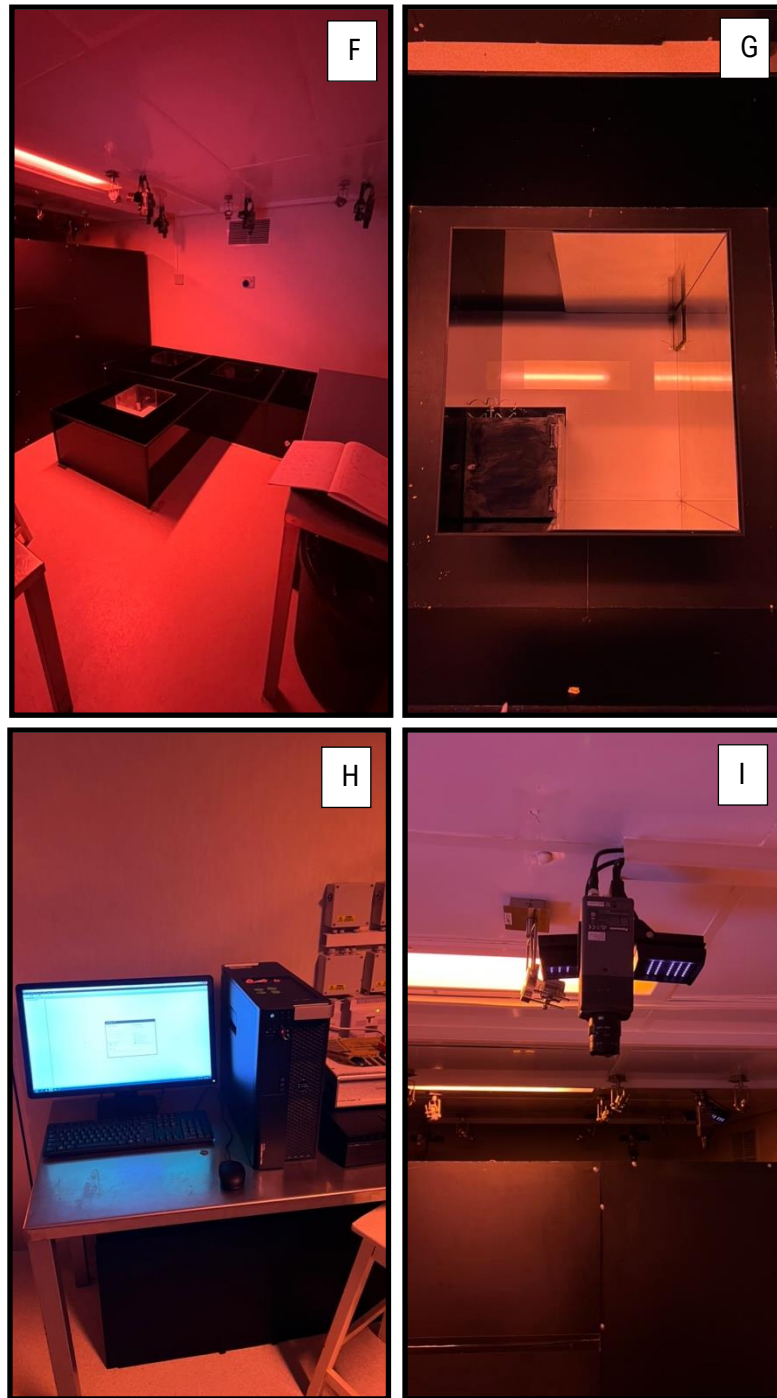


FIGURE A-3: (F, H & I): APPARATUS IN THE ANXIETY TESTING ROOM; (G): THE MIRRORED OPEN FIELD BOX WITH THE DARK CHAMBER

Addendum A

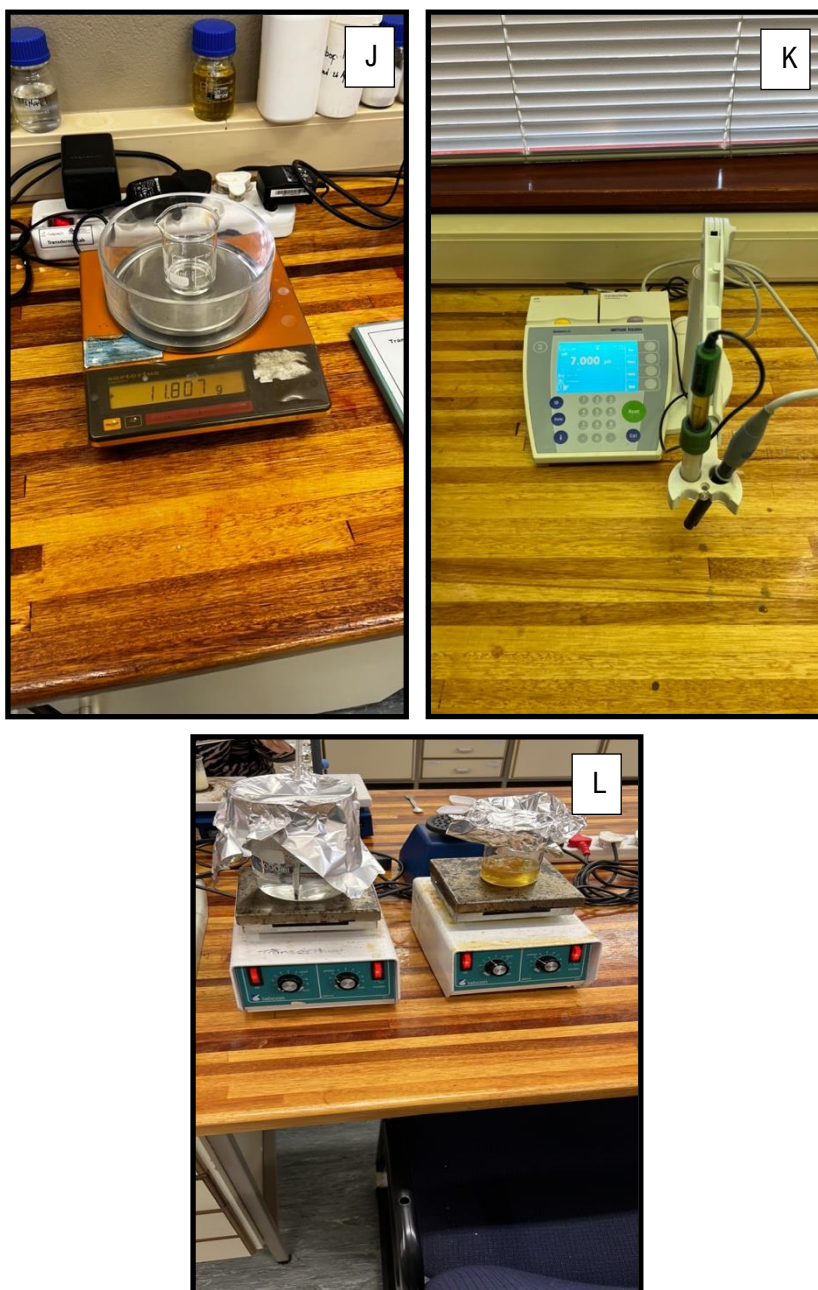


FIGURE A-4: (J) THE BALANCE USED TO WEIGH OF INGREDIENTS DURING THE FORMULATION OF THE VEHICLE EMULSION; (K) PH METER, AND (L) MAGNETIC STIRRER AND CONVECTION PLATES USED DURING THE FORMULATION OF THE AQUEOUS- AND OIL PHASE

References

- de Brouwer G, Engelbrecht J, Mograbi D, Legoabe LJ, Steyn S, Wolmarans D (2021) Stereotypy and spontaneous alternation in deer mice and its response to anti-adenosinergic intervention. *Journal of Neuroscience Research* In Press.
- Wolmarans D, Prinsloo M, Seedat S, Stein DJ, Harvey BH, de Brouwer G (2022) Escitalopram and lorazepam differentially affect nesting and open field behaviour in deer mice exposed to an anxiogenic environment. *Neuroscience Research* 177: 85-93.

Addendum B

In an attempt to elucidate the potential neurobiological differences which may potentially underlie the different phenotypes explored in this investigation, i.e. normal nest building (NNB)¹ and large nest building (LNB)², it was of value to characterize the striatal adenosine A_{2A} receptor expression density in both cohorts. This addendum contains a detailed description of the experimental methods and results which and were applied.

¹ normal nest building

² large nest building

Tissue collection and preparation of homogenates

After completion of the behavioral experimentation, tissues were collected and pooled as per the details provided in **Chapter 3**.

