Title

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Humanized Dopamine D$_{4.7}$ Receptor Male Mice Display Risk-Taking Behavior and Deficits of Social Recognition and Working Memory in Light/Dark-Dependent manner

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Abstract

The dopamine D$_4$ receptor 7 repeat allele (D$_{4.7}$R) has been linked with psychiatric disorders such as attention deficit-hyperactivity disorder, autism, and schizophrenia. However, the highly diverse study populations and often contradictory findings make it difficult to draw reliable conclusions. The D$_{4.7}$R has the potential to explain individual differences in behavior. However, there is still a great deal of ambiguity surrounding whether it is causally connected to the etiology of psychiatric disorders. Therefore, humanized D$_{4.7}$R mice, with the long third intracellular domain of the human D$_{4.7}$R, may provide a valuable tool to examine the relationship between the D$_{4.7}$R variant and specific behavioral phenotypes. We report that D$_{4.7}$R male mice carrying the humanized D$_{4.7}$R variant exhibit distinct behavioral features that are dependent on the light-dark cycle. The behavioral phenotype was characterized by a working memory deficit, delayed decision execution in the light phase, decreased stress and anxiety, and increased risk behavior in the dark phase. Further, D$_{4.7}$R mice displayed impaired social recognition memory in both the light and dark

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phases. These findings provide insight into the potential causal relationship between the human $D_{4.7}R$ variant and specific behaviors and encourage further consideration of dopamine D_4 receptor (DRD4) ligands as novel treatments for psychiatric disorders in which $D_{4.7}R$ has been implicated.

Keywords

Dopamine; D4.7 Receptors; Humanized; Mice; Behavior; Light/Dark

Introduction

The dopamine D_4 receptor (DRD4) is implicated in many psychiatric and neurological disorders, such as attention deficit-hyperactivity disorder (ADHD), schizophrenia, pathological gambling, alcoholism, and substance use disorders (Chen et al., 2011; Emanuele et al., 2010; Kaplan et al., 2008; Langley et al., 2004; Lee et al., 2011; Swanson et al., 2000). In addition, DRD4 has also been associated with behavioral individual differences and personality traits such as novelty seeking, risk-taking, externalizing behavior problems, action and choice impulsivity, and short temper (Bakermans-Kranenburg & van Ijzendoorn, 2006; Benjamin et al., 1996; Bircher et al., 2019; Ding et al., 2002; Dmitrieva, Chen, Greenberger, Ogunseitan, & Ding, 2011; Eisenberg et al., 2010; Eisenegger et al., 2010; Ferre et al., 2022; Kuhnen & Chiao, 2009; Matthews & Butler, 2011; Roussos, Giakoumaki, & Bitsios, 2009; Schilling, Kuhn, Sander, & Gallinat, 2014; Sheese, Rothbart, Voelker, & Posner, 2012). Human DRD4 encoding gene (*DRD4*) is highly polymorphic, and the unusually large number of polymorphisms is assumed to be behind its associations with personality traits and neuropsychiatric disorders.

Human *DRD4* gene is located on chromosome 11p15.5 and contains four exons, wherein the third exon, which encodes the third intracellular loop of the receptor, contains a 48-bp Variable Number Tandem Repeat (VNTR) polymorphism (Van Tol et al., 1992). The length of the polymorphism varies from two (2×16 amino acids) to eleven (11×16 amino acids) repeats, with the most frequent versions in the human population being 4-repeat $D_{4.4}R$ (64%) followed by 7-repeat $D_{4.7}R$ (21%) and 2-repeat $D_{4.2}R$ (8%) (Chang, Kidd, Livak, Pakstis, & Kidd, 1996; Wang et al., 2004). However, there are considerable differences in allele frequencies among the different ethnic populations. For example, the $D_{4.2}$ allele is more frequent (up to 18%) in Asia than globally (8%), whereas the $D_{4.7}$ allele has a low prevalence (<2%) in Asian populations but a high prevalence in Native Americans (48%) (Chang et al., 1996; Wang et al., 2004). Evidence from linkage studies suggests that the D4.7 allele may have arisen after the D4.2 allele during human evolution (Ding et al., 2002). However, more analyses using HapMap data for the DRD4 VNTR data indicated that the D4.7 allele has not undergone strong recent positive selection (Naka, Nishida, & Ohashi, 2011).

Several studies have examined the association between the 7-repeat allele of the *DRD4* gene and behaviors and mental health conditions. These studies have often used genetic association studies, which involve comparing the frequency of the $D_{4.7}R$ allele in individuals with a particular behavior or mental health condition to the frequency of the allele in

a control group (Bakermans-Kranenburg & van Ijzendoorn, 2006; Benjamin et al., 1996; Bircher et al., 2019; Chen et al., 2011; Ding et al., 2002; Dmitrieva et al., 2011; Eisenberg et al., 2010; Eisenegger et al., 2010; Emanuele et al., 2010; Kaplan et al., 2008; Kuhnen & Chiao, 2009; Langley et al., 2004; Lee et al., 2011; Matthews & Butler, 2011; Roussos et al., 2009; Schilling et al., 2014; Sheese et al., 2012; Swanson et al., 2000). Despite the numerous associative studies, the association of $D_{4.7}R$ with personality traits and psychiatric disorders is not simple, and the strength of evidence for the association remains unclear because of the very high heterogeneity among the studies. Humans can express more than one DRD4 variant, and the frequency of allele variants varies among ethnic groups (Chang et al., 1996; Wang et al., 2004), which makes it difficult to determine whether these behavioral changes are exclusively caused by $D_{4.7}R$ expression. Added to the complexity of studying the significance of these polymorphisms is the lack of animal models that can generate causality due to the absence of these repeats in non-primate animals. The rodent gene does not have polymorphisms in the region coding for the third exon of the *DRD4* receptor gene. Wild-type control littermates (WT mice) express a DRD4 with a short third intracellular loop comparable to the human $D_{4.2}R$ (Gonzalez et al., 2012). Therefore, in order to understand the behavioral significance of the 7 repeats of the $D_{4.7}R$, we used a knock-in mouse model with a humanized mouse *DRD4* gene containing seven tandem repeats of the human *DRD4* in the homologous region of the mouse gene, which codes for the third intracellular loop ($D_{4.7}R$ mouse) (Gonzalez et al., 2012). DRD4 mRNA expression has been shown to exhibit significant increases during the dark phase within the retina and pineal gland in rodents (Kim et al., 2010; Klitten et al., 2008), with peaks that are up to 100 times higher compared to the light phase, specifically at times corresponding to day (ZT7) and night (ZT19) (Klitten et al., 2008). We aimed to explore how the D4.7R variant influences behavior, with a specific focus on neuropsychiatric phenotypes known to involve DRD4. To achieve this, we conducted behavioral assessments under both light and dark conditions as distinct experimental setups to observe any potential behavioral differences.

