Cross-Species Studies on the Mechanisms Underlying Abnormal Behavior in Bipolar Disorder: A Dopaminergic Focus

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Cross-Species Studies on the Mechanisms Underlying Abnormal Behavior in Bipolar Disorder: A Dopaminergic Focus

Translationele studies naar de onderliggende mechanismen van afwijkend gedrag in bipolaire stoornissen: Een dopaminerge focus
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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"Normality is a paved road: It's comfortable to walk, but no flowers grow on it"

- Vincent van Gogh

CONTENTS

CHAPTER 1

General introduction

The current use of psychopharmacological interventions in patients with major psychiatric disorders is often characterized by a trial and error approach. Although 'trial and error' is a fundamental method of solving problems, the repeated and varied attempts until success form a poor strategy for chemical substances that can induce serious side-effects in humans.

Bipolar disorder (BD) is one of these major psychiatric disorders, affecting approximately 2% of the global population (1). It often develops in the late teens or early adult years and presents in different forms. As the name suggests, together with its older term 'manic-depressive illness', the disease is characterized by episodes of manic and depressive behavior, where patients in-between these extreme mood states are labeled as euthymic. Additionally, mixed features of both depressive and manic symptoms can occur simultaneously and a state of hypomania exists where one may feel good and function well, but is at serious risk of developing a manic or depressive episode. The DSM-V essentially categorizes BD into BD-I and BD-II, the latter distinguished by an absence of full-blown mania or mixed episodes (2). The opposite extremes of manic and depressive episodes result in markedly different changes in both mood and behavior during these phases (3). Symptoms of mania include long periods of feeling 'high' or overly happy (euphoria), hypersexuality, hyperactivity, reduced sleep, extreme irritability, racing thoughts, aggression, and hedonic behavior. Although irritation and psychotic symptoms such as delusions are often observed during mania, hyperactivity is a conspicuous feature during acute manic states (4). Accordingly, the latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM V) added an emphasis on changes in activity and energy being required for diagnosis of a manic episode on top of an 'increase in goal-directed activity' or 'psychomotor agitation' (2). Symptoms of bipolar depression are largely opposite and include long periods of feeling sad or hopeless (dysphoria), reduced libido, feeling tired or 'slowed down', increased sleep, anhedonia, and increased risk for suicide. It is worth noting that at least one manic or hypomanic episode is required for diagnosis of BD, including bipolar depression (2). Besides changes in mood and behavior, BD is also associated with various significant neurocognitive impairments in domains such as executive dysfunction, vigilance deficits, impulsivity, and poor decision-making characterized by increased risk-taking (5, 6). Although BD is classically viewed as a mood disorder, these neurocognitive deficits are irrefutably important since they correlate closely with the patients' daily living abilities (7, 8).

Because untreated or poorly maintained BD is devastating to the patients' quality of life including that of their families, developing novel therapies is required. The paucity of current treatments is reflected by the fact that no approved treatments have been developed with BD as a target (9). Commonly used treatments approved for BD include the mood stabilizer lithium, anticonvulsants such as valproate and lamotrigine, and

various antipsychotics. None of these treatments improve cognitive functioning or completely stabilize behavior, limiting treatment options for patients. Thus, in combination with the cost of these treatments and the lost productivity of patients, the lifetime costs for patients with BD amounted to $24 billion (≈ €17.5) in the US in 1998 (10). The symptoms and poor treatment options contribute to an increased suicide mortality rate (11) where one in three people with BD attempt suicide (12). Novel therapies that are developed based on the underlying mechanisms of BD would thus likely greatly improve the lives of these patients.

Mechanisms causing the complex umbrella of symptoms in BD are as yet unresolved, complicating the targeted treatment development. Nevertheless, dysfunctional dopamine (DA) neurotransmission is recognized to play a key role in the pathophysiology of BD (13, 14). Polymorphisms in the gene encoding the DA transporter (DAT), the primary reuptake mechanism by which DA homeostasis is maintained, have been associated with BD (15, 16). These polymorphisms can reduce cell surface migration of DAT (17), subsequently down-regulating its functional expression. Indeed, reduced striatal DAT levels have been confirmed in unmedicated euthymic BD patients (18) as well as in postmortem tissue (19). There is also evidence that circadian rhythms are disrupted in BD (20), reflected for example by altered sleep-wake cycles in patients. These abnormal rhythms may actually influence the levels, release, and synthesis of certain neurotransmitters such as DA (21). Taken together, these data provide targets to be tested which can aid the development of novel therapies directed at the biological underpinnings of BD.

Traditionally, both *in vitro* and *in vivo* preclinical studies are a vital tool in the discovery of novel targets to develop useful therapeutic outcomes. The success rate with which new effective medicines are discovered using this approach varies to a great extent between disorders. Medicines to lower blood pressure for instance can be studied relatively easily in a preclinical model animal of essential hypertension called the spontaneously hypertensive rat. Creating cross-species paradigms to assess behaviors relevant to BD however is more challenging (22). As mentioned, BD is characterized by a plethora of symptoms instead of one single major abnormality such as high blood pressure. Moreover, mood symptoms such as grandiosity or racing thoughts, assessed using questionnaires in humans, cannot be derived from rodent behavior. It is because of this difficulty in mimicking affective states present in psychiatric illnesses in rodents that increased motor activity is frequently used as a primary measure to assess models for BD (23, 24), specifically the mania phase.

Hyperactivity in rodents can be induced pharmacologically by stimulants such as the indirect DA agonist amphetamine (25) (with or without the benzodiazepine derivative chlordiazepoxide (26)), while ouabain (27) and the direct DA agonist quinpirole (28) have

also often been used for the purpose of modeling mania. Another approach is to induce mania-like states environmentally. Sleep-deprived rodents for instance exhibit mania-like behaviors that include hyperactivity, increased aggression, and hypersexuality (29). Another environmental technique is the resident-intruder test (or variants thereof) (30) in attempts to model behaviors of aggression observed in patients with BD mania. Finally, genetic models have been created that are based on genes associated with BD. Hyperdopaminergic mutant mice with reduced functioning of the DAT have been created for example (31) and used to model BD mania (32). Other useful animal models for BD mania include mice with a mutation in the circadian gene for D-box binding protein (33) or for the "circadian locomotor output cycles kaput" (CLOCK) protein (34). Collectively, these models exhibit several strengths that include attenuation of the manipulation effect by an approved treatment for BD (i.e., chronic valproate-induced attenuation of hyperactivity) or similar effects of the manipulation in humans (i.e. amphetamine and sleep deprivation can induce mania in susceptible humans) (35). Numerous weaknesses also exist in several of these models however, including observing effects of BD treatment in control animals, the use of acute dosing only, or the use of normal animals for environmental manipulations (normal humans do not become manic under similar conditions) (35).

Instead of focusing on one aspect of BD (e.g. hyperactivity), the use of a comprehensive battery of tests to assess different BD-like behaviors would be helpful in identifying global mechanisms underlying symptoms as well as treatments (36). Combining this approach with the reverse-translation of behavioral tasks characterizing patients would likely yield more relevant information (37). Using this approach, Perry et al. developed a novel method for characterizing the exploratory profile of patients using a behavioral pattern monitor (BPM) that was based on rodent studies. Using this task, a unique behavioral pattern of activity and exploration of manic (32) and euthymic BD patients (38) was identified, that differed from control subjects, adult attention deficit hyperactivity disorder (ADHD) subjects (39), and patients with schizophrenia. This unique pattern included hyperactivity, increased specific exploration, and more linear patterns of movement. Using the rodent BPM, it was possible to examine mechanisms that recreate this abnormal pattern of exploration. Hence, the same phenotype was observed in mice with reduced functioning of the DAT induced by either pharmacological DAT inhibition (40) or constitutive knockdown (KD) (41), but not mice treated with amphetamine. Because lower levels of DAT have been observed in patients with BD, this model has putative etiological relevance and could be useful in the development of novel therapies targeted at the disorder. At the same time, such cross-species studies can help elucidate mechanisms underlying abnormal behavior in patients with BD.

The primary aim of this thesis was therefore to investigate behaviors in the hyperdopaminergic model animal, identifying whether they recreate abnormal behaviors

present in patients with BD, utilizing cross-species tests available in both humans and rodents. Additionally, pharmacological predictive validity was assessed with approved treatments for BD. Underlying mechanisms of behavior were further studied with compounds manipulating different monoamine neurotransmitters. Finally, the importance of depression in BD was also addressed in this thesis, insofar as we examined whether depression-like behaviors may relate to alterations in cholinergic systems. Hence, while this thesis focuses primarily on investigating mechanisms underlying mania symptoms, it also begins the work to understand mechanisms underlying depression as well as cycling between states.

In **chapter 2**, studies are described in which the reverse-translated multivariate exploratory paradigm, the behavioral pattern monitor (BPM), is used to quantify exploratory behavior of the genetic and pharmacological DAT models of mania. We also studied the effects of various pharmacological treatments on the exploratory profile of these models. In **chapter 2.1**, we assessed the pharmacological predictive validity of mice treated with the selective DAT inhibitor GBR12909 and that of genetic DAT KD mice in the BPM. Treatment with chronic valproate attenuated hyperactivity in both models, while hyper-exploration and behavioral organization were not significantly affected. **Chapter 2.2** describes the importance of elevated DA levels in the BD-like phenotype of DAT KD mice by depleting DA with alpha-methyl-p-tyrosine (AMPT). The consistency of the hyper-exploratory pattern of these mice over longer periods of time was also assessed. Through a letter to the editor, **chapter 2.3** includes information on how investigating endocannabinoid/dopaminergic interactions may provide novel treatments for BD. The letter is a brief response to an article regarding cannabis and its abuse potential. We highlighted a study we conducted examining the effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) treatment on the behavioral phenotype of DAT KD mice. We presented how acute Δ^9-THC reduced aspects of hyper-exploration similar to reduced exploration of patients with BD screened positive for cannabis, supporting a self-medication hypothesis for cannabis use in BD.

Chapter 3 includes studies that go beyond assessing exploratory behavior of the DAT models. This chapter investigates the cognitive performance of these models specifically in risk-based decision-making behavior in mice and humans tested in species-specific versions of the Iowa gambling task (IGT). In **chapter 3.1**, we assessed risk-taking behavior of DAT KD mice in a multiple-sessions IGT and observed that these animals exhibited an increased risk-preference compared to wild-type (WT) mice. Using the same multiple-sessions IGT, we assessed the effects of similarly acting drugs on risky choice behavior in mice as described in **chapter 3.2**. We observed that GBR12909 and the atypical stimulant modafinil both increased measures of motor impulsivity and motivation significantly, and risk-preference subtly. The mixed norepinephrine transporter (NET)/DAT inhibitor

amphetamine induced a risk-averse preference in mice without affecting motor impulsivity. In **chapter 3.3**, we describe an optimized single-session version of the rodent IGT more analogous to the human IGT. We observed impaired decision-making in manic BD patients performing the human IGT similar to poor decision-making in both GBR12909-treated and DAT KD mice. Deficits in each population were driven by reward hypersensitivity.

In addition to the described hyper-exploratory profile and impaired decision-making of mice with reduced DAT functioning similar to patients with BD, we also assessed the vigilance performance of these mice. We observed that DAT KD mice exhibited poor vigilance in a cross-species test called the 5-Choice Continuous Performance Test (5C-CPT). Interestingly, patients with BD exhibited the same vigilance deficit in a human 5C-CPT (currently collecting more human data for a combined human/animal paper to be submitted to *Science*).

The final chapters include different studies that further assess environmental, pharmacological, and genetic manipulations in mice and humans on behaviors relevant to BD. As mentioned, another way of potentially inducing a mania-like state is by sleep deprivation. In order to validate a sleep deprivation procedure first, we investigated the effects of sleep deprivation on attentional performance in the 5C-CPT in both humans and mice. **Chapter 4.1** describes this study and details the similarities observed between impaired 5C-CPT performance of humans and mice after 36 hours sleep deprivation. Another important aspect of BD that we have begun to investigate is depression. Hence, we modeled both mania- and depression-like behavior in mice using pharmacological manipulations as described in **chapter 5.1**. We describe how GBR12909 induced mania-like behavior including prepulse inhibition (PPI) deficits, while the acetylcholinesterase (AChE) inhibitor physostigmine induced depression-like behavior. Chronic treatment with lithium treated some, but not all, aspects of these effects. Finally in **chapter 6.1**, we tested another model animal of BD, the *Clock*Δ19 mutant mouse, in cross-species behavioral paradigms. We provided further evidence that *Clock*Δ19 mutant mice model aspects of BD mania, including PPI deficits and hedonia-like behavior.

Collectively, this thesis describes a number of conducted studies and publications that arose from three years of work. The evidence generated supports that hyperdopaminergia via direct or indirect (CLOCK) manipulation of the DA system can recreate the myriad of symptoms observed during a manic phase of BD. In terms of depression however, different mechanisms may be involved. Hence, these studies provide targets for developing therapies for BD, but also highlight that further work is required to model all aspects of BD including the mechanisms underlying switching between states.

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CHAPTER 2

Exploratory behavior of DAT models quantified with the reverse-translated multivariate behavioral pattern monitor

CHAPTER 2.1

Chronic valproate attenuates some, but not all, facets of mania-like behavior in mice

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ABSTRACT

Background

Bipolar Disorder (BD) mania is a psychiatric disorder with multifaceted symptoms. Development of targeted treatments for BD mania may benefit from animal models that mimic multiple symptoms, as opposed to hyperactivity alone. Using the reverse-translated multivariate exploratory paradigm, the behavioral pattern monitor (BPM), we reported that patients with BD mania exhibit hyperactivity as well as increased specific exploration and more linear movements through space. This abnormal profile is also observed in mice with reduced function of the dopamine transporter (DAT) through either constitutive genetic [knockdown (KD)] or acute pharmacological (GBR12909) means.

Methods

Here, we assessed the pharmacological predictive validity of these models by administering the BD-treatment valproic acid (VPA) for 28 d. After 1.5% VPA- or regular-chow treatment for 28 d, C57BL/6J mice received GBR12909 (9 mg/kg) or saline and were tested in the BPM. Similarly, DAT KD and wild type (WT) littermates were treated with VPA-chow and tested in the BPM.

Results

GBR12909-treated and DAT KD mice on regular chow were hyperactive, exhibited increased specific exploration, and moved in straighter patterns compared to saline-treated and WT mice respectively. Chronic 1.5% VPA-chow treatment resulted in therapeutic concentrations of VPA and ameliorated hyperactivity in both models, while specific exploration and behavioral organization remained unaffected.

Conclusions

Hence, the mania-like profile of mice with reduced functional DAT was partially attenuated by chronic VPA treatment, consistent with the incomplete symptomatic effect of VPA treatment in BD patients. Both DAT models may help to identify therapeutics that impact the full spectrum of BD mania.

Keywords

Bipolar mania, Chronic treatment, Dopamine transporter, Mice, Valproate

INTRODUCTION

Patients with Bipolar Disorder (BD) mania are characterized primarily by symptoms of over-active behavior as described in the DSM-IV (1). There are many facets to the over-active behavior exhibited by patients with BD (2). There is a need for therapeutics targeting the multifaceted nature of the disorder, which affects approximately 1% of the worldwide population for BD type-I (3). Current treatments have been found serendipitously, likely in part due to the limited availability of predictive preclinical animal models of BD mania that go beyond treating hyperactivity (4, 5).

We developed a multivariate approach to quantify the abnormal exploration of patients with BD in a novel environment – the behavioral pattern monitor (BPM; (6, 7)). Using the BPM, we evaluated mouse models of mania based on reduced dopamine transporter (DAT) functioning. These models include: (1) DAT knockdown (KD) mice with approximately 10% expression of the DAT; (2) selective inhibition of the DAT using GBR12909 treatment (8). Both models are based on the putative etiology of BD, in which polymorphisms in the human DAT have been associated with the diagnosis (9, 10). Studies *in vitro* suggest that these polymorphisms may result in reduced cell surface expression of the DAT (11). Indeed, lower striatal availability of DAT has been observed in unmedicated patients with BD by using positron emission tomography (12).

In the BPM, patients with BD mania are hyperactive, interact more with objects, and walk in more direct paths, compared to healthy subjects (6). The exploratory profiles of DAT KD and GBR12909-treated mice in the mouse BPM are similarly characterized by hyperactivity, increased specific exploration, and straighter patterns of movement (6, 8, 13). Moreover, the mania-like phenotype in DAT KD mice is attenuated with environmental familiarity but reinstated by environmental novelty and a subthreshold dose of GBR12909 (14), consistent with environmental uncertainty (15) and stimulants (16) deleteriously affecting patients with BD.

One of the core strategies in the development of a valid animal model is the assessment of its pharmacological predictive validity. Lithium and valproic acid (VPA) are among the most commonly used treatments for BD mania, although they are not effective in every subject (17) or in each facet of the disorder (18). Indeed, patients with BD mania remained more active, with increased object interactions and more direct movements in the BPM compared to healthy subjects even when on BD medication for 3 weeks, although effect size differences in activity levels diminished over time (19). Since long-term treatment regimens are required to exert optimal therapeutic effects, chronic treatment regimens should be used when assessing the pharmacological predictive validity of an animal model (4, 20).

To assess the pharmacological predictive validity of the DAT mouse model for BD mania, we examined the effects of chronic VPA medication on the mania-like behavior of these mice in the BPM. We hypothesized that (*a*) DAT KD and GBR12909-treated mice would exhibit a BD mania-like profile in the BPM; and (*b*) chronic treatment with VPA at therapeutic concentrations would attenuate these mania-like profiles.

METHODS

Animals

DAT KD mice were generated by inserting modified embryonic stem cells of the 129Sv/J mouse strain in C57BL/6J blastocysts (21). DAT heterozygous breeders were sent to our laboratory from Columbia University and all the subsequent mice resulted from a breeding colony in the vivarium at the University of California San Diego (UCSD). Male and female DAT KD (20-40 g) and wild type (WT; 20-30 g) littermate mice were generated from heterozygous breeding pairs and tested in the BPM at approximate age 6 months. Male C57BL/6J mice (20-30 g) were obtained from Jackson laboratories and tested at approximate age 3 months for acute GBR12909 studies. All mice were group housed (where possible, maximum four per cage), maintained in a temperature-controlled vivarium (21±1°C) on a reversed day-night cycle (lights on at 7.00 PM), and were tested during the dark phase of the cycle. Mice were brought to the laboratory 60 min before testing between 8.00 AM and 5.00 PM. Food (normal or VPA chow; Harlan Teklab, USA) and water were provided *ad libitum*, except during behavioral testing. During chronic treatment, all mice were inspected twice each week to check for possible drug-induced adverse effects. All procedures were approved by the UCSD Institutional Animal Care and Use Committee. The UCSD animal facility meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care.

Drug treatment

Sodium valproate and GBR12909 dihydrochloride were purchased from Sigma (USA). Rodent chow with VPA was custom produced by Harlan Laboratories (Harlan Teklab, USA). The VPA and vehicle chows were identical with the exception of the added drug in the former, which was produced with a concentration of 15 g VPA/kg chow. This dose was chosen based on a pilot dose-response study indicating that blood VPA concentrations were within the therapeutic concentrations for patients with BD (Fig. 1). Mice were treated for 4 weeks with VPA- or vehicle-chow and then tested in the BPM. GBR12909 was dissolved in saline (0.9%) vehicle after sonicating for 2-4 h at 40 °C; injection volume was 10 ml/kg. Free-base drug weights were used in all drug calculations. GBR12909 (9.0

mg/kg) was administered by i.p. injection immediately before the mouse was placed in the testing chamber and data recording started.

Chemical analysis of VPA blood concentrations

All mice were decapitated and trunk blood collected. Blood was left to clot for approximately 15 min. Samples were then centrifuged (200 *g*; 10 min) and serum was taken to determine drug concentrations. VPA concentrations were quantified with a glucose-6-phosphate dehydrogenase-based enzyme immunoassay technique, performed by UCSD Medical Center (USA).

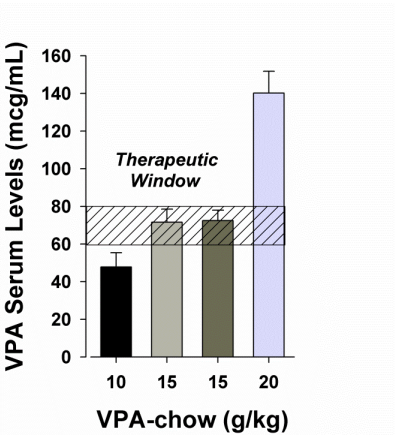

Fig. 1. Serum concentrations of valproic acid (VPA; mcg/mL) after 28 d different doses of VPA-chow treatment. Dose-dependent effects of chronic VPA treatment concentrations were observed on serum concentrations in mice. VPA-chow (1.0 %) resulted in low, while 2.0 % VPA-chow resulted in high/toxic VPA concentrations. The current experiments with 1.5 % VPA chow resulted in optimal therapeutic serum concentrations for bipolar disorder (60-80 μg/mL). Data are presented as mean + S.E.M.

Mouse behavioral pattern monitor

Spontaneous locomotor behavior and specific exploration were examined in nine mouse BPM chambers (San Diego Instruments, USA) as described previously (22). In brief, each Plexiglas chamber consists of a 30.5×61×38 cm area, with three floor holes and eight wall holes (three on each long and one on each short wall; 1.25 cm diameter, 1.9 cm from the floor). Each hole contains an infrared photobeam to detect holepoking. Each chamber is enclosed in an outer box that minimizes outside light and noise, with an internal white house-light above the arena (350 lux in the center and 92 lux in the four corners). Activity was obtained from a grid of 12×24 infrared photobeams 1 cm above the floor (2.5 cm apart along the length and the width of the chamber; 24×12 *X-Y* array), recording the location of the mouse every 0.1 s. Rearing behavior was detected by another set of 16 photobeams, located on the y axis only and placed 2.5 cm above the floor. Position was defined across nine unequal regions (four corners, four walls and center; (23)).

Exploratory assessment

At the start of each session, the mouse was placed in the bottom left-hand corner of the arena and the test session started immediately for 60 min. The primary outcome measures were: locomotor activity as measured by transitions across the defined regions and center entries (cumulative entries into the center region); specific exploration as measured by holepoking and rearing; locomotor pattern as measured by spatial *d*. Spatial

d quantifies the geometric structure of the locomotor path, where a value of 1 represents a path in a straight distance-covering line and 2 highly circumscribed small-scale movements (24).

Exp 1. Effects of chronic vehicle- or VPA-chow treatment on vehicle- or GBR12909-induced exploration in the mouse BPM

Male C57BL/6J mice (*n*=60) were tested first without drug in the BPM for 60 min to baseline-match their behavior. Using baseline measures of activity, specific exploration, and spatial *d*, mice were then counter-balanced into groups that received vehicle- (*n*=28) or VPA-chow (*n*=32). After 28 days treatment, mice in the vehicle-chow group received saline (*n*=14) or GBR12909 (9 mg/kg; *n*=14). Mice in the VPA-chow group received saline (*n*=14) or GBR12909 (9 mg/kg; *n*=15). During this experiment, three mice that received VPA-chow died of unknown causes; all other mice appeared healthy during treatment and testing. The locomotor and behavioral activity of the mice was assessed for 60 min in the BPM.

Exp 2. Effects of chronic vehicle- or VPA-chow treatment on the exploratory profile of WT and DAT KD mice in the mouse BPM

Female (*n*=13) and male DAT KD mice (*n*=11) and their female (*n*=13) and male WT littermates (*n*=15) were tested in the BPM for 60 min to baseline-match their behavior. Using baseline measures of activity, specific exploration, and spatial *d*, mice were then counter-balanced into groups that received vehicle- [female KD (*n*=9), male KD (*n*=6), female WT (*n*=8), male WT (*n*=8)] or VPA-chow [female KD (*n*=4), male KD (*n*=5), female WT (*n*=5), male WT (*n*=7)]. After 28 days treatment, the locomotor and behavioral activity of the mice was assessed for 60 min in the BPM.

Statistics

The outcome measures for each experiment were analyzed using two- or three-way analysis of variance (ANOVA), with sex, genotype, chow treatment, or drug as between-subject variables. Significant interactions and main effects were analyzed using Tukey's *post hoc* analyses. The data were analyzed for 60 min testing period using the Biomedical Data Programs statistical software (Statistical Solutions Inc., USA). The alpha level was set to 0.05.

RESULTS

Exp 1. Exploratory profile of vehicle- or GBR12909-treated C57BL/6J mice after chronic vehicle- or VPA-chow treatment

Locomotor activity

Significant GBR12909 effects were observed for transitions ($F_{(1,53)}$=122.53, p<0.0001) and center entries ($F_{(1,53)}$=33.65, p<0.0001). Significant VPA effects were also observed for transitions ($F_{(1,53)}$=5.06, p<0.05; Fig. 2a) and center entries ($F_{(1,53)}$=9.46, p<0.005; Fig. 2b). A VPA × GBR12909 interaction was found for center entries ($F_{(1,53)}$=4.74, p<0.05), but not for transitions. *Post hoc* analyses revealed that GBR12909 increased transitions compared to saline in both vehicle- and VPA-chow treated mice (p<0.0001). Mice administered GBR12909 after VPA-chow treatment exhibited fewer transitions compared to mice administered GBR12909 after vehicle-chow treatment (p<0.05). VPA-chow treatment did not affect transition levels in the saline-administered mice (p>0.1). Analyses revealed that GBR12909 increased center entries compared to saline in both the vehicle- and VPA-chow treated mice (p<0.005). Mice administered GBR12909 after VPA-chow treatment exhibited fewer center entries compared to vehicle-chow treated mice administered GBR12909 (p<0.005). VPA-chow treatment did not affect the amount of center entries in the saline-treated mice (p>0.1).

Exploratory behavior

A VPA effect was observed for holepoking ($F_{(1,53)}$=6.70, p<0.05; Fig. 2c) with no main effect of or interaction with GBR12909. A significant GBR12909 effect was observed for rearing ($F_{(1,53)}$=23.26, p<0.0001; Fig. 2d) with no main effect of or interaction with VPA. *Post hoc* analyses revealed that GBR12909 increased rearing compared to saline in both the vehicle- (p<0.0005) and VPA-chow treated mice (p<0.01). Analyses revealed that VPA-chow treatment increased holepoking in the saline-treated mice compared to vehicle-chow (p<0.05). Mice administered GBR12909 after VPA-chow treatment exhibited a trend towards increased holepoking compared to vehicle-chow treated mice administered GBR12909 (p<0.1).

Locomotor patterns

A significant GBR12909 effect was observed for spatial *d* ($F_{(1,53)}$=14.07, p<0.0005; Fig. 2e) as well as a trend effect of VPA ($F_{(1,53)}$=3.36, p<0.1) with no GBR12909 × VPA interaction. *Post hoc* analyses revealed that GBR12909 decreased spatial *d* compared to saline in both the vehicle- (p<0.01) and VPA-chow treated mice (p<0.05).

Exp 2. Exploratory profile of WT and DAT KD mice after chronic vehicle- or VPA-chow treatment

Locomotor activity

Significant genotype effects were observed for transitions ($F_{(1,44)}$=33.3, p<0.0001) and center entries ($F_{(1,44)}$=13.1, p<0.001). Significant main effects of VPA were also observed

for transitions ($F_{(1,44)}$=8.4, p<0.01; Fig. 3a) and center entries (F(1,44)=4.2, p<0.05; Fig. 3b). There was a genotype × VPA interaction for transitions ($F_{(1,44)}$=4.3, p<0.05), but not for center entries. Since no sex × genotype, sex × VPA, or sex × genotype × VPA interactions were observed, male and female data were pooled for *post hoc* analyses. These analyses revealed that both the vehicle- (p<0.0001) and VPA-chow treated DAT KD mice (p<0.01) exhibited increased transitions compared to WT mice. DAT KD mice treated with VPA-chow exhibited fewer transitions compared to DAT KD mice treated with vehicle-chow (p<0.05) however. VPA-chow treatment did not affect transitions in the WT mice (p>0.1). DAT KD mice made more center entries compared to WT mice in both the vehicle- (p<0.005) and VPA-chow treated mice (p<0.005). *Post hoc* analyses did not reveal any significant effect of VPA on center entries in the DAT KD or WT mice (p>0.1).

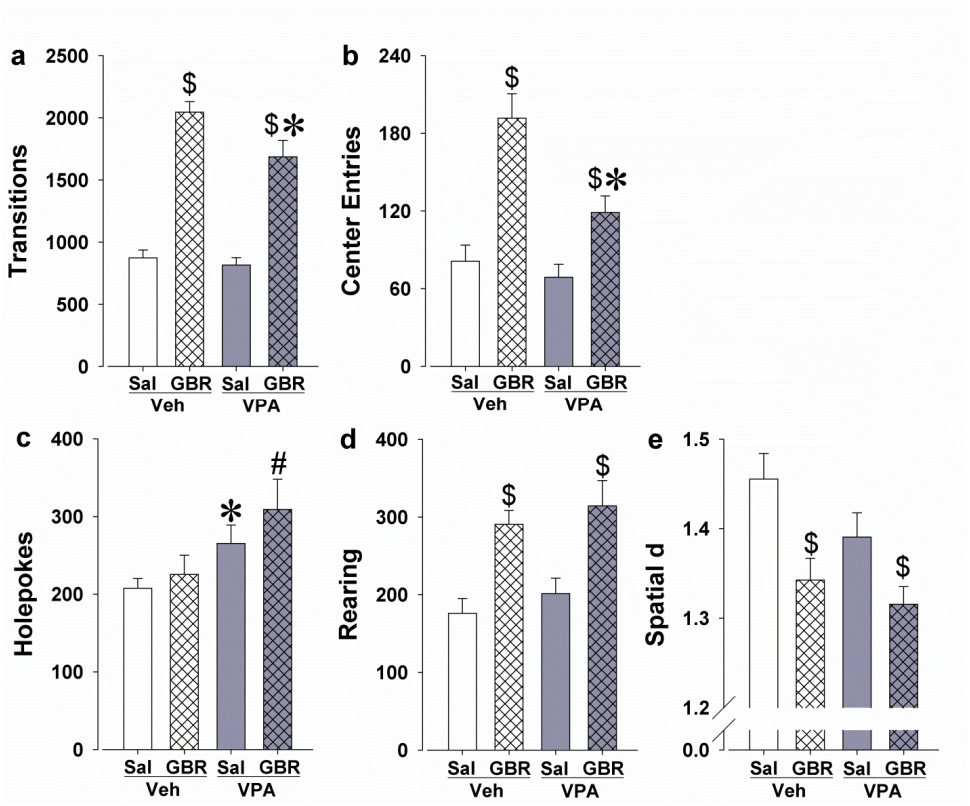

Fig. 2. Effects of chronic treatment with the mood-stabilizer valproic acid (VPA) on the exploratory profile of C57BL/6J mice administered acute GBR12909 (GBR; 9 mg/kg). GBR12909 increased activity as measured by transitions, which was attenuated by VPA (a). VPA also attenuated GBR12909-induced increases in center entries (b). Chronic VPA-chow treatment did not affect activity alone (a-b). GBR12909 did not affect holepoking, while VPA increased holepoking (c). GBR12909 increased specific exploration as measured by rearing, which was not affected by VPA treatment (d). GBR12909 induced more linear patterns of movement (reduced spatial *d*), which also were unaffected by VPA (e). Data are presented as mean + S.E.M. * p<0.05 and $^{\#}$ p<0.1 when compared to the vehicle (Veh)-chow treatment group; $^{\$}$ p<0.05 when compared to saline (Sal).

Exploratory behavior

A significant genotype effect was observed for holepoking ($F_{(1,44)}$=10.3, p<0.005; Fig. 3c) and a trend toward KD mice exhibiting more rearings compared to WT mice ($F_{(1,44)}$=3.2, p<0.1; Fig. 3d). There was no main effect of VPA or genotype × VPA interaction for holepoking or rearing. *Post hoc* analyses on holepoking revealed that DAT KD mice exhibited increased holepoking compared to WT mice in both the vehicle- (p<0.05) and VPA-chow treated mice (p<0.05).

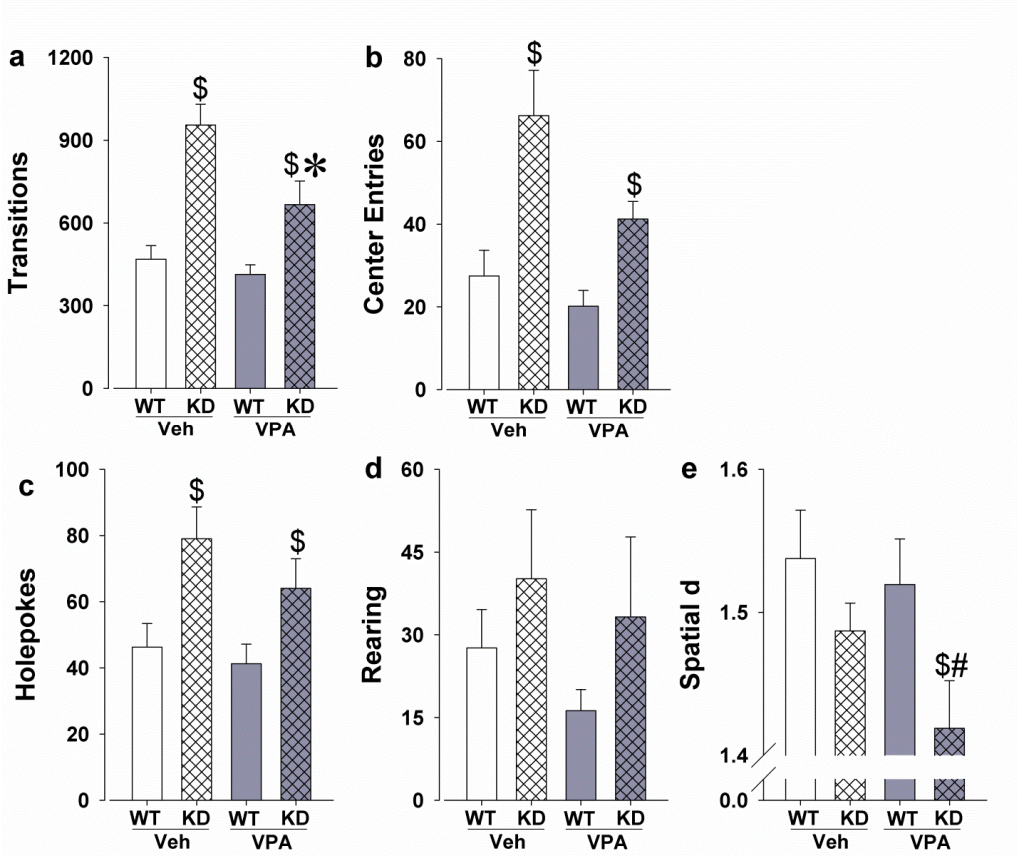

Fig. 3. Effects of chronic treatment with valproic acid (VPA) on the mania-like profile of dopamine transporter (DAT) knockdown (KD) mice in the behavioral pattern monitor (BPM). The DAT KD mice were significantly more active than the wild type (WT) mice as measured by transitions, behavior that was attenuated by VPA treatment (a). DAT KD mice also exhibited more center entries compared to WT mice, which was unaffected by VPA treatment (b). DAT KD mice exhibited increased specific exploration as measured by the amount of holepokes, which was not affected by VPA (c). No genotype or treatment effects were observed on rearing behavior (d). DAT KD mice exhibited straighter paths of movement than the WT mice (reduced spatial *d*), which were further reduced by VPA treatment (e). Data are presented as mean + S.E.M. * p<0.05 and $^{\#}$ p<0.1 when compared to the vehicle (Veh)-chow treatment group; $^{\$}$ p<0.05 when compared to WT mice.

Locomotor patterns

DAT KD mice exhibited straighter path movements compared with WT mice as measured by spatial *d* ($F_{(1,44)}$=5.1, p<0.05; Fig. 3e). Although no main effect of sex was observed, there was a sex × genotype × VPA interaction ($F_{(1,44)}$=5.0, p<0.05). Given the lack of sex effects throughout this study and previous studies, male and female data were combined for *post hoc* analyses. These analyses revealed that VPA-chow treated DAT KD mice exhibited lower spatial *d* compared to VPA-chow treated WT mice (p<0.05). There was a trend towards lower spatial *d* in the VPA-chow treated DAT KD mice compared to the vehicle-chow treated DAT KD mice (p<0.1).

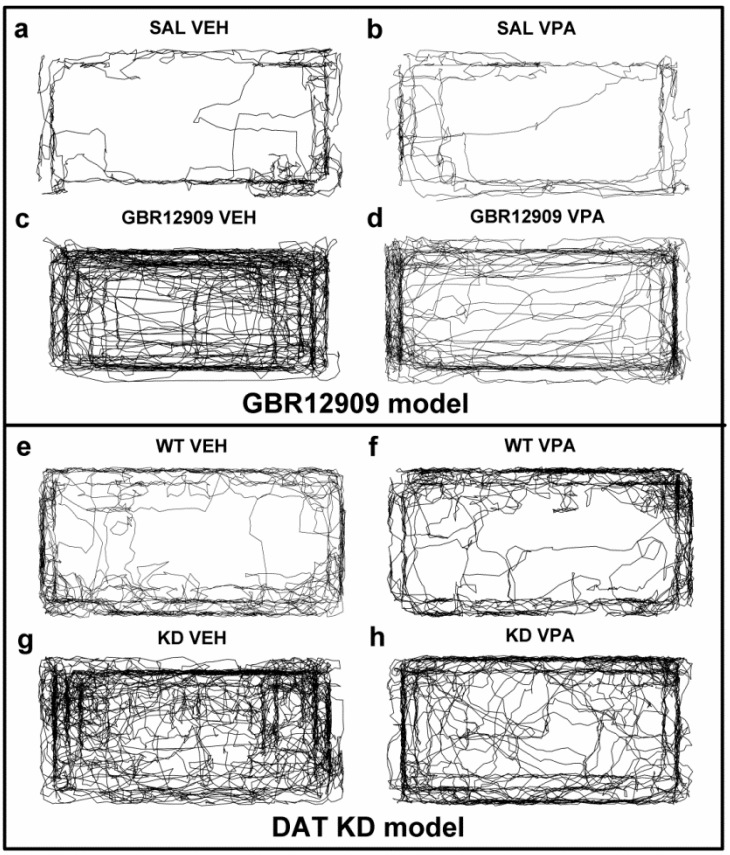

Fig. 4. Representative *X-Y* plots from study of two models of dopamine transporter (DAT) inhibition [GBR12909 model (a-d) and DAT knockdown (KD) model (e-h)] are displayed, showing representative behavioral patterns from mice of each study. As shown, valproic acid (VPA) treatment did not affect the patterns of movement of saline (Sal)-administered mice (a,b). Chronic VPA treatment attenuated GBR12909-induced hyperactivity (c,d), but did not affect specific exploration or sequential organization. VPA treatment did not affect the behavioral patterns of WT mice (e,f). VPA treatment attenuated the hyperactive behavioral pattern of DAT KD mice (g,h), but did not affect specific exploration or sequential organization. Veh, Vehicle.

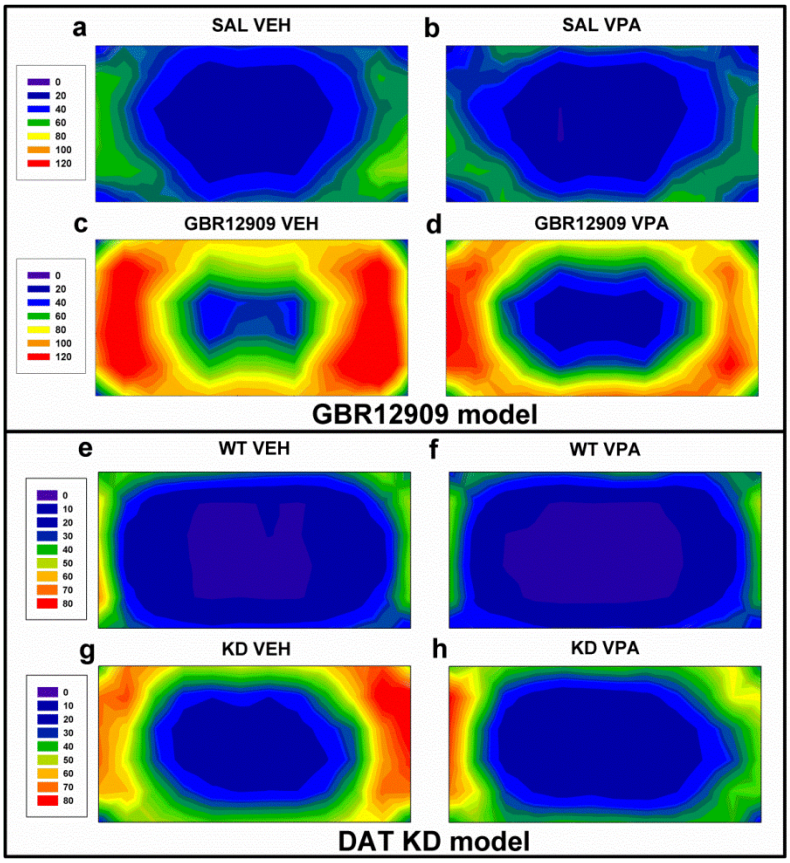

Fig. 5. Heat maps from each study of two models of dopamine transporter (DAT) inhibition [GBR12909 model (a-d) and DAT knockdown (KD) model (e-h) are displayed, representing the average group data based on 72 evenly distributed sector entries. As shown, valproic acid (VPA) treatment did not affect activity levels of saline (Sal)-treated mice (a,b). Chronic VPA treatment attenuated GBR12909-induced hyperactivity (c,d). VPA treatment did not affect activity levels of wild type (WT) mice (e,f), but attenuated the hyperactive behavioral pattern of DAT KD mice (g,h). The scale ranges from 0–120 for (a-d) and from 0 – 80 for (e-h), because of the higher baseline activity of the mice on a C57BL/6J background. Veh, Vehicle.

DISCUSSION

We examined the pharmacological predictive validity of reduced DAT functioning mouse models of BD mania. We observed that VPA attenuated the hyperactivity seen in these models, but did not affect other mania-like behavioral characteristics such as heightened specific exploration and more linear movements. The selectivity of VPA effects could be a reflection of the fact that VPA does not treat every aspect of BD. Thus, these data support the need of measuring beyond hyperactivity alone when developing novel treatments for BD mania (5).

Consistent with previous reports (8, 13, 25), we observed that both DAT KD mice and GBR12909-treated mice exhibit behavioral profiles in the BPM that are similar to those of patients with BD mania (6). The rationale for using a dose of 9 mg/kg GBR12909 came from other studies such as GBR12909-induced increases in motor impulsivity (26) in a five-choice serial reaction time task, increased risk preference in a mouse Iowa gambling task (27), and accelerated choice latency and increased motivation in mice in a progressive ratio breakpoint test (28). Mice administered the specific DAT inhibitor GBR12909 (9 mg/kg) exhibited increased activity, increased specific exploration (as measured by rearing), and straighter patterns of movement. A similar pattern was observed in the DAT KD mice, with levels of holepoking being the increased measure of specific exploration. Consistently, DAT KD mice on a 129Sv/J background exhibited increased holepoking, while GBR12909 administration to C57BL/6J mice resulted in more prominent effects on rearing behavior (6). The divergence in specific exploration is thus likely caused by a difference in background strain and may be in part due to the more active baseline phenotype of the C57 mouse strain (29, 30). Consistent with such baseline differences, 129 mouse strains also exhibit a higher spatial *d* and fewer center entries compared to the C57BL/6 strains (30). These background strain variations are consistent with earlier studies demonstrating that such variations change the effects of the DAT inhibitor modafinil (31).

After pilot studies in which 1.0 and 2.0% VPA-chow treatment for 4 weeks resulted in low-end (≈50 μg/mL) and high-end (≈150 μg/mL) therapeutic serum concentrations respectively (Fig. 1), 1.5% VPA-chow was chosen for investigation. Others have found that 2.5% VPA-chow resulted in therapeutic blood concentrations in mice from N171-82Q and YAC128 strains (≈80 μg/mL) (32). In rats, chronic dietary VPA treatment (2.0%) resulted in low-end blood concentrations (40 μg/mL) in one study (33) and in therapeutic concentrations (≈80 μg/mL) in another (34). Importantly, in the current chronic study, treatment with 1.5% VPA in the animals' chow for 28 days led to optimal therapeutic mood-stabilizing concentrations for the treatment of BD mania [60-80 μg/mL; (2)]. In both models of DAT inhibition, this treatment attenuated their hyperactivity and the frequency of center entries (Figs. 4 and 5). VPA-induced amelioration of center entries was significantly selective in GBR12909- and not saline-treated mice, with a greater effect visually in the DAT KD than WT mice, although a main effect of VPA was observed. Such differences in the models could relate to sample size or background differences described earlier. VPA modestly increased holepoking in the C57BL/6J mice, while VPA led to slightly reduced levels of spatial *d* in the DAT KD mice. The slight variation of effect could reflect background strain differences described above.

Longitudinal VPA treatment studies in the human BPM would assist direct comparisons with the present studies. Similar to the current findings in both DAT models however, 3-

week treatment with various medications reduced effect size differences of activity but not spatial *d* or object interactions in BD patients compared with healthy subjects (19).

Direct cross-species comparisons of treatment effects using similar measures are rarely available. Despite such limitations however, the anticonvulsant drug VPA has been used frequently to test the predictive validity of animal models of BD mania. Several studies have observed that acute VPA treatment attenuated hyperactivity in animals treated with amphetamine (35-37) or a combination of amphetamine and chlordiazepoxide (38). Acute VPA also reversed hyperactivity induced by sleep deprivation in D-box binding protein knockout mice (39). It is important to note however, that acute VPA can also lower basal activity levels in saline-treated animals (35), confounding the interpretation of the 'therapeutic' effects as being specific to the manipulation-induced hyperactivity. Acute VPA attenuated hyperactivity in DAT KD mice while not affecting the activity of WT mice however (13). Acute VPA treatment did not remediate GBR12909-induced hyperactivity in mice, leading to suggestions that the predictive validity of the GBR12909 model for BD mania should be examined using chronic treatment (40). In support of this point, treatment of BD mania requires chronic administration of a mood-stabilizer, nominally at least 3 weeks in clinical trials (41). When chronic treatment is used in animal models to eliminate the risk of false-positive or false-negative effects, the activity-attenuating effects are less striking. Oral treatment for 11 days with 1.2% VPA in drinking water non-significantly attenuated the dopamine D_2/D_3 agonist quinpirole-induced hyperactivity (42), which may have been due to lower end VPA blood concentrations (≈50 μg/mL). Chronic VPA treatment failed to normalize 'mania-like' behaviors in a rat model of mania (43). Nevertheless, chronic VPA treatment normalized hyperactivity in transgenic CN98 mice (44) and VPA microinjections into the nucleus accumbens attenuated amphetamine-induced hyperactivity in rats (45). Thus, positive data for models have been generated using chronic VPA treatment but methodologies have been diverse and few studies examined VPA concentrations to determine whether therapeutic levels were achieved.

In addition to marked hyperactivity in patients with BD mania, other aspects of abnormal exploratory behavior of patients with BD mania were quantified in the human BPM (6). Although therapeutic concentrations of VPA attenuated hyperactivity in the present models, specific exploration and locomotor patterns remained unaffected or worsened. One could speculate that the ineffectiveness of VPA on specific exploration may go beyond this behavior and be related to cognitive impairments that currently go untreated in BD mania as well. For example, holepoking behavior in the mouse BPM correlated with risk preference in the mouse Iowa gambling task (25). Furthermore, greater object interactions in the human BPM correlated with poor human performance in another frontal-mediated task, the Wisconsin Card Sorting Task (46). Chronic VPA treatment increased holepoking in C57BL/6J mice in the present studies. This effect is similar to a

human BPM study in which subjects receiving VPA exhibited significantly more object interactions than those not receiving VPA (6). Although specific pharmacological treatment comparisons would aid such cross-species comparisons, these data reinforce the need for therapeutics that treat the multiple facets of BD (Fig. 6) (4, 5). Accordingly, a drug that would reduce specific exploration in the BPM may also have beneficial effects on cognitive functions. The models based on reduced functioning DAT provide a robust and practical method of investigating the effects of novel compounds.

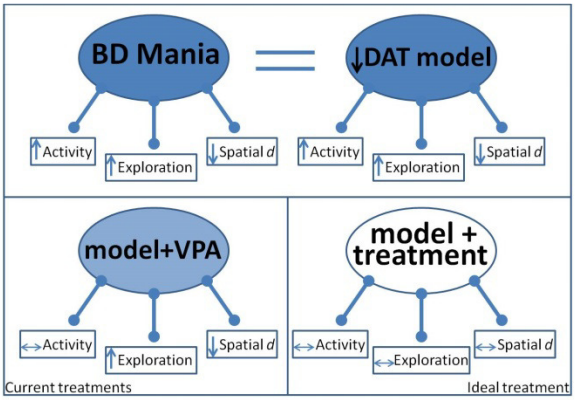

Fig. 6. Schematic on the utility of a multivariate approach for developing treatments for bipolar disorder (BD) mania. In the top panel (left), patients with BD exhibit hyperactivity, more specific exploration, and straighter line movements through space compared to healthy subjects. Both reduced functioning dopamine transporter (DAT) models [knockdown mice and GBR12909-treated mice (right)] exhibit a similarly altered pattern of exploration to that of BD patients. In the lower panel (left), the results of the current study are summarized whereby valproate only attenuated the hyperactivity of these models while not affecting other measures of abnormal exploration. Thus, using these models, an ideal treatment (lower right panel) could be identified that treats each aspect of abnormal exploration. ↑, Increased; ↓ reduced; ↔ attenuated.

The mechanisms by which VPA exerts its therapeutic effects in humans are still not fully understood. VPA affects multiple processes, including glycogen synthase kinase-3 (47), γ-aminobutyric acid (GABA)ergic neurotransmission (48), and N-methyl-D-aspartate glutamatergic signaling (49). One of the biological constructs by which VPA attenuated the mania-like behavior could be by increasing DAT gene expression in the brain (50). Importantly, VPA-induced increase in DAT expression continued to rise with the duration of treatment (50), underscoring the necessity for chronic treatment. Furthermore, VPA down-regulates dopaminergic D_2-like (D_2, D_3, and D_4) receptor signaling (51), supporting a dopaminergic mechanism for VPA. Future studies could target these putative mechanisms.

One of the limitations of this study and others when assessing the pharmacological predictive validity of an animal model for BD mania is the use of suboptimal therapeutics

to validate the models. By using a multivariate approach to quantify exploration however, the effects of a treatment can be assessed on different aspects of exploratory behavior beyond motor hyperactivity alone. Future studies should be performed to assess the effects of other chronic medications such as the mood-stabilizer lithium on the behavioral profile of the DAT KD and GBR12909-treated mice. Additionally, while most treatment studies involve animal models, these data provide a specific testable hypothesis for longitudinal drug testing in humans in the BPM; to wit, VPA treatment for mania would reduce hyperactivity compared to patients in an unmedicated state, but would not affect increased specific exploration or reduced spatial *d*.

In conclusion, mania-like behavior of mice with reduced functional DAT is partially alleviated by chronic treatment with VPA, mimicking the real-life situation in which BD treatment does not alleviate all symptoms. The data presented here provide predictive validation that selective DAT inhibition enables modeling of BD mania-like behavior, although more explicit human medication studies would provide more specific support. The DAT model can be used to screen better therapeutics treating the complete mania-like behavioral profile including cognitive dysfunction associated with mania.

