Neuropeptide S Induces Acute Anxiolysis by Phospholipase C-Dependent Signaling within the Medial Amygdala

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Neuropeptide S (NPS) is an important anxiolytic substance of the brain. However, the signaling pathways downstream of NPS receptor (NPSR) activation, underlying the behavioral effect of NPS, remain largely unknown. Here, we show that bilateral microinfusion of NPS (0.2 nmol/0.5 μ l) into the medial amygdala (MeA) of male adult Wistar rats reduced anxiety-related behavior on both the elevated plus-maze and the open field. Moreover, as shown in amygdala tissue micropunches intracerebroventricular infusion of NPS (1 nmol/5 μ l) (1) evoked phosphorylation and synthesis of CaMKII α in relation to reference protein β -tubulin representing Ca²⁺ influx, and (2) induced phosphorylation of mitogen-activated protein kinase ERK1/2. The NPS-induced anxiolysis was prevented by local inhibition of phospholipase C signaling using U73122 (0.5 nmol/0.5 μ l) in the MeA, indicating the behavioral relevance of this pathway. Conversely, local pharmacological blockade of adenylyl cyclase signaling using 2',5'-dideoxyadenosine (12.5 nmol/0.5 μ l) failed to inhibit the anxiolytic effect of NPS infused into the MeA. Hence, NPS promotes acute anxiolysis within the MeA dependent on NPSR-mediated phospholipase C signaling. Taken together, our study extends the knowledge about the intracellular signaling mechanisms underlying the potent anxiolytic profile of NPS.

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INTRODUCTION

Anxiety disorders have a lifetime prevalence of 28%, and despite substantial research novel therapeutics are still required (Kessler *et al.*, 2005). Neuropeptide S (NPS) represents a potential candidate because of its strong anxiolytic activity following intracerebroventricular (ICV) infusion (Xu *et al.*, 2004; Leonard *et al.*, 2008; Rizzi *et al.*, 2008; Vitale *et al.*, 2008; Wegener *et al.*, 2011; Slattery *et al.*, 2015; Zoicas *et al.*, 2016) in both rats and mice and local infusion into lateral and basolateral amygdala in mice (Jungling *et al.*, 2008; Chauveau *et al.*, 2012). An anxiolytic effect was also described after intranasal application of NPS (Ionescu *et al.*, 2012; Lukas and Neumann, 2012; Dine *et al.*, 2015) in both species. Moreover, NPS has been shown to potently reverse cued fear expression and to promote cued fear extinction in mice and rats (Jungling *et al.*, 2008; Zoicas *et al.*, 2016), also in those selectively bred for extremes in anxiety-related behavior (Slattery *et al.*, 2015), and in a psychopathological mouse model of extinction-deficient 129S1/SvImJ mice (Sartori *et al.*, 2016).

The pericoerulear region and the parabrachial nucleus harbor a cluster of predominantly glutamatergic NPS-immunoreactive neurons in rats (Xu *et al.*, 2007). Moreover, scattered NPS precursor mRNA signals have been detected

in the amygdala and the dorsomedial hypothalamus (Xu *et al.*, 2007). In contrast to the 20-amino acid peptide, the NPS receptor (NPSR) has a wider distribution in the brain. The NPSR is mainly expressed in regions of the limbic system (Xu *et al.*, 2007). The *in vitro* studies in NPSR-transfected human embryonic kidney 293T (HEK293T) cells and Chinese hamster ovary cells revealed increased cyclic adenosine monophosphate (cAMP) concentration by adenylyl cyclase (AC) activation and a rise in intracellular Ca²⁺ levels due to phospholipase C (PLC) stimulation, suggesting that the NPSR is coupled to both G_s and G_q proteins (Reinscheid and Xu, 2005; Camarda *et al.*, 2009; Erdmann *et al.*, 2015; Liao *et al.*, 2016), respectively. More detailed, Pape and colleagues (Erdmann *et al.*, 2015) demonstrated that NPS-evoked Ca²⁺ influx is marked by a biphasic time course in NPSR-transfected hippocampal mouse neurons with the fast component being maintained in an inositol triphosphate- and a ryanodine receptor-dependent manner and the slow component depending on store-operated Ca²⁺ entry from the extracellular space. However, the underlying mechanism(s) mediating the anxiolytic activity following NPSR activation is still unknown.

