




Neuropeptide S-initiated sequential cascade mediated by OX₁, NK₁, mGlu₅ and CB₁ receptors: a pivotal role in stress-induced analgesia

Ming Tatt Lee^{1,2,3†}, Yu-Ting Chiu^{2†}, Yu-Chun Chiu^{2†}, Chia Chun Hor², Hsin-Jung Lee⁴, Remo Guerrini⁵, Girolamo Calo⁶ and Lih-Chu Chiou^{1,2,4,7*} 

Abstract

Background: Stress-induced analgesia (SIA) is an evolutionarily conserved phenomenon during stress. Neuropeptide S (NPS), orexins, substance P, glutamate and endocannabinoids are known to be involved in stress and/or SIA, however their causal links remain unclear. Here, we reveal an unprecedented sequential cascade involving these mediators in the lateral hypothalamus (LH) and ventrolateral periaqueductal gray (vlPAG) using a restraint stress-induced SIA model.

Methods: Male C57BL/6 mice of 8–12 week-old were subjected to intra-cerebroventricular (*i.c.v.*) and/or intra-vlPAG (*i.pag.*) microinjection of NPS, orexin-A or substance P alone or in combination with selective antagonists of NPS receptors (NPSRs), OX₁ receptors (OX₁Rs), NK₁ receptors (NK₁Rs), mGlu₅ receptors (mGlu₅Rs) and CB₁ receptors (CB₁Rs), respectively. Antinociceptive effects of these mediators were evaluated via the hot-plate test. SIA in mice was induced by a 30-min restraint stress. NPS levels in the LH and substance P levels in vlPAG homogenates were compared in restrained and unrestrained mice.

Results: NPS (*i.c.v.*, but not *i.pag.*) induced antinociception. This effect was prevented by *i.c.v.* blockade of NPSRs. Substance P (*i.pag.*) and orexin-A (*i.pag.*) also induced antinociception. Substance P (*i.pag.*)-induced antinociception was prevented by *i.pag.* blockade of NK₁Rs, mGlu₅Rs or CB₁Rs. Orexin-A (*i.pag.*)-induced antinociception has been shown previously to be prevented by *i.pag.* blockade of OX₁Rs or CB₁Rs, and here was prevented by NK₁R or mGlu₅R antagonist (*i.pag.*). NPS (*i.c.v.*)-induced antinociception was prevented by *i.pag.* blockade of OX₁Rs, NK₁Rs, mGlu₅Rs or CB₁Rs. SIA has been previously shown to be prevented by *i.pag.* blockade of OX₁Rs or CB₁Rs. Here, we found that SIA was also prevented by *i.c.v.* blockade of NPSRs or *i.pag.* blockade of NK₁Rs or mGlu₅Rs. Restrained mice had higher levels of NPS in the LH and substance P in the vlPAG than unrestrained mice.

(Continued on next page)

* Correspondence: lcchiou@ntu.edu.tw

†Ming Tatt Lee, Yu-Ting Chiu and Yu-Chun Chiu contributed equally to this work.

¹Graduate Institute of Brain and Mind Sciences, College of Medicine, National Taiwan University, Taipei 10051, Taiwan

²Graduate Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan

Full list of author information is available at the end of the article



(Continued from previous page)

Conclusions: These results suggest that, during stress, NPS is released and activates LH orexin neurons via NPSRs, releasing orexins in the vPAG. Orexins then activate OX₁Rs on substance P-containing neurons in the vPAG to release substance P that subsequently activates NK₁Rs on glutamatergic neurons to release glutamate. Glutamate then activates perisynaptic mGlu₅Rs to initiate the endocannabinoid retrograde inhibition of GABAergic transmission in the vPAG, leading to analgesia.

