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ORIGINAL INVESTIGATION

Cognitive performance in neurokinin 3 receptor knockout mice

R. E. Nordquist · M. Delenclos · T. M. Ballard · H. Savignac · M. Pauly-Evers · L. Ozmen · W. Spooren

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Abstract
Rationale The neurokinin 3 (NK_3) receptor is a novel target under investigation for improvement of the symptoms of schizophrenia due to its ability to modulate dopaminergic signaling. However, research on effects of NK_3 antagonism with animal models has been hindered because of species differences in the receptor between humans, rats, and mice.
Objectives The aim of the present study is to further knowledge on the role of NK_3 in cognitive functioning by testing the effect of knockout of the NK_3 receptor on tests of working memory, spatial memory, and operant responding.
Materials and methods NK_3 knockout mice generated on a C57Bl/6 background were tested in delayed matching to position (DMTP), spontaneous alternation, Morris water maze, and active avoidance tasks.
Results NK_3 knockout mice showed better performance in the DMTP task, though not delay dependently, which points to an effect on operant performance but not on working memory. No differences were seen between the groups in spontaneous alternation, another indication that working memory is not affected in NK_3 knockouts. There was no impairment in knockout mice in Morris water maze training, and the mice also showed faster response latency in the active avoidance task during training.
Conclusions Collectively, these results support a role for the NK_3 receptor in performance of operant tasks and in spatial learning but not in working memory.

Keywords Neurokinin · Tachykinin · Morris water maze · Delayed match to place · Delayed matching to position · Spontaneous alternation · Active avoidance · Working memory · Operant conditioning · Antipsychotic

Introduction

Schizophrenia is a debilitating psychiatric disorder characterized by positive symptoms such as psychosis and delusions, negative symptoms such as flattened affect and apathy, and cognitive deficits. The most common form of treatment for symptoms of schizophrenia is treatment with dopamine D_2 receptor antagonists. This form of treatment effectively counteracts the positive symptoms but causes debilitating side effects. Furthermore, D_2 antagonism leaves negative and cognitive symptoms largely untreated or, in some cases, worsened (Gardner et al. 2005; Lieberman et al. 2005). Recognition of the essential need to alleviate cognitive deficits in schizophrenia has been underscored by the recent creation of the Measurement and Treatment Research to Improve Cognition in Schizophrenia program by the US National Institute of Mental Health. Looking to new drug targets to optimize treatment of all symptoms of schizophrenia is currently an important area of focus for drug development.

R. E. Nordquist · M. Delenclos · T. M. Ballard · H. Savignac · M. Pauly-Evers · L. Ozmen · W. Spooren (✉)
Psychiatry Disease Area, PRBD-N,
F. Hoffmann-La Roche,
Building 72–148, 4070 Basel, Switzerland
e-mail: will.spooren@roche.com

R. E. Nordquist
e-mail: r.e.nordquist@uu.nl

Present address:
R. E. Nordquist
Emotion and Cognition Program,
Department of Farm Animal Health,
Veterinary Faculty, Utrecht University,
Yalelaan 1, 3584CL Utrecht, The Netherlands

The neurokinin 3 (NK_3) receptor is a novel receptor target under investigation to combat schizophrenia (Spooren et al. 2005). The NK_3 receptor is prominently expressed in limbic areas of the brain (Ding et al. 1996), many of which have been implicated in schizophrenia. It is important to note that the NK_3 receptor is also located on dopaminergic neurons in the midbrain (Lévesque et al. 2007; Whitty et al. 1997; Whitty et al. 1995). Activation of the midbrain NK_3 receptors with the endogenous ligand neurokinin B or the highly specific agonist succinyl-[Asp6, Me-Phe8]SP(6–11) (senktide) causes increased dopamine release in the striatum and prefrontal cortex (Humpel et al. 1991; Marco et al. 1998; Tremblay et al. 1992), as well as increased electrophysiological activity of dopaminergic neurons (Nalivaiko et al. 1997; Nordquist et al., submitted; Overton et al. 1992). This effect of NK_3 stimulation on the dopaminergic neurons and dopamine release implies that the NK_3 receptor may provide a means for antipsychotic action by modulating catecholamines, which would avoid the strong adverse side effects produced by D_2 receptor antagonists. Indeed, two NK_3 receptor antagonists have shown promise in clinical testing: SR-142801 (Osanetant) and SB-223412 (Talnetant; Spooren et al. 2005).

Given the strong interest in the NK_3 receptor as a target for antipsychotic drugs, the effect of altered transmission at this receptor on cognition is highly relevant. However, investigation of the effects of NK_3 receptor neurobiology has been seriously hindered by species differences between humans, rats, and mice in the NK_3 receptor that necessitate use of pharmacological agents primarily in gerbils or guinea pigs. Current animal models of cognitive functioning are not optimized to these species. To further the knowledge of the role of the NK_3 receptor in cognition, we tested NK_3 knockout mice in two paradigms that tax working memory, in a spatial learning task, and in an aversively reinforced operant task.

Materials and methods

Generation of NK_3 knockout mice

NK_3 knockout mice were generated and bred as described below (and in Nordquist et al., submitted).

Cloning of the targeting vector

A 3.7-kb (A) and a 2-kb fragment (B) of the Tacr3 (NK_3) mouse gene containing the 5′ region, exon 1, and a part of intron 1 were amplified by long-range polymerase chain reaction (PCR) using the primer combinations fw: CGGGG TACCA AATAA AGGAC CAACA GATCT GACAC, rev: CGGCT CGAGT TGGAC ACCAG TAGTG ATGGC and fw: CGGCT CGAGC TGCCA TCACT ACTGG TGTCC, rev: CGCGG ATCCA TATAA AAGCT ATCTC AGATA GGG, respectively.

