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# Abnormal response to stress and impaired NPS-induced hyperlocomotion, anxiolytic effect and corticosterone increase in mice lacking NPSR1

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## Summary

NPSR1 is a G protein coupled receptor expressed in multiple brain regions involved in modulation of stress. Central administration of NPS, the putative endogenous ligand of NPSR1, can induce hyperlocomotion, anxiolytic effects and activation of the HPA axis. The role of NPSR1 in the brain remains unsettled. Here we used NPSR1 gene-targeted mice to define the functional role of NPSR1 under basal conditions on locomotion, anxiety- and/or depression-like behavior, corticosterone levels, acoustic startle with prepulse inhibition, learning and memory, and under NPS-induced locomotor activation, anxiolysis, and corticosterone release. Male, but not female, NPSR1-deficient mice exhibited enhanced depression-like behavior in a forced swim test, reduced acoustic startle response, and minor changes in the Morris water maze. Neither male nor female NPSR1-deficient mice showed alterations of baseline locomotion, anxiety-like behavior, or corticosterone release after exposure to a forced swim test or methamphetamine challenge in an open-field. After intracerebroventricular (ICV) administration of NPS, NPSR1-deficient mice failed to show normal NPS-induced increases in locomotion, anxiolysis, or corticosterone release compared with WT NPS-treated mice. These findings demonstrate that NPSR1 is essential in mediating NPS effects on behavior.



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**Keywords**

neuropeptide S; neuropeptide S receptor; NPSR1; stress; anxiety; depression; locomotor activity; corticosterone

## Introduction

NPSR1 (also known as GPR154, GPRA) is a G protein coupled receptor (GPCR) expressed in multiple brain regions suggested to be involved in the modulation of anxiety, arousal, energy balance, and hormonal homeostasis, as well as learning and memory (Xu et al., 2007). Human NPSR1 mRNA encodes 8 variants by alternative splicing, only 2 of which, hNPSR1-A and hNPSR1-B, are functional receptors supported by the evidence that they are located on the cell membrane (Vendelin et al., 2005). In rodents, one single NPSR1 exists and its amino acid sequence shows higher similarity to hNPSR1-A. A susceptibility locus for panic disorder was mapped to the human NPSR1 locus (chromosome 7p15) (Knowles et al., 1998; Crowe et al., 2001 and Logue et al., 2003) and an NPSR1 SNP ($Asn/Ile^{107}$) was associated with panic disorder in male Japanese patients (Okamura et al., 2007).

By a reverse pharmacological strategy, neuropeptide S (NPS) was identified as a putative ligand for NPSR1 since binding causes increased intracellular calcium mobilization and cAMP accumulation (Xu et al., 2004). An $Asn/Ile^{107}$ SNP in NPSR1 increases both the agonist efficacy as well as intracellular trafficking of the receptor to the cell membrane without affecting binding affinity (Reinscheid et al., 2005 and Bernier et al., 2006). Real-time PCR shows NPS to be mainly expressed in the brain and endocrine tissues (Xu et al., 2004). NPS precursor mRNA is co-expressed with excitatory or stimulatory transmitters, such as glutamate, acetylcholine, and corticotrophin-releasing factor (CRF) in the brainstem (Xu et al., 2007); all these neurotransmitters have an important role in emotional regulation. Central administration of NPS modulates locomotor activity, arousal, anxiety, the hypothalamic-pituitary-adrenal (HPA) axis, and food intake (Xu et al., 2004; Beck et al., 2005; Niimi, 2006; Smith et al., 2006; Leonard et al., 2008; Rizzi et al., 2008; Vitale et al., 2008). Together, these data suggest that NPSR1 may be involved in these activities induced by NPS. Although several groups developed NPSR1 antagonists to characterize the physiological function of the NPS/NPSR1 system (Okamura et al., 2008; Camarda et al., 2009 and Guerrini et al., 2009), the role of NPSR1 in the brain is still unclear.

The current investigation was undertaken to elucidate the functional role of NPSR1 in locomotor activity, anxiety and/or depression, startle reactivity, corticosterone release, spatial learning ability, and response to methamphetamine challenge at baseline (without extraneous NPS stimulation). Animals were also examined to determine if NPSR1 mediates NPS-induced locomotor activation, anxiolytic effects, or corticosterone increases using a novel NPSR1-targeted mouse in which exon 2 was deleted.

## Materials and Methods

### Mice

NPSR1-deficient mice were established by gene targeting using Velocigene™ technology (Regeneron Pharmaceuticals, Inc. Tarrytown, NY) (Valenzuela et al., 2003). The main strategy was based on replacing the 3' 128 bp of exon 2 and the first 1510 bp of intron 2 with a translational-fusion β-Gal reporter gene and a neomycin resistance gene. Mice with the genetic background of 129Sv/J × C57BL/6 cross were backcrossed 7 generations with C57BL/6 mice. Genotypes were determined by PCR of tail DNA using primers specific for the wild type (WT) allele (sense, 5'-CATCCAGGAAACGT GAGCAC-3'; antisense, 5'-

TCTAGCATCGGCACAGTCTG-3') and the gene deleted allele (sense, 5'-CATCCAGGAAACGTGAGCAC-3'; antisense, 5'-GTCTGTCCTAGCTT CCTCACTG-3'). Behavioral testing under baseline conditions were carried out on 8- to 10-week-old NPSR1-deficient mice and WT littermates derived from crossing NPSR1 heterozygous mice. The NPSR1-deficient mice used for behavioral studies after NPS ICV injection were generated using NPSR1 homozygous female and male matings, and age and sex matched C57BL/6 WT mice bred in-house were used as control. All the mice were maintained and housed 4 per cage by sex under pathogen-free conditions. At least 1 week prior to behavioral testing, NPSR1-deficient and WT mice were transferred to conventional housing under a 14 h light/10 h dark cycle (lights on at 600 h) with temperature (19 ± 1°C) and humidity (50 ± 10%) controlled. All procedures were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Research Foundation. The number of animals used in each test was given in the figure captions and results.

## Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The mRNA expression in the brain from male NPSR1-deficient mice and WT littermates was examined using RT-PCR. Briefly, total RNA was isolated from the tissue, treated with DNAase I and reverse-transcribed using oligo (dT) and Superscript II reverse-transcriptase (Invitrogen, Carlsbad, CA) in a 20 μl reaction. The cDNA produced by these reactions (2 μl) was amplified by PCR using Taq DNA polymerase and the primers used in the PCR reaction are indicated below: full length NPSR1 sense, 5'-TCGTCAGGCAGAACTCTTCA-3' specific to 5'UTR and antisense, 5'-ATCTGCTAGGTGAGGCAGGA-3' specific to 3'UTR (1300 bp product); LacZ sense, 5'-ATACACTTGCTGATGCGGTGC-3' and antisense, 5'-AGATGGCGATGGCTGGTTTC-3' (460 bp product).

## LacZ detection

Brains from male NPSR1-deficient and WT littermates were perfused with 4% PFA and fixed overnight in 4% PFA. Fixed tissues were cryoprotected overnight in cold 30% sucrose in PBS and embedded in OCT embedding medium and frozen on dry ice. These cryostat sections were stored at −80°C until assessment.

β-Gal staining was performed on 20 μm thick cryostat coronal sections of frozen fixed brain (Steele-Perkins et al., 2005). The slides were allowed to dry at room temperature for 2 h and fixed in 0.2% glutaraldehyde on ice for 10 min, washed in PBS with 2 mM $MgCl_2$ 3 times for 10 min, and in PBS with 0.02% NP-40 and 2 mM $MgCl_2$ once for 15 min on ice. Sections were subsequently incubated in the X-gal staining solution (PBS; pH7.3) containing 5 mM potassium hexacyanoferrate, 5 mM potassium ferrocyanide trihydrate, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate, and 1 mg/ml X-gal solution (stock 40 mg/ml X-gal in DMF) at room temperature for 8 h. Stained sections were washed 2 times in PBS with 2 mM $MgCl_2$, cleared in distilled water for 5 min, and then counterstained with 0.1% nuclear fast red in 5% aluminum sulfate for 2 min, washed in running water, dehydrated, and topped with coverslips for analysis.

## Behavioral studies under baseline conditions

The behavioral tests under baseline conditions were carried out between 1300–1700 h on 2 groups of 8- to 10-week-old NPSR1-deficient mice and WT littermates. Both male and female mice were used. One group of mice was used to do behavioral tests as follows: Week 1: Day 1, elevated zero maze; Day 2, light-dark exploration; Day 3, open-field locomotor activity immediately followed by marble burying; Day 4, acoustic startle with prepulse inhibition; Week 2, Day 1–6, cued version of the Morris water maze (MWM); Week 3, Day 1–7, acquisition phase of MWM spatial version; Week 4, Day 1–7, reversal phase of MWM spatial version; Week 5, Day 1–7, shift phase of MWM spatial version; Week 6, Day 1,

locomotor activity with (+)-methamphetamine challenge. The second group of mice was used to test depression-like behavior in the tail suspension test (TST) and 2 days later in the forced swim test (FST).

### Elevated zero maze

Mice were tested in an elevated zero maze as a test of anxiety (Shepherd et al., 1994). The ring-shaped maze was 105 cm in diameter and 72 cm in height with a 10 cm path width. The maze was equally divided into 4 quadrants, including 2 enclosed areas with black walls 28 cm in height and 2 open areas with a clear acrylic curb 1.3 cm in height. Each mouse was placed in the center of one of the closed areas and behavior was video recorded for 5 min as described previously (Williams et al., 2003). The room was lit with a single halogen lamp turned to a dim setting. The apparatus was cleaned with 70% ethanol between animals. The number of head dips, the latency to first open area entry, and time in the open areas in 5 min was scored for each mouse from the video recordings.

### Light-dark exploration

Light-dark exploration was measured in Accuscan activity monitors (41 cm L×41 cm W×30 cm H, Accuscan Instruments, Inc., Columbus, OH) with an inserted box that made half of the chamber dark (Schramm et al., 2001). An opening (5.5 × 7 $cm^2$) allowed mice to pass freely between the two sides. Mice were placed individually in the corner of the lighted side and tested for 10 min with fluorescent overhead room lights illuminating the chambers. Chambers were cleaned with 70% ethanol between animals. Latency to enter the dark side and total time spent in the light area were recorded.