The *in situ* hybridization studies in mice revealed NPSR mRNA expression in the medial nucleus of the amygdala (MeA) as well as the lateral and basolateral amygdala nuclei (Clark *et al.*, 2011), whereas NPSR mRNA expression in rats is restricted to the MeA and intercalated nucleus of the amygdala (Xu *et al.*, 2007). As the amygdala regulates fear and anxiety-related behavior (Tovote *et al.*, 2015), we analyzed, whether (1) NPS promotes anxiolysis within the MeA, (2) central NPS infusion activates Ca²⁺-dependent

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signaling pathways in the amygdala *in vivo*, and (3) acute NPS-induced anxiolysis is mediated in a PLC- and/or AC-dependent manner. These findings will increase our understanding of the involvement of NPS in emotion regulation and in the pathophysiology of anxiety disorders.

MATERIALS AND METHODS

Animals

Male Wistar rats (230–250 g, Charles River Laboratories, Germany) were housed in groups of 3–4 under standard laboratory conditions (food and water *ad libitum*, 12:12 h light/dark cycle, lights on at 0600 h, 21–23 °C, 55% humidity). Rats were allowed at least 1 week of habituation before they were used for surgical procedures. All experiments were performed between 0800 and 1100 h in accordance with the Guide for the Care and Use of Laboratory Animals by the NIH, and the ARRIVE guidelines (Kilkenny *et al*, 2010), and were approved by the government of the Oberpfalz.

Surgical Procedures

Rats were injected subcutaneously with the analgesic Buprenorphin (Bayer, 0.05 mg Buprenovet/kg) and the antibiotic Baytril (Baxter, 10 mg Enrofloxacin/kg) 30 min before surgery. All stereotaxic procedures were performed under isoflurane anesthesia and semisterile conditions. For ICV infusions, a 12 mm long 21-G guide cannula was stereotactically placed 2 mm above the lateral ventricle (AP: −1.0 mm, ML: −1.6 mm, DV: −2.0 mm; (Paxinos and Watson, 1998). For bilateral intra-MeA infusions, 12 mm long 23-G guide cannulas were implanted 2 mm above the left and right MeA (AP: −2.3 mm, ML: ± 3.2, DV: −7.5 mm).

The implants were fixed to two stainless steel screws using dental cement. Rats were housed singly after surgery, allowed to recover for at least 5 days and handled daily to habituate them to the infusion procedure and to minimize nonspecific stress responses at the day of experiment. Guide cannulas were closed using dummy cannulas that were cleaned daily during the handling procedure.

Experimental Design

Drugs and drug infusions. For central infusions, NPS (Bachem) was diluted in sterile Ringer's solution (vehicle (Veh); B. BRAUN Melsungen AG). NPS was infused ICV at 1 nmol/5 μ l, or locally into the MeA at 0.2 nmol/0.5 μ l.

For local inhibition of either AC or PLC before NPS infusion, the AC inhibitor 2',5'-dideoxyadenosine (DDA) or the PLC blocker U73122 were infused into the MeA. The infused doses of DDA (12.5 nmol/0.5 μ l, EnzoLifeScience) and U73122 (0.5 nmol/0.5 μ l, EnzoLifeScience) were selected on the basis of previous *in vitro* studies and their half-maximal inhibitory dose (Ogawa *et al*, 1995; Feisst *et al*, 2005). As both DDA and U73122 stocks were diluted in dimethyl sulfoxide (DMSO, Merck), Veh₁ was composed of 20% DMSO and 80% sterile Ringer's solution. Veh₂ (NPS control) was composed of sterile Ringer's solution.

The infusion systems (ICV: 14.7 mm, 25 G; local: 14 mm, 27 G) were connected to a 10- μ l Hamilton syringe via PE20 tubing. After infusion at a rate of 1 μ l/min, the infusion system was kept in place for 10 s to allow local substance diffusion. None of the drug-infused rats showed any signs of tremor, convulsions, or wet-dog shaking in their home cage.