The primers introduced restriction sites for subsequent cloning into a targeting vector. A loxP element was inserted 68 bp upstream of the exon 1 in fragment A by site-directed mutagenesis. A diphtheria toxin (DT) gene was cloned downstream of fragment B. A neo/TK selection cassette with flanking loxP sites was introduced into the *Xho*I site downstream of exon 1.

Generation of Tacr3tm1.1 mice

The targeting vector was linearized and electroporated into embryonic stem (ES) cells derived from C57BL/6, which were maintained on subconfluent primary embryonic fibroblasts. Neomycin-resistant and DT-selected colonies were isolated and expanded. The ES cells containing the homologous recombination event were determined by PCR with primers internal and external to the targeting construct. ES cell colonies, which underwent the correct recombination event (homologous integration of neo/TK-selection cassette plus maintenance of loxP sites), were designated as Tacr3tm1.

One targeted tm1 clone was subjected to a second electroporation with a Cre expression plasmid to remove the loxP-flanked exon 1 and neo/TK-selection cassette. Clones with a Cre-mediated deletion of either exon 1 and the selection cassette together or the selection cassette alone were identified by PCR analysis, and the resulting alleles were designated Tacr3tm1.1 and Tacr3tm1.2, respectively. Only Tacr3tm1.1 was injected into C57BL/6 blastocysts, which were implanted into pseudopregnant dams. Resulting chimeras were bred with C57BL/6 mice, and offspring were selected for the transmission of the Tacr3tm1.1 allele. Mice used in the present studies were produced by intercrossing heterozygous mice for the Tacr3tm1.1 allele, then determination of progeny genotype by PCR of tail samples.

Delayed matching to position

The protocol used to train mice on the delayed matching to position (DMTP) task was a modification of a previously described protocol (Woolley and Ballard 2005).

Apparatus

Testing was performed in eight standard operant chambers (Med Associates, St. Albans, VT, USA) equipped with two retractable levers positioned either side of a central food tray. A single stimulus light was positioned above each lever. The equipment was run by K-Limbic software (Conclusive Solutions, Harlow, UK) operating on an IBM-compatible personal computer.

Pre-training

At 2.5 months of age, male NK_3 knockout (N=18) and wild-type (N=17) mice were initially pretrained to lever press for food reward (20 mg Formula P pellet, Noyes, NH, USA) in 30-min daily sessions under a continuous reinforcement schedule (CRF-1) in which each lever was presented singly an equal number of times (total 30 min run time). Mice were trained at this level until they were consistently pressing the levers (9 days) and thus had associated lever pressing with food reward. At this stage, mice continued training under a modified CRF schedule (R-CRF), which served to habituate the animals to repeated presentation and retraction of the levers. During this schedule, as soon as the lever was pressed, it was retracted, and a pellet was delivered into the central food tray. Five seconds after the mouse had collected the food reward, the next trial commenced. Mice were again trained until they were consistently pressing the levers under this schedule (11 days) at which point the number of pellets received was restricted to 60.

Training on delayed matching to position

Once mice had attained consistent performance during the R-CRF schedule, they were trained on the matching-to-position rule. This task consisted of a single lever being inserted into the chamber and the illumination of the appropriate stimulus light (sample stage). The mouse was required to press the sample lever, which immediately retracted and to nose poke into the central food tray with a single nose poke resulting in the presentation of both levers and stimulus lights. Pressing the lever previously presented at the sample stage resulted in the delivery of a single food reward (choice stage). If the mouse pressed the other lever, it was recorded as an incorrect response and was unrewarded. An incorrect response or failure to respond to either the sample or choice levers during the 20-s limited hold (i.e., an omission) resulted in a timeout period of 30 s. The next trial was signaled by illumination of the house light for a 5-s period, after which the sample lever was extended. The number of such trials per session was limited to 60. Initially, the delay between the sample and choice stage was 0 s after the first magazine nose poke. Once the animals had learned the matching rule (>80% correct choices for each delay), the delay period was increased in the following way:

Delay set 1: 20 trials at each of 0, 1, and 2-s delay
Delay set 2: 12 trials at each of 0, 1, 2, 3, and 4-s delay
Delay set 3: 12 trials at each of 0, 1, 2, 4, and 6-s delay
Delay set 4: 12 trials at each of 0, 1, 2, 4, and 8-s delay
Delay set 5: 10 trials each of 0, 1, 2, 4, 8, and 12-s delay
Delay set 6: 10 trials each of 1, 2, 4, 8, 16, and 24 s delay

At this stage, the first nose poke after the end of the delay led to the presentation of the two choice levers. Delay intervals were presented in a pseudorandom manner forcing mice to continuously nose poke during the delay period to avoid mediating behavior. The number of days to criterion was recorded for each mouse for each delay set as a score for acquisition of the DMTP task. Mice were trained daily until they demonstrated a consistent performance at criterion (>80% correct responses) on the final delay set for three consecutive sessions. Plateau-level performance was then assessed across 14 days of performance. All measures obtained over this asymptote-level response period were averaged to provide single values of each performance variable per delay for each mouse.

Parameters measured included the total number of correct responses, total number of incorrect responses, percentage correct responses (choice accuracy, measured as total correct responses/total correct responses+total incorrect responses) for each delay time period. Number of missed trials indicates the total number of missed trials during the session, i.e., no response to the lever or magazine during the delay period.