### Open-field locomotor activity

Spontaneous locomotor activity was measured in Accuscan activity monitors (41 cm L×41 cm W×30 cm H, Accuscan Instruments, Inc., Columbus, OH) in 5 min intervals for 60 min. Movement patterns were scored using VersaMax software (Pan et al., 2008). Dependent measures included horizontal activity, vertical activity (rearing), total distance, central and margin distances, and time spent in the central and margin areas. Chambers were cleaned with 70% ethanol between animals.

### Marble Burying

Each mouse was brought to an adjacent suite and placed in a standard mouse cage with fresh hardwood chip bedding (5 cm deep). On the surface of the bedding there were 15 evenly spaced marbles placed in three rows (4.5 cm apart, 4.5 cm from the long edge, and 3.5 cm from the short end). A filter top was placed over the cage. Testing was conducted for 20 min under fluorescent room lighting. Latency to start marble burying and number of marbles at least 2/3$^{rd}$ buried were counted (Pan et al., 2008).

### Acoustic startle reactivity with prepulse inhibition

Acoustic startle reactivity was performed in an SR Lab apparatus (San Diego Instruments, San Diego, CA, USA) as previously described (Brunskill et al., 2005 and Pan et al., 2008). Mice were placed in an acrylic cylindrical test chamber scaled for mice. The test chamber was mounted on a platform with a piezoelectric force transducer attached to the underside. The platform was located inside a sound-attenuated test chamber. A 5 min acclimation period preceded test trials. Each animal received a 12 min test using a 4 × 4 Latin square design that balanced for the 4 trial types: no stimulus, startle stimulus alone, 70 dB or 76 dB prepulse plus startle stimulus. Each set of 16 trials was presented three times for a total of 48 trials. Trials of the same type were averaged together. The intertrial interval was 8 s. The startle signal was a 20 ms, 110 dB sound pressure level mixed-frequency burst and the

startle recording window was 100 ms. Pre-pulses preceded the startle-eliciting stimulus by 70 ms from pre-pulse onset to startle signal onset.

### Morris water maze (MWM)

Animals were tested for spatial learning and memory using the MWM as described previously (Vorhees et al., 2006). The Morris water maze apparatus was 122 cm in diameter and filled with 17.5 cm depth room temperature water (21 ± 1°C). For facilitating tracking, the maze interior was painted white and the water was colored with white tempera paint. Proximal cued learning was evaluated using the cued version; in which mice were required to find a platform (10 cm diameter, submerged 1 cm below the water) marked with a ball (40 mm diameter) affixed to a brass rod mounted 7 cm above the platform. Curtains were drawn around the maze to reduce visibility of extra maze cues, and the mice were given 6 trials on day 1 and 2 trials per day from day 2 to day 6. Both the platform and start position were rotated to different locations on each trial. The time limit for each trial was 1 min and the intertrial interval (ITI) was 5 s on the platform plus 30 s in the home cage during platform changes. Mice not finding the platform within 1 min were placed on the platform for 5 s. Mice were observed on a closed-circuit television, and latency to reach the platform was recorded for each trial. The cued procedure introduced mice to task requirements of the MWM (swimming, the fact that the platform is not near the perimeter, and that climbing on the platform provides escape from the water).

Subsequent to the cued version, mice were tested in the spatial (hidden platform) version to assess spatial acquisition (Vorhees et al., 2006). In this version, the animal learns to use distal cues to navigate a direct path to the hidden platform when started from different locations around the perimeter of the tank. The wall of the room had a variety of prominent cues, including geometric shapes that were visible from the maze. There were three phases: acquisition, reversal, and shift. In brief, each phase consisted of 4 hidden platform trials/day and 6 training days and a single probe trial given with the platform removed 24 h after the last training day of each phase with the curtains open. In each hidden platform trial there was a 1 min trial limit and a 15 s ITI spent on the platform. If a mouse failed to locate the platform, it was removed from the water and placed on the platform for 15 s. Throughout each phase the platform that was submerged 1 cm below water surface remained stationary while start positions (i.e., acquisition: N, E, SE, and NW; reversal: S, W, NW, and SE; shift: S, E, NE, and SW) were quasi-randomized daily with the condition that no position could be used more than once a day. The size and position of the platform in the maze were changed for each phase (i.e., acquisition: 10 cm diameter and SW quadrant; reversal: 7 cm diameter and NE quadrant; and shift: 5 cm diameter and NW quadrant). Mice were tracked using a video tracking system (Anymaze, Stoelting, Wood Dale, IL). Path length, latency, speed, and cumulative distance to the platform were recorded. On the seventh day of each phase, the platform was removed and mice were given a single 30 s probe trial starting from NE in acquisition, SW in reversal, or SE in shift to determine whether the animals remembered the location of the platform sufficiently to be able to swim back to where it was previously located. For probe trials, the measures recorded were average distance to the platform, time and distance in the target quadrant, initial heading to target error, and number of platform site crossovers.

### Locomotor activity with methamphetamine challenge

The week after MWM, mice were reintroduced to the open-field for 30 min of habituation and then injected subcutaneously with 1 mg/kg (+)-methamphetamine (expressed as the freebase) and replaced in the arena. Locomotor activity was recorded for another 2 h (Brunskill et al., 2005). Immediately upon removal from the open-field, mice were

transferred to an adjacent suite, quickly decapitated, and blood was collected for later plasma corticosterone determination.

### Tail suspension test (TST)

A second group of 8–10 week old mice was used for assessment of depression–like behavior using the tail suspension test (Cryan et al., 2003). The mice were moved to the testing room at least 1 h prior to testing. Mice were individually suspended from a plastic platform (distance from floor = 16 cm) by pulling the tail through a hole in the platform as far as possible and fastening the tail to the top of the platform with adhesive tape. The tail was secured in this fashion to prevent tail grabbing by the mice. Time spent immobile over the entire 6 min testing time was recorded. Mice were disqualified if they grabbed their tail but retained if they held onto one foot.

### Forced swim test (FST)

Two days after the TST, the same mice were tested using the Porsolt forced swim test (Porsolt et al., 1977a and Porsolt et al., 1977b). Mice were moved to the test room at least 1 h prior to testing. Each mouse was placed individually in an acrylic cylinder (45 cm in height and 17 cm in diameter) with 30 cm deep water (26°C) for 10 min and time spent immobile in the last 6 min was recorded. The water was changed between subjects. Immobility was defined as the absence of vigorous movements (small paddling motions used by the animal to remain level or to keep from sinking were not considered escape movements).

### Plasma corticosterone levels without NPS stimulation

Immediately following the locomotor activity with challenge or 15 min after the forced swim test, mice were decapitated and blood was collected in polypropylene tubes containing 2% EDTA (0.05 ml) and placed on ice. Samples were centrifuged at 1399 RCF at 4°C for 25 min and plasma was removed and stored at −80°C until assayed. Corticosterone levels were assessed by EIA (IDS Inc., Fountain Hills, AZ).

### Behavioral tests and plasma corticosterone after intracerebroventricular administration of NPS

Separate groups of mice were used for these experiments. Mice at the age of 8 weeks were anesthetized with isoflurane and placed in a stereotaxic apparatus. A C313GS-4 guide cannula (Plastic One Inc., Roanoke, VA, USA) was implanted in the right lateral ventricle under aseptic conditions through a hole drilled in the skull at the following coordinates relative to bregma: anterior–posterior, +0.6 mm; lateral, 1.1 mm; vertical, −2.1 mm beneath the surface of the skull, according to coordinates from Paxinos and Franklin (Paxinos and Franklin, 2004). The cannula was fixed to the skull with surgical glue. A C313DCS-4 cannula dummy was inserted into the guide cannula to prevent blockage of the guide cannula prior to intracerebroventricular (ICV) injection. Each mouse was housed individually after cannula implantation. Mice were allowed a 7-day recovery period before experiments were initiated.

For NPS ICV injection, 3 μl of artificial cerebrospinal fluid (aCSF at PH 7.4: NaCl 124 mM; KCl 3.0 mM; $NaHCO_3$ 26 mM; $CaCl_2$ 2.0 mM; $MgSO_4$ 1.0 mM; $KH_2PO_4$ 1.25 mM; D-glucose 10.0 mM), 0.1, 1, or 10 nmol NPS (Phoenix Pharmaceuticals, Inc. Burlingame, CA USA) in 3 μl of aCSF were administered with a C313IS-4 internal cannula (Plastic One Inc., Roanoke, VA USA) connected to a Hamilton syringe with cannula tubing (C313C, Plastic One Inc., Roanoke, VA). The injection was administered over a period of 15 s. The injection cannula was left in place for another 60 s before being slowly withdrawn to avoid backflow.

In order to reduce the stress from injection, ICV injection was conducted under light isoflurane anesthesia.

Four groups of male C57BL/6 WT mice for NPS dose-response experiments and three groups of male NPSR1-deficient and age matched C57BL/6 WT mice for comparing KO and WT were used in the behavioral tests and assessing plasma corticosterone levels after NPS ICV injection. Wildtype male C57BL/6 mice were transported from their housing room to the testing suite 1 h before the test started. Dose-response effects of ICV NPS were examined in the elevated zero maze, light-dark exploration, and open-field tests between 2000–2400 h. One group of WT mice (n=5–6/dose) was tested in the elevated zero maze for 5 min after receiving a single ICV injection of aCSF, 0.1, 1, or 10 nmol of NPS, and immediately upon removal from the elevated zero maze, the animals were tested for light-dark exploration for 10 min. Another group of WT mice (n=5–6/dose) was tested in the open-field 5 min after receiving an ICV injection of aCSF, 0.1, 1, or 10 nmol of NPS. Following the results with the WT animals, a dose of 1 nmol of NPS was used to determine if NPSR1 is involved in NPS-induced changes in locomotion and anxiety. Furthermore, the effectiveness of NPS (1 nmol)-induced locomotion in the open-field was tested over a 3 h period in a third group of male C57BL/6 mice 5 min after NPS ICV injection. One group of male NPSR1-deficient and age matched C57BL/6 WT mice was tested in the elevated zero-maze for 5 min after NPS ICV injection and then tested for light-dark exploration for 10 min. A second group of male NPSR1-deficient and age matched C57BL/6 WT mice was tested for 60 min in the open-field test 5 min after NPS ICV injection.