Experiment 1: Effect of bilateral infusion of NPS into the MeA on anxiety-related behavior. To examine the local effect of NPS within the MeA on anxiety-related behavior,

Figure 1 Anxiolytic effect of NPS infusion (0.2 nmol/0.5 μ l) bilaterally into the MeA of male rats as assessed on the EPM (a–c) and the OF (d–f). During the 5 min of testing on the EPM, (a) the percentage of time spent on the EPM, (b) the percentage of entries into the open arms, and (c) number of closed arm entries were monitored. During the 5 min of testing in the OF, (d) the time spent in the OF, (e) the entries into the center zone, and (f) traveled distance were analyzed. Data represent mean $\pm$ SEM, * p < 0.05, ** p < 0.01 vs Veh; group sizes: n = 7–9; except outsider n = 2. (g) Schematic drawing of the MeA in gray (opt: optic tract, 3V: third ventricle, Paxinos and Watson, 1998) and (h) representative microphotographs of bilateral MeA infusion sites in a coronal brain slice (H1 and H2). H3 represents an infusion site considered as outsider.

rats were bilaterally infused with either NPS (0.2 nmol) or Veh, and were tested on the elevated plus-maze (EPM; Supplementary Information 1) 15 min thereafter. After 2 days, the same rats were tested in the open field (OF; Supplementary Information 1) 15 min after they received a randomized infusion of either NPS or Veh. Behavioral effects of NPS infusion outside the MeA (outsider, $n=2$) are displayed in Figure 1.

Experiment 2: Effects of ICV NPS on CaMKII α and ERK1/2 in the amygdala. In order to assess NPS-induced activation of intracellular signaling pathways, rats were infused ICV with either NPS (1 nmol/5 μ l) or Veh (5 μ l). Animals were killed by rapid decapitation 15 min after drug infusion, brains were removed, and amygdala tissue from both hemispheres was harvested using a 2 mm-wide micropuncher accordant to the brain atlas. Relative levels of phospho-Ca²⁺/calmodulin-dependent kinase II α (pCaMKII α) and pERK1/2 in relation to respective total protein and the reference protein β -tubulin were analyzed by western blot analysis (Supplementary Information 2).

Experiment 3: Effects of simultaneous blockade of both PLC and AC on NPS-induced anxiolysis. In order to reveal the relevance of NPSR-induced cAMP formation and rise in intracellular Ca²⁺ levels on NPS-induced anxiolysis within the MeA, PLC and AC were inhibited simultaneously by infusion of an inhibitor-cocktail (Inh-cock) composed of U73122 (0.5 nmol) and DDA (12.5 nmol). Consequently, four groups of conscious rats received the following two subsequent intra-MeA infusions of Veh₁/Veh₂, Veh₁/NPS, Inh-cock/Veh₂, or Inh-cock/NPS with a 20 min interval between infusions. Anxiety-related behavior was assessed on the EPM and OF (Supplementary Information 1), respectively, 15 min after the last infusion.

Experiment 4: Effects of specific blockade of either PLC or AC on NPS-mediated anxiolysis in the MeA. To assess whether the anxiolytic activity of NPS is mediated by either NPS-evoked PLC or AC activation, or via both membrane-bound proteins within the MeA, we separately infused U73122 (0.5 nmol), DDA (12.5 nmol), or Veh₁ 20 min before infusion of either NPS (0.2 nmol) or Veh₂ into the MeA. Anxiety-related behavior was assessed on the EPM (Supplementary Information 1) 15 min after the last infusion. After 2 days, the same rats were tested in the OF (Supplementary Information 1) following the same treatment, ie, they received a randomized preinfusion of Veh₁, U73122, or DDA followed by an infusion of NPS 20 min later. They were tested on the OF 15 min after the last infusion.

Verification of Cannula Placements

After the behavioral experiments, placement of cannulas was verified on 40- μ m Nissl-stained coronal cryosections. Only animals with correctly positioned cannulas were included for statistical analyses.

Statistical Analyses

Two-tailed *t*-test or nonparametric Mann-Whitney *U*-test was used in order to analyze differences in the state of anxiety following single infusion of either Veh or NPS, and to analyze NPS-induced phosphorylation and synthesis of CaMKII and ERK1/2. To examine the effect of PLC and/or AC inhibition on NPS-induced anxiolysis, two-way analysis of variance has been used (ANOVA, factors first infusion $\times$ second infusion). Any overall statistical differences, which were set at $p < 0.05$, were further analyzed using Tukey's *post hoc* test. Data are expressed as group mean $\pm$ SEM. Statistical analyses were performed using SigmaPlot 13 (Systat).