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CHAPTER 2.2

Dopamine depletion attenuates some behavioral abnormalities in a hyperdopaminergic mouse model of bipolar disorder

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ABSTRACT

Background

Patients with BD suffer from multifaceted symptoms, including hyperactive and psychomotor agitated behaviors. Previously, we quantified hyperactivity, increased exploration, and straighter movements of patients with BD mania in the human behavioral pattern monitor (BPM). A similar BPM profile is observed in mice that are hyperdopaminergic due to reduced dopamine transporter (DAT) functioning. We hypothesized that DA depletion through alpha-methyl-*p*-tyrosine (AMPT) administration would attenuate this mania-like profile.

Methods

Male and female DAT wild-type (WT; *n*=26) and knockdown (KD; *n*=28) mice on a C57BL/6 background were repeatedly tested in the BPM to assess profile robustness and stability. The optimal AMPT dose was identified by treating male C57BL/6 mice (*n*=39) with vehicle or AMPT (10, 30, or 100 mg/kg) at 24, 20, and 4 h prior to testing in the BPM. Then, male and female DAT WT (*n*=40) and KD (*n*=37) mice were tested in the BPM after vehicle or AMPT (30 mg/kg) treatment.

Results

Compared to WT littermates, KD mice exhibited increased activity, exploration, straighter movement, and disorganized behavior. AMPT-treatment reduced hyperactivity and increased path organization, but potentiated specific exploration in KD mice without affecting WT mice.

Limitations

AMPT is not specific to DA and also depletes norepinephrine.

Conclusions

KD mice exhibit abnormal exploration in the BPM similar to patients with BD mania. AMPT-induced DA depletion attenuated some, but potentiated other, aspects of this mania-like profile in mice. Future studies should extend these findings into other aspects of mania to determine the suitability of AMPT as a treatment for BD mania.

Keywords

AMPT, Bipolar disorder, Mice, Mania, Dopamine transporter

INTRODUCTION

Dysregulated dopamine (DA) neurotransmission is thought to contribute to several psychiatric disorders including bipolar disorder (BD) (1, 2). Polymorphisms in the DA transporter (DAT) gene have been associated with BD (3, 4). These polymorphisms likely result in reduced cell surface expression and hence function of the DAT in patients (5). Indeed, reduced striatal levels of DAT have been observed in unmedicated patients with BD by using positron emission tomography (6) as well as in postmortem tissue (7). Hyperdopaminergia caused by reduced DAT function may therefore underlie many of the behavioral abnormalities observed in patients with BD.

Previously, we reported that patients with BD mania are hyperactive, exhibit increased object interactions, and walk in straight paths as quantified by the human behavioral pattern monitor (BPM) (8, 9). This increased motor and exploratory activity is a cardinal feature of a manic episode and is described in the DSM-IV as an "increase in goal-directed activity" or "psychomotor agitation" (10, 11). Using a cross-species translational approach, we observed consistent behavioral patterns of mice with reduced functional DATs via either pharmacological or genetic manipulations (8, 12-14). Specifically, mice treated acutely with the DAT inhibitor GBR12909 or constitutive DAT knockdown (KD) mice exhibit hyperactivity, increased specific exploration, and straighter paths of movement compared to controls in the mouse BPM. The hyperactivity of each model was attenuated after chronic treatment with the mood-stabilizer valproate (15). Moreover, we observed that the mania-like behavior of these mice is not limited to altered motor and exploratory activity since these animals also exhibited increased risk preference in a gambling task (16, 17).

Hence, DAT KD mice have proven to be a useful model for BD mania. The original DAT KD mice were on a mixed 129/S background however, and their phenotype was less stable than that produced by acute administration of GBR12909 to C57BL/6 mice (12). Despite the fact that one technique used pharmacological and the other genetic manipulations, we hypothesized that the discrepancy in stability was likely as a result of background strain (12). Indeed, the 129/S line of DAT wild-type (WT) and KD mice exhibited lower levels of activity than C57BL/6 mice as expected from these lines (18). To better compare stability of the genetic model to that presented in the C57BL/6 pharmacological model, we have assessed the phenotypic stability of DAT KD mice on a C57BL/6 background.

Better models for BD are required in order to develop treatments targeted at the underlying mechanisms, as opposed to the serendipitous discovery of treatments as has occurred until now. For instance, aberrant motor and exploratory behavior are still observed in patients with BD, despite being on medication for 3 weeks (9). Moreover,

euthymic BD patients also still exhibit a hyperexploratory profile (19), poor risk learning (20), and poor cognitive function, particularly memory difficulties and impaired executive function (21, 22) compared with healthy subjects. Given the reduced DAT levels and hyperdopaminergic state of people with BD, reducing DA availability may theoretically normalize their neurochemical state and perhaps their behavior. DA depletion can be induced by administration of alpha-methyl-*p*-tyrosine (AMPT). AMPT is a competitive inhibitor of tyrosine hydroxylase, the rate limiting enzyme in the synthesis of catecholamines from tyrosine (23, 24). Supporting this idea, pretreatment with AMPT blocked chlordiazepoxide/(+)-amphetamine-induced hyperactivity in mice without affecting activity of control mice (25). Moreover, AMPT treatment can reduce symptoms in patients with BD mania, while an increase in depression was observed in depressed patients treated with AMPT (26). More recently, DA depletion with AMPT did not affect mood in patients with BD during treatment, but patients experienced a relapse of hypomanic symptoms post-depletion (27).

To assess whether DA depletion could rescue some of the cross-species quantified behavioral deficits relevant to BD mania, we examined the effects of AMPT in the DAT KD mouse model for BD mania, which have approximately 10% expression of the DAT and exhibit increased extracellular DA compared to control mice (28). We hypothesized that (*a*) DAT KD mice on a C57BL/6 background would exhibit a BD mania-like profile in the BPM consistent with DAT KD mice on a 129/S background; (*b*) repeated testing of these mice would demonstrate a robust and stable phenotypic profile; and (*c*) catecholamine depletion by AMPT treatment would attenuate this mania-like behavioral profile.

METHODS

Animals

Male C57BL/6J mice (*n*=39), DAT KD (male, *n*=37; female, *n*=28), and DAT WT (male, *n*=32; female, *n*=34) littermate mice were used throughout the three studies. DAT heterozygous breeders backcrossed onto a C57BL/6 background for more than 10 generations were sent to our laboratory from the University of Chicago. Male and female DAT KD and WT mice were generated from heterozygous breeding pairs. All mice were group housed (four/cage) and maintained in a temperature-controlled vivarium (21±1°C) on a reversed day-night cycle (lights on at 7.00 PM). Mice were 3-6 months old at the time of testing, weighed between 20 and 40 g, and were tested during the dark phase between 8.00 AM and 5.00 PM. Mice had *ad libitum* access to water and food (Harlan, Madison, WI, USA). All procedures were approved by the UCSD Institutional Animal Care and Use Committee. The UCSD animal facility meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care.

Drug treatment

Alpha-methyl-*p*-tyrosine methyl ester hydrochloride (AMPT) (Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline (10 ml/kg). AMPT or saline was administered to mice in three equal i.p. injections 24, 20, and 4 h prior to testing (25). Previous studies have shown that mouse brain DA and norepinephrine (NE) levels are reduced ≈30–40% 4 h after administration of 40–80 mg/kg (i.p.) AMPT (29, 30).

Mouse behavioral pattern monitor

Locomotor behavior and exploration were examined using eight mouse BPM chambers (San Diego Instruments, San Diego, CA) as described previously (31, 32). Each Plexiglas arena consists of a 30.5 × 61 × 38 cm area with three floor holes and eight wall holes (three along each long side and one in each of the short sides; 1.25 cm in diameter, 1.9 cm from the floor), each equipped with an infrared photobeam to detect holepoking. An outer box with an internal white house-light above the arena (350 lux in the center and 92 lux in the four corners) minimized external light and noise. Activity was obtained from a grid of 12 × 24 infrared photobeams 1 cm above the floor (2.5 cm apart; 24 × 12 *X*-*Y* array), recording the location of the mouse every 0.1 s, with its position defined across nine unequal regions (four corners, four walls, and center (33)). Another set of 16 photobeams, placed 2.5 cm above the floor, was used to detect rearing behavior. Mice were placed in the bottom left-hand corner of the arena and the test session started immediately. The primary outcome measures were transitions across the defined regions and center entries (locomotor activity), holepoking and rearing (exploratory behavior), and entropy (*h*) and spatial *d* (locomotor patterns). Lower entropy reflects predictable, ordered sequences of activity, while higher entropy indicates a greater disorder of movement. Spatial *d* quantifies the geometric structure of the locomotor path, where a value of 1 reflects a straight path, and 2 highly circumscribed small-scale movements (34).

Exp 1.

Female (*n*=11) and male (*n*=17) DAT KD mice and their female (*n*=12) and male (*n*=14) WT littermates were tested in the BPM to assess their exploratory profile. Activity was measured in the BPM for 60 min during the first test (Exp 1a), then one week later for 60 min (Exp 1b), and finally 10 days later for 180 min (Exp 1c).

Exp 2.

The effects of AMPT on the exploratory profile of male C57BL6/J mice were assessed in the BPM for 60 min. Mice received saline or 10, 30, or 100 mg/kg AMPT 24, 20, and 4 h prior to testing (*n*=10/group).

Exp 3.

The effects of 30 mg/kg AMPT were assessed on the exploratory behavior of female (*n*=17) and male (*n*=20) DAT KD mice and their female (*n*=22) and male (*n*=18) WT littermates in the BPM for 60 min. These mice were not BPM naïve and had been treated with the antipsychotic risperidone six weeks earlier. The mice were baseline-matched based on transitions, holepoking, and spatial *d*, and counter-balanced with previous risperidone experience. Mice were treated with AMPT or saline 24, 20, and 4 h prior to testing.

Statistics

The primary measures for each experiment were analyzed using two- or three-way analyses of variance (ANOVA), with sex, genotype, or drug as between-subject variables. In Exp 3, an ANCOVA was used to assess whether prior risperidone treatment affected the outcome of the study. Statistically significant or relevant interactions and main effects were analyzed using Tukey's *post hoc* analyses where applicable. The data were analyzed for 60 min testing periods using the BMDP statistical software (Statistical Solutions Inc., USA). The α level was set to 0.05.

RESULTS

Exp 1a. Stability of DAT WT and KD exploration in the BPM

Because no sex × genotype interactions were observed for any measures, male and female data were pooled and analyzed together.

Locomotor activity

KD mice were hyperactive as reflected by increased transitions ($F_{(1,52)}$=46.3, p<0.0001; Fig. 1a) and center entries ($F_{(1,52)}$=16.8, p<0.0001; Fig. 1b).

Exploratory behavior

KD mice exhibited more exploration as reflected by increased rearing ($F_{(1,52)}$=4.9, p<0.05; Fig. 1c), but not holepoking (F<1, ns; Fig. 1d).

Locomotor patterns

KD mice moved in straighter lines as reflected by reduced spatial *d* ($F_{(1,52)}$=6.0, p<0.05; Fig. 1e) and exhibited greater disorder of movement as reflected by higher entropy ($F_{(1,52)}$=52.4, p<0.0001; Fig. 1f).

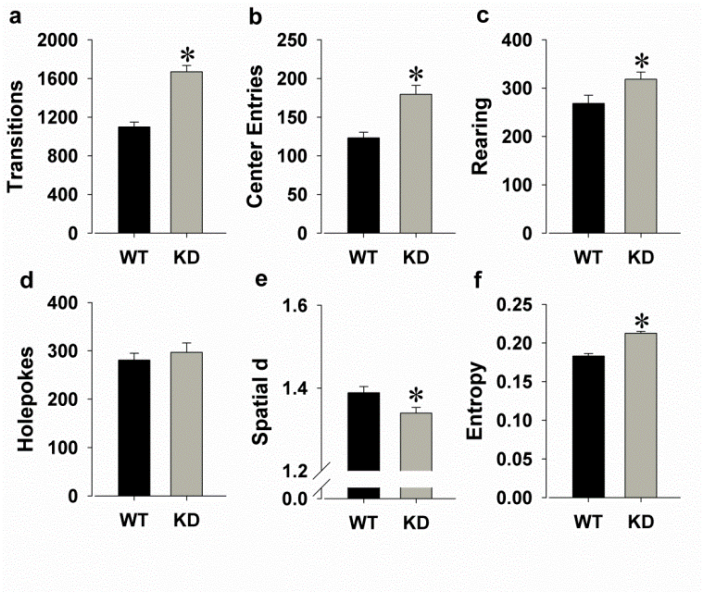

Fig. 1. The exploratory profile of DAT KD and WT mice on a C57BL/6J background in the BPM. KD mice were hyperactive compared to WT mice as measured by increased transitions (a) and more center entries (b). KD mice also exhibited increased exploration compared to WT mice as reflected by increased rearing (c), but not holepoking (d). KD mice moved in straighter paths than WT mice as measured by a lower spatial *d* (e) and exhibited disordered movement organization as measured by higher entropy (f). Data are presented as mean + S.E.M. **p*<0.05 when compared to WT mice.

Exp 1b. Repeated testing of DAT WT and KD mice in the BPM

The exploratory profiles of mice from Exp 1a were assessed in the BPM one week later.

Locomotor activity

KD remained hyperactive as reflected by increased transitions ($F_{(1,52)}$=49.3, *p*<0.0001; Fig. 2a) and center entries ($F_{(1,52)}$=25.4, *p*<0.0001; Fig. 2b).

Exploratory behavior

KD mice exhibited more exploration as reflected by increased rearing ($F_{(1,52)}$=5.8, *p*<0.05; Fig. 2c) and now holepoking ($F_{(1,52)}$=7.1, *p*<0.05; Fig. 2d).

Locomotor patterns

KD mice moved in straighter lines as reflected by reduced spatial *d* ($F_{(1,52)}$=9.6, *p*<0.005; Fig. 2e) and exhibited greater disorder of movement as reflected by higher entropy ($F_{(1,52)}$=56.2, *p*<0.0001; Fig. 2f).

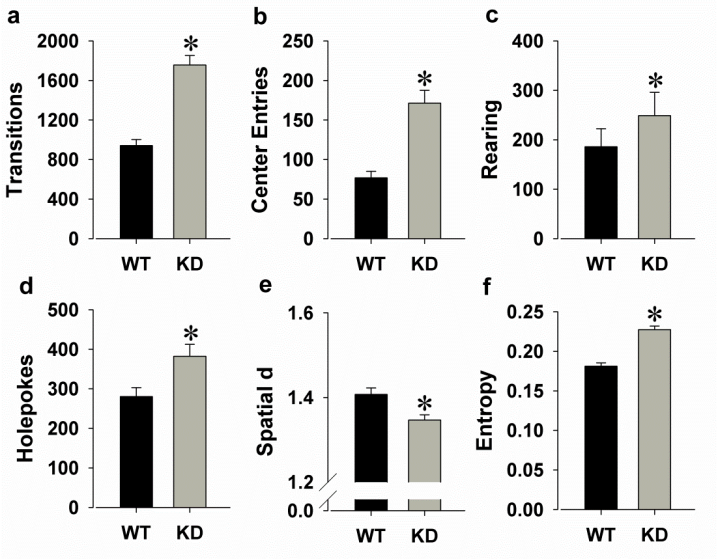

Fig. 2. The exploratory profile of DAT KD and WT mice on a C57BL/6J background tested in the BPM a second time, one week after their initial testing. KD mice remained hyperactive compared to WT mice upon repeated testing as measured by increased transitions (a) and more center entries (b). KD mice remained more exploratory than WT mice as measured by increased rearing (c), and now also increased holepoking (d). KD mice still moved in straighter paths than WT mice as measured by a lower spatial *d* (e) and exhibited disordered movement organization as measured by higher entropy (f). Data are presented as mean + S.E.M. **p*<0.05 when compared to WT mice.

Exp 1c. Prolonged testing for three hours of DAT WT and KD mice in the BPM

Mice from Exp 1b were tested 10 days later for three hours and data were analyzed in three 60 min time bins.

Locomotor activity

KD remained hyperactive as reflected by increased transitions ($F_{(1,52)}$=46.0, *p*<0.0001; Fig. 3a) and center entries ($F_{(1,52)}$=36.3, *p*<0.0001; Fig. 3b). There was a main effect of time and a genotype × time bin interaction for transitions [$F_{(2,104)}$=50.7, *p*<0.0001 and $F_{(2,104)}$=3.4, *p*<0.05 respectively] and center entries [($F_{(2,104)}$=36.8, *p*<0.0001 and $F_{(2,104)}$=3.4, *p*<0.05 respectively]. *Post hoc* analyses revealed that KD mice had increased transitions and center entries compared to WT mice at each time bin (*p*<0.0005) and both WT and KD mice decreased activity over time (*p*<0.0001).

Exploratory behavior

KD mice remained more explorative as reflected by increased rearing ($F_{(1,52)}$=23.5, *p*<0.0001; Fig. 3c) and holepoking ($F_{(1,52)}$=8.2, *p*<0.01; Fig. 3d). There was a main effect of

time for holepoking ($F_{(2,104)}$=5.1, p<0.01) and rearing ($F_{(2,104)}$=12.7, p<0.0001) but only a trend towards a genotype × time bin interaction for rearing ($F_{(2,104)}$=2.9, p<0.1). *Post hoc* analyses revealed that WT mice decreased rearing over time (p<0.0001), but made similar amount of holepokes over time. In contrast, KD mice increased holepoking over time (p<0.05), while their rearing behavior did not change.

Locomotor patterns

KD mice moved in straighter paths as reflected by reduced spatial *d* ($F_{(1,52)}$=10.6, p<0.005; Fig. 3e) and exhibited greater disorder of movement as reflected by higher entropy ($F_{(1,52)}$=64.1, p<0.0001; Fig. 3f). There was a main effect of time for spatial *d* ($F_{(2,104)}$=24.6, p<0.0001) and entropy ($F_{(2,104)}$=83.8, p<0.0001) and a genotype × time bin interaction for entropy ($F_{(2,104)}$=6.2, p<0.005). *Post hoc* analyses revealed that spatial *d* increased and entropy decreased over time in both WT and KD mice (p<0.0001).

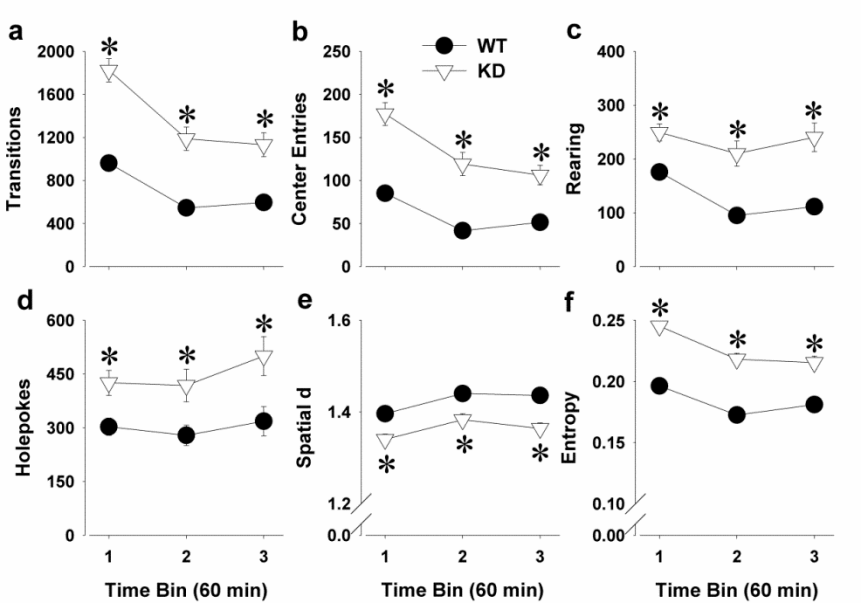

Fig. 3. The exploratory profile of DAT KD and WT mice on a C57BL/6J background tested in the BPM a third time, 10 days after their second testing. The profile of mice was assessed in the BPM for a prolonged period of 3-h and divided in three 60-min time bins. Although both WT and KD mice habituated over time, KD mice remained hyperactive throughout the 3-h period as measured by increased transitions (a) and more center entries (b). While WT mice decreased rearing over time, KD mice did not and exhibited increased rearing at each time bin (c). WT mice did not change in holepoking behavior over time, while KD mice exhibited increased holepoking behavior compared to WT mice and over time (d). Although both WT and KD mice exhibited increased spatial *d* and reduced entropy over time, KD mice moved in straighter paths (e) and exhibited disordered movement organization (f) compared to WT mice throughout the 3-h period. Data are presented as mean ± S.E.M. *p<0.05 when compared to WT mice.

Exp 2. Dose-response study of AMPT in C57BL6/J mice

The effects of sub-chronic AMPT on the exploratory profile of male C57BL6/J mice (*n*=39) were investigated for 60 min in the BPM.

Locomotor activity

There was a significant main effect of AMPT on transitions ($F_{(3,35)}$=3.4, p<0.05; Fig. 4a), but not on center entries (Fig. 4b). *Post hoc* analyses revealed that the highest dose of AMPT reduced activity compared to the lowest dose (p<0.05) and tended to compared to saline (p<0.1).

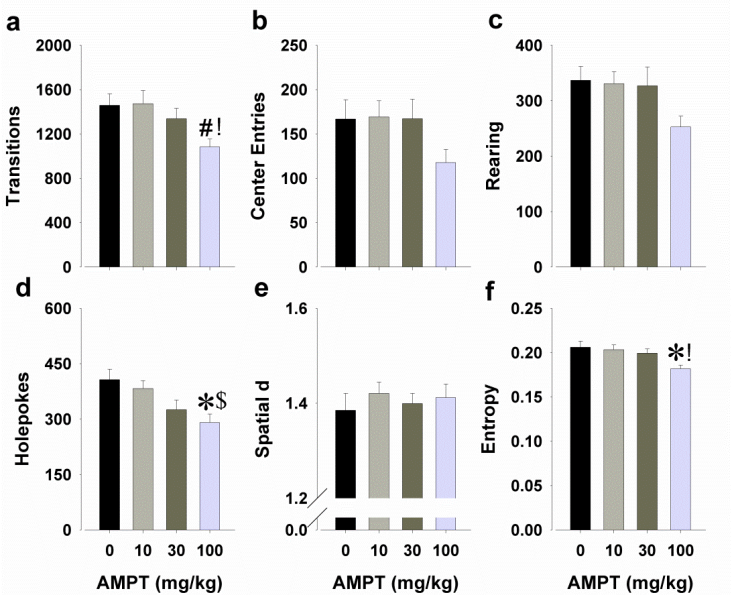

Fig. 4. Effects of AMPT on exploratory behavior in male C57BL6/J mice in the BPM. The tyrosine hydroxylase inhibitor AMPT (10, 30, and 100 mg/kg) was administered to mice at 24, 20, and 4 h prior to their assessment in the BPM. AMPT at the highest dose reduced activity as measured by reduced transitions (a), but not center entries (b). AMPT at the highest dose reduced exploratory behavior as measured by a non-significant drop in rearing (c) and a significant drop in holepoking (d). AMPT did not affect spatial d (e), but reduced entropy at the highest dose (f). Data are presented as mean + S.E.M. * p<0.05 and $^{\#}$ p<0.1 when compared to saline; $^{!}$ p<0.05 and $^{\$}$ p<0.1 when compared to AMPT 10 mg/kg.

Exploratory behavior

A main effect of AMPT was observed on exploration, specifically on holepoking ($F_{(3,35)}$=4.5, p<0.01; Fig. 4d) and a trend effect on rearing ($F_{(3,35)}$=2.4, p<0.1; Fig. 4c). *Post hoc* analyses revealed that the highest dose of AMPT reduced holepoking compared to saline (p<0.05) and tended to compared to the lowest dose (p<0.1).

Locomotor patterns

AMPT treatment affected entropy ($F_{(3,35)}$=4.2, p<0.05; Fig. 4f) but not spatial *d* (Fig. 4e). *Post hoc* analyses indicated that the highest dose of AMPT resulted in more ordered sequences of activity (lower entropy) compared to saline and the lowest dose of AMPT (p<0.05).

Exp 3. Low dose AMPT in DAT WT and KD mice

The effects of 30 mg/kg AMPT (a dose that was ineffective in C57BL/6 mice; Exp 2a) were assessed on the exploratory behavior of WT and KD mice in the BPM. Since there were no effects of or interactions with sex for any of the measures, male and female data were pooled and analyzed together. Moreover, covarying for prior risperidone experience did not affect the statistical findings.

Locomotor activity

Significant genotype effects were observed for transitions ($F_{(1,73)}$=83.3, p<0.0001; Fig. 5a) and center entries ($F_{(1,73)}$=36.0, p<0.0001; Fig. 5b). A significant effect of AMPT was observed for center entries ($F_{(1,73)}$=5.1, p<0.05) and tended to affect transitions ($F_{(1,73)}$=3.9, p<0.1). AMPT treatment and genotype tended to interact on transitions ($F_{(1,73)}$=3.5, p<0.1) and center entries ($F_{(1,73)}$=3.2, p<0.1). *Post hoc* analyses revealed that AMPT did not affect activity in WT mice, but significantly reduced transitions and center entries in KD mice compared with saline (p<0.05).

Exploratory behavior

Main effects of genotype were observed for holepoking ($F_{(1,73)}$=13.6, p<0.0005; Fig. 5d) and rearing ($F_{(1,73)}$=45.7, p<0.0001; Fig. 5c). AMPT did not affect exploration alone (F<1, ns), but tended to interact with genotype on holepoking ($F_{(1,73)}$=3.2, p<0.1). Interestingly, *post hoc* analyses revealed that while saline-treated KD mice did not differ from saline-treated WT mice in holepoking, AMPT-treated KD mice made significantly more holepokes compared to AMPT-treated WT mice (p<0.05), but not compared to saline-treated KD mice.

Locomotor patterns

Significant effects of genotype were observed for spatial *d* ($F_{(1,73)}$=23.1, p<0.0001; Fig. 5e) and entropy ($F_{(1,73)}$=79.2, p<0.0001; Fig. 5f). There were no main effects of AMPT on these variables (F<1, ns), but there was a significant AMPT × genotype interaction for entropy ($F_{(1,73)}$=4.8, p<0.05). *Post hoc* analyses revealed that AMPT did not affect entropy in WT mice, but significantly reduced entropy in KD mice compared with saline-treated KD mice (p<0.05).

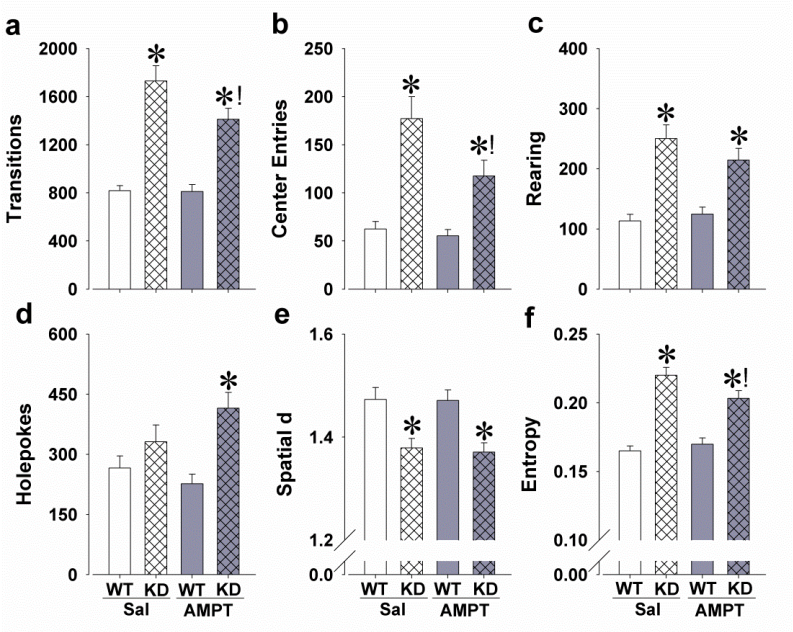

Fig. 5. Effects of 3 × 30 mg/kg AMPT on the mania-like profile of DAT KD mice in the BPM. Hyperactivity of KD mice compared to WT mice was attenuated, but not completely abolished, by AMPT treatment as measured by reduced transitions (a) and less center entries (b). KD mice exhibited increased exploration compared to WT mice as measured by increased rearing without an effect of AMPT (c). AMPT potentiated specific exploration as measured by increased holepoking in the KD mice compared to WT mice (d). KD mice exhibited straighter paths of movement compared to WT mice, which was unaffected by AMPT treatment (e). Disordered movement organization of KD mice compared to WT mice was attenuated by AMPT treatment as measured by a reduction in entropy (f). Data are presented as mean + S.E.M. * $p<0.05$ when compared to WT mice; ! $p<0.05$ when compared to saline.

DISCUSSION

DAT KD mice on a C57BL/6J background exhibit a robust BD mania-like profile in the BPM, which did not wane with repeated testing. Furthermore, AMPT-induced DA depletion attenuated some, but potentiated other, mania-like behaviors, without affecting control animals. Consistent with previous reports, KD mice exhibited hyperactivity, increased exploration, and disrupted behavioral organization in the BPM similar to patients with BD (8, 17). Increased exploration was driven more by increased rearing compared to holepoking behavior, consistent with GBR12909-induced exploration in C57BL/6 mice (14). Interestingly, DAT KD mice on a 129/S background primarily exhibited increased holepoking behavior (8), replicated in 129/SvJ mice administered GBR12909 (14). With the prolonged testing period, the DAT KD mice on the C57BL/6 background also exhibited increased holepokes compared with WT controls. As hypothesized, the mania-like

phenotype of KD mice was consistent even after repeated testing, reflecting a more robust and stable phenotype when compared with KD mice on the 129/S background. Taken together, the behavioral profile of KD mice observed here matches the effects of acutely administered GBR12909 in C57BL/6J mice and that of BD mania patients in the human BPM.

The dose-response study of AMPT in C57 mice revealed that 3 doses of 100 mg/kg decreased activity, exploration, and behavioral organization, while the other doses had no effect. Previously however, Davies et al. reported that pretreatment with 3 doses of 100 mg/kg AMPT did not reduce the holepoking activity of female control mice (Porton strain), but completely abolished increased holepoking induced by a mixture of chlordiazepoxide and (+)-amphetamine (25). The strain difference or the fact that only female mice were used by Davies et al. may have caused this difference in dose-response. When administered to female WT and KD mice, AMPT (3 × 100 mg/kg) also reduced activity in the BPM independent of genotype (unpublished observations). Hence, we attempted to reverse the mania-like behavior of KD mice using 30 mg/kg under the hypothesis that this dose would preferentially affect KD and not WT mice in accordance with the requirement for treatment development (35).

Indeed, catecholamine depletion after 3 × 30 mg/kg AMPT did not affect exploratory behavior in WT mice, consistent with the effects observed in C57BL/6J mice. In DAT KD mice however, this dose of AMPT attenuated some facets of their mania-like behavior, including activity levels (transitions and center entries) and movement organization (entropy h). AMPT potentiated the increased holepoking observed in KD mice however. Perhaps importantly, holepoking behavior in the mouse BPM correlated modestly with risk preference in the mouse Iowa gambling task (17). Moreover, increased object interactions in the human BPM (human analogue for specific exploration) correlated with impaired performance in the Wisconsin Card Sorting Task (36). AMPT may therefore negatively affect aspects of cognitive functioning. In fact, several studies have recently demonstrated that AMPT treatment can impair reward processing and probabilistic learning in patients with remitted major depressive disorder (37, 38) and bulimia nervosa (39). Given that cognitive performance correlates with a patients' ability to live independently (40), any treatment that negatively impacts cognition may not be useful therapeutically (35).

There are some limitations to this study that warrant attention. First, the generalized effects of AMPT may relate to its lack of specificity for DA. As was shown previously, AMPT can also deplete NE (29, 30, 41). Thus, although studies suggest that AMPT causes slightly more DA than NE depletion in rodents (30) and humans (24), it will be important to test whether selective DA depletion affects cognition. Ultimately, although

catecholamine depletion attenuated some facets of the mania-like behavior in KD mice, further investigation of the neurocognitive effects of AMPT are required.

In humans, AMPT is also being used in order to elucidate catecholaminergic pathways underlying brain disorders and as a potential therapeutic in clinical research (24, 42). Although AMPT has only been approved as a therapeutic agent to treat the symptoms of phaeochromocytoma, beneficial effects have been studied in various neuropsychiatric disorders, including dystonia, dyskinesia, Huntington's disease, substance abuse, and schizophrenia (42). Interestingly, some studies have investigated the utility of AMPT as a treatment in BD mania. In one study, AMPT rapidly decreased manic symptoms in patients, while it increased depression symptoms in patients with unipolar depression (26). In another study, AMPT did not affect mood in euthymic BD patients on lithium therapy (27). However, soon after catecholamine depletion ceased, all patients experienced a relapse of hypomanic symptoms. Rebound synthesis of catecholamines and/or postsynaptic DA receptor supersensitivity after administration of AMPT (43) may have led to these relapses of mania. Cognitive performance was not assessed in any of these studies however, and would be very useful in future studies. Additionally, it is important to note that AMPT can produce serious dose-related side effects, including crystalluria and acute dystonia (44, 45). Future studies should therefore also investigate low-dosage regimens, which have been demonstrated to be a suitable alternative that is free from severe side effects (46). Thus, it is possible to conduct cross-species relevant studies in animals and humans with previous studies supporting our findings that AMPT can reduce mania-like behavior.

In summary, DAT KD mice on a C57BL/6J background exhibited a robust and stable mania-like phenotype in the BPM. Catecholamine depletion attenuated some facets of mania-like behavior in KD mice, but also potentiated abnormal exploration. These studies shine new light on a forgotten putative treatment for BD mania. Future studies should investigate whether AMPT in low doses could potentially reduce symptoms in patients with BD mania as well as determine its neurocognitive effects in patients and animal models.

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CHAPTER 2.3

Cannabis use for reward or symptom relief in psychiatric disorders

In response to Katsidoni et al. 2013 "Biphasic effects of delta9-tetrahydrocannabinol on brain stimulation reward and motor activity"

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In the recently published article by Katsidoni et al. (1), the authors investigated the effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive ingredient of cannabis, on brain stimulation reward and motor activity in rodents. With great interest, we read how the authors describe biphasic effects of Δ^9-THC on intracranial self-stimulation (ICSS) thresholds and motor activity including its blockade by the cannabinoid type 1 (CB1) receptor antagonist SR141716A (rimonabant) in rats. A low dose of Δ^9-THC (0.1 mg/kg) decreased ICSS thresholds (reward-facilitating effect) and increased activity and exploration. In contrast, a higher dose (1 mg/kg) increased ICSS thresholds and decreased activity. The authors discuss the ambiguous and inconsistent results observed with Δ^9-THC to date, being highly dependent on animal strain used, methodology, and experimental design. Nevertheless, based on their results, they suggest that Δ^9-THC induces behaviors that are typically seen with other drugs of abuse (i.e., reward-facilitation and hyperactivity), supporting the premise that cannabis is used for reasons similar to other drugs of abuse.

Δ^9-THC and other cannabinoids are increasingly investigated as promising therapeutic targets for a variety of symptoms. Hence, we would like to add some data and discussion relating to research on cannabis use in major psychiatric disorders, specifically bipolar disorder (BD). Indeed, individuals with a mental disorder are about 10 times more likely to use cannabis weekly compared to healthy individuals (2). Cannabis use is particularly prevalent among patients with BD, although the cause remains unknown. While persons with BD may be more liable to use substances of abuse, they may use cannabis as a means to self-medicate, i.e. symptom relief (3). Taking this possibility into consideration, we have begun conducting cross-species studies investigating the relationships between the endocannabinoid system, BD, their cannabis use, and symptom manifestation.

Previously, we established and quantified an exploratory behavioral pattern of patients with BD mania and euthymia in a test called the human behavioral pattern monitor (hBPM). Manic and euthymic BD patients exhibited a different profile from both healthy comparison (HC) subjects as well as SCZ patients (4, 5). In brief, BD patients are consistently hyperactive, exhibit greater object interactions / exploration, and move in more direct paths compared to HC subjects (4). Additionally, we have consistently observed the same phenotype in hyperdopaminergic mice expressing only 10% of the dopamine transporter (DAT) when tested in a smaller version of the BPM; the mouse BPM (mBPM) (4, 6). These behavioral similarities are important because DAT polymorphisms associated with BD likely reduce functional DAT expression, resulting in lower levels of DAT in unmedicated patients with BD (7) and in postmortem tissue. We have used the cross-species comparison of profiles in the BPM to investigate mechanisms of the disorder, the effects of therapeutics (6), and their links to other symptoms. Similarly, we are investigating the effects of Δ^9-THC on this BPM profile in DAT KD mice.

We assessed the effects of vehicle or 3 mg/kg Δ^9-THC (i.p. injection, 30 min prior to testing) on the exploratory behavior of female DAT wild-type [WT, vehicle (n=10), Δ^9-THC (n=10)] and KD [vehicle (n=8), Δ^9-THC (n=7)] mice on a C57BL/6J background by using the mBPM. In brief, the mBPM consists of a 30.5 cm × 61 cm Plexiglas chamber with three floor holes and eight wall holes to stimulate exploratory behavior. Photobeams were used to record and quantify motor activity, exploratory behavior, and patterns of activity for 45 min. All animal procedures were approved by the UCSD Institutional Animal Care and Use Committee.

Consistent with previous studies, KD mice were hyperactive ($F_{(1,31)}$=14.0, p<0.001), hyper-exploratory as indicated by increased rearing ($F_{(1,31)}$=31.3, p<0.001), and moved in more direct paths compared to WT mice ($F_{(1,31)}$=13.6, p<0.001; Fig. 1). DAT KD mice treated with Δ^9-THC however, were not hyperactive or hyper-exploratory. Importantly, Δ^9-THC reduced activity only in the KD mice compared with vehicle (p<0.05) and not in WT mice compared with vehicle. Although Δ^9-THC reduced exploration in both WT and KD mice (p<0.01), a Δ^9-THC × genotype interaction ($F_{(1,31)}$=9.8, p<0.01) indicated stronger effects of Δ^9-THC in KD mice. Δ^9-THC did not affect spatial organization in either genotype.

Katsidoni et al. described motor inhibition in rats by Δ^9-THC at 1 mg/kg. We did not observe any effects on activity in control mice even at 3 mg/kg, although decreased rearing behavior was observed. The inconsistency in results could be due to using different species, doses, or measurement of activity. The major difference however, lies in the interpretation of our findings. We observed that activity was specifically normalized by Δ^9-THC and effects on rearing were greater in KD mice compared to WT controls. Hence, these data support an interpretation that cannabis use may alleviate some symptoms in psychiatric patients and so patients may use cannabis to self-medicate as opposed to simply for its rewarding effects. These findings are supported by preliminary results in our human studies, which support that BD patients screened positive for cannabis use exhibited fewer object interactions compared to BD patients screened negative for cannabis (p=0.06). These data are therefore consistent with our rearing findings in our mouse model described above. These clearest results of Δ^9-THC-induced reduction in exploration across species may have important consequences for other functional domains. In an earlier report, we found that exploration in the mBPM correlated modestly with risk preference in a mouse decision-making test (8). Moreover in humans, greater object interactions in the hBPM correlated with poorer executive functioning in the Wisconsin Card Sorting Task (9). Hence, the effects of Δ^9-THC may go beyond our observed amelioration of exploration alone and may beneficially impact cognitive domains as well. Because BD patients suffer from cognitive deficits that correlate with their functional outcome (10), the potential of cannabis and cannabis-related treatments to alleviate such symptoms should be further investigated.

In conclusion, while data published by Katsidoni et al. indicates that Δ^9-THC can induce behaviors typical of abuse in normal rats, our data support a self-medicating hypothesis for cannabis use in BD. Although cannabis may have abuse potential, the possible therapeutic effects in psychiatric disorders should not be underestimated and future studies should investigate such putative benefits.

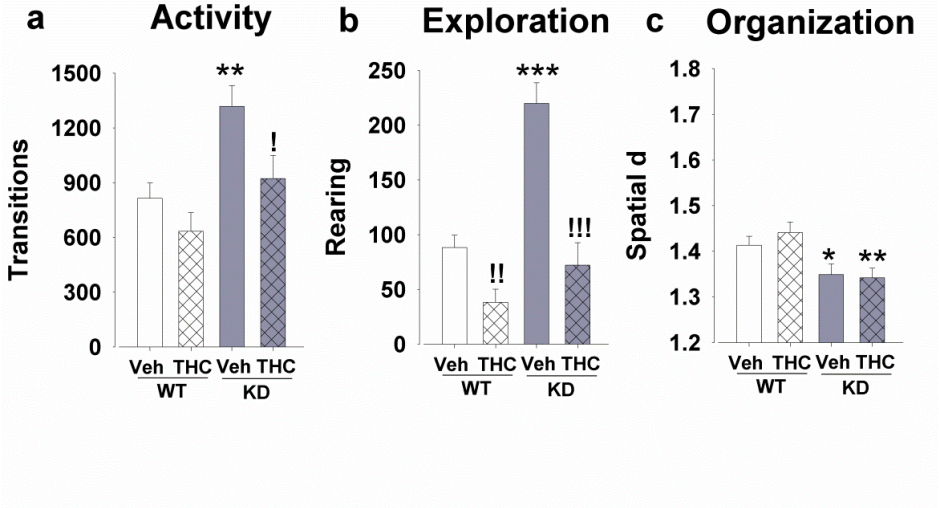

Fig. 1. The exploratory profile of DAT KD mice in the mBPM and the effects of acute Δ^9-THC administration. KD mice exhibited a pattern of hyperactivity (a), hyper-exploration (b), and more direct paths of movement (indicated by lower spatial *d*) (c) compared to wild-type (WT) littermate controls. Δ^9-THC attenuated the hyperactivity (a) and hyper-exploration (b) of KD mice. While Δ^9-THC also reduced the exploration of WT mice, the KD mice were reduced to vehicle- (Veh) treated WT, as represented by a significant Δ^9-THC × genotype interaction (b). Data are presented as mean + S.E.M., $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ when compared with WT, $^{!}p<0.05$, $^{!!}p<0.01$, and $^{!!!}p<0.001$ when compared with Veh.

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CHAPTER 3

Assessment of risk-based decision-making in mice and patients with bipolar disorder in species-specific Iowa gambling tasks

CHAPTER 3.1

Increased risk-taking behavior in dopamine transporter knockdown mice: further support for a mouse model of mania

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ABSTRACT

Background

Reduced functioning of the dopamine transporter (DAT) has been linked to bipolar disorder (BD). Mice with reduced DAT functioning (knockdown, KD) exhibit a behavioral profile in the mouse behavioral pattern monitor (BPM) consistent with patients with BD mania in the human BPM. Patients with BD also exhibit increased risk taking, which can be quantified using the Iowa gambling Task (IGT). We hypothesized that DAT KD mice would exhibit increased risk-taking behavior in a novel mouse version of the IGT.

Methods

DAT KD and wild-type (WT) littermates were trained in the mouse IGT.

Results

In session 1, KD mice initially made riskier choices, but later performed comparably to WT mice. Once trained to stable choice performance, DAT KD mice continued to exhibit a trend to choose the riskier options more than WT mice. Finally, we confirmed that these DAT KD mice also exhibited an exploratory profile in the BPM consistent with patients with BD mania, where risky choice behavior modestly correlated with specific exploration.

Conclusions

These data demonstrate that DAT KD mice chose the riskier options more than WT mice, providing further support for the use of DAT KD mice as a model of BD mania.

Keywords

Dopamine transport, Iowa gambling task, Mania, Mice, Model

INTRODUCTION

Bipolar disorder (BD) is a prevalent and debilitating psychiatric disorder, affecting 1-5% of the population from BD-I to BD-II (1). The prognosis of patients with BD remains poor, with 15% committing suicide, despite treatment options being available. This poor prognosis may be because treatments for BD have been discovered serendipitously, or were originally designed for other psychiatric disorders (2). Most current models of BD derive from observations of similarities as opposed to a rationale based on an understanding of the neuropathology of BD. For example, the amphetamine administration model of mania was first used based upon the observed behavior of rats after drug administration, not as an *a priori* hypothesis related to the neurobiological underpinnings of the disorder (3-5). We proposed a model of mania based on the putative reduced functioning of the dopamine transporter (DAT) in patients with BD. The DAT has been implicated in the neuropathology of BD via genetic linkage studies (6-8), with lower DAT levels (9) and reduced DAT expression (10) being reported in BD patients. Consistent with these neuropathological observations, mice with reduced functioning DAT levels (via either genetic or pharmacological manipulation) exhibit a profile in the mouse behavioral pattern monitor (BPM) that is consistent with that of acutely manic BD patients in a human BPM (11-13), and is exacerbated by subthreshold psychostimulant administration (14). The BPM quantifies exploration in animals across species (11, 15, 16) along three separable factors of activity levels (diverse exploration), investigatory behavior (specific exploration), and patterns of movement (17). The abnormal exploratory behavior of patients with BD mania includes increased activity levels combined with increased specific exploration and abnormal patterns of movement as reflected in reduced spatial *d* (11). Most models of BD utilize hyperactivity as their only outcome measure (18). In contrast, the use of the BPM has revealed that hyperactivity is accompanied by increased specific exploration and reduced spatial *d* both in patients with BD mania and in DAT knockdown (KD) mice (11-13). These DAT KD mice exhibit only 10% DAT levels when compared with wildtype (WT) mice (19). Thus the BPM may provide a behavioral profile by which novel treatments can be developed for mania that affect global behavior (see below) and not simply increased activity.

Mania is the cardinal feature of BD, as exemplified by the fact that it is a core symptom in the diagnosis of both Type I and Type II BD according to the DSM IV. While the BPM has proven to be a valuable tool to establish novel animal models of BD mania with cross-species translational validity (11-13), the BPM only assesses exploratory behavior in a novel environment. Other aspects of mania have been identified and have been quantified utilizing neurocognitive tests with real-world translational validity. Such tests include the Iowa gambling task (IGT) developed by Bechara et al. (20), which can quantify (21) the impulsive gambling trait of patients with BD that can be so detrimental to these

patients (22). In brief, during the IGT the subject is asked to repeatedly choose from four decks of cards from which they may be rewarded (gain) or punished (loss). Two decks provide large immediate rewards but larger punishment, while the other two offer smaller rewards but smaller punishment. Across a series of 100 trials, healthy subjects tend to select cards from the two decks that do not provide large rewards, but also offer the lowest punishment. Patients with BD mania take longer to begin selecting from these two 'safer' decks (21). The IGT has provided a means by which to identify the putative cognitive construct underlying gambling behavior in BD mania (23, 24), as well as to examine the relationship of gambling behavior to other aspects of mania (25).

Given the real-world implications of assessing gambling behavior in the IGT (26), several researchers have attempted to create an animal version of the IGT (27-30). These tasks have begun to be used to investigate the neurobiological underpinnings of performance in the task, including dopaminergic (30) and serotonergic (27, 30) manipulations, as well as relationships to other behaviors (28). We have utilized the strategy developed by Zeeb et al (30) to determine whether our mouse model of BD mania exhibits risk-taking behavior consistent with that of BD mania. We also examined the exploratory behavior of the same cohort of DAT KD mice in the BPM. We hypothesized that DAT KD mice would: (*a*) exhibit a preference for the riskier options; (*b*) exhibit a BD mania-like profile in the BPM; and (*c*) that risk preference would correlate with hole-poking, given that increased specific exploration is one of the most prominent features of abnormal BPM exploration in manic BD patients (11, 31).

METHODS

Animals

Female DAT KD and WT littermate mice (*n*=15 per group) were trained in the mouse IGT. The mice were generated by inserting modified embryonic stem cells of the 129Sv/J mouse strain in C57BL/6J blastocysts. Alteration of these stem cells was detailed by Zhuang et al. (19). DAT heterozygous breeders were sent to our laboratory from Columbia University. All the mice used resulted from a breeding colony in the vivarium at the University of California San Diego (UCSD). The DAT KD and WT mice were approximately 3-5 months old at the time of first training and weighed between 15-26 g (average 20.3 ± 0.6 SEM). No difference in weight was observed between the two genotypes.

All animals were group housed (where possible, maximum four per cage), maintained in a temperature-controlled vivarium (21 ± 1 ºC) on a reversed day-night cycle (lights on at 8.00 PM, off at 8.00 AM), and were tested during the dark phase of the cycle. All mice had *ad libitum* access to water, were food-restricted, and maintained at 85% of their free-feeding weight during the periods of testing as described below. Mice were brought to

the testing area 45 min before testing between 2.00 PM and 5.00 PM. All procedures were approved by the UCSD Institutional Animal Care and Use Committee. The UCSD animal facility meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care.

Apparatus

Mice were trained and tested in 16 five-hole operant chambers (25 cm × 25 cm × 25 cm, Med Associates Inc., St. Albans, VT, USA). Each chamber consisted of an array of five square holes (2.5 cm × 2.5 cm × 2.5 cm) arranged horizontally on a curved wall 2.5 cm above the grid floor with, on the opposite panel, a food-delivery magazine (Lafayette Instruments, Lafayette, IN, USA) at floor level and a houselight near the ceiling. The chamber was enclosed in a sound-attenuating box, ventilated by a fan that also provided a low level of background noise. An infrared camera installed in each chamber enabled the monitoring of performance during training and testing. The animals were trained to respond with a nose-poke to an illuminated LED recessed into the holes. Infrared beams, mounted vertically and located 3 mm from the opening of the hole, were used to detect the responses. The food-delivery magazine opposite to the middle hole contained a well in which liquid reinforcement utilized in the form of strawberry milkshake (Nesquik® plus non-fat milk, 30 µL) was delivered by a peristaltic pump (Lafayette Instruments, Lafayette, IN, USA). An infrared beam mounted horizontally, 5 mm from the floor and recessed 6 mm into the magazine, was used to detect the magazine entries. The control of stimuli and recording of responses were managed by a SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates Inc., St. Albans, VT, USA) using custom programming.

Behavioral handling and training

The mice were given a daily ration of rodent chow such that body weight was maintained at 85% of their free-feeding weight. After approximately 3 days of weighing and thus handling the mice, they were around 85% of their free-feeding weight. The day prior to the initiation of training, mice were acclimated to the food reinforcement by an overnight exposure to the strawberry milkshake in the absence of water. On training days 1-3, mice were placed in the five-hole boxes for 10 min, during which liquid reinforcement was dispensed every 15 s into the well of the magazine, while the magazine was lit (Hab1). Entry into the magazine caused the light to go out until the next reinforcement was delivered. The entries were taken as evidence of learning to associate the lit up magazine with the liquid food reinforcement. At the end of each session the wells were inspected to ensure no liquid was present. On day 4, in order to obtain reinforcement, mice were required to nose-poke in any one of the four lit holes at the side of the chamber opposite to the magazine (central hole was never lit to maintain four options for responding; maximum of 120 trials were possible). This training session (Hab2) was repeated daily

(Moday-Friday) until mice were able to make 40 responses to the light cue within a 30 min session on two consecutive days (days 4-18). Upon attainment of criterion, mice were trained in Hab2 on Tuesday and Friday only in order to maintain responding while training continued for other mice. This methodology has been successfully utilized in the past to avoid over-training on what is an intermediary stage (32-34). Failure to attain this criterion resulted in removal from further analyses.

