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The orphan receptor GPR88 controls impulsivity and is a risk factor for Attention-Deficit/ Hyperactivity Disorder

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Abstract

The neural orphan G protein coupled receptor GPR88 is predominant in the striatum and cortex of both rodents and humans, and considered a potential target for brain disorders. Previous studies have shown multiple behavioral phenotypes in *Gpr88* knockout mice, and human genetic studies have reported association with psychosis. Here we tested the possibility that GPR88 contributes to Attention Deficit Hyperactivity Disorder (ADHD). In the mouse, we tested *Gpr88* knockout mice in three behavioral paradigms, best translatable between rodents and humans, and found higher motor impulsivity and reduced attention together with the reported hyperactivity. Atomoxetine,

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Author Contribution

S.B.H. and BLK designed and led the project, analyzed and interpreted the data and wrote the manuscript. S.B.H., E.C., M. M., E.M. performed mouse behavioral experiments. E.D. contributed to the project design and edited the manuscript. S.M.S., M.T.S., N.G., and R.J. conceived and designed the genetic/clinical study. S.M.S., M.E.F., M.T.S., N.G. and R.J. performed and analyzed the genetic data. S.M.S. and M.T.S. interpreted the genetic/clinical data and edited the manuscript. All authors gave final approval of the version submitted.

Conflict of interests

S.B.H., S.M.S., E.C., M.M., E.M., M.M., E.D., M.T.S., M-E. F. and B.L.K. report no biomedical financial interests or potential conflicts of interest. Dr. Natalie Grizenko reports receiving research funding from CIHR and is a member of the advisory board for Purdue and Shire. Dr. Ridha Joober reports having received research funding from CIHR. He is on the advisory boards and speakers' bureaus of Pfizer, Janssen Ortho, BMS, Sunovion, Otsuka, Lundbeck, Perdue and Myelin. He has received grant funding from them and from AstraZeneca and HLS. He has received honoraria from Janssen Canada, Shire, Lundbeck, Otsuka, Pfizer and from Perdue for CME presentations and royalties for Henry Stewart talks.

a typical ADHD drug, reduced impulsivity in mutant mice. Conditional *Gpr88* knockout mice in either D1R-type or D2R-type medium spiny neurons revealed distinct implications of the two receptor populations in waiting and stopping impulsivity. Thus, animal data demonstrate that deficient GPR88 activity causally promotes ADHD-like behaviors, and identify circuit mechanisms underlying GPR88-regulated impulsivity. In humans, we performed a family-based genetic study including 567 nuclear families with DSM-IV diagnosis of ADHD. There was a minor association for SNP rs2036212 with diagnosis, treatment response and cognition. A stronger association was found for SNP rs2809817 upon patient stratification, suggesting that the T allele is a risk factor when prenatal stress is involved. Human data therefore identify *GPR88* variants associated with the disease, and highlight a potential role of life trajectories to modulate GPR88 function. Overall, animal and human data concur to suggest that GPR88 signaling should be considered a key factor for diagnostic and treatment of ADHD.

Brief summary

Mutant mouse behavior and human genetic data concur to suggest that the orphan receptor GPR88 contributes to Attention-Deficit/ Hyperactivity Disorder.

Introduction

GPR88 is a brain orphan G protein coupled receptor (GPCR), with predominant striatal and cortical expression in both rodents and humans^{1, 2}. In preclinical research, current knowledge of GPR88 function is mostly based on behavioral analyses of genetically modified mice lacking the *Gpr88* gene constitutively (or *Gpr88* knockout mice). These mutant mice show a complex endophenotype, and most prominent deficits include motor hyperactivity and motor coordination deficits³⁻⁵, failure to habituate and risk-taking behavior⁵⁻⁷, impaired sensorimotor gating³ and sensory processing¹, reduced working memory and cognitive flexibility⁷, as well as increased alcohol seeking⁸. Resting-state functional Magnetic Resonance Imaging of these mutant mice shows broad remodeling of brain connectivity⁹. In brief, major modifications were identified for the retrosplenial cortex, central to the Default Mode Network, as well as somatosensory and corticomesolimbic networks, likely related to their hyperactivity, sensory deficits and high alcohol drinking behavior. Finally, stronger correlation of motor cortex and caudate putamen activities was consistent with impaired motor coordination and altered habit learning⁵ in these animals. Altogether, both behavioral studies and neuroimaging concur to demonstrate multiple GPR88 functions in the brain, hence the potential of GPR88 as a target for neuropsychiatric disorders is promising. Pharmacology is still at an early stage, and a first GPR88 agonist showed *in vivo* efficacy in reducing alcohol drinking¹⁰, consistent with the high drinking phenotype of *Gpr88* knockout mice.

Cellular mechanisms underlying GPR88 function have focused on the striatum. In this brain structure, GPR88 is almost equally distributed between medium spiny neurons (MSNs) of the direct (expressing dopamine D1 receptors or D1R-MSNs) and indirect (expressing dopamine D2 receptors or D2R-MSNs) pathways⁴, known to exert opposing influences on motor output systems. We previously showed that conditional deletion of the *Gpr88* gene in either D1R- or D2R-MSNs demonstrates dissociable roles of GPR88 in the two pathways.

D2R-MSN GPR88 mutant mice recapitulated most locomotor phenotypes of constitutive knockout animals, while the D1R-MSN mutants specifically showed lack of habituation and motor skill learning deficits¹¹. The cell-type specificity of GPR88 function also extends to sensory gating¹² and anxiety-like behaviors¹³. Thus, distinct cellular and circuit mechanisms subserve the complex GPR88 function in the brain.

Studies in humans remain limited. A study identified a deleterious homozygous mutation in the *GPR88* gene in a consanguineous family, affecting four sisters with childhood chorea, learning disabilities and speech retardation¹⁴. Another genetic study performed in three different populations found a positive association between single nucleotide polymorphisms (SNPs) of the *GPR88* gene and bipolar disorder in Sardinian and Palestinian triads, and with schizophrenia in triads from the Xhosa population in South Africa^{15, 16}. Also, a genome wide bioinformatics analysis of miRNA-mRNA pairs identified a 3'UTR *GPR88* variant possibly associated with intellectual disability¹⁷. These studies therefore reveal the relevance of *GPR88* as a risk factor for brain development and psychosis, supporting the search for association with perhaps other neuropsychiatric disorders.

In this report, we examine the possibility that GPR88 may represent a risk factor for Attention-Deficit/Hyperactivity Disorder (ADHD), a highly prevalent developmental psychiatric condition¹⁸. ADHD affects children and, when left untreated, is associated to adverse consequences in adulthood, including drug abuse, anxiety, depressed mood, disturbance of emotions, and social rejection^{19–21}. The disease is characterized by three main symptoms, namely hyperactivity, inattention and impulsivity (Fig. 1). In animal research, several behavioral models with face and/or predictive validity have been proposed to address these ADHD-like core symptoms²². Here, we first tested *Gpr88* knockout mice in three behavioral paradigms considered best translatable between rodents and humans for ADHD-like symptoms (Fig. 1), and found deficits in all the tests. Second, we found that a typical ADHD drug used in the clinic, atomoxetine, reduces the ADHD-like phenotype of mutant mice. Third, we tested conditional D1R-MSN and D2R-MSN *Gpr88* knockout mice and discovered pathway-specific mechanisms of GPR88 function in the regulation of motor impulsivity. Fourth, we performed a family-based genetic study in a clinical sample of children with ADHD, covering six *GPR88* SNPs, and found a significant association with multiple dimensions of ADHD, particularly under conditions of prenatal stress.

