Performance deficits of NK1 receptor knockout mice in the 5 choice serial reaction time task: effects of d Amphetamine, stress and time of day.

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Abstract

**Background:** The neurochemical status and hyperactivity of mice lacking functional substance P-preferring NK1 receptors (NK1R-/-) resemble abnormalities in Attention Deficit Hyperactivity Disorder (ADHD). Here we tested whether NK1R-/- mice express other core features of ADHD (impulsivity and inattentiveness) and, if so, whether they are diminished by d-amphetamine, as in ADHD. Prompted by evidence that circadian rhythms are disrupted in ADHD, we also compared the performance of mice that were trained and tested in the morning or afternoon.

**Methods and Results:** The 5-Choice Serial Reaction-Time Task (5-CSRTT) was used to evaluate the cognitive performance of NK1R-/- mice and their wildtypes. After training, animals were tested using a long (LITI) and a variable (VITI) inter-trial interval: these tests were carried out with, and without, d-amphetamine pretreatment (0.3 or 1 mg/kg i.p.). NK1R-/- mice expressed greater omissions (inattentiveness), perseveration and premature responses (impulsivity) in the 5-CSRTT. In NK1R-/- mice, perseveration in the LITI was increased by injection-stress but reduced by d-amphetamine. Omissions by NK1R-/- mice in the VITI were unaffected by d-amphetamine, but premature responses were exacerbated by this psychostimulant. Omissions in the VITI were higher, overall, in the morning than the afternoon but, in the LITI, premature responses of NK1R-/- mice were higher in the afternoon than the morning.

**Conclusion:** In addition to locomotor hyperactivity, NK1R-/- mice express inattentiveness, perseveration and impulsivity in the 5-CSRTT, thereby matching core criteria for a model of ADHD. Because d-amphetamine reduced perseveration in NK1R-/- mice, this action does not require functional NK1R. However, the lack of any improvement of omissions and premature responses in NK1R-/- mice given d-amphetamine suggests that beneficial effects of this psychostimulant in other rodent models, and ADHD patients, need functional NK1R. Finally, our results reveal experimental variables (stimulus parameters, stress and time of day) that could influence translational studies.

Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is a heritable, developmental disorder that affects between 2–5% of children in the UK but is prevalent worldwide [1]. Its core diagnostic features are hyperactivity, inattentiveness and impulsivity. The prominence and combination of these abnormalities define the diagnostic subtype, viz. Predominantly Inattentive, Predominantly Hyperactive/Impulsive or Combined Type [2]. Perseveration is also common in this disorder [3] but is not a diagnostic criterion.

Only three compounds are licensed to treat ADHD in the UK (d-amphetamine, methylphenidate and atomoxetine) [4] but guanfacine and the produg, liselamfetamine, are also available in the USA [5]. All these compounds augment monoamine transmission in the brain and periphery. However, their predictable hemodynamic side-effects, the unease about long-term use of the psychostimulants, d-amphetamine and methylphenidate (especially in children), and their lack of efficacy in approximately 20–25% of patients (e.g., [6]), justify the need for a better understanding of the neurobiological abnormalities underlying ADHD and development of alternative drug treatments.

Mice lacking functional substance P-preferring, neurokinin-1 (NK1) receptors, through either functional ablation of the tachykinin-1 receptor (tacr1) gene (NK1R-/-), [7] or receptor antagonism, display locomotor hyperactivity that is prevented by d-amphetamine or methylphenidate [8,9,10]. There are also striking abnormalities in the regulation of noradrenergic [8,9] dopaminergic [10,11] and serotonergic [12] transmission in the prefrontal cortex and dorsal striatum of NK1R-/- mice. All these findings are consistent with evidence for dysfunctional corticostriatal brain circuits in ADHD (e.g., [13]). Our proposal that NK1R-/- mice offer a mouse model of this disorder [11] is
Impaired cognitive performance of NK1R-/- mice tested with a long inter-trial interval (LITI)

Again, there were no differences in the performance of mice from Cohort 1 and Cohort 2 and so the data were pooled for evaluation of the main effects of genotype and time of day. When tested with the LITI, % omissions (F(1,39) = 7.63, P < 0.01), perseveration (F(1,43) = 5.41, P < 0.05) and latency to collect the reward (F(1,43) = 27.1, P < 0.001) were all greater in NK1R-/- mice than the wildtypes (Fig. 2A–C). Other behavioral measures did not differ in the two genotypes (data not shown).

