

## Neuropeptide S Encodes Stimulus Salience in the Paraventricular Thalamus

Celia Garau,<sup>a†</sup> Xiaobin Liu,<sup>a‡</sup> Girolamo' Calo,<sup>b</sup> Stefan Schulz<sup>c</sup> and Rainer K. Reinscheid<sup>c\*</sup>

<sup>a</sup> Department of Pharmaceutical Sciences, University of California Irvine, Irvine, CA 92617, USA

<sup>b</sup> Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Italy

<sup>c</sup> Institute of Pharmacology and Toxicology, Friedrich-Schiller University, Jena, Germany

**Abstract**—Evaluation of stimulus salience is critical for any higher organism, as it allows for prioritizing of vital information, preparation of responses, and formation of valuable memory. The paraventricular nucleus of the thalamus (PVT) has recently been identified as an integrator of stimulus salience but the neurochemical basis and afferent input regarding salience signaling have remained elusive. Here we report that neuropeptide S (NPS) signaling in the PVT is necessary for stimulus salience encoding, including aversive, neutral and reinforcing sensory input. Taking advantage of a striking deficit of both NPS receptor (NPSR1) and NPS precursor knockout mice in fear extinction or novel object memory formation, we demonstrate that intra-PVT injections of NPS can rescue the phenotype in NPS precursor knockout mice by increasing the salience of otherwise low-intensity stimuli, while intra-PVT injections of NPSR1 antagonist in wild type mice partially replicates the knockout phenotype. The PVT appears to provide stimulus salience encoding in a dose- and NPS-dependent manner. PVT NPSR1 neurons recruit the nucleus accumbens shell and structures in the prefrontal cortex and amygdala, which were previously linked to the brain salience network. Overall, these results demonstrate that stimulus salience encoding is critically associated with NPS activity in the PVT. © 2022 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

**Key words:** paraventricular thalamic nucleus, salience, fear extinction, memory, neuropeptide S, GPCR.

### INTRODUCTION

During processing of sensory input and encoding of memories, humans and animals are evaluating the importance of their content. As a basic cognitive activity, stimulus salience detection influences a multitude of brain functions that involve information processing, response execution, and subsequent memory formation. On the other hand, dysfunctions in salience processing might be a contributing factor in disorders involving associative memory formation or sensory information filtering. For example, it has been suggested that over-interpretation of aversive memories (i.e. “catastrophizing”) can contribute to the development of panic disorder or phobias (Reiss and McNally, 1985;

Clark, 1986; Bouton et al., 2001). Similarly, excessive stimulus evaluation during traumatic events can be a contributing factor in the etiology of post-traumatic stress disorder (PTSD) and individual differences in salience processing might underlie individual vulnerability (Brewin, 2001; Layton and Krikorian, 2002). Different perception of reward salience may also determine substance abuse vulnerability and subsequent relapse probability (Berridge, 2007; Robinson and Berridge, 2008; Robinson and Flagel, 2009; Flagel et al., 2010, 2008). Furthermore, impaired salience interpretation may compromise an individual's ability to prioritize between competing sensory inputs, as seen in schizophrenic patients (Kapur, 2003; Kapur et al., 2005).

The paraventricular thalamic nucleus (PVT) has recently been identified as a critical anatomical substrate involved in stimulus salience processing. PVT glutamatergic principal neurons regulate dynamic stimulus salience processing required for associative learning (Zhu et al., 2018) and were found to promote wakefulness and arousal as a state of heightened sensory alertness (Ren et al., 2018). PVT neurons also appear to attribute incentive valence to reinforcing stimuli (Haight et al., 2015; Campus et al., 2019; Haight et al., 2020) and control behavior during motivational conflict (Choi

\*Corresponding author. Address: Institute of Pharmacology and Toxicology, University Hospital Jena, Drackendorfer Str. 1, D-07747 Jena, Germany.

E-mail address: [rainer.reinscheid@med.uni-jena.de](mailto:rainer.reinscheid@med.uni-jena.de) (R. K. Reinscheid).

† Current address: Department of Neuroscience, Psychology and Behaviour, University of Leicester, Leicester, UK.

‡ Current address: Department of Pharmaceutical Science, University of North Texas Health Sciences Center, Fort Worth, TX, USA. **Abbreviations:** CR, conditioned response; LC, locus coeruleus; NPS, neuropeptide S; NPSR1, neuropeptide S receptor; PVT, paraventricular thalamic nucleus.

et al., 2019). Within the PVT, at least two functionally distinct neuronal clusters seem to modulate valence and arousal in a bidirectional manner. Neurons with highest density in the posterior PVT are activated by salient aversive stimuli, whereas a second group with highest density towards the anterior PVT is inhibited by arousal and salient stimuli (Gao et al., 2020). These neuronal populations also mediate passive vs. active defensive behaviors, respectively (Ma et al., 2021). While orexin/hypocretin input to PVT glutamatergic neurons has been associated with arousal and wakefulness (Ren et al., 2018), the afferent input and neurochemical basis of stimulus salience processing in the PVT remain elusive.

The PVT receives the highest density of neuropeptide S (NPS) projections (Liu et al., 2011) and contains one of the biggest clusters of NPS receptor (NPSR1)-expressing neurons in the mouse or rat brain (Xu et al., 2004, 2007; Clark et al., 2011). In the mouse brain, NPS is produced in only two brainstem nuclei, the peri-coerulear region (periLC) and the lateral parabrachial (LPB) nucleus (Clark et al., 2011; Liu et al., 2011). Previous retrograde tracing studies have identified neuronal projections from the LC area as well as the LPB area to the PVT (Li and Kirouac, 2012; Liang et al., 2016), however, it is currently unknown which of the two brainstem nuclei is the main source of NPS innervation of the PVT. In preclinical animal models, post-training administrations of NPS enhance memory consolidation by interacting with noradrenergic systems, for both aversive (inhibitory avoidance) and neutral (novel object recognition) memory contents (Okamura et al., 2011). Conversely, NPSR1 knockout mice (Okamura et al., 2011) and NPS precursor knockout mice (Liu et al., 2017) display significant deficits in those memory paradigms, indicating that the endogenous NPS system is required for efficient memory processing. In light of the anatomical and behavioral evidence we hypothesized that these and other NPS-mediated physiological effects might as well be interpreted as NPS-dependent enhancement of stimulus salience. For example, central NPS administration was reported to produce arousal (Xu et al., 2004; Adori et al., 2016), increased wakefulness (Xu et al., 2004; Camarda et al., 2009), reduced anxiety (Xu et al., 2004; Leonard et al., 2008; Rizzi et al., 2008; Pulga et al., 2012; Wegener et al., 2012; Cohen et al., 2018), reinstatement of drug-seeking behaviors (Badia-Elder et al., 2008; Cannella et al., 2009, 2013; Pañeda et al., 2009; Kallupi et al., 2010; Ruggeri et al., 2010), and reversal of psychosis-like symptoms produced by NMDA receptor antagonists (Okamura et al., 2010).

The present study, therefore, attempts to establish a unifying mechanistic explanation for these apparently unrelated physiological effects, by demonstrating that NPS acts as a molecular salience regulator in the brain. To test this hypothesis, we used pharmacological, anatomical, and genetic approaches to assess the contribution of NPS signaling for stimulus salience encoding in animal models and identify neuronal substrates of PVT NPSR1 efferents. We discuss the current findings in view of a functional polymorphism found in the human NPSR1 gene (Okamura et al.,

2007) and its potential evolutionary implications for human salience perception.