Plasma corticosterone levels under NPS stimulation were examined 40 min after NPS ICV injection between 800–1000 h as described by Smith et al. (2006). The dose-response effect of a single ICV injection of NPS (aCSF, 0.1, 1, or 10 nmol) was first examined in WT male C57BL/6 mice. Then to test the role of NPSR1 in NPS-induced corticosterone release, aCSF or 10 nmol of NPS was applied to NPSR1-deficient and age matched C57BL/6 WT mice.

After testing, cannula placement was confirmed for each mouse by histological examination of the brain after Evans blue injection. Animals with incorrect placement were not considered in the analysis.

### Statistical analysis

Behavioral data were analyzed using mixed linear ANOVA models (SAS Proc Mixed, SAS Institute, Cary, NC). For each data set, the covariance matrix was checked using best fit statistics. In most cases the best fit was to the autoregressive-1 (AR (1)) covariance structure. This statistical model calculates adjusted degrees of freedom using the Kenward-Roger method, and therefore do not match those obtained from general linear model ANOVAs and can be fractional. Measures taken repetitively on the same animal, such as trial, interval, or day, were repeated measure factors. For clarity of presentation, only significant main effects of genotype or NPS treatment or the highest order interactions involving these variables are presented. Sex was included as a factor when both sexes were tested. When warranted, separate ANOVAs were performed on each sex. Significance was set at $p \leq 0.05$. Data are presented as mean ± SEM.

## Results

### Generation of NPSR1-deficient mice and confirmation of NPSR1 null allele

Genomic organization of the murine NPSR1 gene (Fig. 1*A*, GenBank accession no. NC_000075) contains 10 exons and 9 introns spanning 218 Kb of genomic sequence. The open reading frame encodes 371 amino acids. Exon 2 encodes the majority of extracellular N terminal part of the NPSR1 gene. The main strategy of NPSR1 gene targeting was

replacement of the 3' 128 bp of exon 2 and the first 1510 bp of intron 2 with a translational-fusion β-galactose reporter gene and a neomycin resistance gene (LacZ and Neo, respectively, in Fig. 1*B*). PCR genotyping of tail DNA (Fig. 1*C*) revealed that the NPSR1-deficient allele was inherited to progeny in Mendelian ratios without bias by sex. To confirm that the targeted NPSR1 allele was deleted as described, RT-PCR analysis of brain RNA, isolated from littermates of each NPSR1 genotype, was performed to amplify the full length NPSR1 using primer pairs specific to the 5'UTR (forward) and 3'UTR (reverse) (Fig. 1*D*) and LacZ transcripts (Fig. 1*E*). The full length NPSR1 transcript was present in the brain of WT and heterozygous mice, but absent in the brain of NPSR1-deficient mice. The expression of LacZ was found in the brain of NPSR1 heterozygous and deficient mice, but not in the brain of WT mice (Fig. 1*D* and *E*). These data indicate that replacement of exon 2 with the fusion protein generates a null allele of NPSR1. NPSR1-deficient mice did not display apparent differences from their WT littermates in growth, weight, or reproduction capability.

## Expression of NPSR1 in the brain by X-gal staining

The homologous integration positioned the LacZ gene under the control of the NPSR1 endogenous promoter, which allowed us to monitor NPSR1 expression in the brain (Supplemental Fig. 1) by X-galactosidase staining. The brain of NPSR1-deficient mice did not exhibit obvious structural or morphological abnormalities and X-galactosidase staining was observed in several limbic regions, such as the amygdala, thalamus, and hypothalamus, as well as the motor cortex (Supplemental Fig. 1) (other expression sites not shown). Our X-gal staining results in the NPSR1-deficient mouse brain are consistent with the *in situ* results of NPSR1 based on the Allen Brain Atlas (http://www.brain-map.org/welcome.do). As a control, no X-galactosidase staining was observed in the brain from NPSR1 WT littermates (not shown).

## Behavioral assessment in NPSR1-deficient mice under basal conditions

In view of the expression pattern of NPSR1 in brain regions involved in locomotion, stress response, and learning and memory (Xu et al., 2007), we assessed the effect of the NPSR1 gene deletion on locomotion, anxiety- and/or depression-like behavior, acoustic startle with prepulse inhibition, and learning and memory in KO and WT mice.

**Unaltered anxiety-like behavior and locomotion activity in NPSR1-deficient mice—**Anxiety-like behavior was examined in the elevated zero maze (Fig. 2A), marble burying test (Fig. 2B), light-dark exploration (Fig. 2C), and open-field (central region entries) (Fig. 2D) (Van Meer et al., 2005). Both male and female NPSR1-deficient mice showed similar performance to sex- and age-matched WT littermates in these tests.

Locomotor activity was assessed in an open-field under baseline conditions and after (+)-methamphetamine challenge. Methamphetamine was used since locomotor activity is strongly modulated by monoamines, especially dopamine, and methamphetamine blocks dopamine reuptake and facilitates dopamine release. NPSR1-deficient mice did not show an alteration in spontaneous locomotor activity during the pre-methamphetamine phase (Fig. 3*A*). Methamphetamine markedly increased the activity level of both WT control and NPSR1-deficient mice, but no significant differential response was observed.

**Increased depression-like behavior in the forced swim test, but not the tail suspension test in NPSR1-deficient mice—**The FST and TST were employed to evaluate depression-like behavior. In the FST (Fig. 3*B*) there was a significant interaction of genotype × sex ($F(1, 73.5) = 4.32$, $p < 0.05$). Male NPSR1-deficient mice had significantly increased immobility compared to the male WT littermates ($P<0.05$), whereas female

NPSR1-deficient mice did not show this difference. Overall, the female animals remained immobile longer than the males (p = 0.01). In the tail suspension test, both male and female NPSR1-deficient mice did not differ from age and sex matched WT littermates in immobility (not shown, n=18–21 per group).

**Reduced acoustic startle response in NPSR1-deficient mice—**Assessment of acoustic startle response revealed that there were no significant main effects of genotype or sex or the interaction of genotype × sex, but there was a significant prepulse × sex interaction ($F(2, 134) = 4.2$, $P < 0.02$). Examination of males and females separately showed that male NPSR1-deficient mice had decreased acoustic startle responses on all trials compared to male WT littermates ($F(1, 26) = 4.11$, $P = 0.05$)(Fig. 3*C*). There was no differential change in prepulse inhibition of the startle response as a function of prepulse intensity. Female NPSR1-deficient mice and WT littermates had no differences in the startle response on startle-only or prepulse trials.

**Spatial learning and memory in NPSR1-deficient mice—**Spatial learning and memory was tested using the Morris water maze (MWM). Two versions of the MWM test were used: cued platform, which relies on proximal cues, and the hidden platform, which relies on distal cues. No genotype differences were noted in locating the cued platform (not shown). During hidden platform testing, three different phases of learning were used in which the platform position was changed for each phase. In all 3 phases, there were no differences in the path length, latency, speed, or cumulative distance to the platform between NPSR1-deficient and WT littermates on platform trials. After each learning phase, one probe trial was given with the platform removed, and the animals started from a novel position to assess memory for the position of the platform. In the probe trial after reversal, average heading to target error demonstrated a significant genotype × sex effect ($F(1, 32) = 4.29$, $P < 0.05$). Male NPSR1-deficient mice had an increased average heading error compared with WT littermates (mean ± SEM: male NPSR1-deficient mice=82.8 ± 1.5, male WT mice=78.4 ± 1.4, n=8–9 each group); there were no differences noted among females. No other indexes of probe trial performance, such as platform crossovers, average distance to the target, percent time in target quadrant, percent distance in target quadrant, initial heading to target error after reversal showed genotype effects, nor were genotype differences found on probe trials given after acquisition or shift trials. Figure 3*D* shows path length per testing day in the acquisition phase (left) and average distance from the platform site (right) (the best index of memory performance (Maei et al. 2009) in the probe trial after acquisition training).

## Plasma corticosterone levels

Immediately on removal from locomotor testing with (+)-methamphetamine challenge (Fig. 4*A*) or 15 min after forced swim (Fig. 4*B*), plasma corticosterone was examined. The plasma corticosterone in the NPSR1-deficient mice did not differ from those in WT littermates.

## Plasma corticosterone and behavioral assessment in NPSR1-deficient mice following NPS stimulation

**NPSR1 is required for increased corticosterone induced by NPS—**Smith et al. (2006) reported that ICV NPS could activate the HPA axis and up-regulate plasma corticosterone. Here we first confirmed the dose-response effects of ICV NPS on plasma corticosterone 40 min after NPS ICV injection in WT mice. In male WT mice, ICV administration of 0.1, 1, or 10 nmol NPS caused a significant dose-dependent increase in plasma corticosterone 40 min after injection compared with aCSF ($F(3,39) = 10.7$, $P<0.0001$). Post hoc group comparisons showed that both the 1 and 10 nmol NPS groups had significantly increased corticosterone compared to aCSF controls (mean ± SEM (ng/ml):

aCSF = 95.3 ± 13.2; NPS 0.1 nmol = 86.2 ± 13.1; NPS 1 nmol = 136.2 ± 11.6; NPS 10 nmol = 179.8 ± 13.2; n=10–13 per group; aCSF vs. NPS 0.1 nmol = ns; aCSF vs. NPS 1 nmol = $p < 0.05$; aCSF vs. NPS 10 nmol = $p < 0.001$). Accordingly, the 10 nmol dose of NPS was used for examining if NPSR1 was involved in NPS-induced corticosterone release. There was no significant main effect of genotype on plasma corticosterone; the main effect of NPS was also not significant but showed a trend ($p < 0.06$), however, there was a significant genotype × NPS interaction ($F(1, 34) = 4.3$, $p < 0.05$). As can be seen in Fig. 4*C*, 10 nmol of NPS induced a significant increase in plasma corticosterone in NPS-treated WT mice ($p < 0.01$) but not in NPS-treated NPSR1-deficient mice.