Methods

The mouse study

Mice.—Total *Gpr88*^{-/-} mice were produced as described in Ref⁵. Conditional A2AR-*Gpr88* and D1R-*Gpr88* mice lacking GPR88 in D2R- and D1R-MSNs, respectively, were produced as described in Ref¹¹. The mice were bred at the Douglas Research Centre. All animals were group-housed with littermates (2–5 animals per cage) under a 12h light/dark cycle and given food and water ad libitum unless otherwise stated.

Behavior.—Procedures used for the 5-Choice Reaction Time Task (5-CSRTT), Attentional Set Shifting Task (ASST), Go-NoGo task, atomoxetine administration and statistical

analysis of the animal study are detailed in the Suppl Methods section. Statistical analysis of the data is detailed in Suppl Tables S1 and S2.

The human study

Participants and evaluations: ADHD.—Children (between 6 and 12 years of age, mean=9 years) with a diagnosis of ADHD, were recruited from the Disruptive Behavior Disorders Program and the child psychiatry outpatient clinics at the Douglas Mental Health University Institute (DMHUI) in Montreal. They were referred to these specialized care facilities by schools, community social workers, family doctors and pediatricians. Parents were explained the study and provided written consent. Children (affected child and unaffected siblings) were explained the study and gave their assent to participate. Children with an IQ less than 70, and/or a diagnosis of either Tourette syndrome, pervasive developmental disorder, or psychosis were excluded from the study.

Details of the pharmaco-behavioural-genetic study and the different assessments conducted have been previously described²³. Briefly, the diagnosis (based on DSM-IV criteria) was based on clinical interviews between the child psychiatrist, the affected child and at least one parent. This clinical examination was supplemented with a structured clinical interview of parents using the Diagnostic Interview Schedule for Children-version IV, DISC-IV²⁴ and school reports including the Conners' Global Index-Teacher version questionnaire (Conners'-T)²⁵. The child's behaviour at home was evaluated using the Conners' Global Index-Parents (Conners'-P)²⁶ and the Child Behaviour Checklist (CBCL)²⁷. In the majority of cases, mothers were the primary informants. These evaluations were completed while the child was not taking any medication.

Cognitive dimensions, specifically those related to executive function (EF), were included as quantitative traits in the genetic association analyses. EF encapsulates the range of cognitive abilities that are required for completing a given task, and include response inhibition, sustained attention, working memory, set-shifting, planning and organization. The neuropsychological tests conducted were: Conners' Continuous Performance Test (CPT: measures attention, response inhibition, and impulse control)²⁸, Self-Ordered Pointing Task (SOPT: visual working memory, planning, and response inhibition)²⁹, Wisconsin Card Sorting Test (WCST: measure of cognitive flexibility and set-shifting) (Heaton, Chelune, Talley, Kay, & Curtiss, 1993), and Tower of London test (TOL: planning, organization, and problem-solving capacity)³⁰. These tests were performed as described^{23, 31}. These neuropsychological assessments were carried out at the end of a one-week washout period if the child was on medication prior to inclusion in the study. In addition to the measures of EF, IQ (full scale, verbal, and performance IQ) was evaluated using the Wechsler Intelligence Scale (WISC-III/IV)³².

Obstetrical (pregnancy, delivery and perinatal) complications were assessed using the Kinney Medical and Gynecological Questionnaire and scored using the McNeil-Sjöström scale³³. Mothers were also asked to describe stressful life events experienced during the pregnancy, if any. This information was used to score maternal stress levels from 1 to 4 based on the DSM-III and DSM-III-R axis IV scales (1 = no stress, 2 = mild, 3 = moderate, 4 = severe). Examples of mild stress include events such as arguments with

friends; moderate/ severe stressors include separation from partner, repeated physical or sexual abuse, imprisonment of a spouse, or death of a very close relative. Since information on environmental factors was collected retrospectively (and may therefore be subject to recall bias), the information provided by the mother was corroborated in an independent interview with a second individual close to the mother wherever possible.

Response to treatment with MPH was assessed in a double-blind, placebo-controlled, within-subject (crossover) randomized control trial conducted over a two-week period, as described²³ (trial registration number: [NCT00483106](#)). Briefly, subjects received one week of treatment with placebo (PBO) and one week of treatment with 0.5 mg/kg of MPH in a divided b.i.d. dose (0.25 mg/kg, morning and noon), following a wash-out period. At the end of each week of treatment, the parents and teacher were asked to evaluate the behaviour of the child using the Conners'-parents and Conners'-teachers respectively. In addition, the clinical staff completed the Clinical Global Impression (CGI)-overall improvement during half day of observation of overall behaviour of the child while completing various tasks in the clinic.

Genetics.—The affected child, parents and unaffected siblings were invited to participate in the genetic component of the study. DNA was extracted from a blood or saliva sample for each participant, if the subject was only amenable to the latter. A panel of 8 SNPs (rs2036212, rs2809823, rs2809822, rs2809819, rs2809818, rs2030048, rs2809817, rs2030049) were genotyped using Sequenom iPLEX Gold Technology³⁴, based on the markers previously examined¹⁶. Two SNPs failed the genotyping (rs2809822, rs2809819). For each of the rest of the SNPs, genotyping error was estimated by including duplicates of two reference samples on every plate. Genotypes for these samples were read with 100% accuracy on each of the plates, and each SNP was genotyped in more than 95% of the patients.

Statistics.—Family based tests of association were conducted using the FBAT statistical package (version 2.0.3)³⁵. Family-based association analysis has two major advantages over population-based (case/control) association studies: it is not affected by population stratification, and it may have increased statistical power³⁶. Further, because the non-transmitted parental alleles are the control alleles, this method controls for other possible sources of bias, such as socio-economic status. All the analyses were performed under the assumption of an additive model, with a null hypothesis of no linkage and no association.

In a first step, analysis was conducted with the total sample. Further analysis was conducted in two distinct groups: (1) Families where mother was exposed to no/mild stress during pregnancy (examples of mild stressors include minor financial worries and arguments with friends); (2) Families where mother was exposed to moderate/extreme stress during pregnancy (examples of these stressors include major financial problems, physical/sexual abuse, and imprisonment/abandonment of partner). Significance level was set at $P < 0.05$. Due to the exploratory nature of this study, no correction for multiple-testing was performed. When the total sample was stratified based on maternal stress during pregnancy, SNP rs2036212 was not informative due to lack of the minimum number of families ($n=10$) required.