## EXPERIMENTAL PROCEDURES

### Animals and surgery

Male mice (8–12 weeks) were used for all experiments. All knockout mice were derived from stable heterozygous breeding pairs. C57Bl/6J mice were obtained from the National Cancer Institute (Frederick, MD, USA). NPSR1 knockout mice were generated as described before and were on a 129S6/SvEvTac background (Duangdao et al., 2009). NPS precursor knockout mice were generated as described (Liu et al., 2017) and backcrossed for > 10 generations to C57Bl/6J. For guide cannula implantations, mice were anesthetized by intraperitoneal injection with a mixture of ketamine/xylazine (100 mg/kg; 5 mg/kg). Guide cannula (O.D., 0.48 mm; I.D., 0.34 mm; length, 3.5 mm; Plastics One, Roanoke, VA) were inserted by stereotaxic surgery and attached to the skull by dental cement. Stereotaxic coordinates (Franklin and Paxinos, 2007) for PVT were as follows:  $-1.0$  mm AP,  $+0.55$  mm ML,  $-3.0$  mm DV with a  $10^\circ$  angle toward midline to avoid damage to the superior sagittal sinus. For stereotaxic injections into the 3rd ventricle above the PVT the cannula tip was placed at  $-2.8$  mm DV. Mice were allowed to recover from surgery for at least 7 days. Injections were targeting the anterior-to-medial PVT (AP from bregma  $-0.5$  to  $-1.1$  mm), as this is also the part of the PVT with highest NPSR1 expression and highest density of NPS-immunopositive fiber projections in the mouse brain (Clark et al., 2011; Liu et al., 2011). At the end of experiments, cannula placement was checked macroscopically by visual inspection and scored as either “correct” or “incorrect”. Briefly, mice were sacrificed by decapitation at the end of experiments. Skullcaps were carefully removed while leaving the guide cannula inserted in the brain. Using a fresh scalpel, the brain was cut coronally along the guide cannula and the position of the previously inserted injection needle tip was inspected visually. If the injection had occurred outside the PVT, the animal was excluded from analysis. In the anterior-to-medial part of the PVT, the structure is easily distinguished from the bilateral anterodorsal thalamic nuclei or more ventrally located structures.

Implanted animals were individually housed and kept under controlled conditions ( $21^\circ\text{C}$ , 50–60% relative humidity, 12:12 h light–dark cycle with lights on at 7 am) with free access to food and water. All other mice were group-housed (2–5 animals/cage). All experiments were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council, 2003) and approved by the local Institutional Animal Care and Use Committee at the University of California, Irvine (protocol 2005-2582). Studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny et al., 2010). A total of 245 mice were used in the experiments, with 14 mice being excluded due to cannula misplacement.

### Drug administration

NPS was custom synthesized at the Peptide Array Facility of the Brain Research Centre, University of British Columbia (Vancouver, BC, Canada), and stock solutions were dissolved in water. For central administration, NPS was dissolved in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich). The peptidergic NPSR1 antagonist [D-Cys(tBut)<sup>5</sup>]-NPS was synthesized at the University of Ferrara, Italy, as described (Camarda et al., 2009) and dissolved in PBS, 0.1% BSA. Microinjections of NPS (1 pmole) or [D-Cys(tBut)<sup>5</sup>]-NPS (100 pmole) were carried out using an automated syringe pump (CMA; Harvard Apparatus, Holliston, MA, USA) equipped with a 5  $\mu$ l Hamilton gas-tight syringe. Drug concentrations for intra-PVT microinjections were 100-fold less than the minimally-active concentration after intracerebroventricular administration (Xu et al., 2004; Camarda et al., 2009). Drug solutions were delivered at a total volume of 0.2  $\mu$ l and a speed of 0.5  $\mu$ l/min. Injection cannulae were left in place for an additional minute to prevent backflow. After injections, guide cannulae were capped with a dummy cannula and mice were returned to their home cages. For conditioned place preference, morphine sulfate (Sigma-Aldrich) was dissolved in 0.9% sterile saline and injected intraperitoneally (i.p.) at a total volume of 100  $\mu$ l.

### Auditory fear conditioning

Auditory fear conditioning, extinction, recall and renewal of extinction was carried out as described before (Jüngling et al., 2008) (Suppl. Fig. S1) using an automated fear conditioning system (TSE, Bad Homburg, Germany). Briefly, single-housed mice were adapted (A) on the first day by two presentations of 6 CS– (2.5 kHz tone, 85 dB, duration 10 s, interstimulus interval 20 s; intertrial interval 6 h). On day 2, fear conditioning (FC) was initiated by two exposures of three randomly presented CS+ (10 kHz tone, 85 dB, 10 s, randomized interstimulus interval 10–30 s; intertrial interval 6 h), each of which was terminated by US (scrambled foot shock of 0.2–1.0 mA, duration 1 s). 24 h later (day 3), trained mice were transferred to the retrieval environment (novel visual context) and habituated for 30 min, before initiating six retrieval sessions (R1–R6) for extinction training (intertrial interval 30 min), each consisting of a set of 4 CS– followed by a set of 4 CS+ (stimulus duration 10 s, interstimulus interval 20 s). On day 4, recall of extinction (E1, E2) was tested by exposing mice to two sessions (30 min apart) of one set of 4 CS– followed 40 s later by a set of 4 CS+ (stimulus duration 10 s, interstimulus interval 20 s). For renewal of extinct fear (RN), mice were returned to the initial shock context and received a set of 4 CS– and 40 s later a set of 4 CS+. Drugs were administered 10 min after the last training session (FC) to ensure a drug-free state of the animals during acquisition or recall 24 h later. In some experiments, groups of naive mice were conditioned to the auditory cue with increasing foot shock intensities (0.2–1.0 mA/1 s) and then tested in a single recall session 24 h later in order to assess initial

memory strength. Conditioned freezing to the shock-paired conditioning tone (CS + ) was recorded and corrected for freezing to a non-paired neutral tone (CS–).

### Morris water maze

Spatial learning in the Morris water maze (MWM) was conducted as described before (Köster et al., 1999). Briefly, mice were trained to find a platform (7-cm diameter, 1 cm above or below surface) in a circular pool (diameter, 80 cm; height, 30 cm) filled with milky water (depth, 20 cm; 22  $\pm$  1  $^{\circ}$ C). External visual cues were placed around the pool to facilitate navigation of the animals. Each mouse was placed in the water facing the wall of the pool in a fixed starting position randomly chosen from four quadrants and allowed to swim for 1 min to reach the platform. Mice failing to complete the task were placed on the platform manually. All mice were allowed to rest there for 20 s. Mice performed three sessions per day (2-h interval between sessions) and escape latencies were averaged. For the first 3 days the platform was visible, then submerged for 7 days, then placed submerged in a different quadrant (reversed). Escape latencies, swim speed and time spent in each quadrant were recorded automatically (Ethovision; Noldus, Leesburg, VA, USA).