Material and Methods

Animals

Homozygous $D_{4.7}R$ knockin mice ($D_{4.7}R$ mice) and their wild-type C57BL/6J male littermates were used for the experiments. Animals were obtained from the National Institute on Drug Abuse Intramural Research Program (NIDA IRP) breeding facility. $D_{4.7}R$ mice were characterized previously, as indicated in (Gonzalez et al., 2012). Mice were housed in groups of five per cage within a room maintained at a temperature of $21 \pm 1°C$ and humidity-controlled at $55 \pm 10\%$. A 12:12-hour light/dark cycle was maintained, with lights on at 08:00 (ZT 0) and off at 20:00 (ZT 12). Food and water were provided ad libitum. To study the significance of $D_{4.7}R$ in nocturnal behavioral phenotype, a series of behavioral experiments were conducted in the dark phase. The behavioral assessments conducted during the dark phase used different sets of animals from those tested in the light phase. Prior to dark phase testing, mice designated for these experiments underwent an adaptation period for circadian rhythm reversal to simulate nocturnal conditions. In these experiments, the sleep cycle was reversed for wild-type and transgenic $D_{4.7}R$. After two weeks of adaptation, both groups were subjected to behavioral assays in the dark for

two weeks. Behavioral assessments were conducted between 4–7 hours post-transition into both the light and dark phases, aligning with time frames identified by previous studies as having marked differences in DRD4 mRNA expression levels between these phases (Kim et al., 2010; Klitten et al., 2008). Therefore, behavioral experiments conducted during the light phase took place between ZT 4-ZT7, while those conducted during the dark phase occurred between ZT16-ZT19. In the experiments performed during the dark phase, a red light, provided by a 60-watt bulb, was used to facilitate visibility without disrupting the animals' dark-phase conditions. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine, and were performed in compliance with national and institutional guidelines for the care and use of laboratory animals.

Genotyping

DNA extracted from the mouse tail was used for polymerase chain reaction (PCR). The following primers were used: forward, 5'-ACTCGTCCGTCTGCTCCTTCTTC-3' and reverse, 5'- GCAGGACTCTCATTGCCTTG-3'. FastStartTaq kit (Roche 4738357001) was used along with Accustart II Taq DNA Polymerase (Quantabio 95141) and the samples were run on a 1% agarose gel with a 100bp ladder. $D_{4.7}R$ Heterozygous mice showed two bands at 380 and 580 while homozygous mice showed a single band at 580 (Fig. 1a,b).

Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR)

Eyes were harvested from male mice at two distinct time points, Light phase (ZT 4) and dark phase (ZT 16), immediately following euthanasia. mRNA was extracted using TRI Reagent® solution (Sigma-Aldrich) and reverse-transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche) following manufacturer's instructions. RT-qPCR was performed using LightCycler® 480 SYBR Green I Master reagents (Roche), and analyzed by the LightCycler® 480 System (Roche) with gene specific primers; The following primers were used: D4-For: 5'-CTGCC TCTCTTTGTCTACTCCG-3'; D4-Rev: 3'-ACAGGTTGAAGATGGAGGCGGT-5'; GAPDH-For: 5'-CATCACTGCCACCCAGAAGACTG-3'; GAPDH-Rev: 3'-ATGCCAGTGAGCTT CCCGTTCAG-5'. Group sizes for mRNA analysis were: WT Light (7), WT Dark (7), D4.7R Light (6), and D4.7R Dark (6). GAPDH served as an internal control for quantification. The relative abundance of target gene expression was normalized to the reference gene GAPDH for each sample. Values were calculated using the following equation: Fold change = $2^{\wedge\ [CT(experimental,\ target)\ -CT(experimental,\ ref))-(CT(control,\ target)-CT(control,\ ref)]}$.

Behavioral Experiments

Mice were tested with a battery of behavioral paradigms in the following order (Fig. 1a): locomotion and stereotypy/open field, social interaction and novelty, spontaneous T-maze alternation, novel place preference, elevated plus maze, and contextual fear conditioning. The sequence of specific assays spaced by 3–6 days inter-assay intervals was adapted from previously published reports (Alachkar et al., 2018; Alhassen et al., 2021; Paylor, Spencer, Yuva-Paylor, & Pieke-Dahl, 2006).

Each behavioral experiment group initially comprised 8 mice. This number was determined through statistical power analysis based on our previous studies, to detect the smallest scientifically relevant difference between group means at a significance level of <0.05 with 80% power. In the locomotion and open field test, two WT mice tested in the light phase were excluded due to jumping out of the chamber, thus removed from further studies, and were subsequently replaced with two new mice. Consequently, one WT mouse in the light phase was removed from the novelty-induced place preference and social interaction tests, and a WT mouse was excluded from the dark-phase fear conditioning test. For the novel chamber test, a new addition to your lab's behavioral assays, we included more animals despite power calculations suggesting 7 mice would be sufficient.

Locomotor Activity and Open Field Tests

For these tests, animals were placed in a 40×40 cm locomotion chamber. The chamber for the open field test was divided into two zones: a central 24×24 cm square and a surrounding peripheral area. Mice were observed for 10 minutes, during which the time spent in the central and peripheral zones was recorded. The center-to-periphery exploration time ratio was calculated using Activity Monitor 5 software (Med Associates, Inc.). The locomotor activity was monitored in 5-minute intervals over a 60-minute period, following a 30-minute habituation phase that included the 10-minute open field test. The locomotor activity was analyzed using Activity Monitor 5 software (Med Associates, Inc.)

T-maze Spontaneous Alternation

The T-maze assay was conducted as described in our previous study (Alachkar et al., 2018). Mice were placed in the entrance at the base of the T-maze. Mice were acclimated at the base of the T for 30 seconds. After acclimation, the doors opened, and animals were free to explore the maze's left or right arm. After a choice had been made, the door was closed, allowing the animal to explore the sidearm chosen for 30 seconds. Mice were then returned to the maze to start the subsequent trial. Eight total trials were carried out with 7 possible alternations, and the alternation percentage was calculated as 100*(number of alternations/7). The time taken to make the alternation decision was recorded as well.

Social Interaction and Social Novelty

The 3-chamber box used for these assays is a rectangular Plexiglass box consisting of a left, middle, and right chamber with removable doors, which separate the chambers (Alachkar et al., 2018). Empty mesh wire cups were placed in the middle of both the left and right chambers. In the social interaction assay, mice were allotted 5 minutes to explore the middle chamber. After 5 minutes, a control mouse of the same gender, age, and strain as the experimental mouse was placed inside one of the cups in either the right or left chamber. The doors are then removed, allowing the experimental mice to explore all three chambers for 10 minutes. The total time experimental mice spent interacting with both the empty and control mouse cups was recorded.

Immediately following the social interaction assay, the social novelty assay began. The experimental mouse was returned to the middle chamber, and a new control mouse was placed underneath the empty cup. Doors are removed, and the experimental mouse is

allotted 10 minutes to explore all three chambers. The total time experimental mice spent interacting with the mouse from the social interaction assay and the novel mouse was recorded. ANY-MAZE software (Stoelting, Wood Dale, IL, USA) was used to record and analyze these interactions.