Mouse Iowa gambling task

For the actual mouse IGT sessions, we utilized the protocol as described previously for rats (30). The first three days, mice were tested by means of a forced-choice version of the mouse IGT to acquaint the mice with the different reinforcement and punishment schedules associated with each hole. In brief, only one of the four holes was illuminated randomly, and after nose-poking this hole the appropriate reinforcement and/or punishment schedule followed. For both the mouse IGT program as well as for its forced-choice version two different forms were used to counterbalance for possible hole preferences.

After three days of forced-choice mouse IGT (days 19-21), the mice were moved onto the full mouse IGT. The task lasted for 30 min or for a maximum of 100 trials, whichever was completed first. The session began with the magazine being illuminated, and each trial was initiated by the mouse nose-poking, then removing its nose from the magazine. An inter-trial interval (ITI) of 5 s preceded illumination of the light stimuli in the array. If the mouse nose-poked at the array during this 5 s ITI, a 'premature' response was recorded; such a trial did not count as a completed trial and the response stimuli were not presented. Following a premature response was a time-out period, in which all holes were unresponsive and so the mouse could not earn reward or initiate a new trial, signaled by illumination of the houselight. The next trial began when the houselight was extinguished and the mouse entered and then exited the magazine. If the mouse withheld from responding during the ITI period, holes 1, 2, 4, and 5 were illuminated. These lights remained lit until the mouse nose-poked in one of these holes or until the stimulus duration period of 10 s had passed. Failure to respond in any hole during the light stimuli was registered as an 'omission'. Omissions did not trigger a time-out period, but rather resulted in the lights going out and the magazine being illuminated so that another trial could be started. If the animal did nose-poke in one of the four lit holes during the stimulus, this resulted in a 'correct' response and the hole choice was recorded. All the light stimuli then extinguished and the mouse was rewarded or punished depending on the reward schedule (Table. 1). If the mouse was rewarded, the magazine light illuminated and delivered the appropriate level of liquid food reinforcement. When the mouse nose-poked to receive the food reward, a new trial was initiated automatically. If, on the other hand, a punishment occurred, no reward was given and a punishing time-

out was triggered. The punishing time-out consisted of the light stimulus of the chosen hole starting to flash at a frequency of 0.5 Hz for the duration of the time-out period, during which time all apertures were unresponsive. After the time-out period, the flashing light was extinguished and the magazine light lit up, giving the mouse the opportunity to start a new trial. Repeated nose-poke responses at the hole the mouse had just chosen were counted as 'perseverative' responses. While these were recorded, they were not punished in any way. Repeated nose-pokes in the same hole when rewarded were counted as 'perseverative rewards' while when punished were recorded as 'perseverative punishments'. Finally, the times taken to choose a hole (mean choice latency; MCL) and obtain reward (mean reward latency, MRL) were also recorded. As to the different response options, data were grouped by advantageous (P1 and P2) and disadvantageous options (P3 and P4) and were measured as a percentage of the advantageous choices (P1+P2)/(P1+P2+P3+P4)*100. Mice were trained continuously on the mouse IGT until they exhibited stability in their preference (no main effect of day when analyzed over four consecutive days).

Table 1. The gain-loss structure in the human and mouse Iowa gambling tasks.

	Human Iowa gambling task				Mouse Iowa gambling task			
Sequence	**A**	**B**	**C**	**D**	**P1**	**P2**	**P3**	**P4**
1	100	100	50	50	1	2	3	4
2	100	100	50	50	1	2	-30	4
3	-50	100	0	50	1	2	3	-40
4	100	100	50	50	1	-10	-30	4
5	-200	100	0	50	1	2	3	-40
6	100	100	50	50	1	2	-30	-40
7	-100	100	0	50	1	2	-30	-40
8	100	100	50	50	1	-10	3	4
9	-150	-1150	0	50	1	2	3	-40
10	-250	100	0	-200	-5	2	-30	-40
Gain-loss	5 +	9 +	5 +	9 +	9 +	8 +	5 +	4 +
Frequency	5 -	1 -	5 -	1 -	1 -	2 -	5 -	6 -
Outcome	-250	-250	+250	+250	9+/5s -	16+/20s-	15+/150s-	16+/240s-

Mouse behavioral pattern monitor

Spontaneous locomotor and exploratory behavior was examined in 10 mouse BPM chambers as described previously (16). Each chamber consists of a 30.5 × 61 × 38 cm area with a Plexiglas holeboard floor equipped with floor holes in the front, middle, and rear parts of the floor and eight wall holes (three along each of the long walls, and two holes in the front and back walls). Hole-poking behavior was detected via infrared photobeams located at each hole. The location of the mouse was monitored from a grid of 12 × 24 infrared photobeams 1 cm above the floor (2.5 cm apart along the length and the width of the chamber; 24 × 12 *X*–*Y* array), recording the location of the mouse every 0.1 s, with its position defined across nine unequal regions [four corners, four walls and center; (14,

35)]. Infrared photobeams (16) located 2.5 cm above the floor and aligned with the long axis of the chamber record rearing behavior. The chamber was illuminated from a single light source above the arena (producing 350 lux in the center, and 92 lux in the four corners). Mice were placed in the bottom left-hand corner of the chamber, facing the corner, and the 60-min test session began immediately. The primary dependent variables were transitions (movement across defined regions), exploratory behavior (hole-poking and rearing), and locomotor pattern (spatial *d*). Spatial *d* quantifies the geometrical structure of the locomotor path, where a value of 2 represents highly circumscribed, small-scale movements, while 1 represents straight-line distance-covering movements (36).

Statistical analysis

Operant learning during Hab2 training was analyzed utilizing a repeated measure analysis of variance (ANOVA) with session as a within-subject factor and genotype as a between-subject factor. Time taken to attain criterion was compared utilizing a t-test. Within-session performance in the mouse IGT was analyzed using a repeated measure ANOVA with session half as a within-subject factor and genotype as a between-subject factor. Stable performance in the mouse IGT was compared using a one-way ANOVA with genotype as the between-subject factor. For the mouse BPM, transitions, hole-pokes, and spatial d were subjected to one-way ANOVAs with genotype as the between-subjects factor. Tukey post hoc analyses of statistically significant main or interaction effects were performed where applicable. The level of probability for statistical significance was set at 0.05. Mouse IGT statistics and correlations were performed using SPSS (14.0, Chicago, IL, USA), while mouse BPM data were analyzed using the Biomedical Data Programs (BMDP) statistical software (Statistical Solutions Inc., Saugus, MA, USA).

RESULTS

Operant learning in DAT KD and WT mice

We tested the genetically modified DAT KD mice to investigate the influence of reduced DAT functioning on decision-making behavior. A significant effect of day demonstrated that both KD and WT mice that met criterion learned to nose-poke for a single reward over a 15-day period ($F_{(14,84)}$=6.7, p<0.0001; Fig. 1a). Moreover, there was no effect of genotype on the number of days taken to attain criterion (> 40 responses on two consecutive days; $F_{(1,13)}$=1.2, ns; Fig. 1b). Only 60% of the WT mice attained criterion within 15 days however, in comparison with 100% of the KD mice (Fig. 1c).

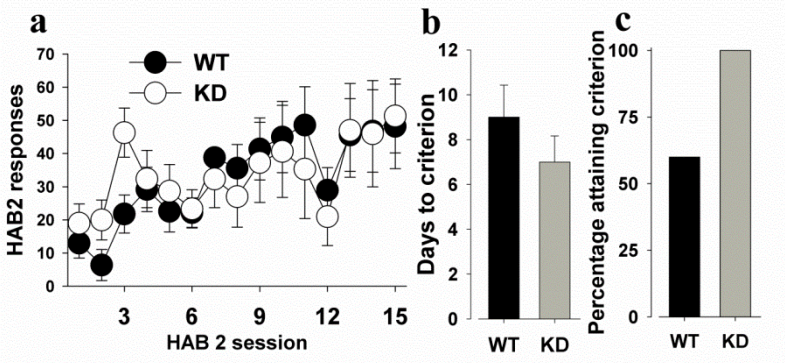

Fig. 1. Instrumental learning of dopamine transporter mutant mice. The learning rate of dopamine transporter (DAT) knockdown (KD) and wildtype (WT) mice were compared. The mice were trained to nose-poke in one of four holes at the rear of an operant chamber with responses measured across sessions (a). No differences in days to criterion were observed (b), although not all WT mice achieved criterion in the allotted time-frame (c). Data presented as mean ± S.E.M.

Initial risk preference of DAT KD and WT mice in the mouse IGT

When the percentage of advantageous choice performance for session 1 was divided by session-half, we observed a significant genotype by session-half interaction ($F_{(1,18)}$=7.0, p<0.05; Fig. 2). *Post hoc* analyses revealed that DAT KD mice exhibited a preference for the 'riskier' cues during the first half of training compared with WT littermates (p<0.05), but by the second session-half they selected the 'safe' cues as often as their littermates (p>0.1). Neither group exhibited an overall preference that differed from chance, however (p>0.2). No main effect of genotype was observed for premature responses (F<1, ns), MCL (F<1, ns), perseveration during punishment (F<1, ns), perseveration during rewards ($F_{(1,18)}$=1.5, ns), % omissions (F<1, ns), MRL ($F_{(1,18)}$=1.4, ns), or total trials ($F_{(1,18)}$=1.8, ns) during session 1.

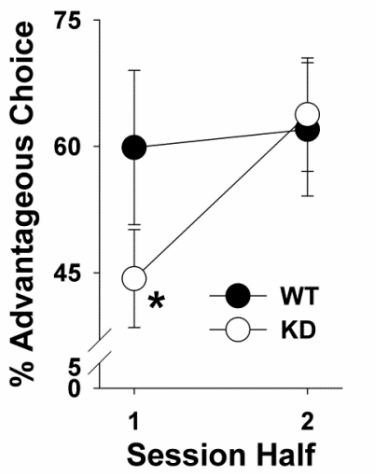

Fig. 2. Initial learning of the mouse Iowa gambling task. The risk preferences of dopamine transporter wildtype (WT) and knockdown (KD) mice were assessed in session 1 of the mouse Iowa gambling task. KD mice exhibited increased risk preference compared to WT mice in the first half of session 1. By the second half, however, the risk preferences of the groups were no longer different. Data presented as mean ± S.E.M., *p<0.05 when compared with WT mice within that session half.

Stable risk preference of DAT KD and WT mice in the mouse IGT

Training in the task continued (Fig. 3) until a stable cue preference was observed. Once stable, we again examined performance by session-half. At this stage, no genotype by session-half interaction was observed for % advantageous choice (F<1, ns; Fig 4a). When performance was examined over the entire session, DAT KD mice exhibited a trend toward risky choice when compared with WT mice (65% vs. 88% advantageous choice, respectively; $F_{(1,18)}$=2.4, p<0.1; Fig. 4b). Further examination revealed that while WT mice exhibited significantly increased performance when compared with chance (50%; t=7.3, p<0.0001), KD mice only exhibited a trend toward a preference for the advantageous holes when compared with chance (t=1.8, p=0.096). KD mice also exhibited a trend toward more premature responses compared with the WT mice, although this was not significant (12 vs. 4; $F_{(1,18)}$=2.6, p<0.1; Fig. 4c). KD mice exhibited increased perseverative responses after punishments compared with their WT littermates ($F_{(1,18)}$=5.4, p<0.05; Fig. 4d), while only a trend was observed for perseverations after a reward ($F_{(1,18)}$=3.0, p=0.10). DAT KD mice exhibited a lower MRL than WT mice ($F_{(1,18)}$=6.1, p<0.05; Fig. 4e). No effect of genotype was observed for MCL, however (F<1, ns; Fig. 4f). No effect of genotype was observed for % omitted trials ($F_{(1,18)}$=1.8, ns; Fig. 4g), or total trials completed ($F_{(1,18)}$=1.0, ns).

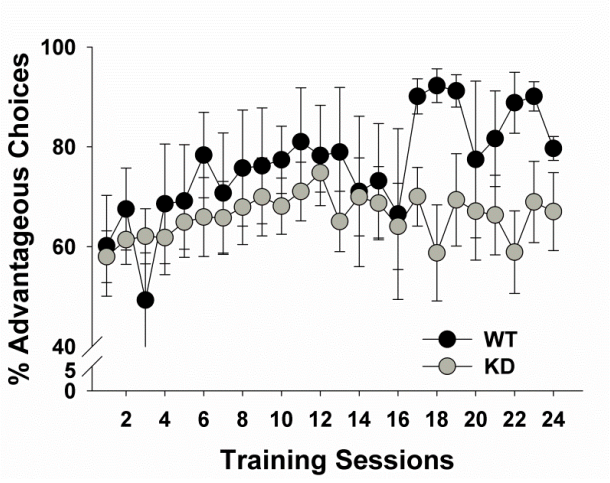

Fig. 3. Alterations in risk preference with continued training. Dopamine transporter (DAT) wildtype (WT) and knockdown (KD) mice were trained in the Iowa gambling task for a further 24 days. While the risk preference of DAT WT and KD mice did not differ greatly after training session 1, as training continued WT mice exhibited increased preference for the safer options compared with KD mice. Data presented as mean ± S.E.M.

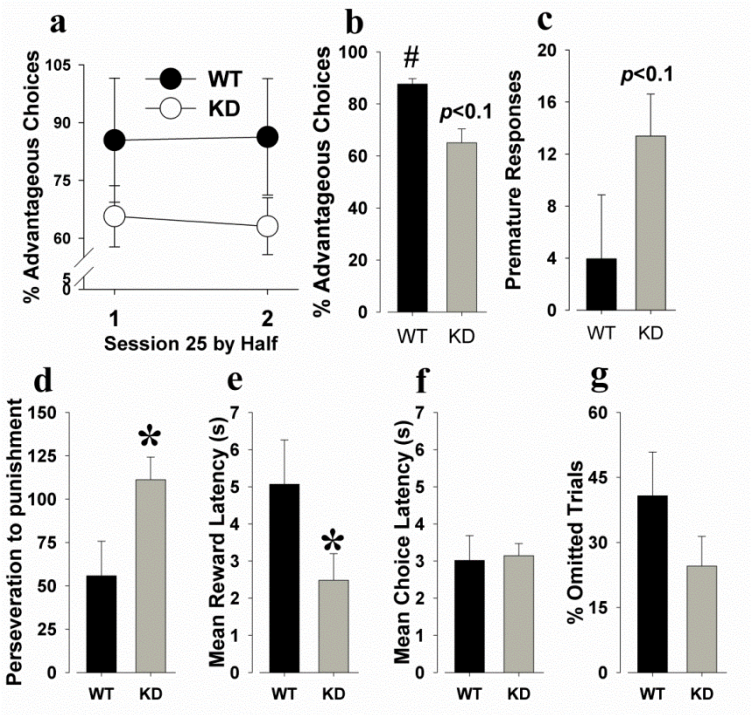

Fig. 4. Stable performance of mice in the Iowa gambling task. After 24 sessions of training, the stable risk preferences of dopamine transporter wildtype (WT) and knockdown (KD) mice were compared. Within the final session, WT and KD mice exhibited stable performance from the first to second halves of the session (a). A trend toward riskier performance in KD mice was observed over the entire session (b), as was premature responses (c). KD mice exhibited increased perseverative responses to punishing stimuli (d), as well as shorter mean reward latencies (e). Despite shorter reward latencies, KD did not differ from WT mice in choice latency (f), nor were differences observed in % omitted trials (g). Data presented as mean ± S.E.M., *p<0.05 when compared with WT, #p<0.05 when compared with chance.

Mania-like exploratory profile of DAT KD mice

Finally, we also tested the exploratory behavior of these mice in the mouse BPM. Consistent with previous reports, DAT KD mice exhibited increased activity as measured by transitions, when compared with WT littermates ($F_{(1,23)}$=16.0, p<0.001; Fig. 5a). Moreover, DAT KD mice also exhibited increased specific exploration as measured by hole-pokes ($F_{(1,23)}$=8.7, p<0.01; Fig. 5b). Finally, a trend toward reduced spatial *d* locomotor pattern was also observed in DAT KD mice compared with their WT littermates ($F_{(1,23)}$=3.8, p=0.06; Fig. 5c). Choosing the riskier option did not correlate with transitions, spatial *d*, or weight (p>0.1) in the BPM, but did exhibit a trend for a modest correlation with specific exploration (hole-poking; r=0.36, p=0.084).

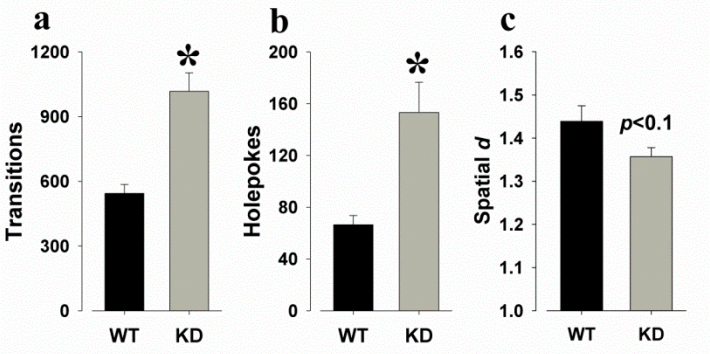

Fig. 5. Mania-like exploratory profile of dopamine transporter knockdown mice in the behavioral pattern monitor. The exploratory behavior of dopamine transporter wildtype (WT) and knockdown (KD) mice was assessed in the behavioral pattern monitor. Consistent with previous reports, KD mice exhibited increased transitions (a), increased hole-poking (b), and reduced spatial *d* (c), when compared with WT littermate mice. Data presented as mean + S.E.M., **p*<0.05 when compared with WT mice.

DISCUSSION

DAT KD mice exhibited increased risky behavior in the mouse IGT. This risky behavior was measured by an increased preference for disadvantageous choices. This increase in risky behavior was observed during initial learning in session 1, with a trend toward risk preference even after prolonged training. Moreover, consistent with previous reports, these DAT KD mice exhibited an exploratory profile in the mouse BPM consistent with that exhibited by patients with BD mania. Thus, these data provide further support for the use of DAT KD mice as a mouse model of BD mania.

The present data are consistent with previous reports that DAT KD mice exhibit normal learning in operant-based tasks (Hab2 performance (37)). In contrast to previous reports, however, WT mice exhibited poorer operant learning than KD mice. The reasons for poorer operant learning in WT mice remain unclear, but could reflect reduced activity in these mice. DAT KD mice backcrossed onto C57BL/6J mice are now available (38) and future studies will examine operant learning in these mice. When the mice in the present study were switched from simple responding to the IGT, the performance of DAT KD and WT mice also differed. From session 1, KD mice exhibited a preference for the disadvantageous holes, only learning to select from the advantageous holes by the second half of the session. While deficient learning in DAT KD mice compared with WT mice could contribute to this observation, we feel that previous reports of normal learning elsewhere in these DAT KD mice (37), as well as superior learning during the Hab2 phase in the present studies, support the interpretation that these mice select the high-risk holes due to preference for larger rewards. Further, these data are consistent with risk preference in the IGT in patients with BD mania (21, 23-25). With a further 24 days of continued

training however, DAT KD mice acquired a preference for the advantageous holes, although this preference was only a trend toward being significantly different from chance. Moreover, DAT KD mice still exhibited a lower preference for advantageous holes when compared with WT mice. These findings were not due to changes in preference over a session as seen in session 1. Furthermore, WT mice exhibited a preference for the advantageous holes that differed from chance. Thus, even with continued training, DAT KD mice continued to exhibit a preference for the riskier choices. Therefore, it appears that chronic increased extracellular dopamine levels increases risk preference.

The present study explored risk preference in female DAT KD mice. Previous studies supporting these mice as a model for BD mania have investigated both male and female mice and found no sex effects or interactions in spontaneous exploration reported (11, 14). Similarly, the spontaneous exploration of BD mania patients was unaffected by gender (11, 31). Gender can interact with genes to influence performance the IGT, although this gender effect was only apparent in the first stage of the IGT where decisions were made under ambiguity and not risk (39). Gender was not reported to influence BD mania performance in the IGT, however (21, 23). In a direct gender comparison in the IGT, it was noted that men chose the safe options more rapidly than women (40), results which have been reflected in earlier rodent IGT versions (29). Thus the learning of the mice in the current study could reflect slower learning rates in WT female mice, in part due to slower learning rates in females, although this slower rate can be influenced by anxiety levels (41). Another putative confound of using female mice in the present study is that the estrus cycle can affect performance of rodents in operant-based tasks such as cocaine (42) or alcohol (43) self-administration. Further, food deprivation can alter the estrus cycle of rodents that vary by background strain (44). The effect of food deprivation on the estrus cycle of DAT KD mice is currently unknown, as is the effect of the estrus cycle on performance of the IGT reported here. Fig. 3 details the development of preference toward safe options during training, with no obvious differences between WT and KD mice on specific days. The mice in the present study were all housed close together, thus their estrus cycle was likely synchronized. Given the possible complication of estrus cycle, however, and the better within-session learning exhibited by men in the human IGT, future studies will focus on assessing risk-preference in male DAT KD mice in the mouse IGT. Moreover, altering reinforcement and punishment schedules may provide a better opportunity to observe within-session learning as has recently been described for another rat IGT version (28).

It was interesting to note that DAT KD mice also exhibited a trend toward more impulsive-like behavior in the task, as measured by premature responses. Previous reports suggest that this measure is linked to elevated 5-HT levels as observed in rats performing the 5-choice serial reaction time task (5CSRTT) (45). This increased motor impulsivity could,

however, simply reflect the increased activity in these DAT KD mice, because psychostimulants such as amphetamine also increase measures of motor impulsivity of rats in the 5CSRTT (46). Such motor impulsivity could also be linked to the increased perseverative responding observed in these mice (46, 47). Future studies could determine the full extent of impulsive behaviors in these mice (48).

DAT KD mice did not exhibit shorter MCLs when compared to WT mice, despite increased activity levels. Thus it would appear that during goal-directed behavior, DAT KD mice respond as quickly as their WT littermates. It is interesting to note, therefore, that DAT KD mice were significantly faster than their WT littermates when returning to the magazine to gain their reward. Since this MRL has been linked to motivational factors for food reward (46), the data support previous reports of increased motivation in DAT KD mice (37). This hedonic behavior supports the use of DAT KD mice as a mouse model of BD mania, because patients with mania also exhibit hedonic behavior (49).

Finally, the DAT KD mice used in the current study exhibited an exploratory profile consistent with that of previous reports and patients with BD mania (11-13). Thus the IGT behavior presented here may well reflect the behavior of patients with BD during their manic phase. Increased risk preference is also observed in patients with attention deficit hyperactivity disorder (ADHD) (50). In contrast with ADHD but consistent with BD mania, subthreshold doses of stimulants reinstate the hyperactive phenotype of DAT KD mice (14). These converging lines of evidence from the BPM and IGT provide further support for the use of these mice as a model of BD mania (14). Moreover, these findings suggest that chronic reductions in DAT functioning result in a preference for riskier options, and may therefore support the idea that reduced DAT functioning may contribute to the risky behavior exhibited by BD manic patients (9, 10). The observed relationship between risk preference in the IGT and hole-poking in the BPM suggests that targeting treatments that reduce hole-poking in the BPM may provide a high-throughput screen to develop treatments to improve a broad range symptomatology in BD. It would prove useful in future studies to examine impulsive and cognitive performance of these mice in cross-species translational tasks (51) to further assess the relevance of DAT KD mice as a model of BD mania. Tasks assaying executive functioning, such as the attentional set-shifting task (52, 53), or vigilance and response inhibition, such as the 5-choice continuous performance test (34) may prove useful given the deficits in performance observed in BD patients in these tasks. Future studies will also begin to examine the effects of current treatments on the exploratory profile of these mice in the BPM.

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CHAPTER 3.2

Differential effects of dopamine transporter inhibitors in the rodent Iowa gambling task: Relevance to mania

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ABSTRACT

Background

The Iowa gambling task (IGT) can be used to quantify impulsive and risky choice behaviors in psychiatric patients, e.g. bipolar disorder (BD) sufferers. Although developing treatments for these behaviors is important, few predictive animal models exist. Inhibition of the dopamine transporter (DAT) can model profiles of altered motor activity and exploration seen in patients with BD. The effect of DAT inhibition on impulsive choices related to BD has received limited study however. We used a rodent IGT to elucidate the effects of similarly acting drugs on risky choice behavior. We hypothesized that (1) C57BL/6 mice could adopt the "safe" choice options in the IGT and (2) DAT inhibition would alter risk preference.

Methods

Mice were trained in the IGT to a stable risk-preference and then administered the norepinephrine/DAT inhibitor amphetamine, or the more selective DAT inhibitors modafinil or GBR12909.

Results

Mice developed a preference for the "safe" option, which was potentiated by amphetamine administration. GBR12909 or modafinil administration increased motor impulsivity, motivation significantly, and risk preference subtly.

Conclusions

The rodent IGT can measure different impulse-related behaviors and differentiate similarly acting BD-related drugs. The contrasting effects of amphetamine and modafinil in mice are similar to effects in rats and humans in corresponding IGT tasks, supporting the translational validity of the task. GBR12909 and modafinil elicited similar behaviors in the IGT, likely through a shared mechanism. Future studies using a within-session IGT are warranted to confirm the suitability of DAT inhibitors to model risk-preference in BD.

Keywords

Iowa gambling task, Dopamine transporter, Bipolar disorder, Risk-taking, Mice, Modafinil, GBR12909, Amphetamine, Mania, Impulsivity

INTRODUCTION

Mania is a cardinal feature of bipolar disorder (BD), a debilitating psychiatric disorder affecting approximately 1% of the US population for BD-I and BD-II combined (1). Impulsive behavior is a critical aspect of mania and one of the diagnostic criteria according to the DSM-IV. Self-report questionnaires identify higher levels of impulsivity or poor risk assessment in depressed, euthymic, and manic phases of BD (2, 3), corroborated using laboratory-based assessments (4). Treatment options for patients with BD, especially those directed at symptoms such as impulsivity and poor risk assessment, are limited and require development.

To quantify such impulsive aspects of mania and develop treatments, laboratory tests with real-world translational validity are now being utilized. Such tests include the Iowa gambling task (IGT) (5), which measures decision-making for risk, choosing between "high yield/high risk options vs. low risk/low yield options". In short, subjects in the IGT have to deduce and select the best of four options that vary in both the size and probability of reward and losses (6, 7) (Table 1). As the session continues, healthy subjects readily select the safe options after initially trying all decks (8), whereas patients with BD take longer to adopt this strategy irrespective of state (4, 9). Thus, the IGT quantifies the uncontrolled risk-taking trait of patients with BD across the spectrum of the disorder (4, 10). The IGT enables examination of the putative mechanisms underlying risk-taking behavior in BD mania by linking performance with self-report questionnaires (11, 12). The IGT can differentiate between clinical populations whereby BD patients take longer to adopt a safe strategy, substance-abusers learn at different rates (13), and less clear impairments are observed in patients with schizophrenia (14).

Table 1. The gain-loss structure in the human and mouse Iowa gambling tasks.

	Human Iowa gambling task				**Mouse Iowa gambling task**			
Sequence	**A** (risky)	**B** (risky)	**C** (safe)	**D** (safe)	**P1** (safe)	**P2** (safe)	**P3** (risky)	**P4** (risky)
1	100	100	50	50	1	2	3	4
2	100	100	50	50	1	2	-30	4
3	-50	100	0	50	1	2	3	-40
4	100	100	50	50	1	-10	-30	4
5	-200	100	0	50	1	2	3	-40
6	100	100	50	50	1	2	-30	-40
7	-100	100	0	50	1	2	-30	-40
8	100	100	50	50	1	-10	3	4
9	-150	-1150	0	50	1	2	3	-40
10	-250	100	0	-200	-5	2	-30	-40
Outcome	-250	-250	+250	+250	9+/5s-	16+/20s-	15+/150s-	16+/240s-
+/- rate	5 + 5 -	9 + 1 -	5 + 5 -	9 + 1 -	9 + 1 -	8 + 2 -	5 + 5 -	4 + 6 -

To better understand these discrepancies and neurobiological underpinnings of risk-taking/gambling behavior, researchers have developed animal versions of the IGT (15-18); see (19). These paradigms may facilitate development of therapeutics for risk-taking behavior and have already been used to study both dopaminergic (18) and serotonergic (16, 18) manipulations in rats. Based initially on the rat paradigm (18), we developed a mouse IGT to investigate the genetic underpinnings of performance (20). In short, analogous to the human IGT, the rodent version presents the animal with a choice between four distinct options with different probabilities and magnitudes of expected gains and losses (Table 1). Two options are ultimately advantageous whereas the other two ultimately have a disadvantageous outcome. Motor impulsivity as measured by premature responses (before choice stimuli appear) and risk-taking behavior as measured by percentage advantageous choices (propensity of an animal to choose advantageous/safe options or disadvantageous/risky options) can be measured simultaneously in the rodent IGT (19). The rodent IGT therefore provides measurements of impulsive activity and impulsive choice, respectively (21), the latter of which more closely resembles the choice/risk-taking measured in the human IGT. Maximizing reward requires learning the gain/loss contingencies available and selecting safe options.

Dysregulation of dopaminergic homeostasis plays an important role in the mechanisms underlying mania in BD (22, 23). Specifically, genetic linkage studies have identified abnormalities in the human dopamine transporter (DAT) in BD (24-26). Such mutations lead to reduced cell surface expression of DAT in human cells (27), corroborated by lower striatal DAT levels in unmedicated BD patients (28). Consistent with reduced DAT functioning producing mania-like behavior, we observed that mice with reduced DAT functioning (via either genetic or pharmacological manipulation) exhibit complex exploratory profiles similar to those of acutely manic patients with BD in the cross-species translational behavioral pattern monitor (BPM) (29-33). In our recently described rodent IGT, we observed that DAT knockdown (KD) mice exhibited increased risk preference, both within the first session and across sessions (20), providing further support for the DAT KD mouse as a model for mania.

Besides genetic models, pharmacological agents have also been used to model BD. Traditionally, the non-selective DAT inhibitor amphetamine is a widely used pharmacological model of BD mania (34-37), despite having several limitations (38). The more selective DAT inhibitor GBR12909 has also been investigated as a model of mania. In contrast with amphetamine, GBR12909 mimicked the behavioral exploratory profile of patients with BD mania as assessed by the BPM (30, 32).

The effects of amphetamine or GBR12909 have not yet been compared in the rodent IGT. Amphetamine reduced risk choice preference in the rodent IGT, leading to a more

conservative choice strategy (18). Assessing the effects of amphetamine in mice may provide evidence of cross-species translational validity of the rodent IGT between rats and mice. Investigating the effects of GBR12909 in this paradigm will be useful to further determine the suitability of this treatment as a model of mania. Modafinil, a drug used to treat narcolepsy, also inhibits the DAT in rodents, monkeys, and humans (39-41) and can increase risk-taking in the human IGT (42).

These DAT inhibitors may help in delineating (*a*) the effects of acute increases in extracellular dopamine levels on risk-taking behavior and (*b*) the pharmacological predictive validity of the IGT across species. Hence, we investigated the effects of d-amphetamine, GBR12909, and modafinil administration on mouse performance of the IGT. We hypothesized that: (*1*) C57BL/6 mice would readily learn to choose the less "risky" options; (*2*) amphetamine would increase the choice of less punishing stimuli (make the mice risk aversive) consistent with effects on rats; and (*3*) GBR12909 and modafinil would increase risk preference in the IGT similar to patients with BD.

METHODS

Animals

Male C57BL/6N mice (Charles Rivers Laboratories) were trained in the IGT (*n*=21), and were approximately 3 months of age and weighed between 20 and 30 g at the start of the studies. All animals were group housed (maximum of 4 per cage), maintained in a temperature-controlled vivarium (21±1 ºC) on a reversed day-night cycle (lights on at 8.00 PM, off at 8.00 AM), and were tested during the dark phase of the cycle. All mice had *ad libitum* access to water, were food-restricted, and maintained at 85% of their free-feeding weight during the periods of training and testing described below. Mice were brought to the testing area 45 min before testing between 2.00 PM and 5.00 PM. All procedures were approved by the UCSD Institutional Animal Care and Use Committee and the "Principles of laboratory animal care" were followed. The UCSD animal facility meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care.

Apparatus

Mice were trained and tested in 16 five-hole operant chambers (25×25×25 cm; Med Associates Inc., St. Albans, VT). Each chamber consisted of an array of 5 square holes (2.5×2.5×2.5 cm) arranged horizontally on a curved wall 2.5 cm above the grid floor. A food delivery magazine (Lafayette Instruments, Lafayette, IN) was on the opposite panel at floor level. The chamber had a house-light near the ceiling and was enclosed in a sound-attenuating box, which was ventilated by a fan that provided a low level of background noise. An infrared camera installed in each chamber enabled the monitoring

of performance during training and testing. The animals were trained to respond with a holepoke to an illuminated LED recessed into the holes. Infrared beams, mounted vertically and located 3 mm from the opening of the hole, were used to detect the responses. The food delivery magazine opposite to the middle hole contained a well in which liquid reinforcement utilized in the form of strawberry milkshake (Nesquik® plus non-fat milk, 30 µl) was delivered by a peristaltic pump (Lafayette Instruments, Lafayette, IN). An infrared beam mounted horizontally, 5 mm from the floor and recessed 6 mm into the magazine, was used to detect magazine entries. The control of stimuli and recording of responses were managed by a SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates Inc., St. Albans, VT) using custom programming.

Behavioral handling and training

Prior to training, mice were acclimated to the reinforcer. Training on days 1-3 lasted 10 min and reinforcement was dispensed every 15 s into the magazine well while the magazine was lit. Magazine entry extinguished the magazine light until the next reinforcement delivery. Magazine entries were counted. On day 4, to obtain reinforcement mice had to holepoke in 1 of the 4 lit holes opposite the magazine (central hole was never lit; Fig. 1). This session was repeated daily (Monday-Friday) until >70 responses were recorded within 30 min for 2 consecutive days. Upon attainment of criterion, mice were trained in this session only on Tuesday and Friday in order to maintain responding while training continued for other mice to avoid overtraining on what is an intermediary stage (43).

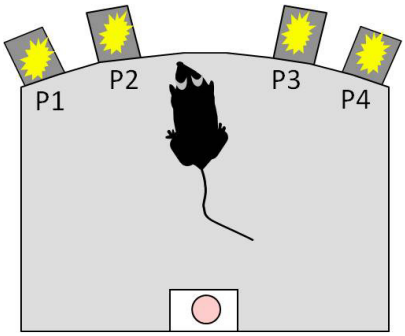

Fig. 1. A visual schematic of the rodent IGT. Mice can holepoke among 4 illuminated holes (P1-P4) during the 10-s stimulus duration to obtain food reward (strawberry milkshake, delivered opposite the holes) or punishment (cue light flashing and all apertures unresponsive for a predetermined period) depending on a predefined schedule (see Table 1 for an example). Each session lasts for 100 trials or 30 min, whichever is completed first. Two options (P1, P2) deliver small rewards and low punishments, ultimately leading to an advantageous outcome, while the other two options (P3, P4) deliver high rewards and high punishments, ultimately leading to disadvantageous final outcome.

Rodent Iowa gambling task

For the IGT sessions we utilized the protocol described previously (20). The first 3 days, mice were presented with a forced choice version of the IGT to acquaint the mice with the different reinforcement and punishment schedules associated with each hole, where only 1 of the 4 holes was illuminated randomly and after holepoking, the appropriate reinforcement or punishment schedule followed. For both the IGT program as well as for its forced choice version, 2 different forms were used to counterbalance for possible hole preferences.

After 3 days of forced choice IGT, the mice were moved onto the full IGT lasting 30 min or 100 trials, whichever was completed first. The session began with the magazine being illuminated and each trial was initiated by holepoking then exiting the magazine. An intertrial interval (ITI) of 5 s preceded illumination of the cue array. If the mouse holepoked in any cue hole during this 5 s ITI, a *premature* response was recorded and did not count as a completed trial, stimuli were not presented, and the house light illuminated for a 5-s time-out period in which all holes were unresponsive (Table 2). The next trial began when the house light was extinguished and the mouse holepoked the magazine. If the mouse withheld from responding during the ITI period, holes 1, 2, 4, and 5 were illuminated. These lights remained lit until the mouse holepoked in one of these holes or until 10 s had passed. Failure to respond in any hole during the light stimuli was registered as an *omission*. Omissions did not trigger a time-out period, but rather resulted in the cue lights being extinguished and the magazine being illuminated so that another trial could be started. If the animal did holepoke in 1 of the 4 lit holes during the stimulus, a "correct" response and the hole choice were recorded. All cue lights were then extinguished and the mouse rewarded or punished depending on the reward schedule (Table 1). For rewards, the magazine light was illuminated and delivered the appropriate level of reinforcer. Retrieving the reward initiated the next trial. If a punishment occurred however, no reward was given and a punishing time-out was triggered whereby the light stimulus of the chosen hole flashed at a frequency of 0.5 Hz for the duration of the time-out period, during which all apertures were unresponsive. After the time-out period, the flashing light was extinguished and the magazine light illuminated for a new trial to begin. Repeated holepoke responses were counted as response *perseveratives* but not punished. Repeated poking in the same hole when rewarded were counted as *reward perseveratives* and when punished as punishment *perseveratives*. Finally, the latency to collect rewards *(mean reward latency)* was also recorded**.** As to the different response options, data were collected as P1, P2, P3, and P4 responses corresponding to different reward schedules. Responses were measured as a percentage of the total trials completed. Where appropriate due to reduced sample size, data were grouped by advantageous (P1 and P2) and disadvantageous options (P3 and P4) and response options were measured as a percentage of the advantageous choices (P1+P2)/(P1+P2+P3+P4)*100 as described

previously (20). Mice were trained continuously on the IGT until they exhibited stable performance after 20 sessions (no main effect of day when analyzed over 4 consecutive days). Because drug and genetic effects on IGT performance can be observed within a session (20, 42), with individual differences in performance interacting with drug effects (44), within-session performance of good and poor performing animals were also analyzed (see statistics).

Table 2. Summary of measures used to assess different behaviors in the IGT.

Measure and description	Outcome
Trials completed: number of trials completed per session (maximum = 100)	Degree of session completion
Premature responses: response in any cue hole during the 5 s intertrial interval (ITI) preceding illumination of the cue array → 5 s time-out period	Motoric impulsivity
Omissions (%): failure to respond in any hole during the light stimulus duration of 10 s	May reflect inattention or amotivation
'Correct' response: response in any of the four lit cue holes during the stimulus of 10 s	A choice between the four cues is made
Responses (%): response to P1, P2, P3, or P4 as a percentage of total holepokes	Preference for 1 cue compared to others
Advantageous choices (%): advantageous response options (P1+P2)/total (P1+P2+P3+P4)*100	Represents choice preference
Punishment perseverative responses: repeated responses in the same hole when punished	Perseveration during punishment
Reward perseverative responses: repeated responses in the same hole when rewarded	Perseveration after being rewarded
Mean reward latency: the latency to collect rewards in seconds	Reflects aspects of motivation

Drug testing procedure

Each mouse received saline for the 2 training days prior to drug testing to habituate the animals to being injected. In the amphetamine study, mice were given their assigned amphetamine dose (1.0 and 2.5 mg/kg) (30, 45, 46) or saline on 3 drug testing days with saline days in between, 10 min prior to assessing performance in the IGT (Fig. 2). After amphetamine testing, a wash-out of 16 days followed until the mice were again stable. For the modafinil study, mice received 3 different doses of modafinil (16, 32, and 64 mg/kg) (47, 48) or vehicle on 4 drug testing days, again with saline days in between. A wash-out of 9 days followed after modafinil testing. For the last challenge, the animals received three different doses of GBR12909 (9.0, 16.0, and 28.5 mg/kg) (32, 49) or saline on 4 drug testing days, with saline days in between. In each study, the range of doses were chosen based on cognitive effects described in the literature, as well as their effects on exploratory behavior we have described previously.

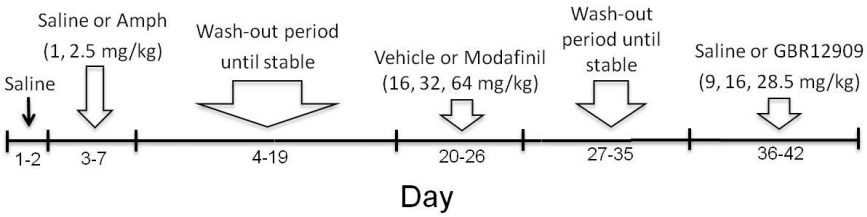

Fig. 2. Timeline of drug testing procedure for amphetamine, modafinil, and GBR12909. Each mouse received saline for 2 training days prior to drug testing to habituate the animals to being injected. Before each drug study, mice were allocated to drug dose or vehicle in randomized order. Following a within-subject design, mice were administered their assigned drug dose or vehicle on 3 (amphetamine) or 4 (modafinil and GBR12909) drug testing days with saline administration training days in between testing.

Drugs

D-amphetamine sulfate, modafinil, and GBR12909 dihydrochloride were purchased from Sigma Aldrich (St Louis, MO USA). D-amphetamine sulfate was dissolved in saline, GBR12909 dihydrochloride was dissolved in saline after heating (45ºC for 60 min), and modafinil was dissolved in vehicle (5% Tween and 1% methylcellulose dissolved in saline). Drugs were injected intraperitoneally, with amphetamine at a volume of 5 ml/kg body weight, and modafinil and GBR12909 at a volume of 10 ml/kg due to poor solubility. Free-base drug weights were used in all drug calculations. Drug solutions were prepared just prior to testing.

Statistics

Stable performance was compared using a repeated measure analysis of variance (ANOVA) with days as a within-subject factor. Data obtained were subjected to a repeated measure ANOVA with dose and response option as within-subject factors. To assess within-session performance, sessions were divided into the first and second halves by trials completed (50) and analyzed using a repeated measure ANOVA with session half, dose, and response option as within-subject factors. Because different within-session performances were observed across the group, mice were further separated into good and poor performers using a median split on their learning performance (% advantageous choice session-half 2 – % advantageous choice session-half 1) during saline treatment of each drug study. Subjects with less than 15 responses in a half were excluded from each within-session analysis. Where appropriate, planned comparison paired *t*-tests were conducted between groups. Pearson *r* correlation coefficients measured the relationship between risk-taking and motor impulsivity measures in each drug study. Tukey *post hoc* analyses of statistically significant main or interaction effects were performed where applicable. The level of probability for statistical significance was defined at 0.05. All statistics were performed using SPSS (19.0, Chicago, IL, USA).

RESULTS

Baseline performance in the IGT

After 20 days of training on the IGT, the C57BL/6 mice exhibited a stable and significant preference for a response option ($F_{(3,171)}$=3.1, p<0.05; Fig. 3), choosing P2 more than P3 and P4 (p<0.05), and tended to choose P2 more than P1 (p<0.1). No significant effect of day was observed for response option or any other measure (F<1, ns).

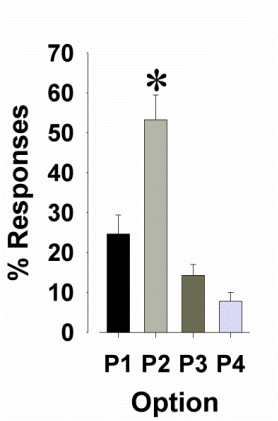

Fig. 3. Baseline choice preference of C57BL/6 mice in the IGT after 20 days of training. Mice (n=21) consistently showed a preference for the safe option P2 over options P3 and P4 as indicated by a higher % of responses in P2. Data are shown as the mean + S.E.M., *p<0.05 when compared with P3, and P4.

Exp 1. The effects of amphetamine on IGT performance

Amphetamine was administered to mice to determine whether this drug would affect their risk-taking behavior in the IGT. Significant main effects of response option were observed ($F_{(3,60)}$=6.4, p<0.01), as was a dose by response-option interaction ($F_{(6,120)}$=3.2, p<0.01; Fig. 4a). No main effect of dose was observed (F<1, *ns*). *Post hoc* analyses revealed that within P1 there was a trend toward 2.5 mg/kg amphetamine inducing more responses than saline (p=0.056). Within P2, amphetamine at 2.5 mg/kg resulted in fewer responses than saline (p<0.05), similarly for P3 (p<0.05). Although an increase in response to P4 was observed at the highest dose, this difference was not significant (p>0.1).

Significant main effects of amphetamine were observed for trials completed ($F_{(2,40)}$=18.5, p<0.001; Fig. 4c), punishment perseveratives ($F_{(2,40)}$=4.6, p<0.05; Fig. 4d), and percentage omissions ($F_{(2,34)}$=21.0, p<0.001; Fig. 4g). *Post hoc* analyses revealed that the highest dose of amphetamine reduced trials completed compared to both saline and 1 mg/kg amphetamine (p<0.01). A decrease in punishment perseveratives at both 1 and 2.5 mg/kg amphetamine compared to saline was also observed (p<0.05). Increased percentage omissions was observed at 1 mg/kg amphetamine compared to saline (p<0.05) and at 2.5 mg/kg compared to both saline and 1 mg/kg (p<0.01). No main effects of dose were observed for premature responses ($F_{(2,40)}$=2.2, p>0.1; Fig. 4b), reward perseveratives (F<1, ns; Fig. 4e), or mean reward latencies ($F_{(2,36)}$=1.3, p>0.1; Fig. 4f).

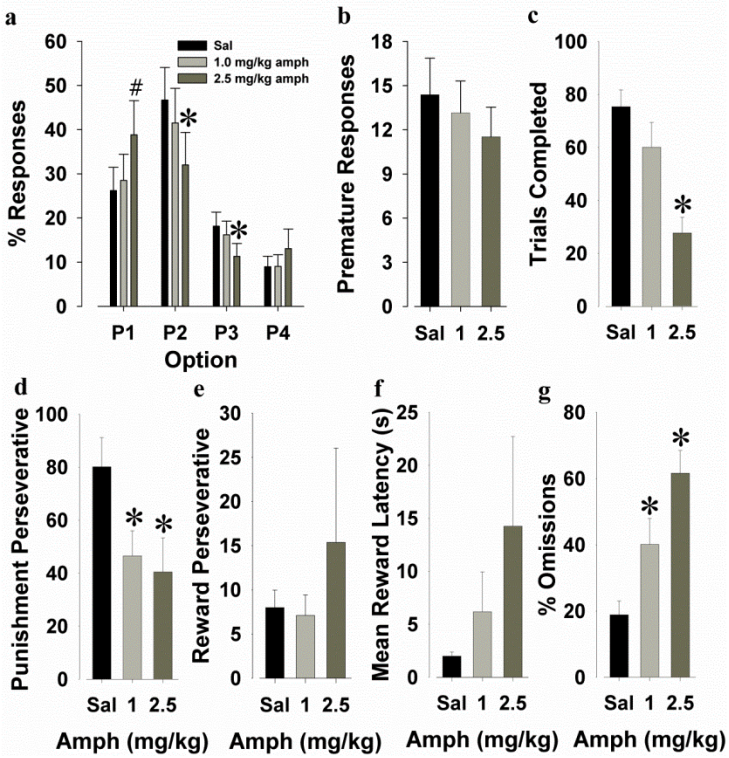

Fig. 4. Effects of amphetamine on performance of C57BL/6 mice in the IGT. Amphetamine (1 and 2.5 mg/kg) administration changed choice preference at the highest dose to the lower punishing P1 option (a), did not affect premature responding (b), and significantly decreased trials completed at the highest dose (c). Both doses of amphetamine decreased punishment perseveratives (d), but had no significant effect on reward perseveratives (e). The time animals took to collect their reward was slowed by the highest dose of amphetamine although not significantly (f). At both doses, amphetamine increased the percentage of omitted trials (g). Data are shown as the mean + S.E.M., **p*<0.05 when compared with saline, #*p*<0.1 when compared with saline.

Within-session risk preference

To determine whether the animals' initial choice strategy was affected by drug, choice preference within the drug-challenged session was examined. Because the highest dose decreased trials completed, we excluded animals given amphetamine at 2.5 mg/kg in this analysis. When performance was split by session half, 1 mg/kg amphetamine significantly increased safe option preference of mice ($F_{(1,12)}$=6.0, p<0.05; Fig. 5a). Planned comparisons revealed that 1 mg/kg amphetamine resulted in a trend to more safe choices compared to saline during the first half (t=-2.1, p<0.1) and significantly more safe choices compared to saline in the second half (t=-2.3, p<0.05). No main effect of session-half or interaction with amphetamine was observed (F<1, *ns*).

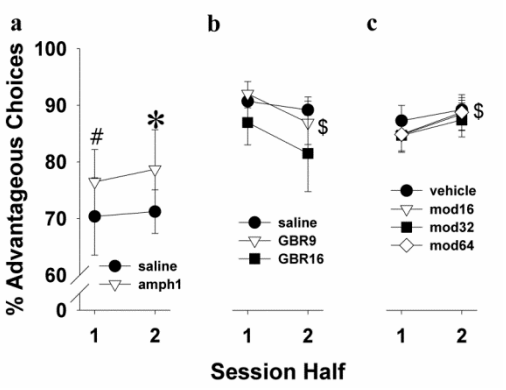

Fig. 5. Effects of treatments on choice strategy within the testing sessions. Amphetamine at 1 mg/kg resulted in mice selecting significantly more safe options compared to saline when examined across the session (a). GBR12909 at 9 mg/kg increased risky choice preference of mice in the second half of the session compared to the first, but was not significantly different from saline (b). Modafinil at 64 mg/kg decreased risk choice preference in the second half of the session compared to the first half driven by their riskier preference in the first half of the session (c). Reductions in risk preference across the session induced by modafinil were predominantly observed in poor learners. Data are shown as the mean ± S.E.M., *p<0.05 when compared with saline, #p<0.1 when compared with saline, \$$p$<0.05 when compared with the first session-half.

Exp 2. The effects of GBR12909 on IGT performance

The selective DAT inhibitor GBR12909 was administered to mice to examine its effects on risk-taking behavior. A main effect of response option was observed ($F_{(3,27)}$=22.5, p<0.001), with no interaction between response option and drug (F<1, ns; Fig. 6a).

Significant main effects of GBR12909 were observed for premature responses ($F_{(3,60)}$=11.9, p<0.001; Fig. 6b), trials completed ($F_{(3,60)}$=33.1, p<0.001; Fig. 6c), punishment perseveratives ($F_{(3,60)}$=7.9, p<0.001; Fig. 6d), reward perseveratives ($F_{(3,60)}$=4.1, p<0.05; Fig. 6e), mean reward latencies ($F_{(3,36)}$=3.3, p<0.05; Fig. 6f), and percentage omissions ($F_{(3,60)}$=38.9, p<0.001; Fig. 6g). *Post hoc* analyses revealed that both lower doses (9 and 16 mg/kg) increased premature responding compared to saline, while 28.5 mg/kg resulted in fewer premature responses than saline (p<0.05). Decreased trials completed was observed at 28.5 mg/kg compared to saline and all other doses (p<0.001), with similar 28.5 mg/kg-induced reductions in punishment perseveratives (p<0.05) and reward perseveratives (p<0.05) compared to saline. Further analyses revealed that the lowest dose (9 mg/kg) decreased the percentage of omissions whereas the highest dose increased percentage omissions compared to saline (p<0.01). There was a trend toward 9 mg/kg inducing shorter mean reward latencies than saline (p<0.1). In contrast, the highest dose resulted in a trend towards inducing longer reward latencies compared to 9 (p<0.1) and 16 mg/kg (p<0.1).