Statistics for delayed matching to position

Acquisition of the DMTP in the form of days to criterion for each set of delays was tested statistically using a repeated-measures analysis of variance (ANOVA), with genotype as the between-subjects factor and delay set as the within-subjects factor. Asymptote level responses of performance (for the 14 sessions conducted after animals reached criterion for delay set 6) were tested statistically using a repeated-measures ANOVA with genotype as the between-subjects factor and both session (14 sessions) and delay length (six delays) as within-subjects factors. If significant main effects or interactions were seen, a repeated-measures ANOVA for each delay length was conducted as post-hoc to determine differences between genotypes for each delay length, with genotype as the between-subjects and session as the within-subjects factors.

Spontaneous alternation

Male and female wild-type (n=20), hetereozygote (n=20), and knockout (n=19) mice were placed in the center of a Y-maze (arms=53×15×30 cm $l \times w \times h$). An observer in an adjacent room viewed the mice through a video camera and recorded the number and sequence of arm entries (entry of the whole body into an arm) during a 5-min period. Alternation is defined as entry into the three arms in any nonrepeating order (e.g., ABC, BAC, CBA, etc.). Percent alternation was calculated as the total number of alternation divided by the possible alternations given the number of

arm entries (total number of arm entries−2). A one-way ANOVA was used to compare percentage of alternations between genotypes.

Morris water maze

A drug- and task-naive group of male NK_3 knockout (N=11) and wild-type (N=14) mice was trained in the Morris water maze task as previously described (Freichel et al. 2007). For both cued and place tasks, sessions consisted of three trials with a maximum trial duration of 60 s. Two sessions per day were conducted, separated by a minimum of 3 h. Briefly, in the cued task, the ability of male wild-type (n=14) and NK_3 knockout (n=11) mice to learn to swim to and climb onto a visible, flagged platform (7 cm in diameter) in varying locations in a water maze (1 m in diameter) was assessed in four sessions. Subsequently, in the place task, mice were trained for eight sessions to locate a submerged, unflagged platform whose position remained static (position balanced within groups) by allowing them to locate the hidden platform from one of three start positions. Assessment of spatial learning was conducted in probe trials performed both 60 min after block 4 and 24 h following the final session. In each probe trial, the platform was removed from the pool, and the path swum by each mouse was recorded over a 60-s period.

All data were captured and analyzed by a video tracking software (HVS Systems, UK). Data were statistically tested using a t test comparing the percent time spent in the platform quadrant between knockout and wild-type mice, as well as a Friedman ANOVA followed by a Wilcoxon post-hoc test when appropriate comparing the quadrant where the platform had been located to the other quadrants.

Active avoidance

Prior to active avoidance training, this cohort was tested for spontaneous alternation as described above. Wild-type (n=20), hetereozygote (n=20), and knockout (n=19) male and female mice were placed into the avoidance boxes running on the software provided with these boxes (San Diego Instruments, San Diego, CA, USA) for one session (20 trials) daily on weekdays. Each trial began with the illumination of a light (CS) on the side currently occupied by the mouse for 10 s, signaling a foot shock (0.2 mA) of 20 s maximum duration, followed by a variable timeout period (mean 20 s, range 15–25 s). Shock can be avoided either by a shuttle to the next compartment during the CS (i.e. avoidance) or escape during the shock presentation. Percentage of trials per session in which shocks were avoided and average latency to shuttle following onset of the CS session were recorded. Repeated-measures ANOVAs for each of these variables were followed by post-hoc testing with two-tailed t tests to compare between genotypes when appropriate.

Results

Delayed matching to position

Acquisition

Mice from both genotypes learned to press a lever to obtain food reinforcement during pretraining, and there was no significant difference between the two genotype in the number of rewards obtained during CRF-1 ($F_{1,\ 216}$=0.45, not significant [NS]) or R-CRF training ($F_{1,\ 341}$=0.02, NS). Notably, five mice (three wild-type and two knockout) did not learn to press the lever to obtain food consistently during this pretraining period. These mice were therefore excluded from the DMTP training and from the rest of the study, meaning that there were 16 knockout and 14 wild-type mice for the remainder of the DMTP task.

Mice from both genotypes learned the DMTP 0-s delay to criterion and were trained further with the sets of delays as described in "Materials and methods." The progressive difficulty of the task is seen in the increase in the number of the days required to reach the criteria from the first to the last delay set (main effect delay set $F_{5,\ 140}$=13.24, p<0.001; Table 1). The NK_3 knockout and their wild-type cohorts acquired the task at similar rates, as seen in the lack of genotype effect ($F_{1,\ 28}$=0.03, NS) as well as the lack of genotype×delay set interaction ($F_{5,\ 140}$=0.19, NS) on the number of days to criterion for each delay set.