**NPSR1 is required for NPS-induced hyperlocomotion and exploration—**To determine if NPSR1 was a non-redundant receptor for NPS in the brain, male NPSR1-deficient and WT mice were exposed to NPS by ICV injection and examined for locomotor activity. In order to identify the appropriate dose, a separate group of WT mice was tested first. ICV NPS in WT mice dose-dependently (0.1, 1, and 10 nmol) increased locomotor activity compared to mice receiving aCSF with the 1 nmol dose of NPS having the clearest effect (horizontal activity (mean ± SEM) over 60 min testing time in open-field test, aCSF=645.9 ± 45.5, NPS 0.1 nmol=1115.9 ± 41.2, NPS 1 nmol=1482.3 ± 44.0, NPS 10 nmol=1364.3 ± 38.0, n=6 per group). Accordingly, we used 1 nmol of NPS to examine the role of NPSR1 in NPS-induced hyperlocomotion in NPRS1-deficient and WT mice. Central administration of NPS (1 nmol) induced increased vertical (rearing) and horizontal activity (Fig. 5*A*, first two panels) and interacted with genotype for both vertical (genotype × NPS, $F(1, 36.7) = 25.77$, $P < 0.0001$) and horizontal activity (genotype × NPS, $F(1, 36) = 21.44$, $P < 0.0001$). In WT animals, ICV NPS (1 nmol) increased vertical and horizontal activity compared to animals given aCSF. In contrast, NPSR1-deficient mice exhibited complete insensitivity to NPS (Fig. 5*A*) relative to WT mice given NPS and instead performed similarly to NPSR1-deficient mice that were administered aCSF. In order to determine how long the NPS stimulation lasted, a separate group of C57BL/6 WT mice was administered ICV NPS (1 nmol) and observed for 3 h. NPS-stimulated animals showed increased vertical and horizontal activity as previously demonstrated (Fig. 5*B*). For vertical activity the effect peaked sharply at about 40 min then gradually tapered off showing an inflection at about 100 min. For horizontal activity the effect peaked almost immediately and then gradually tapered off over the course of the entire 180 min test session. For both measures, there was an NPS × interval interaction ($F(17,295) = 3.60$, $P < 0.0001$ and $F(17,297) = 1.91$, $P < 0.02$, respectively). With the exception of intervals 110 and 180 for vertical activity, NPS continued to significantly stimulate activity greater than aCSF alone at all intervals measured, whereas for horizontal activity all intervals were significantly different between NPS and aCSF.

**Impaired anxiolytic effect induced by NPS in NPSR1-deficient mice—**To determine if the anxiolytic effect of NPS was NPSR1-dependent, male NPSR1-deficient mice were analyzed for anxiety related behaviors in the elevated zero maze, light-dark exploration, and the aforementioned open-field test. A separate group of WT mice was used to identify the appropriate dose in the elevated zero maze and light-dark exploration tests. ICV NPS in WT mice dose-dependently (0.1, 1, and 10 nmol) decreased anxiety-like behaviors by increasing time in the open (time in the open area (mean ± SEM) in elevated zero maze, aCSF=34.8 ± 8, NPS 0.1 nmol=34.9 ± 6.6, NPS 1 nmol=108.5 ± 10.1, NPS 10 nmol=108.1 ± 17.1, n=5–6 per group; for horizontal activity in the light area (mean±SEM) in light-dark exploration test, aCSF=1014 ± 157, NPS 0.1 nmol=1179 ± 126, NPS 1 nmol=1912 ± 146, NPS 10 nmol=1491 ± 155, n=5–6 per group); 1 nmol of NPS had the clearest effect. A 1 nmol dose of NPS was applied to NPSR1-deficient and WT mice to examine the role of NPSR1 in the NPS-induced anxiolytic effect. In the elevated zero maze,

no effect of NPS was observed on latency to enter the open, although there was a genotype effect ($F(1, 39) = 17.06$, $P < 0.002$) that showed the NPSR1-deficient mice took longer to initially enter the open areas (latency to enter the open (s) (mean ± SEM): WT-aCSF=38.1 ± 10.3, KO-aCSF=61.3 ± 8.4, WT-NPS=24.5 ± 8.4, KO-NPS=76.0 ± 8.8, n=8–12 per group) . For time in the open (Fig. 5*C*, *first panel*), there was an effect of NPS ($F(1, 39) = 28.87$, $P < 0.0001$), genotype ($F(1, 39) = 22.85$, $P < 0.0001$), and the interaction of NPS × genotype ($F(1, 39) = 20.01$, $P < 0.0001$). Slice effect ANOVAs by drug showed a genotype effect in NPS ($F(1, 39) = 47.01$, $P < 0.0001$), and slice effect ANOVAs by gene showed a drug effect in WT animals ($F(1, 39) = 44.51$, $P < 0.0001$). The WT animals that were administered NPS had increased time in the open area compared to WT aCSF-administered animals, whereas no differences were noted between the NPSR1-deficient mice receiving NPS versus aCSF. NPS-treated NPSR1-deficient mice were insensitive to NPS stimulation which was seen in WT NPS-treated mice. Similarly for head dips (Fig. 5*C*, *second panel*), there was an NPS ($F(1, 39) = 16.65$, $P < 0.0002$), genotype ($F(1, 39) = 16.53$, $P < 0.0002$) and NPS × genotype effect ($F(1, 39) = 8.90$, $P < 0.005$). Slice effect ANOVAs by drug showed a genotype effect in NPS ($F(1, 39) = 27.28$, $P < 0.0001$), and slice effect ANOVAs by gene showed a drug effect in WT animals ($F(1, 39) = 22.90$, $P < 0.0001$). The WT mice given NPS showed increased head dips compared to WT mice given aCSF; whereas the NPSR1-deficient mice given NPS did not respond to NPS stimulation showing similar performance as the NPSR1-deficient mice given aCSF. In the open-field test (Fig. 5*A*, third panel) for the time in the center, there was a significant genotype × NPS interaction ($F(1, 36.2) = 9.04$, $P < 0.005$). ICV NPS increased time in the central zone in the WT mice compared with aCSF, but not in the NPSR1-deficient mice. No differences were observed in the NPSR1-deficient mice between those receiving NPS versus aCSF. Margin time was the exact opposite (not shown) showing that NPS-treated NPSR1-deficient mice to be insensitive to NPS compared to NPS-treated WT animals. In the light-dark exploration test for the transition between the light and dark areas (Fig. 5*C*, *third panel*), there was an effect of NPS ($F(1, 39) = 11.45$, $P < 0.002$) and the interaction of NPS × genotype ($F(1, 39) = 7.78$, $P < 0.008$). Slice effect ANOVAs by drug showed a genotype effect in NPS ($F(1, 39) = 8.54$, $P < 0.006$), and slice effect ANOVAs by gene showed a drug effect in WT animals ($F(1, 39) = 19.45$, $P < 0.0001$) (mean ± SEM: WT-aCSF=19 ± 5, KO-aCSF=27 ± 5, WT-NPS=50 ± 5, KO-NPS=30 ± 5, n=8–12 per group); For horizontal activity in the light area, there was only a significant effect of NPS ($F(1, 43) = 13.96$, $P < 0.0006$) (mean ± SEM: WT-aCSF=1228 ± 172, KO-aCSF=1657 ± 172, WT-NPS =2083 ± 158, KO-NPS=2049 ± 165). For horizontal activity in the dark area, there was an effect of genotype ($F(1, 39) = 4.50$, $P < 0.04$), but no effect of NPS or an NPS × genotype interaction (mean ± SEM: WT-aCSF=1857 ± 301, KO-aCSF=1724 ± 301, WT-NPS=2796 ± 275, KO-NPS=1695 ± 287). For time spent in the light area or in the dark area there was no effect of NPS or genotype or an NPS × genotype interaction (for time (s) in light area (mean ± SEM): WT-aCSF=260 ± 40, KO-aCSF=295 ± 40, WT-NPS=244 ± 37, KO-NPS=348 ± 38; for time(s) in dark area (mean ± SEM): WT-aCSF=340 ± 40, KO-aCSF=305 ± 40, WT-NPS=356 ± 37, KO-NPS=252 ± 38).

## Discussion

We assessed the functional role of NPSR1-deficiency in locomotion, stress reactivity, and learning and memory, and tested the role of NPSR1 in NPS-stimulated locomotion, anxiety, and HPA axis response through NPSR1 deletion. The principal finding in NPSR1-deficient mice was that males exhibited increased depression-like behavior in the forced swim test and reduced acoustic startle reactivity, both indicative of sensorimotor reactivity down-regulation to aversive stimuli. Why this effect was restricted only to males and was not seen in females is unknown, but it is of interest that an NPSR1 SNP ($Asn/Ile^{107}$) was associated with panic disorder in male Japanese patients (Okamura et al., 2007). After NPS ICV injection, NPSR1-deficient mice did not respond to NPS-induced stimulation during tests of

locomotion, anxiety, or corticosterone release compared with WT NPS-treated mice, identifying a non-redundant role of NPSR1 as the receptor for NPS. It should be noted that NPSR1-deficient mice were tested in a battery of tests to characterize their basic phenotype. Possible carry-over effects of one test on another may be a factor in influencing the observed effects, however, we arranged the tests in order from least to most apparent stress to minimize such influences and it is evident by the findings that the effects were highly specific and hence unlikely to be attributable to experience-related transference.

The FST and TST were used to evaluate depression-like behavior. The development of immobility disengages the animal from active coping when confronted with an inescapable stressor (Lucki et al. 2001) and these tests have proven predictive for antidepressant efficacy. NPSR1 deletion in and of itself did not affect locomotor activity or swimming in the MWM or TST immobility. These data suggest that the depressive phenotype in the FST in male NPSR1-deficient mice is not attributable to reduced locomotor activity or a generalized suppression effect. The FST and TST findings are not necessarily contradictory. Cryan et al. (2005) have reviewed the literature and found that some drugs and gene deletions show divergent outcomes on these two tests. While the reasons for such divergence on tests thought to assess similar functions remains unknown, divergence itself does not imply the presence of a false positive finding in the FST.

Elevated zero maze, light-dark exploration, open-field locomotor activity, and marble burying are the most frequently employed behavioral methods to determine anxiety states in response to novelty (Van Meer and Raber, 2005). Our data show that NPSR1-deficient mice did not have a changed response when evaluated in these tests indicating that absence of the receptor does not alter anxiety under basal conditions. Clinically, antidepressants are often effective in treating anxiety and it is well established that anxiety and mood disorders exhibit significant comorbidity. Yet the link between these functions remains poorly understood. The lack of anxiety differences in NPSR1-deficient mice may represent a mechanism with different effects on anxiety-like versus depression-like behaviors. In reviewing the literature, based on neurotransmitter changes and dysregulation of HPA axis in humans and animals, Boyer (2000) suggested that anxiety and depressive disorders showed differences in regulating the release of several peptides or hormones of the HPA axis except for increases corticotrophin-releasing factor (CRF) in the cerebrospinal fluid which was common across conditions. Another possibility for explaining this may be the compensatory effect of lifelong NPSR1 gene deletion. Thus, for future studies an antagonist specific for NPSR1 could be used to confirm the role of NPSR1 in anxiety and depression.