In a specific case of the FBAT (where both parents are known, and when the additive model is used), the Z^2 statistic can be considered equivalent to a χ^2_{TDT} statistic (N. Laird, personal communication). FBAT is an extension of McNemar's test used to calculate transmission disequilibrium in a pedigree, where $\chi^2_{\text{TDT}} = (T - NT)^2 / (T + NT)$. T and NT denote the number of transmissions and non-transmissions of a specific allele from the parent to the affected offspring. In order to obtain an estimate of effect size, we applied this generalization and calculated the effect size Φ as for a χ^2 test, where the following formula is used $\Phi = \text{square root} [\chi^2 / N(k-1)]$, where N=sample number, and k=number of rows or columns or 2 in the McNemar's test. The number of informative families was used to calculate N. Effect sizes of 0.1, 0.3, and 0.5 are considered small, medium and large respectively (Suppl. Table S3).

Compliance with ethical standards.

All animal procedures in this report were conducted in accordance with the guidelines of the Canadian Council of Animal Care and by the Animal Care Committees of McGill University/Douglas Mental Health University Institute. The human research protocol was approved by the DMHUI Research Ethics Board.

Results

***Gpr88* knockout mice (or *Gpr88*^{-/-} mice) are impaired in the Five-Choice Serial Reaction Time Task (5-CSRTT).**

The 5-CSRTT (Suppl. Fig. S1) evaluates all three features of ADHD, namely hyperactivity, inattention and impulsivity³⁷, and is considered most translatable between humans and rodents³⁸. In this test, *Gpr88*^{-/-} mice were able to learn the task normally (Suppl. Fig. S2). However, at testing (Fig. 2A and statistics in Suppl. Table S1), mutant mice displayed reduced accurate responding, which requires spatial and temporal attention, and this was observed across the four stimulus presentation times. Further, more omission errors were found at shorter intervals, indicating that attention deficits relate to task difficulty. *Gpr88*^{-/-} mice also made more responses prior to signal presentation (premature responses) during the 5s-period preceding stimulus presentation, an index of higher motor impulsivity. Finally, *Gpr88*^{-/-} mice showed increased beam breaks (activity), confirming hyperactivity in these mice. Otherwise, reward collection latency was higher or unchanged, suggesting intact motivation to retrieve the food. The absence of GPR88 in mice therefore produces hyperactivity, and seems to decrease attention and increase motor impulsivity in the 5-CSRTT. The two latter phenotypes were further confirmed using the ASST and Go/NoGo task.

***Gpr88* knockout mice are impaired in the Attentional Set Shifting task (ASST).**

The ASST, based on human cognitive testing³⁹, is also used in rodents⁴⁰. The paradigm involves seven successive steps of increasing difficulty, where an odor and/or a medium are associated with a reward (Suppl. Fig. S3). Data for three parameters are shown in Fig. 2B and statistics are in Suppl. Table S1. In the first three steps of the procedure, mutant mice made more wrong trials in compound discrimination (CD) and reversal 1 (R1) steps, and showed higher average time to choose in the simple discrimination (SD) task, suggesting lower capability to maintain attention. Importantly, *Gpr88*^{-/-} mice were slower in

acquiring the last task (extra-dimensional shift, EDS) considered most meaningful, and this was observed for all the parameters (number of trials, wrong trials and latency to choice). Lack of GPR88 in mice therefore reduces attentional performance in the ASST. This finding was further confirmed in the maternal stress experiment (Suppl. Fig. S9).

Gpr88 knockout mice are impaired in the Go/No-Go task

This task⁴¹ was designed to detect deficient inhibitory processes, which characterize exacerbated impulsivity in children with ADHD, and was successfully transposed to rodents⁴² (Suppl. Fig. S4). In the conditioning phase (Suppl. Fig. S5), Gpr88^{-/-} mice showed a learning pattern similar to Gpr88^{+/+} controls, suggesting that the motivational state was intact. In the Go phase (Fig. 3A and statistics in Suppl. Table S1), the two groups similarly used fewer nose pokes to earn more rewards between first and last trials, indicating normal learning of the task. However, Gpr88^{-/-} mice showed significantly more premature responses, demonstrating lower ability to refrain from initiating an action. Despite higher premature responding, mutant mice eventually reached the criterion and moved to the next Go/No-Go step. In this phase success rates for the Go trials remained stable (Suppl. Fig. S6), however, Gpr88^{-/-} mice showed higher number of premature responses, more commission errors (inability to refrain from inappropriate responding) and a higher impulsivity index (Fig. 3B and statistics in Suppl. Table S1). Thus, the absence of GPR88 in mice increases motor impulsivity in the Go/No-Go task.

We also treated Gpr88^{-/-} mice and their controls with atomoxetine (Suppl. Fig. S7), a noradrenaline reuptake blocker used clinically to treat both adult and childhood ADHD⁴³. Atomoxetine was tested in the Go phase. In the saline groups, as expected, the percent of premature responses was increased in Gpr88^{-/-} mice compared to Gpr88^{-/-} controls. Atomoxetine (10 mg/kg) caused a drastic reduction in the percentage of premature responses in both genotypes, and the increased impulsivity detected in Gpr88^{-/-} mice was brought down to the level of Gpr88^{-/-} controls. The drug had no effect on accuracy indicating a preserved instrumental memory performance in atomoxetine treated mice. These data confirm predictive validity of the Go/No-Go task under our experimental conditions, and supports the notion that lack of GPR88 activity produces an ADHD-like impulsive phenotype.

A2A-Gpr88 and D1-Gpr88 knockout mice are impaired in waiting and stopping impulsivity, respectively

We previously showed dissociable roles of GPR88 at the level of MSN of the direct and indirect pathways for a number of behaviors¹¹. To investigate whether GPR88 in D2R- and D1R-MSNs plays differential roles in the regulation of impulsive behaviors, we tested A2AR-*Gpr88* (D2R pathway), and D1R-*Gpr88* mice and their corresponding controls (A2AR-*Ctl* and D1R-*Ctl*) in the Go/No-Go task. A2AR-*Gpr88*, and D1R-*Gpr88* mice did not differ from their controls in the conditioning phase (Suppl Fig. S8), suggestive of intact motivational state in the conditional mutants. In the Go phase, all the groups used fewer nose pokes to earn more rewards between first and last trials, indicating normal learning of the task (not shown). During testing however (Fig. 4A and Suppl. Table S1), A2AR-*Gpr88* mice showed significantly more premature responses over all sessions, as well as between

first and last sessions, indicating lower ability to wait during the pre-trial period. This was not the case for *D1R-Gpr88* mice.

Despite higher premature responding, *A2AR-Gpr88* mutant mice eventually reached the criterion of accuracy and moved to the next Go/No-Go step. In this phase (Fig. 4B–D and statistics in Suppl. Table S1), accuracy for the Go trials remained stable (not shown). However again, *A2AR-Gpr88*, but not *D1R-Gpr88*, mice showed higher number of premature responding (Fig. 4B), confirming lower inhibition control. Interestingly, the percentage of commission errors decreased normally along sessions in *A2AR-Gpr88* mice, similar to their controls, but remained stable in *D1R-Gpr88* mice. Thus, commission errors were higher than controls in several sessions, over the last 5 sessions and when comparing first and last session, indicating failure for *D1R-Gpr88* mice to refrain their impulsive behavior. This was confirmed by the impulsivity index (Fig. 4D) that was higher across sessions for *D1R-Gpr88* but not *A2AR-Gpr88* mice.

