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The influence of test experience and NK1 receptor antagonists on the performance of NK1R-/- and wild type mice in the 5-Choice Serial Reaction-Time Task

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Abstract
Genetically-altered mice, lacking functional NK1 receptors (NK1R-/-), express abnormal behaviours that are prominent in Attention Deficit Hyperactivity Disorder: namely, inattentiveness and impulsivity (indicated by their greater *% omissions* and *premature responses* in the 5-Choice Serial Reaction-Time Task (5-CSRTT) and locomotor *hyperactivity*. We investigated how behaviour in the 5-CSRTT is affected by repeated testing and whether the abnormalities expressed by NK1R-/- mice are mimicked by treating wild type mice with a NK1R antagonist (L 733060 or RP 67580; 5 or 10 mg/kg). Repeated testing with a variable (VITI) or fixed, prolonged (LITI) intertrial interval reduced *% omissions*. *Premature responses* also declined, but only in NK1R-/- mice, in the VITI test. By contrast, *perseveration* increased in both genotypes. RP 67580 (10 mg/kg) increased the *% omissions* in both genotypes in the VITI, an action which cannot be attributed to NK1R antagonism. Neither drug affected *perseveration*. However, for *premature responses*, the response profile suggested that the low and high doses of RP 67580 (VITI) and L 733060 (LITI) had opposing effects on this behaviour. We infer that the effect of NK1R antagonists in the 5-CSRTT is confounded by animals' test experience and non-specific drug effects at sites other than NK1R, possibly L-type $Ca^{2+}{}_{v}$ channels.

Keywords
Attention deficit hyperactivity disorder, calcium channels, 5-Choice Serial Reaction-Time Task, impulsivity, inattentiveness, locomotor hyperactivity, mouse study, NK1 receptor, NK1 receptor antagonist, perserveration, substance P receptor

Introduction

Mice with functional ablation of the substance P-preferring (NK1) receptor gene (NK1R-/-) (De Felipe et al., 1998) express several abnormal behaviours when compared with their wild types (NK1R+/+). These include locomotor *hyperactivity*, which is ameliorated by the psychostimulants, *d*-amphetamine and methylphenidate (Yan et al., 2010), together with a greater incidence of *premature responses* (a form of impulsivity), *% omissions* (failure to respond in the task, which can indicate inattentiveness) and *perseveration* in the 5-Choice Serial Reaction-Time Task (5-CSRTT) (Yan et al., 2011). Hyperactivity, impulsivity and inattentiveness are core features of Attention Deficit Hyperactivity Disorder (ADHD) in humans. *Perseveration* has been reported in ADHD patients, but is neither common nor a diagnostic criterion. Regulation of dopaminergic, noradrenergic and serotonergic transmission in corticostriatal brain regions is also disrupted in these mutant mice (Froger et al., 2001; Herpfer et al., 2005; Yan et al., 2011), as is thought to be the case in ADHD. Our proposal that NK1R-/- mice express the key signs of this disorder is supported by evidence for an association between polymorphisms in, or near, the *TACR1* receptor gene (the human equivalent of the *nk1R* gene) and increased vulnerability to ADHD (Yan et al., 2010).

Acute administration of an NK1R antagonist (either RP 67580 or L 733060) increases locomotor activity of wild type, but not NK1R-/-, mice (Yan et al., 2010), suggesting that the *hyperactivity* of NK1R-/- mice is a direct consequence of their lack of functional NK1R, rather than any secondary developmental or adaptive change in the phenotype. Here, we investigated whether deficits in cognitive performance of NK1R-/- mice can similarly be attributed to an acute lack of NK1R-mediated transmission. To that end, we tested whether treatment with the NK1R antagonists, RP 67580 or L 733060, impairs performance of wild type mice in the 5-CSRTT, at doses that induce locomotor *hyperactivity.*

After training wild type and NK1R-/- mice in the 5-CSRTT (the latter serving to reveal non-specific drug effects unrelated to antagonism of NK1R), we tested them using two different test parameters: a prolonged inter-trial interval (long inter-trial interval (LITI) of 7 s) and a randomised, variable inter-trial interval (VITI) of 2 – 15 s, during which animals were required to withhold their motor response. These two test conditions challenge the performance of

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mice in the 5-CSRTT in different ways (Humby et al., 2005; Patel et al., 2006; Sanchez-Roige et al., 2012). In particular, in the VITI, it is not possible to predict when the light cue will appear after trial initiation, and so this test avoids the potential confound of animals using interval-timing to prompt their responses, which is thought to occur with the LITI (Praamstra et al., 2006; Sanchez-Roige et al., 2012). Because these two test conditions seem to reveal different performance deficits (Yan et al., 2011), we thought it advisable to test mice under both conditions.

We took into account that, in the course of testing the effects of a control vehicle and two drugs (each at two doses), the animals would be exposed repeatedly to the LITI and VITI tests. To investigate whether the animals' baseline performance changes during repetition of the tests (Economidou et al., 2012), we monitored the behaviour of untreated animals (i.e. no injection of vehicle or drug) in two successive LITI and VITI tests (termed NI-1 and NI-2) and then, again, in a third LITI and VITI test (NI-3). This final test was incorporated into a counterbalanced series of tests of the animals' performance after treatment with NK1R antagonists and vehicle.

Finally, results from our earlier study suggested that certain performance scores in the 5-CSRTT, especially *premature responses*, depended on whether animals were tested in the morning or the afternoon (Yan et al., 2011). To explore this further and to ensure that this factor did not confound interpretation of our study results, the behavioural scores were again compared in different groups of mice that were tested at either of these times of day.

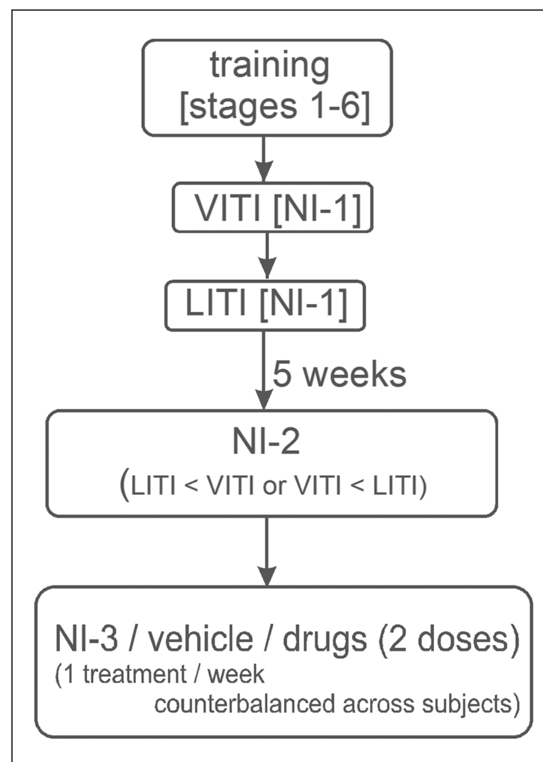

Figure 1. Chronology of the experimental protocol. The test treatments comprised: non-injected mice (NI-1 to NI-3); vehicle injection (Tween 80 in 0.9% saline); L 733060 (5 or 10 mg/kg i.p.); RP 67580 (5 or 10 mg/kg i.p.). i.p.: Intraperitoneal injection; LITI: long inter-trial interval; NI: non-injected; VITI: variable inter-trial interval.