NPSR1 mRNA is expressed in the input and output pathways of the hippocampus, which is involved in regulation of learning and memory. Recently Han et al. (2009) reported that ICV administration of NPS facilitated spatial memory in the MWM without altering latency to the target or swimming speed. We also examined spatial learning and memory in NPSR1-defficient and WT mice using the MWM. Our data showed mild changes in NPSR1-deficient mice, suggesting that endogenous NPS may not modulate spatial learning and memory under basal conditions. Garau et al. (2009) examined fear conditioning in NPSR-deficient mice and showed differences in NPSR-deficient mice compared to WT mice. Factors resulting in these different results may be related to differences in endogenous NPS release under different testing conditions or different mechanisms between the two types of learning and memory processes.

When NPS was administered ICV, clear anxiolytic effects were induced that are consistent with recent data (Xu et al., 2004; Leonard et al., 2008). What is unique in the present experiment is the demonstration that the anxiolytic, locomotor facilitation, and corticosterone response to NPS are dependent on NPSR1 since NPSR1-deficient mice were

unchanged from aCSF-treated WT controls on these measures in contrast to the activating effects of NPS in WT mice. These data therefore provide independent evidence that NPSR1 is the primary and perhaps sole receptor mediating NPS effects on anxiety, locomotor activation, and a significant contributor to stress-induced corticosterone release. These findings are also consistent with other lines of evidence. For example, by reverse pharmacology, NPS has previously been suggested as the endogenous ligand for NPSR1 but whether other receptors existed was unclear. Several groups reported that compounds that bind to NPSR1 inhibit the stimulatory effect of NPS on locomotor activity (Okamura et al., 2008 and Guerrini et al., 2009) and the arousal promoting effect during the righting reflex recovery test (Camarda at al., 2009) but the specificity of these effects was not entirely clear since these antagonists could be binding to other yet unidentified receptors. By using NPSR1 gene targeting, the current experiment provides more specific evidence that NPSR1 is the receptor for NPS-induced locomotor activation and anxiolytic effects at least to the extent that is reflected by the tests used herein.

In the elevated zero maze, NPS-treated WT mice had increased time in open areas and head dips and shorter latencies to enter the first open quadrant, all consistent with a reduced anxiety phenotype. In addition, NPS-treated WT mice showed increased rearing (vertical activity) and central time activity, also consistent with reduced anxiety. Each of these NPS-induced effects was absent in NPSR1-deficient NPS-treated mice. We also examined the effect of NPS on light-dark exploration. At the beginning of the test we put the mice in the lighted area. NPS-treated mice showed increased horizontal activity in both light and dark areas and increased transitions between the light and dark sides. This pattern suggests the predominance of the locomotor activating effect of NPS rather than a specific anxiolytic effect. However, Leonard et al. (2008) have shown that the locomotor activating and anxiolytic effects of NPS can be distinguished by comparing the effects of NPS to the effects of the indirect dopaminergic agonist (+)-amphetamine and to the benzodiazepine type GABAergic anxiolytics. For example, they showed that (+)-amphetamine, while increasing locomotor activity does not increase open time in the elevated zero maze or increase punished crossings in the four-plate test, as does NPS. Therefore, locomotor activation per se is not a confounder when the two effects co-occur but originate from distinct processes. In addition, Leonard et al. (2008) showed that NPS inhibits rectal probe stress-induced hyperthermia, suggesting that the effects of NPS are selective. The weakness of the tests we used here to identify activity of NPSR1 in anxiety is that it is not completely excluded that the reduced anxiety phenotype in the elevated zero maze and open-field test observed in NPS-treated WT mice is partially due to the activation of general locomotion. Future experiments should test NPSR1-deficient NPS-treated mice using tests such as the four-plate test to further evaluate the role of NPSR1 in stress reactivity.

We also examined the effect of NPS on anxiety in the marble burying test (not presented) but saw no NPS-induced anxiolytic effect and hence had no basis for testing NPSR1-deficient mice with this procedure. This is in contrast to Xu et al. (2004) who reported that ICV NPS reduced marble burying. The reason for this discrepancy is not known but may be the result of methodological differences. Xu et al. (2004) tested the mice for 30 min whereas here, the mice were tested for 20 min. It is also unclear whether Xu et al. (2004) performed other tests before marble burying which could contribute to outcome differences. More importantly than this one difference is the fact that overall our anxiety test data are consistent with those of Xu et al. (2004) and the more recent data of Leonard et al. (2008), suggesting that the role of NPS in anxiety and locomotor stimulation are robust.

The HPA axis is activated in response to stressors, and when the stress effect is sufficient results in an increase in plasma corticosterone levels in rodents. Corticosterone regulates a variety of adaptations at the level of neuroendocrine, autonomic, immunological and

behavioral responses. A physical stressor (forced swim) and some drugs (methamphetamine) can activate the HPA axis (Müller et al., 2000 and Prickaerts et al., 2006). After forced swim or methamphetamine challenge and open-field exposure, NPSR1-deficient mice and WT littermates had similar corticosterone levels, suggesting that NPSR1 deletion did not disrupt HPA axis response mechanisms. Smith et al. (2006) reported that NPS stimulates the HPA axis and we showed this effect also. These data are consistent with the finding of Leonard et al. (2008) that ICV NPS attenuates stress-induced hyperthermia and demonstrates the importance of NPS in stress adaption as well as in anxiety.

Genetic variants of NPSR1 have been linked with inflammatory diseases, such as asthma and inflammatory bowel disease (Laitinen et al., 2004 and D'Amato et al., 2007). Our data suggest that a central mechanism in these inflammatory disorders may be operational. Indeed, stress is a known trigger for asthma and inflammatory bowel disease (Chen et al., 2007 and Santos et al., 2008).

In conclusion, the present findings provide the first direct evidence that NPSR1 is the dominant central receptor mediating NPS-induced locomotor activation, anxiolysis, and interaction with corticosterone release and supports the view expressed by Leonard et al. (2008) that the NPS-NPSR1 pathway represents a novel pharmacological target for therapeutic agents for the treatment of anxiety-related disorders.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

Beck B, Fernette B, Stricker-Krongrad A. Peptide S is a novel potent inhibitor of voluntary and fast-induced food intake in rats. Biochem Biophys Res Commun 2005;332:859–865. [PubMed: 15919054]

Bernier V, Stocco R, Bogusky MJ, Joyce JG, Parachoniak C, Grenier K, Arget M, Mathieu MC, O'Neill GP, Slipetz D, Crackower MA, Tan CM, Therien AG. Structure-function relationships in the neuropeptide S receptor: molecular consequences of the asthma-associated mutation N107I. J Biol Chem 2006;281:24704–24712. [PubMed: 16790440]

Boyer P. Do anxiety and depression have a common pathophysiological mechanism? Acta Psychiatr Scand Suppl 2000;406:24–29. [PubMed: 11131467]

Brunskill EW, Ehrman LA, Williams MT, Klanke J, Hammer D, Schaefer TL, Sah R, Dorn GW 2nd, Potter SS, Vorhees CV. Abnormal neurodevelopment, neurosignaling and behaviour in Npas3-deficient mice. Eur J Neurosci 2005;22:1265–1276. [PubMed: 16190882]

Camarda V, Rizzi A, Ruzza C, Zucchini S, Marzola G, Marzola E, Guerrini R, Salvadori S, Reinscheid RK, Regoli D, Calò G. In vitro and in vivo pharmacological characterization of the neuropeptide s receptor antagonist [D-Cys(tBu)5]neuropeptide S. J Pharmacol Exp Ther 2009;328:549–555. [PubMed: 18971372]

Chen E, Miller GE. Stress and inflammation in exacerbations of asthma. Brain Behav Immu 2007;21:993–999.

Cryan JF, Kelly PH, Neijt HC, Sansig G, Flor PJ, van Der Putten H. Antidepressant and anxiolytic-like effects in mice lacking the group III metabotropic glutamate receptor mGluR7. Eur J Neurosci 2003;17:2409–2417. [PubMed: 12814372]

Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 2005;29:571–625. [PubMed: 15890404]

Crowe RR, Goedken R, Samuelson S, Wilson R, Nelson J, Noyes R Jr. Genomewide survey of panic disorder. Am J Med Genet 2001;105:105–109. [PubMed: 11424978]

D'Amato M, et al. Neuropeptide s receptor 1 gene polymorphism is associated with susceptibility to inflammatory bowel disease. Gastroenterology 2007;133:808–817. [PubMed: 17854592]

Garau C, Okamura N, Duangdao DM, Reinscheid RK. The role of neuropeptide S and its receptor in fear memory consolidation and extinction. 2009 Society for Neuroscience meeting abstract. 2009

Guerrini R, Camarda V, Trapella C, Calò G, Rizzi A, Ruzza C, Fiorini S, Marzola E, Reinscheid RK, Regoli D, Salvadori S. Synthesis and biological activity of human neuropeptide S analogues modified in position 5: identification of potent and pure neuropeptide S receptor antagonists. J Med Chem 2009;52:524–529. [PubMed: 19113861]

Han RW, Yin XQ, Chang M, Peng YL, Li W, Wang R. Neuropeptide S facilitates spatial memory and mitigates spatial memory impairment induced by N-methyl-D-aspartate receptor antagonist in mice. Neurosci Lett 2009;455:74–77. [PubMed: 19429110]

Knowles JA, et al. Results of a genome-wide genetic screen for panic disorder. Am J Med Genet 1998;81:139–147. [PubMed: 9613853]

Laitinen T, et al. Characterization of a common susceptibility locus for asthma-related traits. Science 2004;304:300–304. [PubMed: 15073379]

Leonard SK, Dwyer JM, Sukoff Rizzo SJ, Platt B, Logue SF, Neal SJ, Malberg JE, Beyer CE, Schechter LE, Rosenzweig-Lipson S, Ring RH. Pharmacology of neuropeptide S in mice: therapeutic relevance to anxiety disorders. Psychopharmacology (Berl) 2008;197:601–611. [PubMed: 18311561]