Asymptotic performance

Analysis of asymptotic performance showed an increase in the number of correct responses at all delay times in NK_3 knockout animals (Fig. 1a), as reflected in the significant genotype effect (ANOVA, $F_{1,\ 23}$=8.55, p<0.05). Both genotypes displayed reductions in accuracy as the delay

Table 1 Acquisition of delayed matching to position task

Delay set	Delays (s)	Days to criterion	
		Wild type (N=14)	Knockout (N=16)
1	0, 1, 2	3.3±0.7	3.5±0.7
2	0, 1, 2, 3, 4	3.2±0.5	1.7±0.5
3	0, 1, 2, 4, 6	2.5±0.3	1.7±0.3
4	0, 1, 2, 4, 8	3.8±0.8	7.4±1.7
5	0, 1, 2, 4, 8, 12	6.7±1.8	7.4±1.7
6	0, 1, 2, 4, 8, 16, 24	10.6±2.6	10.7±2.4

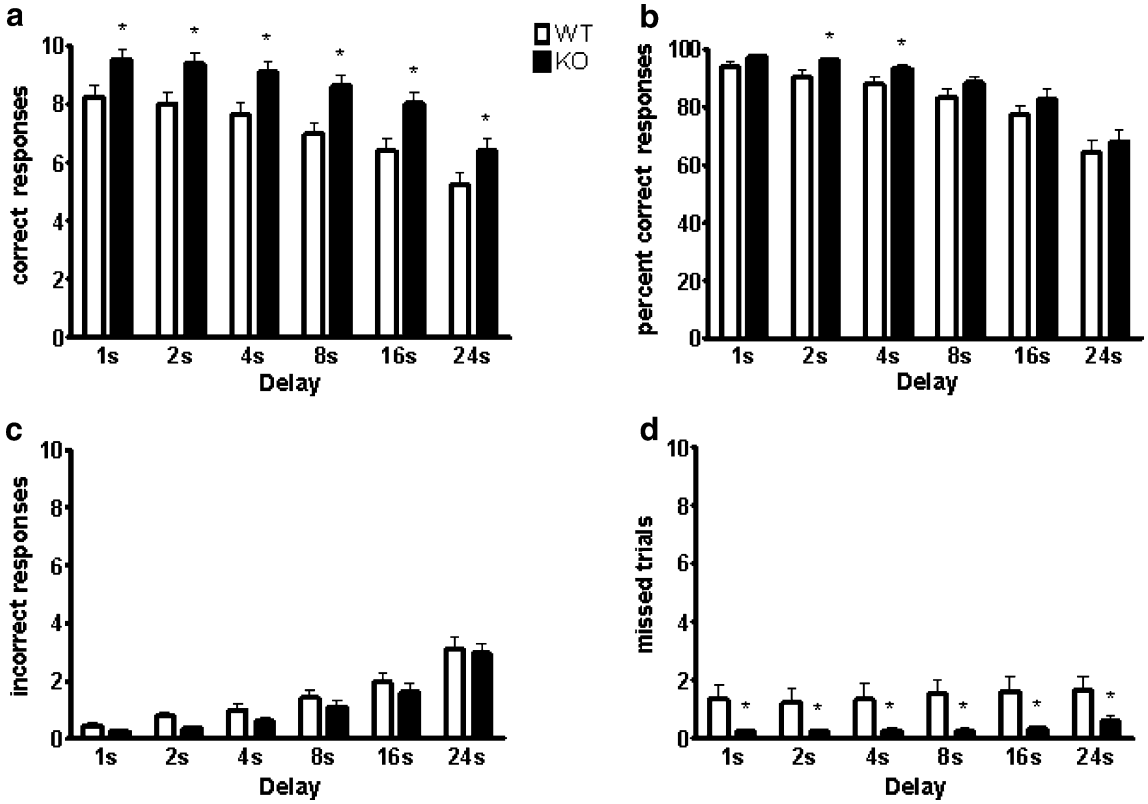

Fig. 1 Performance of NK_3 knockout (N=16) and wild-type (N=14) mice in the delayed matching to position task. For all graphs, bars are grouped by delay length on the x-axis. Performance is averaged across 14 sessions performed after all animals had reached criterion in the final stage of the DMTP task (see "Materials and methods"). The average number of correct responses (maximally ten per delay) is represented in **a**. The average percentage of correct responses is represented in **b**. Percentage correct responses is calculated as correct responses divided by correct plus incorrect responses and thus does not take missed trials into account. The average number of incorrect responses (maximally ten per delay) is represented in **c**. The average number of missed trials, defined as a trial in which any of the lever presses needed to obtain a reward is omitted, is represented in **d** (maximally ten per delay). *White bars* represent group means for wild-type mice, and *black bars* represent group means for knockout mice. *Error bars* indicate SEM. *Asterisk* represents $p<0.05$ in post-hoc testing of wild-type vs. knockout

interval increased from 1 to 24 s, evidenced by the significant delay set ($F_{5,\ 115}=65.6$, $p<0.05$). There was a significant main effect of session ($F_{13,\ 299}=1.53$, $p<0.05$) resulting from daily variation in responding (range of daily average=44.3 to 48.3 correct responses). Furthermore, a session×genotype interaction was observed ($F_{13,\ 299}=2.60$, $p<0.05$) as the knockout mice remained stable at approximately 50 correct responses (range=48.5–51.9), while the wild-type gradually increased their number of correct responses across the 14 sessions (range=37.8–45.7). In post-hoc testing, a significant main effect of genotype was found at each of the delays (1 s: $F_{1,\ 23}=5.4$, $p<0.05$; 2 s: $F_{1,\ 23}=6.4$, $p<0.05$; 4 s: $F_{1,\ 23}=7.1$, $p<0.05$; 8 s: $F_{1,\ 23}=9.0$, $p<0.05$; 16 s: $F_{1,\ 23}=7.8$, $p<0.05$; 24 s: $F_{1,\ 23}=4.7$, $p<0.05$).