Keywords: Neuropeptide S, Orexin, Substance P, Metabotropic glutamate receptor, Endocannabinoid, Periaqueductal gray

Background

Stress-induced analgesia (SIA) is an evolutionarily protective system in mammals for coping with environmental stressors [1]. Several neuropeptides released during stress, such as orexins [2, 3], neuropeptide S (NPS) [4] and substance P [5], may contribute to SIA. However, how these neuropeptide-mediated signals interact to elicit SIA remains unknown.

Orexins, consisting of orexin-A and orexin-B [6], also known as hypocretin-1 and hypocretin-2 [7], are processed from prohypocretin in hypothalamic neurons in the perifornical area (PFA), lateral hypothalamus (LH) and dorsomedial hypothalamus (DMH) [6, 7]. Orexin receptors, OX₁ receptors (OX₁Rs) and OX₂ receptors (OX₂Rs), belong to the G-protein coupled receptor (GPCR) family [8]. In addition to being involved in arousal and reward regulation [9], orexins are antinociceptive [10–12] and are involved in SIA [2, 3, 12, 13]. Previously, we have shown that orexins can be released during stress and contribute to SIA, at least in part, via opioid-independent and endocannabinoid (eCB)-dependent signaling [11, 12] in the ventrolateral periaqueductal gray (vPAG), a crucial midbrain region for the initiation of descending pain inhibition [14, 15]. Specifically, orexins are released during stress [12], and orexins are known to induce antinociception by activating postsynaptic OX₁Rs to generate 2-arachidonoylglycerol (2-AG) [16, 17], an eCB, through a Gq protein-coupled enzymatic cascade mediated by phospholipase C (PLC) and diacylglycerol lipase (DAGL) [18], culminating in retrograde inhibition of GABA release (disinhibition) in the vPAG [11, 12].

NPS is an icosa-peptide named due to its conserved N-terminal residue, serine, in all species [4]. Central administration of NPS (intra-cerebroventricular, *i.c.v.*) is antinociceptive [19–21]. The site of this antinociceptive action could be the PAG, where the mRNA transcript of NPS receptors (NPSRs) is abundant [22, 23], or other NPSR-rich brain regions, such as the amygdala and hypothalamus [22]. All three areas are commonly associated with emotional behaviours, and NPS is therefore implicated in stress-related behaviours. Indeed, forced swimming or restraint stress significantly activated NPS

neurons in the pericoerulear region (peri-LC) and the Kölliker-Fuse nucleus of the lateral parabrachial area (KF-PBN) [24]. Intra-paraventricular nucleus (PVN) or *i.c.v.* administration of NPS in mice increased their locomotor and rearing activity, and plasma adrenocorticotrophic hormone (ACTH) and corticosterone levels, suggesting that NPS can activate the arousal system and the hypothalamus-pituitary axis (HPA) [25].

The findings that both NPS and orexins are involved in the regulation of arousal, reward and pain suggest an interaction between the NPS [26] and orexin systems [9]. Indeed, it has been demonstrated that NPS (*i.c.v.*) can activate orexin neurons in the LH, PFA and DMH of rats [27, 28], where NPSRs are abundantly expressed [23]. Moreover, NPS has been reported to be an upstream activator of hypothalamic orexin neurons in feeding [27] and addiction [28, 29] behaviours. This suggests that NPS can activate orexin neurons and exert its biological functions, possibly including SIA, indirectly, by promoting the release of orexins.

Substance P is an undecapeptide belonging to the neuropeptide (tachykinin) family [30] and exerts its effects mainly via NK₁ receptors (NK₁Rs) [31], a member of the GPCR family. Substance P is a well-known peripheral pronociceptive mediator [32] while it is antinociceptive at the supraspinal level [33]. In fact, intra-PAG microinjection (*i.pag.*) of substance P induces antinociception [34]. This effect may be mediated by the NK₁Rs in the PAG since it is blocked by an NK₁R antagonist and NK₁Rs are densely distributed in pain-modulating brain regions including the PAG [35].