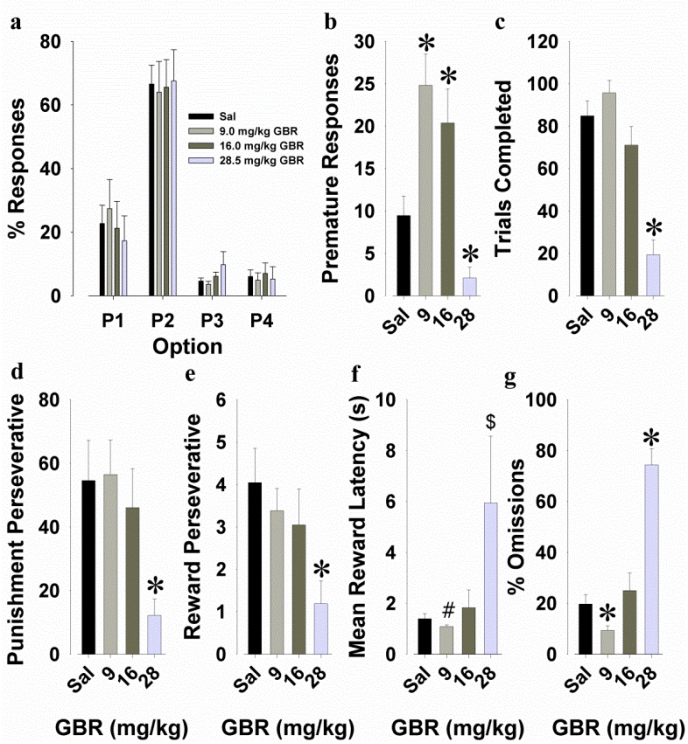

Fig. 6. Effects of GBR12909 on performance of C57BL/6 mice in the IGT. GBR12909 (9, 16, and 28 mg/kg) did not impair the animals' choice preference (a). GBR12909 decreased premature responding at the highest dose, while increasing it at the lower doses (b). At the highest dose, GBR12909 decreased the total trials completed (c), as well as punishment (d) and reward perseveratives (e). GBR12909 at the highest dose tended to slow the latency to collect reward compared to 9 and 16 mg/kg while 9 mg/kg tended to speed latencies compared with saline (f). GBR12909 decreased the percentage of omitted trials at the lowest dose but increased it at the highest dose (g). Data are shown as the mean + S.E.M., *p<0.05 when compared with saline, #p<0.1 when compared with saline, \$$p$<0.1 when compared with 9 and 16 mg/kg GBR12909.

Within-session risk preference

Mice administered 28.5 mg/kg GBR12909 were discarded from this analysis because of their low level of responding. There was a trend towards a main session-half effect ($F_{(1,14)}$=3.1, p<0.1; Fig. 5b). No main effect of GBR12909 ($F_{(2,28)}$=2.1, ns) nor interaction with session-half (F<1, ns) was observed. Planned comparisons revealed that GBR12909 at 9 mg/kg induced significantly more risky choices during the second half compared to the first (t=2.5, p<0.05).

Exp 3. The effects of modafinil on IGT performance

We tested the effects of modafinil on risk-taking behavior in the IGT to determine whether it would have effects similar to GBR12909. For response option, a main effect was observed ($F_{(3,60)}$=21.1, p<0.001), with no dose by response-option interaction (F<1, ns; Fig.

7a). Main effects of modafinil were observed for premature responses ($F_{(3,60)}$=23.7, p<0.001; Fig. 7b), reward perseveratives ($F_{(3,60)}$=4.1, p<0.05; Fig. 7e), and mean reward latencies ($F_{(3,60)}$=3.5, p<0.05; Fig. 7f). *Post hoc* analyses revealed that all doses increased premature responses compared to vehicle (p<0.001), with 32 mg/kg and 64 mg/kg increasing premature responses more than 16 mg/kg modafinil (p<0.005). Analyses revealed that modafinil induced a decrease in reward perseveratives at 16 mg/kg (p<0.05) and 64 mg/kg (p<0.01) compared to vehicle. A significant decrease in mean reward latency was observed following modafinil administration at 64 mg/kg compared to vehicle (p<0.005), 16 mg/kg (p<0.05), and 32 mg/kg (p<0.1). No main effects of drug were observed for trials completed ($F_{(3,60)}$=1.1, p>0.1; Fig. 7c), or punishment perseveratives ($F_{(3,60)}$=1.9, p>0.1; Fig. 7d). Modafinil tended to reduce percentage omissions ($F_{(3,60)}$=2.5, p<0.1; Fig. 7g), with *post hoc* analyses revealing that modafinil at 32 mg/kg reduced omissions (p<0.05), while 64 mg/kg tended to reduce omissions (p<0.1) compared to vehicle.

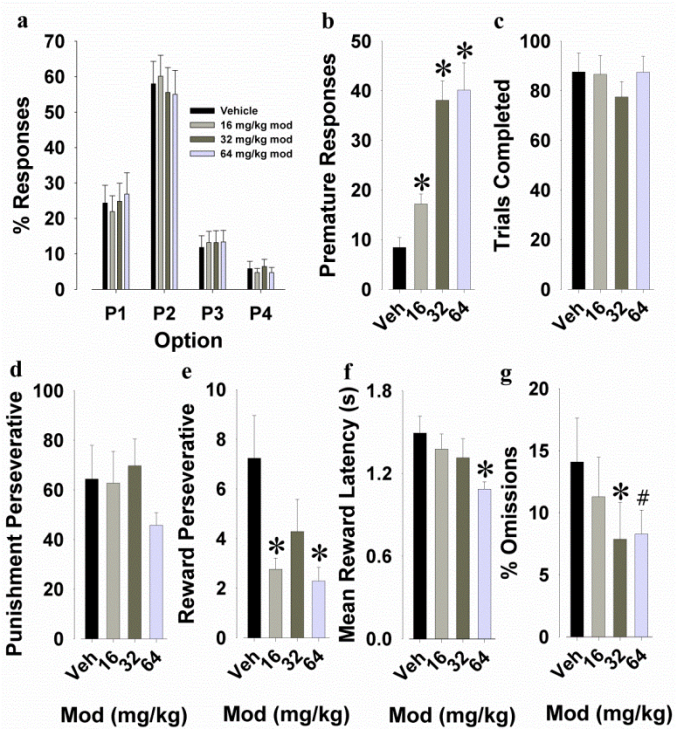

Fig. 7. Effects of modafinil on performance of C57BL/6 mice in the IGT. Modafinil (16, 32, and 64 mg/kg) did not affect the animals' overall choice preference (a). Premature responding was significantly increased after administration of modafinil at all doses (b). Modafinil had no effect on the animals' total trials completed (c), nor did it affect punishment perseveratives (d). Mice exhibited less reward perseveratives after treatment with modafinil at both the lowest as well as the highest doses (e), while the latency to collect rewards was significantly decreased by modafinil at the highest dose (f). Modafinil reduced the percentage of omitted trials at the highest doses (g). Data are shown as the mean + S.E.M., *p<0.05 when compared with vehicle, #p<0.1 when compared with vehicle.

Within-session risk preference

A main effect of session-half ($F_{(1,17)}$=4.9, p<0.05; Fig. 5c) was observed. No main effect of modafinil or interaction with session-half was observed however (F<1, ns). *Post hoc* analysis revealed that mice treated with the highest dose of modafinil exhibited more risky choices in the first half of the session compared to the second session-half where they selected more from the safe cues (t=-2.4, p<0.05).

"Poor learners" vs. "good learners"

In a recent human placebo-controlled IGT study it was observed that modafinil increases risky choice in "low-impulsive" gamblers, but decreases risky choice in "high-impulsive" gamblers (42). We separated our subjects into poor and good learners using a median split on the learning performance of the vehicle-administered mice of the particular drug study (% advantageous choice session-half 2 – % advantageous choice session-half 1).

Amphetamine - good/poor learners

We observed a main effect of amphetamine ($F_{(1,11)}$=5.8, p<0.05; Fig. 8a) and a session-half × poor/good learners interaction ($F_{(1,11)}$=5.1, p<0.05). The good learners (n=7) chose significantly more safe options during the second vs. the first half (70% vs. 64%) (saline, t=-3.1, p<0.05). During the first half, amphetamine at 1 mg/kg increased safe choices compared to saline (70 % vs. 64%) in the good learners (t=-2.9, p<0.05). No effects of amphetamine were observed in the poor learners (n=6).

GBR12909 - good/poor learners

We observed a trend effect of half ($F_{(1,13)}$=3.3, p<0.1; Fig. 8b) and half × poor/good learners interaction ($F_{(1,13)}$=4.4, p<0.1). Poor learners (n=8) chose fewer safe options during the second vs. the first half (84 % vs. 90%) (saline, t=2.6, p<0.05). GBR12909 at 9 mg/kg led to fewer safe choices during the second vs. the first half in the poor learners (82 % vs. 91%) (t=2.9, p<0.05). The good learners (n=7) chose significantly more safe options during the second vs. the first half (96 % vs. 92%) (saline, t=-4.1, p<0.05). During the second half, GBR12909 at 9 mg/kg decreased safe choice preference compared to saline in the good learners (93 % vs. 96%) (t=2.2, p<0.1).

Modafinil - good/poor learners

A main effect of half ($F_{(1,16)}$=6.1, p<0.05; Fig. 8c) and half × poor/good learners interaction ($F_{(1,16)}$=5.2, p<0.05) and a trend effect of modafinil × half × poor/good learners interaction ($F_{(3,48)}$=2.4, p<0.1) was observed. Further analyses revealed that the poor learners (n=9) chose significantly fewer safe options during the second vs. the first half (90 % vs. 96%) (saline, t=3.6, p<0.05). All three doses of modafinil induced a trend towards fewer safe choices compared to saline (90 %, 90 %, and 92 % from low to high dose vs. 96% for

saline) during the first half of the session in the poor learners (p<0.1). During the second half however, modafinil at 64 mg/kg led to more safe choices compared to saline (94 % vs. 90%) in the poor learners (t=-2.8, p<0.05). The good learners (n=9) chose significantly more safe options during the second vs. the first half (88 % vs. 79%) (saline, t=-3.6, p<0.05). Modafinil did not affect the choice performance of good learners.

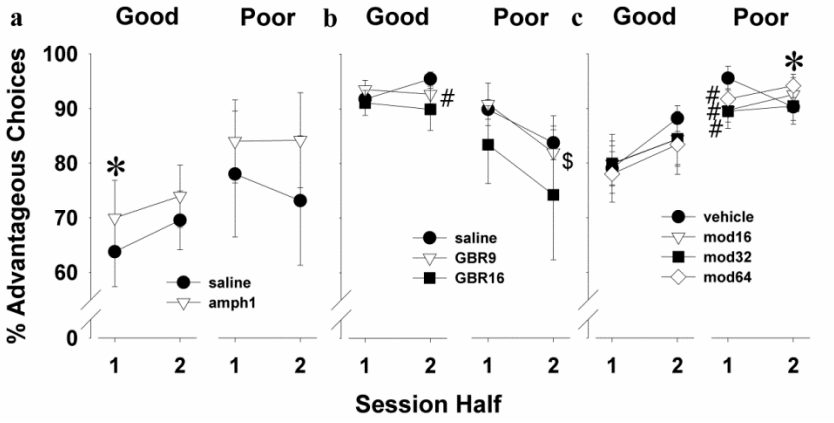

Fig. 8. Effects of DAT inhibitor treatments on choice strategy of good and poor IGT learners. Amphetamine significantly increased safe choice preference of the good learners in the first half of trials compared to saline (a). GBR12909 at 9 mg/kg increased risk choice preference of the good learners in the second half compared to saline and of the poor learners in the second half compared to the first half, but not compared to saline (b). All three doses of modafinil increased risk choice preference of the poor learners in the first half compared to saline, while the highest dose increased safe choice preference in the latter half (c). Data are shown as the mean ± S.E.M., *p<0.05 when compared with saline, #p<0.1 when compared with saline, \$$p$<0.05 when compared with the first session-half.

Correlation of impulsivity measures: risk preference and premature responding

Analyses revealed that there were individual differences between the animals' performance on different parameters. No correlation (r=0.07, p>0.1) was observed between the individual differences on % advantageous choices (risk-taking behavior) and "premature responses" (motoric impulsivity). At effective doses of modafinil (r=0.26, p>0.1) and GBR12909 (r=0.19, p>0.1), still no correlations were observed. A correlation between % advantageous choices and premature responses was observed for mice during amphetamine administration (r=0.47, p<0.05), but this did not pass the Bonferroni correction (p>0.0125).

DISCUSSION

With repeated training, C57BL/6 mice learned to choose the less risky options in the IGT resulting in frequent small rewards and little punishment. Importantly, we observed no correlation between risk choice preference and premature responding, suggesting that these two variables measure two different forms of impulsive behavior, impulsive choice and motoric impulsivity, respectively (21). Moreover, we have demonstrated that performance in the IGT can be differentially influenced by drugs with similar dopaminergic mechanisms. Administration of the mixed norepinephrine (NET)/DAT inhibitor amphetamine resulted in a risk-averse preference with no effect on premature responding. In contrast, the more selective DAT inhibitors GBR12909 and modafinil modestly increased risk preference in the IGT, but only when within-session learning rates were controlled for. Unlike amphetamine, these more selective DAT inhibitors also increased measures of motivation and motor impulsivity.

Amphetamine altered the risk preference of mice in a manner that was consistent with its effect in rats (18), supporting the cross-species translational validity of the rodent IGT. The shift to the more likely rewarded and less risk-prone option P1 indicates a more conservative punishment-averse choice strategy. Zeeb et al. (18) suggest that amphetamine could make animals hypersensitive to punishments, leading to this increased preference of the small reward/punishment option. This interpretation is supported by amphetamine-induced anxiogenic effects in rodents (51, 52). Moreover, Rivalan et al. (17) observed that punishment-averse strategies in the IGT correlated with risk-averse (high anxiety) behaviors in other rat paradigms such as the light/dark emergence and elevated plus-maze test. In approach/avoidance conflict situations, which engender a tradeoff between risk-taking and anxiety, the anxiogenic effects of amphetamine may result in more avoidance behavior. The effects of amphetamine may depend on dose, strain, the presence of a conditioned stimulus during a delayed reward (53), or the specific cost-benefit decisions the animal is faced with (54). The use of signaled punishing time-outs in the rodent IGT may thus have promoted an amphetamine-induced conservative strategy. In the present studies, we have also examined within-session task performance in an attempt to track the subject's ongoing decision-making behavior as is performed in human IGT testing and as we have presented previously (20). These analyses demonstrated that amphetamine increased the preference for safe options across the whole session. Because the highest dose – tested for comparability with exploratory studies (30, 32) – also reduced trials completed, decreasing perseveration and increasing omissions, future studies could examine the effects of lower doses to determine whether there is a dose-dependent effect on risk preference. The current data underscore the limited aspects of BD that can be modeled by amphetamine

(38, 55), since patients with BD exhibit increased risk preference in the IGT (11, 12), opposite to what was observed here in mice.

In contrast with amphetamine administration to rats in the IGT or five-choice serial reaction time task (5CSRTT) (18, 56, 57), amphetamine administration did not increase premature responding in mice in the IGT. This result may reflect species differences because previous studies in mice have reported an increased although less robust effect of amphetamine on premature responding than in rats (58, 59). Amphetamine has 5- to 9-fold less potency at the DAT than the NET in mice and humans (60), but with greater selectivity to the DAT over NET in rats (61). Given that selective NET inhibition reduces premature responding (62-64), while selective DAT inhibition increases premature responding (58), combined NET and DAT inhibition may obfuscate the effect of DAT inhibition alone, supporting this rat/mouse species difference of amphetamine effects.

Selective DAT inhibition with GBR12909 (9 mg/kg) increased risk preference in mice, albeit only in one session-half. Treatment with the highest dose, 28.5 mg/kg, decreased the number of trials and increased omissions consistent with amphetamine at 2.5 mg/kg [reflecting stereotypy; (65)], and supporting similar effects on exploratory behavior in the mouse BPM at these doses (30, 66). Despite the greater selectivity for the DAT, at a high dose GBR12909 is also likely to inhibit the NET (67), thus producing amphetamine-like effects such as no increase in premature responding. At low to moderate doses however, GBR12909 induced more premature responses, indicative of motor impulsivity consistent with 5CSRTT studies (58). GBR12909 at 9 mg/kg also decreased the amount of omitted trials and the time to obtain reward, supporting drug-induced increases in motivation (68) as seen previously in mice in a progressive ratio breakpoint study (PRBS) (69). This increased motivation could be interpreted as hedonic-like behavior (70, 71), which would support the use of selective DAT inhibition to model mania since patients with BD mania also exhibit hedonic behavior (72). Increased risk preference in the IGT induced by GBR12909, would further support its use as a model of BD (9). The subtlety of the effect observed here limits the possible utility of this model however, given the striking difference between patients with BD and healthy subjects (4, 9).

Consistent with GBR12909, modafinil induced a subtle increase in risk preference when analyzed across session halves. Effects of modafinil on other IGT measures were also consistent with the effects of GBR12909 at low to moderate doses, supporting the interpretation of a similar underlying DAT inhibitory mechanism of these two drugs (39, 41). In support of DAT inhibition-mediated effects of modafinil, this drug produces the same behavioral effects as GBR12909 in rat activity (40), the mouse BPM (32, 73), and in a mouse PRBS (69). In the human IGT, “low-impulsive” gamblers treated with modafinil chose more risky options compared to placebo, whereas the opposite was observed in

"high-impulsive" gamblers within one IGT session (42). When we separated our subjects in a similar manner, we observed that modafinil increased safe choice preference in the poor within-session learners, similar to the human findings. In the good within-session learners, modafinil did not increase safe choice preference. Hence, these data provide some limited support for the pharmacological predictive validity of the rodent IGT for testing in humans.

We recently reported that DAT KD mice chose riskier options than their WT littermates during initial learning of the IGT (session 1) (20). Moreover, DAT KD mice exhibited increased risk preference throughout acquisition of the IGT. Furthermore, DAT KD mice exhibited the same decreased reward latencies and omissions and increased premature responding (20) as was observed after modafinil and low-dose GBR12909 administration to C57BL/6 mice. These similar increases in hedonic-like behavior and motor impulsivity support the use of selective pharmacological DAT inhibition in an attempt to model BD mania (30, 32). The consistent risk preference of DAT KD mice during training contrasts with the subtlety of effects observed with acute DAT inhibition once the task was acquired. Differences may therefore exist between affecting performances during within-session learning vs. once trained (19, 74), an important aspect for cross-species translational research because the human IGT assesses within-session learning of risk preference.

Modafinil- and GBR12909-induced increases in risk preference were only detected by analysis of changes in performance across the session. Specifically, modafinil induced more risky choices in the first half of the session, while GBR12909 increased the risk preference of the mice during the second half. This disparity in timing of effects may relate to the pharmacokinetics of the two drugs. GBR12909 did not affect exploration in mice for the first 10 min of a BPM session, while modafinil affected several aspects of exploration (32, 73). Alternatively, these drugs' ability to change choice preference within a session may have been influenced by repeated training in the rodent IGT given that a single session is used in the human version. The GBR12909 study was conducted last in these mice after re-baselining performance (Fig. 2), which may have reduced the sensitivity of the mice to DAT inhibition on this behavior. Although performance was stable prior to each drug study, the animals gradually increased advantageous choices, from approximately 70% in Exp. 1 to 90% in Exp. 3. Thus, repeated testing in this rodent IGT may not provide the best means with which to assess the effects of drug manipulation on risk choice. Future studies will examine the effects of these drugs within an extended single training session to determine whether they affect risk learning behavior within a session. Risk preference learning in rats has been measured in a single session in the rodent IGT without prolonged training (17, 19). Experimental effects can differ between these within- and between-session risk learning paradigms. Lesions of the orbitofrontal

cortex (OFC) before acquisition in a between-session risk learning rodent IGT led to delayed development of safe choice preference, but with no effect on decision making if lesioned after repeated training (74). In a within-session rodent IGT however, lesions of the OFC decreased the amount of good decision-making rats (75), highlighting the difference between learned and within-session learning risk behaviors. Future studies in mice will track the ongoing decision process - like the human IGT - in one session in order to develop a robust cross-species translational task and animal models of BD. Besides DAT inhibition, testing the effects of dopamine agonists on IGT performance may further elucidate the role of the dopaminergic system in risk-taking.

In summary, the present data demonstrate that C57BL/6 mice can be trained to perform the IGT. Moreover, this task can discriminate between two different aspects of impulsive behavior, impulsive choice and motor impulsivity. These two behaviors are differentially influenced by very similar drugs that modulate norepinephrine and dopamine levels and are used to model BD. Both modafinil and GBR12909 induced similar increases in motor impulsivity, motivation, and a modest increase in risk-preference, consistent with, but less striking than, those observed in patients with BD mania. Repeated training in the IGT may impede drug-induced risk-taking effects however. The rodent IGT is a novel tool for investigating the genetic and neurobiological basis of risk-taking behavior. With further refinement, it could be used to screen other animal models of neuropsychiatric disorders and test novel treatments.

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CHAPTER 3.3

Reduced dopamine transporter functioning induces high-reward risk-preference consistent with bipolar disorder

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ABSTRACT

Background

Individuals with bipolar disorder (BD) exhibit deleterious decision-making, negatively impacting their lives. Such aberrant decision-making can be quantified using the Iowa gambling task (IGT), which requires choosing between advantageous and disadvantageous options based on different reward/punishment schedules. The mechanisms underlying this behavioral deficit are unknown, but may include the reduced dopamine transporter (DAT) functioning reported in BD patients. Using both human and mouse IGTs, we tested whether reduced DAT functioning would recreate patterns of deficient decision-making of BD patients.

Methods

We assessed the IGT performance of 16 BD subjects (7 female) and 17 healthy control (HC) subjects (12 female). We recorded standard IGT performance measures and novel post-reward and post-punishment decision-making strategies. We characterized a novel single-session mouse IGT using C57BL/6J mice (*n*=44). The BD and HC IGT performance were compared with the effects of chronic [genetic knockdown (KD; *n*=31) and wild-type (WT; *n*=28) mice] and acute [C57BL/6J mice (*n*=89) treated with the DAT inhibitor GBR12909] reductions of DAT functioning in mice performing this novel IGT.

Results

BD patients exhibited impaired decision-making compared to HC subjects. Both DAT KD and GBR12909-treated mice exhibited poor decision-making in the mouse IGT. The deficit of each population was driven by reward hypersensitivity.

Conclusions

The single-session mouse IGT measures dynamic risk-based decision-making similar to humans. Chronic and acute reductions of DAT functioning in mice impaired decision-making consistent with poor IGT performance of BD patients. Hyperdopaminergia caused by reduced DAT may therefore underlie the poor decision-making induced by reward hypersensitivity in BD patients.

Keywords

Bipolar disorder, Dopamine transporter, Mice, Decision-making, GBR12909, Iowa gambling task

INTRODUCTION

Several psychiatric disorders are associated with impaired decision making (1-3), deleteriously impacting the patients' quality of life (4). Neural networks mediating decision making have been identified (5, 6), but clarification of the neurotransmitter activity underlying decision making in psychiatric patients needs to be delineated in order to develop therapeutic interventions. The Iowa gambling task (IGT) utilizes high-yield/high-risk options versus low-yield/low-risk options to measure decision making with real-world translational validity in a single test-session (7). Among psychiatric populations, patients with bipolar disorder (BD) exhibit poor IGT performance (8, 9). Moreover, a diagnosis-specific pattern can be detected with manic BD patients being hypersensitive to rewards in accordance with symptoms of increased hedonia (10). In contrast, schizophrenia patients exhibit disrupted contingency learning (11) and depressed patients are more sensitive to punishment (8, 12).

Model animals for impaired decision making are required for treatment development (13). Based on the human IGT, animal analogues have been created (14). In the rodent IGT, animals are presented with four options with different reward/punishment probabilities and magnitudes. Consistent with the human IGT, two options offer small rewards and little punishment (safe/advantageous choices), while the other two options offer larger rewards and more punishment (risky/disadvantageous choices). The effects of dopaminergic, serotonergic, and noradrenergic manipulations have been investigated in rats and mice using a rodent IGT in which learning was acquired and examined across multiple test sessions (15-18). These studies revealed the involvement of different neurotransmitters in learned decision-making processes and their relationship with motor impulsivity, including indications that dopamine (DA) is relevant to rodent IGT performance. In contrast to the gambling tasks used in these studies however, the human IGT examines dynamic learning in a single session, limiting the translational validity of findings in the multiple-sessions rodent IGT (14). In support of this assertion, different results from similar experimental manipulations have been observed in multiple- versus single-session rodent IGTs. For example, lesions of the orbitofrontal cortex in rats impaired (19) or did not affect (20) decision making in a single- or multiple-sessions IGT respectively. In the single-session rodent IGT, good, intermediate, and poor decision-making rodents can be identified based on their risk-learning performance (21). Similarly, inter-individual differences are observed among healthy humans performing the IGT (22, 23). Assessment of mechanisms that may contribute to impaired IGT performance in BD could therefore be conducted in mice by using a single-session IGT that allows direct comparison with human IGT findings.

Although mechanisms underlying BD symptoms remain poorly defined, increasing evidence suggests that both circadian abnormalities (24) and aberrant DA levels most likely play key roles (25, 26). One of the central mechanisms by which DA homeostasis is maintained is its reuptake from the synaptic cleft into presynaptic nerve terminals by the DA transporter (DAT). Because several studies support an important role of DA in regulating risk-based decision-making in rodents (6, 27), altered DAT functioning may contribute to abnormal decision making in individuals with BD. Supporting this assumption, polymorphisms in the DAT gene have been linked with BD (28, 29). More recently, reduced striatal DAT levels have been measured in unmedicated BD patients (30) as well as in postmortem tissue (31). Thus, the relationship between reduced DAT functioning and its contribution to impaired risk-based decision-making in BD patients may be important, but remains undetermined.

In order to determine whether reduced DAT functioning could induce comparable impairments in decision making under risk in the IGT, we compared the single-session IGT performance of patients with BD and mice with chronic [genetic knockdown (KD)] and acute (DAT inhibitor GBR12909) reductions of DAT functioning. A single-session IGT was used for consistency with the human IGT and to measure dynamic changes in decision making after rewards or punishments. We predicted that: (*a*) decision making would be impaired in patients with BD; (*b*) subpopulations of mice would be identifiable in the mouse IGT based on risk learning; and (*c*) both chronic and acute reductions of DAT functioning would impair IGT performance similar to deficits observed in BD.

METHODS

Participants

16 participants between the age of 18 and 55 who met SCID (Structured Clinical Interview for DSM-IV) criteria for BD were recruited from inpatient and outpatient psychiatric clinics located at the University of California San Diego (UCSD) Medical Center. Nine participants met criteria for a current manic episode (Young Mania Rating Scale (YMRS) score ≥20) and seven participants were classified as hypomanic (YMRS score 12 to 15) (32). The majority of patients were taking mood stabilizers and/or atypical antipsychotics. The most common antipsychotic medication prescribed was risperidone and the most common mood stabilizers prescribed were valproate and lithium. 17 healthy comparison (HC) participants who had never met SCID criteria for any Axis I psychiatric disorder and did not have first degree relatives with BD were recruited from advertisements in the San Diego community. BD and HC groups were matched for age, gender, education, and ethnicity, and had equivalent premorbid IQ as assessed by the Peabody Picture Vocabulary Test (33) (Table 1). Participants were excluded for: (*1*) current alcohol or substance dependence; (*2*) a history of neurological conditions, head trauma, or seizures; (*3*) treatment with

electroconvulsive therapy; (*4*) stroke or myocardial infarction; and (*5*) a positive result for cocaine, amphetamine, or phencyclidine on a urine toxicology Rapid Drug screen (Pharmatic Inc., San Diego, CA) administered during the test session. All subjects provided written informed consent to the current protocol approved by the UCSD institutional review board known as the Human Research Protections Program.

Human Iowa gambling task

Participants were administered a computerized version of the IGT where individuals were required to select from 4 decks of cards (A, B, C, & D) (7). After selecting a card, a theoretical amount of money was displayed on the screen. Decks A or B resulted in high monetary gains, but also high unpredictable penalties (disadvantageous). Decks C & D paid smaller amounts of money but incurred smaller losses (advantageous). The task included 100 trials and participants were informed that the goal was to avoid losing money and win as much money as possible. Decision making was measured using a net score calculated by subtracting disadvantageous choices (A+B) from the advantageous choices (C+D).

Table 1. Demographic and clinical characteristics of bipolar disorder (BD) patients and healthy control (HC) subjects

Parameter	HC	BD	Group differences
	(*n*=17)	(*n*=16)	
Age (years)	33.9 ± 3.0	33.8 ± 2.8	ns
Gender (male/female)	5 M, 12 F	9 M, 7 F	ns
Education (years)	14.8 ± 0.6	13.9 ± 0.6	ns
Ethnicity (% Caucasian)	47%	75%	ns
Peabody Picture Vocabulary Test	104.7 ± 2.7	96.9 ± 4.2	ns
BD Age of Onset (years)		23.0 ± 1.8	
BD Duration of Illness (years)		10.1 ± 1.9	
Number of BD Hospitalizations		2.7 ± 0.5	
YMRS Score	0.9 ± 0.8	22.3 ± 2.3***	BD > HC
HDRS Score	0.9 ± 0.4	9.3 ± 1.2***	BD > HC
Medication			
Antipsychotic alone		3	
Mood stabilizer alone		1	
Antipsychotic + Mood stabilizer		9	
Other medications		0	
Not medicated		3	

HDRS, Hamilton Depression Rating Scale; YMRS, Young Mania Rating Scale; *** $p < 0.001$

Mice

Male C57BL/6J (*n*=133), DAT KD (*n*=31), and wild-type (WT) littermate (*n*=28) mice were used throughout the experiments. DAT KD mice express ≈10% DAT levels compared with WTs (34). DAT heterozygous breeders backcrossed onto a C57BL/6J background for >10 generations were sent to UCSD from the University of Chicago. All mutant mice used resulted from heterozygous breeding pairs. All mice were 3-5 months old at the time of testing and weighed between 21-34 g. All animals were group housed (maximum four/cage) and maintained in a temperature-controlled vivarium (21±1 °C) on a reversed day-night cycle (lights on at 7.00 PM, off at 7.00 AM). All mice had *ad libitum* access to water and were food-restricted at 85% of their free-feeding weight during the periods of testing (during the dark phase of the day-night cycle between 8.00 AM and 6.00 PM). All procedures were approved by the UCSD Institutional Animal Care and Use Committee. The UCSD animal facility meets all federal and state requirements for animal care.

Drugs

GBR12909 dihydrochloride was purchased from Sigma Aldrich (St Louis, MO, USA) and dissolved in saline after heating (45 °C, 60 min) (35, 36). GBR12909 was injected intraperitoneally with a volume of 10 ml/kg, immediately prior to testing. Free-base drug weight was used in drug calculations.

Mouse Iowa gambling task

Sixteen five-hole operant chambers were used for the IGT (Supplementary Methods). In short, mice had 10 s to holepoke in one of four illuminated holes. Mice were rewarded with strawberry milkshake or punished with a time-out period depending on the reward schedule (Fig. 1). Two options delivered large rewards or long time-out penalties (disadvantageous). The other two options delivered smaller rewards or shorter time-out penalties (advantageous). Decision making was measured as *%advantageous choices*. Several other measures were recorded and presented (Table 2).

Characterization of the single-session mouse IGT

After stabilization of responding on a simple fixed-ratio schedule (Supplementary Materials and Methods), single-session performance of C57BL/6J mice (*n*=44) was assessed in the IGT.

Effects of chronic reductions of DAT functioning on IGT performance

DAT KD (*n*=31) and WT littermates (*n*=28) were trained to holepoke. After stabilization of responding, the IGT performance of these mice were assessed and compared.

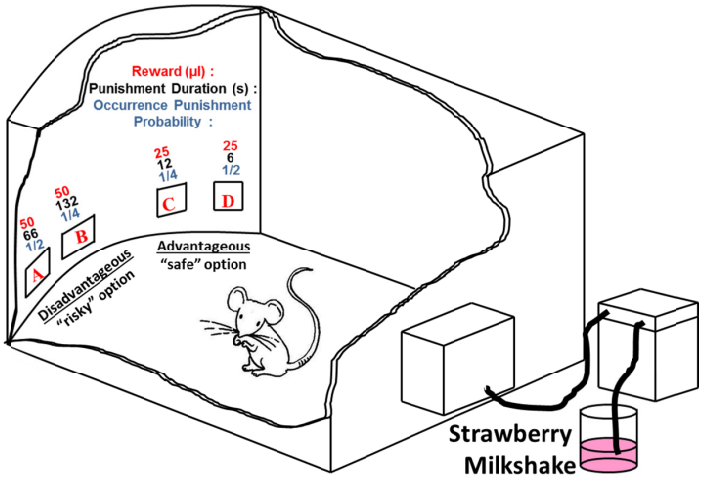

Fig. 1. An illustration of the single-session mouse Iowa gambling task (IGT). After stable responding to 1 of 4 lit holes for a single reward (A-D), mice were tested in the single-session 1-hour long IGT. After trial initiation and a 5-s delay, holes A-D were illuminated for a 10-s period and the mouse could holepoke in 1 hole. The selected hole determined the given food reward value (strawberry milkshake; in red), or punishment value (cue light flashing and all apertures unresponsive; in black), according to the associated probability (in blue). Consistent with the human IGT, two small reward/low punishment options (C-D) were ultimately advantageous, while the other two high reward/high punishment options (A-B) were disadvantageous. Two different versions, one with the opposite hole locations as described above, were used to control for possible location bias.

Effects of acute reductions of DAT functioning on IGT performance

After stabilization of responding, C57BL/6J mice (*n*=89) naïve to the IGT received saline (*n*=29), GBR12909 at 9 (*n*=30) or 16 mg/kg (*n*=30) (16, 35) and were challenged in the IGT.

Quantifying individual differences in learning performance

Based on Rivalan et al. (21), we examined the performance of individual mice and identified three different subpopulations. These subpopulations were quantified by subtracting % advantageous choices of trial period 1 from % advantageous choices of trial period 3. Good, intermediate, and poor decision-makers were stratified as 1) >0.5, 2) between 0.5 and -0.5, and 3) <0.5 standard deviations from the mean respectively. This stratification was made for each genotype or drug treatment group separately.

Post-reward/punishment decision-making: win-stay/lose-shift strategies

The likelihood of a subject repeatedly choosing a card/stimulus following a reward from the advantageous options (safe-stay) and disadvantageous options (risky-stay) was compared with their likelihood of selecting a different choice following punishment from the advantageous (safe-shift) and disadvantageous options (risky-shift) (Supplementary Methods).

Table 2. Description of the behavioral measures used in the single-session mouse Iowa gambling task.

Measures	Description
%Advantageous choices	Advantageous response options [(C+D) / total (A+B+C+D)]*100
%Disadvantageous choices	Disadvantageous response options [(A+B) / total (A+B+C+D)]*100
(p) Safe-stay	Probability of choosing advantageous options after being rewarded from advantageous options
(p) Risky-stay	Probability of choosing disadvantageous options after being rewarded from disadvantageous options
(p) Safe-shift	Probability of choosing disadvantageous options after being punished from advantageous options
(p) Risky-shift	Probability of choosing advantageous options after being punished from disadvantageous options
Omissions (%)	Failure to respond in any hole during the light stimulus duration of 12s (motivation)
Premature responses	Response in any cue hole during the 5s inter-trial interval (ITI) preceding illumination of the cue array (motor impulsivity)
Mean choice latency (s)	The latency to holepoke in one of the four holes (reaction time)

Statistical analyses

Human and mouse choices were analyzed over three equal blocks of trials. Human IGT net score was analyzed using analysis of variance (ANOVA) with group (BD, HC) as a between-subject factor and trial block as a within-subject factor. Animal choices were analyzed using ANOVAs with trial period as a within-subject factor and genotype, drug, and group as between-subject factors. Group was determined by quantification of learning as described above. Subjects with ≤ 10 completed trials per trial period were excluded from analysis. The % advantageous choice preference was compared with chance (50%) using a one-sample *t*-test. Tukey *post hoc* analyses of statistically significant main or interaction effects were performed where applicable and Cohen's *d* effect sizes were calculated. Where appropriate, planned comparison paired *t*-tests were conducted between groups. The α level was set at 0.05. All analyses were performed using SPSS (19.0, Chicago, IL, USA).

RESULTS

Human IGT performance

There was a significant difference in decision-making ability between HC and BD subjects ($F_{(1,31)}$=6.7, p<0.05; Fig. 2A), indicating that BD subjects made less advantageous choices compared to HC subjects (p<0.05; effect size [d]=0.91). Net score increased significantly over three trial blocks in the HC group ($F_{(2,32)}$=3.5, p<0.05; Fig. 2B) with only a trend in the BD subjects ($F_{(2,30)}$=2.9, p=0.070). Traditional IGT analyses over five trial blocks indicated that net score increased significantly over time in both the HC ($F_{(4,64)}$=3.3, p<0.05) and BD

subjects ($F_{(4,60)}$=3.1, p<0.05; Fig. S1). Interestingly, despite the majority of HC subjects increasing their net score over time, some individuals did not, while others decreased advantageous preference over time (Fig. 2E).

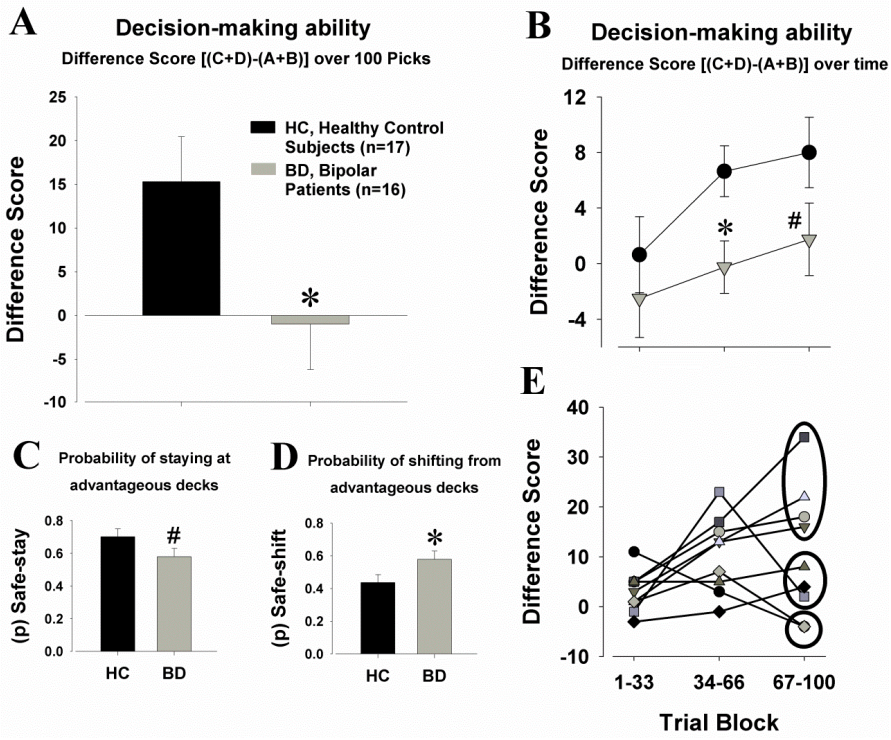

Fig. 2. Iowa gambling task (IGT) performance of bipolar disorder (BD) and healthy control (HC) subjects. Over 100 card picks, the BD group had a significantly lower net difference score compared to HC subjects (A). When analyzed over three trial blocks, BD subjects performed poorly compared to HC subjects in blocks two and three (B). Compared to HC subjects, BD subjects tended to choose less from the safe decks directly after being rewarded from the safe decks (C). Compared to HC subjects, BD subjects switched more to the risky decks directly after losing at the safe decks (D). Inter-individual differences of a subset of HC subjects (n=9) are displayed, indicating that the majority increases their net score over time, while others remain the same or decrease their net score over time (E). Data are presented as the mean ± SEM, * p<0.05 and # p<0.1 when compared with HC subjects.

Win-stay/lose-shift strategies in BD subjects

All subjects increased safe-stays over time ($F_{(2,62)}$=5.3, p<0.05), but the BD subjects tended to make less safe-stays compared to HCs ($F_{(1,31)}$=4.0, p=0.054; Fig. 2D). No significant effects were observed for risky-stays (Fig. S2A). BD subjects made more shifts from the safe options after punishment compared to HCs ($F_{(1,31)}$=5.0, p<0.05; Fig. 2D), but there were no effects for risky-shifts (Fig. S2B). Hence, BD subjects were less likely to stay at the safe low-reward options but as likely to stay at the risky high-reward option.

Inter-individual differences of C57BL/6J mice in a single-session IGT

Mice were grouped into good (32%), intermediate (48%), or poor (20%) decision-makers based on their IGT learning performance (see Methods).

Advantageous choices

A group × trial period interaction ($F_{(4,82)}$=19.1, p<0.001; Fig. 3A) indicated that good decision-makers increased advantageous choices ($F_{(2,26)}$=12.0, p<0.01), while poor decision-makers decreased advantageous choices ($F_{(2,16)}$=16.9, p<0.001) over time.

Other behavioral measures (Table 3)

Independent of group, mice reacted faster ($F_{(2,82)}$=4.8, p<0.05), while omissions and premature responses did not differ (F<1, ns) over time.

Table 3. Motivational/motor impulsivity measures of mice in the Iowa gambling task.

Measure	Group	C57	WT	KD	Saline	GBR 9	GBR 16
Mean choice latency (s)	Good	2.86±0.24	4.09±0.24	3.37±0.20*	4.31±0.36	3.72±0.30	4.91±0.30
	Interm	3.11±0.20	4.07±0.21	3.11±0.24*	4.88±0.26	4.04±0.32*	4.25±0.27#
	Poor	3.30±0.30	4.70±0.41	3.37±0.34*	4.57±0.42	4.59±0.29	4.45±0.33
Omissions (%)	Good	7.60±3.05	17.21±2.82	6.31±2.30*	18.75±4.31	7.65±3.60*	24.17±3.60
	Interm	10.79±2.49	14.23±1.80	7.62±2.14*	20.72±3.16	17.84±3.80	23.68±3.29
	Poor	14.93±3.80	17.77±2.47	7.26±2.02*	23.84±5.10	21.29±3.44	17.10±4.03
Premature responses	Good	5.21±1.82	18.13±10.83	47.00±8.84#	1.05±1.55	2.23±1.30	2.90±1.30
	Interm	9.10±1.48	17.79±6.01	47.70±7.11*	3.69±1.14	1.52±1.37	3.44±1.19
	Poor	6.67±2.27	10.83±8.63	32.33±7.05#	2.40±1.84	1.42±1.24	4.50±1.45

Interm, intermediate; WT, wild-type; KD, knockdown

*p<0.05 and #p<0.1 when compared to WT/Saline

Win-stay/lose-shift strategies of C57BL/6J mice

Good decision-makers exhibited more safe-stays over time ($F_{(2,26)}$=3.8, p<0.05; Fig. 3B) and compared to poor decision-makers at the end of the session (p<0.01). Poor decision-makers however, tended to exhibit more risky-stay choices over time ($F_{(2,16)}$=3.1, p=0.074; Fig. S3A) and compared to good decision-makers at the end of the session (p<0.05). Poor decision-makers exhibited more safe-shifts compared to good decision-makers in trial period 3 (p<0.01; Fig. 3C), while they made less risky-shifts over time ($F_{(2,16)}$=5.1, p<0.05; Fig. S3B).

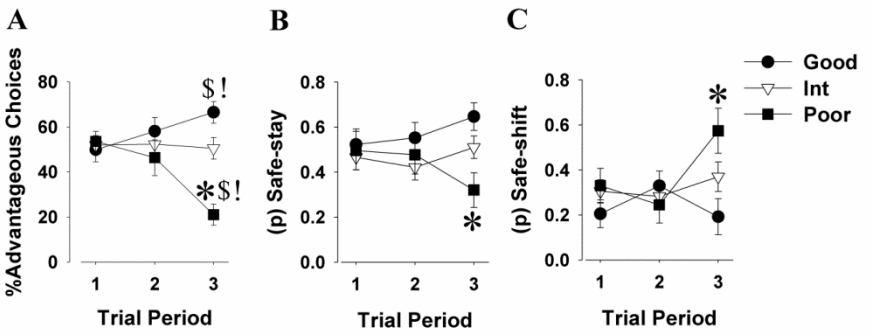

Fig. 3. Iowa gambling task (IGT) performance of C57BL/6J mice identified as good, intermediate, and poor decision-makers. Good decision-makers chose advantageous options, whereas poor decision-makers preferred disadvantageous options as the session progressed (A). Good decision-makers stayed more at the advantageous options after being rewarded there (safe-stay), and more compared to poor decision-makers at the end of the session (B). By the final trial period, poor decision-makers shifted more from the advantageous options compared to good decision-makers after punishment (safe-shift) (C). Data are presented as the mean ± SEM, **p*<0.05 when compared with good decision-makers, $*p*<0.05 when compared with intermediate decision-makers, and !*p*<0.05 when compared with chance.

IGT performance of DAT WT and KD mice

A main group effect ($F_{(2,53)}$=4.4, p<0.05), trial period × group interaction ($F_{(4,106)}$=30.2, p<0.001), and trends toward trial period × genotype ($F_{(2,106)}$=3.1, p=0.058) and trial period × genotype × group interactions ($F_{(4,106)}$=2.3, p=0.078) were observed. Similar to C57BL/6J mice, good decision-makers increased and poor decision-makers decreased advantageous choices over time.

Good decision-makers

Both WT and KD mice made increased advantageous choices over time ($F_{(2,36)}$=27.6, p<0.001). A trial period × genotype interaction ($F_{(2,36)}$=5.0, p<0.05) indicated that KD mice made significantly less advantageous choices than WT mice during trial period 2 (p<0.05; effect size [d]=1.22; Fig. 4A). Importantly, WT mice performed above chance in trial periods 2 and 3 (p<0.05), while KD mice did not differ from chance. No genotype differences were observed in either the intermediate or poor decision-makers.

Other behavioral measures (Table 3)

KD mice exhibited fewer omissions ($F_{(1,53)}$=24.1, p<0.001), increased premature responses ($F_{(1,53)}$=15.4, p<0.001), and faster reaction times ($F_{(1,53)}$=20.7, p<0.001) compared to WT mice. No group differences or interaction with genotype were observed.

Win-stay/lose-shift strategies of DAT KD and WT mice

Although both WT and KD tended to make more safe-stays over time ($F_{(2,106)}$=3.0, p=0.055; Fig. 4B), a trial period × genotype ($F_{(2,106)}$=4.2, p<0.05) and trial period × group ($F_{(4,106)}$=8.8,

p<0.001) interaction indicated that among good performing mice, KD mice exhibited less safe-stays than WT mice during trial period 3 (*p*<0.01). No other differences in genotype were observed for any of the measures (Fig. 4C, S4A, and S4B).

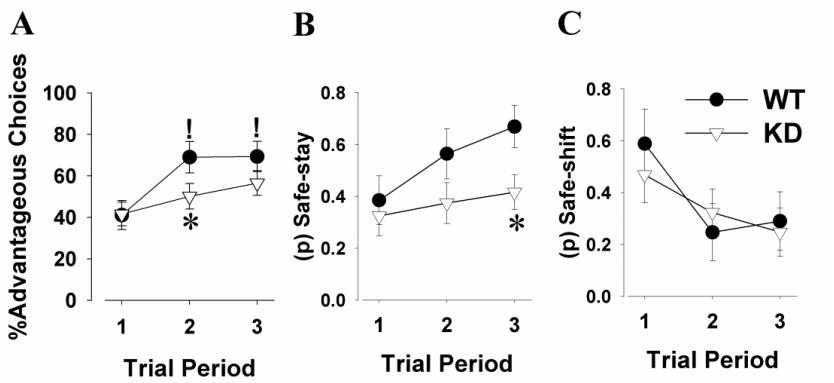

Fig. 4. Iowa gambling task (IGT) performance of dopamine transporter (DAT) knockdown (KD) and wild-type (WT) littermates identified as good decision-makers. WT mice rapidly increased advantageous choices over time, while KD mice increased more gradually and performed poorer in trial period 2 (A). Over time, both WT and KD mice stayed more at the advantageous options after being rewarded there (safe-stay), although KD mice stayed significantly less compared with WT mice by the final trial period (B). Over time, both WT and KD mice shifted less from the advantageous options after punishment (safe-shift) (C). Data are presented as the mean ± SEM, **p*<0.05 and #*p*<0.1 when compared with WT, ! *p*<0.05 when compared with chance.

IGT performance of mice treated with the acute DAT inhibitor GBR12909

Overall, a trial period × group ($F_{(4,152)}$=40.0, *p*<0.001) and trial period × GBR12909 ($F_{(4,152)}$=3.2, *p*<0.05) interaction indicated similar overall group differences as above.

Good decision-makers

Although both saline- and GBR12909-treated mice learned to select advantageous choices over time ($F_{(2,48)}$=32.1, *p*<0.001), mice receiving GBR12909 at 16 mg/kg made significantly less advantageous choices than saline-treated mice overall (*p*<0.05; effect size [*d*]=0.95; Fig. 5A). Moreover, saline-treated mice performed above chance in trial periods 1 and 3 (*p*<0.05), while mice receiving GBR12909 at 16 mg/kg did not differ from chance and mice receiving 9 mg/kg GBR12909 were only above chance in trial period 3 (*p*<0.05). No differences in treatment were observed in both the intermediate and poor decision-makers. These findings indicate deleterious effects of GBR12909 on risk-based decision-making of mice.

Other behavioral measures (Table 3)

No overall effects of GBR12909, group differences, or interaction were observed, although a main effect of GBR12909 in the good decision-makers ($F_{(2,24)}$=8.1, *p*<0.01) indicated that

mice receiving 9 mg/kg GBR12909 exhibited fewer omissions compared to saline-treated mice (p<0.05).

Win-stay/lose-shift strategies of GBR12909-treated mice

Although all good decision-makers made more safe-stays over time ($F_{(2,46)}$=8.6, p<0.01), mice receiving GBR12909 at 16 mg/kg exhibited less safe-stays than saline-treated mice overall (p<0.05; Fig. 5B). No other differences in GBR12909 treatment were observed for any other measures (Fig. 5C, S5A, and S5B).