Asymptotic performance of wild-type and knockout mice on the parameter choice accuracy (percent correct) is shown in Fig. 1b. Both genotypes had high levels of performance at the 1-s delay, averaging 94% choice accuracy for wild-type and 97% for knockout mice. There is no significant main effect of genotype (ANOVA, $F_{1,\ 23}=3.50$, $p>0.05$), but there is a significant main effect of delay ($F_{5,\ 115}=60.62$, $p<0.05$). A main effect of session was also observed ($F_{13,\ 299}=2.61$, $p<0.05$), precipitated by a gradual increase in the percentage correct from sessions 1 (82.1±2.2) to 14 (88.4±1.3). As with the number of correct responses, a session×genotype interaction ($F_{13,\ 299}=2.39$, $p<0.05$) was seen, reflecting the relatively stable responses of the knockouts (range=85.3–89.4) compared to a gradual increase in the percentage correct responses in the wild-type mice (range=76.9–88.2). A delay×session interaction ($F_{65,\ 1,495}=1.39$, $p<0.05$) was seen as well, caused by the small but steady increase in accuracy following the 14 sessions at longer delays (24-s delay: session 1, 60.8±3.8; session 14, 72.3±3.1), with shorter delays increasing more modestly (1-s delay: session 1, 93.9±2.4, session 14, 99.0±0.7). Post-hoc testing showed a genotype main effect at the 2- ($F_{1,\ 23}=7.48$, $p<0.05$) and 4-s ($F_{1,\ 23}=4.43$, $p<0.05$) delays.

Although there was a significant main effect of delay length ($F_{5,\ 115}=62.42$, $p<0.05$) and of session ($F_{13,\ 299}=2.64$, $p<0.05$) on the number of incorrect trials, knockout of

the NK_3 receptor did not influence this measure (main effect genotype: $F_{1, 23}=1.48$, NS), nor did genotype interact with the other factors (delay length×genotype: $F_{5, 115}=0.16$, NS; session×genotype: $F_{13, 299}=1.75$, NS; Fig. 1c).

NK_3 knockouts missed significantly fewer trials than wild types, illustrated by the significant main effect of genotype ($F_{1, 23}=5.68$, $p<0.05$) on this parameter (Fig. 1d). The number of missed trials increased with delay length, as seen in the significant main effect of delay length ($F_{5, 115}=8.31$, $p<0.001$). No main effect of session was observed ($F_{13, 299}=0.62$, NS), nor did session interact other factors (session×genotype: $F_{13, 299}=0.62$, NS; session×delay length: $F_{65, 1,495}=0.95$, NS; session×genotype×delay length: $F_{65, 1,495}=0.82$, NS). Post-hoc testing per delay shows a genotype effect for each delay (1 s: $F_{1, 23}=5.0$, $p<0.05$; 2 s: $F_{1, 23}=4.4$, $p<0.05$; 4 s: $F_{1, 23}=4.6$, $p<0.05$, 8 s: $F_{1, 23}=7.4$, $p<0.05$; 16 s: $F_{1, 23}=6.9$, $p<0.05$; 24 s: $F_{1, 23}=4.8$, $p<0.05$).

Spontaneous alternation

The wild-type, heterozygote, and knockout mice showed similar levels of spontaneous alternation, with percentages of 60.7±1.8, 59.8±1.8, and 62.4±1.9, respectively (Fig. 2). There was no significant effect of genotype on spontaneous alternation ($F_{2, 117}=0.52$, NS).

Morris water maze

Both the knockout and wild-type animals readily acquired the cued version of the Morris water maze (Fig. 3a). The wild-type mice found the platform in an average of 41.0±2.9 s and the knockouts in 35.5±2.9 s during the first session. The latency decreased steadily during the four trials, reaching an average latency of 7.49±1.0 and 6.8±0.8 s for wild types and knockouts, respectively, during the fourth and final session. There were no significant differences between the two groups during cued training with respect to latency ($F_{1, 23}=0.96$, NS) or swim speed ($F_{1, 23}=0.35$, NS).

The mice also readily acquired the place version of the test (Fig. 3b). Latency to find the platform decreased in wild-type mice from 20.9±3.3 s during the first session to 12.0±2.3 s during the eighth session and in knockouts from 27.8±4.3 to 8.9±1.6 s. There were no significant differences between genotypes during training with respect to latency ($F_{1, 23}=0.04$, NS) or swim speed ($F_{2, 23}=0.39$, NS).

During the first probe trial, tested immediately following the fourth training session, no significant difference was seen between the percentage of time spent in the platform quadrant between knockout and wild-type mice ($t_{23}=0.53$, NS). However, there was a significant difference in the amount of time that the NK_3 knockout mice spent in each quadrant ($\chi^2_{(3)}=16.31$, $p<0.001$, Fig. 3c). Post-hoc analysis revealed a significant difference between the time spent in the quadrant that had contained the platform and the opposite quadrant ($p<0.05$). In contrast, the wild-type mice did not show a significant difference between the time spent in the various quadrants ($\chi^2_{(3)}=6.63$, NS).

During the second probe trial, conducted 24 h following the eighth training session, again, no significant difference was seen between the knockout and wild-type mice in terms of percentage of time spent in the probe quadrant ($t_{23}=0.26$, NS). Additionally, both the wild-type and knockout mice spent significantly more time in the quadrant that had previously held the platform compared to all other quadrants (wild type: $\chi^2_{(3)}=34.73$, $p<0.001$; knock-out: $\chi^2_{(3)}=16.43$, $p<0.001$; post-hoc testing: $p<0.05$ for platform quadrant compared to all other quadrants; Fig. 3d).