Using an electrophysiological approach, Drew et al. (2009) [36] have investigated how substance P modulates synaptic transmission in brain slices containing the vPAG. They demonstrated that substance P decreased evoked GABA release in vPAG slices. This effect was abolished by an inhibitor of DAGL, a degradation enzyme of 2-AG, and an antagonist of mGlu₅ receptors (mGlu₅R). Importantly, a glutamate transporter inhibitor mimicked the GABA-reducing effect of substance P, but also occluded such action of substance P [37]. However, substance P markedly increased action potential-driven spontaneous glutamate release. It is suggested that

substance P induces an enormous release of glutamate that may activate perisynaptic mGlu₅R to initiate the eCB-mediated retrograde disinhibition mechanism in the vPAG. They suggested this effect may contribute to the substance P-induced analgesic effect in the vPAG [37], however no pain behaviors were not evaluated. Substance P in the PAG may also contribute to SIA since restraint stress [38] and LH stimulation [39] increased the substance P level in the PAG and *i.pag.* blockade of NK₁R abolished LH-stimulation-induced antinociception. However, there are no direct *in vivo* studies supporting the involvement of PAG substance P in SIA. Taking into consideration the the complexity of the aforementioned neuropeptides in SIA, a scheme depicting the possible relationships among NPS, orexins, substance P, mGlu₅R and eCB (2-AG) during SIA, based on the available literature, is illustrated in Fig. 1.

Substance P-induced vPAG disinhibition, which is mediated through mGlu₅R-initiated eCB signaling, highly resembles the OX₁R-initiated 2-AG/CB₁R signaling we reported previously [11], which contributes to SIA [12]. Moreover, mGlu₅R [40] and eCBs [41, 42] are also involved in SIA. These events, all taking place in the vPAG, prompted us to hypothesize the involvement of NK₁R and mGlu₅R

in orexin-induced antinociception, and subsequently their involvement in SIA, possibly as downstream effectors of NPS. In this study, via behavioral, pharmacological and neurochemical approaches, we first examined the involvement of NK₁R, mGlu₅R and CB₁R in substance P-induced antinociception. Next, we investigated whether orexins are upstream to substance P in the vPAG in eliciting antinociceptive effects. Then, we examined whether NPS is an upstream modulator of LH orexin neurons. Lastly, we studied the involvement of the NPSR-OX₁R-NK₁R-mGlu₅R-CB₁R pathway in SIA.

Materials and methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University following ARRIVE guidelines. Male C57BL/6 mice of 8–12 weeks were housed in groups of 10 in plastic cages and maintained in a holding room with a 12 h light-dark cycle with free access to food and water *ad libitum*. On the experimental day, mice were moved in their home cages to the behaviour room and acclimated there for at least 1 h before testing.