Novelty-induced Place Preference

The three-chamber box was slightly modified by introducing two dividers perpendicular to the two doors in the center of the middle chamber, creating a narrow path between the two side chambers. One of the side chambers was decorated with striped wallpapers, while the other had dotted wallpapers. The experimental animals of both groups were randomly assigned so that the chamber with the striped wallpapers for half of the tested mice was the 'novel' chamber. For the other half, the chamber with the dotted wallpapers was the 'novel' chamber. The acclimatization period included placing the mice in the center of the middle chamber for 25 minutes in three consecutive days, with only the door that allows access to the designated familiar chamber being removed. On the test day, the mice were placed in the middle chamber and allowed to explore both the 'familiar' and 'novel' chambers for 10 minutes, with the doors between the two compartments being removed. The total time the mouse spent in the 'familiar' chamber and the 'novel' chamber during the 10-minute test was recorded. The mice were considered to have entered a chamber when their two front paws and head crossed the threshold.

The Elevated Plus Maze (EPM)

A standard elevated plus-maze, made of grey Plexiglass, was placed in a sound-proof observation room with controlled light (200 Lux) on the central platform of the maze (Sanathara et al., 2018). Animals were given a 30-minute period to habituate to the room before being tested. During the test, the mice were placed in the center of the plus facing an open arm, and were given 5 minutes to explore. The behavior was recorded and scored by two independent observers blind to the animal treatments. The animals were scored based on time spent in the closed and open arms and the number of entries to the closed and open arms.

Contextual Fear Conditioning

This assay consists of a training and a testing session 24 hours following the training session (Alachkar et al., 2018). On day 1, mice were placed in the conditioning chamber for 3 minutes, received a 2-second 0.7 mA foot shock at 2.5 minutes, and returned to their home cage. On day 2, animals are returned to the same chamber for 5 minutes without shock. Freezing behavior is measured pre and post-shock sessions and was scored as freezing (1) or not (0) within a 5-second interval and calculated as 100* (the number of intervals of freezing/total intervals).

Statistical Analysis

GraphPad Prism (version 9.3.1, GraphPad Software, Inc.) was used for the statistical analysis of the behavioral data. Data were presented as means±SD. The normal distribution of the sample data was confirmed using the D'Agostino-Pearson normality test. Behavioral

comparisons between WT and D4.7R mice in total distance traveled, elevated maze performance, and T-maze data, were analyzed using two-way ANOVA, with genotype (wild-type vs. D4.7R variant) and environmental conditions (light vs. dark) as the two factors, followed by appreciate post-tests. Assessments of the open field activity, place preference, fear conditioning, social interaction, and social novelty were analyzed using three-way ANOVA with Bonferroni corrections for multiple comparisons. DRD4 mRNA expression data were analyzed using two-way ANOVA, followed by Tukey's post hoc tests for detailed group comparisons.

Results

Consistent with established patterns, DRD4 mRNA levels were higher in the eyes of both WT and $D_{4.7}R$ male mice during the dark phase (ZT 16) compared to the light phase (ZT 4). Notably, $D_{4.7}R$ mice exhibited lower DRD4 mRNA levels in the light phase than WT mice. Furthermore, there was a trend toward decreased DRD4 mRNA expression in the dark phase for $D_{4.7}R$ mice compared to WT, although this difference did not reach statistical significance ($P = 0.08$). This suggests a potential modulation of DRD4 expression by the $D_{4.7}R$ allele throughout the daily light-dark cycle (Fig. 1c).

Mice were subjected to a series of behavioral assessments, beginning with locomotion and open field tests, and ending with fear conditioning (Fig. 2a).

$D_{4.7}R$ mice displayed normal spontaneous locomotor activity during the light and dark phases. This is reflected by comparable total distance traveled in 60 minutes by $D_{4.7}R$ mice was not significant from that in the WT mice in the light and dark phase (Fig. 2b). Comparable levels of horizontal, vertical, and stereotypic activities were observed in both WT and D4.7R mice (Fig. 2c–h).

$D_{4.7}R$ mice also displayed normal responses in the open field test, novelty-induced place preference, and fear conditioning.

In the open field test, which measures anxiety levels in a novel environment, WT and $D_{4.7}R$ mice spent comparable time in the peripheral and central zones in the light and dark phases (Fig. 3a). Further, both groups spent more time in the peripheral zone than the central zone (Fig. 3a).

Similarly, WT and $D_{4.7}R$ mice spent more time in the novel chamber in the novelty-induced place preference test, which measures novelty seeking, and spatial memory (Fig. 3b).

WT and $D_{4.7}R$ mice displayed similar freezing scores 24 hours after exposure to the electric foot shock in the fear conditioning assay, indicating normal contextual memory in $D_{4.7}R$ mice in the light and dark phases (Fig. 3c). However, the immediate post foot-shock freezing response was lower in the $D_{4.7}R$ mice in the dark phase, indicating reduced shock-induced fear or stress in these animals (Fig. 3c). Further, when compared with the WT mice, $D_{4.7}R$ mice spent more time in the open arms and less time in the closed arms of the elevated plus maze in the dark phase but not in the light phase (Fig. 4a,b), indicating a reduced fear and

anxiety in the dark phase in these animals. However, the percentage of entry to the open and closed arm was not different between the WT and $D_{4.7}R$ mice (Fig. 4c).

In the T-maze, $D_{4.7}R$ mice displayed a lower score of correct choices compared to the WT animals during the light phase (Fig. 5a), indicating an impaired working memory. Associated with impaired working memory, $D_{4.7}R$ mice displayed longer onset of decision-making. In the dark phase, however, $D_{4.7}R$ and WT mice showed comparable scores, indicating normal short-term memory. Further, $D_{4.7}R$ and WT mice exhibited a similar onset of time for decision-making (Fig. 5b).

When tested for their social behavior in the three-chamber social test, WT and $D_{4.7}R$ mice spent more time exploring the stranger mouse than the empty cup in the light phase. It should be mentioned that $D_{4.7}R$ exhibited a trend for spending more time with the stranger mouse than the empty cup in the light phase; however, the difference was not significant (Fig. 6a). In the dark phase, WT and $D_{4.7}R$ mice spent more time with the strange mouse than the empty cup, indicating a normal sociability of the $D_{4.7}R$ mice in the dark phase.

In the social novelty assay conducted during the light phase, WT mice spent more time with the new mouse than the familiar mouse, whereas $D_{4.7}R$ mice displayed similar time with the familiar and new mice, indicating an impairment in social novelty. Similarly, in the dark phase, $D_{4.7}R$ mice exhibited a deficit in social novelty (Fig. 6b).