Active avoidance

All genotypes acquired the active avoidance task, as evidenced by the significant decrease in latency to shuttle ($F_{28, 1,456}=138.63$, $p<0.001$; Fig. 4a) and significant increase in the percentage of shocks avoided ($F_{28, 1,456}=220.35$; $p<0.001$; Fig. 4b).

The NK_3 knockout mice shortened the latency to respond to the conditioned stimulus earlier during training than the wild type animals, evidenced by the genotype×session interaction in latency to shuttle ($F_{56, 1,456}=1.42$, $p<0.05$) and the significantly lower latency times found in knockout mice compared to wild types in sessions 9 through 13, as well as 15 and 16. No significant differences were seen between heterozygotes and the other two genotypes during any session.

No significant differences in percent shocks avoided were observed between the genotypes ($F_{2, 52}=1.054$, NS), and no genotype×session interaction was observed ($F_{56, 1,456}=0.86$, NS).

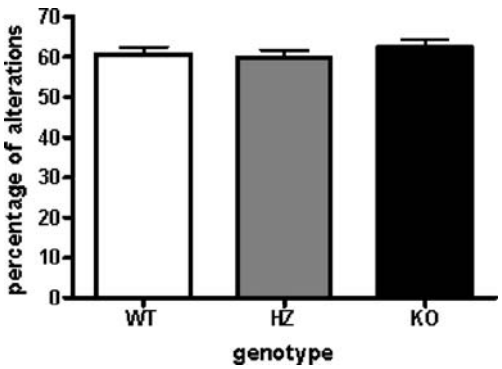

Fig. 2 Spontaneous alternation in wild-type ($N=20$), heterozygote ($N=20$), and NK_3 knockout ($N=19$) mice. *Bars* represent average percentage of alternations per genotype; *y*-axis indicates percentage of alternations during a 5-min period. *Error bars* indicate SEM

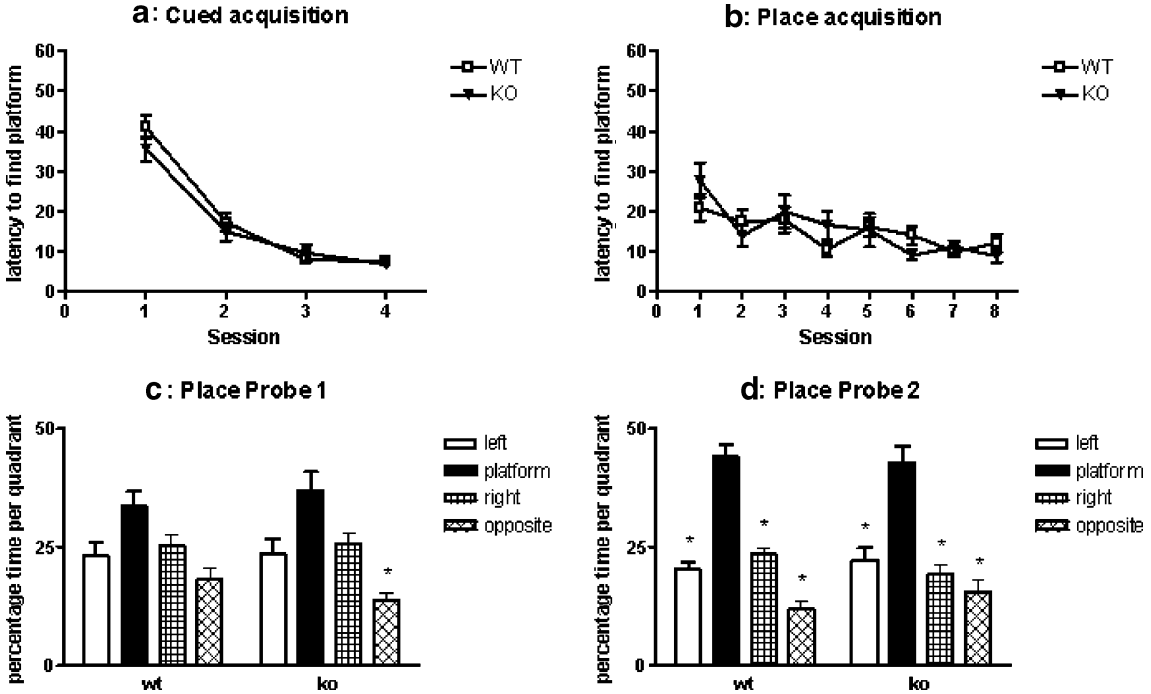

Fig. 3 Performance of NK_3 knockout (N=11) and wild-type (N=14) mice in the Morris water maze as measured by latency to find platform during acquisition of cued (**a**) and place (**b**) tasks, during probe testing immediately after training session 4 (**c**) and probe testing 24 h after training session 8 (**d**) of training. Note that the NK_3 knockout mice show a significant difference between platform and opposite quadrants during the first probe test, an effect not seen in wild-type animals. For all graphs in the figure, *error bars* indicate SEM. For **a** and **b**, the *x*-axis represents training session and the *y*-axis represents the latency to find the platform in seconds. *Squares* represent wild-type (*WT*) animals, and triangles represent knockouts (*KO*). In **c** and **d**, the *y*-axis represents the percentage of time spent in each quadrant, while the *bars* represent the quadrants as described in the legend. *Bars* are grouped by genotype on the *x*-axis. *Asterisk* represents $p<0.05$ in post-hoc testing vs. the platform quadrant

Discussion

The present results demonstrate that knockout of the NK_3 receptor leads to enhanced performance of the DMTP task and improved performance in the Morris water maze task early in training and that knockout mice reach plateau-level performance earlier in training of active avoidance. There is very limited data on the role of NK_3 in cognition in animal models and no peer-reviewed published data from clinical trials on effects of NK_3 antagonism on cognition. Thus, the present data are an important step forward for understanding the role of NK_3 in tests of cognition relevant to schizophrenia.