Impaired cognitive performance of NK1R-/- mice tested with a variable inter-trial interval (VITI)

The overall incidence of certain behaviors differed in the two cohorts when tested in the VITI (Table 1). However, no behavior was influenced by an interaction between Cohort and either genotype or time of day and so the data from the two cohorts were pooled for statistical analysis of the main effects of these two factors.

% Omissions were higher overall in NK1R-/- mice than wildtypes (F(1,43) = 24.59, P = 0.001), as were perseveration (F(1,43) = 4.95; P < 0.05) and latency to correct response (F(1,43) = 13.0, P < 0.001) (Fig. 3A–C). The % premature responses was also greater in NK1R-/- mice (F(1,39) = 14.9, P < 0.001) (Fig. 3D), especially with the longer ITIs (c.f. wildtypes at 10 s and 15 s post hoc tests: P < 0.001 and P < 0.05, respectively). Accuracy was also impaired in NK1R-/- mice, albeit to a small extent (3%; F(1,39) = 7.96, P < 0.01; Fig. 3E). There was no genotype difference in latency to collect the reward (Fig. 3F).

Saline injection and d-amphetamine modify behavior in the 5-CSRTT

In the LITI, perseveration was the only behavioral abnormality expressed by NK1R-/- mice to be ameliorated by d-amphetamine (Fig. 4A). Specifically, d-amphetamine restored baseline performance by preventing an increase in perseveration in NK1R-/- mice following an i.p. injection (c.f., d-amphetamine and saline: F(2,35) = 5.5, P < 0.05). This pattern of changes differed strikingly from that in wildtypes in which perseveration was reduced by an i.p. injection (c.f., saline and NI-2: F(1,22) = 9.8, P < 0.01, t11 = 2.6, P < 0.05) and unaffected by d-amphetamine.

Saline injection increased % omissions in wildtypes but did not affect NK1R-/- mice and so abolished the genotype difference seen in uninjected subjects (c.f., saline and NI-2: F(1,22) = 4.7, P < 0.05; Fig. 4B); d-Amphetamine did not reduce % omissions in either genotype (Fig. 4B). Latency to collect the reward was increased by saline injection in NK1R-/- mice but unaffected by d-amphetamine whereas, in wildtypes, the opposite occurred: this behavior was unaffected by saline injection in either genotype and was increased by the higher dose of d-amphetamine (Fig. 4C). The latency to correct response was not affected by saline injection in either genotype but was increased by the higher dose of d-amphetamine in both (Fig. 4D). Neither saline nor d-amphetamine had any effect on accuracy or premature responses (data not shown).

In the VITI, saline injection did not affect any behavioral measure in either genotype whereas both doses of d-amphetamine abolished the genotype differences in % omissions (Fig. 5A), perseveration (Fig. 5B) and latency to collect the reward (Fig. 5C). These effects were a consequence of drug-induced changes in both genotypes (a reduction in NK1R-/- mice and an increase in wildtypes), rather than a selective action in NK1R-/- mice, but there was no statistically significant interaction between drug treatment and genotype. The higher dose of d-amphetamine

Further supported by the identification of disease susceptibility haplotypes in the human tarf gene of patients with ADHD [10,14]. Here, we investigated whether NK1R-/- mice also display inattentiveness and impulsivity. We compared their behavior with that of wildtypes in the 5-Choice Serial Reaction-Time Task (5-CSRTT), which enables evaluation of several aspects of animals’ cognitive performance and response control [13,16,17]. These include: premature responses (an index of one type of impulsivity; see: [19]) and perseveration, as well as % incorrect responses and % omissions (failure to respond in the task), both of which indicate inattentiveness.

After training the animals to criterion, they were tested under conditions that increased attentional demand in two different ways. The first prolonged the inter-trial interval (7 s: ‘LITI’) during which animals were required to withhold their motor response. The second used a randomised, variable inter-trial interval (2–15 s: ‘VITI’). Both procedures increase measures of inattentiveness and premature responding (see: [19]), but the latter prevents the time elapsed since the start of the trial from serving as a cue that would confound measures of animals’ performance. We then went on to investigate whether any deficits in cognitive performance and response control in either of these tests are ameliorated by d-amphetamine.