Discussion

In this study, we evaluated the behavioral phenotype of a mouse model carrying the humanized $D_{4.7}R$ variant. We report that $D_{4.7}R$ mice exhibit specific behavioral features that are light-dark-phase dependent. The behavioral phenotype was characterized by working memory deficit and social impairment in the light phase, an impaired social novelty in the light and dark phases, and increased risk behavior in the elevated plus maze in the dark phase.

While the *DRD4* gene has been widely studied in psychiatric disorders, the high heterogeneity among these studies and the very controversial findings makes the results inconclusive. Further, whether the *DRD4* gene is causally linked to the etiology of psychiatric disorders is still largely ambiguous. Therefore, the humanized $D_{4.7}R$ mice, with the long intracellular domain of the human $D_{4.7}R$ (Gonzalez et al., 2012), provide a valuable tool to examine the causal relationship of the $D_{4.7}R$ variant with specific behavioral responses. The WT mouse, which expresses a DRD4 with a shorter third intracellular loop comparable to the human $D_{4.2}R$ was used as the control mouse (Bonaventura et al., 2017; Gonzalez et al., 2012).

DRD4 mRNA expression has been shown to exhibit significant fluctuations between light and dark phases in the retina and various brain regions such as the amygdala, pineal gland, retinal pigment epithelium, and substantia nigra in primates (Mure et al., 2018). Specifically, DRD4 mRNA levels in the retina and pineal gland of rodents are markedly higher during the dark phase (Klitten et al., 2008). Our analysis of DRD4 mRNA levels in the eye supports these observations, showing a significant but less pronounced variation

between light and dark phases. Recognizing the remarkable impact of light-dark changes on behavioral responses, we assessed the behavioral effects of carrying the D4.7R variant in the dark and light phases.

DRD4's highest levels in the brain are found in the prefrontal cortex, amygdala, hypothalamus, and hippocampus (Ariano, Wang, Noblett, Larson, & Sibley, 1997; Matsumoto, Hidaka, Tada, Tasaki, & Yamaguchi, 1995). Significant differences in the behavioral responses related to these brain regions could result from the presence of the DRD4 polymorphic variant. Interestingly, the behaviors altered in the $D_{4.7}R$ mice (i.e., working memory, decision-making, social function, and anxiety-like behavior) are governed by brain regions that express the highest levels of DRD4 expression.

Of the nine behavioral assays used in this study, we found that the presence of the $D_{4.7}R$ variant affected three different behavioral responses, working memory monitored by T-maze, social recognition monitored by the 3-chamber assay, and anxiety and risk-taking monitored by the elevated plus maze and acute foot shock.

The working memory impairment observed in $D_{4.7}R$ mice in the T-maze assay was associated with a delayed decision latency, reflecting an impairment of working memory. Noteworthy, $D_{4.7}R$ did not exhibit deficits in other types of memory tested, such as place memory and contextual memory. Further, the working memory impairment observed in $D_{4.7}R$ mice was associated with delayed decision latency, indicating a role for $D_{4.7}R$ in regulating brain circuits that control working memory and decision execution. In the prefrontal cortex, DRD4s are particularly expressed in the pyramidal glutamatergic neuron and in the GABAergic interneurons (Bonaventura et al., 2017; Gilsbach et al., 2012; Lauzon & Laviolette, 2010). DRD4 modulates glutamatergic corticostriatal neurotransmission by eliciting an inhibitory control of glutamate release from the striatal glutamatergic terminals where DRD4s are co-localized with D_{2S} isoform (Gonzalez et al., 2012; Thomas, Grandy, Gerhardt, & Glaser, 2009). The cortico-striatal circuit is known to control various executive functions, including decision-making and working memory (Forstmann et al., 2010; Gelskov, Madsen, Ramsoy, & Siebner, 2016; Middleton & Strick, 2000; Mills et al., 2012). The presence of DRD4 in the glutamatergic corticostriatal neurons puts this receptor in an excellent location to modulate working memory and decision execution. Given a hypothesized role of working memory deficits in ADHD, schizophrenia, and autism spectrum disorder (Carnahan, Aguilar, Malla, & Norman, 1997; Chey, Lee, Kim, Kwon, & Shin, 2002; Demeter et al., 2013; Dittrich & Johansen, 2013; Dutschke et al., 2018; Mills et al., 2012; Pennington & Ozonoff, 1996; Possin, Filoteo, Song, & Salmon, 2008; Possin et al., 2017; Shafer, Lewis, Newell, & Bodfish, 2021; Steele, Minshew, Luna, & Sweeney, 2007; Stoit, van Schie, Slaats-Willemse, & Buitelaar, 2013; van der Wee et al., 2003), the effect of $D_{4.7}R$ variant on working memory identified in the current study provides a causal link between $D_{4.7}R$ polymorphism and the etiology of these psychiatric disorders.

Adaptive social behaviors such as bonding, sociability, social recognition memory, and face recognition require the coordinated action of several brain areas. Remarkably, some of the most important regions in social behavior regulation, such as the prefrontal cortex, hippocampus, amygdala, and hypothalamus (Andari et al., 2010; Bartz, Zaki, Bolger, &

Ochsner, 2011; Baskerville & Douglas, 2008; Burri, Heinrichs, Schedlowski, & Kruger, 2008; Herbert, 1994; Insel, 2010; Moadab, Bliss-Moreau, Bauman, & Amaral, 2017; Pedersen, Ascher, Monroe, & Prange, 1982; Walum et al., 2012), all contain DRD4s. Furthermore, social functioning impairments are a core feature of several psychiatric and neurodevelopmental disorders, such as autism spectrum disorder, ADHD, schizophrenia, and depression. Thus, the impairment of social behaviors in the $D_{4.7}R$ mice, particularly their lack of interest in interacting with a novel mouse, may implicate this variant in the pathophysiology of the disorders associated with social function impairments.

Interestingly, while $D_{4.7}R$ mice exhibited normal novelty-induced place preference behavior, they displayed higher levels of exploration of the open arm in the elevated plus maze. The two behavioral assays share a common feature: they examine the exploration of a novel environment, which is often associated with anxiety and potential risks. However, in the novelty-induced place preference test, the novelty-seeking and long-term spatial memory domains are prominent since mice are acclimated to the place for three days, and the novel place is not associated with aversive condition or danger. On the other hand, the elevated plus-maze examines anxiety-related behavior, assessed by measures of open-arm avoidance (Hagenbuch, Feldon, & Yee, 2006; Walf & Frye, 2007; Wall & Messier, 2001), which reflects a conflict between the mouse preference for protected areas (closed arms) and the instinctive motivation for exploring novel environments (open arms). When mice are introduced into an open field or elevated maze, they tend to explore mainly the peripheral zone of the open field and the closed arm of the elevated maze. Therefore, a higher exploration of the open arm is interpreted as a higher degree of risk-taking behavior. However, in the open field, $D_{4.7}R$ mice exhibited normal anxiety behavior, contrary to their low reaction to the electric foot-shock.