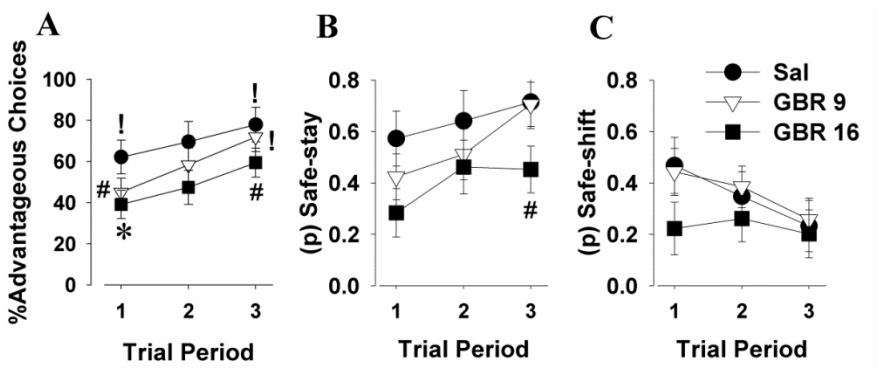

Fig. 5. Iowa gambling task (IGT) performance of C57BL/6 mice, identified as good decision-makers, treated with the acute dopamine transporter (DAT) inhibitor GBR12909. Both saline-treated (Sal) mice and GBR12909-treated (GBR 9 and GBR 16) mice increased advantageous choices over time (A). However, GBR12909-treated mice exhibited impaired decision making compared to saline-treated mice, especially at 16 mg/kg. Over time, all mice stayed more at the advantageous options after being rewarded there (safe-stay), although mice receiving GBR12909 at 16 mg/kg stayed significantly less compared with saline-treated mice (B). No effect of GBR12909 treatment was observed on the animals' decrease in shifting from the advantageous options after punishment (safe-shift) over time (C). Data are presented as the mean ± SEM, * p<0.05 and # p<0.1 when compared with saline, ! p<0.05 when compared with chance.

DISCUSSION

Impaired IGT performance was observed in BD patients compared to healthy subjects. This impairment was driven by a hypersensitivity to high rewards. In the single-session IGT task for mice, we demonstrated that dynamic decision making could be assessed when the animals were exposed to risk and reward contingencies similar to those in the human IGT. Stable subgroups were identified among mice, consistent with rats (21) and inter-individual differences in humans [(22); illustrated in Fig. 2E]. Importantly, good decision-makers increased safe-stays and decreased risky-stays over time, a pattern consistent with human IGT studies (37). As hypothesized, both chronic and acute reduced DAT functioning via genetic KD and GBR12909 respectively affected IGT performance deleteriously, resulting in impaired IGT performance. In the mice, as in humans, this

deficit was driven by a hypersensitivity to high rewards. Hence, the impaired decision-making profile of these mice was consistent with that of BD patients (8, 9).

The IGT performance deficits of BD patients are consistent with previous studies (38-40), where deficits were observed across several phases of BD [euthymia, depression, and mania; (8)]. Consistent with subtle differences observed across three phases of BD (8), the manic BD patients studied here tended to pick less from the safe decks repeatedly and instead switched more often to the risky decks. Understanding such differences in punishment- and reward-related learning is critical given that depressed subjects make punishment-sensitive decisions in the IGT (8), while substance dependent (37) and remitted BD subjects (11) are hypersensitive to rewards.

This hypersensitivity to reward of BD patients and the development of the single-session mouse IGT enabled our primary investigation, determining whether this pattern is recapitulated in mice that are hyperdopaminergic due to reduced functional DAT levels. Using the single-session mouse IGT, we observed that among regular C57BL/6J mice, good decision-makers developed a preference for advantageous options. In contrast, poor decision-makers developed a bias for the disadvantageous options, while intermediate decision-makers remained indecisive. Such inter-individual differences have been observed in rat (21) and human single-session IGT studies (see Fig. 2E), although greater proportions of humans exhibit preference for advantageous choices (22, 23). Despite subgroup differences in decision-making over time, these groups did not differ on secondary measures of omissions, premature responses, and reaction times. This dissociation of performance measures emphasizes the selectivity of risk-learning performance, suggesting that it is unrelated to motor impulsivity or motivational features. Together, these data support the use of the single-session rodent IGT to examine risk-based decision-making and highlight the inter-individual differences in risk-preference (21).

Both chronic and acute reductions of functional DAT in mice negatively affected risk-based decision-making. Within the good decision-makers, KD mice exhibited poor risk-related learning compared to WT mice, similar to impaired IGT performance of BD patients compared to HCs. Similar risk-preference of KD mice has been observed before in the multiple-sessions rodent IGT using long-term memory for learning (41), but it is important to note that the present study utilizes a single-session IGT, in accordance with the human IGT. Thus, here we could conduct *post hoc* analyses of dynamic decision-making after rewards or punishment as was conducted in humans. Poorer KD performance was likely mediated by their hypersensitivity to reward, reflected by less likelihood of staying with low-reward options. The effects of acute DAT inhibition via GBR12909 treatment on decision making were similar to performance in KD mice. GBR12909 treatment at 16

mg/kg significantly increased risk-preference compared to saline, the effect being driven by a reduced tendency to stay at the low-reward option. Supporting these effects, we previously observed that reduced DAT via GBR12909 modestly increased risk-preference in the multiple-sessions mouse IGT (16). Utilizing the same multiple-sessions task in rats however, simultaneous administration of GBR12909 and the norepinephrine transporter (NET) inhibitor atomoxetine was required in order to disrupt decision making (15). This disparity in results may reflect task differences or species differences because potencies of DAT and NET inhibitors vary between rats compared to mice and humans (42, 43). In humans, limited pharmacological IGT studies have been performed, although reduced DA activity impaired decision making in one study (44). Interestingly, treatment with modafinil (an atypical stimulant with DAT inhibition properties) also impaired decision making in low pathological gamblers in the IGT (45). Modafinil improved performance in high pathological gamblers however, which may relate to ceiling effects in these gamblers. Overall, the findings of reduced DAT functioning in mice are consistent with BD patients making less repeated picks from low-reward decks.

The importance of these findings is highlighted from studies indicating that polymorphisms in the DAT gene may induce lower levels of DAT observed in patients with BD (28, 30). Lower striatal DAT levels have also been observed in people with ADHD (46) and seasonal affective disorder (47). People with ADHD also exhibit increased risky choices in the IGT (1), albeit to a lesser extent when compared with BD (9). Although impaired decision making of the reduced DAT model animals here could therefore resemble other clinical populations, the consistencies of BD patient IGT performance including the sensitivity for rewards, to our reduced DAT functioning in mice are striking. These findings are reinforced by previous observations of parallels between behavior of these DAT models and that of BD patients in other paradigms (48-50). Moreover, supporting the increased reward-seeking trait of DAT KD mice, chronic DAT reduction also resulted in faster reaction times, fewer omissions, and increased premature responses in both the present and previous studies (41). Similarly, and as observed previously (16), GBR12909 at 9 mg/kg reduced omissions, although no significant effects were found on motor impulsivity in contrast to the increased motor impulsivity with this dose that has been observed repeatedly in mice (16, 51) and rats (15). Indeed, increased motivation and motor impulsivity of both DAT KD and to a lesser degree GBR12909-treated mice may be interpreted as consistent with the exaggerated hedonia-like symptomatology observed in BD (10). Therefore, both DAT model animals resemble patients with BD in both behavior and putative etiology.

The differences on motivational and impulsivity measures seen between the chronic and acute DAT inhibition models may not be unsurprising. Indeed, acute DAT blockade with GBR12909 previously did not affect reaction times in mice (16, 51). In contrast to selective

pharmacological DAT inhibition, constitutive DAT KD mice possibly have altered neurotransmission besides hyperdopaminergia [e.g., DAT KD mice exhibit reduced expression of choline transporters (52)], putatively contributing to these behavioral differences. In mice completely lacking DAT expression, there are numerous compensatory mechanisms including altered D_1 and D_2 receptor expression (53, 54). Developmental changes resulting from reduced DAT expression in mice may reproduce altered receptor levels in patients with BD (30). Hence, chronic reductions of DAT expression in mice may model more aspects of BD than acute DAT inhibition alone. The etiological validity of the chronic DAT KD model is likely limited however by the fact that these animals express only 10% of the transporter (34), whereas only a ≈20% reduction of DAT availability is observed in euthymic BD patients (30). As yet, DAT levels in BD patients in the manic phase have yet to be established. Moreover, as a rule, these experimental animals are kept in stable and controlled environments (i.e. circadian rhythms/light exposure), whereas evidence suggests that environmental factors such as long day-lengths contribute to BD symptoms (55). Interestingly, long day-lengths may further induce a hyperdopaminergic state (56), which theoretically could exacerbate the reduced DAT levels of BD patients. Future tests will therefore include mice with ≈40-50% expression of the DAT and concurrent environmental manipulations such as aberrant light exposure to assess the relevance of these manipulations in modeling BD or other potentially DAT-mediated disorders such as ADHD. Cross-species translational studies can further help elucidate the differences in decision making and other measures between individuals with ADHD and BD. Examining a selective NET blocker may prove useful, given its role in the treatment of ADHD and previous implication in ameliorating prepulse inhibition deficits in DAT knockout mice (57, 58). Future studies in humans and other model animals for BD (59, 60) will help delineate the mechanism(s) underlying impaired decision making and contribute to developing therapeutics aimed to treat these deficits.

In summary, BD patients exhibit impaired decision making in the IGT. Using post-reward/punishment decision-making measurements developed from animal studies, we identified evidence to support a high reward-sensitivity in these patients. The development of a dynamic single-session mouse IGT, wherein mice increase advantageous choices over time and post-reward/punishment measurements can be examined aids our translational work. Chronic and acute reductions of DAT functioning in mice deleteriously impacted risk-based decision-making, making mice hypersensitive to high rewards and mimicking deficits of BD patients. DAT reductions may therefore contribute to some of the symptoms of BD. Finally, the single-session IGT may be used to assess other model organisms and test putative treatments for impaired decision making.

SUPPLEMENTARY MATERIAL

Apparatus

Mice were trained and tested in 16 five-hole operant chambers (25×25×25 cm, Med Associates Inc., St. Albans, VT, USA), each of which consisted of a horizontal array of five square holes (2.5×2.5×2.5 cm) on a curved wall 2.5 cm above the grid floor, a food-delivery magazine (Lafayette Instruments, Lafayette, IN, USA) on the opposite panel at floor level, and a house light near the ceiling. Animals were trained to holepoke to an illuminated LED recessed into the holes. The food-delivery magazine contained a well in which liquid reinforcement (strawberry milkshake; Nesquik® plus non-fat milk, 25 µl) was delivered by a peristaltic pump. Infrared beams were used to detect holepoke responses and magazine entries. Chambers were enclosed in sound-attenuating boxes, ventilated by fans that also provided a low level of background noise. Performance was monitored during training and testing using an infrared camera installed in each chamber. The control of stimuli and recording of responses were managed by a SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates Inc., St. Albans, VT, USA) using custom programming.

Training

Prior to training, mice were acclimated to the food reward by an overnight exposure to strawberry milkshake. During the first training phase on days 1-8, mice were placed in the five-hole chambers for 10 min with milkshake dispensed every 15 s into the well of the lit magazine. Magazine entry resulted in the light being extinguished until the next reinforcement was delivered. Acquisition criterion was ≥ 30 entries in the reward magazine per session for two consecutive days. After initial training, mice were required to holepoke in one of the four lit holes opposite the magazine (central hole was never lit) (maintenance training) in order to obtain reinforcement. To minimize biased responses in specific holes, five consecutive holepokes in one hole resulted in that hole being extinguished and inactive until two other holes were poked. This session was repeated daily until >70 responses were recorded within 30 min for two consecutive days.

Mouse single-session IGT – behavioral measurements

For the IGT session, we utilized a protocol based on one used for rats. The session began with the magazine being illuminated and each trial was initiated by holepoking then exiting the magazine. An inter-trial interval (ITI) of 5 s preceded illumination of the cue array. A holepoke during this ITI was recorded as a 'premature' response, which resulted in a 5 s time-out period signaled by the illuminated houselight, during which all holes were unresponsive. After withholding from responding during the ITI, holes 1, 2, 4, and 5 were illuminated. A holepoke in one of the four lit holes during the stimulus duration (SD) of 10 s was recorded as a 'correct' response. All cue lights were then extinguished and the

mouse rewarded or punished depending on the reward schedule. Rewards were delivered in an illuminated magazine and recorded as the total amount of rewards earned. For punishments, no reward was given and a time-out was triggered whereby the light stimulus of the chosen hole flashed at a frequency of 0.5 Hz, during which all apertures were unresponsive. These data were recorded as the total punishment duration in seconds. After the time-out period, the magazine light illuminated to start a new trial. Failure to respond in any of the four holes during the SD was recorded as an 'omission' and resulted in illumination of the magazine so that a new trial could be started. Finally, the time taken to make a choice (mean choice latency; MCL) was also recorded. The 60 min session thus allowed the mouse to choose between four holes (A-D), each associated with a different reward/punishment schedule. Choices A and B (disadvantageous) delivered more reward than choices C and D (advantageous), but could be followed by longer time-out penalties compared with choices C and D. The probability of penalty was low for choices B and C and high for A and D. Two different versions were used to control for possible location bias. Response options were measured as a percentage of the total trials completed. Data were grouped by advantageous (C and D) and disadvantageous options (A and B) and measured as $\%advantageous\ choices = \frac{\mathrm{C+D}}{(\mathrm{A+B+C+D})} * 100$.

Win-stay and lose-shift calculations for mice and humans

For win-stay/lose-shift analyses, advantageous and disadvantageous options were combined. For each trial, *t*,

$$win(t) = \begin{cases} 0\ for\ an\ omitted\ or\ punished\ trial \\ 1\ for\ a\ rewarded\ trial \end{cases}$$
$$loss(t) = \begin{cases} 0\ for\ an\ omitted\ or\ rewarded\ trial \\ 1\ for\ a\ punished\ trial \end{cases}$$

Following a rewarded trial, *t*, a win-stay event for trial *t+1* was recorded if a subject made a response at the same side (advantageous or disadvantageous) that was rewarded for trial *t*,

$$stay(t+1) = \begin{cases} 0\ for\ an\ omission\ or\ switching\ to\ the\ side\ opposite \\ to\ that\ rewarded \\ 1\ for\ staying\ on\ the\ same\ rewarded\ side \end{cases}$$

Following a punished trial, *t*, a lose-shift event for trial *t+1* was recorded if a subject made a response at the opposite side from that which was punished on trial *t*.

$$shift(t+1) = \begin{cases} 0\ for\ an\ omission\ or\ staying\ on\ the\ punished\ side \\ 1\ for\ switching\ to\ the\ side\ opposite\ to\ that\ punished \end{cases}$$

The probabilities of win-stay and lose-shift events following advantageous and disadvantageous options were calculated as follows:

$$P(stay_{adv}|win_{adv}) = \frac{\sum stay_{adv}}{\sum win_{adv}}$$
$$P(stay_{dis}|win_{dis}) = \frac{\sum stay_{dis}}{\sum win_{dis}}$$

$$P(shift_{adv}|loss_{adv}) = \frac{\sum shift_{adv}}{\sum loss_{adv}}$$
$$P(shift_{dis}|loss_{dis}) = \frac{\sum shift_{dis}}{\sum loss_{dis}}$$

Win-stay and lose-shift probabilities were calculated separately for each trial period. When looked over three trial periods, wins on the last trial of the first two thirds and win-stays on the first trial of the last two thirds were not recorded, as these wins and win-stays would not be recorded in the same third as the following win-stay or preceding win, respectively.

Supplementary figures

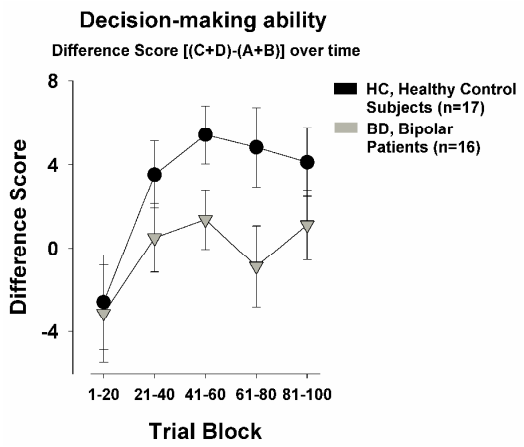

Fig. S1. Iowa gambling task (IGT) performance of bipolar disorder (BD) and healthy control (HC) subjects over time. When analyzed over five trial blocks, both HC and BD subjects increase their net score over time, although BD subjects make significantly more risky choices compared to HC subjects. Data are presented as the mean ± S.E.M.

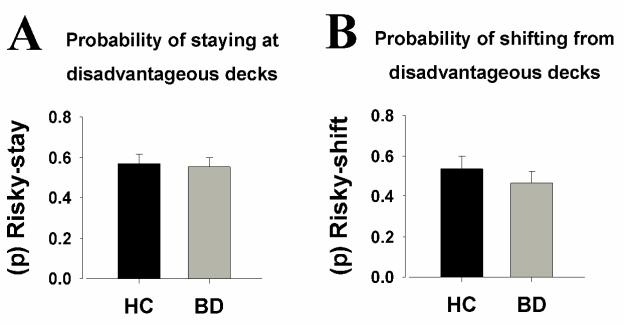

Fig. S2. There were no differences between healthy control (HC) subjects and bipolar disorder (BD) patients on choosing from the risky decks directly after being rewarded from the risky decks (A). No effects were observed for shifts to the safe decks directly after losing at the risky decks (B). Data are presented as the mean ± S.E.M.

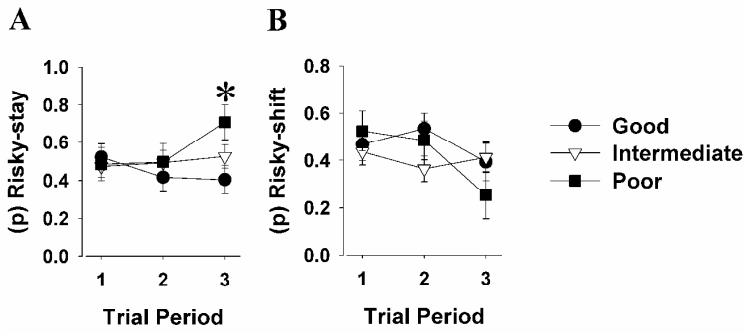

Fig. S3. Poor decision-makers stayed more at the risky options after being rewarded there (risky-stay), and more compared to good decision-makers at the end of the session (A). Poor decision-makers shifted less to the safe options directly after punishment was obtained at the risky options (B). Data are presented as the mean ± SEM, **p*<0.05 when compared with good decision-makers.

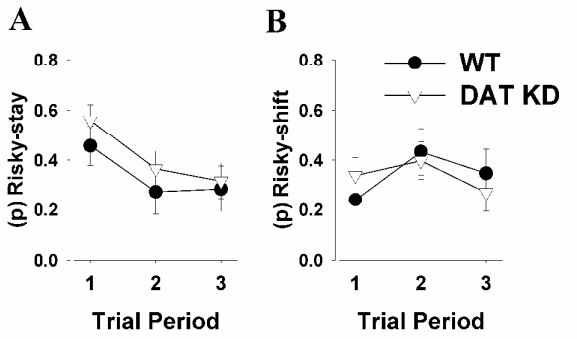

Fig. S4. Both WT and KD mice stayed less at the risky options after being rewarded there over time (risky-stay) (A). No difference between genotypes was observed for shifts to the safe options after punishment from the risky options (B). Data are presented as the mean ± S.E.M.

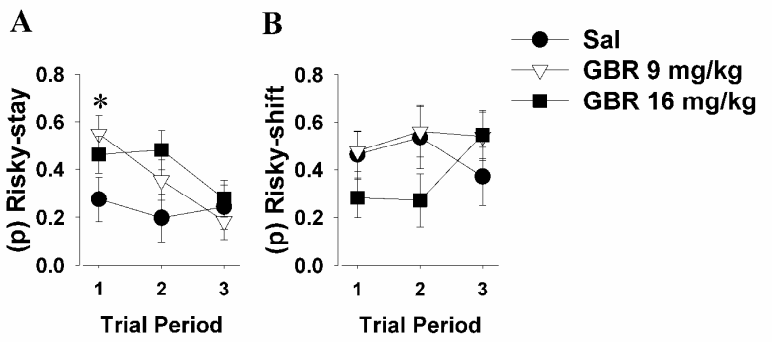

Fig. S5. Compared to saline-treated mice, mice treated with 9 mg/kg started off staying significantly more at the risky options directly after being rewarded from there (risky-stay), but this normalized over time (A). No effect of GBR12909 was observed for shifts to the safe options directly after punishment from the risky options (B). Data are presented as the mean ± SEM, **p*<0.05 when compared with saline.

ACKNOWLEDGEMENTS

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CHAPTER 4

The effects of sleep deprivation on attentional performance in both mice and humans

CHAPTER 4.1

Sleep deprivation impairs performance in the 5-choice continuous performance test: Similarities between humans and mice

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ABSTRACT

Background

Several groups undergo extended periods without sleep due to working conditions or mental illness. Such sleep deprivation (SD) can deleteriously affect attentional processes and disrupt work and family functioning. Understanding the biological underpinnings of SD effects may assist in developing sleep therapies and cognitive enhancers. Utilizing cross-species tests of attentional processing in humans and rodents would aid in mechanistic studies examining SD-induced inattention.

Methods

We assessed the effects of 36 h of: (1) Total SD (TSD) in healthy male and female humans (*n*=50); and (2) REM SD (RSD) in male C57BL/6 mice (*n*=26) on performance in the cross-species 5-choice continuous performance test (5C-CPT). The 5C-CPT includes target trials on which subjects were required to respond and non-target trials on which subjects were required to inhibit from responding. TSD-induced effects on human psychomotor vigilance test (PVT) were also examined. Effects of SD were also examined on mice split into good and poor performance groups based on pre-deprivation scores.

Results

In the human 5C-CPT, TSD decreased hit rate and vigilance with trend-level effects on accuracy. In the PVT, TSD slowed response times and increased lapses. In the mouse 5C-CPT, RSD reduced accuracy and hit rate with trend-level effects on vigilance, primarily in good performers.

Conclusions

In conclusion, SD induced impaired 5C-CPT performance in both humans and mice and validates the 5C-CPT as a cross-species translational task. The 5C-CPT can be used to examine mechanisms underlying SD-induced deficits in vigilance and assist in testing putative cognitive enhancers.

Keywords

Attention, Vigilance, CPT, Psychomotor vigilance test, Bipolar disorder

INTRODUCTION

All species, including humans, require some state of sleep (1). Despite the ubiquity of this phenomenon, much of the underlying mechanisms, long-term effects, and the actual function that sleep provides are still poorly understood. Nevertheless, it is well known that deprivation from sleep negatively affects general health and cognition in humans (2-4). The extent to which sustained wakefulness impairs cognitive performance in particular seems to depend on the task at hand. For example, sleep deprivation (SD) has a more profound effect in tasks requiring the maintenance of attention than in tasks assessing working memory and executive functions (5).

The increasingly fast-paced nature of society requires people to work longer hours resulting in sleeping fewer hours per day with irregular patterns of sleep (6). For example, several professions including piloting or the military require vigilance (attending to relevant stimuli over time), yet involve extended periods without sleep, which impairs vigilance (7, 8). Moreover, certain psychiatric populations exhibit abnormal sleeping patterns, which may further impact their already deficient cognitive performance and possibly impair efficacy of some treatments (e.g. cognitive behavioral therapy). Patients with bipolar disorder for instance are well known for experiencing disrupted sleep patterns, SD, and concomitantly suffer from cognitive symptoms (9). Furthermore, SD can precipitate manic and hypomanic episodes (10), yet benefit patients in depressive episodes (11, 12). Investigating the mechanisms of SD-induced effects on behaviors including vigilance would aid in developing cognition-enhancing pharmaceuticals or behavioral countermeasures to cognitive deficits for certain professions and psychiatric disorders. While humans can be experimentally sleep deprived, animal models are more suitable for investigating underlying mechanisms of SD-induced deficits in vigilance. Additionally, SD may serve as an environmental challenge in animal models of psychiatric disorders (13, 14). The limited cross-species tests of attention/vigilance in humans and animals hampers such investigations however.

Attentional performance during SD in humans has commonly been assessed using the psychomotor vigilance test (PVT) (15). This reaction time (RT) task requires responding to a visual cue (target stimulus) presented at pseudo-random intervals. Generally, RTs are slowed and more variable, while omissions are increased in humans subjected to SD (7). SD-induced impaired performance has been observed in rats in a PVT analog (16) and the 5-choice serial reaction time task (5CSRTT), the latter of which requires responding in varied locations (17). These tasks require only responses to target stimuli however, despite the important and distinct role that inhibiting from responding to irrelevant (non-target) stimuli has in attentional processes (18). Specifically, with only target stimuli, separating attentional lapses from response fatigue is difficult. By including non-target

stimuli, one can determine whether response rates are globally or specifically diminished due to inattention to relevant stimuli. Likewise, treatments that increase global responsiveness may not be useful when one's environment is littered with irrelevant (non-target) stimuli. Hence, cross-species studies are required on the effects of SD on attentional performance that is specific to responding to relevant (target) stimuli.

The combination of both target and non-target stimuli is the hallmark of tests labeled as continuous performance tests [CPT; (18)]. With the inclusion of non-target stimuli, CPTs measure vigilance and are the gold-standard tests of attention in psychiatric populations (19). In the limited studies conducted on the effects of SD on CPT performance, several days of sleep restriction increased misses to target stimuli and reduced responses to non-target stimuli, thereby overall impairing vigilance and reducing responsiveness (20, 21). Other studies using total SD (TSD) report modest but non-significantly increased misses to targets but no change in non-target responses after TSD in healthy subjects; however stronger attentional disruption is reported in methadone-maintained subjects (22, 23). TSD primarily increased non-target responses compared to target responses in a go/no-go task however, despite this task not being a true CPT (24). Determining the effects of SD on a cross-species vigilance task is required however, for examining putative underlying mechanisms.

The 5-choice (5C-CPT), based on the 5CSRTT, was developed to assess vigilance in mice (25-27) and rats (28, 29), and is now available in humans (30), including in an fMRI setting (31). Consistent with other CPTs, the 5C-CPT presents target stimuli to which the subject is required to respond as well as non-target stimuli, to which the subject is required to inhibit from responding. To date, no studies have assessed whether SD affects mouse or human performance in this cross-species CPT. Thus, the present studies investigated whether SD would affect 5C-CPT performance similarly in both mice and humans. We hypothesized that: (*a*) 36 h of TSD in humans; and (*b*) 36 h of rapid eye movement (REM) SD (RSD) in mice would similarly impair 5C-CPT performance. Since inter-individual differences were expected on mice 5C-CPT performance (27), and treatments can affect rodent performance differentially dependent upon baseline performance (32, 33), we split the animals in good and poor performers. Finally, to ensure the validity of our TSD protocol, we also assessed TSD-induced effects in the human PVT.

METHODS

Humans

Fifty human subjects (23 female) aged between 18 and 39 years were recruited through flyers, newspaper, and radio from the general San Diego community to participate in this study. Subjects were initially screened via telephone for eligibility. Informed consent was signed at an in-person screen, which included a complete medical history and a Structured Clinical Interview for DSM-IV. Inclusion criteria were at least 12 years of education, a consistent sleep-wake schedule (7-9 h sleep each night), and for women to be tested in the early follicular phase of their menstrual cycle. Exclusion criteria were history of any sleep disorder, Axis I psychopathology or immediate family history of mood or psychotic disorders; head injury followed by unconsciousness, migraine headaches requiring treatment, seizures, neurological symptoms of the hand, wrist, or arm; current use of nicotine or in the past 2 years; current use of psychotropic medications, hormone-based birth control; high caffeine (>400 mg/day) or alcohol (>2 ounces/day) use; positive urine toxicology screen for illegal substances; hearing threshold above 45 dB(A) at 500-6000 Hz; non-responsiveness to startling stimuli or any other medical condition which might pose a health risk for the subject. Subjects were instructed to maintain a regular sleep-wake schedule at home for at least one week prior to the study, which was monitored with sleep diaries and actigraphy. Sleep monitoring on the first night of the study screened for unreported sleep disorders. This study was conducted at the VA San Diego with the approval of the IRBs of UCSD and VA and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Total sleep deprivation

Subjects spent four nights and days in the laboratory: (*a*) adaptation to the lab (night/day 0); (*b*) normal sleep followed by a battery of testing including the PVT and then the 5C-CPT (night/day 1); (*c*) sleep or TSD followed by a similar battery of testing (night/day 2); and (*d*) sleep or TSD followed by a similar battery of testing as night/day 2 (night/day 3). Subjects were randomly assigned to one of three groups. Group 1 received normal sleep throughout the study; group 2 was sleep deprived for 36 h prior to day 2; and group 3 was sleep deprived for 36 h prior to day 3. Subjects assigned to group 1 were included in the 'normal sleep' group (n=18). Post-deprivation night data for subjects in groups 2 and 3 were collapsed into the TSD group (n=32). The data from group 1 used for analysis was taken from day 2 or 3 in order to match with subjects from groups 2 and 3 therefore minimizing practice effects as a putative confound. Sleep schedules were made as similar to those maintained at home as possible with sleep being monitored with a standard overnight polysomnogram, including EEG, EOG, and EMG. At each point, subjects were free to engage in activities such as reading, watching television, or socializing. No exercise more strenuous than walking was allowed, nor any form of stimulant. Light snacks and

meals were provided. Lights were kept at a constant low level, with no sunlight introduced. Wakefulness was documented through (1) a staff-completed monitoring log every 15 min with subjects' activities and mental status and (2) actigraphy.

Psychomotor vigilance test

During the PVT, subjects were presented with a blank box in the middle of a screen. At pseudo-random intervals ranging from 2 to 10 s, a bright red light millisecond (ms) counter started to scroll, and subjects had to press the space bar to stop the counter as quickly as possible. After pressing the button, the counter displayed the achieved RT for 1 s, providing the subject with feedback on performance. The PVT task lasted 10 min and was programmed in E-prime (Psychology Software Tools (Sharpsburg, PA, USA)). Median RT, fastest and slowest 10% of RTs, and number of lapses (RTs > 500 ms) were measured.

Human 5C-CPT apparatus

The task appeared on a 56 cm CRT computer screen (60 cm from subject). Subjects used an arcade joystick to make responses. The joystick was spring-mounted so that it would return to the center after each response. A Dell PC with E-Prime2 software (Psychology Software Tools) was used for stimulus presentation and data acquisition.

Human 5C-CPT

A schematic of the paradigm is presented in Fig. 1 and described elsewhere (30). In brief, participants were briefly introduced to the task and were told that they would see 5 white lines (3 cm) in an arc on a black background. Subjects were instructed that if a white circle (≈2 cm) appeared behind a line (target stimuli), the joystick should be moved in that direction, but if circles appeared behind every line (non-target stimuli) they should inhibit from responding. Stimuli appeared for 100 ms with a response window of 1 s after the stimuli disappeared. A variable inter-trial interval (ITI; 0.5, 1, or 1.5 s) occurring 1 s after the stimulus of the previous trial was presented in a pseudo-random order between trials. Before the actual task, subjects were given a practice session, which consisted of 12 trials (10 target and 2 non-target stimuli randomly presented). The full task consisted of 270 trials (225 target and 45 non-target stimuli pseudo-randomly presented). Several measures were determined from this task (Table 1) and calculations based on hit rates (HR), false alarms (FA), FA rates (FAR), and correct rejections (CR) were made accordingly:

$$\text{Accuracy} = \frac{\text{Hit}}{\text{Hit} + \text{Incorrect}}$$

$$\%\ \text{Omissions} = \left(\frac{\text{Miss}}{\text{Total trials}}\right) \times 100$$

$$\text{Reaction Time} = \frac{\text{Cumulative correct latency}}{\text{Corrects}}$$

$$\text{HR} = \frac{\text{Hit}}{\text{Hit} + \text{Miss}} \qquad \text{FAR} = \frac{\text{FA}}{\text{FA} + \text{CR}}$$

Signal detection indices were calculated based upon these basic parameters to assess both sensitivity and responsivity indices:

$$d' = \text{z}(\text{HR}) - \text{z}(\text{FAR}) \qquad \text{RI} = \frac{\text{HR} + \text{FAR} - 1}{1 - [\text{FAR} - \text{HR}]^2}$$

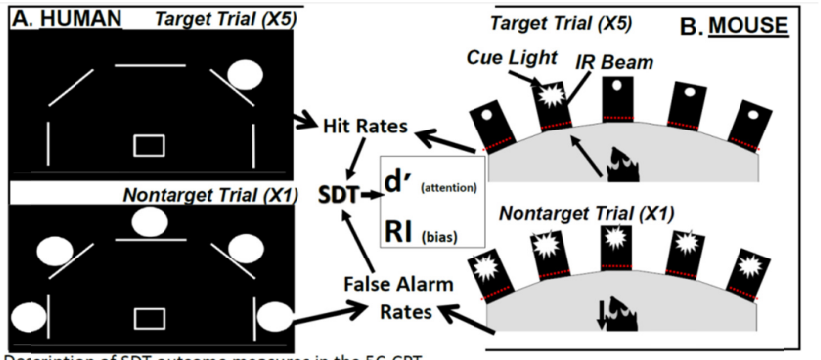
d' provides a parametric assessment of sensitivity to appropriate responding. The non-parametric response bias measure RI provides a measure of the 'tendency to respond'. Low numbers indicate a conservative response strategy, while high numbers indicate liberal responding (34, 35).

Animals

Male C57BL/6 mice (*n*=26) were 12-14 months old at the time of testing and weighed between 23-30 g. All animals were group housed (maximum 4/cage) and maintained in a temperature-controlled vivarium (21±1 °C) on a reversed day-night cycle (lights on at 7.00 PM, off at 7.00 AM) and tested during the dark phase of the day-night cycle between 8.00 AM and 11.00 AM. All mice had *ad libitum* access to water and were food-restricted at 85% of their free-feeding weight during periods of testing. All procedures were approved by the UCSD Institutional Animal Care and Use Committee. The UCSD animal facility meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care.

REM sleep deprivation

Mice receiving normal sleep (*n*=13) and mice on RSD (*n*=13) were baseline matched on training performance as measured by their average *d'* 3 days prior to testing. The conventional 'inverted flower pot' technique was used, originally designed by Jouvet et al. in 1964 (36) and still used in RSD studies in animals (13). In brief, group-housed mice were sleep deprived by placing the same number of small inverted cups (4 cm diameter) as there were mice in the cage in a pool of water (37 C°; 2 cm height) for 36 h prior to testing. Control animals had bigger inverted cups (7 cm diameter), which because of its size allowed for sleep, in a pool of water for the same period.



Description of SDT outcome measures in the 5C-CPT

d'	Responsivity Index (RI)	Hit Rate	False Alarm Rate	Explanation of performance
+5	0	1.0	0.0	Perfect attention: Responding to every target and inhibiting from responding to all non-target stimuli
0	+1	1.0	1.0	Chance: Responding to every stimulus irrespective of whether it is a target or non-target
	-1	0.0	0.0	Chance: Not responding to any stimulus irrespective of whether it is a target or non-target
-5	0	0.0	1.0	Rule reversed: Inhibition of responding to all targets while responding to all non-target stimuli

Fig. 1. Schematic of the human and mouse 5C-CPT. In both the human and mouse 5C-CPTs, there are 5 stimuli locations. For humans, stimuli are presented in 1 of 5 locations arrayed in an arc on a computer screen, and subjects respond using a 5-way joystick (A). For mice, stimuli are presented in 1 of 5 holes located in an arc at the rear of a 5-hole operant chamber and responses are recorded by infrared beams in each hole (B). The task design is the same in both cases, whereby: (1) a single stimulus represents a target trial to which subjects must respond; and (2) all 5 stimuli being presented simultaneously represents a non-target trial to which the subject must inhibit from responding. Target trials generate measures of hits and misses (target responses and omissions), which are used to calculate a subjects' hit rate, while non-target trials generate measures of correct rejections and false alarms, which are used to calculate a subjects' false alarm rate. Using signal detection theory (SDT), the non-parametric measure of vigilance (*d'*) and bias (responsivity index (RI)) are generated. The table provides examples of what permutations of hit and false alarm rates result in various *d'* and RI levels and its interpretation.

Mouse 5C-CPT

A schematic of the paradigm is presented in Fig. 1 and described elsewhere (25, 27). Consistent with the human task, mice were required to make a hole poke if 1 of the 5 holes lit up (target trials) in order to obtain a food reward, but inhibit from responding when all 5 holes lit up (non-target trials) in order to obtain a reward (see Supplemental Material and Methods). In brief, mice were progressively trained to conduct this task using simple choice progressing to use the entire 5-hole array and until performance was stable on *d'*, % omissions, and RTs when tested for baseline performance over 3 days before SD (≈70 5C-CPT training sessions). Measures were calculated as described for the human 5C-CPT (see Table 1 for measures).

Statistics

Human 5C-CPT performance was analyzed using the general linear model (GLM) with TSD and gender as between-subject factors and trial period as a within-subjects factor. Mouse 5C-CPT performance was analyzed using a repeated measure ANOVA with stimulus duration as a within-subject factor and RSD as a between-subject factor. Where

appropriate, planned comparison Tukey *post hoc* analyses were conducted between groups and Cohen's *d* effect sizes were calculated. Two animals from the RSD group were removed from statistical analyses because of a lack of responding (>95% omissions). In order to explore the effects of SD on individual differences in performance, a median split was conducted on vigilance performance (*d'*) measured during 3 days of baseline testing to group subjects into good and poor performers. Performance group was entered into the model as a between subject factor. The level of probability for statistical significance was set at 0.05. All statistics were performed using SPSS (19.0, Chicago, IL, USA).

Table 1. Description of the behavioral measures used in the human and rodent 5C-CPTs.

Measure	Description
Hit	Response to target stimulus in correct location
Miss	Non-response to target stimulus
Incorrect	Response to target stimulus but in wrong location
Correct rejection (CR)	Correct non-response to non-target stimulus
False alarm (FA)	Incorrect response to non-target stimulus
Premature response	Response to no stimuli during the inter-trial interval
Mean reaction time (RT)	Mean latencies to correct responses
Variable RT	Standard deviation of the RT
Hit rate (HR)	Proportion of correct responses to target stimuli
False alarm rate (FAR)	Proportion of incorrect responses to non-target stimuli
Vigilance (*d'*)	Parametric measure examining the difference between hit and false alarm rates to determine performance
Responsivity index (RI)	Non-parametric measure examining the combination of hit and false alarm rates to determine responsivity to stimuli
Accuracy	Proportion of correct compared to incorrect responses
% Omissions	Percentage of misses/lapses

RESULTS

Humans

Effects of TSD on PVT performance

The effects of TSD on PVT performance in humans are detailed in Table 2. In brief, TSD slowed overall RTs, including the fastest and slowest 10% of responses during the task. TSD also increased the number of attentional lapses (RTs > 500 ms).

Table 2. Means, standard errors of the mean, and statistical comparison of human PVT performance after normal sleep *vs.* TSD.

Variable	Normal sleep mean (SEM)	TSD mean (SEM)	d.f.	*F*	*p*-value
Lapses	0.4 (1.2)	5.2 (0.9)	1,48	10.5	<0.005
Median RT	278.2 (8.2)	312.2 (6.2)	1,48	11.0	<0.005
Fastest 10% RT	230.2 (5.6)	245.6 (4.2)	1,48	4.8	<0.05
Slowest 10% RT	389.9 (154.8)	816.0 (116.1)	1,48	4.8	<0.05

RT, reaction time (in milliseconds); TSD, total sleep deprivation.

Effects of TSD on human 5C-CPT performance

Because there were no interactions of TSD with trial period, gender, or baseline performance ($F<1.8$, ns), data were pooled and analyzed. As hypothesized, TSD impaired vigilance as measured by reduced *d'* ($F_{(1,42)}=5.7$, $p<0.05$; Cohen's $d = 0.6$; Fig. 2a). TSD also reduced hit ($F_{(1,42)}=4.8$, $p<0.05$; Cohen's $d = 0.5$; Fig. 2b) and false alarm rates ($F_{(1,42)}=4.2$, $p<0.05$; Cohen's $d = 0.15$; Fig. 2c). TSD tended to reduce responsivity as measured by reduced RI ($F_{(1,42)}=3.5$, $p<0.1$; Cohen's $d = 0.2$; Fig. 2d), and tended to decrease accuracy ($F_{(1,42)}=3.4$, $p<0.1$; Cohen's $d = 0.1$; Fig. 2e). There was no effect of TSD on omissions ($F_{(1,42)}=2.5$, ns; Fig. 2f). Interestingly, TSD did not affect RT ($F_{(1,42)}=2.2$, ns; Fig. 2g), but tended to increase variability of RT ($F_{(1,42)}=4.0$, $p<0.1$; Fig. 2h).

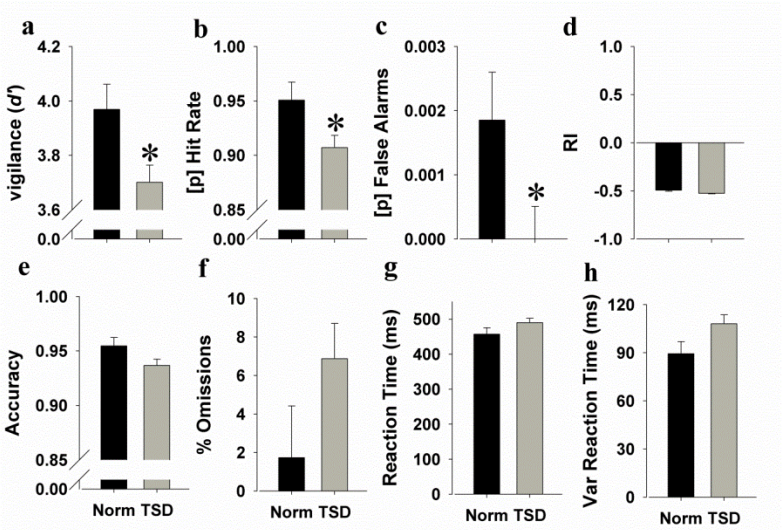


Fig. 2. Effects of TSD on 5C-CPT performance in humans. TSD impaired vigilance as measured by reduced *d'* (a) with a large effect size (Cohen's $d = 0.6$). This TSD-impaired vigilance was partially driven by reduced overall hit rate (b; Cohen's *d* effect size = 0.5) and lower non-target responses (c; Cohen's *d* effect size = 0.15). Humans during TSD were slightly less responsive (d) and also made slightly less target responses (e) compared to humans after normal sleep. No significant difference between normal sleep and TSD was observed on the number of omitted trials (f). Mean RTs did not differ (g), but humans during TSD exhibited slight increased variable RTs compared to humans after normal sleep (h). Data are presented as the mean ± S.E.M., **p*<0.05 when compared with humans after normal sleep.

Mice

Effects of RSD on mouse 5C-CPT performance

Interestingly, while longer stimulus durations improved hit rate in control mice (stimulus duration; $F_{(2,22)}$=12.3, p<0.0001), this effect was not present in the RSD mice (stimulus duration; F<1, ns). *Post hoc* analyses revealed that RSD mice exhibited a reduced hit rate at the 2 s stimulus duration compared with control mice (p<0.05; Fig. 3b). Similar benefits to lengthening the stimulus duration were observed in fewer omissions in control mice (stimulus duration; $F_{(2,22)}$=12.0, p<0.0001) and again this effect was not present in the RSD mice (stimulus duration; F<1, ns). *Post hoc* analyses revealed that RSD mice exhibited increased omissions at the 2 s stimulus duration compared with control mice (p<0.05; Fig. 3f). RSD tended to reduce accuracy ($F_{(1,21)}$=3.5, p<0.1; Fig. 3e), without interacting with stimulus duration. Overall, RSD did not affect *d'*, false alarms, RI, RTs, or vRTs (Fig. 3). RSD also did not affect premature responses, but reduced the amount of trials completed in mice ($F_{(1,21)}$=4.6, p<0.05), without interacting with stimulus duration (see Supplemental Table 1).

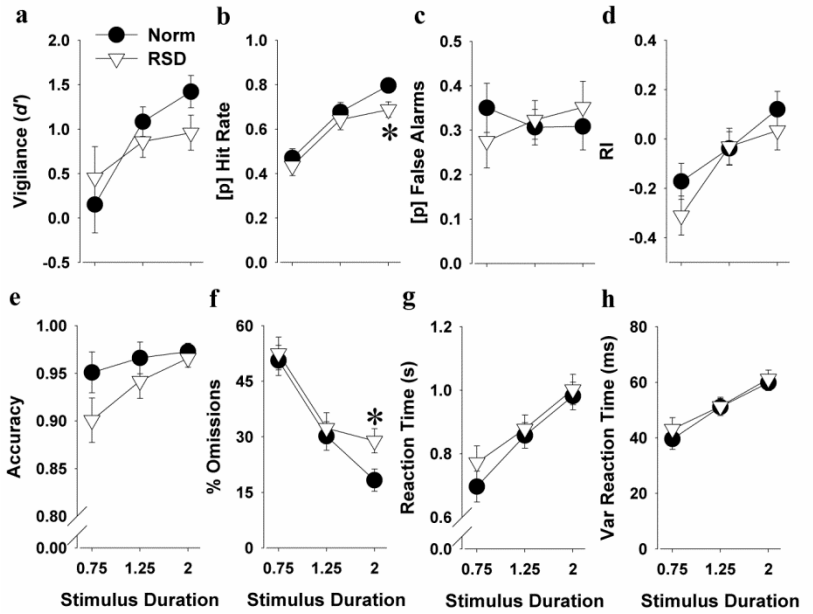


Fig. 3. Effects of RSD on 5C-CPT performance in all C57BL/6 mice. RSD had only subtle effects when looked at the overall group performance of mice in the 5C-CPT. Overall, mice seemed to perform better with longer stimulus duration (a-h). However, this effect was less pronounced in mice during RSD, where RSD decreased hit rate (b) and increased the amount of omissions (f) at the longest stimulus duration of 2 s. Data are presented as the mean ± S.E.M., *p<0.05 when compared with mice after normal sleep.

Good and poor performing mice

Consistent with previous reports (27), inter-individual differences in performance were observed in mice, with several subjects performing at a low baseline level. Treatments can differentially affect rodent performances in operant tasks dependent upon baseline level of performance (32, 33). Therefore, we investigated the effects of RSD in good *vs.* poor performing mice. Good and poor performers (n=12/12) were identified as described above (see Statistics).

The effects of RSD on good performing mice in the 5C-CPT

In good performing mice, RSD deleteriously affected accuracy ($F_{(1,9)}$=5.7, p<0.05), with its effect tending to interact with stimulus duration ($F_{(2,18)}$=4.6, p=0.051). RSD specifically reduced accuracy at the 0.75 s stimulus duration (p<0.05; Cohen's d = 1.34; Fig. 4e). For percentage omissions there was a trend effect of RSD ($F_{(1,9)}$=3.5, p<0.1). Again, longer stimulus durations resulted in fewer omissions in control mice (stimulus duration; $F_{(2,10)}$=12.3, p<0.005), but this effect was not present in the RSD mice (stimulus duration; F<1, ns), who exhibited more omissions at the 2 s stimulus duration compared with control mice (p<0.05; Cohen's d = 1.79; Fig. 4f). No main effect of RSD or interaction with stimulus duration was observed for *d'*. Longer stimulus durations tended to result in increased *d'* in control mice however (stimulus duration; $F_{(2,10)}$=3.3, p<0.1), with this effect not being present in RSD mice (stimulus duration; F<1, ns), who tended to exhibit reduced *d'* at the 1.25 s stimulus duration compared with control mice (p<0.1; Cohen's d = 1.18; Fig. 4a). There was a trend effect of RSD impairing hit rate ($F_{(1,9)}$=4.7, p<0.1). Although longer stimulus durations improved hit rate in control mice (stimulus duration; $F_{(2,10)}$=17.3, p<0.005), this effect was not present in RSD mice (stimulus duration; $F_{(2,6)}$=2.0, ns), whom exhibited a reduced hit rate at both the 0.75 (p<0.05; Cohen's d = 1.28; Fig. 4b) and the 2 s stimulus durations (p<0.05; Cohen's d = 1.89) compared with control mice. For the RI, a trend stimulus duration by RSD interaction was observed ($F_{(2,18)}$=3.6, p<0.1; Fig. 4d), but no *post hoc* effect of RSD was observed. RSD did not affect RTs, vRTs, false alarms (Fig. 4), trials completed, or percentage premature responses (See Supplemental Table 2).

The effects of RSD on mice performing at low baseline levels in the 5C-CPT

The effects of RSD on poor performing mice in the 5C-CPT are detailed in Supplemental Table 3. In brief, RSD did not affect trials completed, percentage premature responses, RTs, vRTs, accuracy, false alarms, *d'*, or RI of these mice, overall, nor at any specific stimulus duration.

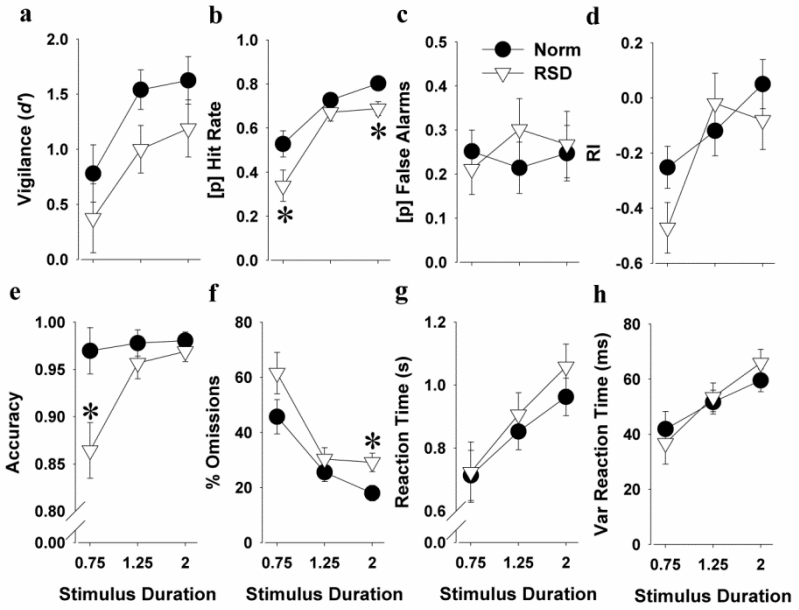


Fig. 4. Effects of RSD on 5C-CPT performance in good performing mice. In the good performing subgroup of mice, RSD more severely impaired 5C-CPT performance. RSD negatively impacted vigilance as measured by slight reduced *d'* at the 1.25 s stimulus duration (a). RSD decreased hit rate, specifically at the 0.75 s and 2 s stimulus durations (b), while leaving non-target responses unaffected (c). No effect of RSD was observed on responsiveness (d), but after RSD, mice made fewer target responses compared to mice after normal sleep, specifically at the 0.75 s stimulus duration (e). Although longer stimulus durations reduced the number of omitted trials in control mice, this effect was less pronounced in the mice after RSD, where RSD increased omissions at the highest stimulus duration (f). No effect of RSD was observed on both mean and variable RTs (g,h). Data are presented as the mean ± S.E.M., **p*<0.05 when compared with mice after normal sleep.

DISCUSSION

We report that 36 h of TSD and RSD impaired 5C-CPT performance in humans and mice respectively. This SD-impaired 5C-CPT performance was driven by more misses of target stimuli, consistent with previous CPT studies in humans (20, 21). TSD-induced attentional lapses in the PVT confirm the efficacy of the TSD procedure in humans. Importantly, the present data reveal that despite SD-induced reduced responsiveness of humans and mice overall, inattention specific to relevant stimuli was still observed, particularly in good performing subjects.