Finally, there is a great deal of evidence linking disruption of circadian rhythms with ADHD. For instance, there are reports of: a polymorphism in the circadian gene, CLOCK [20]; disruption of sleep rhythms (e.g., [21]) and fluctuation of inattentiveness with time of day [22] in ADHD patients. NK1R are prevalent in the rat intergeniculate leaflet, an area implicated in circadian control, and in the dorsolateral margin of the suprachiasmatic nucleus [23], which has an undisputed role in regulation of circadian rhythms. Furthermore, the NK1R antagonist, aprepitant (used clinically as an anti-emetic), can cause daytime fatigue and insomnia in humans, while another NK1R antagonist, GR 205 171, disrupts circadian rhythms of motor activity in rodents [24]. Prompted by all this evidence, the experimental design enabled us to investigate whether the performance of NK1R-/- and wildtype mice in the 5-CSRTT is influenced by the time of day during which the mice are trained and tested.

Results

Training

We compared the behavior of the two genotypes in two batches of mice (Cohort 1 and Cohort 2; see Methods). Because no differences between the two cohorts emerged, the data were pooled for evaluation of the main effects of genotype and time of day.

% Omissions (F(1,39) = 8.2, P < 0.01) and perseveration (F(1,39) = 23.3, P < 0.001) were greater in NK1R-/- mice than wildtypes (Fig. 1A & 1B). Latency to collect the reward was also slightly greater in the knockouts (F(1,39) = 22.8, P < 0.001) (Fig. 1C). Accuracy and latency to correct response were not affected by genotype (Figs. 1D & 1E). Paradoxically, the incidence of premature responses across Stages 1–6 was greater in wildtype mice than NK1R-/- mice, overall (F(1,43) = 11.5, P < 0.001) (Fig. 1F) and increased transiently in both genotypes during Stage 3 of training, as has been reported previously [19].

The number of sessions needed for the mice to match the baseline criteria for testing depended on genotype. NK1R-/- mice needed more (c.15%) training sessions than wildtypes, overall (F(1,43) = 4.14, P < 0.05), but this depended on time of day to some extent (see below).
actually increased premature responses in NK1R-/- mice (F(2,42) = 3.6, P<0.05) (Fig. 5D) and slightly reduced the accuracy of wildtypes (Fig. 5E). There were no drug effects on latency to correct response (data not shown).

Circadian influences on behavior

Several aspects of animals’ behavior depended on time of day. During training, wildtypes and NK1R-/- mice trained in the morning needed more sessions to stabilize at the baseline criterion for testing than did wildtypes trained in the afternoon (F(1,43) = 5.7, P<0.05) (Fig. 6A). Moreover, premature responses during Stage 3 were lower in NK1R-/- mice trained in the morning than all other groups [F(1,43) = 16.3, P<0.001] (Fig. 6B).

In the LITI, premature responses were influenced by an interaction between genotype and time of day [F(1,43) = 6.6, P<0.05]: their incidence in the NK1R-/- group that were tested in the morning was only 36% of that in wildtypes but, in the afternoon, this behavior increased in NK1R-/- mice and no longer differed in the two genotypes (Fig. 6C).

% Omissions in the VITI was higher (25%), overall, in the morning than the afternoon [F(1,43) = 5.4, P<0.05] but there was no interaction with genotype (Fig. 6D).

Discussion

Mice lacking functional NK1 receptors are capable of learning the 5-CSRTT, as has been reported for their background strain (C57BL/6x129Sv: [25]). However, NK1R-/- mice needed more training sessions overall and expressed deficits in their cognitive performance that resembled those found in ADHD patients [see: Figure 1. The performance of wildtype and NK1R-/- mice during training in the 5-CSRTT. % Omissions, perseveration and latency to collect the reward are all greater in NK1R-/- mice than wildtypes, regardless of time of day (A–C). There was no difference in accuracy (D) or latency to correct response (E), but premature responses (F) were greater in wildtypes, especially during Stage 3 of training. Points show mean ± s.e.m. * P<0.05, ** P<0.01, *** P<0.001. N = 23–24 per group.
doi:10.1371/journal.pone.0017586.g001]
Inattentiveness has been attributed to abnormal (deficient or excessive) phasic release of norepinephrine in the prefrontal cortex. The optimal phasic response depends on background tonic activity [27]. Norepinephrine transmission has also been linked with attention in the 5-CSRTT [28], especially when the stimulus/reward contingency is altered [29]. It follows that the greater tonic release of norepinephrine in corticostriatal brain regions of NK1R-/- mice [9,11] could contribute to their inattentiveness.