Preparation of homogenates

Equipment

TABLE B-1: EQUIPMENT USED FOR THE PREPARATION OF THE TISSUE HOMOGENATES

Equipment used	Originating company
Sartorius microbalance	Sartorius lab instruments GmbH & Co.KG 37070 Goettingen, Germany
Optima XPN-100 Ultracentrifuge	Beckman coulter, USA
Kinematica® Polytron (homogenizer)	Kinematica, Kriens, Luzern, Switzerland
Teflon bit homogenizer	Self-made, NWU ¹
Erlenmeyer flasks	-
Water bath	-

Procedure

1. First, frozen tissues were weighed and pooled to a minimum of 90 mg of tissue per phenotype. No more than three tissue samples were pooled.
2. Tissue pools were added to Erlenmeyer flasks to which approximately 10 mL of ice-cold TRIS-buffer was added (see later).
3. Samples were homogenized by Polytron® (2 x 5 sec intervals).
4. Homogenates were added to tubes suitable for ultra-centrifugation and centrifuged for 10 min at 49 000 *g*.
5. After 10 min, tubes were removed from the centrifuge and the supernatant discarded.
6. Fresh buffer was no added to the pellets at a concentration of 100 volumes.
7. Tissue pellets were dislodged and added with the buffer content to a glass laboratory tube in a cold room.
8. Pellets were homogenized using a Teflon® drill bit while still in the cold room.
9. Centrifugation was repeated and the process repeated once more.
10. After a final round of homogenization, homogenates were left in a shaking water bath at 25 °C for 1 h.

¹ North-West University

TRIS-buffer preparation

TABLE B-2: CHEMICALS NEEDED FOR THE CONSTITUTION OF THE BUFFER SOLUTION

Compound	Manufacturer
TRIS HCl	Merck KGaA, Darmstadt, Germany
TRIS Base	Merck KGaA, Darmstadt, Germany
Magnesium Chloride	USB, Cleveland, Ohio, US

To make 5 L of buffer, 31.52 g of Tris-HCL was dissolved in 4 L of milliQ® water. Next, 6.05 g of the TRIS base was dissolved in 1 L of milliQ® water, while a separate 2.12 g of TRIS base was dissolved in 350 mL of water. Next, 4.76 g MgCl₂ was added to the 4 L TRIS-HCL solution. Then, the 4 L and 1 L solutions were combined in an appropriately sized container. According to the method, the pH of the resultant mixture was to be tested and adjusted by step wise addition of small volumes of the 350 mL TRIS base solutions prepared beforehand, to a target pH of 7.7. The prepared buffer was stored at 4 °C until further use.

CPA solution preparation

CPA (N⁶-Cyclopentyladenosine)¹ is a selective adenosine A₁ agonist that is used to block the binding of ³H-NECA² to the A₁ receptors. During this assay 20 µL of 50 nM CPA was added to every series. The calculations for the 50 nM CPA were made as follow:

- CPA molecular weight = 335.364 g/mol
- Dilution factor: 500 µL x 50 nM/20 µL = 1.25 x 10⁻⁶ M
- 335,364 mg in 1 mL = 1 M
- X mg in 1 mL = 1.25 x 10⁻⁶ M
- Equals 0.000419 mg in 1 mL OR 4.19 mg in 10 mL DMSO³
- Dilute x 100 to reach 50 nM concentration

¹ N⁶-Cyclopentyladenosine

² 5'-ethylcarboxamido adenosine

³ dimethyl sulfoxide

Cold ligand (NECA) preparation

The cold ligand was prepared in a concentration of approximately 1500 times more than the highest concentration of the hot ligand. Therefore, the cold ligand was constituted as a 30 μM mother solution. Calculations were made as follow:

- NECA¹ MM = 308.29g
- Use 1 % DMSO² to dissolve NECA
- Dilution factor: $500\ \mu\text{L} \times 30 \times 10^{-6}\ \text{M} / 10\ \mu\text{L}$
- Equals $1.5 \times 10^{-3}\ \text{M}$
- $308.29\ \text{mg in } 1\ \text{mL} = 1\ \text{M}$
- $X\ \text{mg in } 1\ \text{ml} = 1.5 \times 10^{-3}\ \text{M}$
- 0.4624 mg in 1 mL of 4.6 mg in 10 mL of 2.3 mg in 5 ml DMSO

Hot ligand (³H-NECA) preparation

The stock solution of [³H] NECA should be: $500\ \mu\text{L} \times 30 \times 10^{-9}\ \text{M} / 25\ \mu\text{L} = 6 \times 10^{-7}\ \text{M}$

- ³H-NECA specific activity = 29.6 Ci/mmol
- $0.001/29.6\ \text{Ci}$
- 0.00003385 M
- $3.38 \times 10^{-5}\ \text{M}$ of 33.8 μL of the ³H-NECA in 250 μL solution
- Therefore, the amount of ligand needed to create the stock solution with a volume of 1400 μL and a concentration of $6 \times 10^{-7}\ \text{M}$ was calculated as follows:
 - $1500\ \mu\text{L} \times 6 \times 10^{-7}\ \text{M} / 3.38 \times 10^{-5}$
 - 26.62 μL (27 μL) + (1400 μL – 25 μL)