Materials and methods

Ethics statement

These experiments were licensed under the UK Animals (Scientific Procedures) Act of 1986, after ethical approval at University College London and the University of Sussex, UK (PPL number 70/6837).

Animals

Male wild type (NK1R+/+) and NK1R-/- mice, with the same genetic background, were used (129/Sv *x* C57BL/6J, crossed with an outbred MF1 strain many generations ago (described fully in De Felipe et al., 1998). The colonies were bred at UCL in a facility held at 21 ± 2 °C, 45 ± 5% humidity, with a 12 h : 12 h light : dark cycle (where lighting increased gradually, from 07.00 – 08.00 h).

Four mice were taken, at random, from three homozygous breeding pairs for each genotype. Littermates were housed together, such that every home cage contained up to four wild type or four NK1R-/- mice. The home cages incorporated environmental enrichment and were cleaned twice a week. Water was freely available, but the food supply was adjusted to stabilise each subject at 90% of free-feeding body weight. The subjects weighed 25.7 – 37.2 g and were 6 – 8 weeks of age before the start of food restriction. They were brought into the laboratory at the same time every day (Monday to Friday, at 09:30 h) and weighed before training / testing in the 5-CSRTT. Two mice of each genotype, from each cage, were trained and tested in the morning (10.00 – 11.30 h: i.e. after 18 – 19h of food deprivation) while the remainder were trained and tested in the afternoon (13.00 – 14.30 h: i.e. after 21 – 22 h of food deprivation). All animals were returned to the housing facility at 16.00 h and provided with their quota of food. One wild-type mouse died during training.

Apparatus (5-CSRTT)

The 5-CSRTT apparatus (Med Associates, St. Albans, VT, USA) comprised four operant chambers with five equally-spaced apertures incorporated in the rear wall: each of these holes could be illuminated independently. A magazine in the opposite wall delivered a liquid reward (0.01 mL of a 30% condensed milk solution). Interception of an infrared photo-cell beam scored mouse head-entries into either the test holes or the magazine. The entire procedure was controlled by a Smart Ctrl Package 8IN/16OUT with an additional interface, MED-PC for Windows (supplied by Med Associates, St. Albans, VT, USA).

5-Choice Serial Reaction-Time Task

The procedure is based on that developed by Oliver et al. (2009) and is described fully in Yan et al. (2011). The schedule for training and testing of the mice is illustrated in Figure 1. Briefly, after habituation to the apparatus and training to baseline criterion at Stage 6, the mice were tested with a VITI of 2, 5, 10 or 15 s, in

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which the ITI was delivered on a random schedule (compared with a fixed interval of 5 s, at Stage 6). This was followed by a single test with a LITI in which the interval remained constant (7 s). After a *premature response, omission*, or incorrect response, the house-light was switched off for 5 s before starting the next trial. For each *premature response*, one additional trial was added to the series, to ensure that the performance of the mice was tested over a full complement of 100 trials. The testing sessions were first carried out on uninjected mice (i.e. no pre-treatment with either vehicle or test drug (no-injection 1: NI-1)). Mice were always tested on Friday. In between (Monday to Thursday), they were retrained to ensure that the criteria for baseline performance were restored, before retesting at the end of the week.

Stable baseline criteria (> 75% accuracy; < 25% *omission* errors, for at least 3 consecutive days) follow equation (1):

$$\text{Total trials} - \textit{premature responses} = 100 \quad (1)$$

After NI-1, a free-feeding diet was restored during a 1-week closure of the establishment. Animals' body weight was reduced to 90% of their free-feeding weight over the next 2 weeks and then the criteria for baseline behavioural performance were restored over a 3-week period of retraining at Stage 6 (Figure 1). All the mice were then tested for a second time (with no vehicle or drug injection: NI-2) in the LITI and VITI.

One week after testing in NI-2, the mice started a final series of once-weekly tests. This incorporated a third session in which the mice were not injected with either vehicle or drug (NI-3), which was embedded within the series of counterbalanced, Latin-square tests carried out 30 min after an intraperitoneal (i.p.) injection of either vehicle (10 mL/kg; Tween 80 in 0.9% saline), or RP 67580 (5 or 10 mg/kg; 'RP-5 / RP-10'), or L 733060 (5 or 10 mg/kg; 'L-5 / L-10'). Inclusion of NI-3 within this sequence enabled us to control for changes in baseline behavioural scores (including possible carry-over from a previous drug treatment) during this prolonged series of 5-CSRTT tests, and also to control for an interaction between the effects of the drugs and the injection procedure on behaviour. We chose these drug doses because they induced locomotor *hyperactivity* in wild type mice, in a previous study (Yan et al., 2009). Every mouse experienced each test (no treatment, vehicle, RP 67580 and L 733060 (each at 2 doses: 5 mg/kg and 10 mg/kg)) in the LITI and VITI, only once (i.e. 12 tests in all). Individual mice served as a blocking factor with the sequence of within-subject treatments counterbalanced across subjects.

Only two of the 24 mice did not complete the study. One died during training and one NK1R -/- did not complete all the tests for all drug doses. Sample numbers for each test condition are given in Table 1.

Table 1. Sample number for each fixed factor in the study.

Time of testing	LITI		VITI	
	wild type	NK1R-/-	wild type	NK1R-/-
AM	6	6	6	6
PM	5	5 or 6	5	5 or 6

AM: a.m., i.e. before noon; LITI: fixed inter-trial interval; NK1R-/-: genetically-altered mice, lacking functional NK1 receptors; PM: p.m., i.e. after noon; VITI: variable inter-trial interval

Behavioural scoring

The following performance variables in the 5-CSRTT tests were scored, with online capture and storage of the data:

- ***Total number of sessions required to pass the training phase:*** the sum of all the sessions completed over Stages 1 – 6 of training.
- ***Total number of trials completed in each test session:*** total correct responses + total incorrect responses + total test (maximum = 100 or 45 min, whichever occurs first).

$$\text{Total trials per test session} = \text{Total correct} + \text{total incorrect} + \text{total omissions} \quad (2)$$

- $$\textit{\% Accuracy} = \left(\frac{\text{correct responses}}{(\text{correct} + \text{incorrect responses})}\right) \times 100 \quad (3)$$
- ***Latency to correct response:*** latency to nose-poke into the correct hole after the onset of stimulus.
- ***Latency to collect the reward (reach the magazine):*** latency to collect the reinforcer after a correct response.
- ***% Omissions*** = $$\left(\frac{\text{total omissions}}{(\text{correct} + \text{incorrect responses} + \text{omissions})}\right) \times 100 \quad (4)$$
- ***Perseveration score:*** The total number of responses (per 100 trials) into the same, correct hole during the interval between a correct response and collection of the reinforcer.
- ***Premature responses:*** The number of premature responses (per 100 trials). An additional trial was added to the total after each premature response, to ensure that the animals would complete 100 trials.