d-Amphetamine had no appreciable effect on % omissions of NK1R-/- and even tended to increase it in wildtypes, as in outbred rats [30]. This exacerbation of inattentiveness is unlikely to be due to any anorectic effect of d-amphetamine because there were no consistent changes in latency to correct response or latency to collect the reward in either the LITI or the VITI and the effects of d-amphetamine on these measures did not differ in the two genotypes.

If excessive norepinephrine transmission in NK1R-/- mice underlies their inattentiveness, then it is not surprising that d-amphetamine, a potent norepinephrine releasing-agent, did not diminish their inattentiveness. d-Amphetamine would be expected to be beneficial only in subjects with a deficit in norepinephrine transmission in the prefrontal cortex. This proposal is supported by reports that d-amphetamine reduces the inattentiveness of the Spontaneously Hypertensive Rat (SHR) [31], which is the benchmark rodent model of ADHD and has a lower concentration of extracellular norepinephrine in the prefrontal cortex than their control strain [32]. Therefore, a lack of a therapeutic response to d-amphetamine might serve as a marker for patients with polymorphism(s) in the region of the tacr1 gene. The NK1R-/- mouse model of ADHD predicts that such patients would benefit from treatments that would augment, or mimic, neurotransmission governed by activation of NK1R.

Table 1. Behavior of the two cohorts of untreated mice in the VITI test.

<table>
<thead>
<tr>
<th></th>
<th>Wildtype</th>
<th>NK1R-/-</th>
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<tbody>
<tr>
<td>% Accuracy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 1 **</td>
<td>98.0±0.5</td>
<td>95.1±1.1</td>
</tr>
<tr>
<td>Cohort 2</td>
<td>94.7±0.9</td>
<td>90.9±1.6</td>
</tr>
<tr>
<td>Perseveration score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 1 *</td>
<td>13.3±2.8</td>
<td>18.7±3.8</td>
</tr>
<tr>
<td>Cohort 2</td>
<td>4.7±1.0</td>
<td>20.7±7.9</td>
</tr>
<tr>
<td>% Premature responses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 1 **</td>
<td>16.0±2.5</td>
<td>25.2±3.6</td>
</tr>
<tr>
<td>Cohort 2</td>
<td>21.2±2.7</td>
<td>38.5±4.9</td>
</tr>
</tbody>
</table>

Animals’ accuracy, perseveration and premature responses in the VITI test differed in the two cohorts, but there was no interaction between ‘cohort’ and ‘genotype’ or ‘time of day’ for any of these behaviors. N = 23–24 per group. * P < 0.05, ** P < 0.01 (c.f., Cohorts 1 and 2).

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Perseveration

Perseveration of NK1R-/- mice was consistently greater than that of wildtypes during training in the LITI and VITI. This behavior is not a diagnostic feature in ADHD but is a common co-morbid complication [33,34].

A potentially important caveat is that saline injection increased perseveration of NK1R-/- in the LITI but reduced that of wildtypes. This suggests that provocation of perseveration by stress is prevented by activation of NK1R. When d-amphetamine, rather than saline, was injected there was a dose-dependent attenuation of perseveration in wildtypes, possibly because of a floor effect. A similar, albeit less clear-cut, pattern of changes in perseveration emerged in the VITI.

Perseveration is typically linked with a deficit in dopaminergic transmission in the neuronal circuit linking the ventral tegmental area (VTA), the prefrontal cortex, nucleus accumbens and dorsomedial striatum [35,36,37]. The reduced extracellular dopamine in the prefrontal cortex of NK1R-/- mice [10] is consistent with this proposal. Stress increases release of dopamine in corticostriatal regions [38] and so would be predicted to reduce perseveration in wildtypes, as was found here.