### Novel object recognition

Novel object recognition memory was tested exactly as described before (Okamura et al., 2011). Briefly, single-housed mice were tested in their home cage. A total of four sets containing either two identical (A-A) or two different objects (A-B) were used. All objects were made of plastic, similar in size (6–7 cm high) but different in color and shape. A crossover design was used in all recognition memory experiments, by using different sets of objects for training in order to exclude potential confounding influences of preference for particular objects. The test consisted of two sessions: a training session followed by a retention trial 24 h later. During the training session, a plexiglass board with two identical objects (A-A) was placed in the center of the cage and the board was covered with bedding. Each animal was allowed to explore the objects for 5 min. The mouse was considered exploring the object when the head of the animal was facing the object or the animal was touching or sniffing the object. Total time spent exploring each object was recorded by a trained observer blind to treatment or genotype condition using ODLog 2.0 (Macropod Software). After training, the board with objects was immediately removed from the home cage. NPS or vehicle (PBS, 0.1% BSA) was administered by i.c.v. injection 10 min after the training and mice were tested for retention 24 h later with one familiar object (A') and one novel object (B). Exploration times were normalized [(time exploring object A or A') + (time exploring object B) = total exploration time] and exploration of each object was expressed as percentage of total exploration time. 50% exploration time was considered as “no preference” during training or “no novel object recognition” during the recall session, respectively.

### Conditioned place preference

An unbiased two-compartment conditioned place preference (CPP) paradigm was used to study the influence of disrupted NPS signaling on morphine-induced reinforcement. Compartments differed by bedding texture, visual wall decoration and smell (banana vs. vanilla flavor), and were connected by a door. Animals were habituated to both compartments on three consecutive days for 10 min each. Conditioning was carried out by pairing one compartment with injections of 5 or 10 mg/kg morphine sulfate (Sigma-Aldrich) while the other compartment was paired with saline injections on three consecutive days. Drug-paired compartments were assigned randomly. Place preference was recorded on the seventh day by monitoring residence time on each side with an automatic activity system (Versamax; Accuscan, Columbus, OH, USA).

### Immunofluorescence

Mice with cannulae targeting the PVT were injected with 1 pmole NPS. 2 h later, mice were anesthetized with ketamine/xylazine and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Brains were removed, postfixed and cryoprotected as described (Liu et al., 2011). Coronal cryostat sections (20  $\mu$ m) were stained with a primary rabbit antiserum against the immediate-early gene product c-fos (# 2250; Cell Signaling, Danvers, MA, USA) at a dilution of 1:200. An Alexa488-conjugated anti-rabbit secondary antiserum (# A32731; Thermo Fisher, Waltham, MA, USA) was used to develop the slices and staining was visualized under a confocal fluorescence microscope with single-photon excitation (LSM 710 Meta; Zeiss, Göttingen, Germany). All digital micrographs were adjusted for brightness and contrast using Photoshop CS3 (Adobe, San Jose, CA, USA).

### Statistical analysis

Data for fear conditioning and extinction were analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test, wherever appropriate (Prism; GraphPad, San Diego, CA, USA). Novel object recognition data were analyzed by one-sample *t*-tests with 50% exploration time as the reference value for no recognition. *P* values <0.05 were considered significant. All data are means  $\pm$  S.E.M.

## RESULTS

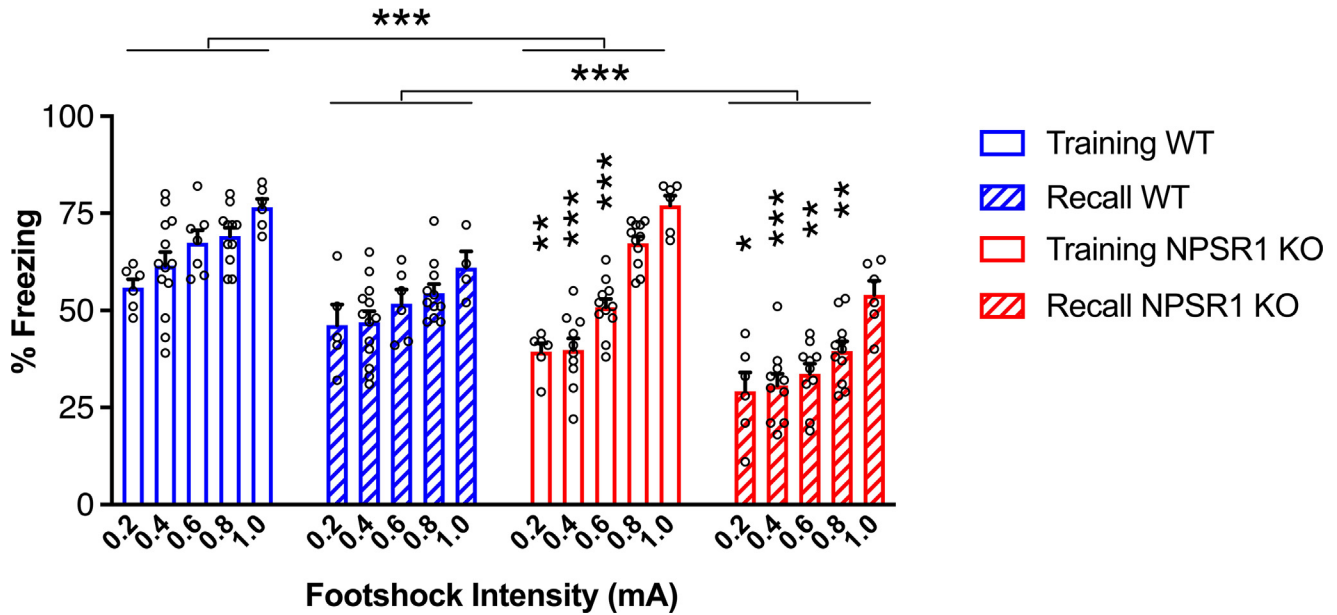
### NPS signaling affects stimulus–response encoding as a function of stimulus intensity

During pilot studies to establish a protocol for auditory fear conditioning and extinction, we observed continuously reduced freezing responses in the first recall session in NPSR1 knockout mice, as compared to their wildtype littermates. Systematic investigation of this phenotype revealed significantly attenuated cue-induced freezing 24 h after auditory fear conditioning as a