Over the last decade, the PVT has been used as the 'gold standard' to assess the effects of SD on alertness (4). Broadly, PVT studies reliably find that SD slows RTs and increases lapses (omissions) of attention (7). Similarly here, 36 h of TSD slowed RTs and increased PVT lapses (RTs > 500 ms). This behavior has been associated with increased activation of the prefrontal region part of the 'default mode network', which is generally activated

when subjects are at rest and not engaged in goal-directed behaviors (37). Our PVT data confirm that our TSD protocol reliably affected attentional performance. Nevertheless, the TSD-induced reduction in PVT responsiveness could reflect generalized reduced responding rather than vigilance *per se*, since this distinction cannot be made using the PVT because it contains only target stimuli.

Specifically examining vigilance requires assessing both accurate responding to target stimuli as well as the inhibition of responding to non-target (irrelevant) stimuli. Using the 5C-CPT, we observed that TSD overall reduced target responding, as in the PVT, and also reduced non-target responding. Importantly, the greater decrease in target responding (as indicated by greater effect sizes) resulted in a lower *d'* score of vigilance. Hence, these 5C-CPT findings indicate that TSD impairs attention beyond simply reducing responding as seen in the PVT, supporting the use of both stimulus types. Furthermore, mood questionnaires (PANAS) (38) completed by the subjects indicated that feelings of alertness correlated significantly with *d'* in the 5C-CPT ($r=0.42$, $p<0.005$), but less with attentional lapses in the PVT ($r=-0.31$, $p<0.05$), whereas pleasantness correlated with PVT ($p<0.05$), but not 5C-CPT performance ($p>0.1$). These data support TSD-induced deficits in 5C-CPT as reflecting attentional dysfunction.

Consistent with the present results, mild cumulative sleep restriction impaired CPT performance of healthy controls and children with ADHD (21). Joo et al. reported that impaired attention in subjects performing a complex CPT after 24 h of TSD was also driven by reduced target responding, which was accompanied by increased non-target responses (20). The discrepancy of SD effects on non-target responding between that report and the present study could have resulted from their small study population of only 6 young male adults and/or the complexity of the CPT used. In healthy and methadone-treated humans, a non-significant reduction in target responding was observed following 36 h of TSD (22), which may have been limited by low sample sizes, practice effects, and/or poor performing subjects. In another study, SD did not significantly affect CPT performance in Korean medical residents and interns (23). In the present studies using healthy subjects from the general population and matching post TSD-testing days to account for possible practice effects, we observed that 36 h of TSD clearly impaired 5C-CPT performance.

Similar to our human study, 36 h of RSD impaired performance in mice in the rodent 5C-CPT, an effect that was not observed in poor performing mice. Interestingly, the TSD-induced reduction in *d'* and target responding in humans was primarily driven by affecting good performing humans, without significantly affecting poor performers (data not shown). RSD in good performing mice decreased their target (correct) responses, resulting in more omitted trials and a trend-level vigilance deficit as measured by reduced *d'*. Comparable results have been observed in the 5CSRTT where 10 h TSD rats made

fewer correct responses and omitted more trials compared to rats with normal sleep (17). Additionally, 24 h of TSD slowed responses and increased lapses in a rat PVT (16). These tasks support our findings in the 5C-CPT. Because these tasks include only target trials however, and no measure of false alarm rates, a simple reduction in responding could not be discounted. When developing treatments by using these tasks, it would be unclear therefore if developed treatments simply increased responding to any presented (even irrelevant) stimuli. In contrast, the 5C-CPT measures both correct responses to target trials and failures to inhibit responding to non-target trials (25). Hence, responsiveness can be dissociated from target responding and our data support that RSD affects attentive responding beyond simply reducing responding. To date, the rodent 5C-CPT has been successfully used to assess genetic and pharmacological manipulations on attentional measures in both rats (28, 29) and mice (25-27, 39). Thus, the current study validates the 5C-CPT as a test suited for translational studies because SD manipulations induce similar 5C-CPT effects in both humans and mice. Consequently, the 5C-CPT will be useful to examine the mechanism(s) underlying SD-induced impairment of attentional performance.

A cross-species comparison between 5C-CPT performance of mice and humans revealed that 36 h of SD decreased hit rate and vigilance and tended to decrease accuracy in humans, whereas it decreased accuracy and hit rate while tending to decrease vigilance in mice, primarily in those with a good baseline performance. The lack of SD-induced deleterious effect in poorly performing mice could be due to a floor effect wherein performance could not be made worse in these mice (see Supplemental Table 3). The stronger overall vigilance deficits observed in humans may have also resulted from the different SD technique used compared to mice. TSD in humans slowed RTs in the PVT and increased variable RTs in the 5C-CPT, but did not affect 5C-CPT RTs in mice. The 'inverted flower pot' technique used here in mice affects various forms of sleep including deep slow wave sleep (40), but primarily deprives animals from REM sleep (41, 42). TSD also affects non-REM sleep and reduces the overall amount of sleep to a greater extent than RSD. Thus, the TSD we administered in humans may have exerted a stronger effect on attention than the RSD we administered in mice. The extended training used in mice, which may have resulted in greater use of procedural memory and hence different circuitry activation patterns, may have also resulted in some of the differences between species.

With cross-species similarity of SD effects on 5C-CPT performance, the mechanism(s) underlying these effects can be investigated. Some putative mechanisms have been tested, *e.g.* that microdialysis perfusion-induced elevation of basal forebrain adenosine, a key mediator of sleep homeostasis, impaired rat PVT performance (43). Similarly, SD increased basal levels of adenosine in rats (44). Furthermore, the adenosine antagonist caffeine is commonly consumed by humans to increase wakefulness. Serotonergic

mechanisms could also be examined given that RSD for 24 h increased serotonergic activity in the hypothalamus in rats (45). Therefore, various mechanisms that may underlie SD-impaired 5C-CPT performance could be targeted in the future to improve attention following sleep loss.

The consistency of SD-induced impaired human and mouse 5C-CPT performance could also prove useful when investigating aspects of psychiatric disorders. As described above, SD can switch people with bipolar disorder into a mania episode. In fact, SD has been used to model mania in rodents (13, 46, 47). Such studies are limited however because healthy humans do not become manic after SD (14). Thus, people with bipolar disorder have an underlying sensitivity to SD-induction of mania (10). Therefore, using the 5C-CPT and SD technique described here may enable the examination of susceptibility genotypes that result in impaired attention in bipolar disorder patients (48).

SD impaired 5C-CPT performance in both humans and mice, primarily by reducing target responding. The SD-induced deficits in mice were only significant in good performers and at longer stimulus durations. Mouse 5C-CPT performance consistently improved with longer stimulus durations. It is clear that SD disrupted the benefit of longer stimulus durations leading to pronounced effects at these durations. With larger sample sizes however, SD would likely impair performance at all stimulus durations. Besides smaller sample sizes after the median split, differences in training and TSD *vs.* RSD techniques discussed above could have also contributed to the limited effects observed in mice. In addition to not affecting all forms of sleep, the 'flower pot' technique can be stressful for animals (49), even more so in combination with food restriction (50). Other techniques such as the gentle handling method (17) may therefore be useful in future studies. Future studies with larger sample sizes will be conducted in order to account for inter-individual differences and SD effects.

In conclusion, 36 h SD deleteriously affected 5C-CPT performance of both humans and mice. Importantly, SD primarily reduced target responding in both species, with a smaller effect on reducing non-target responding, indicating that SD is primarily deleterious to vigilance and not overall responding. These data validate using the 5C-CPT as a cross-species test of vigilance. Therefore, the rodent and human 5C-CPTs can be used in the future across species to examine mechanisms underlying SD effects, susceptibility of psychiatric disorders to such effects, and test pro-vigilance medication for affected groups.

SUPPLEMENTARY MATERIAL

Mouse 5C-CPT apparatus

16 operant chambers (25×25×25 cm, Med Associates Inc., St. Albans, VT, USA) were each equipped with a horizontal array of 5 square response holes (2.5×2.5×2.5 cm) on a curved wall 2.5 cm above the grid floor, a food-delivery magazine with a reward dispenser (strawberry milkshake, Nesquik® (Vevey, Switzerland) plus non-fat milk, 30 µl; Lafayette Instruments, Lafayette, IN, USA) at the opposite wall, a house light near the ceiling, and placed in sound-attenuating ventilated boxes. Both response holes and the food magazine contained LED stimulus lights recessed into the apertures and infrared response detectors. An infrared camera installed in each chamber was used to monitor performance during testing. The control of stimuli and recording of responses were managed by a SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates Inc., St. Albans, VT, USA) using custom programming.

Mouse 5C-CPT training and testing

Mice were trained to holepoke in one of the 5 illuminated holes in order to obtain food reward. The animals were trained daily, with each session lasting 30 min or 120 trials, whichever was completed first. Each trial was initiated by holepoking in the illuminated magazine. Following a constant 4 s ITI, one of the 5 holes opposite the magazine lit up. A response in any cue hole during the ITI was recorded as a premature response (presented here as a percentage of total trials completed) and triggered a 4 s time-out in which all holes were unresponsive. A holepoke in the illuminated stimulus light during the stimulus duration registered a 'correct' response (accuracy) and reward was delivered in the magazine. If the mouse made a holepoke in any other hole during the stimulus duration, an 'incorrect' response was recorded and a time-out occurred. Failure to respond in any hole during the stimulus duration was counted as an 'omission' and also resulted in a time-out. The latency to make a correct response was recorded and presented as the mean RT. The next trial was initiated when the mouse entered and exited the magazine. Animals began the task with a stimulus duration of 20 s, which was reduced to 10, 8, 4, and 2 s following attainment of each criterion (more than 30 correct responses and mean correct latency less than half of the current stimulus duration for 2 consecutive days). After 6 weeks, all mice exhibited stable responding, ±100 correct responses per session on a 2 s stimulus duration. Mice were then moved to a variable 3-7 s ITI for 1 week after which training initiated for the 5C-CPT. For the 5C-CPT with a variable 3-7 s ITI, in addition to the above described 'target' trials where 1 of the 5 holes lit up, 'non-target' trials where all 5 holes lit up, were presented pseudo-randomly (max. 3 sequential non-target trials). Successful inhibition of a response in a non-target trial registered a correct rejection and reward was delivered. If a response was made in a non-target trial however, a false alarm

was recorded and a time-out period occurred. A 30 min session, allowing for a maximum of 120 trials with 80 trials being target trials and 40 non-target trials (2:1 ratio) was used initially to acquaint the animals with the newly introduced non-target trials. Mice were then trained on the final 5C-CPT session with 100 target trials vs. 20 non-target trials (5:1 ratio) following attainment of criterion (less than 50% false alarms and more than 20 correct responses for 2 consecutive days) and the stimulus duration was reduced to 1.75 and 1.5 s based on the same criterion as described above. Hit rate, false alarm rate, *d'*, and RI were calculated as described for the human 5C-CPT. Mice were trained until exhibiting stable responding in the 5C-CPT with 5 target vs. 1 non-target trials, a variable 3-7 s ITI, and a 1.5 s stimulus duration. Mice then received either normal sleep or RSD and performance was challenged in a 60 min session (max. 250 trials) with a variable stimulus duration (0.75, 1.25, and 2 s).

Supplemental Table 1. Means, standard errors of the mean, and statistical comparison of trials completed and premature responses of mice after 36 h of RSD vs. control-treated mice in the 5C-CPT.

Variable	SD (s)	Normal sleep mean (SEM)	RSD mean (SEM)	d.f.	*F*	*p*-Value
Trials completed	0.75	74.4 (3.5)	62.8 (3.8)	1,21	5.1	<0.05
	1.25	73.6 (3.5)	63.2 (3.8)	1,21	4.1	<0.1
	2.0	74.7 (3.7)	62.8 (4.1)	1,21	4.6	<0.05
% Premature responses	0.75	4.3 (1.3)	4.6 (1.4)	1,21	<1	Ns
	1.25	4.5 (1.0)	5.3 (1.1)	1,21	<1	Ns
	2.0	5.6 (1.2)	4.8 (1.4)	1,21	<1	Ns

SD, stimulus duration; RSD, REM sleep deprivation; Ns, not significant.

Supplemental Table 2. Means, standard errors of the mean, and statistical comparison of trials completed and premature responses of good performing mice after 36 h of RSD vs. control-treated mice in the 5C-CPT.

Variable	SD (s)	Normal sleep mean (SEM)	RSD mean (SEM)	d.f.	*F*	*p*-Value
Trials completed	0.75	72.5 (5.2)	58.7 (6.2)	1,9	2.7	Ns
	1.25	72.2 (5.1)	58.7 (6.1)	1,9	2.7	Ns
	2.0	73.2 (5.2)	57.9 (6.2)	1,9	3.3	Ns
% Premature responses	0.75	3.9 (1.0)	2.9 (1.2)	1,9	<1	Ns
	1.25	3.5 (1.0)	3.4 (1.2)	1,9	<1	Ns
	2.0	4.9 (1.0)	2.6 (1.2)	1,9	2.1	Ns

RT, reaction time; SD, stimulus duration; RSD, REM sleep deprivation; Ns, not significant.

Supplemental Table 3. Means and standard errors of the mean of poor performers in the mouse 5C-CPT after normal sleep or 36 h of RSD.

Variable	SD (s)	Normal sleep mean (SEM)	RSD mean (SEM)	d.f.	*F*	*p*-Value
Accuracy	0.75	0.95 (0.04)	0.92 (0.04)	1,9	<1	Ns
	1.25	0.95 (0.03)	0.93 (0.03)	1,9	<1	Ns
	2.0	0.97 (0.02)	0.96 (0.02)	1,9	<1	Ns
% Omissions	0.75	54.8 (5.0)	46.5 (5.0)	1,9	1.4	Ns
	1.25	36.8 (7.1)	32.7 (7.1)	1,9	<1	Ns
	2.0	18.9 (6.1)	28.4 (6.1)	1,9	1.2	Ns
Mean RT (s)	0.75	0.67 (0.07)	0.82 (0.07)	1,9	2.6	Ns
	1.25	0.86 (0.07)	0.86 (0.07)	1,9	<1	Ns
	2.0	0.99 (0.07)	0.97 (0.07)	1,9	<1	Ns
Variable RT (s)	0.75	36.9 (4.5)	48.4 (4.5)	1,9	3.3	Ns
	1.25	50.3 (5.0)	49.7 (5.0)	1,9	<1	Ns
	2.0	59.4 (4.6)	58.3 (4.6)	1,9	<1	Ns
d'	0.75	-0.42 (0.57)	0.36 (0.57)	1,9	<1	Ns
	1.25	0.56 (0.21)	0.73 (0.21)	1,9	<1	Ns
	2.0	1.25 (0.29)	0.70 (0.29)	1,9	1.7	Ns
[p] False alarms	0.75	0.44 (0.09)	0.35 (0.09)	1,9	<1	Ns
	1.25	0.40 (0.05)	0.40 (0.05)	1,9	<1	Ns
	2.0	0.36 (0.08)	0.44 (0.08)	1,9	<1	Ns
[p] Hit rate	0.75	0.43 (0.05)	0.49 (0.05)	1,9	<1	Ns
	1.25	0.61 (0.08)	0.63 (0.08)	1,9	<1	Ns
	2.0	0.79 (0.07)	0.69 (0.07)	1,9	1.1	Ns
Responsivity Index	0.75	-0.09 (0.12)	-0.17 (0.12)	1,9	<1	Ns
	1.25	0.02 (0.11)	0.00 (0.11)	1,9	<1	Ns
	2.0	0.18 (0.12)	0.15 (0.12)	1,9	<1	Ns
Trials completed	0.75	75.6 (5.1)	67.3 (5.1)	1,9	1.3	Ns
	1.25	74.1 (5.1)	68.1 (5.1)	1,9	<1	Ns
	2.0	75.2 (5.8)	68.0 (5.8)	1,9	<1	Ns
% Premature responses	0.75	4.7 (2.5)	6.1 (2.5)	1,9	<1	Ns
	1.25	5.7 (1.7)	6.8 (1.7)	1,9	<1	Ns
	2.0	6.7 (2.3)	6.4 (2.3)	1,9	<1	Ns

RT, reaction time; SD, stimulus duration; RSD, REM sleep deprivation; Ns, not significant.

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CHAPTER 5

Pharmacological modeling of both manic and depressive phases of bipolar disorder in mice

CHAPTER 5.1

Reproducing bipolar depression- and mania-like behaviors in mice by increasing acetylcholine or dopamine: Chronic lithium treats most, but not all

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ABSTRACT

Background

Bipolar disorder (BD) is a disabling and potentially life-threatening disease characterized by periods of depression and mania. New and efficacious treatments have remained absent partly due to a lack of well-validated animal models that represent both facets of the BD syndrome. Cholinergic signaling may be involved in depression whereas dopaminergic abnormalities may underlie mania. We hypothesized that the mood-stabilizer lithium would reverse both cholinergic and dopaminergic pharmacological models of depression and mania respectively.

Methods

Male C57BL/6J mice (*n*=40) received the acetylcholinesterase (AChE) inhibitor physostigmine or saline before testing in the tail suspension test (TST) and forced swim test (FST). Physostigmine effects on exploration and prepulse inhibition (PPI) were assessed using the cross-species behavioral pattern monitor (BPM) and acoustic startle test. Other male C57BL/6J mice (*n*=96) received chronic lithium (300, 600, or 1200 mg/l) drinking water before testing acute GBR12909 (dopamine transporter inhibitor) on exploration and PPI and physostigmine in the FST.

Results

Physostigmine (0.03 mg/kg) increased immobility in the TST and FST without affecting activity, exploration, or PPI. GBR12909 (9 mg/kg) decreased PPI and induced mania-like behavior in the BPM. Lithium (600 mg/l) resulted in therapeutic concentrations for BD and normalized the physostigmine-increased immobility in the FST. Although GBR12909-induced PPI deficits were not observed in lithium-treated mice, lithium potentiated GBR12909-induced hyper-exploration.

Conclusions

Increased cholinergic levels induced depression-like behavior while hyperdopaminergia induced mania-like behavior in mice. Lithium treated some, but not all, facets of these effects. These data support a cholinergic-monoaminergic mechanism for modeling BD aspects and provide a way to assess novel therapeutics.

Keywords

Acetylcholine, Dopamine transporter, Lithium, Bipolar disorder, Prepulse inhibition, Depression

INTRODUCTION

Bipolar disorder (BD) is a severely disabling mental illness affecting 1-2 % of the global population for BD type I (1). The seriousness of the disorder is indicated by an increased suicide mortality rate (2) where one of three patients attempt suicides (3) and a lifetime cost amounting to $24 billion (4).

BD is a unique mood disorder defined by periods of depression and mania during which symptoms of patients differ markedly (5). In fact, symptoms can be largely opposite from each other with hyperactivity being a hallmark feature of mania (6), whereas lethargy or psychomotor retardation can characterize depression. Lithium is commonly used to treat aspects of mania and depression as well as maintain a patient's state between periods (7), but its effects are limited and it has a low acceptability profile (8). Greater understanding of the mechanisms underlying and/or overlapping different phases of BD may improve the development of novel and more efficacious therapeutics.

An improved understanding of mechanisms contributing to each aspect of BD may also lead to better animal models with which to test therapeutics targeted at these mechanisms. The currently limited treatments may be due in part to the dearth of valid animal models targeting the etiologies of BD (9, 10). Indeed, animal models being used to reproduce a BD-like phenotype are usually specific only to the manic phase of the disorder (11-13). These models typically reproduce aspects of hyperactivity (14), with some measuring impaired risk-taking (15), and increased reward seeking (16). An awareness exists for the necessity to model the full spectrum of BD (depression and mania) in animals in order to find novel treatments for the full disorder (17), but the complexity of the matter has prevented such an outcome as yet.

Recently, a human imaging study indicated that acetylcholine (ACh) levels are increased in both acutely depressed and recovered subjects (18). Importantly, the same has also been suggested in BD patients during periods of depression (19). Mineur et al. demonstrated that physostigmine, an acetylcholinesterase (AChE; the primary enzyme hydrolyzing ACh) inhibitor which elevates ACh, induces depression-like behavior in mice (20). These animal data are consistent with early evidence of physostigmine-induced severe depression and psychomotor retardation in marijuana-intoxicated humans (21) and increased symptoms of depression in patients with mania, depression, schizoaffective disorder (22), and healthy subjects (23). Thus, increasing ACh levels models the behavior and putative etiology of depression that is relevant to BD, renewing interest in the cholinergic aspect of the cholinergic-adrenergic hypothesis of depression and mania (24).

Given the range of relevant behaviors in BD, the biological underpinnings of mania likely involve other mechanisms than those of depression. We have previously demonstrated that mice with reduced dopamine transporter (DAT) functioning model aspects of BD mania (25). Both DAT knockdown (KD) mice and mice receiving the selective DAT inhibitor GBR12909 exhibit hyperactivity, increased exploration, and straight paths of movement as quantified by the mouse behavioral pattern monitor (BPM) (26, 27) similar to patients with BD mania (28, 29) and euthymia (30) in a human BPM. Although some of the abnormal behavior observed in these GBR12909-treated mice, such as impaired decision-making (31), is also present in depressed patients with BD performing a similar human task (32), their hyperactivity, increased motivation (33), and reduced immobility times in the tail suspension (34) and forced swim tests (35) more closely model the manic phase of BD. These animal data may have etiological relevance to BD because reduced striatal DAT levels are observed in unmedicated patients with BD (36) as well as in postmortem tissue (37). Thus, hyperdopaminergia caused by reduced DAT levels may be used to model mania-like behavior in mice.

Here, we test the putative depression-like effects of physostigmine on inducing "behavioral despair" in mice by using the tail suspension test (TST) (38) and forced swim test (FST) (39). Moreover, we examined the effects of physostigmine in human and rodent cross-species tests of exploration in the BPM and sensorimotor gating of the startle reflex as measured by prepulse inhibition (PPI) (40). For example, reduced PPI has been observed in manic patients with BD (41), but not in depressed subjects (42, 43), suggesting that dysfunctional PPI is state dependent (44). PPI deficits can be recreated in rodents (45) and have been observed in rodent models of BD mania (16). Moreover, we investigated whether lithium would attenuate any depression-like effects of physostigmine or mania-like effects of GBR 12909.

We hypothesized that: (*a*) physostigmine would induce depression- but not mania-like behavior in mice; (*b*) GBR12909 would induce a mania- but not depression-like state; and (*c*) chronic lithium treatment would attenuate these behaviors.

METHODS

Animals

Male C57BL/6J mice (*n*=136) were used throughout the studies (Fig. 1). Mice were maintained in a temperature-controlled vivarium (21±1°C) on a reversed day-night cycle (lights on at 7.00 PM, off at 7.00 AM) and were tested during the dark phase between 8.00 AM and 5.00 PM. Mice were group housed (four/cage), weighed between 20-40 g, and were 3-4 months old at the time of testing. Mice had *ad libitum* access to water and food (Harlan, Madison, WI, USA) except during testing with lithium (see below). All procedures

were approved by the UCSD Institutional Animal Care and Use Committee. The UCSD animal facility meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care.

Drug treatment

GBR12909 dihydrochloride and physostigmine sulfate (Sigma-Aldrich, St Louis, MO, USA) were both dissolved in 0.9% saline vehicle (10 ml/kg), GBR12909 after sonicating for 2-4 h at 40 °C as done previously (12, 27). GBR12909 (9.0 mg/kg) (12, 31, 46) and physostigmine (0.01, 0.03, 0.1, and 0.3 mg/kg) (47-49) were administered in a single i.p. injection 0 and 30 min prior to the experiment respectively (see Results for details). Lithium chloride (Sigma-Aldrich, St Louis, MO, USA) was dissolved into the drinking water at 300, 600, or 1200 mg/l and given for 10-17 days (Fig. 1). This procedure was chosen based on previous studies using the same procedure with 600 mg/l and 1200 mg/l to achieve serum levels approaching human therapeutic concentrations (Fig. 2) (13, 50). Control animals received tap water. Free-base drug weights were used in all drug calculations, except for lithium chloride.

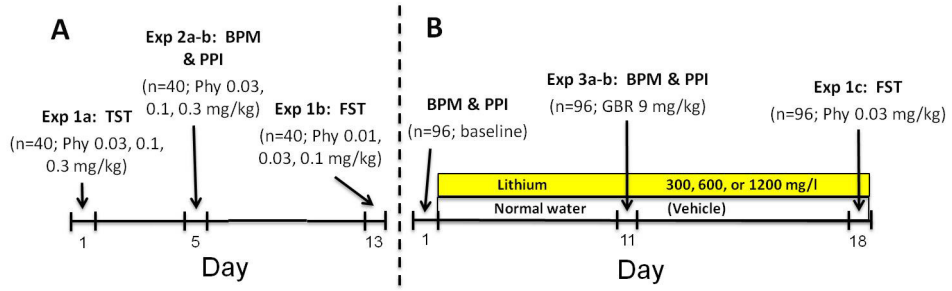

Fig. 1. Timeline of the testing procedures for the current studies. One group of male mice (*n*=40; a) were tested in the TST, BPM, PPI, and FST respectively. Over the four tests, each animal received each drug dose or saline once. Drug or saline was administered 30 min prior to testing in the TST and FST, and 10 and 55 min prior to the BPM and PPI test respectively. Another group of male mice (*n*=96; b) was treated with either lithium in their drinking water (300, 600, or 1200 mg/l) or normal tap water (vehicle) after baseline-matching. After 10 days of treatment, each group received saline or GBR12909 in randomized order immediately prior to testing in the BPM and PPI. After one week wash-out from GBR12909 and 17 days of lithium treatment, each group received saline or physostigmine in randomized order 30 min prior to testing in the FST.

Serum lithium measurements

Mice were decapitated and trunk blood was collected. Blood was left to clot for approximately 15 min and then centrifuged for 10 min. Serum was removed and frozen. Samples were analyzed by using spectrophotometry performed by UCSD Medican Center (USA).

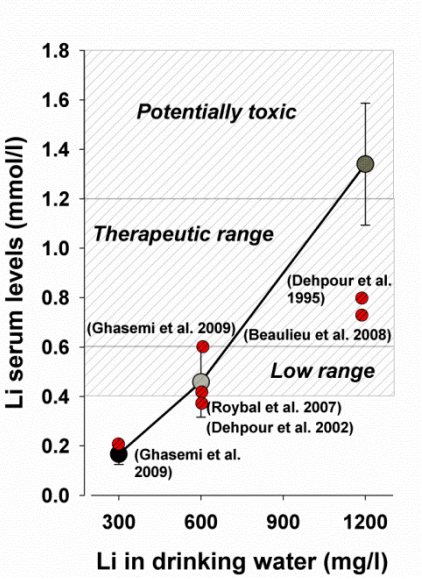

Fig. 2. Serum concentrations of lithium after 17 days of different doses of lithium treatment in drinking water. Lithium (Li) at 300 mg/l resulted in low serum levels (0.17 ± 0.04 mmol/l), while 1200 mg/l resulted in high/toxic Li concentrations (1.34 ± 0.25 mmol/l). Drinking water with Li 600 mg/l resulted in low therapeutic serum concentrations for bipolar disorder (0.46 ± 0.14 mmol/l). Comparative lithium levels are provided from earlier publications, the variability from which is discussed below. Data are presented as mean ± S.E.M.

Mouse behavioral pattern monitor

Locomotor behavior and exploration were examined in eight mouse BPM chambers (San Diego Instruments, USA) as described previously (51, 52). In brief, each Plexiglas arena consists of a 30.5 × 61 × 38 cm area with three floor and eight wall holes (three in each long wall and one in each short wall; 1.25 cm in diameter, 1.9 cm from the floor), each equipped with an infrared photobeam to detect holepoking. Each chamber is enclosed in an outer box with an internal white house-light above the arena (350 lux in the center and 92 lux in the four corners) that minimizes external light and noise. Activity was obtained from a grid of 12 × 24 infrared photobeams 1 cm above the floor (2.5 cm apart; 24 × 12 *X-Y* array), recording the location of the mouse every 0.1 s, with its position defined across nine unequal regions (four corners, four walls, and center (53)). Another set of 16 photobeams, placed 2.5 cm above the floor, was used to detect rearing behavior. At the start of the session, mice were placed in the bottom left-hand corner of the arena and the test session started immediately. The primary outcome measures were transitions across the defined regions and center entries (locomotor activity), holepoking and rearing (exploratory behavior), and spatial *d* (locomotor patterns). Spatial *d* measures the degree to which the animal makes more straight line movements versus more circumscribed paths of movement. It quantifies the geometric structure of the locomotor path, where a value closer to 1 reflects a straighter path, and values closer to 2 indicating highly circumscribed small-scale movements (54).

Acoustic startle testing

Startle and PPI testing were examined in eight startle chambers (SR-LAB, San Diego Instruments, USA), using an experimental session as described previously (16). Each chamber consists of a Plexiglas cylinder, 5 cm in diameter, resting on a platform in a

ventilated sound-attenuating outer box. Speakers mounted 33 cm above the cylinders produced all acoustic stimuli and movements of the animal were transduced by piezoelectric accelerometers mounted under the cylinders and stored and digitized by an interface and computer assembly. Mice were placed into the startle chambers and testing started after a 5 min acclimation period. Mice were exposed to a 65 dB background sound and light, located on the ceiling of the chamber, continuously throughout the session. Startle pulses were 40 ms and prepulses were 20 ms in duration. The inter-trial interval (ISI) between stimulus presentations ranged between 3-12 s (7 s average). The acoustic startle sessions included five blocks. The first block consisted of five 120 dB pulses. The second block included prepulse trials (69, 73, and 81 dB) preceding a 120 dB pulse. The third block included acoustic startle responding only (80, 90, 100, 110, and 120 dB pulses). The fourth block varied the ISI, consisting of 73 dB prepulses preceding 120 dB pulses by 25, 50, 100, 200, and 500 ms. The fifth and final block delivered five 120 dB pulses and together with 120 dB pulses in each block served to assess habituation. PPI was calculated as a percentage score for each prepulse intensity based on the 120 dB pulse within that block: %PPI = 100 – [(startle magnitude for prepulse + pulse / startle magnitude for pulse alone) × 100].

Tail suspension test

Assessing immobility in the TST is commonly used to screen for compounds with antidepressant efficacy and is also used to identify depression-like behavior in mice (38). Mice were gently suspended by the tip of the tail attached with a piece of adhesive tape to a metal bar placed horizontally 50 cm above the tabletop. Videotapes were scored by an experimenter blind to the experimental treatment for time spent immobile over 6 min. The primary outcome measure was immobility defined as no movement except for respiration.

Forced swim (Porsolt) test

Consistent with the TST, the FST was devised to screen for compounds with antidepressant efficacy and is based on observing so-called “behavioral despair”. Mice were placed in a clear glass beaker with a diameter of 15 cm, a height of 24 cm, and 15 cm of tap water at 25 °C. The duration of the test was 6 min and videotapes were scored for all 6 min by a trained investigator blind to the experimental treatment. The primary outcome measure was immobility defined as no movement except minor movement required to keep afloat. After the test, the animal was dried and returned to its home cage. The water was replaced between every 5-6 animals.

Experiments

For a more detailed description of the animals used, and the experimental timeline, see Fig. 1.

Exp 1a. Assessing the effects of physostigmine on immobility in the TST

Mice received saline or 0.03, 0.1, or 0.3 mg/kg physostigmine 30 min prior to testing (*n*=10/group).

Exp 1b. Assessing the effects of physostigmine on immobility in the FST

After observing a strong lethargy-inducing effect with 0.3 mg/kg physostigmine treatment in the BPM (see Exp 2a), we investigated a lower dose of this compound in the FST. Mice received saline or 0.01, 0.03, or 0.1 mg/kg physostigmine 30 min prior to testing (*n*=10/group).

Exp 1c. Assessing the effects of lithium on physostigmine-induced increased immobility in the FST

Mice (*n*=96) received chronic lithium (300, 600, or 1200 mg/l) or regular drinking water (vehicle) for 17 days. Because mice treated with lithium 1200 mg/l had experienced severe side-effects (including some deaths), only mice treated with lithium 300 or 600 mg/l were tested. These mice then received saline or 0.03 mg/kg physostigmine 30 min prior to testing (*n*=12/group).

Exp 2a. Assessing the effects of physostigmine on exploration in the BPM

Mice were tested in the BPM for 45 min and received saline or 0.03, 0.1, or 0.3 mg/kg physostigmine 10 min prior to testing (*n*=10/group).

Exp 2b. Assessing the effects of physostigmine on PPI in the acoustic startle test

Immediately after Exp 2a, mice were tested in the acoustic startle test. Hence, these mice had received saline or 0.03, 0.1, or 0.3 mg/kg physostigmine 55 min prior to testing (*n*=10/group).

Exp 3a. Assessing the effects of lithium on GBR12909-induced hyper-exploration in the BPM

Mice (*n*=96) were tested first without drug in the BPM for 30 min and PPI test to baseline-match their behavior based on transitions, holepoking, rearing, spatial *d*, PPI, and startle. They were then counter-balanced into groups that received regular tap water (vehicle; *n*=24) or lithium solution (*n*=24/dose). After 10 days of treatment, mice received saline or

GBR12909 (9 mg/kg; *n*=12/group) immediately prior to assessing their exploration in the BPM for 60 min.

Exp 3b. Assessing the effects of lithium on GBR12909-induced impaired PPI in the acoustic startle test

Immediately after Exp 3a, both vehicle- and lithium-treated mice were tested in the acoustic startle PPI test. Hence, these mice had received saline or 9 mg/kg GBR12909 60 min prior to testing (*n*=12/group).

Statistical analyses

We first confirmed that all data was distributed normally and displayed equal variances. Data from the BPM and acoustic startle test were analyzed using two- or three-way analyses of variance (ANOVA), with GBR12909 and dose of lithium treatment as between-subject factors and time-period (15 min), prepulse intensity, pulse intensity, ISI, or habituation block as within-subject factors. TST and FST data were analyzed using a one- or two-way ANOVA with drug and dose of lithium treatment as between-subject factors. Tukey *post hoc* analyses of main or interaction effects were performed where applicable. The animals' body weights were compared using an independent samples *t*-test. All BPM and startle data were analyzed using the BMDP statistical software (Statistical Solutions Inc., USA), while TST and FST data were analyzed using SPSS (19.0, Chicago, IL, USA). The α level was set to 0.05.

RESULTS

Exp 1a. The effects of physostigmine on immobility in the TST

Three saline-treated mice were excluded from analyses, due to climbing their tail. Physostigmine significantly increased immobility time ($F_{(3,33)}$=16.3, p<0.001; Fig. 3a), at each dose compared to saline (p<0.001).

Exp 1b. The effects of physostigmine on immobility in the FST

Consistent with the TST, physostigmine increased immobility time in the FST ($F_{(3,36)}$=8.0, p<0.001; Fig. 3b), at 0.03 and 0.1 mg/kg (p<0.001), but not 0.01 mg/kg (p=0.119), compared to saline.

Exp 1c. The effects of lithium on physostigmine-induced increased immobility in the FST

Several mice from the lithium groups were excluded from analyses because of unforeseen death. When analyzed including both doses, there was a main effect of lithium ($F_{(2,60)}$=10.4, p<0.001) and physostigmine ($F_{(1,60)}$=4.2, p=0.042), but no interaction between

both ($F_{(2,60)}$=2.3, p=0.107). When analyzed with only lithium 600 mg/l, a main effect of lithium ($F_{(1,42)}$=15.4, p<0.001) and interaction with physostigmine ($F_{(1,42)}$=4.2, p<0.046; Fig. 3c) was observed, but no main effect of physostigmine ($F_{(1,42)}$=4.2, p=0.168). *Post hoc* analyses revealed that physostigmine increased immobility time compared to saline in the vehicle- (p<0.01), but not the lithium-treated animals (p>0.05). Hence, treatment with lithium 600 mg/l attenuated the physostigmine-induced increased immobility (p<0.001). Lithium did not affect mice receiving saline (p>0.05). We also observed the mice for potential adverse effects of lithium. No adverse effects on health were reported, neither were differences in weight observed between vehicle-treated mice and mice treated with lithium 300 mg/l ($t_{(46)}$=1.0, p=0.328) or lithium 600 mg/l ($t_{(45)}$=1.5, p=0.135).

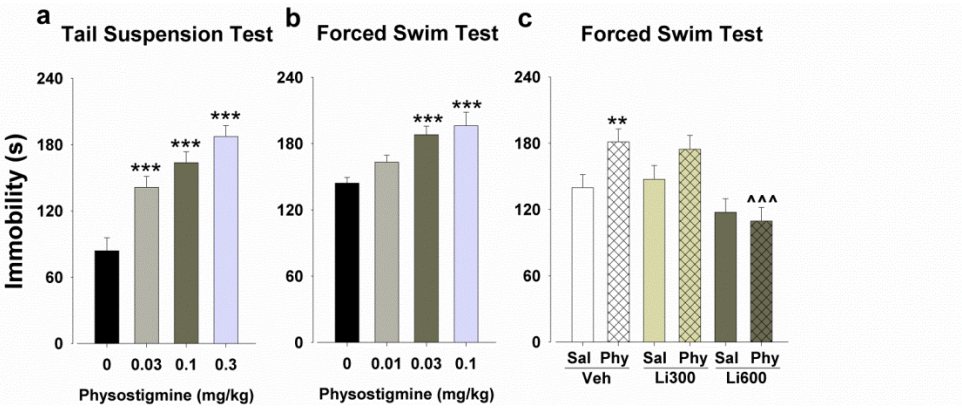

Fig. 3. Time spent immobile in the TST and FST after physostigmine administration and its reversal by lithium in the FST. Each dose of physostigmine increased immobility times compared to saline in the TST (a). Both 0.03 and 0.1 mg/kg increased immobility times compared to saline in the FST, but 0.01 mg/kg had no effect (b). In another group of mice, physostigmine (Phy; 0.03 mg/kg) increased immobility times compared to saline (Sal) in the vehicle-treated mice (Veh), but not in the mice treated with lithium 600 mg/l (c). Data are presented as mean + S.E.M. n=10-12 animals per group. **p<0.01 and ***p<0.001 when compared to saline and ^^^p<0.001 when compared to vehicle.

Exp 2a. The effects of physostigmine on exploration in the BPM

Mice receiving physostigmine exhibited significantly reduced transitions ($F_{(3,36)}$=55.0, p<0.001; Fig. 4a), center entries ($F_{(3,36)}$=26.1, p<0.001; Fig. 4b), holepoking ($F_{(3,36)}$=44.9, p<0.001; Fig. 4c), rearing ($F_{(3,36)}$=33.5, p<0.001; Fig. 4d), and increased spatial *d* ($F_{(3,36)}$=34.1, p<0.001; Fig. 4e). *Post hoc* analyses confirmed that only the two highest doses of physostigmine (0.1 and 0.3 mg/kg) reduced transitions, center entries, holepokes, and rearing (p<0.001). Only 0.3 mg/kg physostigmine increased spatial *d* compared to saline (p<0.001), although the low activity limits the exact measurement of spatial *d*. The lowest dose (0.03 mg/kg) did not affect any of the above measures compared with vehicle (p>0.05). When split by three 15 min time bins, there were significant time by physostigmine interactions for transitions ($F_{(6,72)}$=12.2, p<0.001), center entries ($F_{(6,72)}$=2.3,

p=0.0461), holepoking ($F_{(6,72)}$=7.4, p<0.001), and rearing ($F_{(6,72)}$=5.5, p<0.001), reflecting that mice treated with 0.1 mg/kg physostigmine approached control levels of behavior over time (Fig. 4f).

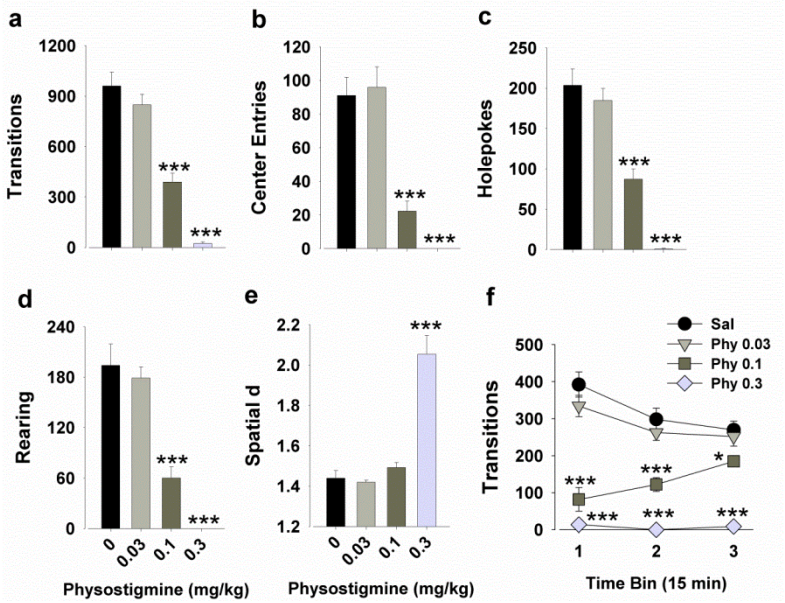

Fig. 4. Effects of physostigmine on the exploratory profile of mice in the BPM. Physostigmine (0.1 and 0.3 mg/kg) decreased activity as measured by transitions (a) and center entries (b). Physostigmine (0.1 and 0.3 mg/kg) decreased exploration as measured by holepoking (c) and rearing (d). The highest dose greatly increased spatial *d* values to the limit of its range, likely due to severely depressed activity, limiting spatial *d*'s calculation (e). Mice administered 0.1 mg/kg physostigmine exhibited increased activity over time, approaching normal levels by the end of the session (f). The lowest dose (0.03 mg/kg) had no effect on any of the measures (a-f). Data are presented as mean ± S.E.M. n=10 animals per group. *p<0.05 and ***p<0.001 when compared to saline.

Exp 2b. The effects of physostigmine on PPI in the acoustic startle test

A main effect of prepulse ($F_{(2,72)}$=129.0, p<0.001; Fig. 5a) revealed that PPI improved with higher prepulse intensities, providing construct validity. A main effect of physostigmine ($F_{(3,36)}$=8.7, p<0.001) and interaction with prepulse ($F_{(6,72)}$=3.3, p=0.007) were observed. *Post hoc* analyses revealed that 0.3 mg/kg physostigmine increased PPI at each prepulse intensity (p<0.01), while 0.03 mg/kg tended to increase PPI only at a 69 dB prepulse intensity (p<0.1). There was also a trend effect of physostigmine on startle amplitude ($F_{(3,36)}$=2.6, p=0.065; Fig. 5b) and a pulse × physostigmine interaction ($F_{(12,144)}$=1.8, p=0.048). No *post hoc* effects were observed however. When split by ISI, there was a main effect of physostigmine ($F_{(3,36)}$=3.6, p=0.022; Fig. 5c). *Post hoc* analyses revealed that consistent with its effects on PPI at varying prepulse intensity levels, 0.3 mg/kg

physostigmine increased PPI at ISI 100 (p<0.01) and tended to at ISI 500 (p<0.1). All mice habituated over time ($F_{(4,144)}$=15.5, p<0.001; Fig. 5d), without effect of physostigmine treatment ($F_{(3,36)}$=1.6, p=0.215). No effect of physostigmine was observed on movement when no stimuli were presented ($F_{(3,36)}$=1.9, p=0.145; Fig. 5e). Finally, in mice matched for startle reactivity (n=14) there was still a main effect of physostigmine on PPI ($F_{(3,30)}$=6.0, p=0.003) with 0.3 mg/kg increasing PPI at each prepulse intensitiy (p<0.05).

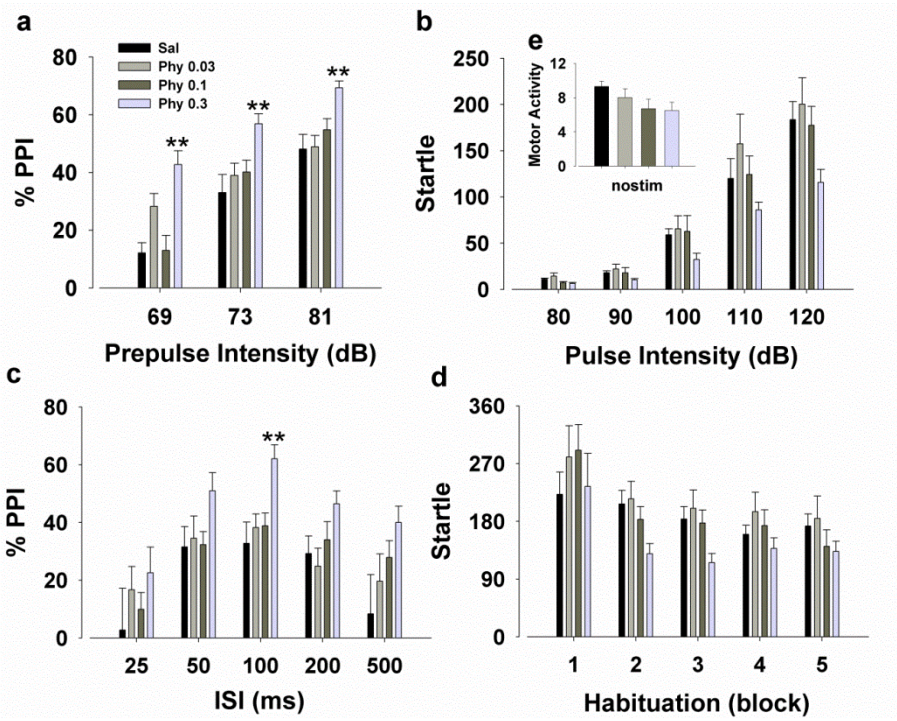

Fig. 5. Effects of physostigmine on sensorimotor gating of the acoustic startle response of mice. The highest dose of physostigmine (0.3 mg/kg) increased PPI compared to saline (Sal) whereas 0.03 mg/kg only tended to increase PPI at the lowest prepulse (a). Physostigmine had no effect on overall amplitude of the startle response (b). When split by ISI, 0.3 mg/kg physostigmine increased PPI at ISI 100 (c). All mice exhibited habituation over time, without effect of physostigmine treatment (d). No difference between physostigmine and saline was observed when no stimulus was presented (e). Data are presented as mean + S.E.M. n=10 animals per group. **p<0.01 when compared to saline.

Exp 3a. The effects of lithium on GBR12909-induced hyper-exploration in the BPM (Table 1)

Locomotor behavior

Main effects of GBR12909 were observed on transitions (Fig. 6a) and center entries (Fig. 6b). Lithium slightly affected transitions, but not center entries. No GBR12909 × lithium interactions were observed for transitions or center entries. *Post hoc* analyses revealed

that GBR12909 subtly increased transitions in the vehicle-treated mice (p<0.1), but robustly in mice treated with each dose of lithium (p<0.05). Mice receiving GBR12909 and lithium 300 or 600 mg/l exhibited increased transitions compared to mice receiving GBR12909 alone (p<0.01). Mice receiving GBR12909 and lithium 600 mg/l tended to exhibit increased center entries compared to their controls or mice receiving GBR12909 alone (p<0.1).

Exploratory behavior

GBR12909 significantly increased holepoking (Fig. 6c) and rearing (Fig. 6d). There was no effect of lithium on holepoking or rearing with no interactions with GBR12909 for holepoking or rearing. *Post hoc* analyses revealed that GBR12909 alone increased holepoking subtly (p<0.1), but not rearing. In mice treated with lithium 600 mg/l, GBR12909 significantly increased both measures (p<0.05). In mice treated with lithium 300 mg/l, GBR12909 increased rearing (p<0.01).

Locomotor patterns

There was a main effect of GBR12909 (Fig. 6e) and lithium on spatial *d*, without interaction. *Post hoc* analyses revealed that GBR12909 reduced spatial *d* in mice treated with lithium 300 and 600 mg/l (p<0.01).

We also examined the potential adverse effect of lithium on the animals' body weights and observed no difference between vehicle-treated mice and mice treated with lithium 300 mg/l [$t_{(46)}$=1.4, p=0.175] or lithium 600 mg/l [$t_{(45)}$=1.7, p=0.101]. However, mice treated with lithium 1200 mg/l had significantly lower body weights compared to vehicle [$t_{(46)}$=3.0, p=0.004].

Table 1. The effects of lithium and GBR12909 in the BPM.

Measure	Group	s.e.m.	d.f.	*F*	*p*-Value
Activity	Transitions	GBR	(1,87)	39.3	**<0.001**
		Lithium	(3,87)	2.7	**0.051**
		GBR × Lithium	(3,87)	2.2	**0.092**
	Center entries	GBR	(1,87)	5.2	**0.025**
		Lithium	(3,87)	1.6	0.202
		GBR × Lithium	(3,87)	0.3	0.804
Exploration	Holepoking	GBR	(1,87)	14.0	**<0.001**
		Lithium	(3,87)	1.5	0.214
		GBR × Lithium	(3,87)	0.6	0.616
	Rearing	GBR	(1,87)	9.4	**0.003**
		Lithium	(3,87)	1.5	0.215
		GBR × Lithium	(3,87)	1.7	0.175
Patterns	Spatial *d*	GBR	(1,87)	29.6	**<0.001**
		Lithium	(3,87)	3.5	**0.019**
		GBR × Lithium	(3,87)	1.4	0.238

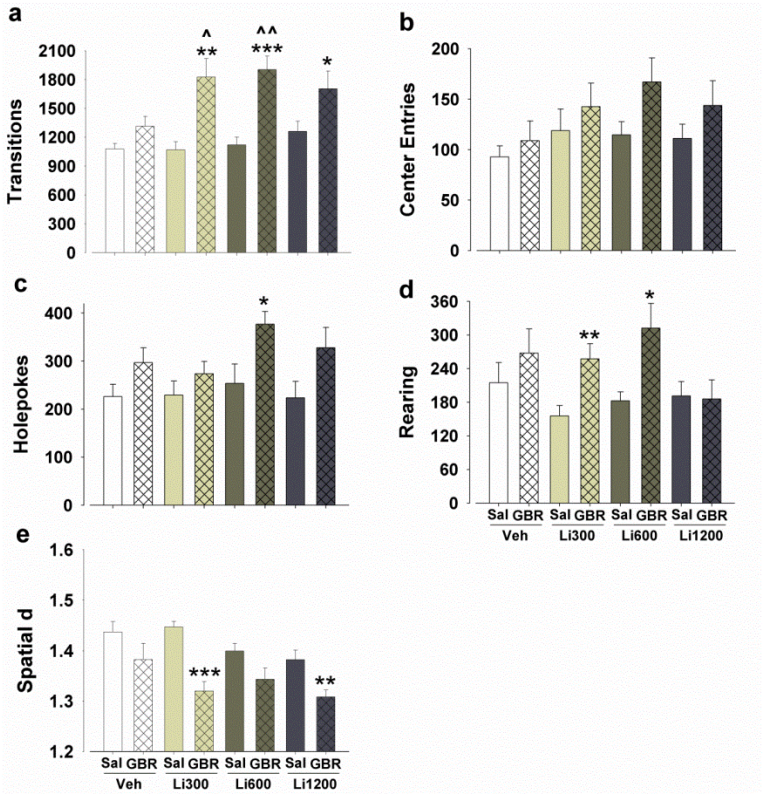

Fig. 6. Effects of chronic treatment with the mood-stabilizer lithium on the exploratory profile of mice administered acute GBR12909 (9 mg/kg). GBR12909 (GBR) slightly increased activity as measured by transitions, which was potentiated by lithium (a). There were no significant effects on center entries (b). The effect of GBR on holepoking, was again potentiated by lithium 600 mg/l (c). The effect of GBR on rearing was also potentiated by lithium 300 and 600 mg/l, but not 1200 mg/l (d). GBR exerted a subtle effect on locomotor pattern by inducing more linear patterns of movement in mice treated with lithium 300 or 1200 mg/l (e). Data are presented as mean + S.E.M. n=12 animals per group. *p<0.05, **p<0.01, and ***p<0.001 when compared to saline and ^p<0.05 and ^^p<0.01 when compared to vehicle.