Drugs and reagents

The NK1R antagonists, L 733060 ((2S,3S)-3-[(3,5-bis (Trifluoromethyl) phenyl) methoxy]-2-phenylpiperidine hydrochloride) and RP 67580 ((3a*R*,7a*R*)-Octahydro-2-[1-imino-2-(2-methoxyphenyl) ethyl]-7,7-diphenyl-4*H*-isoindol) were purchased from Tocris and dissolved/ sonicated in 10 µL Tween 80, with their final volumes adjusted for i.p. injection (10 mL/kg) with 0.9% sterile saline.

Statistics

The experiment adopted a counterbalanced block design with repeated measures. We used SPSS PC$^+$ and InVivoStat (Clark et al., 2011) for statistical analysis of the data. If necessary, the raw data were transformed to ensure homogeneity of the variance in the Levene's test for between-subject factors. Transforms were either:

The square-root of the behaviour score, or

$$\text{Lg10} = \text{Log10(behavioural score} + 1) \quad (5)$$

or, for % *accuracy*:

$$\text{arsin \%accuracy} = \text{arsin}\left[\frac{\sqrt{\text{behaviour score}}}{100}\right] \quad (6)$$

The transformation that produced the data set with the most homogeneous variance was used in the ANOVA.

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We assessed the effects of the main factors, or their interactions, using 2-way repeated measures Analysis of Variance (ANOVA), with '*genotype*' as a between-subjects factor and '*test treatment'* (NI-3 / vehicle / dose of drug) as a within-subjects factor. The mouse-testing time (morning or afternoon) was treated as another between-subjects fixed factor. Mauchly's test was used to confirm sphericity of the variance / covariance matrix for within-subject factors. Because none was significant for any of the comparisons, no ε-correction was necessary. Data across the NI tests, using the LITI and VITI, were evaluated separately. We evaluated the drug effects from the data obtained from tests of vehicle versus the two drug doses. Results for each compound and the VITI and LITI tests were analysed separately. A significant effect of one of the main factors, or a relevant interaction between them, was taken as the criterion for progressing to 1-way ANOVA and *post hoc* multiple group comparisons, which used the Least Significant Difference (LSD) test. Changes in *premature responses* on repeated testing and after each drug treatment were also subjected to linear and quadratic regression analysis (across vehicle and the two drug doses) to calculate the best fit to the data and to estimate the regression parameters. Statistical significance was set at $P < 0.05$.

Results

All mice from both genotypes passed stages 1 – 6 of training and satisfied the established criteria for stable baseline performance before testing in the LITI and VITI (see Equation (1); > 75% accuracy; < 25% *omission* errors) for at least 2 consecutive days, at stages 1 – 5, and for 3 consecutive days of stage 6 parameters.

As described in detail, below, when NK1R-/- mice experienced either the LITI or VITI tests for the first time (NI-1), they expressed greater *% omissions* (and a longer *latency to collect the reward)* than the wild types. A higher incidence of *premature responses, perseveration*, and a longer *latency to the correct response* were also evident in the VITI test.

Genotype-dependent differences in behaviour of mice in the LITI and/or VITI tests depended on past experience of the tests

Premature responses declined overall, on repetition of the VITI test, but the pattern differed in the two genotypes (repetition*genotype: $F(2, 36) = 9.6$; $P < 0.001$), as seen in Figure 2a. Whereas NK1R-/- mice showed a progressive (linear) reduction in *premature responses* ($F(1,10) = 22.8$; $P < 0.001$), the response for the wild types was best fitted by a quadratic regression ((quadratic) $F(2,30) = 4.45$; $P < 0.01$; $P = 0.02$, $b_1 = 0.908$, $b_2 = –0.225$; $R^2 = 0.229$; (linear) $F(1,31) = 0.04$; $P = 0.85$; $_{b1}= 0.009$, $R^2 = 0.001$), with the incidence of *premature responses* in NI-2 being greater than in NI-1 ($F(1,9) = 11.4$; $P < 0.01$; *post hoc* LSD: $P = 0.01$) and NI-3 ($P < 0.05$).

By contrast, there was no overall difference in *premature responses* between the two genotypes, when tested in the LITI, and this did not change upon repetition of the test (Figure 2(b)). However, the incidence of this behaviour in the two genotypes depended on whether they were tested in the morning or afternoon (genotype *time of testing: $F(1, 19) = 5.5$; $P < 0.05$), with NK1R-/- mice showing a higher incidence in the afternoon, overall ($F(1, 10) = 9.2$; $P < 0.05$). Equivalent comparisons for the VITI test were not statistically significant.

Perseveration was exacerbated in both genotypes, overall, by repetition of the VITI (*cf* NI-1 and NI-3: $F(2, 65) = 4.4$; $P < 0.01$), as seen in Figure 2(c), and LITI ($F(2,38) = 5.6$; $P < 0.01$) in Figure 2(d). In the VITI, there was a greater incidence of this behaviour in NK1R-/- mice than in the wild types ($F(1, 18) = 6.0$; $P < 0.05$) but, in the LITI, an apparent genotype difference just missed the criterion for statistical significance ($F(1, 19) = 3.9$; $P = 0.06$) (Figure 2(d)).

The *% omissions* was greater, overall, in NK1R-/- mice than wild types in the VITI (NI-1 – NI-3: $F(2, 36) = 17.3$; $P < 0.001$), as seen in Figure 3(a), and the LITI (NI-1 – NI-3, $F(2, 38) = 7.93$; $P < 0.001$) (Figure 3(b)); and it was unaffected by the time of testing, but fell markedly (30 – 50%) in both genotypes, on repetition of either test (Figure 3(a) and Figure 3(b)).

There was a parallel, small reduction (4 – 15%) in the *latency to correct response* of both genotypes in the VITI: $F(2,36) = 10.8$; $P < 0.001$ (Figure 3(c)) and the LITI: $F(2,38) = 5.89$; $P < 0.01$ (Figure 3(d)), and a slight overall improvement in *% accuracy* (2 – 7%) in the VITI: $F(2,36) = 17.9$; $P < 0.001$ (Figure 3(e)), but none of these changes interacted with the genotype.

The only behaviour to be unaffected by repetition of either test was *latency to collect the reward*, which remained slightly greater in NK1R-/- mice, throughout (LITI: $F(1, 19) = 6.1$; $P < 0.05$; VITI: $F(1, 18) = 12.1$; $P < 0.01$), as seen in Figure 4.

NK1R antagonists modify the performance of both wild type and NK1R-/- mice in the 5-CSRTT

Neither RP 67580 nor L 733060 affected the *total number of trials* (data not shown) or % *accuracy* (see Figure 6(a) and (b)) in either the LITI or the VITI which indicated that they had no actions that obstructed animals' ability to carry out the test, or impaired their performance accuracy, once a cued response was initiated.