Although the NK_3 knockout mice showed a clear enhancement of performance of the DMTP task, this effect was not delay dependent, and no effect of the NK_3 knockout was seen in spontaneous alternation. Together, this points to a facilitated performance of an operant task but a lack of effect specific to working memory. This was surprising, given that one of the few sets of studies addressing the role of pharmacological interventions at the NK_3 receptor in cognition demonstrated that intracerebroventricular infusion of the NK_3 agonist senktide reverses scopolamine-induced deficits in spontaneous alternation (Kameyama et al. 1998; Ukai et al. 1998). The lack of effect of the knockout on working memory measures may be due to ceiling effects in performance masking any improvements. It may also be that the NK_3 receptor is involved in working memory when stimulated or antagonized acutely but that the NK_3 receptor is more involved in instrumental conditioning processes than in working memory in the chronic deprivation state that a knockout represents. The effect on instrumental responding is not specific to appetitively reinforced behaviors, as improved performance was also seen in the aversively motivated active avoidance task.

In light of the interest in NK_3 receptor antagonism as a potential antipsychotic treatment, it is interesting to note that both typical and atypical antipsychotics have been reported to either worsen delayed nonmatch to position performance delay independently (Didriksen 1995 and data generated in-house at Roche) or leave delayed nonmatch to position performance unaffected in rats (Gemperle et al. 2003), though iloperidone has been reported to improve delayed nonmatch to position performance delay dependently (Gemperle et al. 2003), and delay-dependent deficits following haloperidol and clozapine intake have been demonstrated in primates (Murphy et al. 1996). Furthermore,

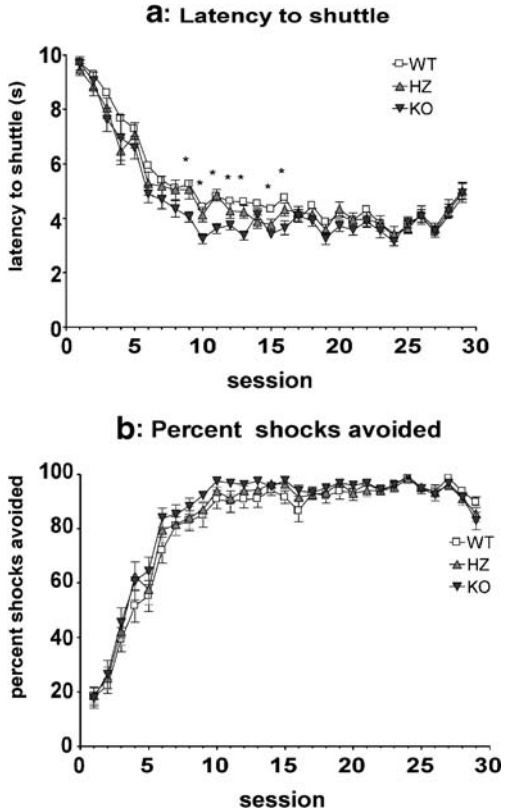

Fig. 4 Performance of NK_3 wild-type (N=20), heterozygous (N=20), and knockout (N=19) mice on active avoidance task. **a** represents the latency to shuttle after onset of the CS light. The x-axis represents training sessions, and the y-axis represents the latency to shuttle in seconds. **b** represents the percent shocks avoided, whereby the x-axis represents sessions and the y-axis represents the percentage of shocks avoided during each session. For both graphs, the *error bars* indicate SEM. Symbols are as follows: *squares* represent wild types, the *upward-pointing triangles* heterozygotes, and the *downward-pointing triangles* knockouts. *Asterisk* represents $p<0.05$ in post-hoc testing for wild type vs. knockout

dopamine D_2 antagonist-based antipsychotic drugs are detrimental to performance of an eight-arm radial maze task for working memory when given acutely, although this detriment is no longer seen after chronic treatment (Ortega-Alvaro et al. 2006). These data collectively stand in contrast to the delay-independent improvement observed in the NK_3 knockouts, indicating that this alternative mechanism of action for modulating dopamine levels may leave working memory unimpaired.

There was no impairment of performance in the Morris water maze task; however, there was a tendency to improvement in acquisition, as demonstrated by a significant difference between the platform and opposite areas during the first probe trial. This is of particular relevance given that schizophrenic patients were recently demonstrated to be impaired in performing a virtual version of this task (Hanlon et al. 2006). This improvement in the NK_3 knockout mice stands in contrast to most currently used antipsychotics, which produce deficits in performance of the Morris water maze task when given acutely and, in some cases, also when given chronically (Didriksen et al. 2006; Hou et al. 2006). The fact that the absence of the NK_3 receptor does not hinder but actually tends to improve performance on this task suggests at the least that blockade of the NK_3 receptor may not produce cognitive deficits, and it also implies that NK_3 antagonism warrants further investigation as a possible target for improvement of cognitive functioning.