Exp 3b. The effects of lithium 600 mg/l on GBR12909-induced PPI in the acoustic startle test

Only the 600 mg/l data are presented in the current results because only this dose was efficacious in the FST and no effects were observed with other doses. A main effect of prepulse ($F_{(2,88)}$=74.3, p<0.001; Fig. 7a) revealed that the sensorimotor gating of all mice improved with higher prepulse intensities. GBR12909 significantly reduced PPI ($F_{(1,44)}$=7.4, p=0.010) and tended to interact with prepulse intensity ($F_{(2,88)}$=3.0, p=0.054). There was no effect of lithium ($F_{(1,44)}$=0.3, p=0.595) or interaction with GBR12909 ($F_{(1,44)}$=0.8, p=0.388). *Post hoc* analyses revealed that GBR12909 induced a PPI deficit compared to saline in the vehicle-treated mice at the 69 and 73 dB prepulse intensities (p<0.05), an

effect not observed in mice treated with lithium 600 mg/l (p>0.05). Neither GBR12909 ($F_{(1,44)}$=0.2, p=0.634) nor lithium ($F_{(1,44)}$=0.1, p=0.792) affected startle amplitude (Fig. 7b) and no interaction was observed ($F_{(1,44)}$=0.9, p=0.339). Similarly, neither GBR12909 ($F_{(1,44)}$=1.7, p=0.204) nor lithium ($F_{(1,44)}$=2.4, p=0.132) affected PPI when split by ISI (Fig. 7c) and no interaction was observed ($F_{(1,44)}$=0.0, p=0.903). The startle amplitude of mice habituated over time ($F_{(4,176)}$=16.1, p<0.001; Fig. 7d), which interacted with GBR12909 and lithium treatment ($F_{(4,176)}$=3.7, p=0.007), but no *post hoc* effects were observed. Finally, GBR12909 increased movement when no stimuli were presented ($F_{(1,44)}$=8.5, p=0.006; Fig. 7*e*), without effect of lithium ($F_{(1,44)}$=0.4, p=0.555) or interaction ($F_{(1,44)}$=1.2, p=0.280).

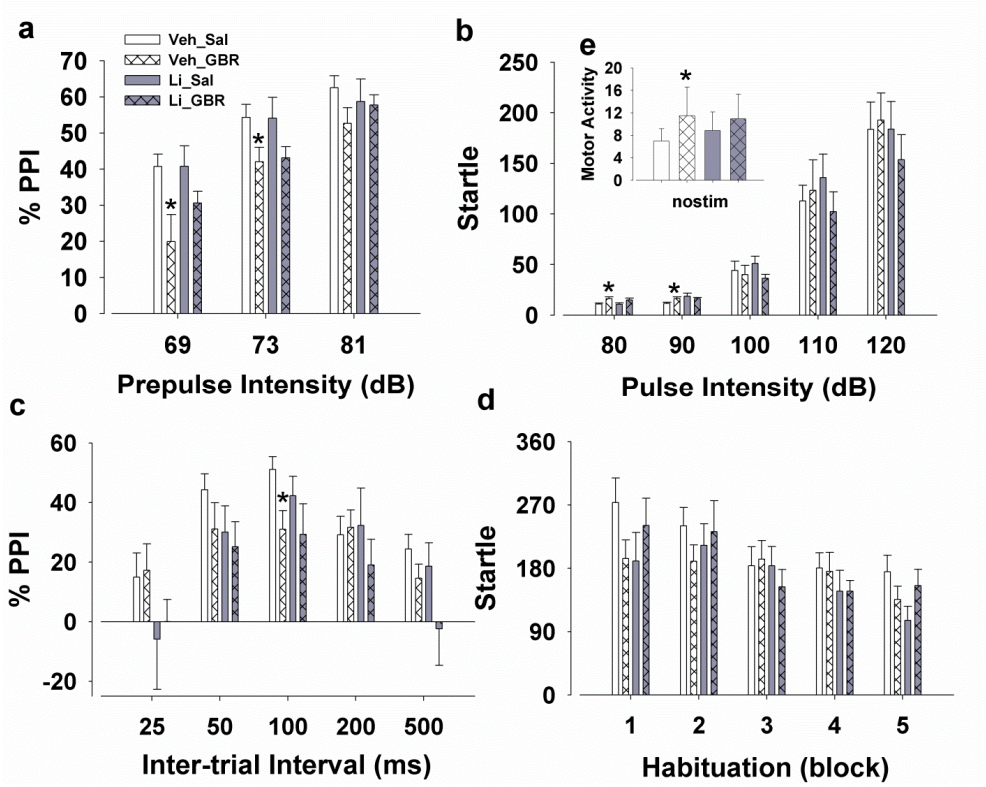

Fig. 7. Effects of chronic treatment with lithium 600 mg/l on sensorimotor gating as measured by prepulse inhibition (PPI) of mice administered acute GBR12909 (9 mg/kg). GBR12909 (GBR) decreased PPI in the vehicle (Veh)-, but not in the lithium (Li)-treated mice (a). GBR increased amplitude of the startle response in vehicle-treated mice at 80 and 90 dB pulses (b). When split by inter-stimulus interval (ISI), GBR decreased PPI in vehicle-treated mice at ISI 100 (c). All mice exhibited habituation to startle pulses over time, without significant effect of GBR or lithium (d). GBR increased motor activity in the vehicle-treated, but not in the lithium-treated mice when no stimulus was presented (e). Data are presented as mean ± SEM. n=12 animals per group. *p<0.05 when compared to saline.

DISCUSSION

We examined the potential use of cholinergic and dopaminergic manipulations to model BD facets of depression and mania in mice respectively. The AChE inhibitor physostigmine induced depression-like behavior in mice similar to human observations and consistent with increased ACh levels observed in the pathophysiology of depressed patients. Importantly for BD research, this increased ACh-induced "behavioral despair" was normalized by the mood-stabilizer lithium. Reducing DAT functioning via GBR12909 treatment induced a mania-like state in mice as measured by hyper-exploration and impaired PPI. Similar to its reversal of physostigmine-induced depression-like behavior, chronic lithium-treated mice receiving GBR12909 no longer differed in PPI from control mice. In contrast however, lithium potentiated GBR12909-induced hyper-exploration. Thus, separate models of both depression and mania that are in part treated by lithium can be generated pharmacologically by targeting differing etiologically relevant mechanisms.

Physostigmine administration induced depression-like behavior in mice, replicating its depression-inducing effects in humans (23). This depression-like behavior was interpreted from physostigmine increasing immobility times in the TST and FST at doses that were not sedative, an effect consistent with previous results of physostigmine-induced immobility in mice (20) and rats (55). This behavior may be hippocampally mediated because both viral AChE knockdown and local physostigmine infusion into the hippocampus induced depression-like behavior in mice (20). The authors reported that 0.5 mg/kg physostigmine induced depression-like behavior without affecting motor activity. In contrast, we observed that 0.03 mg/kg increased immobility without affecting locomotion, while 0.3 mg/kg severely depressed locomotion and immobility. Differences between these dosing reports remain unclear, although reduced locomotor activity has been observed before with physostigmine at doses above 0.1 mg/kg (47), but not as low as 0.03 mg/kg. Here, 0.03 mg/kg physostigmine increased immobility in the TST and FST, without affecting activity in the BPM, supporting the conclusion that physostigmine induced "behavioral despair"/ depression-like behavior opposed to overall depressed activity. While 0.03 mg/kg of physostigmine did not affect any BPM measure, higher doses decreased activity, exploration (0.1 and 0.3 mg/kg), and produced severely localized movements (0.3 mg/kg; Fig. 4). To determine the cross-species relevance of these findings, current studies are investigating the human exploratory profile of patients with both unipolar and bipolar depression in the BPM.

At high doses, physostigmine also increased PPI in the acoustic startle test, consistent with previous results at similar doses (48). Similar increases in PPI in mice matched for startle reactivity support the interpretation that physostigmine-induced increases in PPI do not

result from its sedation-inducing effects. Moreover, physostigmine at higher doses was likely less sedation-inducing due to later testing in PPI as indicated by increases in locomotor activity over time (Fig. 4f). The immobility-inducing dose of 0.03 mg/kg did not affect PPI however. Although PPI has yet to be studied in depressed bipolar patients, PPI is unaffected in unipolar depression (42, 43), to some extent supporting the disease-relevance of these findings. Future studies will investigate sensorimotor gating across the spectrum of BD, but it is likely that active manic symptoms and/or acute psychosis are necessary to exhibit PPI deficits (44).

Presently, we show that the DAT inhibition model reproduced PPI deficits similar to those observed in patients with BD mania (41). This effect is consistent with previous studies reporting impaired PPI in mice caused by GBR12909 at 5 mg/kg (56). Previously, we consistently observed a pattern of increased activity, increased exploration, and more linear patterns of movement in mice administered GBR12909 or DAT KD mice in the mouse BPM (25-27), mimicking the behavior of manic (28) and euthymic BD patients (30). In the current studies, GBR12909 at 9 mg/kg in the vehicle-treated mice did not reach significance levels although the measures were all in the expected direction. Strong significant main effects of GBR12909 on all measures further support its mania-like behavior inducing properties. We have observed small effect sizes with GBR12909 at 9 mg/kg before (27), but prior habituation of mice in the BPM may have also attenuated GBR12909's effects. Recently, we reported on 9 mg/kg GBR12909 inducing a mania-like pattern, which was attenuated by chronic valproate treatment (12). Moreover, the same dose increased measures of motivation (31) and sped responding in mice (33), while even lower doses (5 mg/kg) reduced immobility time in the FST (35). Importantly, GBR12909 exerted biological effects here reflected by reduced PPI in the acoustic startle test consistent with BD mania.

Previously, we showed that chronic valproate treatment in chow resulted in concentrations equivalent to human therapeutic blood levels for BD (12). This treatment only attenuated GBR12909-induced hyperactivity, not specific exploration in mice. We attempted to study chronic lithium in chow (1.2 and 2.4%) but these studies were stopped due to concerns for the health of the mice (e.g. polydipsia and weight loss, despite making saline available; unpublished observations). Lithium 600 mg/l administered chronically in drinking water however, resulted in low therapeutic blood concentrations commonly used for maintenance of mood during euthymia consistent with previous studies of 10-21 days lithium administration (Fig. 2) (13, 57, 58). Lithium at 300 mg/l resulted in serum concentrations below therapeutic levels and was not effective in reversing any BD-like behavior. No adverse health or weight effects were observed with 300 and 600 mg/l. Lithium at 1200 mg/l however, resulted in toxic blood concentrations, reduced the animals' body weights, and resulted in some death. Other studies examining 1200 mg/l of

lithium resulted in lower concentrations likely as a result from measurements taken in the brain (59) and only 10 days of treatment (50). Hence, the only positive effect of lithium in the present study was at 600 mg/l which resulted in serum levels that are prescribed for minimizing switches into mania or depression (maintenance).

Chronic lithium at 600 mg/l attenuated GBR12909-induced PPI deficits (Fig. 7). Although no directly interactive effects of lithium were observed on PPI, the GBR12909-induced PPI deficits were not present after lithium treatment. In contrast however, lithium worsened the effects of GBR12909 on exploratory behavior of mice in the BPM. Hence, lithium at 600 mg/l exerted some beneficial effects on GBR12909-induced alterations of behavior but worsened others. In terms of depression-like behavior, chronic lithium treatment at 600 mg/l normalized the physostigmine-induced immobility without affecting vehicle-treated mice. Physostigmine-induced immobility was similarly reversed by acute treatment with the antidepressant fluoxetine (20), although immobility was also reduced in control mice. The 'antidepressant' effect of lithium observed here may be due to compensation of physostigmine-induced reduction of AChE levels as lithium increased AChE levels in rats (60). Since chronic lithium is required to exert an antidepressant effect in acutely depressed BD patients (7), it is of importance to test chronic dosing regimens when assessing putative treatment effects (9). Although the physostigmine model may also represent components of unipolar depression, lithium is more effective in bipolar depression than unipolar depression (61). Taken together, lithium may treat depression-like symptoms via its ability to reduce ACh levels, but the mechanism underlying lithium's attenuation of GBR12909-induced PPI deficits yet potentiation of hyper-exploratory profile in the BPM remain unclear. This finding may be because lithium only prevents mania relapses in approximately 50% of patients (62), but further investigations are required. Future studies examining other behaviors and possible pharmacokinetic interactions between GBR12909 and lithium are also required.

The present data are consistent with the hypothesis that cholinergic mechanisms may underlie phases of depression, whereas dysregulated dopamine systems may underlie aspects of mania, supporting the cholinergic hypothesis of bipolar depression (24). More recently, lower β_2-subunit-containing nicotinic ACh receptor (β_2*-nAChR) availability has been observed in actively unipolar and BD depressed patients, which may be due to higher endogenous levels of ACh levels (18), yet euthymic BD patients exhibited normal levels (19). Hence, increased ACh levels may partly underlie BD depression and are restored during euthymic phases. During mania phases however, there may be a switch to a hyperdopaminergic mediation of behaviors (63-65). Reduced striatal DAT availability has been observed in euthymic patients (36) and postmortem tissue of BD patients (37). When we modeled this reduced DAT availability in mice, we consistently observe mania-like behaviors across a broad spectrum of behaviors (15, 25), including the current

sensorimotor gating deficits induced by the DAT inhibitor GBR12909. Because there is an important relationship between dopamine and cholinergic systems in the brain (66), the mechanisms underlying BD may comprise a cholinergic-monoaminergic interaction (67). Research is required investigating how the same subjects may cycle between putative different neurobiological states of hypercholinergia and hyperdopaminergia in depression and mania respectively. Altered light exposure has been theorized to contribute to these extreme behaviors (68) and indeed changing light exposure can switch neurotransmitter levels in rats (69). Utilizing this environmental manipulation alongside animals with a genetic susceptibility to BD (10) may provide a model animal with which to test novel therapeutics to treat the entire spectrum of BD. Thus, instead of reproducing depression- and mania-like behaviors using two separate manipulations, these combined challenges may mimic BD more accurately in a single model animal.

For future testing, improving the techniques used to measure depression-like behavior would prove useful. Although increased immobility times are commonly used to identify depression-like behavior, there is a debate on whether the FST and TST can only be used to screen for antidepressant-like activity. Future studies could assess more complex behaviors related to cognition given its importance to functional outcome for patients with BD (70). For example, examining dynamic decision-making in models of BD mania and depression could be useful. Patients across the spectrum exhibit poor decision-making (32), but this effect may be driven by a hypersensitivity to rewards during the manic phase (71), yet a hypersensitivity to punishments during the depressed phase (32). Future tests should also assess other treatments available such as antipsychotics and antidepressants in these putative models of BD depression- and mania-like behavior.

In conclusion, we described separate model animals of both depressive- and mania-like facets of BD by manipulating the cholinergic and dopaminergic pathways respectively. These models support the hypotheses that the neurobiological underpinnings may vary dependent upon the phases of BD. Understanding the mechanism(s) under which the neurobiology of patients change will be vital for blocking the cycling that occurs in patients. Meanwhile, the currently described models provide a way of testing novel therapeutics targeted at symptoms exhibited during different stages of BD.

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CHAPTER 6

A model animal for bipolar disorder based on dysfunctional circadian rhythms assessed in cross-species behavioral paradigms

CHAPTER 6.1

Further evidence for ClockΔ19 mice as a model for bipolar disorder mania using cross-species tests of exploration and sensorimotor gating

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ABSTRACT

Background

Bipolar disorder (BD) is a pervasive neuropsychiatric disorder characterized by episodes of mania and depression. The switch between mania and depression may reflect seasonal changes and certainly can be affected by alterations in sleep and circadian control. The circadian locomotor output cycles kaput (CLOCK) protein is a key component of the cellular circadian clock. Mutation of the *Clock* gene encoding this protein in *Clock*Δ19 mutant mice leads to behavioral abnormalities reminiscent of BD mania. To date, however, these mice have not been assessed in behavioral paradigms that have cross-species translational validity.

Methods

In the present studies of *Clock*Δ19 and wild-type (WT) littermate mice, we quantified exploratory behavior and sensorimotor gating, which are abnormal in BD manic patients. We also examined the saccharin preference of these mice and their circadian control in different photoperiods.

Results and Conclusions

*Clock*Δ19 mice exhibited behavioral alterations that are consistent with BD manic patients tested in comparable tasks, including hyperactivity, increased specific exploration, and reduced sensorimotor gating. Moreover, compared to WT mice, *Clock*Δ19 mice exhibited a greater preference for sweetened solutions and greater sensitivity to altered photoperiod. In contrast with BD manic patients however, *Clock*Δ19 mice exhibited more circumscribed movements during exploration. Future studies will extend the characterization of these mice in measures with cross-species translational relevance to human testing.

Keywords

Clock, Bipolar disorder, Mania, Cycling, PPI, Hedonia, Circadian

INTRODUCTION

Bipolar disorder (BD) in its various forms affects approximately 3% of the population and is a debilitating illness that impacts every aspect of the lives of sufferers and their loved ones (1). Current treatments for BD have been found serendipitously as no treatments have been developed specifically to target the mechanism(s) underlying this disorder (2). This lack of treatment development could reflect the simplicity of behavioral models used to date (2, 3) that neither recreate the mechanism underlying BD (4) nor reflect the complexity of BD symptoms. BD is a unique disorder in that it is characterized by sufferers cycling through periods of mania and depression, the symptoms of which differing markedly in these phases (4, 5). Mania is associated with hyperactivity, hypersexuality, risk-taking, less need for sleep, aggression, and hedonic behavior (6). Depression is largely the opposite however, with symptoms including low sex-drive, increased sleep, lethargy, and anhedonia (6). Surprisingly, sufferers of BD can cycle between these two states, often linked to the seasons of the year (7, 8). Such cycling may be explained by the evolutionary origin theory of BD, postulating that BD may have first arisen in people from the northern hemisphere where lengthening and shortening photoperiods (daylight length) in the summer and winter respectively induced mania- and depressive-like behaviors (9). This theory provides an avenue by which BD may be modeled since this theory suggests that alterations in the photoperiod length underlie BD. Hence, by examining mechanisms regulating circadian rhythms, it may be possible to model aspects of BD.

The basic molecular loop that regulates circadian rhythms consists of transcription factors regulating their own expression over 24 h (10). The circadian locomotor output cycles kaput (CLOCK) protein binds to brain and muscle ARNT-like protein 1 (BMAL1). The heterodimer then regulates the expression of the period (Per) and cryptochrome genes, which bind together as proteins, enter the nucleus, and inhibit CLOCK and BMAL1 activity (11). These systems are entrained by light *via* the suprachiasmatic nucleus (12), which may explain why light therapy works for sufferers of seasonal affective disorder (13), a depression that occurs during short photoperiod seasons (*i.e.*, winter). Moreover, it is recognized that there is a disruption in the circadian rhythm in people with BD (14). Social rhythm therapy – generating rhythms of behavior that are consistent from day to day (15) – or using extended bed rest and darkness (16) reduced some symptoms of BD. Interestingly, sleep deprivation can alleviate depression symptoms (17), but can also induce a manic episode in people with a predisposition for BD (18). Thus, altered circadian rhythm can impact the current state of people with BD.

Because behaviorally augmenting the circadian rhythm is beneficial for aspects of BD, it will be useful to investigate whether disrupting these rhythms produces BD-relevant behaviors. Mice with a deletion of exon 19 in the CLOCK gene (*Clock*Δ19 mice) exhibit

abnormal behaviors that have been interpreted as 'mania-like'. For example, *Clock*Δ19 mice are hyperactive, exhibit an altered circadian rhythm, spend less time immobile in a forced swim test, exhibit a preference for sweet sucrose solution and cocaine, and have lower reward thresholds identified using intra-cranial self-stimulation (19, 20). Importantly, some of these behaviors are attenuated by lithium treatment (19), a common treatment for BD. The mania-like behavior of these mice may be mediated by increased dopamine firing in the ventral tegmental area, which can also be reversed by lithium treatment (21). Moreover, genetic associations of a polymorphism in the 3' flanking region of the CLOCK (3111 T to C) in people with BD was linked with more frequent episodes of mood disturbances and reduced need for sleep (22, 23). Hence, it has been postulated that *Clock*Δ19 mice model aspects of BD.

While evidence continues to be collected that *Clock*Δ19 mice may be a viable model for aspects of BD, as yet no studies have directly utilized cross-species tests to examine the validity of this model in terms of behaviors quantified in people with BD. Previously, we utilized measures of behaviors that are available in both humans and rodents, *e.g.*, exploration in the behavioral pattern monitor (BPM) and sensorimotor gating measured by prepulse inhibition (PPI) of the startle reflex, to model aspects of psychiatric disorders (24). For example, using the BPM we identified that acutely manic patients with BD exhibit hyperactivity (25), increased specific exploration (26), and more direct movements through space (27, 28). Moreover, this abnormal exploration is consistent over time (29), and can be recreated in mice by selectively reducing the function of the dopamine transporter (DAT) *via* genetic or pharmacological means (27, 28, 30-33). Reduced PPI has been observed in people with BD (34), a behavior that can also be modeled in rodents (35, 36). Similarly, PPI is impaired in mice with a hyperdopaminergic tone due to a lack of DAT, an impairment that can be attenuated with antipsychotic treatment (37, 38). Assessing *Clock*Δ19 mice in tests having cross-species translational validity would test the appropriateness of these mice as a model for BD.

Herein, we utilized the cross-species BPM and PPI paradigms to examine the similarity of profiles of *Clock*Δ19 mice to BD mania. Moreover, we examined the behavior of these mice in the saccharin preference test (39), in order to assess hedonia-like behavior, and their circadian rhythm in response to altered photoperiod lengths. In parallel with BD, we hypothesized that *Clock*Δ19 mice would exhibit: (1) an abnormal exploratory profile of increased activity and specific exploration; more straight-line movements through space; (2) impaired sensorimotor gating; (3) hedonia-like preference for rewarding stimuli; and (4) less control over their circadian rhythm in response to altered photoperiod lengths.

METHODS

Animals

*Clock*Δ19 mutant mice with a dominant-negative CLOCK protein defective in transcriptional activation activity were created through *N*-ethyl-*N*-nitrosourea mutagenesis as described (40). Male (*n*=20) and female (*n*=13) *Clock*Δ19 mutant mice and male (*n*=17) and female (*n*=22) wildtype (WT) littermate controls on a mixed BALBc:C57BL/6 background were used throughout the different studies. *Clock*Δ19 heterozygous breeders were sent to our laboratory from David Welsh, (University of California San Diego; UCSD). All *Clock*Δ19 WT and mutant mice used in the present studies resulted from a heterozygous breeding colony in the vivarium at UCSD. Mice were group housed (maximum 4/cage, 2/cage for the saccharin and circadian rhythm tests), maintained in a temperature controlled vivarium (21±1 ºC) on a reversed day-night cycle (lights on at 19:00, off at 07:00 h), and tested during the dark phase between 8:00 and 13:00 h. Mice were 3 - 5.5 months old at the time of all tests except for the saccharin and circadian rhythm tests, at which time mice were 11 months old. Mice had *ad libitum* access to water and food (Harlan, Madison, WI, USA) except during testing. All procedures were approved by the UCSD Institutional Animal Care and Use Committee. The UCSD animal facility meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care.

Mouse behavioral pattern monitor

Locomotor behavior and exploration was examined in eight mouse BPM chambers (BPM; San Diego Instruments, San Diego, CA) as described previously (41-43). In brief, each Plexiglas chamber consists of a 30.5 cm × 61 cm × 38 cm area, equipped with three floor holes and eight wall holes (three along each side of the long walls and one in each of the short walls; 1.25 cm in diameter, 1.9 cm from the floor; see Fig. 1), containing infrared photobeams to detect holepoking behavior. Each chamber is enclosed in an outer box to minimize external light and noise, with an internal white light above the arena (producing 350 lux in the center and 92 lux in the four corners). Subject activity was obtained from a grid of infrared photobeams 1 cm above the floor (2.5 cm apart along the length and the width of the chamber; 24 × 12 *X*-*Y* array), recording the location of the mouse every 0.1 s. Rearing behavior was detected by another set of 16 photobeams, located on the *Y*-axis only and placed 2.5 cm above the floor. The subject's position was defined across nine unequal regions (four corners, four walls and center (44)). At the start of the session, the mouse was placed in the bottom left-hand corner of the arena and the test session started immediately for a period of 60 min. Primary measures obtained were transitions across the defined regions and center entries (locomotor activity), holepoking, rearing, and center duration (exploratory behavior), and entropy (*h*) and scaling measures (locomotor patterns). Lower values of *h* suggest predictable, ordered sequences of activity, while

higher values of *h* indicate greater variety or disorder of movement. Spatial *d* quantifies the geometric structure of the locomotor path (see Fig. 1), where a value of 1 represents a path in a straight distance-covering line, and 2 highly circumscribed small-scale movements (45). The spatial coefficient of variation (CV) is a measure of the *X-Y* pattern representing the variation of transitions across the nine regions. Spatial CV increases when the mouse repeats certain transitions across the chamber regions. The temporal CV measures the amount of time spent in each region, where a high temporal CV indicates a substantial preference for some region(s) over others (44).

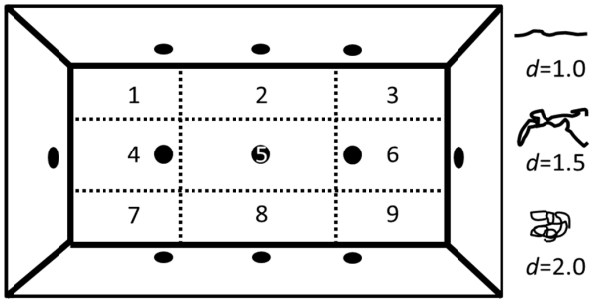

Fig. 1. Schematic of the mouse behavioral pattern monitor. The arena was divided into nine unequal regions (1 – 9) on which transitions, center time, center duration, and the coefficient of variation calculations are based. The quantifiable measure spatial d was used to describe the subject's pattern of movement with values represented in the schematic. The location of the mouse was obtained from a grid of infrared photobeams (24 × 12 *X-Y* array) located 1 cm above the floor. Another set of 16 photobeams located 2.5 cm above the floor on the *Y*-axis only was used to detect rearing behavior. The chamber is equipped with three floor holes and eight wall holes (1.2 cm diameter), each containing an infrared photobeam to detect holepoking behavior.

BPM – initial assessment

Male (*n*=20) and female (*n*=13) *Clock*Δ19 mutant mice and male (*n*=17) and female (*n*=22) WT littermate controls were tested in the BPM to examine the exploratory profiles of these mice.

BPM – repeated test to examine reproducibility of effect

A subgroup of mice from experiment 1 (*Clock*Δ19 mutant male *n*=6; female, *n*=4; WT male, *n*=7; female, *n*=7) were retested in the BPM one week after their initial testing. This test was conducted to determine whether any abnormal exploratory behavior exhibited by mutant mice would be reproducible.

Sensorimotor gating of the acoustic startle response

Sensorimotor gating of the acoustic startle response of a behaviorally naïve cohort of *Clock*Δ19 mice (WT male, *n*=7; female, *n*=7; mutant male, *n*=6; female, *n*=4) was examined in eight startle chambers (SR-LAB, San Diego Instruments, San Diego, CA), each consisting of a Plexiglas cylinder, 5 cm in diameter, resting on a platform in a ventilated sound-

attenuating chamber as described previously (37, 46). Speakers mounted 33 cm above the cylinders produced all acoustic stimuli and an interface and computer assembly stored and digitized movements of the animal transduced by piezoelectric accelerometers mounted under the cylinders. A 65 dB background sound and light delivered by an incandescent bulb located on the ceiling of the chamber were presented continuously throughout the session. Mice were placed into the startle chambers and testing was initiated after a 5 min acclimation period. Startle pulses were 40 ms and prepulses were 20 ms in duration. The inter-trial interval between stimulus presentations ranged between 3 and 12 s (7 s average) for both experiments. The acoustic startle sessions included five blocks. The first block included only five 120 dB pulses. The second block consisted of three different prepulse trials: 69, 73, and 81 dB prepulses preceding a 120 dB pulse. The third block included acoustic startle responding only and included stimulus intensities of 80, 90, 100, 110, and 120 dB. The fourth block varied the inter-stimulus interval (ISI), consisting of 73 dB prepulses preceding pulses at 120 dB by 25, 50, 100, 200, and 500 ms. The fifth and final block delivered five 120 dB pulses and together with the first block served to assess habituation. This test session has been used and described previously (46). The amount of PPI was calculated as a percentage score for each prepulse intensity based on the 120 dB pulse within that block: %PPI = 100 – [(startle magnitude for prepulse + pulse / startle magnitude for pulse alone) × 100].

Sweet solution preference

The design of this test was based on previous mania-modeling studies of (39). A subset of male *Clock*Δ19 mutant (*n*=8) and WT (*n*=8) mice from the BPM experiment were supplied with a bottle of 1.0% saccharin sodium dihydrate solution (Sigma, St, Lewis, MI), dissolved in tap water, on top of the regular supply of water and food. Both the regular water bottle and the saccharin solution bottle were available to the mice throughout the entire sweet solution preference test. Both bottles were weighed at the beginning of the study and 24 h thereafter, for 4 days. Sweet solution preference was calculated daily as a percentage of saccharin solution out of total liquid consumption.

Measuring running wheel activity to assess circadian rhythm

After the sweet solution preference experiment, male *Clock*Δ19 mutant (*n*=8) and WT (*n*=8) mice were housed by genotype with 2 mice per cage. Mice had access to a running wheel (Silent Spinner; Forest city, Iowa). The level of running wheel activity was measured using a cyclcomputer (Easton-Bell Sports; Van Nuys, CA) to determine the distance traveled by mice over time. Measurements were taken at 07:00 and 19:00 h daily for 10 days, coinciding with the time room lights were turned off and on respectively as a surrogate measure of circadian rhythm. Mice were initially exposed to a 12 hr light/dark (LD) cycle. After stable running wheel activity was established (Day 7, see Results), the lighting of the room was altered to increase the inactive light period. Thus, while lights

continued to be turned off at 07:00 h, lights were turned back on at 08:00 h (LD 23:1). The running wheel activity of mice continued to be measured at 07:00 and 19:00 h.

Statistical analyses

Data from the BPM were analyzed using two- or three-way analyses of variance (ANOVA), with sex and genotype as between-subject factors and trial period (three 20 min periods) as a within-subject factor. PPI data were analyzed using a two- or three-way ANOVA, with prepulse intensity as a within-subject factor and genotype and sex as a between-subject factor. Further assessments used startle-matched subgroup comparisons to assess PPI. Sweet solution preference was analyzed using a repeated measure ANOVA with day as a within-subject factor and genotype as a between-subject factor. Running wheel activity was assessed using a three-way ANOVA with genotype as a between subject factor while time of measurement and day were within-subject factors. Tukey *post hoc* analyses were performed where applicable. When no effect of sex or interaction with sex was observed, data were pooled and reanalyzed. Pearson *r* correlation coefficients measured the relationship between BPM measures from the first to the second test. All BPM and PPI data were analyzed using Biomedical Data Programs statistical software (Statistical Solutions Inc., USA), while sweet solution preference and running wheel activity levels were analyzed using SPSS (19.0, Chicago, IL, USA). The α level was set to 0.05.

RESULTS

BPM Exploration: initial characterization

To assess the exploratory profile of *Clock*Δ19 mice, mutant (*n*=33) and WT littermate (*n*=39) mice were tested in the BPM for 60 min. There were no interactions with sex for any of the measures. Male and female data were therefore pooled and analyzed together. Data are presented with variables grouped into domains of locomotor activity, specific exploration (holepoking) and diversive expoloration (rearing), and locomotor patterns based on the primary variables affected in people with BD mania (28), as well as factor analyses of rat and mouse BPM behavior (41, 47).

Locomotor activity

*Clock*Δ19 mutant mice were hyperactive with representative *X-Y* patterns and average activity level heat maps, presented in Fig. 2. The hyperactivity of mutant mice was quantified by increased transitions ($F_{(1,70)}$=19.8, p<0.0001; Fig. 3a) and increased center entries ($F_{(1,70)}$=18.6, p<0.0001; Fig. 3b) compared to WT mice. A trend towards a time by genotype interaction was observed for transitions ($F_{(2,140)}$=2.5, p<0.1). *Post hoc* analyses revealed that mutant mice exhibited more transitions compared to WT mice in each time period however (p<0.05).

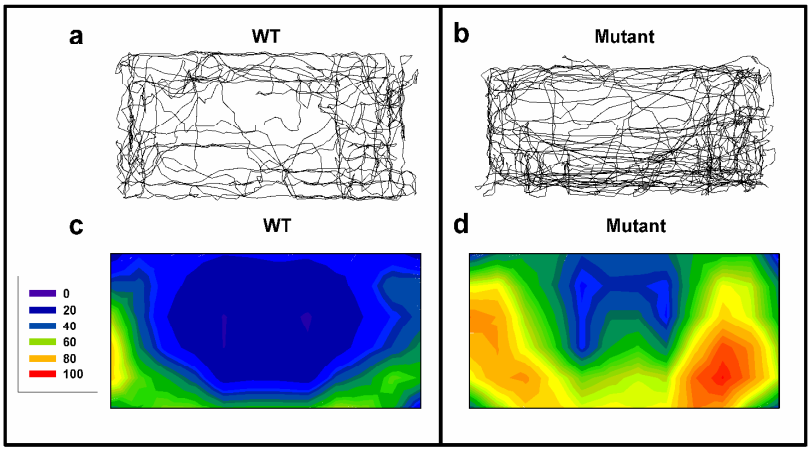

Fig. 2. *X-Y* plots and heat maps of *Clock*Δ19 WT and mutant mice. Representative *X-Y* plots of wild-type (WT) and mutant mice (a,b) as well as heat maps representing the average group data based on 72 evenly distributed sector entries (c,d) are displayed. *Clock*Δ19 mutant mice (b,d) exhibited increased activity and center entries compared to WT mice (a,c). Moreover, more disordered patterns of movement were noticeable in the mutant mice compared to WT mice.

Exploratory behavior

Over 60 min, *Clock*Δ19 mutant mice exhibited greater exploration as reflected by increased rearing ($F_{(1,70)}$=5.9, p<0.05; Fig. 3c), but not holepoking (F<1, ns; Fig. 3d) compared to WT mice. Analyzed within time however, interactions with genotype interactions were observed for both rearing ($F_{(2,140)}$=4.0, p<0.05) and holepoking ($F_{(2,140)}$=5.4, p<0.01). *Post hoc* analyses revealed that mutant mice made fewer holepokes than WT mice only in time period 1 (p<0.05), while exhibiting increased rearing compared with WT mice in the latter 2 time periods (p<0.05). Mutant mice also spent significantly more time in the center compared to WT mice (center duration; $F_{(1,70)}$=6.6, p<0.05; Fig. 3e), indicative of higher specific exploration (41).

Locomotor patterns

*Clock*Δ19 mutant mice moved in more circumscribed patterns compared to WT mice as reflected by increased spatial *d* ($F_{(1,70)}$=4.6, p<0.05; Fig. 3f). Mutant mice also exhibited a higher entropy ($F_{(1,70)}$=5.0, p<0.05; Fig. 3g) and lower temporal CV ($F_{(1,70)}$=7.0, p<0.05; Fig. 3h). Although there was a time × genotype interaction for temporal CV ($F_{(2,140)}$=3.5, p<0.05) *post hoc* analyses revealed that mutant mice exhibited a lower temporal CV in each time period however (p<0.05). No differences between genotypes were observed for spatial CV (F=1.1, ns; Fig. 3i).

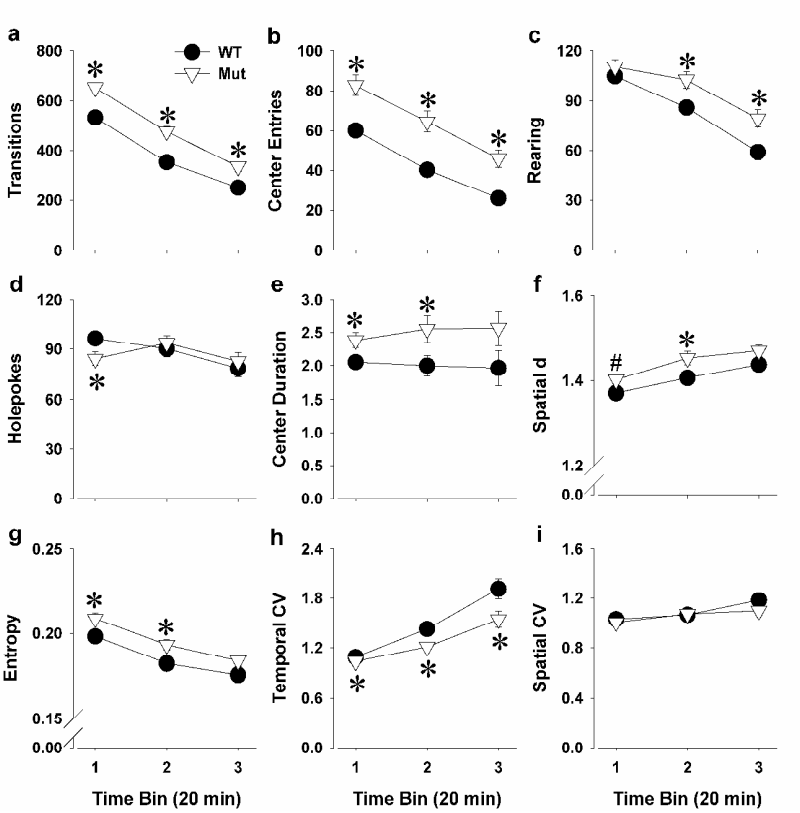

Fig. 3. The exploratory profile of *Clock*Δ19 WT and mutant mice in the BPM. *Clock*Δ19 mutant (Mut) mice were hyperactive compared to wild-type (WT) littermate mice as measured by increased transitions (a) and center entries (b). Mutant mice exhibited more specific exploration compared to WT mice as measured by increased rearing (c), but not holepoking (d). Mutant mice also spent significantly more time in the center of the arena (e). Mutant mice also exhibited more circumscribed or disordered patterns of movement compared to WT mice as reflected by a higher spatial *d* (f) and entropy *h* (g). Compared to WT mice, mutant mice exhibited less preference for specific regions in the arena as reflected by lower temporal CV (h), without an effect on spatial CV (i). Data are presented as mean +S.E.M. *$p<0.05$ and $^{\#}p<0.1$ when compared to WT mice.

BPM exploration: examining the consistency of the exploratory profile.

To assess the consistency of the altered exploratory profile of *Clock*Δ19 mice, mutant (*n*=10) and WT littermate (*n*=14) mice were tested in the BPM for 60 min a second time one week later. Again, because there were no interactions with sex for any of the measures, data from male and female were pooled and analyzed together. Intra-subject comparisons between the two tests revealed significant correlations for all primary measures (Table 1).

Locomotor activity

*Clock*Δ19 mutant mice were hyperactive as reflected by increased transitions ($F_{(1,22)}$=26.7, p<0.0001; Fig. 4a) and increased center entries ($F_{(1,22)}$=19.7, p<0.0005; Fig. 4b) compared to WT mice. Time by genotype interactions were observed for transitions ($F_{(2,44)}$=3.2, p<0.05) and center entries ($F_{(2,44)}$=4.1, p<0.05). *Post hoc* analyses revealed that mutant mice exhibited increased transitions and center entries compared with WT mice in each time point (p<0.05).

Exploratory behavior

*Clock*Δ19 mutant mice exhibited higher exploration as reflected by increased rearing ($F_{(1,22)}$=22.1, p<0.0001; Fig. 4c), but again not for holepoking (F<1, ns; Fig. 4d) compared to WT mice. Genotype interacted with time period to affect holepoking ($F_{(2,44)}$=3.3, p<0.05), and *post hoc* analyses revealed that mutant mice exhibited a trend toward increased holepoking compared with WT mice in the last time period (p<0.1). Support for increased exploration in mutant mice was also observed with these mice spending more time in the center compared to WT mice (center duration; $F_{(1,22)}$=25.1, p<0.0001; Fig. 4e). A trend toward a time × genotype interaction was observed for center duration ($F_{(2,44)}$=2.9, p<0.1), with *post hoc* analyses revealing that mutant mice spent more time in the center than WT mice at each time point (p<0.05)

Locomotor patterns

Spatial *d* did not differ between genotypes (F<1, ns; Fig. 2f), but *Clock*Δ19 mutant mice exhibited a higher entropy ($F_{(1,22)}$=16.3, p<0.0005; Fig. 4g) and lower temporal ($F_{(1,22)}$=15.6, p<0.001; Fig. 4h) and spatial CV ($F_{(1,22)}$=8.8, p<0.01; Fig. 4i). A time × genotype interaction was observed for temporal CV ($F_{(2,44)}$=4.1, p<0.05). *Post hoc* analyses revealed that mutant mice exhibited a lower temporal CV in each time period however (p<0.05).

Table 1. Test-retest reliability of all primary measures in the BPM between testing days 1 and 2 as determined by correlation coefficients.

Measure	*R*-value	*p*-value
Transitions	0.76	<0.001
Holepoking	0.63	<0.005
Rearing	0.70	<0.001
Spatial *d*	0.84	<0.001
Entropy (*h*)	0.71	<0.001
Spatial CV	0.59	<0.005
Temporal CV	0.45	<0.05
Center duration	0.52	<0.05
Center entries	0.81	<0.001

CV = coefficient of variation.

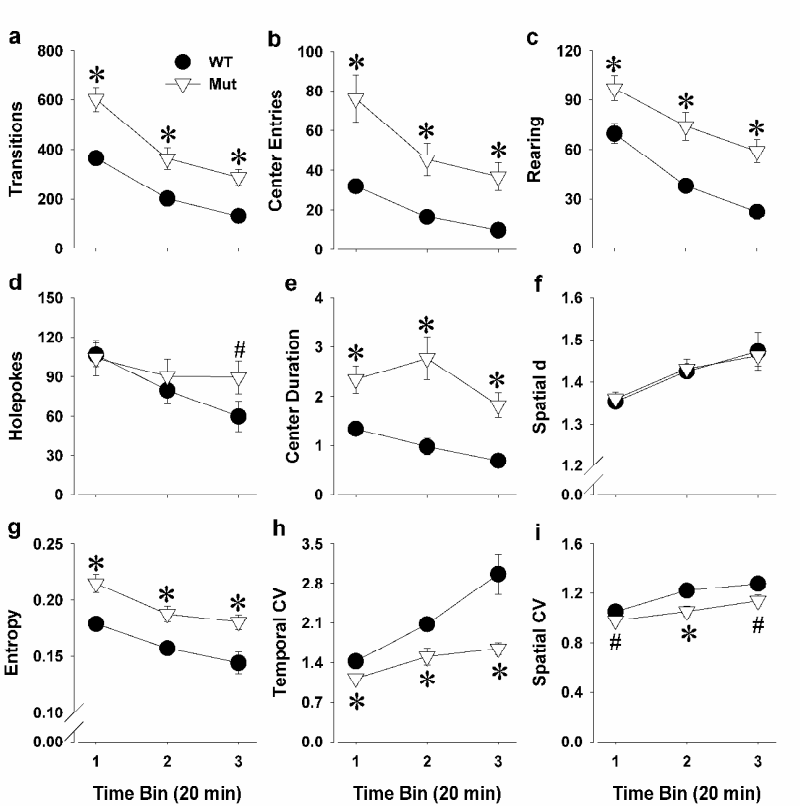

Fig. 4. The exploratory profile of *Clock*Δ19 WT and mutant mice tested in the BPM a second time, one week after their initial testing. *Clock*Δ19 mutant (Mut) mice remained hyperactive compared to wild-type (WT) littermate mice even upon retesting as measured by increased transitions (a) and center entries (b). More specific exploration was observed in mutant mice compared to WT mice as again reflected by increased rearing (c), but not so much holepoking (d). Mutant mice spent more time in the center of the arena compared to WT mice (e). Spatial *d* did not differ by genotype (*f*) in this second test, while mutant mice still exhibited disordered patterns of movement compared to WT mice as reflected by higher entropy *h* (g). Compared to WT mice, mutant mice exhibited a lower temporal and spatial CV, reflecting less preference for and reduced repetitive transitions between specific regions. Data are presented as mean +S.E.M. $*p<0.05$ and $^{\#}p<0.1$ when compared to WT mice.

Sensorimotor gating

To assess the sensorimotor gating of *Clock*Δ19 mice, mutant (n=10) and WT littermate (n=14) mice were tested on prepulse inhibition (PPI) in the acoustic startle test. There were no interactions with sex for any of the acoustic startle measures. Male and female data were therefore pooled and analyzed together.

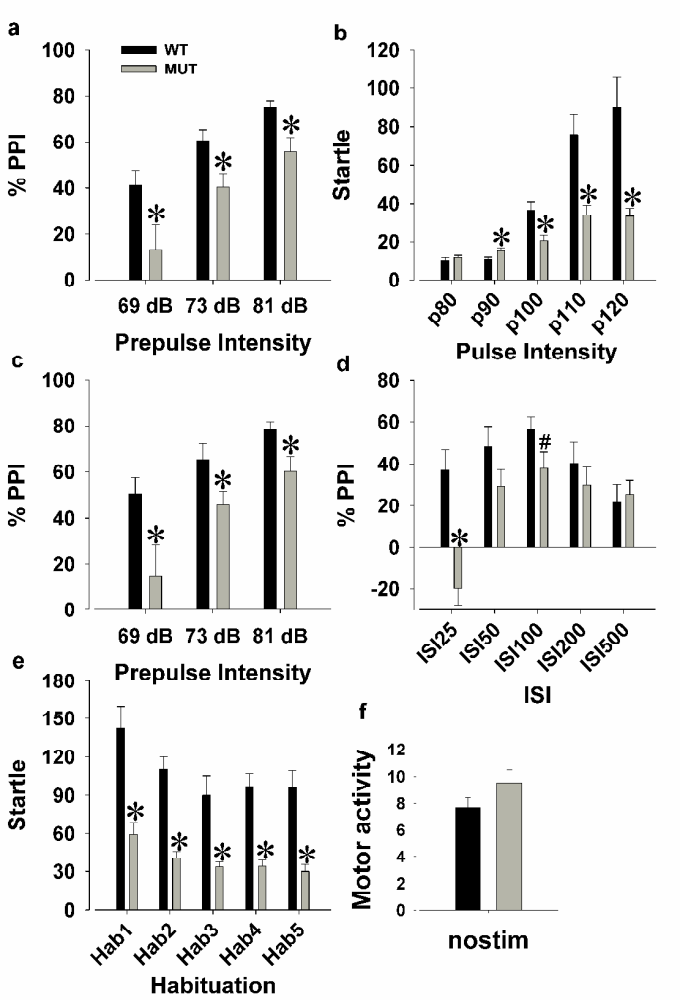

Fig. 5. Evaluation of the sensorimotor gating of the acoustic startle response of *Clock*Δ19 WT and mutant mice. *Clock*Δ19 mutant (Mut) mice exhibited significantly lower prepulse inhibition (PPI) compared to wild-type (WT) littermate mice (a), but also exhibited reduced overall amplitude of the startle response (b). When mice were matched by startle amplitude and compared, mutant mice still exhibited significantly lower PPI compared to WT mice (c). When split by inter-stimulus interval (ISI), mutant mice exhibited a PPI deficit compared to WT mice at ISI 25 and ISI 100 (d). Both WT and mutant mice exhibited habituation over time, although mutant mice had lower startle amplitude compared to WT mice at each habituation phase (e). No difference between genotypes was observed when no stimulus was presented (f). Data are presented as mean ± S.E.M. *$p<0.05$ and $^{\#}p<0.1$ when compared to WT mice.

A main effect of prepulse ($F_{(2,44)}=51.9$, $p<0.0001$) and no interaction with genotype ($F<1$, ns) revealed that the sensorimotor gating of mice improved with higher prepulse intensities. Importantly, mutant mice exhibited a significant PPI deficit compared with WT mice ($F_{(1,22)}=8.4$, $p<0.01$; Fig. 5a) at every prepulse intensity ($p<0.05$). Mutant mice

exhibited a lower startle amplitude than WT mice ($F_{(1,22)}$=13.5, p<0.005; Fig. 5b), with a pulse × genotype interaction ($F_{(4,88)}$=7.4, p<0.005). *Post hoc* analyses revealed that mutant mice exhibited lower startle than WT mice at pulse intensities 90-120 (p<0.05). An increased startle amplitude with higher pulse intensities was observed for both genotypes ($F_{(4,88)}$=22.9, p<0.0001). Consistent with previous studies when startle differences were observed (48), PPI was re-examined in WT and mutant mice matched for startle reactivity. Following baseline matching (WT, n=7; mutant, n=8), *Clock*Δ19 mutant mice still exhibited a significantly lower PPI compared to WT mice ($F_{(1,13)}$=5.8, p<0.05; Fig. 5c). We also addressed the potential influence of weight on startle measures and observed no difference in weight between WT (M=24.1 g) and mutant (M=27.1) mice (T=-1.7, ns), including the subgroup matched for startle reactivity (WT; M=24.1 g, mutant; M=27.3 g, T=-1.4, ns). Furthermore, weight did not influence PPI or startle reactivity as measured by linear regression (overall, $F_{(1,22)}$<1, ns; in WT only, $F_{(1,12)}$<1, ns; or in mutant only, $F_{(1,8)}$<2, ns). There was a trend effect of mutant mice exhibiting lower PPI than WT mice when split by ISI ($F_{(1,22)}$=3.6, p<0.1; Fig. 5d), with an ISI × genotype interaction ($F_{(4,88)}$=8.9, p<0.0001). *Post hoc* analyses revealed that mutant mice exhibited a PPI deficit at ISI 25 (p<0.05) and a trend towards a deficit at ISI 100 (p<0.1). Although both WT and mutant mice habituated over time ($F_{(4,88)}$=5.5, p<0.001), mutant mice again exhibited significantly lower startle levels ($F_{(1,22)}$=34.6, p<0.0001; Fig. 5e), with *post hoc* analyses revealing the presence of lower startle at each habituation phase. No difference between genotypes was observed for movements when no stimulus was presented (F=2.5, ns; Fig. 5f).

Sweet solution preference

Both WT (±75%) and ClockΔ19 mutant mice (±89%) exhibited a high sweet solution preference, which decreased over the four testing days ($F_{(3,42)}$=6.7, p<0.005; Fig. 6) independent of genotype. These preference levels are a little higher compared to previously described data of various mouse strains (39). No main effect of genotype was observed when analyzed over all four testing days ($F_{(1,14)}$=2.8, p=0.116). Given higher sucrose preference in these mice having been observed before, we examined their preference over individual days. When examined over days, mutant mice exhibited a higher sweet solution preference compared with WT mice on day 1 (p=0.05) and tended to be higher on day 4 (p<0.1).