The effects of vehicle or drug treatments on *premature responses* in the VITI depended on genotype (treatment*genotype: $F(3, 60) = 3.9$; $P < 0.05$), but were unaffected by the time of testing (Figure 5(a)). RP-10 reduced the incidence of this behaviour, compared with vehicle ($F(2, 32 = 7.4$; $P = 0.01$), but the reduction compared with response to RP-5 was even more consistent (LSD: $P < 0.001$). Moreover, the best fit to the response of wild types to the two doses of RP 67580 was a quadratic, rather than a linear, regression ($F(2, 30) = 7.6$; $P < 0.01$; $b_1 = 1.23$, $R^2 = 0.34$ versus (linear) $F(1, 31) = 4.08$; $P = 0.052$); $b_1 = – 0.14$, $b_2 = – 0.34$, $R^2 = 0.116$).

In the LITI, the *premature responses* depended on whether the mice were tested in the morning or afternoon (genotype * 'time of testing': $F(1, 18) = 13.2$; $P < 0.01$), as seen in Figure 5(b). L 733060 strongly affected *premature responses* of wild types tested in the morning. As with RP 67580, the response to L 733060, across vehicle and the two drug doses, was best described by a quadratic regression ($F(2, 15) = 5.51$; $P < 0.05$; $b_1 = 1.89$, $b_2 = – 0.51$; $R^2 = 0.42$), rather than linear regression $F(1, 16 = 1.46$; $P = 0.24$, $b_1 = – 0.146$, $R^2 = 0.08$). Consistent with this, the incidence of *premature responses* after treatment with the L-10 dose was less than after L-5 ($F(3, 23 = 5.39$; $P < 0.007$; LSD: $P < 0.01$), but did not differ from vehicle. However, an apparent increase in *premature responses* after treatment with the L-5 dose just failed to reach the criterion for statistical significance (LSD: $P = 0.06$). Compared

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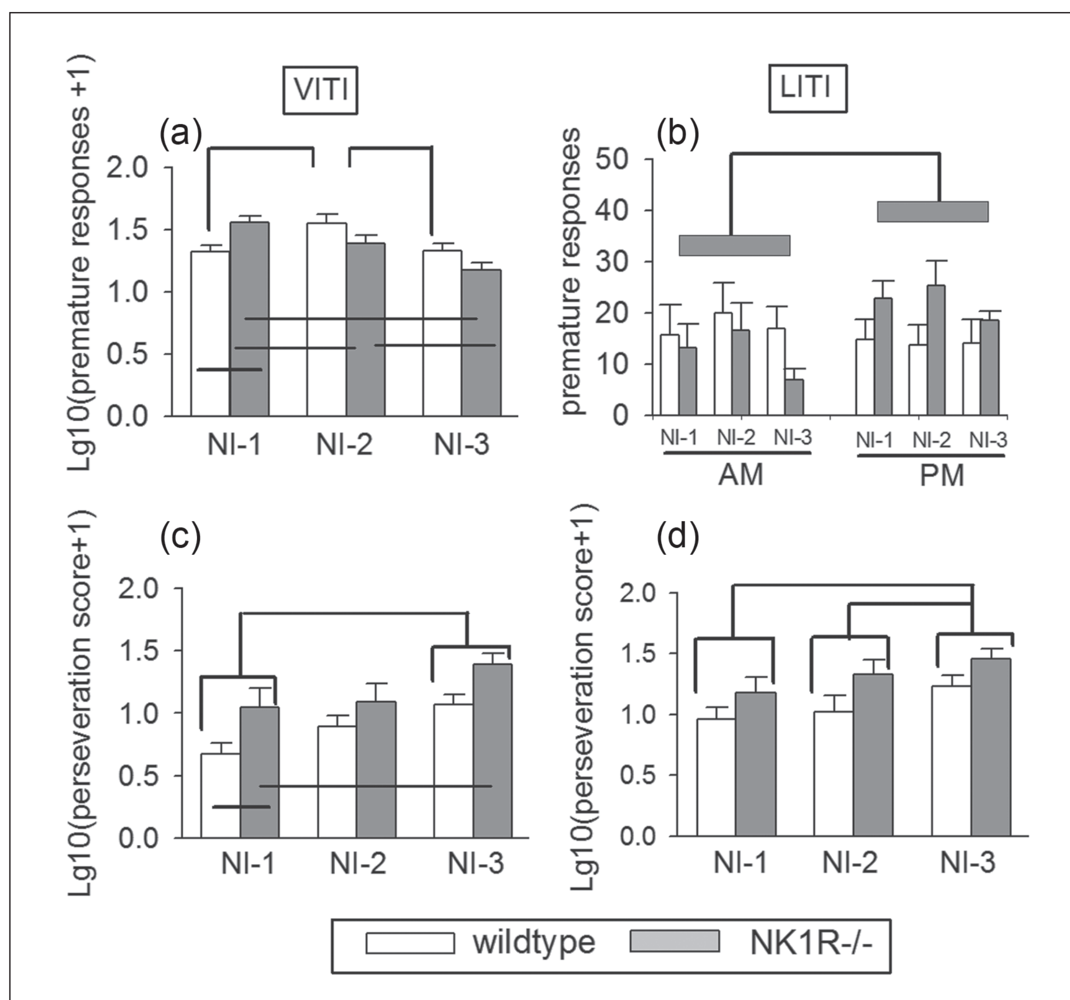

Figure 2. Repeated testing with the VITI and LITI modified *premature* responses and *perseveration*. Bar charts indicate mean ± s.e.m. transformed scores (as applied in the statistical analysis) for uninjected mice tested for the first (NI-1), second (NI-2) and third (NI-3) time. The NI-3 test condition is embedded in the counterbalance series of tests of drug or vehicle, in the LITI and VITI, in which animals receive a total of 12 tests in all. Lines linking boxes (open or filled) indicate a difference across the clusters of test groups indicated, and solid lines link groups which differ at statistical significance of $P < 0.05$, at least. *Premature responses* in the VITI declined in NK1R-/- mice, but not wild types, and did not change in either genotype when tested repeatedly in the LITI. *Perseveration* increased in both genotypes, on repetition of either test. When mice were tested in the LITI in the afternoon, *premature responses* by NK1R-/- mice were greater than in wild-type mice were *perseveration* of the NK1R-/- mice was greater than when they were tested in the morning. For sample 'N', see Table 1.

AM: before noon; Lg10: Log10 ; LITI: fixed intertrial interval ; NI: non-injected; NK1R-/-: genetically altered mice that lack functional NK1 receptors; PM: after noon; s.e.m.: standard error of the mean; VITI: variable intertrial interval.

with vehicle, the higher dose of RP 67580 (RP-10) also reduced *premature responses* in the wild types tested with the LITI in the morning (F (3, 23) = 8.24; $P < 0.001$; *c.f.* vehicle: LSD: $P < 0.01$), as seen in Figure 5(b), but the equivalent comparison for NK1R-/- mice just missed the criterion for significance (*c.f.* R-5 and R-10, LSD: $P = 0.06$).