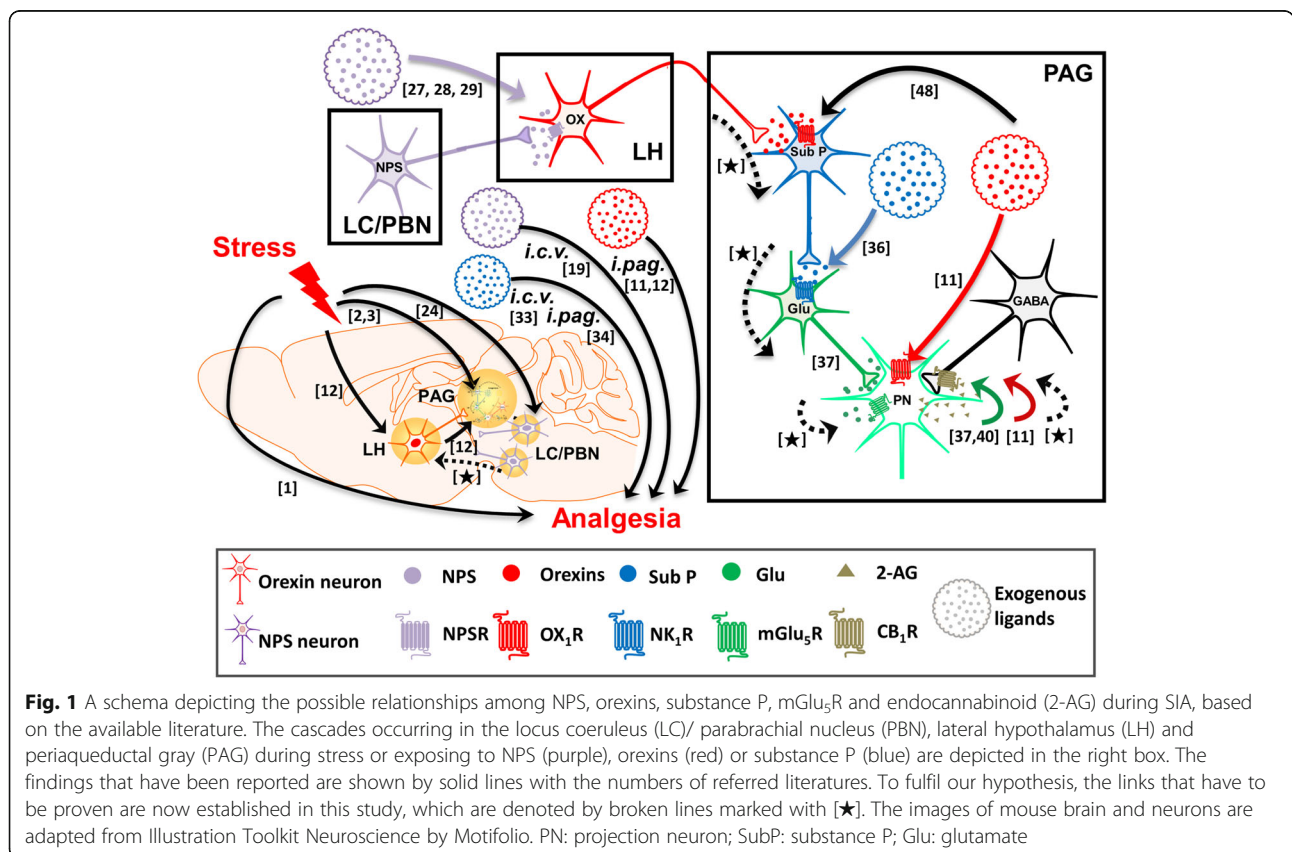


Fig. 1 A schema depicting the possible relationships among NPS, orexins, substance P, mGlu₅R and endocannabinoid (2-AG) during SIA, based on the available literature. The cascades occurring in the locus coeruleus (LC)/ parabrachial nucleus (PBN), lateral hypothalamus (LH) and periaqueductal gray (PAG) during stress or exposing to NPS (purple), orexins (red) or substance P (blue) are depicted in the right box. The findings that have been reported are shown by solid lines with the numbers of referred literatures. To fulfil our hypothesis, the links that have to be proven are now established in this study, which are denoted by broken lines marked with [★]. The images of mouse brain and neurons are adapted from Illustration Toolkit Neuroscience by Motifolio. PN: projection neuron; SubP: substance P; Glu: glutamate

Hot-plate test

The hot-plate test in mice was performed as reported previously [12]. Briefly, the mouse was placed on a hot-plate maintained at 50 °C and paw withdrawal latency was recorded with a cut-off time of 60 s to prevent tissue damage. The antinociceptive effect in each mouse at each time point was calculated as the % of the maximal possible effect (MPE) by the equation: $\%MPE = 100 \times (\text{Latency}_{\text{after treatment}} - \text{Latency}_{\text{before treatment}}) / (60\text{s} - \text{Latency}_{\text{before treatment}})$. The AUC of withdrawal latencies during the 60 min-recording period was calculated as the total antinociceptive effect in each mouse.