RESULTS

Experiment 1: Effect of Bilateral Infusion of NPS into the MeA on Anxiety-Related Behavior

Bilateral infusion of NPS into the MeA significantly reduced anxiety-related behavior of male rats on both the EPM and OF compared with Veh infusion (Figure 1). NPS-treated rats spent a higher percentage of time on the open arms ($t_{15} = -2.46$, $p = 0.027$) without effects on the percentage of entries into the open arms ($t_{15} = -0.37$, $p = 0.72$) and on the number of closed arm entries ($t_{15} = -0.32$, $p = 0.75$). Notably, the anxiolytic effect of NPS in the MeA was confirmed in the OF test 2 days later, as NPS increased the time the animals spent in the center zone ($U = 11.0$, $p = 0.034$) and almost double the number of center zone entries ($t_{14} = -2.58$, $p = 0.022$), when compared with Veh-treated rats. Moreover, NPS increased the distance rats traveled in the OF ($t_{14} = -3.44$, $p = 0.004$) indicative of a hyperlocomotor effect.

Experiment 2: Effects of ICV NPS on CaMKII α and ERK1/2 in the Amygdala

Western blot analysis of amygdala tissue micropunches revealed increased amounts of both pCaMKII α ($t_{20} = -2.48$, $p = 0.022$ Figure 2b) and total CaMKII α ($t_{20} = -2.10$, $p = 0.049$, Figure 2c) relative to β -tubulin following ICV infusion of NPS. The phospho-CaMKII α levels compared with total CaMKII α were not altered ($U = 57.00$, $p = 0.84$, Figure 2a).

Moreover, NPS increased cytoplasmic pERK1/2 levels. This applied to both the pERK1/2/ERK1/2 (pERK1/ERK1: $U = 23.00$, $p = 0.027$; pERK2/ERK2: $U = 21.00$, $p = 0.018$, Figure 2d) and the pERK1/2/ β -tubulin ratios (pERK1/ β -tubulin: $t_{18} = -2.12$, $p = 0.048$; pERK2/ β -tubulin: $t_{18} = -2.18$, $p = 0.043$; Figure 2e). Total ERK1/2 levels were not altered by ICV NPS (ERK1/ β -tubulin: $t_{19} = 0.92$, $p = 0.37$; ERK2/ β -tubulin: $t_{18} = -0.11$, $p = 0.91$, Figure 2f).

Experiment 3: Effects of Simultaneous Blockade of both PLC and AC on NPS-Induced Anxiolysis

Preinfusion of an inhibitor cocktail consisting of both the PLC blocker U73122 and the AC inhibitor DDA bilaterally into the MeA prevented the anxiolytic effect of subsequent locally infused NPS, substantiating that PLC and/or AC mediate the anxiolytic effect (Figure 3). On the EPM, we found an interaction between the first and second infusion

on the percentage of time the rats spent on the open arms ($F_{(1, 46)} = 4.23$, $p = 0.046$), whereas the percentage of entries into the open arms was not altered ($F_{(1, 46)} = 1.13$, $p = 0.29$).

The *post hoc* analysis revealed that NPS increased the percentage of time on the open arms in Veh₁-pretreated ($p = 0.003$) rats but not in rats pretreated with U73122/DDA

Figure 2 Effects of ICV NPS (1 nmol/5 μ l) on relative amount of phosphorylated CaMKIIα (pCaMKIIα) compared with (a) total CaMKIIα and (b) β-tubulin in the cytosolic fraction of amygdala tissue micropunches of male rats. NPS evoked synthesis of (c) CaMKIIα in relation to β-tubulin. Relative amount of phosphorylated ERK1/2 (pERK1/2) compared with (d) total ERK1/2 and (e) β-tubulin without affecting (f) total ERK1/2 levels relative to β-tubulin following NPS infusion. Data represent mean+SEM, * $p < 0.05$ versus Veh; group sizes: $n = 9-12$. (g) Representative western blots of (p)CaMKIIα, (p)ERK1/2, and β-tubulin.