As before, the incidence of *perseveration* in the VITI was higher in NK1R-/- mice than in the wild types, overall (F (1, 18) = 9.4 $P < 0.01$), but none of the drug treatments, nor the time of testing, affected this behaviour (Figure 5(c)). In the LITI, *perseveration* by NK1R-/- mice was again higher in the afternoon than the morning (genotype*time of testing' LITI: F (1, 18) = 4.5, $P < 0.05$), as seen in Figure 5(d), and was also higher in the NK1R-/- mice than in the wild types, overall (F (1, 8) = 6.8; $P < 0.05$), but neither genotype was affected by either NK1R antagonist.

Compared with vehicle, the RP-10 dose increased *% omissions* (Figure 6(a) and Figure 6(b)), *latency to correct response* (Figure 6(c) and Figure 6(d)); and *latency to collect the reward* (Figure 6(e) and Figure 6(f)) of both genotypes, in the LITI and VITI tests. RP 67580 did not affect *% accuracy* (Figure 6(g) and Figure 6(h)), but after treatment with L 733060, there was a dose * genotype interaction in the LITI (F (2, 40) = 5.14; $P < 0.01$) (Figure 6(h)). Although NK1R-/- showed greater *% accuracy* than wild types at both doses (LSD: $P < 0.001$), the response did not differ from vehicle in either case.

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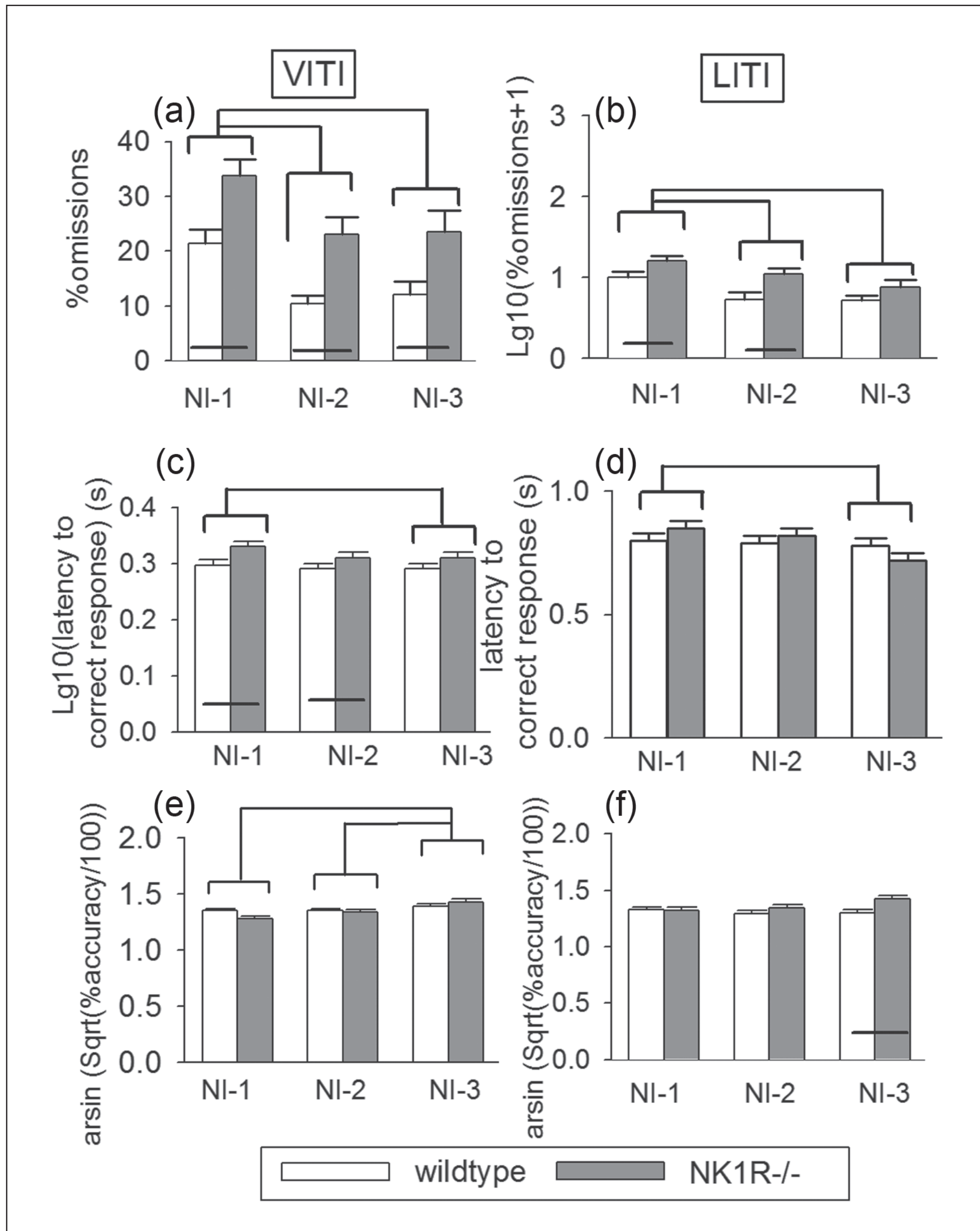

Figure 3. Repeated experience of the LITI and VITI tests reduced *% omissions* and *latency to respond*, and improved *% accuracy*, in the 5-CSRTT. Bars indicate mean ± s.e.m. transformed scores (as applied in the statistical analysis) for uninjected animals, when tested for the first (NI-1), second (NI-2) and third (NI-3) time. The NI-3 test condition is embedded in the counterbalanced series of tests of drug or vehicle in the LITI and VITI, in which animals received a total of 12 tests in all. Solid lines link test groups for which the statistical significance of differences is $P < 0.05$, at least. For sample 'N', see Table 1.

AM: a.m., i.e. before noon; arsin: arcsine; Lg10: Log10; LITI: fixed inter-trial interval; NI: non-injected; NK1R-/-: genetically-altered mice, lacking functional NK1 receptors; PM: p.m., i.e. after noon; s.e.m.: standard error of the mean; sqrt: square-root; VITI: variable inter-trial interval.

Discussion

The two genotypes reached the criteria for testing with the VITI and LITI, which confirmed that they were both able to learn the task to the standard baseline.