Overall therefore, *A2AR-Gpr88* mice were unable to withhold from starting the task (higher premature anticipatory responding, failure to wait) while *D1R-Gpr88* mice were unable to refrain from performing the task (higher commission errors, failure to refrain from acting). Together these results clearly differentiate two distinct GPR88-mediated mechanisms regulating impulsivity.

The human GPR88 gene is associated with ADHD in children

Because ADHD-like behaviors were detectable in *Gpr88*^{-/-} mice across three gold standard animal models, we next moved to humans and investigated whether genetic variants in the *GPR88* gene show an association with ADHD dimensions in a clinical sample of children with ADHD (clinical characteristics in Methods).

We tested a selected panel of 6 single nucleotide polymorphisms (SNPs) for association with overall DSM-IV diagnosis, quantitative measures of behavior and cognition, and response to treatment with a fixed dose of methylphenidate (MPH) using family-based association tests (see Methods). The study included 567 nuclear families having one or more child with a DSM-IV diagnosis of ADHD⁴⁴. Of the total number of children included in this family-based study, 78.1% were male; 52.9% were diagnosed with the combined subtype, while 38.1% and 9% had the inattentive and hyperactive subtypes of ADHD respectively. Among comorbid disorders, 41.9% had oppositional defiant disorder, 17.3% had conduct disorder, 43% had anxiety disorder (including phobias), and 7% had a mood disorder.

In the total sample, a significant but minor association was observed with one of the 6 SNPs in the selected panel (rs2036212, 5' gene¹⁶) with overall DSM-IV diagnosis and dimensions of behaviour, treatment response and cognition (Table 1 and statistics in Table S3).

More interestingly, however, when the total sample was stratified based on maternal stress during pregnancy, a highly significant association was observed with tag SNP rs2809817 (3' UTR¹⁶) in the group where mothers experienced moderate to severe stress during pregnancy. Here, a significant over-transmission of the T allele was observed with categorical DSM-IV diagnosis ($Z=3.41$, $P=0.0006$), suggesting that this allele carries risk for

ADHD. Further association was observed in the quantitative FBAT analysis. The T allele showed association with: (a) total number of ADHD items ($Z=2.74$, $P=0.006$); (b) number of inattention ($Z=2.67$, $P=0.008$), hyperactivity ($Z=2.73$, $P=0.006$), and impulsivity ($Z=2.70$, $P=0.007$) items on DISC-IV; (c) Conners' -P ($Z=2.25$, $P=0.02$) and Conners' -T ($Z=2.85$, $P=0.004$) scores at baseline; (d) dimensional scores on the Child Behaviour Checklist (CBCL). This suggests that this allele is associated with a more severe symptom profile as assessed in the home (Conners' -Parents, CBCL, DISC), school (Conners' -T), and clinic (DSM-IV diagnosis).

In terms of cognitive function, association was observed with several dimensions, with the T allele over-transmitted to higher number of errors: (a) spatial working memory, planning and interference control, as measured by the SOPT; (b) commission errors on the CPT; (c) non-perserverative errors on the Wisconsin Card Sorting Test. It is interesting that this tag SNP shows association not only with clinical dimensions of ADHD, but also with Research Domain Criteria (RDoC) within the domain of cognitive function. Interestingly, significant association was also observed with response to treatment with MPH (Table), as noted in clinic.

It is also interesting to note that when family-based analysis was conducted in the group where the mothers experienced none/mild stress during pregnancy, the T allele of tag SNP rs2809817 showed significant association with: (a) lower number of inattention items on DISC-IV (under-transmission of T allele; $Z= -1.99$, $P=0.05$); (b) lower scores on specific dimensions on CBCL, particularly withdrawn behaviour ($Z= -2.84$, $P=0.004$); (c) better performance on the SOPT ($Z= -2.37$, $P=0.02$). These results suggest that not having the T allele at this locus may be protective on some dimensions of ADHD.

No correction for multiple comparisons was applied here. If the stringent Bonferroni correction was to be applied (6 SNPs X two exposure strata), the cut-off for significance would be set at $p = 0.05/12$ or 0.003 . At this cut-off, the association in the total sample would not be significant. However, the association observed in the group where the mothers experienced moderate to severe stress during pregnancy would be significant.

Stress influence in the mouse model.

Because of the major influence of environmental conditions in the human association analysis, we back-translated the finding in $Gpr88^{-/-}$ mice, and tested whether maternal stress during pregnancy would influence endophenotype of the offspring. Pregnant $Gpr88^{-/-}$ females and their $Gpr88^{+/+}$ controls were subjected (or not) to unpredictable chronic stress during gestation and their adult offspring tested in the ASST (Suppl Fig. S9). Under no stress condition, $Gpr88^{-/-}$ mice needed more trials to reach criterion (SD, CD, IDS1 and EDS) and made more wrong trials (SD and EDS) compared to $Gpr88^{-/-}$ controls, as expected. Stressed $Gpr88^{-/-}$ mice showed enhanced deficits compared to stressed $Gpr88^{+/+}$ mice, shown by increased number of trials and wrong trials in both CD and EDS (Supplemental Table S1). Maternal stress therefore worsens the pre-existing attentional deficit of $Gpr88^{-/-}$ mice.

Discussion

Here we report a preclinical and a clinical study, which both address the possibility that GPR88 signaling in the brain may regulate behavioral dimensions related to ADHD. In the mouse study, mutant mice lacking the *Gpr88* gene showed hyperactivity, reduced attention and higher motor impulsivity throughout three testing procedures. Deletion of *Gpr88* gene in either indirect or direct pathways of the mesolimbic dopamine circuitry further revealed distinct implications of these two receptor populations in waiting and stopping impulsivity. The animal data therefore demonstrate that deficient activity of the orphan GPR88 receptor causally promotes ADHD-like behaviors, and also identify part of the circuit mechanisms underlying GPR88-regulated motor impulsivity. In the human family-based association study, conducted in a cohort of children affected with ADHD, a minor association was found for the tag SNP rs2036212 with DSM-IV diagnosis, treatment response and cognition. A much stronger association was found for another tag SNP when the patient population was stratified, suggesting that the T allele of tag SNP rs2809817 becomes a risk factor for ADHD when prenatal stress is involved. Finally, the stress effect back-translated into the mouse experimentation.

GPR88 regulates impulsive behaviors

This is the first report indicating that GPR88 activity regulates impulsive behaviors. Prior animal research by others and us showed that the constitutive deletion of GPR88 in mice alters a number of behaviors in the adult¹⁻⁸. In particular, we previously found high risk-taking behavior⁵ and excessive alcohol drinking⁸ in these mutant mice, features associated to ADHD in humans^{45, 46}. These findings led us to hypothesize that deficient inhibitory controls may contribute to their complex phenotypic pattern. In the present study, we found higher impulsivity in these mice in the 5-CSRTT (increased premature responses and omissions), and this was further confirmed in the Go/No-Go paradigm (higher premature responses in Go and NoGo, higher commission errors and impulsivity index in NoGo). These results have implications for psychiatric research, as dysfunctional GPR88 signaling may increase impulsivity, a symptom known to cross-cut a number of disease conditions, including borderline personality, obsessive compulsive behaviors, addiction and ADHD, and even adverse effects of Parkinson Disease treatment⁴⁷.