Figure 3 Effects of preinfusion of an inhibitor-cocktail (Inh-cock) consisting of both U73122 (PLC inhibitor, 0.5 nmol) and DDA (AC inhibitor, 12.5 nmol) on the anxiolytic effect of NPS bilaterally infused into the MeA (0.2 nmol/0.5 μ l) on the EPM (a-c) and OF (d-f). (a) Percentage of time spent on the EPM, (b) percentage of entries performed into the open arms, (c) closed arm entries, and (d) time spent in the OF, (e) entries performed into the center zone, and (f) distance traveled. As Veh₁, 0.5 μ l of Ringer's solution supplemented with DMSO, and as Veh₂, 0.5 μ l of Ringer's solution were used. Data represent mean+SEM, ** $p < 0.01$, *** $p < 0.001$ versus all other groups (a, e, f) or as indicated; group sizes: $n = 13-15$, except Inh-cock/Veh₂: $n = 6-8$.

($p=0.004$). In Veh₂-infused rats, U73122/DDA preinfusion did not influence anxiety on the EPM. Local NPS did not affect locomotor activity, as measured by the number of closed arm entries ($F_{(1,46)}=2.86$, $p=0.098$) in both Veh₁- and U73122/DDA-pretreated rats.

In the OF, the anxiolytic effect of intra-MeA NPS was similarly prevented by preinfusion of U73122/DDA, as assessed by the time the rats spent in the center zone ($F_{(1,50)}=3.36$, $p=0.073$, first infusion: $F_{(1,50)}=13.72$, $p<0.001$, second infusion: $F_{(1,50)}=4.12$, $p=0.048$) and the number of center zone entries ($F_{(1,50)}=7.99$, $p=0.007$). Moreover, statistical analysis revealed increased locomotion ($F_{(1,50)}=8.28$, $p=0.006$), an effect caused by NPS in Veh₁-preinfused rats ($p<0.001$). In the OF, preinfusion of the inhibitor cocktail also blocked NPS-induced hyperlocomotion ($p<0.001$).

Experiment 4: Effects of Specific Blockade of Either PLC or AC on NPSR-Mediated Anxiolysis in the MeA

To assess whether the anxiolytic activity of NPS is mediated via the NPSR-evoked activation of either PLC or AC or via both pathways within the MeA, we next preinfused U73122 (PLC inhibitor), DDA (AC inhibitor), or Veh₁ bilaterally into the MeA before local NPS or Veh₂ infusion. Selective inhibition of PLC signaling by preinfusion of U73122 prevented NPS-induced anxiolysis, whereas specific blockade of the AC by DDA preinfusion failed to inhibit the anxiolytic properties of NPS (Figure 4).

In more detail, specific inhibition of the PLC signaling affected the percentage of time spent on the open arms (first infusion: $F_{(1,44)}=1.75$, $p=0.19$; second infusion: $F_{(1,44)}=4.72$, $p=0.036$; interaction: $F_{(1,44)}=3.01$, $p=0.09$) and the percentage of open arm entries (first infusion: $F_{(1,44)}=7.47$, $p=0.009$; second infusion: $F_{(1,44)}=3.42$, $p=0.072$; interaction: $F_{(1,44)}=0.87$, $p=0.36$) on the EPM. The *post hoc* analysis revealed that NPS-infused rats spent more time on the open arms (% time; $p=0.003$ vs Veh₁/Veh₂ rats) when preinfused with Veh₁. This anxiolytic effect of NPS was prevented in the presence of U73122 ($p=0.024$ vs Veh₁/NPS). Similarly, preinfusion of U73122 reduced the percentage of entries into the open arms compared with Veh₁ ($p=0.009$ vs Veh₁/NPS). In addition, U73122 exerted a slight but significant stimulatory effect on locomotor activity ($p=0.015$ vs Veh₁/NPS), as U73122/NPS animals showed an increased number of closed arm entries.