Stress also increases release of substance P [39] and activation of NK1R is essential for the dopamine response to stress [40]. It follows that a lack of functional receptors in NK1R-/- mice would blunt the dopamine response to injection-stress and so prevent inhibition of perseveration. This leads to the possibility that d-amphetamine mimics the effects of stress by triggering impulse-independent release of dopamine in the terminal field [41].

Suppression of perseveration by stress is consistent with evidence that behavioral control is most impaired in ADHD patients with a blunted (cortisol) response to stress [42].

On the basis of these findings, we infer that relief of perseveration by d-amphetamine does not require functional NK1R.

Premature responses

A higher incidence of premature responses (impulsivity) in NK1R-/- mice was evident during the VITI. Impulsivity has long been associated with abnormal serotonergic transmission, which disrupts functional coupling of corticostriatal regions [43,44]. Premature
**Figure 4. Effects of d-amphetamine on the behavior of wildtype and NK1R-/- mice tested with the LITI in the 5-CSRTT.** The perseveration score of NK1R-/- mice is exacerbated by saline injection but ameliorated by d-amphetamine: the latter has no effect in the wildtypes (A). d-Amphetamine has no effect on % omissions in either genotype (B) but increases latency to reward in wildtypes (C) and latency to correct response (D) in both genotypes. Bars show mean ± s.e.m for the behavior of either untreated mice, tested for the second time with the LITI (NI-2), or mice given an i.p. injection of saline (Sal) or d-amphetamine (0.3 mg/kg, 'A(0.3)' or 1 mg/kg, 'A(1)'). The mice experienced each treatment, once only, at weekly intervals. The sequence of treatments (including NI-2) was pseudo-randomised (latin-square) across the subjects. The black line linking adjacent bars indicates a genotype difference, regardless of time of day, of at least. N = 12 per group. * P<0.05; ** P<0.01; *** P<0.001 for comparisons of group means indicated above the bars.

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**Validation of the NK1R-/- Mouse Model of ADHD**

The incidence of both omissions and premature responses in NK1R-/- mice depended on time of day. Omissions in the VITI were slightly lower in the afternoon but the lack of interaction between genotype and time of day means that NK1R do not influence this circadian change. Nevertheless, it is interesting that inattentiveness in ADHD is more pronounced in patients who orient their behavior towards the evening (‘owls’ or ‘evening types’: [22]), especially in the Predominantly Inattentive subgroup.

By contrast, genotype did affect a circadian influence on premature responding because the transient increase during Stage 3 of training did not occur in NK1R-/- mice trained in the morning. A

responding in the 5-CSRTT correlates positively with serotonin efflux in the prefrontal cortex [45] and is induced by activation of serotonin_{2A} or serotonin_{2C} receptors [46,47]. Although other monoamines can influence impulsivity [e.g., 45, 48, 49, 50], the greater serotonin release in the prefrontal cortex of NK1R-/- mice, compared with the wildtypes [12], is consistent with their impulsivity.

A feature shared by the transition from Stage 2 to Stage 3 of training (when premature responding was increased in both genotypes) and the VITI test is that prolongation of the ITI is unpredictable in both cases (see Table 2). This suggests that animals’ response control is influenced by their anticipation of the light signal and perception of the time that has passed since the start of the trial, as has been found in ADHD patients [51,52,53]. Serotonin has a key role in interval timing [54]: the greater release of this transmitter in NK1R-/- mice [12] might aggravate impulsivity by disrupting their perception of the passage of time.

d-Amphetamine did not diminish premature responses in either the LITI or the VITI: the higher dose even exacerbated this behavior in the latter test. Because serotonergic transmission is increased in NK1R-/- mice at baseline [12], a further increase in serotonin release following administration of a high dose of d-amphetamine [41,55] would be expected to exacerbate impulsivity.