¹ 5'-ethylcarboxamido adenosine

² dimethyl sulfoxide

Saturation binding assay:

Radioactive standard preparation

TABLE B-3: COMPOUNDS NEEDED DURING ASSAY

Compound	Equipment
Homogenates	Whatman GF/B® glass microfiber filters
[3 ^H] NECA ¹ (warm ligand)	scintillation-counter tubes
NECA (cold ligand)	PerkinElmer Tri-Carb 2810 TR®
CPA (N ⁶ -Cyclopentyladenosine)	Vacuum chamber
Adenosine deaminase	
Filter-Count® scintillation fluid (PerkinElmer)	

1. The binding experiment began by thawing the homogenates and transferring 9 mL (445 µL x 20 = 8.9 mL) of the homogenate to 15 mL centrifuge tubes. 4 µL adenosine deaminase was added to each tube to break down any remaining adenosine in the preparation. Mixtures were placed to the side.
2. A series of 8 points containing increasing amounts of radioactive ligand (RAL²; ³H-NECA; Sigma-Aldrich®, South Africa) were prepared according to **Table B-4**. Note that two series of standards were prepared.

TABLE B-4: CONSTITUTION OF RADIOACTIVE STANDARDS

Concentration series (nM)	³ H-NECA Volume from stock solution (µl)	Buffer volume (µl)
0.5	7	393
1	13	387
2	27	373
4	53	347
7	93	307
10	133	267
15	200	200
20	267	133
Total	793 (overage to 1000 µL)	-

¹ 5'-ethylcarboxamido adenosine² radioactive ligand

Total binding series

This comprised a series of 8 points which were prepared by pipetting 10 μL TRIS buffer into a sealable vial. Next, 25 μL of the RAL¹ from the respective range of ten standard points were added to the respective vials. Last, 445 μL of the specific homogenate and 20 μL CPA² were transferred by pipette, resulting in a final assay volume of 500 μL .

Non-specific binding series

These reaction volumes were the same as above, except that buffer was replaced with the cold ligand (NECA)³ into a sealable vial.

Standard series

These reaction volumes simply comprised a series of 10 points, each containing 4 mL of Filter-Count[®] scintillation fluid (Perkin-Elmer[®], South Africa) to which 25 μL of the RAL was added.

After preparation of the reaction mixtures, samples were incubated for sixty minutes at room temperature after which binding was terminated by means of vacuum filtration (**Fig. B-1**) through Whatman GF/B[®] glass microfiber filters. Filters were rinsed twice with 4 mL of ice-cold buffer solution, removed, and carefully placed into scintillation-counter tubes which were filled with 4 mL scintillation fluid (Filter-Count[®]). Samples were left in this state for two hours at room temperature before latent radioactivity trapped in the filters was counted using a liquid scintillation counter (Perkin-Elmer[®] Tri-Carb[®] 2810-TR).

¹ radioactive ligand

² N⁶-Cyclopentyladenosine

³ 5'-ethylcarboxamido adenosine



FIGURE B-1: VACUUM FILTRATION SYSTEM

Statistics

Linear regressions of the counts per minute (CPM)¹ generated by the standard solutions were drawn (**Fig. B-2**) to determine the standard slope. The baseline corrected CPM values for the respective homogenates (**Fig. B-3**) were calculated by subtracting the CPM of the respective total binding (TB)² series from the corresponding non-specific (NS)³ binding series. GraphPad Prism 9 was then used to calculate the respective $B_{\max}$ and K_D values from each binding experiment.