It will be important to know whether GPR88-mediated impulse control is due to tonic receptor activity in the adult, or to compensatory rewiring of GPR88-expressing neural circuits during development. A partial response comes from the use of conditional *Gpr88* knockout mice in the Go/NoGo experiment. In the latter mice, the genetic deletion was limited to one neuron population, namely either D1R-MSNs or D2R-MSNs, and occurred later in development⁴⁸. We found that the two conditional lines together recapitulate the impulsivity-related deficits observed in the germ line *Gpr88* knockout mice, making unlikely the possibility that the constitutive mutant phenotype is entirely due to early developmental compensatory mechanisms. In the future, the novel pharmacological tools under development may definitely answer this question^{10, 49, 50}.

Mechanisms of GPR88-regulated impulsivity

A2AR-*Gpr88* mice (D2R cells) but not D1R-*Gpr88* mice (D1 cells) showed higher premature responses in both Go and NoGo phases, suggesting a specific role of GPR88 expressed in the indirect pathway in “waiting impulsivity” or the inability to withhold from initiating the task^{38, 51}. On the contrary, D1R- but not A2A-*Gpr88* mice showed higher commission errors and impulsivity index in the NoGo phase, indicative of a specific implication of GPR88 expressed in the direct pathway in “stopping impulsivity” or the inability to refrain from performing the task^{38, 52}. These results first demonstrate that GPR88 expressed in MSNs of the striatum are essential in regulating motor impulsivity. Second, the data clearly show dissociable cell-specific roles for the two striatal GPR88 populations.

The direct (D1R-MSNs) and indirect (D2R-MSNs) mesolimbic pathways are known to promote or suppress behaviour, respectively^{53, 54}. A correct balance between the two activities within cortico-basal ganglia-thalamic circuits is key to movement control, action selection and goal-directed behaviors, and has implications for several neuropsychiatric disorders, including notably impulsive-compulsive behaviors in Parkinson Disease or addiction⁵⁵. Because GPR88 is expressed on these two opposing pathways, one may expect that deleting the receptor gene from one or the other circuit has contrasting effects, which we already documented¹¹, and demonstrate again here for impulsive behaviors. At present, waiting and stopping impulsivities⁵⁶ have been associated to ventral and dorsal striatum, respectively³⁸. Our data suggest that, in addition to these anatomical subdivisions, the two main striatal circuits also contribute to regulate distinct aspects of motor impulsivity. Further testing in other tasks (e. g. inter-temporal choice, stop-signal reaction time task) may definitely confirm a dissociation of A2A-GPR88 and D1-GPR88 roles in stopping and waiting impulsivity, respectively. Here we propose that releasing a brake from the indirect (A2AR-*Gpr88* mice) or direct (D1R-*Gpr88* mice) circuits alters the capacity to withhold from responding (waiting) or stop a response that has been initiated (stopping), respectively. Our data are also consistent with findings that D2/3 receptor availability is associated with waiting impulsivity⁵⁷. Finally, beyond the direct/indirect mesolimbic pathway hypothesis, it is likely that other circuits are involved in regulating behaviors engaged in the Go/NoGo task. Other populations of GPR88-expressing neurons within striatal-associated cortico-thalamic loops⁷, somatosensory cortex¹ or amygdala⁵ may be investigated in the future.

Gpr88 mice, an animal model for ADHD?

DSMV criteria for ADHD include three main dimensions, i. e. hyperactivity, inattention and impulsivity, and several behavioral testing paradigms are able to investigate these different aspects of the disease, in both humans and rodents (Fig. 1). In animals⁴², hyperactivity can be detected in most experimental set-ups, levels of attention can be measured in the 5-CSRTT and ASST, and impulsivity is most studied using the Go/NoGo task. Here we show that *Gpr88* knockout mice show deficits across all the tests and, in addition, respond to atomoxetine. Preliminary results also suggest that methylphenidate could reduce locomotor hyperactivity in these mice (data not shown). This genetic line could thus be considered a mouse model for ADHD-like symptoms, with both face and predictive validity²². Only few genetic mouse lines have been proposed as models for ADHD. Mice

lacking the dopamine transporter are hyperactive in new environments, a phenotype reversed by psychostimulants^{58, 59}. Mice lacking the B2 subunit of the nicotinic receptor show ADHD-like deficits in behavioral flexibility and inhibitory controls⁶⁰. Coloboma mice with mutated SNAP-25, a synaptic protein, are also hyperactive possibly due to imbalance between dopamine and noradrenaline transmission⁶¹, and show impulsivity in a delayed reinforcement task⁶². Finally, transgenic mice carrying a mutant human thyroid hormone receptor gene show hyperactivity, inattention and high impulsivity, and are sensitive to treatment with methylphenidate⁶³. *Gpr88* knockout mice therefore represent a novel tool in attempts to model ADHD in basic research⁶⁴, with not only face and predictive validity, but also some construct validity in their alteration of dopamine transmission^{4, 8} and cortico-striatal circuit connectivity^{7, 9}.

A GPR88 risk allele for ADHD

ADHD is a high heritable disorder and genetic associations studies using candidate gene and GWAS approaches have identified a number of genes⁶⁵. Among these, are genes with known roles in neurodevelopment⁶⁶ such as *BDNF* or *SEMA6D* for example^{67, 68} or genes belonging to neurotransmitter systems, including glutamate (*GRM1*, 5 and 7), dopamine- (*DRD4*, *DRD5*), serotonin- (*5HT1B*, *SLC6A1* and 4) and acetylcholine- (*CHRNA7*) related genes. Our study proposes *GPR88* as another gene linked to ADHD, with reported roles in development and neurotransmission. This is the third human study supporting a genetic association between *GPR88* and psychiatric disorders, and the first for ADHD. Confirmation is needed, using larger independent replication cohorts.

Two tag SNPs, located in regulatory regions of the *GPR88* gene¹⁶ were significantly associated with ADHD in multiple parameters of our evaluations. The weak association for the C allele of tag SNP rs2036212 in the total human cohort, versus the strong ADHD-like phenotype observed in mice, is not unexpected as the variant (or a causal variant in linkage disequilibrium with this SNP) in humans may have less drastic consequences than the extreme *Gpr88* mutation (null allele) in a highly homogenous mouse.

The association was much stronger when maternal stress during pregnancy is involved, and here we identified the tag SNP rs2809817 as a risk allele for ADHD upon prenatal stress. Substantial literature has documented the importance of environmental factors in the development and persistence of ADHD^{44, 69, 70}, including maternal mental health⁷¹. The compelling gene x environment interaction (stress x *GPR88*) detected in the human study back-translated in the mouse model, and the data together highlight a potentially key role of life trajectories to modulate *GPR88* function, a factor to be considered for future diagnostic and treatment strategies of ADHD.