Separate inhibition of the AC signaling by preinfusion of DDA revealed an effect for the second infusion on the percentage of time spent on the open arms (first infusion: $F_{(1,44)}=0.09$, $p=0.76$; second infusion: $F_{(1,44)}=9.32$, $p=0.004$; interaction: $F_{(1,44)}=0.68$, $p=0.42$) and on the percentage of open arm entries (first infusion: $F_{(1,44)}=0.76$, $p=0.39$; second infusion: $F_{(1,44)}=8.77$, $p=0.005$; interaction: $F_{(1,44)}=0.16$, $p=0.69$). The *post hoc* analysis indicated that NPS almost doubled the percentage of time the animals spent on the open arms ($p=0.004$) and increased the percentage of open arm entries ($p=0.005$) in comparison with Veh₂-infused rats. More importantly, preinfusion of DDA failed to block this anxiolytic effect of NPS (percentage of time spent on open arms: $p=0.41$ vs Veh₁/NPS; percentage of open arm entries: $p=0.73$), suggesting that the NPS-induced anxiolysis is independent of AC signaling.

None of the drug-infused rats of this experiment showed any signs of hyperlocomotion as the number of closed arm entries was not altered ($F_{(1,45)}=0.016$, $p=0.90$).

In addition, the effect of either Veh₁, U73122, or DDA before NPS infusion was assessed 2 days later in the OF. One-way ANOVA did not reveal any significant changes with respect to time spent in the center ($F_{(2,20)}=2.03$, $p=0.16$), entries into the center (ANOVA on ranks: $H=2.15$, $p=0.34$), or traveled distance ($F_{(2,20)}=0.49$, $p=0.62$). However, when performing separate statistics our previous findings could be confirmed. In detail, specific inhibition of the PLC signaling by U73122 decreased the time spent in the center zone compared with Veh₁/NPS-treated animals ($U=11.0$, $p=0.028$), but inhibition of AC signaling by DDA failed to block NPS-induced anxiolysis ($t_{11}=1.03$, $p=0.32$). None of the drug-infused rats showed any difference in center zone entries (Veh₁/NPS vs U73122/NPS: $U=15.5$, $p=0.08$; Veh₁/NPS vs DDA/NPS: $U=17.5$, $p=0.72$) or locomotor activity, as the traveled distance was not altered (Veh₁/NPS vs U73122/NPS: $t_{14}=0.92$, $p=0.37$; Veh₁/NPS vs DDA/NPS: $t_{11}=-0.10$, $p=0.93$).

DISCUSSION

The present study describes the MeA as an important brain target of NPS to induce anxiolytic effects in male rats and NPSR-evoked PLC signaling as the essential signaling cascade underlying this local anxiolytic effect. More specifically, the PLC inhibitor U73122 prevented NPS-induced anxiolysis in NPS-sensitive amygdalar neurons.

The MeA pointed out as an important relay station and regulator of emotional responses including anxiety-related behaviors (LeDoux, 2007; Keshavarzi *et al*, 2014). It is critically involved in processing of predator odor-induced defensive reactions, ie, freezing and risk assessment, a defensive behavior related to panic attacks (Herdade *et al*, 2006). The MeA receives dense synaptic input from various brain regions involved in sensory processing and neuroendocrine and autonomic regulation including the accessory olfactory bulb, the pericoerulear region, and the parabrachial nucleus (Moore and Bloom, 1979; Saper and Loewy, 1980; Keshavarzi *et al*, 2014), whereas the latter two brain regions harbor a cluster of predominantly glutamatergic NPS-immunoreactive neurons (Xu *et al*, 2007). The NPSR is differentially expressed in amygdala subnuclei in rats and mice (Xu *et al*, 2007; Clark *et al*, 2011). However, both species express the NPSR in the MeA, but not the central nucleus of the amygdala (CeA), the major output nucleus of the amygdala (Tovote *et al*, 2015) regulating, ie, pericoerulear NPS neurons by monosynaptic, GABAergic projections in mice (Jungling *et al*, 2015). How MeA neurons stimulated by NPS regulate the activity of CeA neurons and its output signaling remains elusive.