Logue MW, Vieland VJ, Goedken RJ, Crowe RR. Bayesian analysis of a previously published genome screen for panic disorder reveals new and compelling evidence for linkage to chromosome 7. Am J Med Genet B Neuropsychiatr Genet 2003;121B:95–99. [PubMed: 12898582]

Lucki I, Dalvi A, Mayorga AJ. Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. Psychopharmacology (Berl) 2001;155:315–322. [PubMed: 11432695]

Maei HR, Zaslavsky K, Teixeira CM, Frankland PW. What is the Most Sensitive Measure of Water Maze Probe Test Performance? Front Integr Neurosci 2009;3:4–12. [PubMed: 19404412]

Müller MB, Landgraf R, Preil J, Sillaber I, Kresse AE, Keck ME, Zimmermann S, Holsboer F, Wurst W. Selective activation of the hypothalamic vasopressinergic system in mice deficient for the corticotropin-releasing hormone receptor 1 is dependent on glucocorticoids. Endocrinology 2000;141:4262–4269. [PubMed: 11089561]

Niimi M. Centrally administered neuropeptide S activates orexin-containing neurons in the hypothalamus and stimulates feeding in rats. Endocrine 2006;30:75–79. [PubMed: 17185795]

Okamura N, Hashimoto K, Iyo M, Shimizu E, Dempfle A, Friedel S, Reinscheid RK. Gender-specific association of a functional coding polymorphism in the Neuropeptide S receptor gene with panic disorder but not with schizophrenia or attention-deficit/hyperactivity disorder. Prog Neuropsychopharmacol Biol Psychiatry 2007;31:1444–1448. [PubMed: 17669576]

Okamura N, Habay SA, Zeng J, Chamberlin AR, Reinscheid RK. Synthesis and pharmacological in vitro and in vivo profile of 3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (SHA 68), a selective antagonist of the neuropeptide S receptor. J Pharmacol Exp Ther 2008;325:893–901. [PubMed: 18337476]

Pan D, Sciascia A, Vorhees CV, Williams MT. Progression of multiple behavioral deficits with various ages of onset in a murine model of Hurler syndrome. Brain Res 2008;1188:241–253. [PubMed: 18022143]

Paxinos, G.; Franklin, KB. The Mouse Brain in Stereotaxic Coordinates (Compact 2nd edition). Elsevier Academic Press; 2004.

Porsolt RD, Lepichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. Nature 1977a;266:730–732. [PubMed: 559941]

Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 1977b;229:327–336. [PubMed: 596982]

Prickaerts J, Moechars D, Cryns K, Lenaerts I, van Craenendonck H, Goris I, Daneels G, Bouwknecht JA, Steckler T. Transgenic mice overexpressing glycogen synthase kinase 3beta: a putative model of hyperactivity and mania. J Neurosci 2006;26:9022–9029. [PubMed: 16943560]

Reinscheid RK, Xu YL, Okamura N, Zeng J, Chung S, Pai R, Wang Z, Civelli O. Pharmacological characterization of human and murine neuropeptide s receptor variants. J Pharmacol Exp Ther 2005;315:1338–1345. [PubMed: 16144971]

Rizzi A, Vergura R, Marzola G, Ruzza C, Guerrini R, Salvadori S, Regoli D, Calo G. Neuropeptide S is a stimulatory anxiolytic agent: a behavioural study in mice. Br J Pharmacol 2008;154:471–479. [PubMed: 18376418]

Santos J, Alonso C, Vicario M, Ramos L, Lobo B, Malagelada JR. Neuropharmacology of stress-induced mucosal inflammation: implications for inflammatory bowel disease and irritable bowel syndrome. Curr Mol Med 2008;8:258–273. [PubMed: 18537634]

Schramm NL, McDonald MP, Limbird LE. The alpha (2a)-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety. J Neurosci 2001;21:4875–4882. [PubMed: 11425914]

Shepherd JK, Grewal SS, Fletcher A, Bill DJ, Dourish CT. Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. Psychopharmacology (Berl) 1994;116:56–64. [PubMed: 7862931]

Smith KL, Patterson M, Dhillo WS, Patel SR, Semjonous NM, Gardiner JV, Ghatei MA, Bloom SR. Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake. Endocrinology 2006;147:3510–3518. [PubMed: 16574794]

Steele-Perkins G, Plachez C, Butz KG, Yang G, Bachurski CJ, Kinsman SL, Litwack ED, Richards LJ, Gronostajski RM. The transcription factor gene Nfib is essential for both lung maturation and brain development. Mol Cell Biol 2005;25:685–698. [PubMed: 15632069]

Valenzuela DM, et al. High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. Nat Biotechnol 2003;21:652–659. [PubMed: 12730667]

Van Meer P, Raber J. Mouse behavioural analysis in systems biology. Biochem J 2005;389:593–610. [PubMed: 16035954]

Vendelin J, Pulkkinen V, Rehn M, Pirskanen A, Räisänen-Sokolowski A, Laitinen A, Laitinen LA, Kere J, Laitinen T. Characterization of GPRA, a novel G protein-coupled receptor related to asthma. Am J Respir Cell Mol Biol 2005;33:262–270. [PubMed: 15947423]

Vitale G, Filaferro M, Ruggieri V, Pennella S, Frigeri C, Rizzi A, Guerrini R, Calò G. Anxiolytic-like effect of neuropeptide S in the rat defensive burying. Peptides 2008;29:2286–2291. [PubMed: 18793688]

Vorhees CV, Williams MT. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 2006;1:848–858. [PubMed: 17406317]

Williams MT, Moran MS, Vorhees CV. Refining the critical period for methamphetamine-induced spatial deficits in the Morris water maze. Psychopharmacology (Berl) 2003;168:329–338. [PubMed: 12684734]

Xu YL, Reinscheid RK, Huitron-Resendiz S, Clark SD, Wang Z, Lin SH, Brucher FA, Zeng J, Ly NK, Henriksen SJ, de Lecea L, Civelli O. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. Neuron 2004;43:487–497. [PubMed: 15312648]

Xu YL, Gall CM, Jackson VR, Civelli O, Reinscheid RK. Distribution of neuropeptide S receptor mRNA and neurochemical characteristics of neuropeptide S-expressing neurons in the rat brain. J Comp Neurol 2007;500:84–102. [PubMed: 17099900]

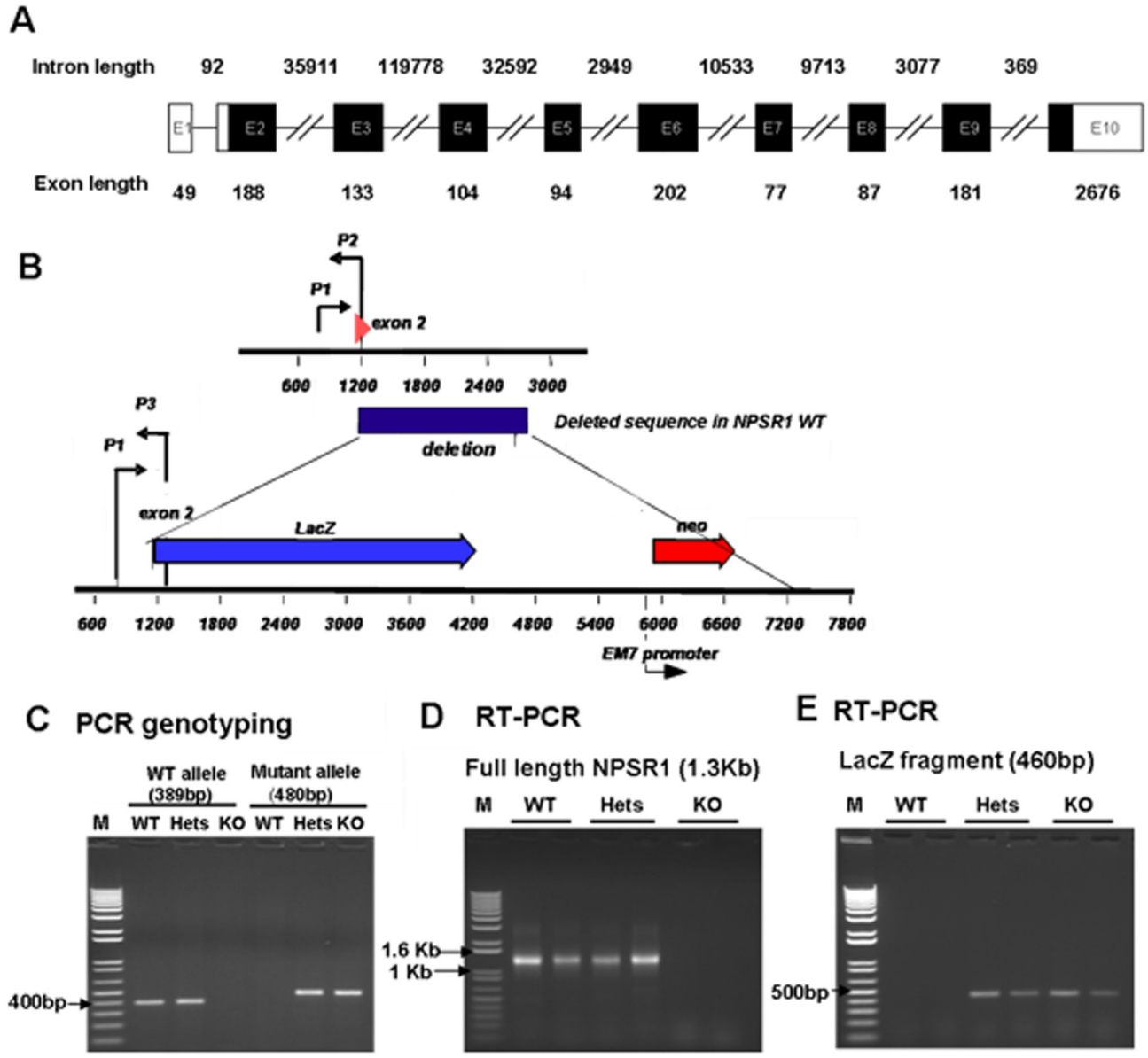


**Fig. 1.**

Generation of NPSR1 gene-targeted mice. (***A***) Schematic representation of the genomic organization of murine NPSR1 is shown according to the GenBank (NC_000075) sequence. Exons are depicted as boxes and introns as lines. Coding regions of exons are shown as shaded boxes. The length of the exon and intron are marked. (***B***) Strategy for disruption of the NPSR1 gene. Most of exon 2 and part of intron 2 were replaced with the coding sequence of LacZ and a neo-selection cassette. Primers for PCR genotyping are also shown. (***C***) PCR analysis of tail genomic DNA using primers specific for the WT (+/+) allele (P1 and P2) and for the recombinant mutant allele (P1 and P3). (***D*** and ***E***) RT-PCR to demonstrate the absence of full length NPSR1 transcripts in the brain from NPSR1-deficient (KO) mice (***D***) and the expression of LacZ in the brain of NPSR1 heterozygous and deficient (KO) mice (***E***).