¹ counts per minute

² total binding

³ non-specific

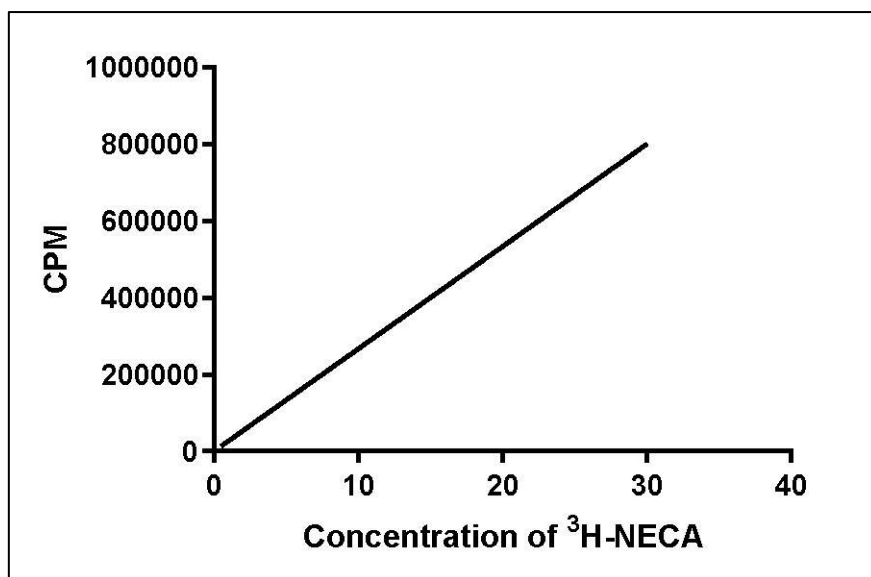


FIGURE B-2: STANDARDS CONCENTRATION CURVE

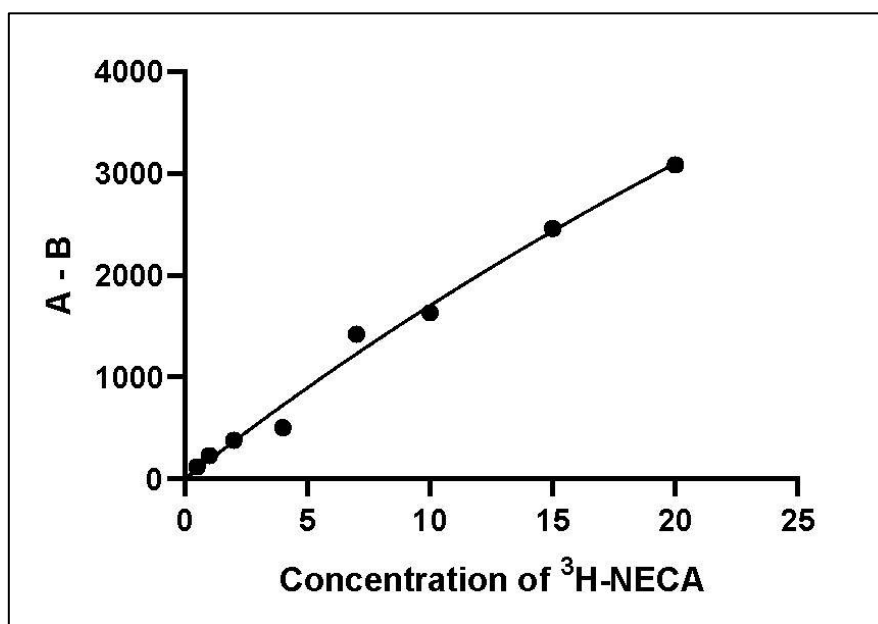


FIGURE B-3: SATURATION BINDING CURVE GENERATED FOR EACH SAMPLE (EXAMPLE OF ONE BINDING EXPERIMENT IN **NNB**-ASSOCIATED STRAITA)

Addendum C

This addendum contains the two letters of permission from co-authors of Chapter 3, that were not formally involved as functionaries of this project, i.e. Prof Gisella Terre'Blanche and Dr Heleen Janse van Rensburg, indicating that this work may be submitted for examination purposes.

LETTER OF AGREEMENT

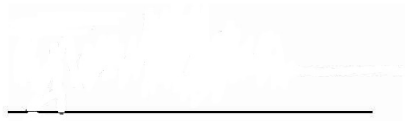
November 2022

To whom it may concern

CO-AUTHORSHIP ON ORIGINAL ARTICLE:

The undersigned is a co-author of the original article entitled "**Striatal adenosine A2A receptor involvement in normal and large nest building deer mice: perspectives on compulsivity and anxiety**" contained in Chapter 3, and hereby gives Mr Devan Saaiman permission to submit this article as part of his dissertation in fulfilment of the requirements for the degree Master of Science in Pharmacology at the North-West University (NWU).

Sincerely,

A handwritten signature in blue ink, appearing to read 'Gisella Terre'Blanche', is written over a horizontal line.

Prof Gisella Terre'Blanche

LETTER OF AGREEMENT

November 2022

To whom it may concern

Co-authorship on original article:

The undersigned is a co-author of the original article entitled **"Striatal adenosine A_{2A} receptor involvement in normal and large nest building deer mice: perspectives on compulsivity and anxiety"** contained in Chapter 3, and hereby gives Mr Devan Saaiman permission to submit this article as part of his dissertation in fulfilment of the requirements for the degree Master of Science in Pharmacology at the North-West University (NWU).

Sincerely,

Dr Helena D. Janse van Rensburg