The increased anti-anxiety/risk-taking behavior in the $D_{4.7}R$ mice was observed only during the dark phase. This is interesting, given the 100-fold higher levels of DRD4 expression in the retina in the dark phase in rodents. However, as mice have poor vision, they use their whiskers to generate a spatial representation of the environment and identify risk and shelter areas (Bosman et al., 2011; Pluta, Lyall, Telian, Ryapolova-Webb, & Adesnik, 2017; Prusky & Douglas, 2004; Voller, Potuzakova, Simecek, & Vozeh, 2014). Therefore, the dark-phase dependent increase in risk-taking behavior in the $D_{4.7}R$ mice is unlikely to be due to their inability to see in the dark. Our results depict a link between $D_{4.7}R$ polymorphism and an increased drive toward exploring unfamiliar environments, irrespective of the harm potentially related to such behavior. This is aligned with the low reaction to the electric foot shock we found in the $D_{4.7}R$ mice. This was predicted from previous studies demonstrating that $D_{4.7}R$ provides a gain of function of the effect of dopamine in inhibiting striatal glutamatergic neurotransmission (Bonaventura et al., 2017), which seems to involve differences in oligomerization with dopamine D2 receptors (D_2R) and the properties of D_2R-$D_{4.7}R$ heteromers (Ferre et al., 2022).

Based on seminal studies from Hikosaka's group (Bromberg-Martin, Matsumoto, & Hikosaka, 2010), we anticipated that the $D_{4.7}R$-associated blunted corticostriatal transmission should affect the activity of both the "Go" and "NoGo" GABAergic striatal efferent pathways, decreasing their respective ability to increase the reactivity to reward-

related stimuli and to suppress the reactivity to non-rewarded- or aversive-related stimuli. We expected the outcome should be an increased drive towards irrelevant stimuli and a reduced inhibition of irrelevant responses, which could be important in explaining the attentional deficit and impulsivity of ADHD (Bonaventura et al., 2017). The decreased reactivity to non-rewarded- or aversive-related stimuli should translate into low levels of harm avoidance and anxiety are reported to be associated with risky activities such as hazardous driving, gambling, and substance abuse (Baker & Galvan, 2020; Elsey et al., 2016; Nash, Leota, & Tran, 2021; Peris & Galvan, 2021; Wills, Vaccaro, & McNamara, 1994). Thus, our findings may provide a causal model for the link between $D_{4.7}$ repeats polymorphism and specific personality traits such as novelty-seeking and risk-taking.

In conclusion, the present study describes, for the first time, different behavioral phenotypes induced by the presence of the humanized $D_{4.7}R$ in mice. These differences were found in working memory deficit and social impairment in the light phase, an impaired social novelty in the light and dark phases, and increased risk behavior in the elevated plus maze in the dark phase. These findings provide an insight into the possible association between the human $D_{4.7}R$ variant and specific behaviors and encourage further consideration of DRD4-specific ligands as novel treatments for psychiatric disorders in which $D_{4.7}R$ has been implicated, such as ADHD, autism, and schizophrenia.

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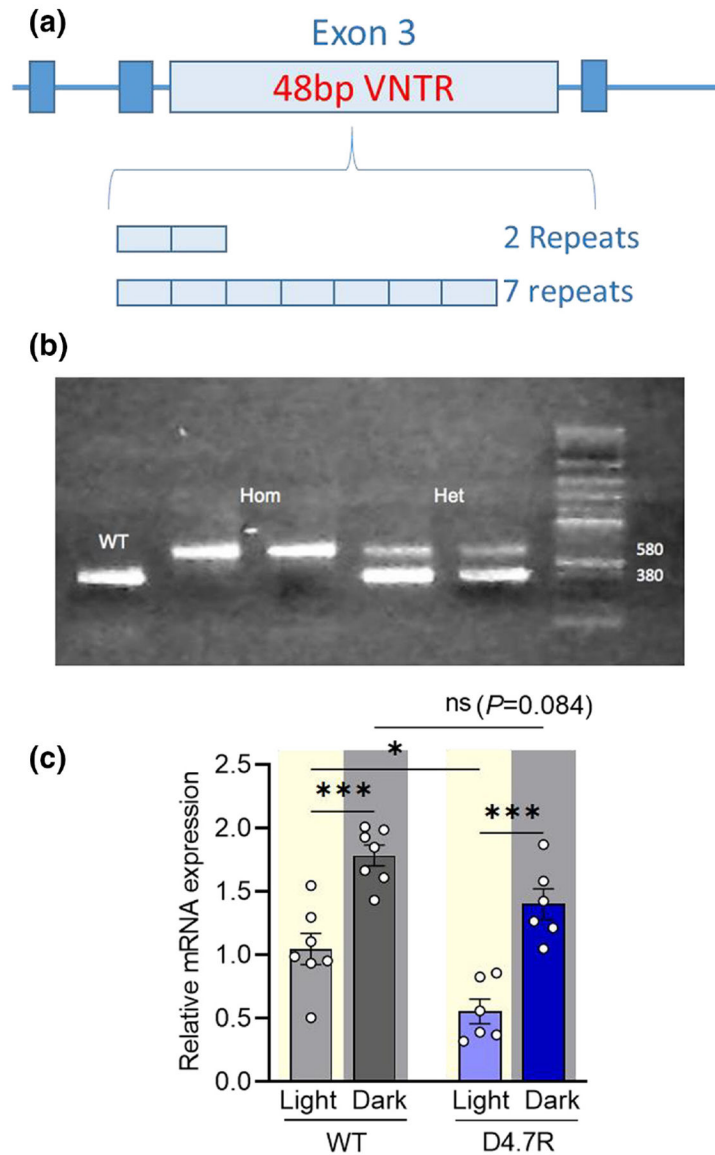
Figure 1. *DRD4* **gene exon 3 with the 48-bp Variable Number Tandem Repeat (VNTR) polymorphism and Light (ZT4)/Dark (ZT16) variation in the whole eye DRD4 expression**
(a) Schematic diagram of the exon 3 tandem repeats of 48bp VNTR**, (b)** Agarose Gel Electrophoresis of DNA after PCR on DNA extracted from WT and $D_{4.7}R$ mice. Lane 1, bp PCR fragment from WT mice; Lane 2 and 3, bp PCR fragment from $D_{4.7}R$ homozygous mice (top); Lane 4 and 5, bp PCR fragment from heterozygous $D_{4.7}R$ mice; Lane 6, DNA ladder 1kb. **(c)** Quantification of relative DRD4 mRNA levels in the whole eye as determined by quantitative RT-PCR. Expression levels were normalized to GAPDH within each group. The graph represents the expression relative to the wild-type (WT) mice measured during the light phase (WT/Light phase group); two-way ANOVA, followed by Tukey's multiple comparisons test (genotype effect: $F_{(1, 22)} = 16.69$, $P<0.001$, light-dark effect: $F_{(1, 22)} = 54.51$, $P<0.001$, Interaction: $F_{(1, 22)} = 0.2704$, $P=0.60$); n = 7 for WT in light and dark phases, n = 6 for $D_{4.7}R$ in the light and dark phases: *$P<0.05$, ***$P<0.001$, ns, not significant. Data are presented as means±SD.
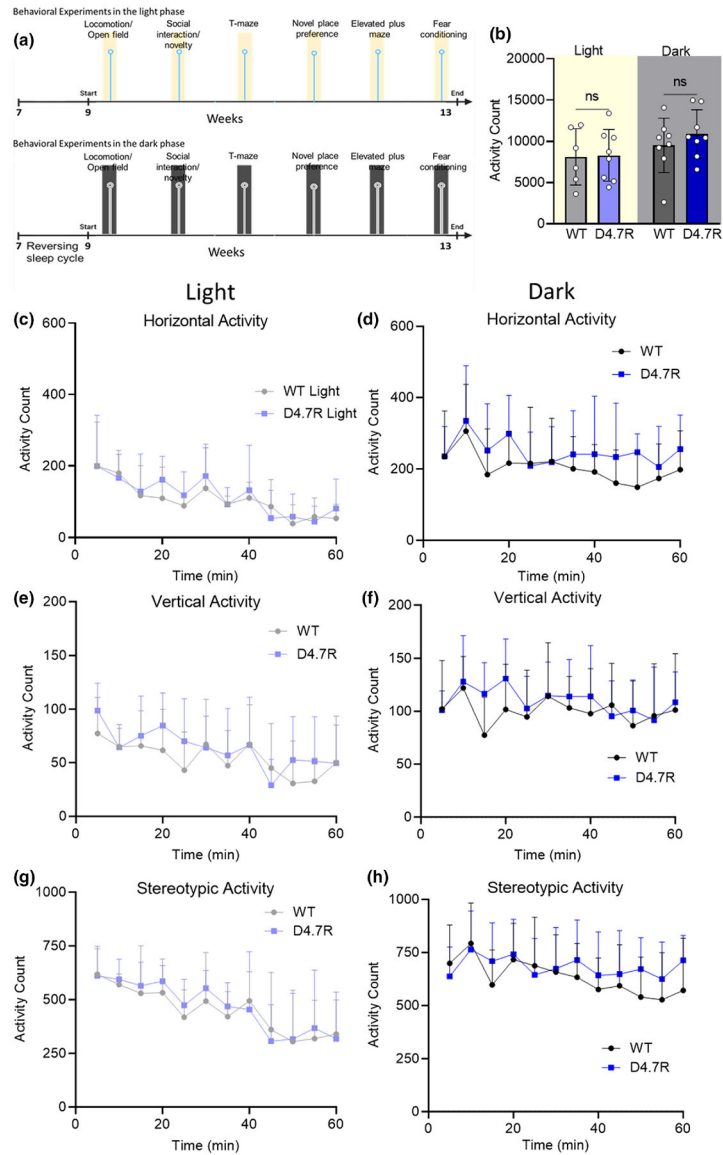
Figure 2. Transgenic D$_{4.7}$R mice display normal locomotor activity in the light (ZT4-ZT7) and dark (ZT16-ZT19) phases.