Our data demonstrating that NPS infusion into the MeA reduced the anxiety-related behavior in both the EPM and OF of male rats confirm the robust anxiolytic profile of NPS described following ICV infusion (Xu *et al*, 2004; Leonard *et al*, 2008; Rizzi *et al*, 2008; Vitale *et al*, 2008; Wegener *et al*, 2011; Slattery *et al*, 2015; Zoicas *et al*, 2016) and intranasal application (Ionescu *et al*, 2012; Lukas and Neumann, 2012; Dine *et al*, 2015) in both rats and mice, and infusion into the

Figure 4 Effects of preinfusion of either U73122 (PLC inhibitor, 0.5 nmol), DDA (AC inhibitor, 12.5 nmol), or Veh₁ (0.5 μ l of Ringer's solution supplemented with DMSO) on the anxiolytic effect of NPS bilaterally infused into the MeA (0.2 nmol/0.5 μ l) on the EPM (a–f) and the OF (g–i). (a, d) Percentage of time spent on the EPM, (b, e) percentage of entries performed into the open arms, and (c, f) closed arm entries (group sizes: $n = 13$ – 15 , except Inh-cock/Veh₂: $n = 6$ – 8). As Veh₂, 0.5 μ l of Ringer's solution were used. (g) Time spent in the center zone, (h) center zone entries, and (i) traveled distance in the OF (group sizes: $n = 5$ – 8). All data represent mean $\pm$ SEM. * $P < 0.05$, ** $p < 0.01$ as indicated, # $p < 0.05$ vs Veh₁/NPS, \$ $p < 0.05$ vs Veh₁/Veh₂.

lateral and basolateral amygdala in mice (Jungling *et al*, 2008). Specifically, intra-MeA infusion of NPS increased the percentage of time the animals spent on the open arms of the EPM and in the exposed center zone of the OF indicating reduced anxiety levels.

The *in vitro* activation of the NPSR has been described to increase intracellular Ca^{2+} levels and to phosphorylate ERK1/2 in NPSR-transfected HEK293T cells (Reinscheid *et al*, 2005). Furthermore, NPS-induced mobilization of intracellular Ca^{2+} has been reported in NPSR-transfected hippocampal mouse neurons *in vitro*, an effect that was strictly dependent on PLC activity (Erdmann *et al*, 2015). As activation of intracellular signaling cascades is cell-type specific and depends on the cellular microenvironment, the NPS-induced increase in intracellular Ca^{2+} levels and activation of ERK1/2 found *in vitro* may not necessarily reflect intracellular signaling upon NPS binding to its receptor within the brain. Our *in vivo* results are the first to show NPSR-induced activation of intracellular signaling

cascades within the brain. Specifically, quantification of pCaMKII α as a biological marker of NPSR-mediated Ca^{2+} influx after ICV NPS revealed increased relative quantities of pCaMKII α reflecting NPS-induced rise in intracellular Ca^{2+} levels. Our results also demonstrate NPS-induced activation of the MAPK pathway in amygdala punches as reflected by increased pERK1/2 levels in relation to both total ERK1/2 and β -tubulin. Thus, NPSR-evoked phosphorylation and synthesis of CaMKII α and the phosphorylation of ERK1/2 provide important confirmation of intracellular actions of NPS in the amygdala. However, our results are based on amygdala micropunches. Therefore, it remains elusive whether the described NPSR-mediated pathway activation is restricted to NPSR-positive neurons. Thus, future studies should address signal amplification at cellular level by fluorescent labeling of neurons using NPSR promoter-based expression of reporter proteins.

As *in vitro* studies demonstrated that NPSR can couple to intracellular Ca^{2+} as well as cAMP pathways, indicating