Behavioural deficits are attenuated by repeated experience of the LITI and VITI tests

NK1R-/- mice displayed greater *% omissions, premature responses* and *perseveration* than wild type mice, when tested in

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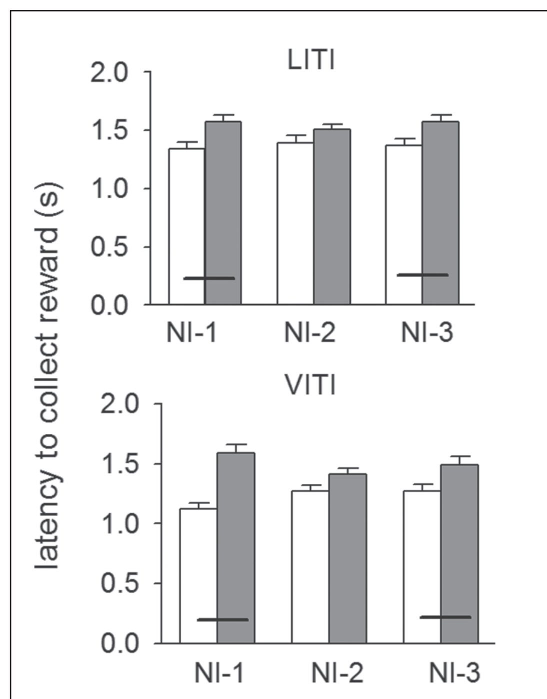

Figure 4. *Latency to collect reward* was unaffected by repetition of either the VITI or the LITI in either genotype. Bars indicate mean ± s.e.m. transformed scores (as applied in the statistical analysis). Solid lines link groups for which the statistical significance of the genotype difference is $P < 0.05$, at least. For sample 'N', see Table 1.
LITI: fixed inter-trial interval; NI: non-injected; s.e.m.: standard error of the mean; VITI: variable inter-trial interval.

the 5-CSRTT for the first time; but in the LITI, there was a greater incidence of *premature responses*, only in the afternoon. Apart from the emergence of a longer *latency to collect the reward* in the VITI, all these findings support those reported in Yan et al., (2011) and Dudley et al. (2013). However, we now add the caveat that *premature responses* by NK1R-/- mice diminish, when they are tested repeatedly with the VITI.

There are strikingly few published studies involving repeated assessment of humans in Choice Reaction-Time Tests. Measures of inattention do seem to change (Castellanos et al., 2000; Kuntsi et al, 2005; Soreni et al., 2009), but no clear pattern has emerged so far. However, there are reports that *premature responses* by outbred (wild type) Lister hooded rats do not change on repetition of the LITI (Besson et al., 2010), as was found here with our mice. By contrast, repetition of the VITI test does reduce *premature responses* by inbred C57BL/6J mice (Walker et al., 2011), as was also found here in the NK1R-/- strain. The transient increase in *premature responses* in NI-2, when wild types were tested in the VITI, could arise from the pause between testing in NI-1 and NI-2; but, if so, it remains to be explained why this did not happen in the LITI or in NK1R-/- mice, as well.

Further changes were a reduction in *% omissions* and an increase in *perseveration* in both genotypes, on repetition of the LITI and the VITI tests. Neither can be explained by a reduction in motivation to carry out the task, because *latency to correct response* was reduced, and *latency to reward* was unaffected, in both tests. Instead, it seems that animals learned to improve their performance, as they do in a 5-hole operant gambling task (Young et al., 2011), and this is evident even within the training sessions. Whether there are within-session genotype differences in learning that could explain why *premature responses* in the VITI only diminished in NK1R-/- mice, will be explored in the future. Nevertheless, 'learning' cannot explain why *perseveration* was exacerbated in both genotypes, in both tests.

The reduction in *% omissions* and *premature responses* and the increase in *perseveration* all occurred in both genotypes, so the underlying mechanism(s) cannot involve NK1R. However, noradrenergic, serotonergic and dopaminergic transmission are all strong candidates. First, as discussed in detail in Yan et al. (2011), these monoamine transmitters modulate attention (Aston-Jones and Cohen, 2005), impulsivity (Cole and Robbins, 1987; Dalley and Roiser, 2012) and perseveration (Pioli et al., 2008). Secondly, their release is abnormal in NK1R-/- mice (Fisher et al, 2007; Froger et al., 2001; Herpfer et al., 2005). Thirdly, monoaminergic adaptation to challenging stimuli is well-known (e.g. Stanford, 1995). Lastly, drugs that augment monoaminergic transmission relieve behavioural deficits in ADHD (Heal et al., 2012). Indeed, a study which combined microdialysis with the 5-CSRTT confirms that noradrenaline efflux in the prefrontal cortex does not change during performance of the 5-CSRTT, but does increase in response to task challenges such as a change in the predictability of the light cue (Dalley et al., 2001).

We cannot distinguish why *premature responses* in the LITI depended on the time of testing, but NK1R-mediated regulation of circadian rhythms is a possibility (Piggins et al., 2001). Cognitive and motor activity both show circadian variation (Beau, 1992; Weinert and Waterhouse, 1998; Winocur and Hasher, 2004). Moreover, this rhythm is abnormal in the Spontaneously Hypertensive Rat (SHR), the established rodent model of ADHD (Adriani et al., 2003), and in ADHD patients (Chiang et al., 2010). In view of the findings discussed below, it is interesting that L-type $Ca^{2+}{}_{v}$ channels are under circadian control (e.g. Colwell, 2011; Ko et al., 2009; 2012; Nahm et al., 2005) and that the SHR and their WKY counterparts express different splice variants of these channels (Tang et al., 2008). Nevertheless, we cannot rule out the possibility that procedural factor(s) account for the differences in behaviour in mice tested in the morning or afternoon. One possibility is that the time of testing affects animals' interval-timing and, as a consequence, performance in Choice-Reaction Time tests (Sanchez-Roige et al., 2012; Smith et al., 2002). Interval-timing is under strong circadian control (Agostino et al., 2011) but it is also disrupted in sleep-deprived subjects (Spati et al., 2009), which could be an important factor in this study, because the mice were tested during the light-phase. The differences are unlikely to be related to the time elapsed since animals were last fed, because that would be expected to reduce *latency to respond* and *latency to collect the reward*, and yet these measures did not differ in the morning and afternoon. Whatever the explanation, we believe that this important finding, together with the dissociation of this behaviour in the LITI and VITI, merits further investigation in future studies, because it clearly affects the animals' performance, so it should be a factor incorporated into the experimental design.