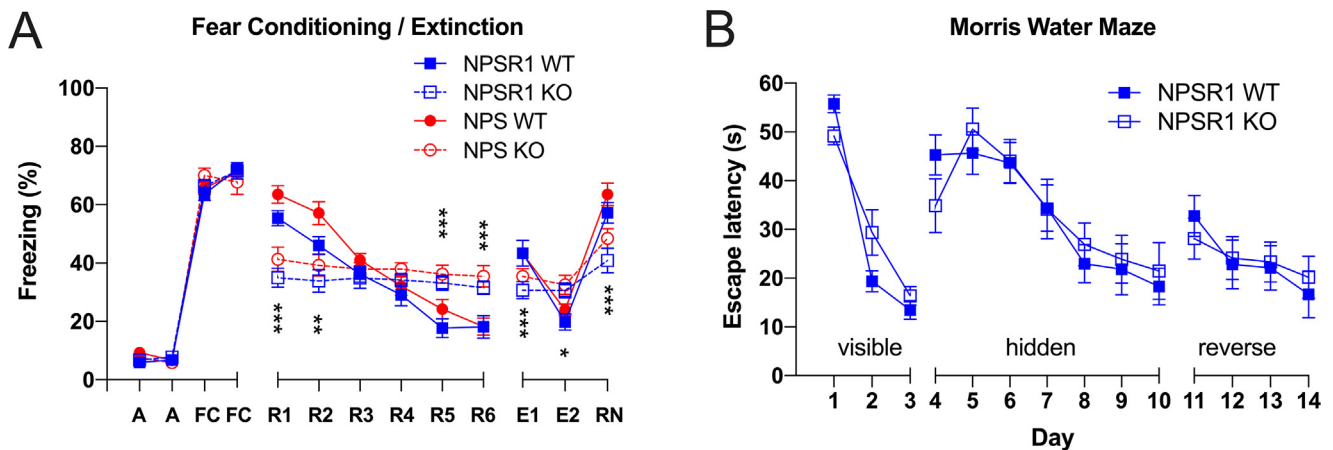
function of foot shock intensity in NPSR1 knockout mice versus their wildtype littermates with significant treatment  $\times$  genotype interaction ( $F_{4,73} = 4.56$ ,  $p = 0.0024$ ) and main effects of genotype ( $F_{1,73} = 28.12$ ,  $p < 0.0001$ ) and shock intensity ( $F_{4,73} = 25.59$ ,  $p < 0.0001$ ) comparing freezing between training and recall (Fig. 1). Compared to WT littermates, NPSR1 knockout mice showed significantly attenuated acquisition of the conditioned freezing response at foot shock intensities below 0.8 mA. Two-way ANOVA revealed significant effects of shock intensity (“training”:  $F_{4,73} = 44.78$ ,  $p < 0.0001$ ), genotype ( $F_{1,73} = 12.3$ ,  $p < 0.0001$ ), and interaction of genotype  $\times$  treatment ( $F_{4,73} = 7.971$ ,  $p = 0.0023$ ). Similarly, recall of conditioned freezing was attenuated in NPSR1 knockout mice at training shock intensities below 1.0 mA (“recall”; genotype:  $F_{1,72} = 39.94$ ,  $p < 0.0001$ ; shock intensity:  $F_{4,72} = 9.49$ ,  $p < 0.0001$ ; interaction:  $F_{4,72} = 0.55$ ,  $p = 0.7029$ ). Similar results were obtained with NPS precursor knockout mice at foot shock intensities between 0.4–0.8 mA (data not shown). These observations suggest that NPS signaling may contribute to the encoding of stimulus intensity and thus influence memory strength for the conditioned response (CR).

### NPS is required for extinction of conditioned fear but not for reversal learning

Next, we investigated fear extinction learning in NPSR1 and NPS precursor knockout mice and selected a shock intensity of 0.8 mA to ensure equal levels of conditioned freezing at the end of training. Due to the inherently lower salience of the extinction training (compared to the highly salient conditioning) we hypothesized that mice with disrupted NPS signaling should display attenuated fear extinction learning if the NPS system is involved in stimulus salience encoding. As shown in Fig. 2A both NPSR1 and NPS precursor knockout mice displayed a complete absence of extinction learning compared to their wildtype littermates (genotype  $\times$  training interaction:  $F_{5,48} = 24.41$ ,  $p < 0.0001$ ; training:  $F_{5,48} = 36.14$ ,  $p < 0.0001$ ; with apparent absence of genotype effects:  $F_{1,48} = 0.00$ ,  $p = 0.9815$ ). Post hoc analysis revealed significant differences in extinction of conditioned freezing in the first two and final two extinction trials (Fig. 2A). Both knockout models were able to form the contingency of the tone/shock pairings, but their freezing responses were significantly attenuated in the first recall session (R1), as described above. Most strikingly, conditioned freezing did not change during the repeated extinction trials (R1–R6) in NPSR1 and NPS precursor knockout mice, whereas wildtype littermates developed normal extinction of the CR. NPSR1 and NPS precursor knockout mice continued to show unaltered freezing behavior upon presentation of the conditioning tone one day later during recall of extinction (E1) tests, whereas their wildtype littermates displayed the typical profile of initial reinstatement of conditioned freezing behavior that was reduced to extinction levels upon a second extinction training (E2). Both genotypes showed normal



**Fig. 1.** Dose-response relationship of conditioned freezing response as a function of foot shock intensity in NPSR1 knockout (KO) mice and their wildtype (WT) littermates. Separate groups of naive animals were used for each shock level; 6–13 animals per group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , versus WT training or recall, respectively; Bonferroni's post hoc test after significant two-way ANOVA. Bar graph data are means  $\pm$  S.E.M.



**Fig. 2.** (A) Auditory fear conditioning and extinction in NPSR1 and NPS precursor knockout (KO) mice and their wildtype (WT) littermates. (B) Spatial learning in the Morris water maze paradigm of NPSR1 KO and WT mice with visible, hidden, and hidden-reversed escape platform.  $N = 6$ –7 mice per group in (A), 11 mice per group in (B). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  KO versus WT in both genetic models, Bonferroni's post hoc test after significant two-way ANOVA. Data are means  $\pm$  S.E.M. Abbreviations in (A): A, acclimatization; FC, auditory fear conditioning; R1–R6, extinction sessions; E1–2, recall of extinction; RN, renewal of conditioning. See methods for details.

renewal of fear conditioning (RN) when a new tone/shock pairing was administered at the end of the session, albeit at significantly lower levels in the two knockout models (Fig. 2A).

Since extinction learning requires the acquisition of new memory that competes with the initially learned content, we wondered if the extinction deficit phenotype in NPSR1 knockout mice might be caused by aberrant reversal learning. In order to test this hypothesis, we performed a classical Morris water maze (MWM) paradigm in which the location of the hidden platform is changed after the animals show stable performance in finding the platform. As shown in Fig. 2B, MWM

performance of NPSR1 knockout mice and their wildtype littermates was indistinguishable, regardless if the platform was visible, hidden or hidden-reversed. Probe trials (without platform) conducted at the end of training days 10 and 14 also demonstrated that both genotypes spent more time searching in the quadrant that previously contained the platform (data not shown). These observations indicate that the NPS system is not involved in spatial learning and not required for reversal learning competing with previously established memory. It should be noted however, that the intensity of sensory stimuli encountered during the MWM training sessions remains constant and can be considered high salience

(exposure to water and swim stress), compared to the relative “dullness” of fear extinction trials in which the animals are just exposed to a box with auditory stimulation, but no further sensory input. We therefore hypothesized that under conditions where NPS signaling is restored, or even enhanced, the relative dullness of fear extinction training can become a salient-enough stimulus to initiate new learning that is required for successful extinction of conditioned responses. In contrast, the strongly salient MWM training might be able to pass the stimulus threshold for successful memory encoding in both wildtype and NPSR1 knockout mice, whereas the relatively low salience of fear extinction training will not surpass threshold levels in mice devoid of NPS signaling.