Conclusion

Although mouse models are highly reductionists, it is remarkable that detection of an ADHD-like phenotype in mice lacking the *Gpr88* gene indeed parallels a significant association in a human cohort. In fact, our mechanistic data from multiple mutant *Gpr88* mice, combined with the association of *GPR88* with multiple dimensions of ADHD in our cohort, strongly support the possibility that *GPR88* may represent a risk factor for ADHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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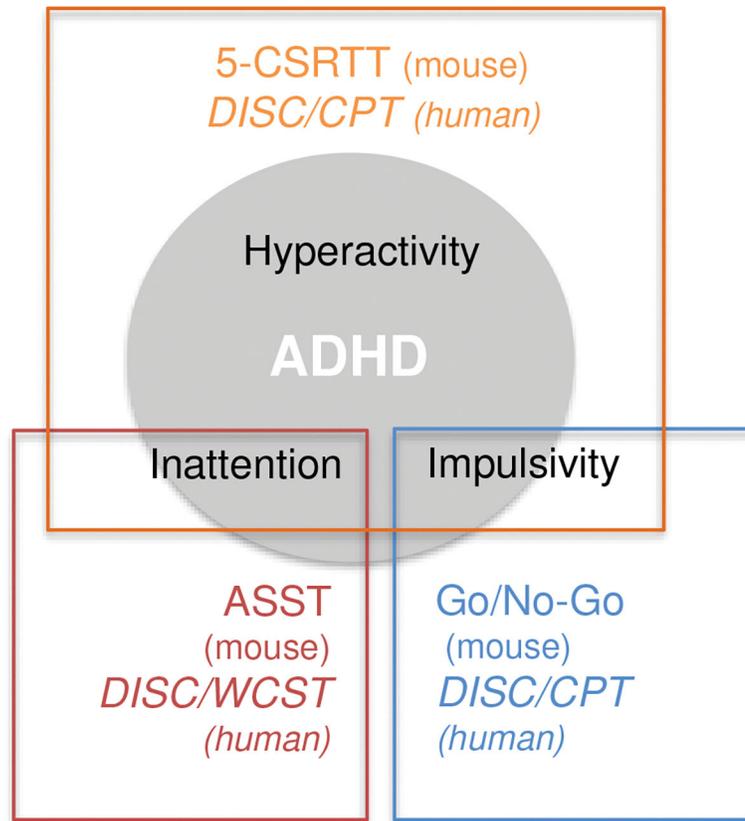


Fig. 1. Cardinal features of ADHD.

Three behavioral paradigms were used in this study to assess ADHD-like phenotypes in *Gpr88^{-/-}* mice. The five-Choice Serial Reaction Time Task (5-CSRTT) evaluates hyperactivity, motor impulsivity and inattention, which characterize clinical features of ADHD. The Go/No-Go task and Attentional Set Shifting Task (ASST) specifically address impulsivity and inattention, respectively. Human testing paradigms, which best parallel animal testing and are used in this study (Table 1), are indicated in *italics*, and include the Diagnostic Interview for Children (DISC), the Continuous Performance Test (CPT) and the Wisconsin Card Sorting Test (WCST).

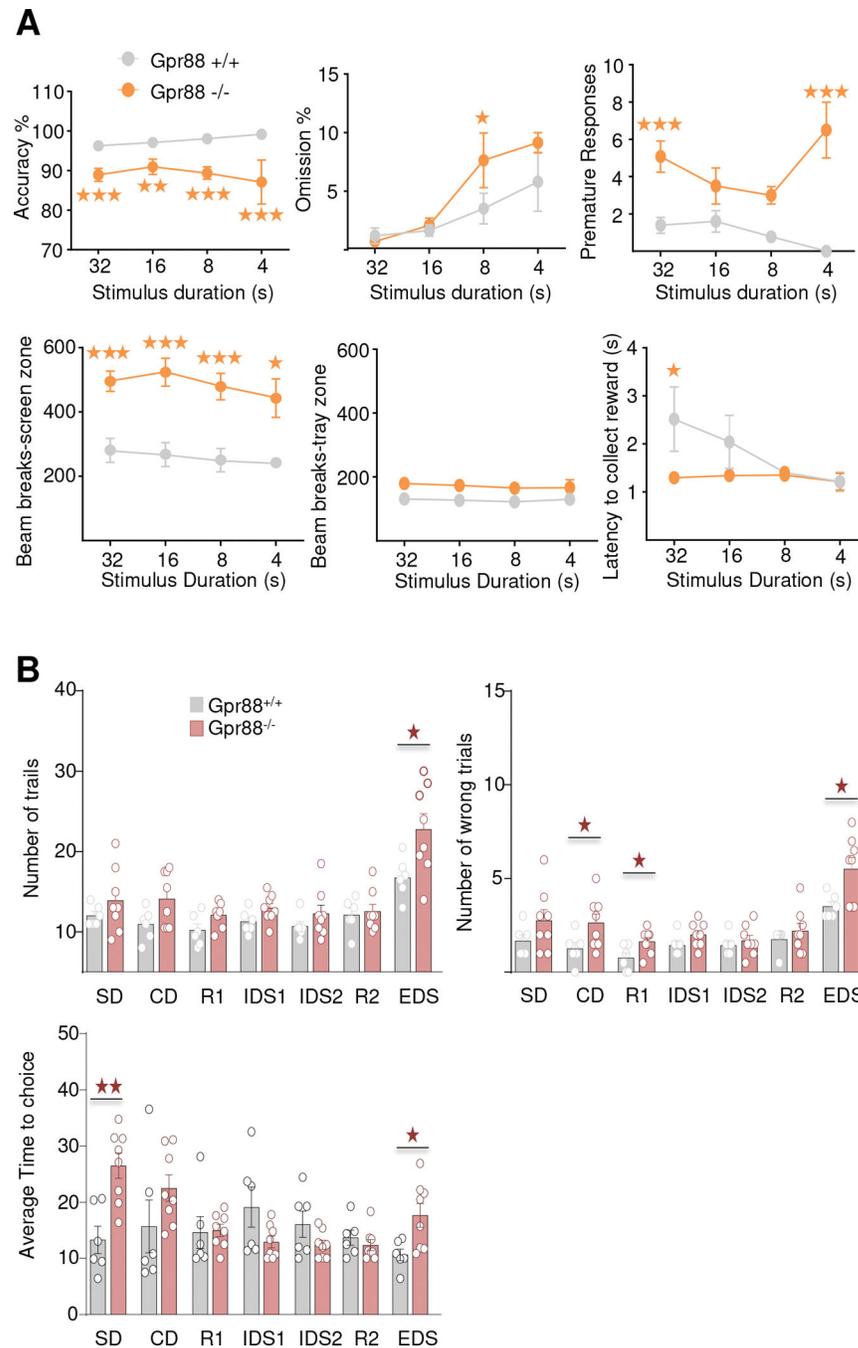


Fig. 2. Gpr88 mutant mice show ADHD-like behavior in the 5-CSRTT and higher inattention in the ASST.