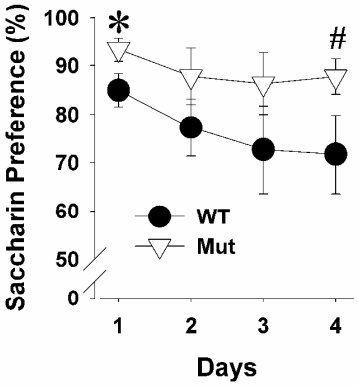

Fig. 6. Preference for 1.0% saccharin solution of *Clock*Δ19 WT and mutant mice across a four days test. Both wild-type (WT) and mutant (Mut) mice exhibited a preference for the sweet solution that decreased over time. *Clock*Δ19 mutant mice exhibited a higher preference compared to WT mice on the first and last day. Data are presented as mean ± S.E.M. $^{*}p<0.05$ and $^{\#}p<0.1$ when compared to WT mice.

Running wheel-based assessment of circadian rhythms

Running wheel activity levels of WT and mutant mice were initially assessed in an LD 12:12 light/dark cycle (12 h light, 12 h dark; Fig. 7). *Clock*Δ19 mutant mice exhibited more activity overall ($F_{(1,14)}=22.9$, $p<0.0001$), in both the dark ($F_{(1,14)}=45.1$, $p<0.0001$) and light ($F_{(1,14)}=17.2$, $p<0.0001$) phases. Mice were more active in the dark period ($F_{(1,14)}=31.3$, $p<0.0001$), but the size of this effect depended on genotype ($F_{(1,14)}=9.5$, $p<0.01$), reflecting a greater increase of dark period activity compared with light period in the mutant mice (large effect size $d=1.02$) compared with WT mice (medium to large effect size $d=0.59$). After introduction of the running wheels, all mice increased activity over the days in LD 12:12 ($F_{(6,84)}=6.7$, $p<0.001$), independent of genotype ($F=2.1$, ns), and reached stable levels by day 5, as days 5, 6, and 7 did not differ in the WT or mutant mice ($p>0.1$).

Subsequently, the light cycle was changed from LD 12:12 to LD 23:1 (23 h light, 1 h dark). Over the next three days, the activity of the mice continued to be measured during the 12 h previously in dark (active phase) and the 12 h previously in light (rest phase). A significant interaction between day, phase, and genotype was observed ($F_{(2,28)}=21.5$, $p<0.0001$), with *post hoc* analyses revealing that in those three days, WT mice continued to exhibit more activity in active phase compared with rest phase for all three days ($F_{(1,7)}=7.2$, $p<0.05$), while *Clock*Δ19 mutant mice only exhibited such a distinct difference on day 1 ($F_{(1,7)}=6.0$, $p<0.05$), but not on days 2 or 3 ($F<1$, ns). Despite the change in lighting, mutant mice remained more active than WT mice irrespective of phase ($F_{(1,14)}=22.6$, $p<0.0001$).

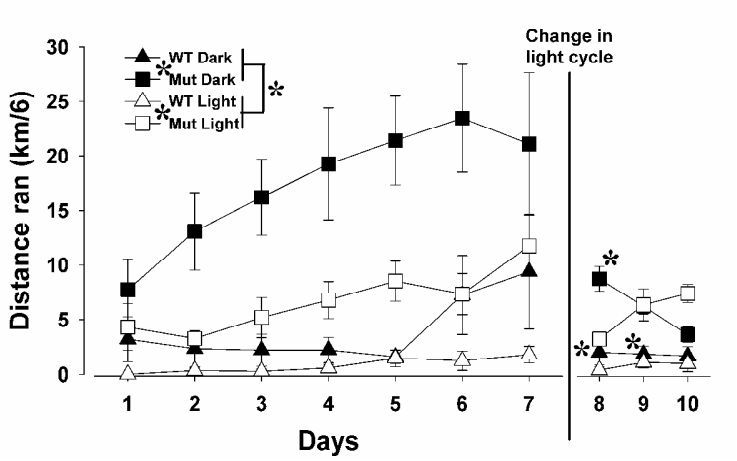

Fig. 7. Home cage running wheel activity of *Clock*Δ19 WT and mutant mice across seven days of LD 12:12 and three days of LD 23:1. Both WT and mutant (Mut) mice were more active during the dark (D) than the light (L) phase during the seven days of LD 12:12. For the first two days in LD 23:1, WT mice maintained greater activity during the 12 h previously in darkness (active phase). Mutant mice however, rapidly lost maintenance of their circadian rhythm by the second day of LD 23:1, exhibiting equal activity during the 12 h previously in darkness (active phase) and the 12 h previously in light (rest phase). Finally, mutant mice were more active than the WT mice in both photoperiods. Data are presented as mean home cage running activity ± S.E.M. *p<0.05 when compared with mutant mice, #p<0.05 when compared with activity during what was the 12 h L cycle.

DISCUSSION

*Clock*Δ19 mutant mice exhibited abnormal behavior in several cross-species tests that measure aspects of BD mania. Mutant mice were hyperactive and exhibited increased specific exploration in the BPM, consistent with patients with BD in a manic (26, 28) and euthymic phase (29). The mutant mice also exhibited altered startle responses and modest sensorimotor gating deficits similar to patients with BD (34). Moreover, we have replicated the preference of mutant mice for sweet solution but using a non-caloric saccharin solution. Finally, we confirmed that these mice are even hyperactive in their home cage and importantly, that mutant mice exert less control of their circadian rhythm of activity in response to altered photoperiods. Thus, here we provide further support that *Clock*Δ19 mutant mice share numerous similarities to patients with BD by using cross-species translational tests.

The present studies of increased transitions and center entries support previous reports of hyperactive behavior in *Clock*Δ19 mutant mice both in a novel environment and in their home cage (19). Importantly, these findings are consistent with the increased activity of patients with BD both in a manic and euthymic state (28, 29). Moreover, because the present studies examined exploratory behaviors of *Clock*Δ19 mutant mice in the BPM, we also quantified increased exploration as measured by increased rearing and center

duration in these mice, which collectively load onto a diversive exploratory factor (41). These findings go beyond simple hyperactivity and provide further consistency to increased object interactions of patients with BD (28). Besides increased exploration, the increased time spent in the center by *Clock*Δ19 mutant mice could be related to their reduced anxiety/increased risk-seeking behavior (19). Future studies on tasks measuring risk-proneness are required however (49, 50). Furthermore, consistent with patients with BD and in contrast to patients with schizophrenia (28), *Clock*Δ19 mice habituated rapidly to their testing environment. In contrast to both patients with BD or schizophrenia however, mutant mice exhibited increased spatial *d*, reflecting more circumscribed exploratory movement, compared with more linear movement in these patients (28). We have previously demonstrated that both pharmacological and genetic reduction of DAT functioning, which increases extracellular dopamine (51), resulted in reduced spatial *d*, consistent with patients with BD and schizophrenia (28, 31-33) that was untreated by chronic valproate (30). Increased spatial *d* can occur however, when there is a unilateral increase of dopamine in the brains of mice, such as the chakragati mouse model of schizophrenia (unpublished observations). *Clock*Δ19 mice exhibit increased dopamine firing from the VTA (21), but it is unclear whether this is bilateral or unilateral. While BD treatments such as lithium (19, 52-54) and valproate (30) can normalize hyperactivity in animal models of BD mania, normalization of spatial *d* has yet to be demonstrated. Thus, while *Clock*Δ19 mutant mice share many characteristics of the abnormal exploration of patients with BD mania, some differences exist that require investigation.

Psychiatric populations, including patients with BD and schizophrenia exhibit impaired sensorimotor gating, as measured by PPI (34, 55, 56). Despite the cross-species availability of PPI testing, to date these are the first studies to assess the PPI of *Clock*Δ19 mutant mice. We used a paradigm designed to quantify PPI across prepulse intensities and inter-stimulus intervals, startle amplitude in response to varying pulses, and startle habituation over time (57). This study revealed that *Clock*Δ19 mutant mice exhibit reduced PPI, complicated by a reduced startle response in these mice. Importantly however, when mice were matched for baseline startle response (48, 58), the PPI deficit of *Clock*Δ19 mutant mice compared with WT mice was still observed. Thus, mutant mice exhibit sensorimotor gating deficits similar to people with BD.

The present data demonstrate that *Clock*Δ19 mutant mice share several characteristics with people with BD, but also some with people with schizophrenia as described above. Another characteristic consistently reported in *Clock*Δ19 mutant mice is increased reward preference as measured by reduced stimulation threshold and increased preference for sugared solutions (19). Hedonia, including increased reward seeking, is a defining characteristic of BD mania as described in the DSM IV, and differs from people with schizophrenia whom traditionally are described as anhedonic, reflected by the need of

greater stimulation and reduced preference for rewards (59). The present findings extend the hedonia-like behavior of *Clock*Δ19 mutant mice, describing their preference even for non-caloric sweetened solutions (*i.e.*, saccharin solution). Hence, despite some characteristics of the mutant mice that overlap with those of schizophrenia, our findings support these mice as modeling mania, including hedonia-like behaviors.

Previous studies have identified an altered circadian rhythm of *Clock*Δ19 mutant mice (60). Indeed, the present studies support more activity of the mutant compared with WT mice during periods in which the mice should be inactive. Mutant mice were more active than WT mice overall however. Hence, more importantly our findings provide evidence for a direct consequence of the dysregulated circadian rhythm of these mice in response to aberrant photoperiod length. When the *Clock*Δ19 mutant and WT mice were challenged with the aberrant LD 23:1 photoperiod, we found increased activity of mutant mice during the rest phase. WT mice continued to exhibit circadian entrainment, suggesting increased resistance to photoperiod changes. Thus, *Clock*Δ19 mutant mice may represent a vulnerability genotype that is more susceptible to changes in photoperiod, which are known to affect mood states in patients with BD (4). Altering photoperiod or putting nocturnal animals in constant light can induce depressive-like behaviors (61) that can be rescued using antidepressants (62). Although shRNA-induced knockdown of CLOCK in the VTA of mice induced both mania- and depression-like behaviors in mice (63), to date no one has demonstrated such behaviors in response to environmental manipulations, such as changes in photoperiod. Future studies will determine whether photoperiod challenges will alter the mania-like behavioral phenotype of these *Clock*Δ19 mutant mice.

*Clock*Δ19 mutant mice exhibit increased dopaminergic firing in the VTA (21). This increased firing rate may underlie many of the behavioral abnormalities observed here and elsewhere, given the similar profile of these mice to hyperdopaminergic mice mediated by reduced DAT expression (27, 28, 31-33). Moreover, additional studies support increased dopamine release and turnover in the striatum of *Clock*Δ19 mutants, resulting in increased dopamine D_1 and D_2 protein expression, with a shift to increased D_2 *vs.* D_1 signaling (64). The dopamine reward hypothesis postulates that striatal dopamine receptors such as D_1 and D_2 play critical roles in all forms of learning (65, 66). Thus, altered dopamine D_1 and D_2 receptor signaling will likely alter learning mechanisms, which can be measured similarly to humans (67). People with depression and mania exhibit numerous neurocognitive deficits (68, 69). Such deficits include impaired probabilistic learning and decision-making behavior and are mediated by hypersensitivity to punishment in depression (70) and reward (71) in mania. Hence, tasks such as the Iowa gambling task (72) could be used to determine putative changes in 'mood state' in these mice resulting from environmental challenges (3). Hence, future studies will determine

the neurocognitive performance of the *Clock*Δ19 mutant mice, such as attention in a continuous performance test (73, 74), spatial working memory (75, 76), and decision-making under risk conditions in an Iowa gambling task (49, 50). Such studies will be vital in the future given the correlation between cognition and a subject's functional capabilities (77, 78).

In conclusion, we provide further evidence that *Clock*Δ19 mutant mice can be used to model aspects of BD mania by using tasks that have been utilized in patients with BD mania. *Clock*Δ19 mutant mice are not only hyperactive, but also exhibit increased specific exploration, a key aspect of abnormal exploration in patients with BD. Mutant mice also exhibited impaired sensorimotor gating, which was still evident after normalizing for the reduced baseline startle amplitude observed in these mice. Such characterization provides a platform for putative treatments tested in this model to be validated in equivalent human tests. The increased preference for saccharin solution extends previous findings of hedonia-like behavior observed in *Clock*Δ19 mutant mice. Finally, the poor circadian control of the *Clock*Δ19 mutant mice in an abnormal photoperiod supports further studies of whether photoperiod challenges can induce depressive-like behaviors in these mice.

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CHAPTER 7

Discussion and conclusions

This thesis describes the testing and validation of animal models for BD based on abnormal behaviors observed in patients using similar test paradigms. We investigated the putative mechanisms underlying symptomatology of BD. Pharmacological and genetic manipulations were done in mice to assess BD-like behavior. A range of dysfunctional behaviors was observed in these model animals similar to those exhibited by patients with BD. Observed mania-like behaviors included hyperactivity, hyper-exploration, and impaired PPI. Other deficits such as poor decision-making, increased motor impulsivity, increased motivation, and reduced vigilance were also identified. Depression-like behavior was inferred from observations of increased immobility ("behavioral despair"). Behaviors related to BD mania were mediated by manipulations of the DAT and CLOCK genes in mice, whereas depression-like behavior was influenced by the cholinergic system. Finally, the pharmacological predictive validity of these models was established using various treatments approved for use in patients with BD.

In treatment development, the research to market success rate is only approximately 1 in 1000 compounds, while the cost of drug development escalates. Optimal efficiency in target development is therefore critical, especially in the low-validation preclinical phase prior to clinical testing. Hence, the putative clinical efficacy of a compound should be determined with as much certainty as possible before moving to human studies. Often however, drugs are moved to clinical phases based on whatever positive proof-of-concept data are available from *in vitro* and *in vivo* tests if high investments have already been made, irrespective of the clinical relevancy of the findings (1). This feed-forward loop, which tends to occur for candidate compounds, severely damages the drug discovery process. Determination of potential clinical relevance/efficacy is therefore especially important. Using an *in vivo* animal model with etiological and predictive validity developed from a translational approach is highly likely to reduce the risk of high-cost late-phase termination of a potential new drug due to a lack of efficacy (1).

In an attempt to meet the etiological criterion, we first identified mechanisms that might contribute to BD at the genetic level [i.e. polymorphisms of the DAT (the plasma membrane protein that modulates the availability of DA in the brain) gene], with support at the physiological level (reduced DAT levels in patients). Subsequently, abnormal behavior of BD patients was characterized with tests that could also be conducted in mice. Mechanisms were identified by which DAT functioning could be reduced in mice, the functional consequences of which were tested using the same tests conducted in patients. Using this approach, we assessed the potential contribution of reduced DAT functioning to abnormal behaviors associated with BD. Additionally, to meet the pharmacological predictive validity criterion, the model animals were validated with approved BD treatments, while novel treatments were also tested.

Previously, it was observed that mice with reduced functioning of the DAT exhibited the same abnormal exploratory pattern in the mouse BPM as patients with BD do in the human BPM (2). Specifically, both BD patients and reduced DAT functioning mice are overly active, explore excessively, and move in more straight, direct patterns compared to healthy subjects. While these observations have been made of mice on a mixed 129/SJ background, the first stage of this thesis was to confirm that similar abnormal behaviors would be observed in DAT KD mice on a C57BL/6J background **(Chapter 2)**. In addition, it was demonstrated that C57BL/6J mice treated with the DAT inhibitor GBR12909 exhibited disrupted sensorimotor gating reflected by PPI deficits **(Chapter 5.1)** consistent with observations of manic BD patients (3) (DAT KD mice also exhibit PPI deficits, unpublished observations). Interestingly, mice with altered circadian transcription factor CLOCK functioning also exhibited PPI deficits **(Chapter 6.1)**. These *Clock*Δ19 mutant mice also exhibited hyperactivity and hyper-exploration in the BPM, although increased straight movements observed in patients with BD were absent. The *Clock*Δ19 mice also exhibited exaggerated hedonia-like behavior, further extending previous findings of mania-like behavior in these mice (4, 5). Because *Clock*Δ19 mice have increased DA release and turnover (6), altered dopaminergic transmission in both mice with reduced DAT functioning and mice with a loss of CLOCK functioning seems to mediate certain aspects of BD mania-like behavior. That elevated DA levels underlie the BD mania-like behavior of mice with reduced DAT functioning was supported by AMPT-induced DA depletion attenuating the hyperactivity and disordered movement organization (entropy) of DAT KD mice without affecting control mice **(Chapter 2.2)**. Because other behaviors such as hyper-exploration were not attenuated by AMPT, there may be other downstream effects contributing to the phenotype of DAT KD mice. Future studies should test the effects of AMPT in *Clock*Δ19 mice and assess to what degree hyperdopaminergia is responsible for the mania-like phenotype of these mice. Furthermore, low-dosage regimens of AMPT could be tested clinically, since earlier negative outcome studies with AMPT in BD patients (7, 8) may have used too high a dose and focused on depressed and remitted patients.

It was therefore possible to alleviate some of the abnormal behaviors resulting from reduced DAT functioning that are similar to those of BD mania patients. Because AMPT is not currently an approved treatment for BD however, we also assessed the pharmacological predictive validity of these models using approved medications. Chronic valproate treatment (1.5% in chow) for 28 days resulted in therapeutic concentration levels in mice. Behaviorally, this treatment ameliorated the hyperactivity of both DAT KD and GBR12909-treated mice **(Chapter 2.1)**, although hyper-exploration and spatial patterns were not significantly affected. Considering that valproate does not fully treat BD mania, this study supported the predictive validity of the DAT model of BD mania. In another study, lithium actually exacerbated the BD-like profile of mice treated with GBR12909 in the BPM, but normalized the GBR12909-induced PPI deficits. Lithium also

attenuated depression-like behavior in mice with elevated acetylcholine (ACh) levels **(Chapter 5.1)**. Hence, mixed results have been observed concerning pharmacological predictive validity of the DAT models. Importantly, chronic treatment regimens resulting in human therapeutic concentrations were used to assess treatment validity of the animal models. Similarly, chronic administration of mood stabilizers is required in patients with BD to be efficacious (e.g. at least 3 weeks in clinical trials (9)). Therefore, despite the frequency of acute treatment studies being conducted in rodent models, chronic studies are necessary to assess treatment validity of a preclinical model as clinically relevant as possible (10, 11). That being said, there is a general difficulty when assessing treatment validity of animal models in psychiatry. For decades, there has been a lack of significant drug discoveries in psychiatric disorders (12). Since current treatments do not treat all symptoms, there is no 'gold standard' available that is expected to treat all abnormal behaviors in an animal model (1). Relevant to the current work, treatments for BD have been found serendipitously (13). The normalizing effect of both chronic valproate and lithium on certain aspects of the BD-like behavior, but not others, is therefore unsurprising. Novel therapies that would treat all abnormal behaviors in these mice will likely result in better efficacy in patients.

In testing other avenues for BD treatments, we referred to epidemiological studies that identified a high prevalence of cannabis use among patients with BD. In fact, patients may be using cannabis as a means to self-medicate. We therefore tested whether THC (the psychoactive component of cannabis) would normalize the behavior of our DAT model of BD mania. Interestingly, we observed that in addition to ameliorating hyperactivity, we observed that acute Δ^9-THC also reduced the hyper-exploration of DAT KD mice **(Chapter 2.3)**. Interestingly, although preliminary, results from patients with BD tested positive for cannabis in the human BPM revealed similar reduced exploration compared to cannabis-naïve patients. Together, these data are in line with cannabis use being highly prevalent among patients with BD (14) and the accompanying self-medication hypothesis that has been suggested in psychiatric disorders (15). Future research should investigate the mechanisms underlying interactions between DA, the (endo)cannabinoid system, and its relationship with BD.

Because neurocognitive deficits are negatively associated with daily living abilities of patients with BD (16), treatments targeted at these cognitive symptoms should greatly benefit patients. We therefore examined multiple aspects of cognitive functioning in a cross-species fashion. Initially, we observed that DAT KD mice exhibited increased risk-taking behavior in a multiple-sessions IGT **(Chapter 3.1)**, as well as increased motivation and motor impulsivity. Using pharmacological inhibitors of DAT and NET, we also observed that acute inhibition of the DAT similarly increased motivation and motor impulsivity of mice **(Chapter 3.2)**. Importantly, motor impulsivity was dissociated from

risk-taking behavior in the IGT (impulsive choice), which was slightly impaired by pharmacological DAT inhibition. Training over time likely influenced the drugs' ability to induce increased risk-taking behavior in mice. Hence, we developed a single-session IGT for mice that is more analogous to the human IGT. Thus, in **chapter 3.3** we described how both DAT KD mice and GBR12909-treated mice exhibited similar poor decision-making in the dynamic single-session IGT in accordance with poor decision-making of BD patients in the human IGT. Overall, poor decision-making guided by a hypersensitivity to rewards occurred in both reduced DAT functioning models and manic BD patients. Moreover, DAT inhibition increased motivation and motor impulsivity in the task. The consistency of such observations likely supports a reduced DAT functioning inducing symptoms that resemble hedonia behaviors observed in BD (17).

Importantly, a relationship between risk-preference and specific exploration in the BPM was observed in mice **(Chapter 3.1)**. Interestingly, specific exploration in the human BPM also correlated with impaired cognition in BD patients (18). It is therefore feasible that treatments that reduce the hyper-exploration displayed by the DAT models may also positively impact cognitive functioning. Valproate and lithium did not normalize this hyper-exploration, but actually worsened this behavior. In support of the relationship with cognition, both mood stabilizers have been associated with cognitive deficits in healthy subjects and BD patients (19, 20). Although these negative side-effects may be underreported due to publication bias, patients have complained about cognitive deficits associated with lithium since the 1950s (personal correspondence). Since patients already suffer from cognitive deficits and this correlates with functional outcome, lithium treatment is not ideal. Novel efficacious treatments should thus not only reduce activity levels, but also normalize poor decision-making and reduce exploration.

Besides the IGT, another typical neuropsychological test to quantify the inhibitory deficits representing a core aspect of cognitive dysfunction in BD, is the CPT. Consistent with BD patients in the human 5C-CPT, we observed that DAT KD mice exhibited inhibitory deficits and reduced vigilance performance (combined human/animal paper to be submitted to *Science*). Using the 5C-CPT in both normal mice and healthy humans, we demonstrated that both species exhibited similar attentional deficits after sleep deprivation **(Chapter 4.1)**. This procedure could be used in future studies to investigate whether genetically susceptible models for BD would show an exaggerated response after sleep deprivation, since it can precipitate (hypo)mania in patients (21). Interestingly, patients with bipolar depression can benefit from sleep deprivation (22). This environmental manipulation may thus also be useful in further validation of models for BD depression. Finally, this testing platform enables the assessment of cognition-enhancing pharmaceuticals for affected populations.

Although the hyperdopaminergic animal models were validated using a broad range of behaviors based on cross-species testing they are not relevant to every aspect of BD. These models mimic behaviors specific to the (hypo)manic phase of BD. The behavior resulting from reduced DAT functioning did not resemble any aspects of depressive behavior however. From earlier studies, we know that treatment with the AChE inhibitor physostigmine (which increases ACh levels) induced depressive symptoms in healthy humans (23). Based on these observations together with increased ACh levels measured in depressed BD patients (24), we tested whether physostigmine would induce depression-like behaviors in mice. Indeed, physostigmine increased behavioral despair consistent with previous results of increased immobility in mice (25), and was normalized by chronic lithium **(Chapter 5.1)**. These data support the hypothesis that cholinergic mechanisms play a key role during phases of depression, in accordance with early theories describing a cholinergic-adrenergic mechanism underlying depression and mania (26). More recent reports have also renewed interest in the cholinergic system underlying (bipolar) depression (27, 28). Thus, dysfunctional cholinergic signaling may underlie phases of BD depression, but be restored during euthymic phases (24). During phases of mania however, aberrant DA signaling may be the predominant etiological factor (29-31). This concept is buttressed by our findings of reduced DAT functioning resulting in mania-like behaviors in mice and altered functioning of DAT observed in BD patients (32, 33). Disruptions in circadian rhythms also play a key role in BD (34), but the associations with altered DA signaling and subsequent presentation of manic symptoms should be further examined. One possibility could be that the molecular clock machinery in patients with BD is susceptible to internal or external stimuli which can influence the homeostasis of DA (transporters) and then evoke a switch into a manic phase. For instance, a hypersensitivity to bright light is thought to underlie the onset of seasonal mania (35). Moreover, altering photoperiod lengths of normal rats switched their behavior into mania-like accompanied by increased immunocytochemical markers of DA (36). Currently used treatments can also inform us about underlying mechanisms. Indeed, chronic valproate can increase DAT levels (37), perhaps underlying the positive effects in BD patients and the DAT model animals. Positive effects of lithium in BD depression and the physostigmine model may result from its ability to increase AChE levels (38). Finally, valproate and lithium may both exert some of their beneficial effects in patients by impacting the molecular clock (39). Exact mechanisms underlying the efficacy of mood stabilizers remain unclear however. Ultimately, the current data from animal models with support from the literature suggest that different mechanisms are underlying different stages of BD. Accordingly, therapies targeted at each state should challenge the underlying mechanism of that state. Alternatively, the ideal treatment would target the 'holy grail' of BD research; *viz.* the mechanism underlying the switching of mechanisms and behaviors that we refer to as BD. The present studies have provided information on what mechanisms might underlie each pole of the disorder. Moreover, cross-species

relevant paradigms with which to test behaviors relevant to each state have been studied. Therefore, once the switch mechanism is identified/hypothesized, this thesis work supplies the means to test these hypotheses.

There are several limitations to this thesis that warrant comment. Although the reduced DAT functioning model has been attributed here to model BD, DAT KD and knockout mice have also been described as ADHD models (40). Indeed, the main characteristics of ADHD, hyperactivity and inattention, were observed in DAT KD mice. Additionally, reduced striatal DAT levels and impaired decision-making have also been observed in ADHD subjects (41, 42). Nevertheless, the similar behavioral profile of these mice and BD patients in the BPM differs from that of ADHD patients in the BPM (43). Moreover, DAT KD mice are hypersensitive to psychostimulants (44) consistent with stimulant-induced mania in BD (45). In ADHD however, stimulants such as amphetamine and methylphenidate comprise the main line of treatment. Because ADHD and BD both present with similar clinical symptoms and a strong comorbidity exists between them (46), it is of major importance that future studies elucidate the differences between disorders (including better diagnostic tools) to avoid potentially harmful treatment of wrongly diagnosed ADHD with stimulants. Another limitation of these studies is the separate modeling of manic and depressive phases in mice, while the same patients can switch from one to the other. Future studies should investigate the mechanisms underlying such switches (47), wherein above-mentioned approaches combining genetic susceptibility with environmental stimuli could prove useful. Indeed, the current work has contributed to examining the effects of altered photoperiod exposures combined with reduced DAT functioning on depression- and mania-like behavior in mice (48). It is important to note that the future treatment for BD may not lie with pharmaceutical compounds, but instead management of the biological clock by manipulating exposure to light (49) (called chronotherapeutics). Although manipulations of the sleep-wake cycle and exposure to light and dark can successfully be performed in rodents, the main positive effects observed with chronotherapy in patients are mood-related and thus generate additional difficulties regarding animal testing. Further clinical studies should be performed to assess the potential benefits of chronotherapy on neurocognitive symptoms in BD. Finally, the inability to mirror human mood states in rodents may be considered as an overall limitation. Future translational tests across BD patients and rodent models may hopefully provide novel therapies with clinical relevancy and efficacy. However, higher mammalian orders such as non-human primates could be more useful when modeling complex mood-associated states present in humans (50).

When conducting psychiatric research, other species may thus have benefits compared to rats and mice (51). Although non-human primates are likely more informative when studying highly complex human traits such as cognitive dysfunction, there are also clear

disadvantages such as ethical concerns, high costs, and practical limitations. Other animals such as the diurnal fat sand rat (*Psammomys obesus*) and the grass Nile rat (*Arvicanthis niloticus*) have been suggested to have benefits compared to nocturnal mice and rats when modeling disorders affected by day-lengths (51). For instance, short photoperiods induced depression- and anxiety-like behaviors in these diurnal rodents (52), similar to increased occurrence of (bipolar) depression during short daylight periods (i.e. winter). In addition to different species being useful, selection of strain may also be important since Black Swiss mice exhibit certain mania-like behaviors compared to other strains (53). Although a battery of tests was used to assess mania-like behavior across strains, these models are limited by the use of normal animals. The *Clock*Δ19 mutant mice may be genetically susceptible and were also previously tested in a comprehensive battery of tests (5), indicating mania-like behavior across several domains. In this thesis we have extended these findings with tests that have relevance to human tests. Ultimately, the assessment of a range of behaviors should replace the observation of just stimulant-induced hyperactivity as 'mania-like' (54). These and other BD models such as sleep deprivation exhibit several weaknesses that limit interpretation of observed behavior. Importantly, the hyperdopaminergic models assessed in this thesis were tested in a unique set of cross-species translational tests. Utilizing a model with a targeted mechanism potentially underlying BD in combination with a battery of tests available in both animals and humans fills a critically important gap existing in preclinical BD research.

In conclusion, the cross-species translational studies described in this thesis contribute to the understanding of mechanisms underlying different phases of BD and put forward a novel way of testing potential BD therapies. Dysfunctional DA signaling and particularly reduced DAT functioning induces mania-like behaviors in rodents, while abnormal cholinergic signaling is likely more involved in depression. Disrupted circadian rhythms also mediate these behaviors and may do this by regulating neurotransmitter levels. Importantly, we have shown that the therapeutically neglected neurocognitive deficits in BD can be addressed in a cross-species manner. Environmental manipulations such as sleep deprivation can be used to assess cognitive-improving medication or be used in combination with models of BD to exacerbate (mania) or treat (depression) symptoms. The extensive validation provides reliable and high-quality preclinical models relevant to the disorder, which minimizes the risk of costly late-phase termination of investigational drugs due to inefficacy. Ultimately, this approach may eventually yield therapeutics that specifically target the underlying circuitry of BD and therefore improve the lives of patients more effectively than current treatments.

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LIST OF ABBREVIATIONS

5C-CPT	5-choice continuous performance test
5CSRTT	5-choice serial reaction time task
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADHD	Attention deficit hyperactivity disorder
AMPT	Alpha-methyl-p-tyrosine
BD	Bipolar disorder
BPM	Behavioral pattern monitor
CLOCK	Circadian locomotor output cycles kaput
CR	Correct rejection
DA	Dopamine
DAT	Dopamine transporter
DSM	Diagnostic and Statistical Manual of Mental Disorders
FA	False alarm
FAR	FA rate
FST	Forced swim test
HC	Healthy control
HR	Hit rate
ICSS	Intracranial self-stimulation
IGT	Iowa gambling task
ITI	Inter-trial interval
KD	Knockdown
NET	Norepinephrine transporter
PPI	Prepulse inhibition
PVT	Psychomotor vigilance test
REM	Rapid eye movement
RI	Responsivity index
RSD	REM sleep deprivation
RT	Reaction time
SD	Sleep deprivation
THC	Tetrahydrocannabinol
TSD	Total sleep deprivation
TST	Tail suspension test
VPA	Valproate or valproic acid
vRT	Variable RT
WT	Wild-type

SUMMARY

Numerous neuropsychiatric disorders suffer from a shortage of efficacious treatments, with multiple treatment regimens often being tried in an attempt to reduce symptoms. Paradoxically, many psychopharmacological interventions can induce anxiety, psychosis, suicidal thoughts, impaired judgment, and clouded thoughts, while being used to treat the same symptoms. Bipolar disorder (BD) is such a severe mental illness, affecting approximately 2% of the worldwide population. BD is characterized by states of extreme euphoria labeled mania and opposite mood states of severe depression. Symptoms of mania include hyperactivity, risk-taking, irritability, racing thoughts, and hedonic behavior. Depression is characterized by increased sleep, anhedonic behavior, and increased suicidal thoughts. BD is devastating to the quality of life of both the sufferers and their families. Current treatments include mood stabilizers, antipsychotics, and antidepressants, none of which fully treat the patient. These poor treatment options may contribute to the fact that 1 in 3 patients with BD attempt suicide. The shortage of efficacious therapeutics is reflected by the fact that no approved treatments have been developed with BD as a target. Hence, neurocognitive symptoms such as impaired decision-making and reduced vigilance often go untreated, while being highly associated with a patient's quality of life. Novel medication targeted at the underlying circuitry of BD is therefore urgently required.

In order to better understand BD and hence develop treatments targeted at its underlying mechanisms, animal models for BD are needed. To achieve such models, the biological underpinnings of BD should be recreated in animals in order to provide etiological validity. Moreover, the predictive validity of the model requires establishment by assessing altered behaviors induced by these manipulations using similar tests in both humans and rodents. Pharmacological validation of such models would also be beneficial, where current treatments may ameliorate some, but not all behavioral abnormalities of the disorder. This thesis describes a combination of these approaches, investigating the putative underlying mechanisms of BD and providing novel targets to aid treatment development.

Key altered mechanisms underlying BD are as of yet unresolved, but dysfunctional dopamine (DA) neurotransmission likely plays a central role in its pathophysiology. Polymorphisms in the DA transporter (DAT) have been associated with BD and reduced DAT levels measured in both live patients and their postmortem tissue. Hyperdopaminergia caused by reduced DAT functioning may therefore be one of the factors resulting in BD symptomatology. Abnormal circadian rhythms are also present in patients and can alter various neurotransmitter levels including DA. Previously, it was observed that patients with BD exhibit a characteristic pattern of increased activity, hyper-exploration, and straighter movements compared to healthy control subjects in a human behavioral pattern monitor (BPM). Selective manipulation of the DAT in mice, either

genetically or pharmacologically, recreated this pattern in the mouse BPM. **Chapter 2** describes studies using this cross-species translational BPM that quantifies these abnormal behavioral patterns. Mice with reduced functioning of the DAT by constitutive knockdown (KD) were repeatedly and robustly hyperactive, hyper-explorative, and moved in straighter movements compared to wild-type (WT) mice. Catecholamine depletion with alpha-methyl-p-tyrosine (AMPT) in these mice attenuated some of these abnormal behaviors, underscoring the importance of elevated DA levels underlying the BD mania-like phenotype of these DAT KD mice **(Chapter 2.2)**. The selective DAT inhibitor GBR12909 also induced this pattern in the BPM and resulted in prepulse inhibition (PPI) deficits in the acoustic startle test similar to patients with BD. Chronic treatment with valproate (an approved mood stabilizing medication for BD) attenuated in part this characteristic exploratory pattern in both DAT KD and GBR12909 models **(Chapter 2.1)**. Chronic lithium however, exaggerated this mania-like behavior in the GBR12909 model, but normalized the PPI deficits **(Chapter 5.1)**. Hence, mixed pharmacological predictive validity of the DAT models was established. Because cannabis use is highly prevalent among patients with BD, we also investigated the effects of acute THC in DAT KD mice **(Chapter 2.3)**. Interestingly, THC reduced the excessive exploratory behavior in these mice similar to reduced exploration observed in BD patients screened positive for cannabis use (preliminary results). Together, these findings support a self-medication hypothesis of cannabis use that has been suggested in psychiatric disorders. Altogether, **chapter 2** supports a reduced DAT functioning hypothesis contributing to the exploratory behaviors of BD patients.

Besides normalizing this abnormal exploratory behavior, potential novel treatments should also target the neurocognitive deficits present in BD. In **chapter 3**, we therefore examined several aspects of cognitive functioning in cross-species tests that go beyond assessing exploratory behavior. Both DAT KD and GBR12909-treated mice exhibited impaired decision-making in a mouse Iowa gambling task (IGT) consistent with impaired IGT performance of patients with BD. Moreover, reduced DAT functioning in mice increased measures of motivation and motor impulsivity consistent with increased hedonic behaviors observed in BD. In another cross-species fashion, we observed that DAT KD mice exhibit reduced vigilance in a mouse 5-choice continuous performance test (5C-CPT) consistent with poor attentional performance of BD patients in a human 5C-CPT. Besides this genetic manipulation, sleep deprivation also impaired 5C-CPT performance in both normal mice and healthy humans **(Chapter 4.1)**. Because sleep deprivation can be used to induce mania-like behavior in rodents, but normal humans do not become manic after loss of sleep, future studies should investigate genetically susceptible models for BD in combination with sleep deprivation. Hence, **chapters 3 and 4** highlight that reduced DAT functioning, as well as environmental factors known to contribute to mania, can reproduce the cognitive deficits associated with BD mania.

Because reduced functioning of the DAT induced behaviors in mice characteristic of symptoms seen in BD mania, other mechanisms may underlie depression. Previously, increases in acetylcholine (ACh) induced depressive symptoms in healthy humans and likewise increased ACh levels were measured in depressed BD patients. Hence, the effects of increased ACh levels induced by the ACh-esterase (AChE) inhibitor physostigmine were assessed in **chapter 5.1**. Indeed, physostigmine induced depressive-like behavior in mice which was attenuated by chronic lithium. Finally, genetically manipulated mice with disrupted circadian rhythms (*Clock*Δ19) were also tested in cross-species tests. Consistent with the DAT animal models and behavior abnormalities observed in BD patients, these mice also exhibited hyperactivity and hyper-exploration in the BPM and PPI deficits in the acoustic startle test **(Chapter 6.1)**. Moreover, *Clock*Δ19 mutant mice had an increased saccharine preference, which could be interpreted as hedonic-like behavior. Interestingly, increased straight movements observed in BD patients in the BPM were absent in these mice, warranting further assessment of these mice as model animals for BD. Ultimately, the described studies support a reduced DAT functioning model as recreating several aspects of BD mania. The final chapters however, highlight the acknowledged need of this research to look beyond DATs and mania alone in investigating and treating BD.

In conclusion, this thesis provides further information on the underlying mechanisms of different phases of BD and offers a way to test novel therapeutics. The cross-species translational studies described here indicate that dysfunctional DAT functioning and disrupted circadian rhythms mediate mania-like behavior, while cholinergic systems are likely more important in depression. Utilizing extensively validated animal models with etiological and predictive validity as described in this thesis may ultimately yield therapeutics that specifically target the underlying circuitry of BD and improve the lives of patients.

NEDERLANDSE SAMENVATTING

Tal van neuropsychiatrische stoornissen lijden aan een tekort aan effectieve behandelingen, waarbij meerdere medicijnen vaak worden geprobeerd in een poging de symptomen te verminderen. Paradoxaal genoeg kunnen veel psychofarmacologische interventies angst, psychose, suïcidale gedachten, verminderd oordeelsvermogen en vertroebelde gedachten veroorzaken, terwijl dezelfde medicatie gebruikt worden om dezelfde symptomen te behandelen.

Bipolaire stoornis (BS) is een van die ernstige psychiatrische stoornissen met een wereldwijde prevalentie van circa 2%. BS wordt gekenmerkt door extreem euforische periodes genaamd manie en tegenovergestelde gemoedstoestanden van ernstige depressie. Symptomen van manie bestaan onder anderen uit hyperactiviteit, verhoogd risico nemen, prikkelbaarheid, snelle gedachten en hedonistisch gedrag. Depressie wordt gekenmerkt door verhoogde slaap, anhedonia en verhoogde suïcidale gedachten. BS vermindert de kwaliteit van leven drastisch voor zowel patiënten als hun directe familie. Huidige medicatie voor BS bestaat uit stemmingsstabilisatoren, antipsychotica en antidepressiva, maar geen van allen helpen de patiënt volledig. Deze ontoereikende behandelingen kunnen bijdragen aan het feit dat 1 op de 3 patiënten met BS een poging tot zelfmoord doet. Het tekort aan effectieve behandelingen wordt weerspiegeld door het feit dat er geen medicatie voor BS is ontwikkeld met de ziekte als aangrijpingspunt. Hierdoor blijven neurocognitieve symptomen zoals verhoogd risico nemen en verminderd concentratievermogen vaak onbehandeld, terwijl deze in sterke mate geassocieerd zijn met de kwaliteit van leven. Nieuwe medicatie gericht op de onderliggende mechanismen van BS is daarom noodzakelijk.

Om BS beter te begrijpen en vervolgens behandelingen te ontwikkelen gericht op de onderliggende mechanismen, zijn diermodellen voor BS nodig. Om dergelijke modellen te realiseren, moet de biologische achtergrond van BS worden nagebootst in dieren om etiologische validiteit te bieden. Daarnaast moet de voorspellende waarde van het model bepaald worden door abnormaal gedrag, dat veroorzaakt wordt door deze manipulaties, te onderzoeken met vergelijkbare testen bij zowel mensen als dieren. Farmacologische validatie van dergelijke modellen is ook zeer nuttig, waarbij huidige medicatie sommige, maar niet alle, gedragsafwijkingen van de aandoening verbeterd. Dit proefschrift beschrijft een combinatie van deze benaderingen en onderzoekt de vermeende onderliggende mechanismen van BS en verschaft nieuwe mogelijkheden voor de ontwikkeling van nieuwe medicatie.

De voornaamste onderliggende mechanismen van BS zijn vooralsnog onopgelost, maar verstoorde dopamine (DA) neurotransmissie speelt waarschijnlijk een centrale rol in de

pathofysiologie. Polymorfismen in het gen voor de DA transporter (DAT) zijn geassocieerd met BS en verminderde DAT concentraties zijn gemeten in zowel patiënten als postmortaal weefsel. Hyperdopaminergie veroorzaakt door verminderde DAT werking kan daarom een van de factoren zijn die betrokken is bij de symptomatologie van BS. Abnormale circadiane ritmes zijn ook aanwezig bij patiënten en deze kunnen verschillende neurotransmitters, waaronder DA, beïnvloeden. Voorheen is gebleken dat patiënten met BS een karakteristiek patroon vertonen van verhoogde activiteit, verhoogd onderzoeksgedrag en in rechtere lijnen bewegen in vergelijking met gezonde proefpersonen in een menselijke "behavioral pattern monitor" (BPM). Dit patroon was nagebootst in de BPM voor muizen door selectieve manipulatie van de DAT in muizen, zowel genetisch als farmacologisch. **Hoofdstuk 2** beschrijft studies die met behulp van deze translationele BPM in muizen en mensen deze abnormale gedragspatronen kwantificeert. Muizen met een verminderd DAT functioneren door genetische "knockdown" (KD) waren herhaaldelijk en robuust hyperactief met verhoogd onderzoeksgedrag en bewegingen in rechtere lijnen vergeleken met normale muizen. Catecholamine depletie met alfa-methyl-p-tyrosine (AMPT) in deze muizen verminderde sommige aspecten van dit abnormale gedrag. Deze bevindingen benadrukken het belang van verhoogde DA niveaus die ten grondslag liggen aan het BS manie-achtige fenotype van deze DAT KD muizen **(Hoofdstuk 2.2)**. Tevens veroorzaakte de selective DAT remmer GBR12909 dit patroon in muizen en resulteerde in verminderde "prepulse inhibition" (PPI) in de akoestische schrikreactie test vergelijkbaar met patiënten met BS. Chronische behandeling met valproïnezuur (goedgekeurde stemmingsstabiliserende medicatie voor BS) verminderde dit karakteristieke onderzoekende gedrag deels in zowel de DAT KD als de GBR12909 modellen **(Hoofdstuk 2.1)**. Chronische behandeling met lithium echter, verergerde dit manie-achtige gedrag in het GBR12909 model, maar normaliseerde de PPI tekorten **(Hoofdstuk 5.1)**. Er is dus gemengde farmacologische voorspellende validiteit aangetoond van deze DAT modellen. Omdat cannabisgebruik veel voorkomt bij patiënten met BS, hebben we ook de effecten onderzocht van THC in de DAT KD muizen **(Hoofdstuk 2.3)**. Interessant genoeg verminderde THC het overmatig verkennende gedrag in deze muizen, wat vergelijkbaar is met verminderd verkennend gedrag dat waargenomen was bij patiënten positief getest voor cannabis (voorlopige resultaten). Samen ondersteunen deze bevindingen een zelfmedicatie-hypothese van cannabisgebruik die is voorgesteld in psychiatrische stoornissen. Al met al ondersteunt **hoofdstuk 2** een verminderd functionerende DAT hypothese die bijdraagt aan het verkennende gedrag van BS patiënten.

Naast het normaliseren van dit abnormale onderzoeksgedrag, moeten potentiele nieuwe behandelingen zich ook richten op de neurocognitieve tekorten aanwezig in BS. In **hoofdstuk 3** hebben we daarom een aantal aspecten van het cognitief functioneren onderzocht in translationele testen in muizen en mensen die verder gaan dan de

beoordeling van enkel verkennend gedrag. Zowel DAT KD als GBR12909-behandelde muizen vertoonden risicovollere besluitvorming in een muizen "Iowa gambling task" (IGT) in overeenstemming met verminderde IGT prestatie van patiënten met BS. Bovendien verhoogde het verminderde DAT functioneren paramaters van motivatie en motorische impulsiviteit vergelijkbaar met verhoogd hedonistisch gedrag waargenomen in BS. In een andere translationele test, zagen we dat DAT KD muizen een verminderd concentratievermogen hadden in een muizen "5-choice continous performance test" (5C-CPT) vergelijkbaar met verminderde aandacht van BS patiënten in een menselijke 5C-CPT. Naast deze genetische manipulatie, is ook aangetoond dat slaaponthouding verminderde 5C-CPT prestatie veroorzaakt in zowel normale muizen als gezonde proefpersonen **(Hoofdstuk 4.1)**. Omdat slaaponthouding kan worden gebruikt om manie-achtig gedrag bij ratten en muizen te veroorzaken, maar gezonde mensen niet manisch worden na slaaptekort, moeten toekomstige studies genetisch modellen voor BD in combinatie met slaaponthouding onderzoeken. **Hoofdstukken 3 en 4** tonen aan dat zowel verminderd DAT functioneren als omgevingsfactoren die bekend zijn bij te dragen aan manie, de cognitieve tekorten in BS kunnen reproduceren.

Omdat verminderd functioneren van de DAT gedrag induceerde in muizen in overeenstemming met manische symptomen, kunnen andere mechanismen ten grondslag liggen aan depressie. In eerder onderzoek resulteerde verhoogde acetylcholine (ACh) concentraties in symptomen van depressie in gezonde personen en werden verhoogde ACh concentraties gemeten in depressive BS patiënten. Hierom zijn de effecten van verhoogde ACh concentraties door de ACh-esterase (AChE) remmer fysostigmine beoordeeld in **hoofdstuk 5.1**. Fysostigmine veroorzaakte inderdaad depressief-achtig gedrag in muizen wat werd verholpen door chronische behandeling met lithium. Tenslotte werden genetisch gemanipuleerde muizen met een verstoord circadiaan ritme (*Clock*Δ19) ook getest in translationele testen. In overeenstemming met de DAT diermodellen en gedragsafwijkingen waargenomen in BS patiënten, waren deze muizen ook hyperactief en excessief verkennend in de BPM en hadden een PPI tekort in de akoestische schrikreactie test **(Hoofdstuk 6.1)**. Bovendien hadden deze *Clock*Δ19 muizen een verhoogde voorkeur voor saccharine, wat kan worden geïnterpreteerd als hedonistisch-achtig gedrag. Interessant genoeg, waren de bewegingen in rechtere lijnen van BS patiënten in de BPM afwezig in *Clock*Δ19 muizen, waardoor verdere beoordeling van deze muizen als diermodel voor BS nodig is. Al met al ondersteunen de bovenstaande studies een verminderd DAT functioneren als model voor diverse aspecten van BS manie. De laatste hoofdstukken benadrukken echter de erkende noodzaak van dit onderzoek om verder te kijken dan alleen de DAT en manie bij het onderzoeken en behandelen van BS.

Concluderend verschaft dit proefschrift nadere informatie over de onderliggende mechanismen van de verschillende fasen van BS en biedt een manier om nieuwe

geneesmiddelen te testen. De translationele studies in zowel mensen als muizen die hier zijn beschreven tonen aan dat verstoord DAT functioneren en abnormale circadiane ritmes manie-achtig gedrag beïnvloeden, terwijl cholinerge systemen waarschijnlijk belangrijker zijn in depressie. Het gebruik maken van uitgebreid gevalideerde diermodellen met etiologische en voorspellende validiteit, zoals beschreven in dit proefschrift, kan uiteindelijk therapieën opleveren die specifiek gericht zijn op de onderliggende circuits van BS en zo het leven van patiënten verbeteren.

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ABOUT THE AUTHOR

Jordy van Enkhuizen was born on May 10 1986 in Ridderkerk, where he spent his childhood. In 1998 he started high school at the Erasmiaans Gymnasium in Rotterdam, where he obtained his gymnasium degree in 2004. In September 2004, he started the Bachelor's program in Pharmaceutical Sciences at the University of Utrecht and moved to Utrecht in 2005. After obtaining his Bachelor's degree in August 2007, he started with the Master's program in Pharmaceutical Sciences at the University of Utrecht in September 2007. During the Master's program, he performed research at the laboratory of M. A. Geyer, Ph.D at the Department of Psychiatry, University of California, San Diego, USA. From June 2010 until August 2010, Jordy worked on HIV-related research at the Kilimanjaro Christian Medical Center in Tanzania. He obtained his Master's degree in August 2010. From October 2010 until April 2011, he worked in the hospital pharmacy of the Erasmus Medical Center in Rotterdam as a pharmacist. In April 2011, he started with his Ph.D studies that are described in this thesis at the Department of Psychiatry, University of California, San Diego, USA in collaboration with the Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences at the University of Utrecht. His research was supervised by B. Olivier, Ph.D, M.A. Geyer, Ph.D, and J.W. Young, Ph.D.

OVER DE AUTEUR

Jordy van Enkhuizen werd geboren op 10 mei 1986 te Ridderkerk en groeide daar op. In 1998 begon hij zijn middelbare schooltijd aan het Erasmiaans Gymnasium te Rotterdam, waar hij in 2004 zijn gymnasium diploma behaalde. Vervolgens begon hij in september 2004 met de bacheloropleiding Farmacie aan de Universiteit Utrecht en verhuisde naar Utrecht in 2005. Na het behalen van zijn bachelordiploma in augustus 2007, begon hij in september 2007 aan de masteropleiding Farmacie aan de Universiteit Utrecht. Tijdens de masteropleiding heeft hij van september 2008 tot maart 2009 onderzoek uitgevoerd in het laboratorium van prof. dr. M. A. Geyer aan het Department of Psychiatry, University of California, San Diego in de VS. Van juni 2010 tot augustus 2010 heeft Jordy gewerkt aan HIV-gerelateerd onderzoek in het Kilimanjaro Christian Medical Center in Tanzania. Het masterdiploma werd in augustus 2010 behaald. Van oktober 2010 tot april 2011 heeft Jordy in de ziekenhuisapotheek van het Erasmus Medisch Centrum in Rotterdam gewerkt als projectapotheker. In april 2011 begon hij met het promotieonderzoek dat beschreven is in dit proefschrift aan het Department of Psychiatry, University of California, San Diego in de VS in samenwerking met de Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences aan de Universiteit Utrecht. Het onderzoek werd uitgevoerd onder begeleiding van prof. dr. B. Olivier, prof. dr. M.A. Geyer en dr. J.W. Young.

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van Enkhuizen, J, Henry, BL, Minassian, A, Perry, W, Milienne-Petiot, M, Higa, K, Geyer, MA, and Young, JW (2013). Poor decision-making under risk of bipolar disorder patients may be due to reduced dopamine transporter functioning: Cross-species evidence using human and mouse Iowa gambling tasks. Neuropsychopharmacology Submitted

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