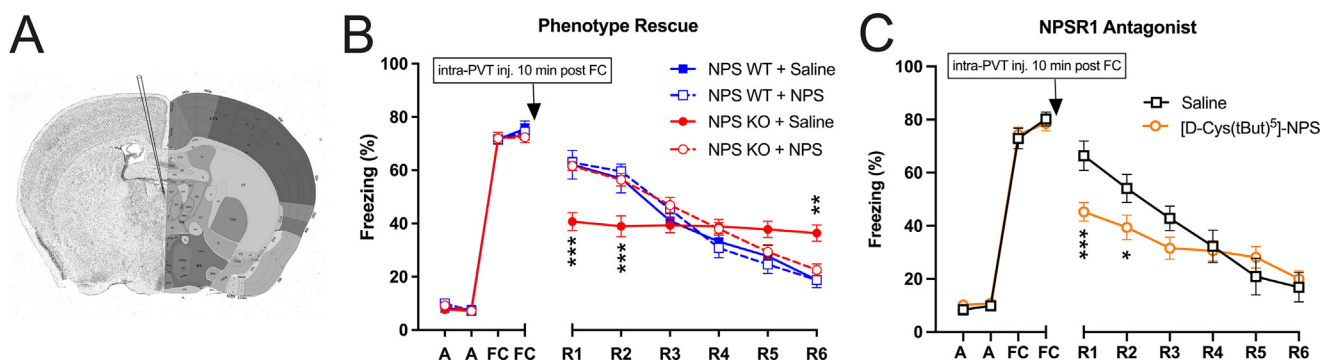
### NPSR1 activation in the PVT is required for stimulus salience encoding

The extinction deficit observed in NPSR1 and NPS precursor knockout mice is the most profound phenotypical change that can obviously not be compensated by other neuronal systems. In order to identify the neuronal substrate(s) mediating this behavioral phenotype, we focused on brain areas showing (1) high levels of NPSR1 mRNA expression, and (2) high density of NPS-containing neuronal fiber projections (Clark et al., 2011; Liu et al., 2011). Fulfilling both criteria is the paraventricular thalamic nucleus (PVT). We performed phenotype-rescue experiments by intra-PVT NPS microinjections in NPS precursor knockout mice (Fig. 3A) as these animals show the same extinction deficit as NPSR1 knockout mice but still possess functional NPS receptors (Liu et al., 2017).

As shown in Fig. 3B, post-training intra-PVT microinjections of 1 pmole NPS were sufficient to completely rescue the extinction deficit phenotype one day later. Intra-PVT NPS-treated NPS precursor knockout mice were indistinguishable from wildtype mice, but showed significantly improved extinction learning compared to saline-treated NPS precursor

knockout mice (drug  $\times$  training interaction:  $F_{5,77} = 12.16$ ,  $p < 0.0001$ ; drug treatment:  $F_{1,77} = 8.76$ ,  $p = 0.005$ ; extinction training:  $F_{5,77} = 19.83$ ,  $p < 0.0001$ ). Post hoc analysis showed significant differences in conditioned freezing responses during the first two and the last extinction trial (Fig. 3B). Interestingly, NPS microinjections into the PVT also restored the deficit in initial fear memory recall during session R1. Intra-PVT NPS treatment did not improve fear extinction in wildtype mice compared to saline-treated animals, suggesting that NPS receptors in the PVT might be already maximally activated in normal animals during a critical period immediately following fear conditioning training. Due to the close proximity of the PVT to the third ventricle, we also tested whether ventricular injections of the same dose of NPS (1 pmole) could produce similar effects, in order to exclude the possibility that leakage of NPS into the ventricular fluid might have occurred, potentially causing activation of NPSR1 in remote brain areas. However, administration of 1 pmole NPS into the third ventricle of NPS precursor knockout mice was unable to rescue the extinction deficit phenotype (Suppl. Fig. S2), indicating that local activation of NPSR1 within the PVT is required for this effect.

To further investigate the role of the PVT in NPS-mediated facilitation of fear expression and extinction, we microinjected the peptidergic NPSR1 antagonist [D-Cys(tBut)<sup>5</sup>]-NPS (100 pmole) in the PVT of C57Bl/6J wildtype mice 10 min after completion of fear conditioning training. As shown in Fig. 3C, transient post-training blockade of NPSR1 signaling attenuated the consolidation of fear memories, compared to saline-treated mice. NPSR1 antagonist-treated mice showed significantly reduced expression of conditioned fear in the first recall session (R1), at levels similar to those seen in NPSR1 and NPS precursor knockout mice. Repeated-measures ANOVA revealed significant drug treatment  $\times$  training interaction ( $F_{5,50} = 10.51$ ,  $p < 0.0001$ ) and main effects of drug treatment ( $F_{1,50} = 5.98$ ,  $p = 0.0345$ ) and training ( $F_{5,50} = 70.65$ ,



**Fig 3.** NPS signaling in the PVT is necessary for acquisition and extinction of conditioned fear. **(A)** Schematic illustration of cannula placement for intra-PVT injections. Allen Brain Atlas, coronal mouse brain reference map (<https://mouse.brain-map.org/static/atlas>), adjusted to black-and-white, AP  $-0.6$  mm from bregma. **(B)** Rescue of fear extinction deficit phenotype in NPS precursor knockout mice by intra-PVT microinjections of NPS (1 pmole) or saline.  $N = 6$ –11 animals per group. **(C)** Blocking of NPS signaling in the PVT attenuates recall of conditioned fear. Intra-PVT microinjections of the NPSR1 antagonist [D-Cys(tBut)<sup>5</sup>]-NPS (100 pmole) or saline in C57Bl/6J mice.  $N = 5$ –8 animals per group.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$  drug-treated versus saline-treated animals; Bonferroni's post hoc test after significant two-way ANOVA. Data are means  $\pm$  S.E.M. Abbreviations: A, acclimatization; FC, auditory fear conditioning; R1–R6, extinction sessions.

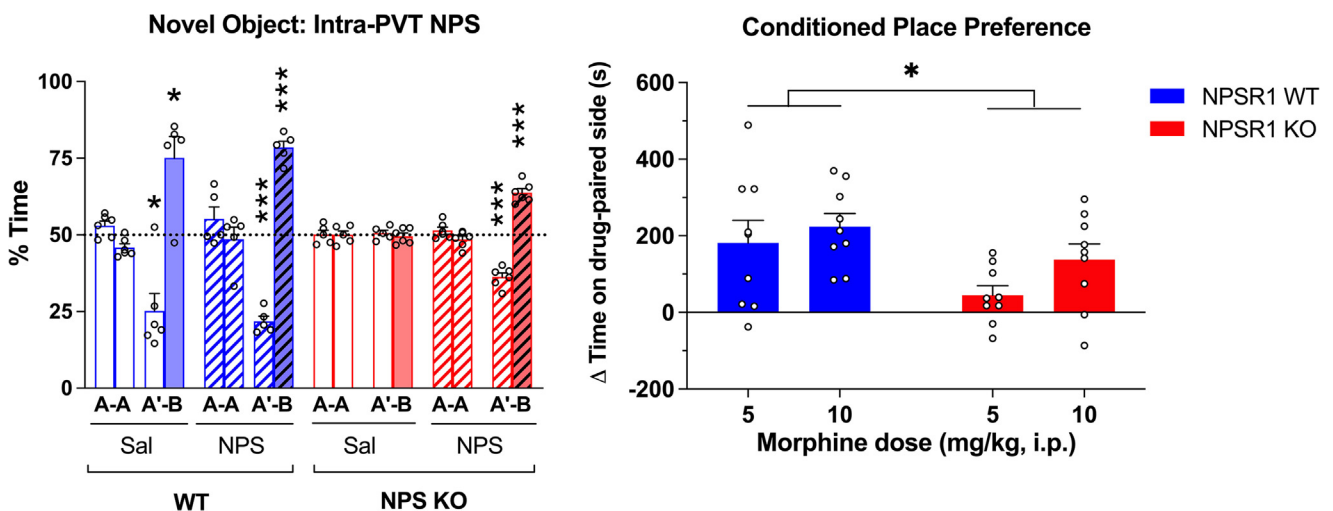
$p < 0.0001$ ). Post hoc analysis showed significant differences in expression of conditioned freezing behavior during the first two extinction sessions in mice that received post-training intra-PVT NPSR1 antagonist administrations. These data suggest, that NPS signaling in the PVT during a critical period after fear conditioning is important for consolidation, affecting the strength of such fear memories. However, transient blockade of NPS signaling in the PVT right after fear conditioning does not affect extinction of such – albeit weaker – fear memories one day later. In view of the previous observations made in NPSR1 and NPS precursor knockout mice (Fig. 2A), or NPS precursor knockout mice treated with intra-PVT NPS (Fig. 3B), a dual role for NPS signaling in the PVT can be postulated: in the immediate phase during memory consolidation NPS affects memory strength, which is possibly related to stimulus salience encoding, while during fear extinction NPS is required for successful acquisition of the less salient extinction content. Since the C57Bl/6J mice used in this experiment have functional NPS systems and duration of antagonist activity is likely less than one hour (Camarda et al., 2009), these animals can use their intact NPS system during extinction training and consequently show normal extinction of the CR on the following day. However, single post-training intra-PVT NPS administrations are able to fully restore fear expression and extinction in NPS precursor knockout mice, which cannot recruit a functional NPS system during fear extinction one day later. These observations suggest that maximal activation of NPS signaling in the PVT right after fear conditioning may induce a particular form of neuronal plasticity that renders the CR sensitive to extinction, with or without further participation of NPS signaling during extinction.