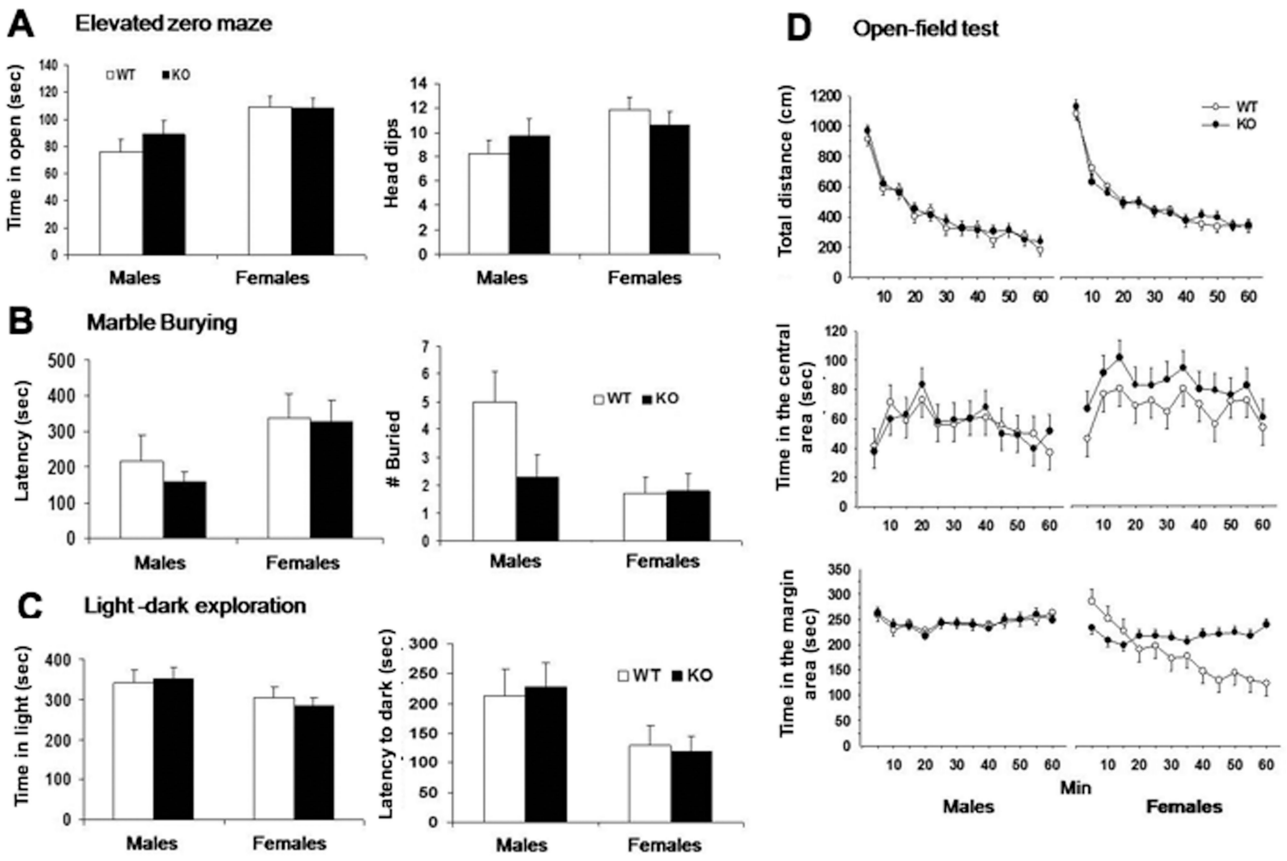


**Fig. 2.**

Anxiety-like behaviors in NPSR1-deficient mice. (***A***) Elevated zero maze: time in open areas and number of head dips in NPSR1-deficient mice (male, n=17; female, n=21) and WT littermates (male, n=16; female, n=21). (***B***) Marble burying test: latency to bury marbles and number of buried marbles in NPSR1-deficient mice (male, n=17; female, n=21)) and WT littermates (male, n=16; female, n=21) in defensive object burying test. (***C***) Light-dark exploration: time in light area and latency to dark side entry in NPSR1-deficient mice (male, n=17; female, n=23) and WT littermates (male, n=15; female, n=22). (***D***) Open-field: total distance, time in the central area, and time in the margin area of NPSR1-deficient mice (male, n=17; female, n=23) and WT littermates (male, n=15; female, n=22) were analyzed in the locomotor activity test and plotted in 5 min intervals. Values are reported as mean ± SEM.

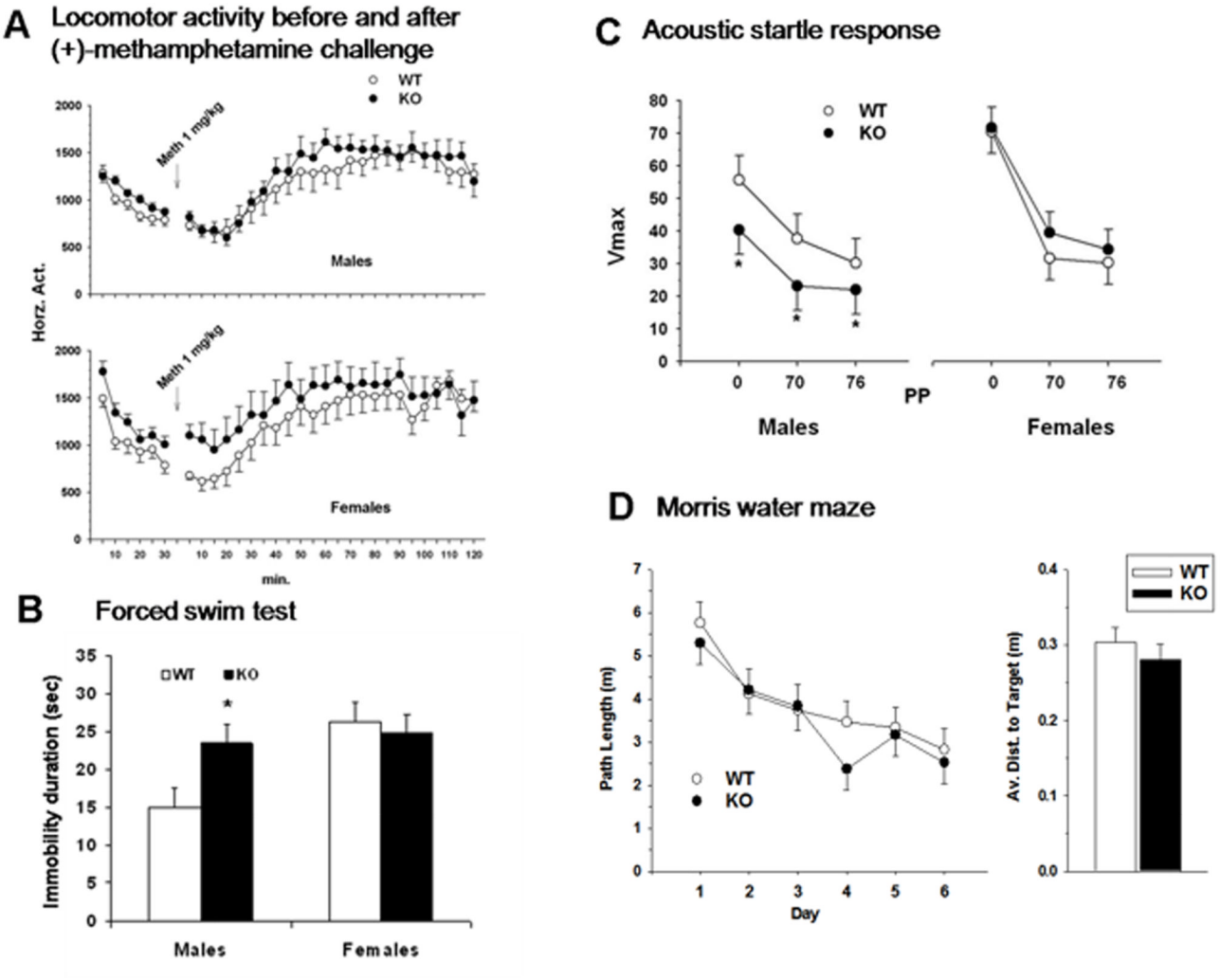


**Fig. 3.**

Methamphetamine regulated locomotion, depression-like behavior, acoustic startle response, and spatial learning and memory in NPSR1-deficient mice. (***A***) Open-field locomotor activity of NPSR1-deficient mice (male, n=10; female, n=10) and WT littermates (male, n=11; female, n=10) was evaluated before and after s.c. injection of 1 mg/kg (+)-methamphetamine. NPSR1 gene deletion did not affect methamphetamine modulated locomotor activity. (***B***) Forced swim test: immobility duration in NPSR1-deficient mice (male, n=21; female, n=19) compared with WT littermates (male, n=18; female, n=19). Values shown here were the averages of immobility duration per minute for last 6 min of the 10 min test session. Male NPSR1-deficient mice showed significantly increased immobility compared to male WT littermates. (***C***) Acoustic startle responses on startle-only trials and on trials with 70 or 76 dB prepulse (PP) intensities in NPSR1-deficient mice (male, n=15; female, n=21) compared with WT littermates (male, n=14; female, n=21). Male NPSR1-deficient mice had decreased acoustic startle responses on all trials compared to male WT littermates, but no change in prepulse inhibition of the startle response. (***D***) Morris water maze: Male NPSR1-deficient mice (male, n=9) showed similar path length in the acquisition phase learning trials (left) and average distance from the platform site on the probe trial after acquisition training (right) on hidden platform trials compared to male WT littermates (male,

n=8). Values are reported as mean ± SEM. *P≤0.05 compared with sex and age matched WT littermates.