(a) Schematic showing the experimental design sequence of behavioral assays

(b-h) Locomotor activity in 60 minutes: n = 6 WT and 8 D4.7R mice in the light phase, and 8 WT and 8 D4.7R mice in the dark phase

(b) Total activity in 60 minutes in the light and dark phases: Two-way ANOVA, followed by Tukey's multiple comparisons test: (genotype effect: F (1, 26) = 0.45, P=0.506, light-dark effect: F (1, 26) = 2.947, P=0.098), Interaction: F (1, 26) = 0.265, P=0.610); ns: not significant. Data are presented as means±SD.

(c) Time-course of the horizontal activity in the light phase: Two-way ANOVA, followed by Bonferroni's multiple comparisons test (genotype effect: F (1, 144) = 0.806, P=0.370, time effect: F (11, 144) = 4.971, P<0.001, Interaction: F (11, 144) = 0.307, P=0.983).

(d) Time-course of the horizontal activity in the dark phase: Two-way ANOVA, followed by Bonferroni's multiple comparisons test (genotype effect: $F (1, 168) = 6.871$, $P=0.01$, time effect: $F (11, 168) = 1.469$, $P=0.147$, Interaction: $F (11, 168) = 0.354$, $P=0.971$).

(e) Time-course of the vertical activity in the light phase: Two-way ANOVA, followed by Bonferroni's multiple comparisons test (genotype effect: $F (1, 144) = 2.755$, $P=0.099$, time effect: $F (11, 144) = 2.518$, $P=0.006$, Interaction: $F (11, 144) = 0.494$, $P=0.904$).

(f) Time-course of the vertical activity in the dark phase: Two-way ANOVA, followed by Bonferroni's multiple comparisons test (genotype effect: $F (1, 168) = 2.889$, $P=0.091$, time effect: $F (11, 168) = 0.960$, $P=0.485$, Interaction: $F (11, 168) = 0.503$, $P=0.899$).

(g) Time-course of the stereotypic activity in the light phase: Two-way ANOVA, followed by Bonferroni's multiple comparisons test (genotype effect: $F (1, 144) = 0.432$, $P=0.512$, time effect: $F (11, 144) = 5.442$, $P<0.001$, Interaction: $F (11, 144) = 0.1818$, $P=0.998$).

(h) Time-course of the stereotypic activity in the dark phase: Two-way ANOVA, followed by Bonferroni's multiple comparisons test (genotype effect: $F (1, 168) = 3.498$, $P=0.063$, time effect: $F (11, 168) = 1.456$, $P=0.153$, Interaction: $F (11, 168) = 0.560$, $P=0.858$).
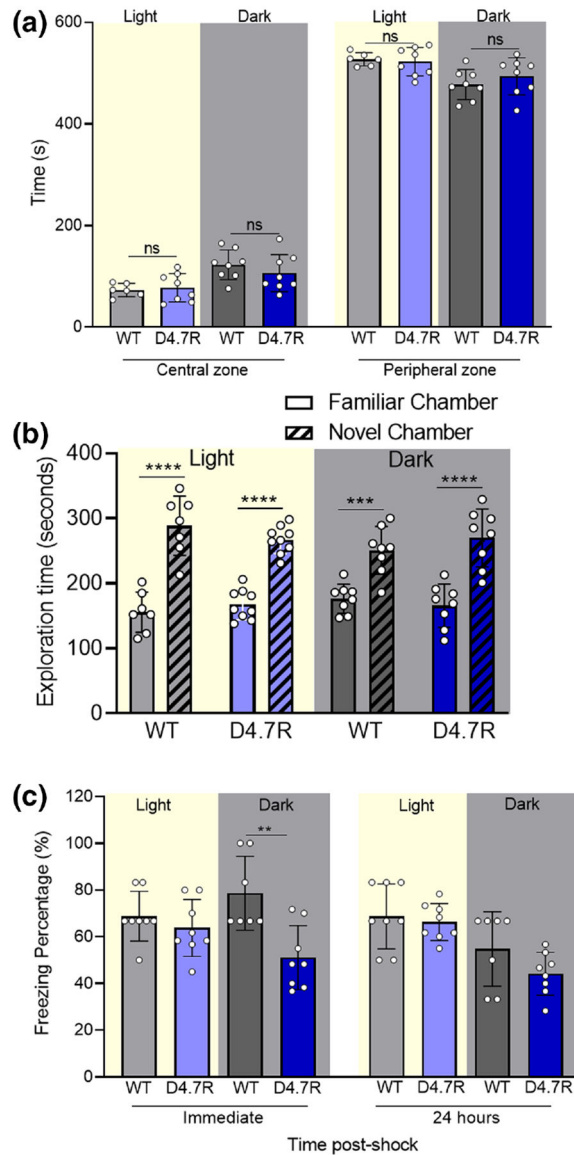
Figure 3. Transgenic D$_{4.7}$R mice normal responses to a novel environment in open field, novelty place preference, and fear conditioning but reduced immediate response to foot-shock in the dark (ZT16-ZT19) phase