interaction with both G_q and G_s types of G proteins (Reinscheid *et al*, 2005), we aimed to reveal the specific involvement of PLC and/or AC signaling in NPS-induced anxiolysis. As an initial approach, we infused a cocktail composed of both the aminosteroidic PLC inhibitor U73122 and the noncompetitive AC inhibitor DDA before NPS infusion into the MeA. NPS failed to induce anxiolysis in rats pretreated with an inhibitor cocktail composed of U73122 and DDA, indicating that either both or one of these intracellular pathways in the MeA are critically involved in NPS-induced behavioral changes. Next, each of the inhibitors was separately applied before local infusion of NPS. Although NPS has a relatively equal potency to Ca^{2+} mobilization and cAMP synthesis *in vitro* (Reinscheid *et al*, 2005), in rats separate preinfusion of U73122 prevented the anxiolytic effect of locally infused NPS in both the EPM and OF, suggesting that activation of PLC is crucial to orchestrate acute NPS-induced anxiolysis. In contrast, separate preinfusion of DDA failed to block NPS-induced anxiolysis, suggesting that the behavioral effect is independent of AC activation. Recently, a truncated version of the NPS peptide, called compound 4, has been demonstrated to evoke anxiolysis and to induce memory-promoting effects with reduced levels of locomotor stimulation following ICV infusion in mice (Clark *et al*, 2017). In HEK293T cells transfected with NPSR107I, compound 4 demonstrated a strong level of bias for the Ca^{2+} mobilization pathway over the cAMP pathway (Clark *et al*, 2017), in line with our results demonstrating that a block of PLC signaling in the presence of NPS prevents its anxiolytic profile. Although NPS induces cAMP accumulation in HEK293T cells *in vitro* (Reinscheid *et al*, 2005), a cAMP-independent mechanism seems to be involved in the anxiolytic effect of NPS in the MeA. Our observation that U73122 and DDA, when infused alone, did not significantly alter anxiety-like behavior compared with control excluded the possibility that the inhibitors changed baseline anxiety.

Based on their chemical structure, both U73122 and DDA are cell membrane permeable. Thus, inhibitor infusion 20 min before local NPS treatment should be sufficient to locally block the respective effector proteins. The different doses of U73122 and DDA used need some considerations: U73122 is reported to reliably inhibit PLC (MacMillan and McCarron, 2010), and usually used at a concentration of 10 μM *in vitro* (Erdmann *et al*, 2015). As the binding domain of PLC isoforms is highly conserved (Rhee *et al*, 1989; Rhee and Bae, 1997), U73122 should inhibit all isoforms of PLC (Carvou *et al*, 2007). Moreover, U73122 reduces Ca^{2+} release, when evoked by direct activation of inositol-1,4,5-triphosphate receptors or ryanodine receptors independent of G_q or PLC activity (MacMillan and McCarron, 2010). Here, we used a dose of 0.5 nmol of U73122 for local infusion into each MeA.

In mammals, nine isoforms of AC are expressed catalyzing the production of the second messenger cAMP (Hanoune and Defer, 2001). A Ca^{2+} /Calmodulin-dependent mechanism was demonstrated to activate the AC isoforms 1, 3, and 8 (MacNeil *et al*, 1985; Halls and Cooper, 2011). Hence, this demonstrates that intracellular pathways do not necessarily act in a linear manner, but can be integrated to bring about the appropriate physiological and behavioral responses. However, whether NPS-evoked cAMP accumulation depends on local rise in intracellular Ca^{2+} levels needs further

investigation. For example, the analysis of NPSR/G protein interactions using fluorescence resonance energy transfer might demonstrate whether NPSR stimulation recruits both G_q and G_s proteins or whether cAMP accumulation is maintained by a Ca^{2+} -dependent activation of AC. Herein, we used DDA to block the membrane-bound AC. The half-maximal inhibitory concentration of DDA is described with 250 μM *in vitro* (Holgate *et al*, 1980; Ogawa *et al*, 1995). Therefore, we infused DDA at a 25-fold higher concentration in order to exert a potentially similar effective inhibition compared with U73122. The total amount of U73122 (0.5 nmol) and DDA (12.5 nmol) applied was selected following diffusion-based calculations in order to maintain at least half-maximal inhibition of the effector proteins within the MeA.

In summary, our *in vivo* data identified another brain region in rats, the MeA, where NPS reliably exerts anxiolytic properties, and demonstrated that this behavioral effect is strictly dependent upon local NPSR-mediated PLC activation. We could further show that central NPS infusion induced phosphorylation and synthesis of CaMKII α in amygdala punches, thereby promoting the activation of the MAPK pathway as another NPSR-mediated intracellular signaling cascade. Our findings contribute to a broader knowledge about the molecular, cellular, and behavioral consequences of NPSR activation in the context of its profound anxiolytic profile that may lead to the development of specific NPSR agonists activating specific intracellular pathways involved in anxiolysis.

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The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

TG and IDN designed the study based on literature searches; TG performed the experiments including the statistical analysis, and wrote the first draft of the manuscript. Both authors contributed to and have approved the final manuscript.

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