### NPSR1 activation in PVT enhances salience of low-intensity stimuli

If activation of NPSR1 in the PVT is critically involved in stimulus salience encoding, it should be possible to enhance formation of memory for otherwise low-salient stimuli after local NPS administration into this brain structure. NPSR1 and NPS precursor knockout mice both show a remarkable deficit in object recognition memory (Okamura et al., 2011). Presentation of a neutral object is obviously a sensory stimulus of low inherent salience. As shown in Fig. 4A, post-training microinjections of 1 pmole NPS into the PVT were able to rescue the object recognition deficit in NPS precursor knockout mice 24 h later, supporting our hypothesis that NPS signaling in the PVT is sufficient to increase the sensory salience of otherwise low salient stimuli. Saline-treated NPS precursor knockout mice fail to recognize a familiar object versus a novel object 24 h after initial presentation of the training objects ( $t = 1.311$ ,  $p = 0.32$ ; one-sample  $t$ -test). However, intra-PVT NPS administration fully reverses this phenotype in NPS precursor knockout mice, bringing their performance in object recognition to levels indistinguishable from wild-type mice (novel vs. familiar object; WT:  $t = 23.62$ ,  $p = 0.0018$ ; NPS KO:  $t = 9.089$ ,  $p = 0.0008$ ; one-sample  $t$ -test). Together with our previous findings on fear extinction learning, these observations demonstrate that NPS signaling in the PVT may be functionally involved in stimulus salience encoding, independent of sensory modality or affective valence of the stimulus.

### NPS signaling influences establishment of morphine CPP

Next, we explored whether NPS signaling may also be required for salience encoding of appetitive or



**Fig. 4.** Encoding of stimulus salience of different modalities is dependent on NPS signaling. **(A)** Rescue of novel object recognition deficit in NPS precursor knockout (NPS KO) vs. wildtype (WT) mice after intra-PVT NPS or saline administration. Mice were allowed to explore two identical objects (A-A) and then microinjected with either saline (Sal) or 1 pmole NPS into the PVT. 24 h later mice were exposed to one familiar (A') and one novel object (B) in the same context. Object exploration times were normalized to total exploration time and are given as means  $\pm$  S.E.M. The dashed line indicates 50% exploration time if none of the objects is preferred. \* $p > 0.05$ , \*\*\* $p > 0.001$ ; one-sample  $t$ -test for both familiar and novel object compared to 50% chance level.  $N = 5-6$  animals per group. **(B)** Expression of morphine conditioned place preference is significantly lower in NPSR1 knockout (KO) mice compared to wildtype (WT) littermates. \* $p < 0.05$  for genotype in two-way ANOVA.  $N = 9-10$  animals per group.

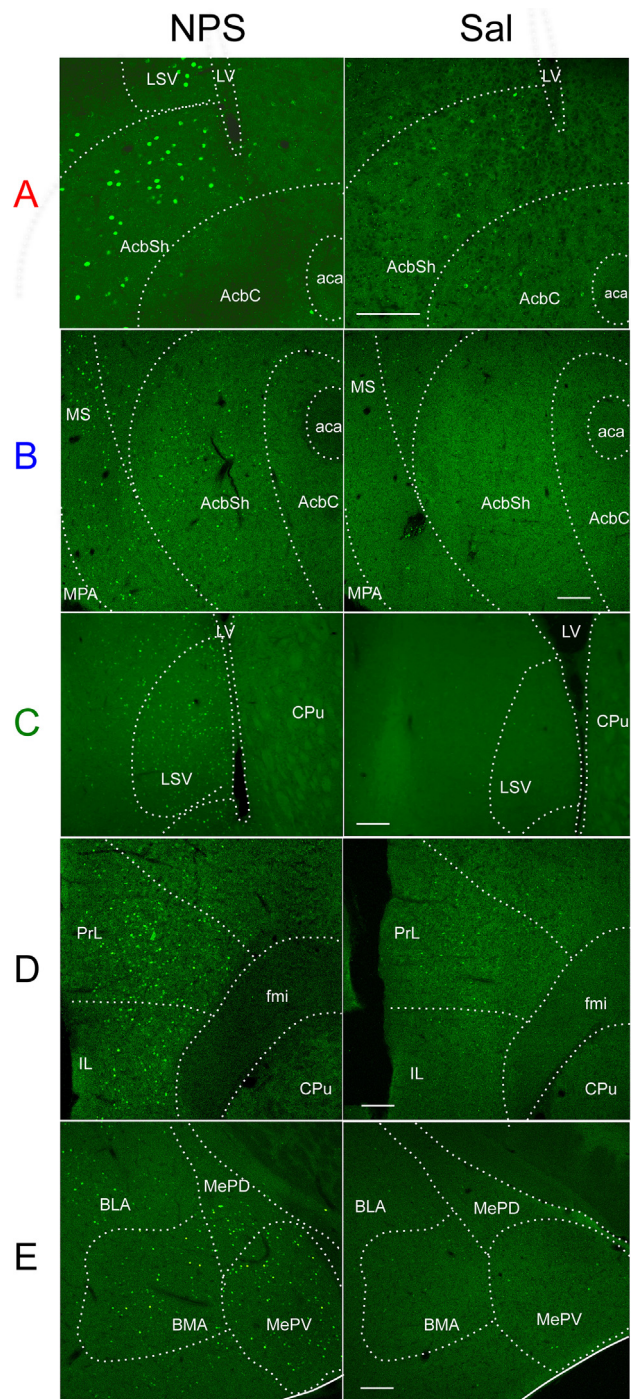
reinforcing stimuli. We used an unbiased morphine conditioned place preference paradigm in which groups of mice received two different doses of morphine on three consecutive days paired with one of the two distinct test chambers (see Methods). Expression of place preference was tested on the seventh day with free access to both chambers. As shown in Fig. 4B, NPSR1 knockout mice display attenuated conditioned place preference (CPP) after training with two doses of morphine, compared to their wildtype littermates. Two-way ANOVA revealed main effects of genotype ( $F_{1,32} = 6.15$ ,  $p = 0.0186$ ) without significant effects of individual morphine doses or genotype  $\times$  treatment interaction. Both doses of morphine produced conditioned place preference in NPSR1 knockout mice, but residence time on the drug-paired side was significantly lower than in wildtype littermates. These results indicate that NPS signaling is involved in encoding stimulus salience independent of sensory modality or affective valence. It should be noted however, that the experimental conditions do not allow for distinction between effects on acquisition or expression of CPP.