(a) Time spent in the peripheral and central zones in the light and dark phase; three-way ANOVA, followed by Bonferroni's multiple comparisons test (genotype: F (1, 52) = 0.000, P>0.999, zone: F (1, 52) = 298, P<0.001, environment light-dark: F (1, 52) = 0.000, P>0.999, zone × light-dark: F (1, 52) = 27.30, P<0.001, zone × genotype: F (1, 52) = 0.604, P=0.440, light-dark × genotype: F (1, 52) = 0.000, P>0.999, zone × light-dark × genotype: F (1, 52) = 1.928, P=0.171). WT vs. D$_{4.7}$R: ns, not significant. n = 6 WT and 8 D4.7R mice in the light phase, and 8 WT and 8 D4.7R mice in the dark phase. Data are presented as means±SD.

(b) Time spent in the old place or novel place in the light and dark phases; three-way ANOVA, followed by Bonferroni's multiple comparisons test (genotype: F (1, 56) = 0.225, P=0.637, chamber: F (1, 56) = 154.7, P<0.001, light-dark: F (1, 56) = 0.003, P=0.959,

chamber × genotype: F (1, 56) = 2.685, P=0.107, chamber × light-dark: F (1, 56) = 0.010, P=0.918, genotype × light-dark: F (1, 56) = 0.312, P=0.578, chamber × genotype × light-dark: F (1, 56) = 3.722, P=0.059). Familiar Chamber vs Novel Chamber: ***P<0.001, ****P<0.0001. n = 7 WT and 9 D4.7R mice in the light phase, and 8 WT and 8 D4.7R mice in the dark phase. Data are presented as means±SD.Data are presented as means±SD **(c)** $D_{4.7}R$ mice display decreased foot shock-induced stress but normal contextual memory in the fear conditioning test during the light and dark phases; three-way ANOVA, followed by Bonferroni's multiple comparisons test: (genotype: F (1, 54) = 12.75, P<0.001, light-dark: F (1, 54) = 9.276, P=0.004, post-shock: F (1, 54) = 4.862, P=0.032, post-shock × light-dark: F (1, 54) = 6.728, P=0.012, post-shock × genotype: F (1, 54) = 2.313, P=0.134, light-dark × genotype: F (1, 54) = 5.740, P=0.020, post-shock × light-dark × genotype: F (1, 54) = 1.272, P=0.264. WT vs. $D_{4.7}R$: **P<0.01. ns, not significant. n = 8 WT and 8 D4.7R mice in the light phase, and 7 WT and 8 D4.7R mice in the dark phase. Data are presented as means±SD.Data are presented as means±SD
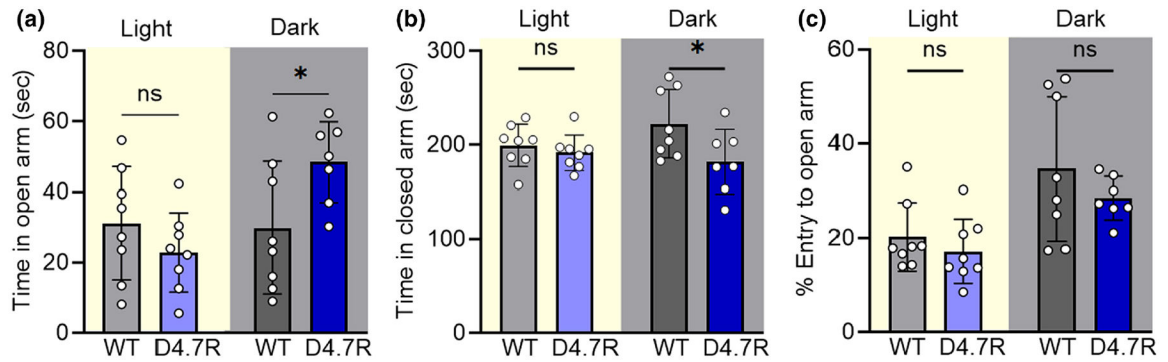
Figure 4. Transgenic D$_{4.7}$R mice display dark-phase-selective low anxiety levels and high risk-taking behavior

Performance in the elevated plus maze in the light and dark phases (n = 8 WT and 8 D$_{4.7}$R mice in the light phase, and 8 WT and 7 D$_{4.7}$R mice in the dark phase):

(a) Time spent in the open arms; two-way ANOVA, followed by Bonferroni's multiple comparisons test: (genotype effect: F (1, 27) = 0.931, P=0.3430, light-dark effect: F (1, 27) = 5.184, P=0.031, Interaction: F (1, 27) = 6.296 P=0.0184); WT vs. D$_{4.7}$R: *P<0.05, ns: not significant.

(c) Time spent in the closed arms; two-way ANOVA, followed by Bonferroni's multiple comparisons test: (genotype effect: F (1, 27) = 5.366, P=0.028, light-dark effect: F (1, 27) = 0.446, P=0.5098, Interaction: F (1, 27) = 2.406, P=0.133); WT vs. D$_{4.7}$R: *P<0.05, ns: not significant.