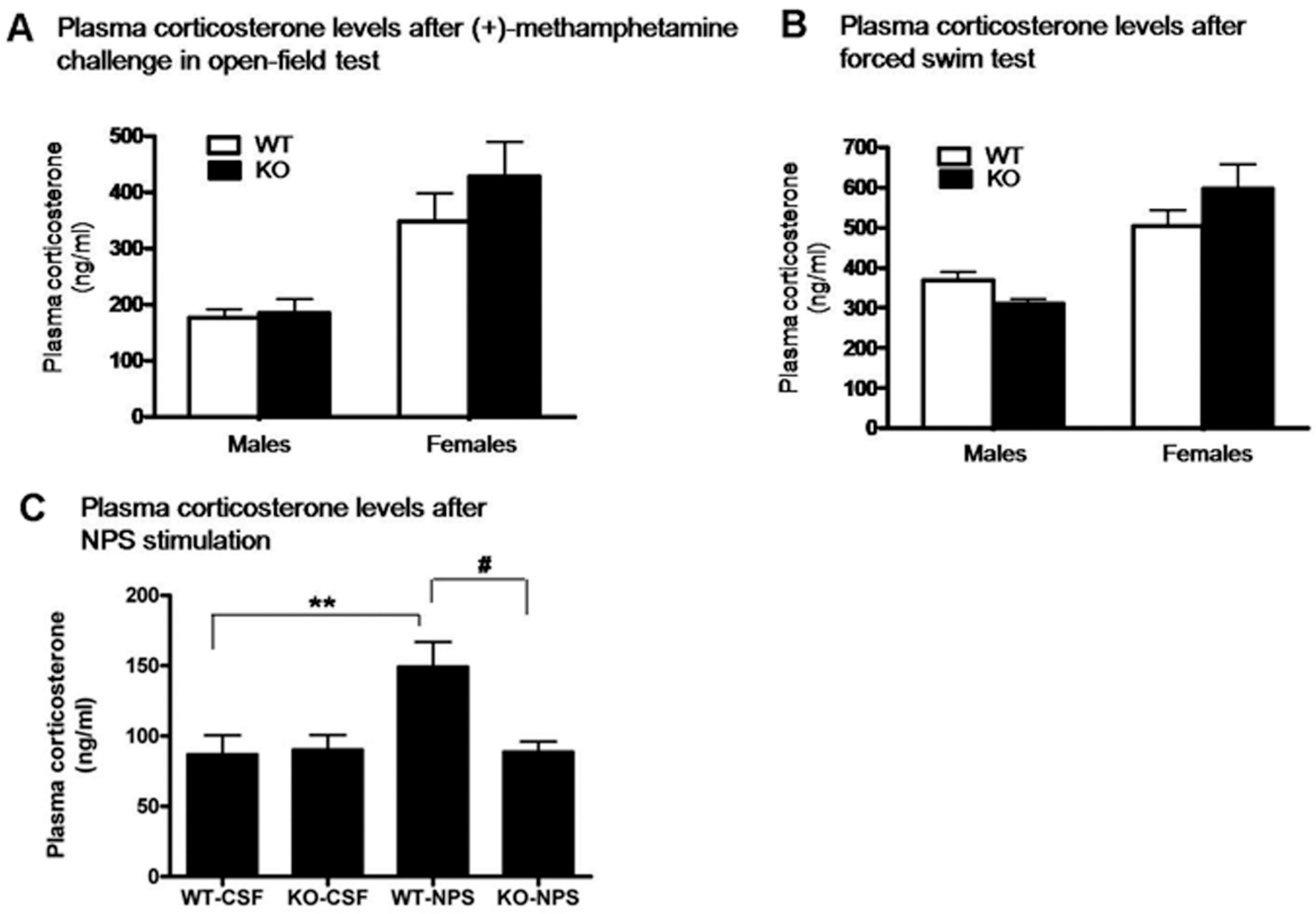


**Fig. 4.**

Plasma corticosterone (***A***) 2 hours after 1 mg/kg (+)-methamphetamine challenge in open-field test, the plasma corticosterone levels in NPSR1-deficient mice (male, n=10; female, n=10) did not differ from those in WT littermates (male, n=10; female, n=10). (***B***) 15 min after forced swim test, NPSR1-deficient mice (male, n=10; female, n=10) had similar levels of plasma corticosterone to WT littermates (male, n=10; female, n=10). (***C***) Plasma corticosterone levels in NPSR1-deficient and WT mice 40 min after 10 nmol NPS ICV injection (WT-CSF, aCSF treated WT mice, n=8; KO-CSF, aCSF treated NPSR1-deficient mice, n=8; WT-NPS, NPS treated WT mice, n=13; KO-NPS, NPS treated NPSR1-deficient mice, n=10). NPSR1-deficient mice receiving 10 nmol ICV NPS did not respond to NPS compared to WT mice given NPS. **P < 0.01 for WT-NPS vs. WT-CSF; $^{\#}$P < 0.05 for WT-NPS vs. KO-NPS. Values are reported as mean ± SEM.

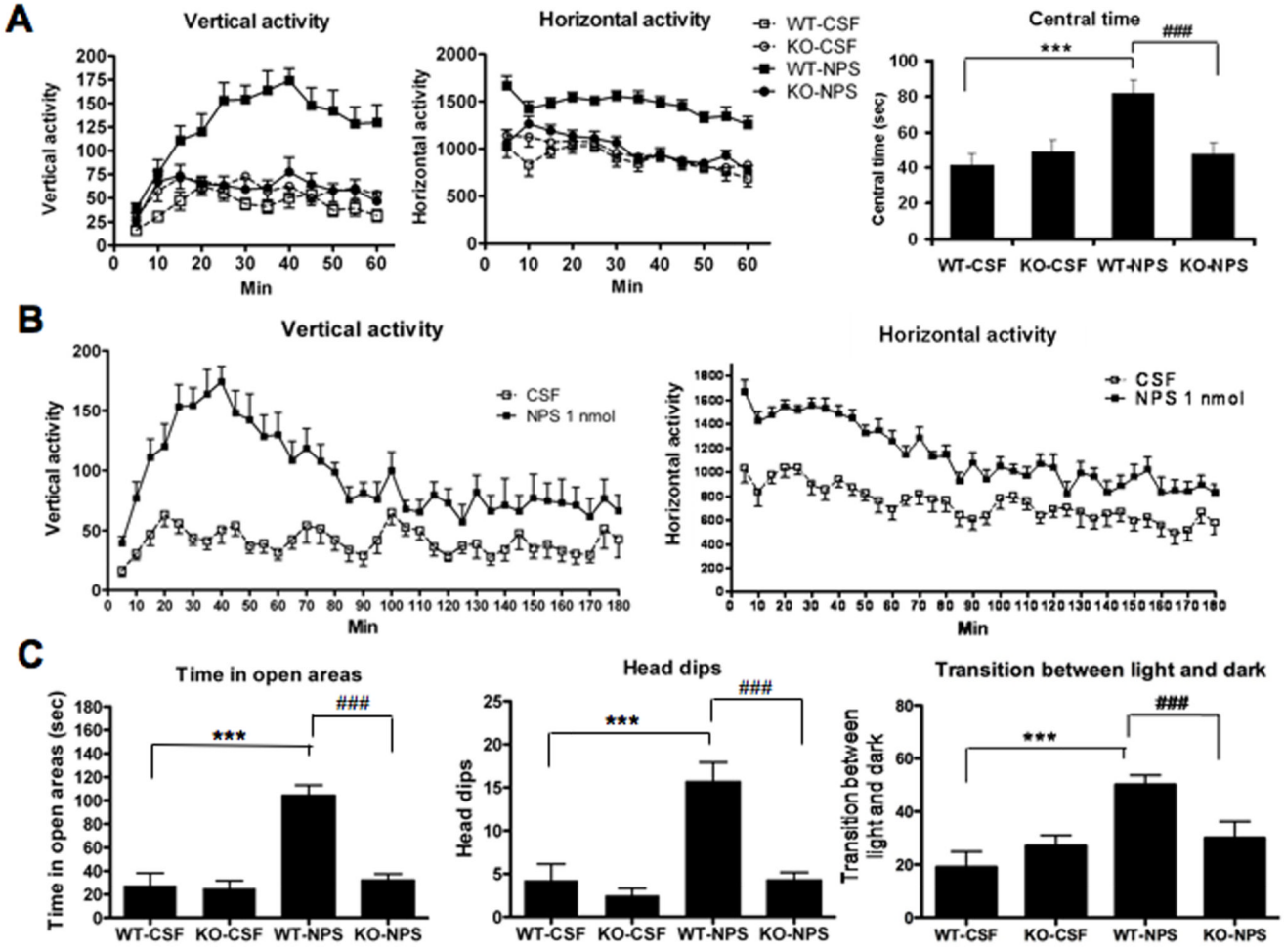


**Fig. 5.**

NPSR1 is required for NPS-induced hyperlocomotion, exploration, and anxiolytic-like effects. (***A***) Open-field: ICV NPS (1 nmol)-treated WT mice had hyperlocomotion, increased exploratory activity, and increased time spent in central area and NPSR1-deficient mice exhibited complete insensitivity to NPS relative to WT mice given NPS (WT-CSF, aCSF treated WT mice, n=10; KO-CSF, aCSF treated NPSR1-deficient mice, n=10; WT-NPS, NPS treated WT mice, n=9; KO-NPS, NPS treated NPSR1-deficient mice, n=11). (***B***) NPS (1 nmol)-induced hyperlocomotion and increased exploratory activity in WT mice was observed in open-field test for 3 hours (CSF, n=11; NPS 1 nmol, n=11). (***C***) Time in open areas and head dips in the elevated zero maze test (WT-CSF, aCSF treated WT mice, n=8; KO-CSF, aCSF treated NPSR1-deficient mice, n=12; WT-NPS, NPS treated WT mice, n=12; KO-NPS, NPS treated NPSR1-deficient mice, n=11), and transitions between light and dark area in the light-dark exploration test (WT-CSF, aCSF treated WT mice, n=10; KO-CSF, aCSF treated NPSR1-deficient mice, n=10; WT-NPS, NPS treated WT mice, n=12; KO-NPS, NPS treated NPSR1-deficient mice, n=11) in WT and NPSR1-deficient mice after 1 nmol NPS ICV injection. ***P < 0.001 for WT-NPS vs. WT-CSF; $^{###}$P < 0.001 for WT-NPS vs. KO-NPS. Values are reported as mean ± SEM.