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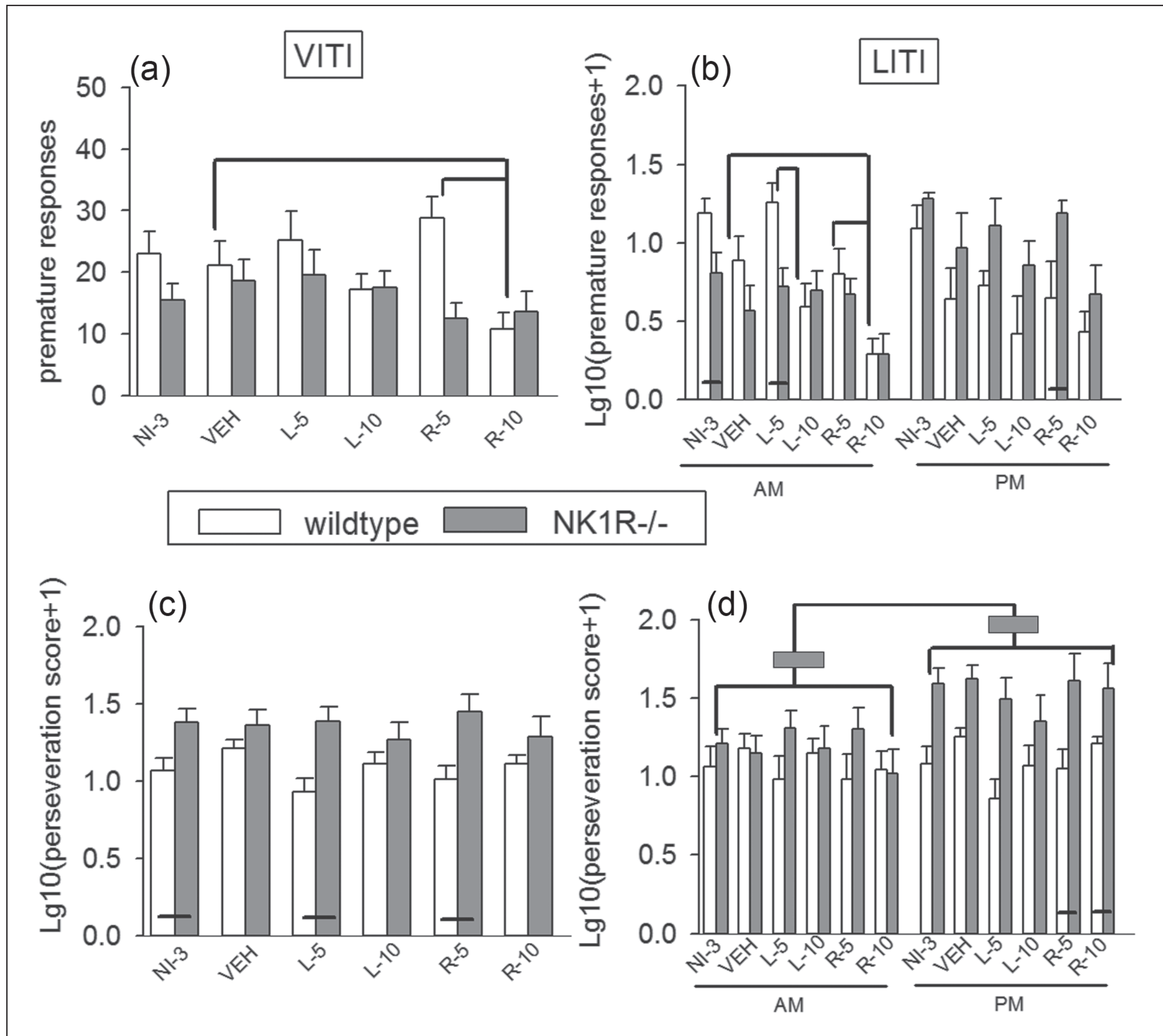

Figure 5. The effect of RP 67580 and L 733060 on *% premature responses* depended on test procedure (LITI or VITI) and, in the LITI, depended on the time of testing (morning (AM) or afternoon (PM). Bars indicate mean ± s.e.m. transformed scores (as applied in the statistical analysis). All test conditions (NI-3, vehicle and both drugs at two doses) were carried out in a sequence defined by a William's (counterbalanced) Latin square. Lines linking boxes (open or filled) indicate a genotype difference across the cluster of test groups indicated; and solid lines link groups which differ at a statistical significance of $P < 0.05$, at least. RP-5 increased *premature responses* in the VITI, but this increase was not evident at the higher dose. Similarly, *premature responses* after treatment with L-5 were greater than after L-10, and lower after RP-10 than RP-5 in both wild types tested in the morning and NK1R-/- mice tested in the afternoon. *Perseveration* was unaffected by either NK1R antagonist in either test. For sample 'N', see Table 1.
AM: a.m., i.e. before noon; L-5 and L-10: the dosage levels of drug L 733060; Lg10: log10; LITI: fixed inter-trial interval; NI: non-injected; NK1R-/-: genetically-altered mice, lacking functional NK1 receptors; PM: p.m. i.e. after noon; R-5 and R-10: the dosage levels of drug RP 67580; VEH: vehicle; VITI: variable inter-trial interval.

NK1R antagonists disrupt behaviour of wild type and NK1R-/- mice in the 5-CSRTT

Prompted by our findings that both L 733060 and RP 67580 increase the locomotor activity of wild type mice (Yan et al., 2010), and that *% omissions, premature responses* and *perseveration* are exacerbated by a lack of functional NK1R in NK1R-/- mice (Yan et al., 2011), we predicted that these behaviours would also be increased in wild type mice by treatment with these two drugs. Indeed, an increase in motor activity could exacerbate (or even underlie) any impairment of the animals' performance in this test, as in ADHD patients. However, both drugs had complex effects on cognitive performance, most likely because their actions are not limited to NK1R antagonism. It should also be borne in mind that epigenetic or developmental changes could influence the behaviour of inbred homozygote mice, through the lifelong lack of NK1R in the breeding pairs and their progeny. Spontaneous mutation(s) in wild type and/or NK1R-/- mice are also possible. Nevertheless, acute antagonism of NK1R does increase locomotor activity of the wild types (Yan et al., 2010) and so this behaviour, at least, is not affected by such confounding influences.

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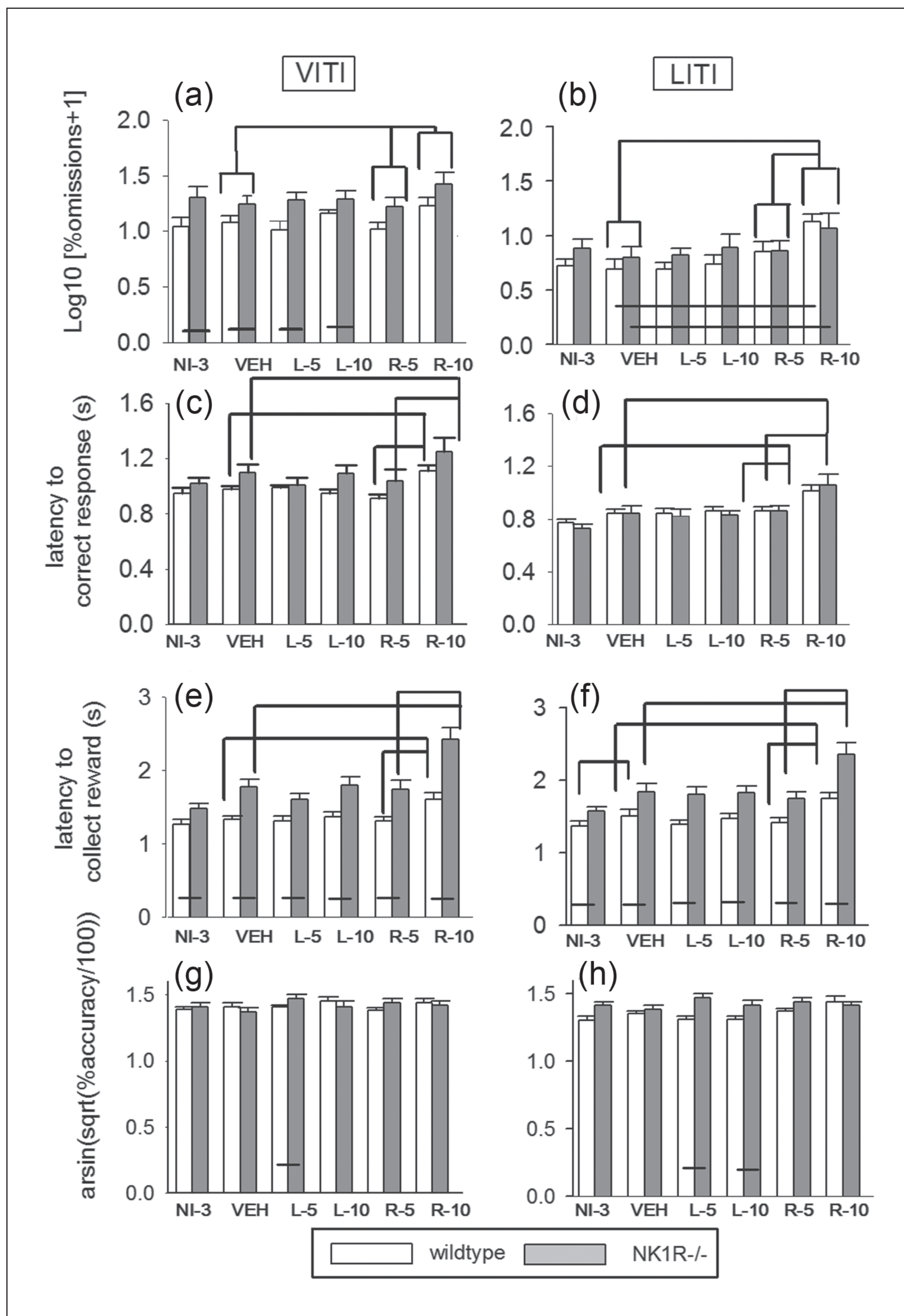