(e) Percentage of entries to the open arms; two-way ANOVA, followed by Bonferroni's multiple comparisons test: (genotype effect: F (1, 27) = 1.796, P=0.1914, light-dark effect: F (1, 27) = 13.9, P<0.001, Interaction: F (1, 27) = 0.2158, P=0.646); WT vs. D$_{4.7}$R: ns: not significant.
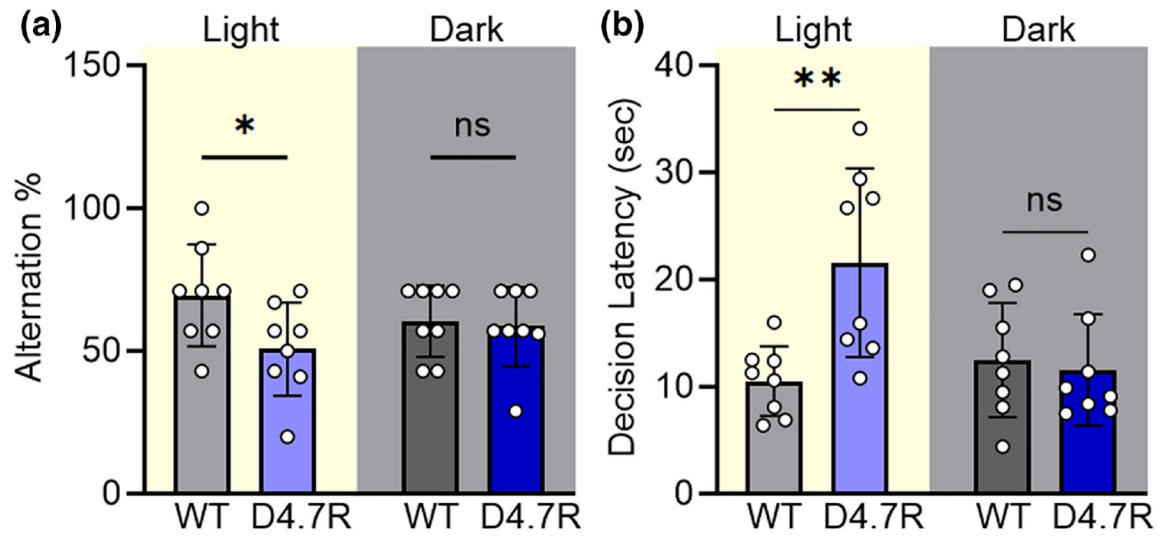
Figure 5. Transgenic D$_{4.7}$R mice display impaired spatial working memory and delayed decision latency during the light (ZT4-ZT7) but not the dark (ZT16-ZT19) phase.

(**a**) Working spatial memory alteration percentage in T-maze in the light and dark phases; two-way ANOVA, followed by Bonferroni's multiple comparisons test: (genotype effect: F $(1, 28) = 3.661$, $P=0.066$, light-dark effect: F $(1, 28) = 0.01089$, $P=0.917$, Interaction: F $(1, 28) = 2.451$, P=0.128); WT vs. D$_{4.7}$R: *$P<0.05$, ns: not significant. n = 8 WT and 8 D4.7R mice in the light phase, and 8 WT and 8 D4.7R mice in the dark phase. Data are presented as means±SD.

(**b**) Decision latency in T-maze test in the light phase, two-way ANOVA, followed by Bonferroni's multiple comparisons test: (genotype effect: F $(1, 28) = 5.729$, $P=0.024$, light-dark effect: F $(1, 28) = 3.554$, $P=0.070$, Interaction: F $(1, 28) = 7.981$, $P=0.009$); WT vs. D$_{4.7}$R: **$P<0.01$, ns: not significant. Data are presented as means±SD.
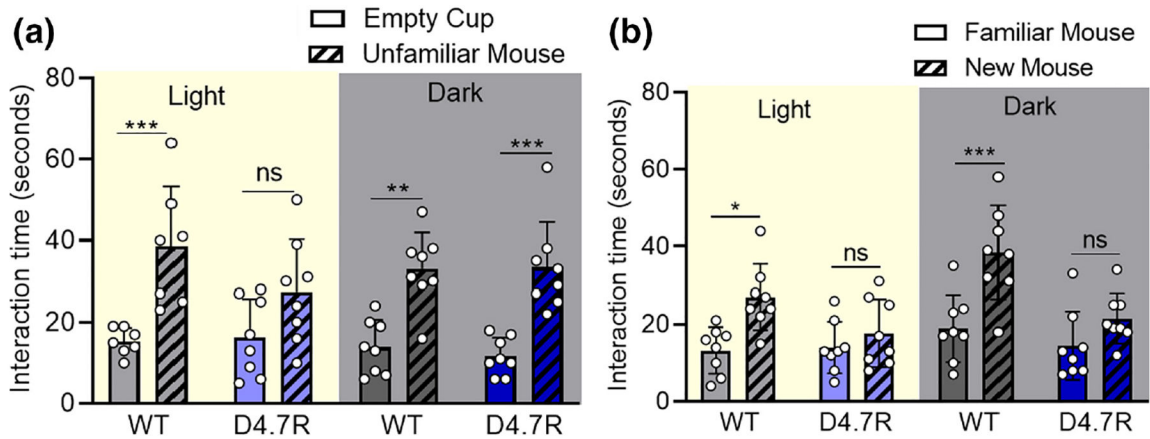
Figure 6. Transgenic D$_{4.7}$R mice display impaired sociability during the light phase and impaired social recognition in the light (ZT4-ZT7) and dark (ZT16-ZT19) phases

(a) Social interaction during the light and dark phases, presented as time spent with the empty cup or the unfamiliar mouse, three-way ANOVA, followed by Bonferroni's multiple comparisons test: (genotype: $F_{(1, 54)} = 0.3198$, $P=0.574$, light-dark: $F_{(1, 54)} = 1.435$, $P=0.236$, cup: $F_{(1, 54)} = 57.99$, $P<0.001$, cup $\times$ light-dark: $F_{(1, 54)} = 0.879$, P=0.353, cup $\times$ genotype: $F_{(1, 54)} = 0.430$, $P=0.515$, light-dark $\times$ genotype: $F_{(1, 54)} = 0.6699$, $P=0.417$, cup $\times$ light-dark $\times$ genotype: $F_{(1, 54)} = 2.158$, $P=0.148$. Empty Cup vs. Unfamiliar Mouse: **$P<0.01$, ***$P<0.001$, ns, not significant. n = 7 WT and 8 D$_{4.7}$R mice in the light phase, and 8 WT and 8 D$_{4.7}$R mice in the dark phase. Data are presented as means±SD

(b) Social novelty during the light and dark phases, presented as time spent with the familiar mouse or the new mouse, three-way ANOVA, followed by Bonferroni's multiple comparisons test: (genotype: $F_{(1, 56)} = 12.46$, $P=0.0008$, light-dark: $F_{(1, 56)} = 6.378$, $P=0.014$, old-new mouse: $F_{(1, 56)} = 27.18$, $P<0.0001$, mouse $\times$ light-dark: $F_{(1, 56)} = 1.162$, $P=0.286$, mouse $\times$ genotype: $F_{(1, 56)} = 7.145$, $P=0.01$, light-dark $\times$ genotype: $F_{(1, 56)} = 2.314$, $P=0.134$, old-new mouse $\times$ light-dark $\times$ genotype: $F_{(1, 56)} = 0.096$, $P=0.758$. Familiar Mouse vs. New Mouse: *$P<0.05$, ***$P<0.001$, ns, not significant. n = 8 WT and 8 D$_{4.7}$R mice in the light phase, and 8 WT and 8 D$_{4.7}$R mice in the dark phase. Data are presented as means±SD