There are inconsistent reports on the effects of d-amphetamine on premature responses when (outbred) rats and mice are studied in the 5-CSRTT: both a reduction (LITI: [30]; LITI or VITI: [56]) and an increase [57,58,59,60] have been reported. Reasons for these disparate findings are not known, but d-amphetamine does have beneficial effects in other measures of impulsivity in rodents (e.g., ‘delay-discounting’: [61]). This could be because different test procedures probe different types of impulsivity [18], which will have different neurobiological substrates.

d-Amphetamine also reduces impulsivity in the SHR model of ADHD [31]. Although, to the best of our knowledge, the SHR has not been tested in the 5-CSRTT, it is striking that basal serotonergic release is not increased in their prefrontal cortex [62]. Evidence suggests that insufficient, as well as excessive, serotonin transmission can provoke impulsivity (see: [48]) and so it is possible that a d-amphetamine-induced increase in serotonin release improves response control in the SHR but not the NK1R-/- mouse. Furthermore, the lack of any improvement in the NK1R-/- mouse suggests that the response to d-amphetamine normally recruits functional NK1R. If so, relief of impulsivity in ADHD patients with impaired NK1R function would need a treatment that either augments activation of these receptors or mimics the downstream response.

**Circadian influences**

The incidence of both omissions and premature responses in NK1R-/- mice depended on time of day. Omissions in the VITI were slightly lower in the afternoon but the lack of interaction between genotype and time of day means that NK1R do not influence this circadian change. Nevertheless, it is interesting that inattentiveness in ADHD is more pronounced in patients who orient their behavior towards the evening (‘owls’ or ‘evening types’: [22]), especially in the Predominantly Inattentive subgroup.

By contrast, genotype did affect a circadian influence on premature responding because the transient increase during Stage 3 of training did not occur in NK1R-/- mice trained in the morning.
similar pattern emerged with the LITI. Circadian fluctuation of impulsivity has been found in humans, also [63]. The lack of any effect of time of day on premature responses in the VITI could suggest that exacerbation of impulsivity by unpredictable, prolonged ITIs masks any circadian influences on this behavior.

NK1R are abundantly expressed in the intergeniculate leaflet of the mouse and, to a lesser extent, by neurons along the dorsolateral border region of the suprachiasmatic nucleus [64]. Both areas are strongly linked with circadian rhythms and their entrainment. Abnormal neurotransmission at either of these sites could disrupt a circadian regulation of premature responses in NK1R−/− mice. Whether or not this is correct, our findings suggest that time of day might be a key variable in studies of ADHD patients and that the effect of an interaction between NK1R function and circadian rhythms on response control merits further investigation.

**Conclusion**

NK1R−/− mice display deficits in cognitive performance and response control that resemble diagnostic features of ADHD: namely, inattentiveness, impulsivity and perseveration. Injection stress increased perseveration in NK1R−/− mice and this increase was prevented by d-amphetamine, which otherwise did not diminish the performance deficits in this genotype. The incidence of omissions (VITI) and premature responses (LITI) were influenced by time of day. Moreover, the incidence of the latter behavior depended on an interaction between genotype and time of day, suggesting coupling between NK1R activation and neuronal circuits that govern circadian rhythms and response control. Collectively, our findings consolidate the NK1R−/− mouse as a model of ADHD, possibly of the Predominantly Inattentive subtype and further suggest that time of day, the test parameters, and stress are variables that could influence the outcome of translational studies.

**Materials and Methods**

**Ethics Statement**

These experiments were licensed under the Animals (Scientific Procedures) Act, 1986 (UK) and had local ethical approval at University College London and the University of Sussex.
Animals

We used male wildtype and NK1R-/- mice (25–40 g and 6–8 weeks of age at the start of each experiment) from a colony based at UCL. Both genotypes derived from a 129/Sv x C57BL/6 genetic background, crossed with an outbred MF1 strain (Harlan OLAC, Bicester, UK), for one generation, many generations ago [7]. The facility was held at 21±2°C, 45±5% humidity, and a 12:12 h light: dark cycle (lighting increased gradually from 07.00–08.00 h). The cages incorporated environmental enrichment and were cleaned twice a week (bedding: Litaspen Premium (Lillico)). Water was freely available throughout the study, from standard water bottles with a nozzle that penetrated the cage lid. Access to food (2018 global Rodent Diet (Harlan)) was adjusted to stabilise each subject at 90% of free-feeding body weight. The mice were weighed every morning before training/testing in the 5-CSRTT.