Although the present study was not designed to allow attribution of the improvement seen to a specific brain area, the hippocampus is well known to play an essential role in performance of the Morris water maze task. It has been suggested that the impaired performance of schizophrenics in this task is related to hippocampal deficits (Hanlon et al. 2006). The stimulation of the NK_3 receptor has been demonstrated to activate cholinergic systems in the hippocampus (Marco et al. 1998) and septohippocampal cholinergic neurons (Morozova et al. 2008), which suggests that antagonism and/or knockout of this receptor might reduce cholinergic activity in the hippocampus. Acute cholinergic antagonism with scopolamine is well known to impair performance in the Morris water maze (Buresova et al. 1986; Paylor and Rudy 1990). Based on this interaction between NK_3 and acetylcholine, one might predict deficits in Morris water maze performance in NK_3 knockout mice, in opposition to what we observed. However, it is important to bear in mind that the NK_3 receptor interacts with dopaminergic and serotinergic systems (reviewed in Spooren et al. 2005) as well as the cholinergic system. The behavioral effects observed in the NK_3 receptor knockouts are the result of interactions between all of these systems, under the influence of congenital NK_3 receptor depletion. Thus, the effects of knockout of the NK_3 receptor or chronic NK_3 receptor blockade will be difficult to predict based on a single neurotransmitter system or acute effects of antagonists.

The enhanced acquisition of the active avoidance task, represented by reaching plateau-level latencies earlier in the task than wild-type animals, further supports enhanced acquisition of instrumental tasks induced by the NK_3 knockout. However, there was no difference observed in the percent of shocks avoided, which is another measure of acquisition. It is important to note that the percentage of shocks avoided reached ceiling levels of responding early in training. During the sessions that showed significant differences in latency, performance of the wild-type mice on percent shocks at or above 85% makes demonstration of improvement nearly impossible for this parameter, emphasizing the importance of taking a variety of parameters into account when testing cognition. The parameter of latency to respond can be potentially confounded by locomotor effects, and these NK_3 knockout mice do show mild

hyperlocomotion (Nordquist et al., submitted). However, it is important to note in this context that although latency times decreased faster in the knockout mice than the wild types, the plateau level reached did not differ, excluding simple motor effects as an explanation for this effect. The NK_3 knockouts also showed mild motor coordination impairment on the rotarod test (Nordquist et al., submitted), further arguing against a motor effect.

The effect of NK_3 knockout on active avoidance seen in the present study stands in contrast to the effects of currently known antipsychotics, which impair performance in the active avoidance task when given both acutely and chronically (Li et al. 2007; Wadenberg and Hicks 1999). Indeed, deficits in active avoidance have been suggested as a screening method for detecting antipsychotic activity. However, this impairment is only seen in combination with dopamine D_2 receptor antagonism and not when dopamine D_4, adrenergic, serotinergic, or glutamatergic receptors alone are (ant)agonized (Wadenberg and Hicks 1999). In the present study, we did not directly manipulate the dopamine D_2 receptor, which may explain the difference between the present results and the performance of traditional antipsychotics on this test.

Ii is interesting to note that the present results with respect to active avoidance and the Morris water maze contrast with a recent report from another group that generated NK_3 knockouts and demonstrated deficits in active avoidance and a mild deficit in Morris water maze performance in their knockout line (Siuciak et al. 2007). There are several differences between the present study and the study by Siuciak et al. that may explain these discrepancies. With respect to the Morris water maze, it is of note that the Siuciak study found a difference between wild-type and knockout mice at one time point during acquisition but that they did not conduct a probe test, which is where differences were observed in the present study. Differences in test protocol may thus have influenced the Morris water maze results.

The main difference, though, may be that our knockout line was generated using ES cells derived from C57Bl/6, while Siuciak et al. used 129P2/OlaHsd ES cells and then injected these into C57Bl/6J blastocytes. Any remaining background from the 129P2/OlaHsd strain could influence the behavioral results strongly and might explain differences observed between the two NK_3 knockout lines. Strain differences between C57Bl6 and 129 lines have been demonstrated to affect performance in active avoidance (Clark et al. 2003) and cued fear conditioning (Bothe et al. 2004), an essential component of the active avoidance paradigm. Strain differences in performance have also been demonstrated in mice in the Morris water maze. 129S6/SvEvTac acquired the Morris water maze faster than C57Bl/6J in one study (Clapcote and Roder 2004), while data generated in-house at Roche show that C57Bl/6J performed a probe trial better than 129/Ola mice at a time period comparable to the second probe trial in the present study. It is interesting to note that another study demonstrated that an F2 generation hybrid of the C57Bl/6J and 129sv strains acquired the task significantly better than either of the parental inbred strains (de Bruin et al. 2006). Thus, the differences observed between the performances of these two NK_3 knockout lines may be related to different genetic backgrounds, which could mean that genetic influences are paramount to determining effects of NK_3 receptor blockade.

In summary, the present paper demonstrates that knockout of the NK_3 receptor produces enhanced performance in operant tasks and in spatial learning but does not support a role for the NK_3 receptor in working memory. The NK_3 receptor has been of strong interest as a target for antipsychotic action and has shown promise in clinical testing in schizophrenics. As outlined in the introduction, antipsychotics relying on dopamine D_2 antagonism do not improve and often worsen cognitive symptoms in schizophrenics. Given the mild improvement or lack of impairment in various cognitive tasks following knockout of the NK_3 receptor as described in the present study, it is tempting to speculate that NK_3 receptor antagonists may not worsen or even may hold promise to mildly improve cognition in schizophrenics. To this end, it is essential to substantiate the current findings and to assess the effects of NK_3 receptor antagonists on cognition in both animal and man.

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