#### PVT NPSR1 stimulation produces neuronal activation in nucleus accumbens shell, prefrontal cortex and amygdala

To determine the projection areas of PVT NPSR1-expressing neurons, we mapped expression of the immediate-early gene marker *c-fos* after local microinjection of 1 pmole NPS into the PVT. Compared to saline-injected animals, significant *c-fos* protein expression was detected by immunostaining in the nucleus accumbens shell (AcbSh), the lateral septal nucleus (LSV), the infralimbic (IL) and prelimbic (PrL) prefrontal cortex (PFC), and structures of the medial amygdala (Fig. 5). These results are consistent with published data showing strong projections of anterior PVT principal neurons to dorsomedial parts of AcbSh and reciprocal connections with PFC (Pinto et al., 2003; Parsons et al., 2006; Li and Kirouac, 2012, 2008; Dong et al., 2017; Li et al., 2021). Unsurprisingly, specific activation of PVT NPSR1-expressing neurons reveals only a subset of PVT projection structures that have been previously identified by anterograde tracing studies. For example, no *c-fos* staining was observed in the central amygdala or bed nucleus of the stria terminalis (Dong et al., 2017; Li et al., 2021). It is however unknown, whether specific NPS-dependent activation of *c-fos* expression in these areas occurs via direct neuronal projections or indirectly, involving other brain areas.

#### DISCUSSION

The current study provides evidence for a significant role of NPS signaling in encoding stimulus salience via the PVT, independent of affective valence or modality of the sensory input. The notion of NPS as a salience integrator also offers a unifying hypothesis for many of the apparently unrelated physiological and behavioral effects of this neuropeptide transmitter in the brain. In



**Fig. 5.** Stimulation of *c-fos* expression after intra-PVT microinjections of 1 pmole NPS or saline (Sal) in specific mouse brain regions. (A–C) nucleus accumbens and lateral septal nucleus, (D) prefrontal cortex, (E) amygdala. Schematic brain maps showing locations of micrographs are provided in Suppl. Fig. 3. Expression of *c-fos* was detected by immunofluorescence as described in the Methods. Approximate borders of brain regions were assigned according to the mouse brain atlas (Franklin and Paxinos, 2007).  $N = 3$  per treatment. Scale bar = 100  $\mu\text{m}$ . Abbreviations in the immunofluorescence micrographs: aca, anterior commissure anterior part; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; BLA, basolateral amygdaloid nucleus anterior part; BMA, basomedial amygdaloid nucleus anterior part; CPu, caudate putamen; fmi, forceps minor of the corpus callosum; IL, infralimbic cortex; LSV, lateral septal nucleus ventral part; LV, lateral ventricle; MePD, medial amygdaloid nucleus posterodorsal part; MePV, medial amygdaloid nucleus posteroventral part; MPA, medial preoptic area; MS, medial septal nucleus; PrL, prelimbic cortex.

addition, our data establish a neurochemical mechanism for salience encoding in the PVT, where at least three excitatory neuropeptide systems converge to modulate interpretation of incoming sensory input, i.e. NPS, orexin/hypocretin and CART (Parsons et al., 2006; Kirouac, 2015; Ren et al., 2018). Activation of NPSR1 in PVT neurons appears to recruit NAc, PFC, and amygdala structures that have been previously implicated in salience encoding and may form a reciprocal network (Pinto et al., 2003; Kirouac, 2015; Dong et al., 2017; Li et al., 2021).

Measuring absolute stimulus salience across different stimulus modalities is impossible, especially in animal models. Intuitively, the inherent salience of a foot shock is assumed to be greater than presentation of a novel object, but there are no tools to objectively quantify subjective salience perception. This dilemma is similar for comparing inherent salience of rewarding stimuli, for example reward produced by reinforcing drugs versus food or social interaction. In order to determine whether any neuronal system encodes a dynamic relationship between stimulus salience and physiological effect, e.g. subsequent memory strength, stimulus intensity must be a controllable and scalable parameter. Foot shock intensity in auditory fear conditioning fulfills these criteria and the magnitude of conditioned freezing behavior is an objective measure to quantify memory strength as a function of stimulus intensity.

Our data presented in Fig. 1 suggest that the NPS system may be dynamically involved in acquisition and expression of the conditioned fear response up to a critical threshold ( $\leq 0.8$  mA). Below this level lies a “dynamic range” in which NPS signaling facilitates perception of the stimulus, resulting in increased training-dependent conditioned freezing. Since stimulus intensity perception is correlated with absence or presence of NPS signaling, significant differences in recall of the CR are observed on the next day. The striking deficit in fear extinction in both NPSR1 and NPS precursor knockout mice can be interpreted as an absence of stimulus salience detection. Previous studies have shown, that extinction learning requires formation of new memory that temporarily competes with pre-existing memory and then suppresses expression of fear or reward memory, rather than erasing previously learned content (Maren and Quirk, 2004). However, multiple exposures to the non-reinforced, and therefore significantly lower salient extinction training, are usually necessary to finally establish extinction of the previously learned CR. In the case of NPSR1 or NPS precursor knockout mice, the lower salience of extinction training might therefore be unable to compete with, or suppress, the highly salient contingency of tone/shock pairings that was acquired during the training. This deficit is, however, not caused by a general inability of these mice to consolidate new memory or an incapacity for reversal learning as we have shown in the MWM paradigm. Since stimulus salience in the MWM is constantly high across trials due to the inherent swim stress, the stimulus content is obviously strong enough to enter memory storage. The “reversed platform” component of the MWM is another

form of extinction learning since previously acquired spatial memory conflicts with new information and needs to be suppressed for a successful escape strategy. Additional support for the hypothesis that the NPS system is an integrator of stimulus salience comes from our observation that the same pharmacological manipulation (intra-PVT NPS microinjections) is able to rescue both the fear extinction and novel object recognition deficit phenotypes, involving sensory stimuli of significantly different nature, and that conversely NPSR1 antagonism in the PVT produces a deficit in fear recall. Together with the demonstration of attenuated morphine CPP in NPSR1 knockout mice, these data strongly suggest that the stimulus salience-encoding activity of the NPS system might be independent of stimulus modality or emotional valence.