A. $Gpr88^{-/-}$ mice show lower performance in all aspects of the 5-CSRTT. This test is performed in a touchscreen apparatus (Suppl Fig. S1). Mice must maintain attention to five spatial locations on a screen, and withhold from responding until a signal (flashlight) is presented randomly at one of the locations. The animal then needs to touch the screen where light was presented to obtain a food reward, a task requiring spatial and temporal attention, and effective inhibitory controls to avoid premature responding. The % accuracy was calculated as the number of correct trials divided by total number of trials \times 100 (correct

and incorrect), and the % omission was calculated as the number of missed trials divided by number of presented trials \times 100. Lower accuracy and higher omissions were found across the four stimulus durations, and the number of premature responses to stimulus presentation was higher. Animal activity (infrared beams) was higher at the screen and on the reward side of the chamber. Latency to collect the reward was increased or unchanged. $n = 6-8$. Stars show genotype effects. * $p < 0.05$. ** $p < 0.01$; *** $p < 0.001$. **B.** $Gpr88^{-/-}$ mice show lower performance in the ASST. In this test series, mice need to associate an odor and/or a medium with a reward in seven steps of increasing difficulty (Suppl Fig. S3). Steps proceed in the following order: simple discrimination (SD), compound discrimination (CD), reversal (R1), intra-dimensional shift IDS1, IDS2, reversal (R2) and extra-dimensional shift (EDS). For each step, mice are required to perform eight consecutive correct responses before moving onto the next step. $Gpr88^{-/-}$ mice needed more trials to achieve EDS, made more wrong trials in CD and EDS, and showed higher latencies to correct choice in SD and EDS. Data are represented as mean (\pm SEM) per session. $n = 6-8$. Stars show genotype effects * $p < 0.05$.

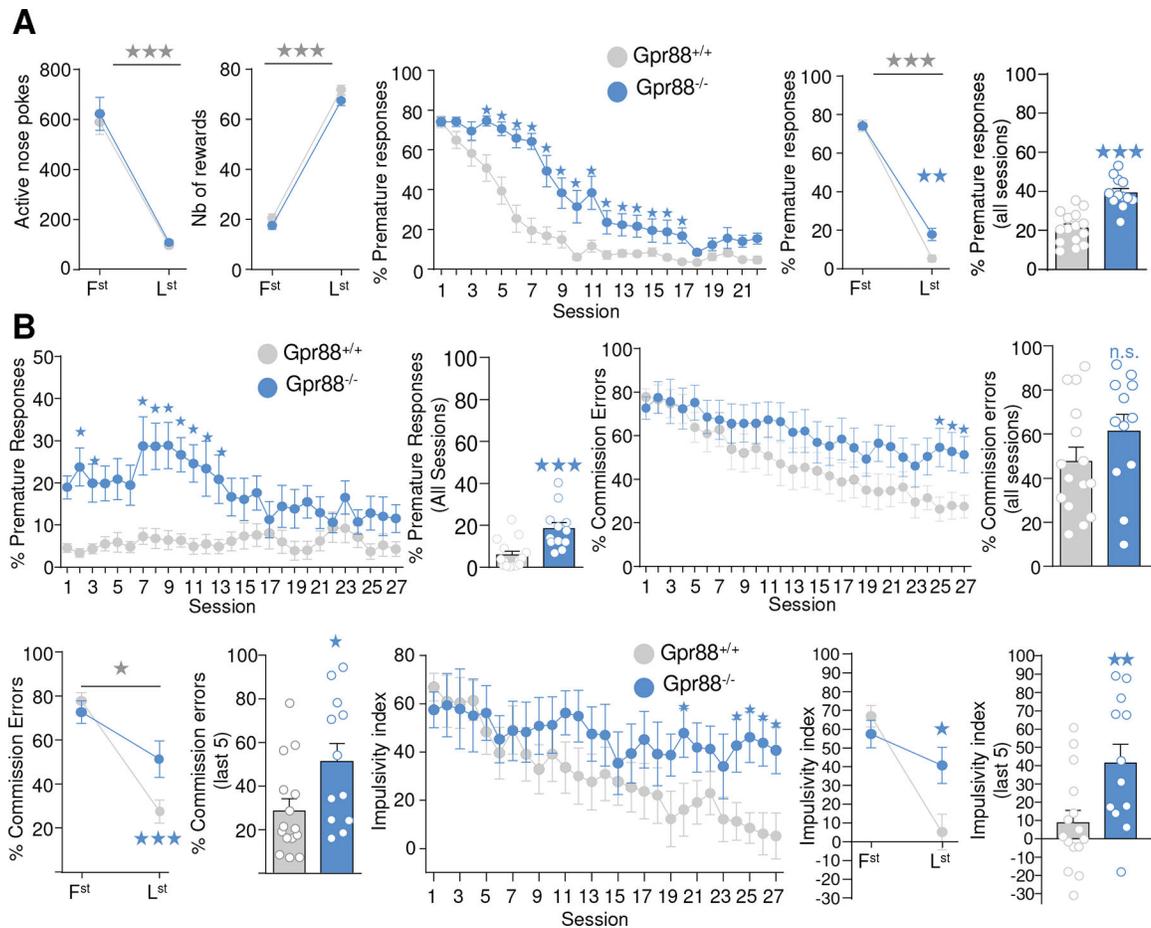


Fig. 3. Gpr88 mutant mice show higher motor impulsivity in the Go/No-Go task.

Mice are initially trained to nose poke for food reward, signaled by a light cue in operant boxes (autoshaping Suppl Fig. S4). Ability to inhibit the already initiated response was then measured in two successive experimental series. **A. Go phase.** Intertrial (no light) and pretrial (house light) periods are introduced before the cue light is illuminated, to test whether animals are able to withhold responding. Gpr88^{-/-} mice and their controls showed similar learning patterns, shown by same numbers of active nosepokes and earned rewards during the first and last session. The % premature responses (number of premature responses divided by number of pre-trials \times 100) was increased in Gpr88^{-/-} mice in sessions 4 to 11, and between first and last session. **B. No-Go phase.** Animal's ability to inhibit inappropriate behavior was further tested. A light cue only (Go, omission error) or a paired light/sound cue (no-Go, commission error) are randomly presented and rewarded. The % premature responses was increased for Gpr88^{-/-} mice in sessions 1, 7, 8, 9, 10 and 11, and over all sessions. The % commission errors (number of commission errors divided by number of No-Go trials, \times 100) was increased, with a significant effect in the last session compared to first session, and over the last 5 sessions. The impulsivity index (percentage of correct go trials minus percentage of correct No-Go trials) was increased, with significant difference during the last session comparing to first session and over the last 5 sessions. Data are represented as mean (\pm SEM) per session. n = 12–15. Grey stars show session effects; colored stars show genotype effects. * p < 0.05. ** p < 0.01; *** p < 0.001.