In two separate experiments, using the same training/testing procedures, four mice (in each experiment) were taken, at random, from three breeding pairs for each genotype. These groups of four mice were housed together such that every ‘home cage’ contained four wildtype or four NK1R-/- mice. Two mice of each genotype from each cage were trained and tested in the morning while the remainder were trained and tested in the afternoon. These cage groups were maintained throughout the experiments. One mouse from each cohort died before the end of the experiment, leaving N = 11 for the remainder of the experiment.

Apparatus (5-CSRTT)

The apparatus comprised four mouse operant chambers, each housed within a ventilated sound-attenuating box (Med Associates, St. Albans, VT, USA). The rear wall of the chamber was curved and incorporated five equally-spaced apertures. Inside each of these was a stimulus light, used to illuminate the hole, and an infrared detector for monitoring nose-pokes by the mouse. A hole in the front wall provided access to a magazine that delivered a liquid reward (0.01 mL of 30% condensed milk solution), which was signalled by illumination of the magazine. Head entries into the magazine, to collect the reward, were scored following interception of an infrared photo-cell beam. A house-light, to illuminate the test chamber, was mounted above the magazine. The presentation of the light stimuli and recording of the animals’ responses were controlled by a Smart Ctrl Package 8IN/16OUT with an additional interface by MED-PC for Windows (Med Associates, St. Albans, VT, USA).

5-Choice Serial Reaction-Time Task

Subjects were consistently brought into the laboratory (Monday to Friday) at 09.30 h and were trained/tested, as described below, either between 10.00–12.00 h or 13.00–15.00 h. This enabled us to study circadian influences on behavior. To eliminate any influence of ‘cage effect’ on behavior, half the mice in each cage were trained and tested in the morning: the remainder were

Figure 6. Behaviors of NK1R-/- and wildtype mice in the 5-CSRTT that depend on time of day. (A) the number of sessions needed to train mice to baseline criteria for testing; (B) % premature responses during stage 3 of training; (C) % premature responses in the LITI test; (D) % omissions in the VITI test. Bars show mean ± s.e.m. * P < 0.05, ** P < 0.01 for comparisons indicated. N = 23–24 per group.
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Table 2. Schedule for stimulus parameters during Stages 1 to 6 (training) and testing in the 5-CSRTT.

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<th>Pretraining</th>
<th>Parameters used</th>
<th>Progression criteria</th>
</tr>
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<tbody>
<tr>
<td>Habituation</td>
<td>All apparatus lights switched on</td>
<td>&gt;30 correct trials for 2 consecutive days</td>
</tr>
<tr>
<td>Reward from magazine</td>
<td>Reward continuously available from magazine</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Stimulus holes illuminated constantly</td>
<td>All stimulus holes illuminated: reward offered on nose-poke through any hole</td>
<td>Unchanged</td>
</tr>
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<table>
<thead>
<tr>
<th>Training</th>
<th>Parameters used</th>
<th>Progression criteria</th>
</tr>
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<tbody>
<tr>
<td>Only one (of five) stimulus holes is illuminated in any trial. A nose-poke into this hole triggers reward</td>
<td>&gt;50 correct trials for 2 consecutive days</td>
<td></td>
</tr>
<tr>
<td>Unchanged</td>
<td>Unchanged</td>
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<thead>
<tr>
<th>Tests</th>
<th>Parameters used</th>
<th>Progression criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long ITI (LITI)</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>Variable ITI (VITI)</td>
<td>1.8</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug testing</th>
<th>Parameters used</th>
<th>Progression criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice were tested with no treatment (NI-1) and then retested with neither vehicle nor drug treatment (NI-2), or after injection of either vehicle or 3-d-amphetamine (0.3 or 1 mg/kg i.p.). Mice experienced each test condition once, only. The sequence was semi-randomised (Latin-square) with a one-week interval between each test.</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

1 SD: stimulus duration, LH: limited hold, ITI: inter-trial interval.
rehearsing the task, and was embedded randomly (Latin square) within the series of once-weekly assessments the effects of saline or d-amphetamine. Every mouse experienced each test condition, once only. During the intervening week, animals were subject to once-daily sessions at Stage 6 to ensure that their behavior was restored to the stable baseline before the next test. This series of tests was then repeated, substituting a variable ITI (VITI; see Table 2) for the LITI. The VITI could be any one of four alternatives (2, 5, 10 or 15 s), delivered on a random schedule.