The present study also provides a unifying hypothesis for many of the apparently unrelated behavioral effects produced by central NPS administration, or absence thereof in genetic models. Enhanced exploratory activity in a novel environment (open field, hole board) (Xu et al., 2004; Liu et al., 2017), reduced anxiety-like behaviors (plus maze, open field, light–dark box) (Xu et al., 2004; Leonard et al., 2008; Rizzi et al., 2008; Wegener et al., 2012; Adori et al., 2016), increased memory consolidation (inhibitory avoidance, novel object recognition) (Okamura et al., 2011; Lukas and Neumann, 2012; Han et al., 2013), accelerated fear extinction (Jüngling et al., 2008), restored prepulse inhibition after pharmacologically-induced disruption (Okamura et al., 2010), or facilitation and precipitation of drug-seeking behavior (Pañeda et al., 2009; Schmoutz et al., 2012; Kallupi et al., 2013, 2010; Cannella et al., 2016, 2009), have all been reported after central NPS injections. Most knockout mouse models display the corresponding phenotype deficits (Duangdao et al., 2009; Zhu et al., 2010; Liu et al., 2017; Si et al., 2021) with the exception of an NPSR1 knockout on a CD1 background (Ruzza et al., 2012). All these behavioral effects can indeed be interpreted as NPS-induced increased salience of sensory input provided by novel environments and tasks, or presentation of otherwise low-salience stimuli that now become important. With respect to NPS-dependent modulation of anxiety-like behavior, a direct anxiolytic-like effect is observed after increased NPS neurotransmission in the amygdala, without stimulation of locomotion (Jüngling et al., 2008; Pape et al., 2010; Grund and Neumann, 2018). Behavioral responses produced by ICV NPS administrations might therefore be a composite of amygdala-mediated anxiolysis, paired with enhanced salience of the novel environment, thus leading to increased exploratory activity.

The hypothesis of NPS as a mediator of stimulus salience can also be extended to humans. A common polymorphism in human NPSR1 (rs324981; T/A, Ile<sup>107</sup>Asn) renders NPSR1-Asn<sup>107</sup> 5–10fold less sensitive to the endogenous ligand than the ancestral NPSR1-Ile<sup>107</sup> (Reinscheid et al., 2005; Bernier et al., 2006; Acevedo et al., 2017). Due to a high worldwide frequency of the polymorphism, about 25% of humans are homozygous for the less active NPSR1-Asn<sup>107</sup> (Reinscheid et al., 2021). A study in human volunteers

revealed significant differences in their evaluation of conditioned fear memory, depending on their NPSR1 genotype at rs324981. Carriers of the more sensitive NPSR1-Ile<sup>107</sup> isoform rate their subjective fear significantly higher than individuals homozygous for the low sensitivity variant (Raczka et al., 2010). These results can be interpreted as increased NPSR1 signaling that enhances fear stimulus salience perception. Presence of the NPSR1-Ile<sup>107</sup> variant was also associated with delayed bedtime, in a gene dosage-dependent manner (Gottlieb et al., 2007). Again, it is conceivable that increased attention to distracting environmental stimuli in NPSR1-Ile<sup>107</sup> carriers keeps those individuals longer awake. An additional hypomorphic polymorphism exists in the NPS peptide itself (rs4751440, G/C, Val<sup>6</sup>Leu) that reduces agonist potency about 20-fold (Deng et al., 2013). The mutation may have resulted from Neandertal introgression and is found at low frequency only in European and Indian subcontinent populations (Reinscheid et al., 2021). Only 0.4% of Europeans are assumed to be homozygous for the low potency NPS-Leu<sup>6</sup> variant and therefore no phenotypical analyses have been carried out so far. However, we predict a similar attenuation of stimulus salience detection in such individuals, which could be assessed by suitable conditioning tasks. Hypomorphic mutations in the NPS system that attenuate salience detection may have become adaptive during the human transition from essentially life-threatening prehistoric habitats to predominantly social environments by providing serenity to cope with social stress. Indeed, the NPSR1-Ile<sup>107</sup> variant has been found associated with maladaptive social behaviors, such as impulsivity, hyperactivity, or alcohol abuse (Laas et al., 2014a,b; 2015a,b).

Numerous studies have reported anxiolytic-like effects after central NPS administration in animal models, while NPSR1 or NPS precursor knockout mice display mildly increased anxiety-like behaviors. Counter intuitively, the more active NPSR1-Ile<sup>107</sup> variant has been associated with anxiety disorders in humans while the less active NPSR1-Asn<sup>107</sup> variant appears to provide some protective effects from developing e.g. panic disorder or generalized anxiety disorder (Okamura et al., 2007; Donner et al., 2010; Domschke et al., 2011). These apparently conflicting observations may point at a dual role of NPS, producing acutely anxiolytic-like effects but also mediating salience of aversive stimuli that will influence long-term memory formation, as we have demonstrated in animal models (Okamura et al., 2011; Liu et al., 2017). It is well established that many forms of anxiety disorders involve aberrant conditioning to non-threatening stimuli, thus facilitating disease progression (Clark, 1986; Bouton et al., 2001; Behar et al., 2009; Love and Robinson, 2020; Locke and Robinson, 2021). The NPSR1 genotype-dependent over-interpretation of fear stimuli – facilitated by NPSR1-Ile<sup>107</sup> – may lie at the root of such a psychopathological mechanism (Raczka et al., 2010).

Our current studies support the central role of the PVT as a salience integrator, independent of stimulus modality or affective valence, and identify NPS as a critical neurochemical agent. Anterograde tracing studies

described numerous output structures of the PVT, some of which become activated after presentation of salient stimuli. NPSR1 activation in the PVT revealed a specific subset of neural projections, recruiting for example the dorsomedial AcbSh, the pre- and infralimbic cortex, and parts of the medial amygdala. These structures have been previously identified as parts of the “brain salience network” which become activated by either appetitive or aversive stimuli (Kirouac, 2015; Peters et al., 2016). Our results now offer a neurochemical and functional basis for further investigations into this network.

#### Note added in proof

During preparation of this manuscript, preliminary data from this study were made available to guide a study by Bengoetxea et al. The authors used a humanized NPSR1-Asn<sup>107</sup> transgenic mouse model, thus creating attenuated NPSR1 signaling, and confirmed our findings that stimulus salience, i.e. variable foot shock intensities, during auditory fear conditioning influence memory strength, and that NPSR1 genotypes affect fear extinction (Bengoetxea et al., 2021).

#### ACKNOWLEDGEMENTS

This study was supported in part by grants from the National Institute of Mental Health (MH-71313, to R.K. R.). The authors would like to thank Drs. Marcelo Wood, James McGaugh, Benno Roozendaal, Lawrence Cahill and Olivier Civelli (all UCI) for access to equipment and critical discussions of data and experimental strategies. We would also like to thank Dr. Gilbert Kirouac (University of Manitoba) for technical advice regarding PVT injections.

#### AUTHOR CONTRIBUTIONS

Conceptualization and study design: CG, XL, RKR.  
 Critical materials: XL, GC.  
 Data analysis: CG, RKR.  
 Manuscript writing: RKR.  
 Draft editing and illustration: XL, GC, SS, RKR.

#### DECLARATIONS OF INTEREST

None.

#### DATA ACCESSIBILITY

All experimental data are presented in this paper and the [Supplementary Material](#).

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuroscience.2022.06.013>.

(Received 13 March 2022, Accepted 7 June 2022)  
(Available online 13 June 2022)