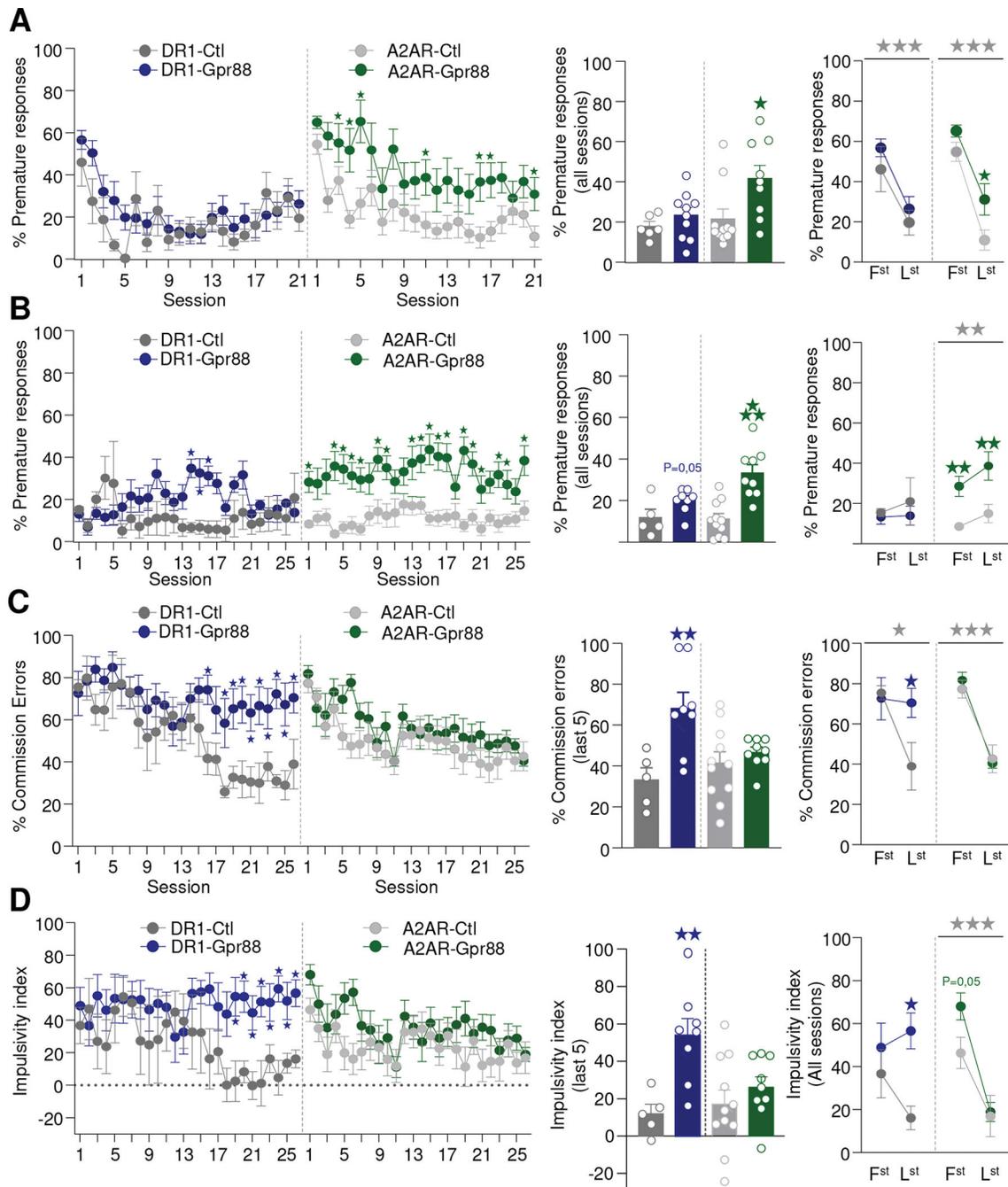


Fig. 4. A2A-GPR88 and D1-GPR88 show higher waiting and stopping impulsivity, respectively, in the Go/No-Go task.

The experiment was performed as in Fig. 3. A2AR- and D1R-Gpr88 mice and their respective controls (A2A-Ctl and D1R-Ctl) were initially trained to nose poke for food reward and all animals reached the criterion at the end of the AutoShaping phase (autosshaping Suppl Fig. S4). Next, mice underwent the Go phase, followed by the No-Go phase. **A. Go phase.** Intertrial (no light) and pretrial (house light) periods are introduced before the cue light is illuminated, to test whether animals are able to withhold responding.

The % premature responses (number of premature responses divided by number of pre-trials \times 100) was increased in A2AR-Gpr88 but not D1R-Gpr88 mice in sessions 3–5, 11, 16–17 and 21, over all the sessions, and between first and last session. **B-D No-Go phase. B.** The % premature responses was again increased A2AR-Gpr88 mice, with higher significance compared to the Go phase. D1R-Gpr88 mice showed higher premature responses in 3 session (14–16) only. **C.** The % commission errors (number of commission errors divided by number of No-Go trials, \times 100) and **D.** The impulsivity index (percentage of correct go trials minus percentage of correct No-Go trials) were increased in DR1-Gpr88, but not D1R-Gpr88, mice over the last 5 sessions and when comparing first and last session. Data are represented as mean (\pm SEM) per session. $n = 5-11$. Grey stars show session effects; colored stars show genotype effects. * $p < 0.05$. ** $p < 0.01$; *** $p < 0.001$.

Table 1.

Association of *GPR88* tag SNPs with ADHD, clinical/behavioural traits, response to treatment and cognition.

	Total (Panel a)				Stress (Panel b)				No stress (Panel c)				
Allele	T rs2030048	A rs2030049	C rs2036212	T rs2809817	T rs2030048	A rs2030049	C rs2036212	T rs2809817	T rs2030048	A rs2030049	C rs2036212	T rs2809817	T rs2809823
ADHD Diagnosis													
• Total number DISC ADHD items													
• Number of DISC inattention items													
• Number of DISC hyperactivity items													
• Number of DISC impulsivity items													
Conners' Parents													
Conners Teachers													
CBCL Total score													
• CBCL Internalizing behaviour													
- CBCL Withdrawn													
- CBCL Somatic complaints													
- CBCL Anxious/depressed													
- CBCL Social problems													
- CBCL Thought problems													
• CBCL Externalizing behaviour													
- CBCL Attention problems													
- CBCL Aggressive behaviour													
Treatment response													
CGI - improvement PBO													
CGI - improvement active													
Conners P (placebo-active)													
Conners T (placebo-active)													
RDoC measures within cognition domain													
SOPT Total Score													
CPT													
• Omission errors													
• Commission errors													
• Hit Reaction Time													
• Detectability													
• Response Style													
• Hit reaction time block change													
• Hit SE block change													
• Overall index													
WCST													
• Number of categories completed													
• Failure to maintain set													
• Perseverative errors standard score													
• Non-perseverative errors standard score													

Schematic representation of family-based association tests (FBAT) analyses conducted in total sample (**panel a**), and in subgroups of families stratified based on whether the mother experienced severe/extreme stress during pregnancy (**panel b**), or the mother experienced minimal (or mild) stress during pregnancy (**panel c**). (**a**) In the total sample, minor association was observed between tag SNP rs2036212 (*C* allele) with DSM-IV diagnosis of ADHD⁴⁴, clinical/behavioral measures and some cognitive measures (CPT²⁸). (**b**) In the stress group, highly significant association was observed between tag SNP rs2809817 (*T* allele) with all the dimensions, i.e. clinical/behavioural traits, treatment response and cognitive dimensions, suggesting that this allele (or a variant in linkage disequilibrium with this SNP) may be a risk factor for ADHD. (**c**) In the no stress group, under-representation of the same rs2809817 *T* allele was significantly associated with better performance in diagnostic, behavioural and cognitive (SOPT²⁹) measures. All methods are described in the Supplemental Methods section. *P* value ranges are indicated for each measure and are color-coded (light orange, 0.01–0.049; light blue, 0.001–0.009; dark blue, 0.0001–0.0009). Under transmission is indicated by hatched boxes. DISC = Diagnostic Interview Schedule for Children; CBCL = Child Behavior Checklist; SOPT, Self-Ordered Pointing Task; CPT, Continuous Performance Test; SE, standard error; WCST = Wisconsin Card Sorting Test.