In a second cohort of mice (‘Cohort 2’), the procedures were the same with the exception that uninjected mice (NI-1) were tested with the VITI before the LITI, so as to counterbalance the sequence experienced by Cohort 1, before going on to test the effects of a different compound at weekly intervals (not reported here).

Behavioral scoring

The following performance variables in the 5-CSRTT training and tests were scored and stored online:

- **Total number of sessions required to pass the training phase**: the sum of all the sessions completed over Training Stages 1–6.
- **Total number of trials completed in each test session**: total correct responses + total incorrect responses + total omissions during the LITI or VITI test.
- **% Accuracy**: [correct responses/(correct + incorrect responses)] \times 100.
- **% Omissions**: [total omissions/(correct + incorrect responses + omissions)] \times 100.
- **% Premature responses**: [premature responses/(correct + incorrect + omissions + premature responses)] \times 100.
- **Latency to correct response**: latency to nose-poke into the correct hole after the onset of the stimulus.
- **Latency to collect the reward (reach the magazine)**: latency to collect the reinforcer after a correct response.
- **Perseveration score**: total number of responses into the same, correct hole during the interval between a correct response and collection of the reinforcer.

Statistical analysis

Statistical analyses were carried out on the raw data, log10-transformed, (score + 1)log10-transformed or square-root-transformed data, according to whichever produced the least significant value in the Levene’s test. We pooled data from the two cohorts if the influence of the factor(s) of interest on behavior did not differ, as in the training sessions and the LITI. In the VITI, there were differences in the incidence of certain aspects of behavior of the two cohorts (Table 1). However, there was no interaction between the factor ‘Cohort’ and either ‘genotype’ or ‘time of day’ (i.e., the influence of neither ‘genotype’ nor ‘time of day’ depended on cohort) and so we again pooled the data when looking for main effects of these two factors on behavior. The results of all the statistical comparisons, for all parts of this study, are given in Supporting Information (Tables S1, S2, S3, S4, S5, S6, S7 and S8).

Raw or transformed data were first analyzed using 3-way repeated measures ANOVA (SPSS PC+) with ‘cohort’, ‘genotype’ and ‘time of day’ as between-subjects factors, and ‘training stage’ or ‘test treatment’ as within-subjects factors. In tests of repeated measures, the Greenhouse-Geisser ‘ε’ correction was applied routinely to data sets that showed statistical significance in Mauchley’s sphericity test. A significant effect of one of the main factors, or a relevant interaction between them, was used as the criterion for progressing to 2-way or 1-way ANOVA with post hoc comparisons of the data (LSD test or matched-pair and/or independent-samples t-test, or the non-parametric Mann-Whitney U-test, as appropriate). Statistical significance was set at \( P<0.05 \).

Supporting Information

Table S1 Statistical comparisons of behaviour in NK1R-/- and wildtype mice during training stages 1–6. (DOC)

Table S2 Number of training sessions needed to match the baseline criteria for testing. (DOC)

Table S3 Statistical analysis of the effect of genotype and time of day on behavior of uninjected mice, tested for the first time (NI-1), with a long ITI (LITI). (DOC)

Table S4 Statistical analysis of the effect of genotype and time of day on behavior of uninjected mice, tested for the first time (NI-1), with a variable ITI (VITI). (DOC)

Table S5 Statistical comparisons of behavior during the LITI: NI-2 versus vehicle-injected mice. (DOC)

Table S6 Statistical comparisons of behavior in vehicle- and d amphetamine (0.3 mg/kg or 1 mg/kg (i.p.)) treated mice in the LITI. (DOC)

Table S7 Statistical comparisons of behavior during the VITI: NI-2 versus vehicle-injected mice. (DOC)

Table S8 Statistical comparisons of behavior in vehicle- and d amphetamine (0.3 mg/kg or 1 mg/kg (i.p.)) treated mice in the VITI. (DOC)

Author Contributions

Conceived and designed the experiments: TCY DNS YP-O SCS. Performed the experiments: TCY JAD RKW EMG. Analyzed the data: TCY JAD RKW SCS. Contributed reagents/materials/analysis tools: YPO TLR SPH DNS SCS. Wrote the paper: SCS.

References

attention is mediated through antagonisms of adenosine A2A receptors. Behav Brain Res 185: 32–42.