Figure 6. RP 67580 (10 mg/kg) increased *% omissions, latency to correct response* and *latency to collect the reward* in both genotypes, in both the VITI (left column) and LITI (right) tests. Neither dose of L 733060 affected these behaviours. Bars indicate mean ± s.e.m. transformed scores (as applied in the statistical analysis). All test conditions (NI-3, vehicle and both drugs at two doses) were carried out in a sequence defined by a William's (counterbalanced) Latin square. Solid lines link test groups for which the statistical significance of group differences is at $P < 0.05$, at least. For sample 'N', see Table 1.

AM: a.m. or before noon; arsin: arcsine; L-5 and L-10: the dosage levels of drug L 733060; Lg10: log10; LITI: fixed inter-trial interval; NI: non-injected; NK1R-/-: genetically-altered mice, lacking functional NK1 receptors; PM: p.m. or after noon; R-5 and R-10: the dosage levels of drug RP 67580; s.e.m.: standard error of the mean; sqrt: square-root; VEH: vehicle; VITI: variable inter-trial interval.

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RP-10 increased *% omissions* in both genotypes, which indicated a drug-induced increase in inattentiveness. Although *latency to correct response* and *latency to collect the reward* also increased, it is unlikely that the increase in *% omissions* was caused by either sedation or reduced motivation to respond, because neither drug affected the *total number of trial*s completed or *perseveration*. Also, this drug dose increased locomotor activity in our previous study (Yan et al., 2010), an action that is not consistent with sedation; and sedation has not been reported by patients treated with the antiemetic NK1R antagonist, Apripetant. Nevertheless, the increased *% omissions* occurred in both genotypes, so they cannot be attributed to NK1R antagonism.

Contrary to our prediction, RP-10 reduced *premature responses* in wild type mice. However, this reduction was more consistent when compared with the response to RP-5, rather than for vehicle, which could suggest that RP-5 exacerbated this behaviour, whereas the higher dose reduced it. This possibility is supported by the quadratic regression fit to the data for dose versus response. There was a similar (quadratic) response to L 733060, when wild-type mice were tested in the LITI in the morning.

It is certain that the drug effects are not explained by any change in motivation to respond, because:

1. There was no change in *the latency to respond* or *latency to collect the reward;*
2. Unlike *premature responses*, neither of these latencies was affected by time of testing;
3. There was no change in *% omissions*; and
4. There was no reduction in the number of trials completed by the mice.

However, it remains to be explained why L 733060 did not affect *premature responses*, when wild type mice were tested in the afternoon.

Recently, we reported that the L-type Ca^{2+}_{v} channel blocker, nifedipine, increased *% omissions* and reduced *premature responses* in both genotypes (Dudley et al., 2012). Many, if not all, NK1R antagonists block L-type Ca^{2+}_{v} channels, which can confound the effects of NK1R antagonism on behaviour (Garret et al., 1991; Guard et al., 1993; Rupniak et al., 1993; Seabrook et al., 1996). It follows that a possible explanation for the complex effects of the two NK1R antagonists on *premature responses* in this study is that they are exacerbated by NK1R antagonism at low drug doses, and that this increase is masked by L-type channel blockade at higher doses. If so, our finding that both doses of NK1R antagonists induced *hyperactivity* in wild type mice, but had no effect on the hyperactive NK1R-/- mice (Yan et al., 2009), implies that L-channel blockade by NK1R antagonists does not affect locomotor activity. This proposal is consistent with reports that nifedipine does not affect spontaneous locomotor activity in the mouse (Maiolini et al., 2003; Zhang et al., 1994).

Neither antagonist affected *perseveration*, which could suggest that this behaviour arises from a lifelong deficit in NK1R. Alternatively, NK1R occupancy by these antagonists was insufficient to modify behaviour. The latter is more likely, because the L-type channel blocker, nifedipine, did increase *preservation* in the VITI (Dudley et al., 2012), and RP 67580 is a comparatively weak L-type channel blocker (*c.f.* Murphy et al., 1990; Rupniak et al., 1993). To the best of our knowledge, the binding affinity of L 733060 to L-type channels has not been evaluated.

We cannot be certain about the explanation for the differences in the effects of the two antagonists on premature and perseverative responses in the LITI and the VITI. However, our findings suggest that RP 67580 has a higher affinity for L-type channels than does L 733060, so it is more likely to mask any effects of NK1R antagonism on these behaviours. These pharmacological differences will be complemented by any differences in the influence of NK1R and L-type channels in the neuronal pathways that mediate these behaviours.

There was a small increase in *% accuracy* when NK1R-/- were tested in the LITI after treatment with L 733060, but no other test nor treatment condition affected this measure. This finding reflects the lack of any consistent evidence for impaired task accuracy in ADHD patients (Epstein et al., 2011) and also the lack of any beneficial effects of drugs used to treat this disorder (Koffarnus and Katz, 2011; Prasad et al., 2012).

In conclusion, the test experience and/or time of testing affected measures of performance of mice in the 5-CSRTT, which is likely to be relevant to assessments of ADHD patients (Kuntsi and Klein, 2012). As well as being influenced by these factors, the effects of NK1R antagonists are confounded by their lack of selectivity for NK1R. As in preclinical screens for antidepressants (Rupniak et al., 1993), the complex actions of NK1R antagonists need to be taken into account when assessing their effects on cognitive performance in the 5-CSRTT and other behaviours.

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Conflict of interest

UCL Business is the owner of an EU patent (An Animal Model of ADHD) with SCS and SPH as named inventors.

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