SIA

To induce SIA, mice were restrained in a 50-ml centrifugal tube with several small holes for 30 min as reported previously [12]. The control non-stress group of mice remained at their home cages for the same 30 min before being subjected to the hot-plate test.

Spontaneous locomotor activity

Locomotor activity was assessed by an open-field test in a 48 × 48 × 40 cm³ acrylic chamber with the arena floor divided into 36 squares, as described previously [43]. The mouse was placed in the center of the chamber, and the number of squares the mouse transpassed with forepaws (number of crossing) and the number of times the mouse stood up with forepaws on the floor (number of rearing) were counted for 5 min.

Intra-vlPAG (*i.pag.*) and intra-cerebroventricular (*i.c.v.*) microinjection

When drugs were given by *i.pag.* or *i.c.v.* microinjection, mice received the *i.pag.* or *i.c.v.* cannulation surgery 1 week before the microinjection experiment, as reported previously [12]. Briefly, under anesthesia with 50 mg/kg Zoletil® 50 (a mixture of tiletamine and zolazepam) and xylazine (10 mg/kg), the mouse was placed in a stereotaxic apparatus and a 24-gauge, 10 mm stainless-steel guide cannula was implanted into the right vlPAG (−4.8 mm caudal, −0.5 mm lateral, −2.8 mm ventral from bregma, Additional file 1: Figure S1A) or the right ventricle (−0.5 mm caudal, −1.0 mm lateral, −2.2 mm ventral from bregma, Additional file 1: Figure S1B), according to stereotaxic coordinates of mice [44]. On the day of experiments, *i.pag.* or *i.c.v.* microinjection was performed through a 30-gauge injection needle (10 mm) connected to a Hamilton syringe (1.0 µl) on a microinfusion pump (KDS311, KD Scientific Inc., Holliston, MA, USA). The drug solution (0.1 µl) was delivered in 60 s, followed by a 240 s-residual time to avoid back-flow of drug solution. Nociceptive responses were measured 5 mins before as well as 5, 10, 20, 30 and 40 mins after *i.pag.* or *i.c.v.* microinjections. For the mice that

underwent restraint stress, *i.pag.* or *i.c.v.* microinjections of the antagonists were performed 5 mins before stress, and nociceptive responses were measured immediately, 5, 10, 20, 30 and 40 mins after stress. After the final behavioural evaluation, the animals were microinjected with 0.5 µl of 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) through the guide cannula to verify the injection tract location. Animals were then sacrificed by decapitation, coronal brain sections (300 µm) were prepared on a vibratome (DSK microslicer DTK-1000, Dosaka, Japan). The injection site was identified by the presence of trypan blue stain diffusion in the vlPAG tissue. Only animals with the cannula correctly targeting the ventricle or vlPAG were included in data analysis.

Measurements of substance P in vlPAG and NPS in LH of brain tissue homogenates

The preparation of the mouse vlPAG and LH homogenate is the same as previously reported [12]. Briefly, immediately after restraint stress, the mouse was sacrificed. Its brain was removed, placed in a pre-cooled adult mouse brain slicer matrix (Roboz Surgical Instrument, Gaithersburg, MD, USA), and sliced into 1 mm-thick coronal sections. vlPAG or LH brain tissues were bilaterally punched out with a 0.5 mm-tip according to a mouse brain atlas [44]. Each vlPAG sample was collected from one mouse brain, whereas each LH sample was from two mouse brains. After ultrasonication in lysis buffer, the lysates were homogenized and centrifuged (1900 g, 14,000 rpm, 15 min) and supernatants collected. The protein concentration in the supernatant was measured by the Bradford method [45].

The substance P level in the vlPAG homogenate was measured with an EIA kit (Cat. No. 583751, Cayman Chemical, Ann Arbor, MI, USA) with a detection range of 3.9–500 pg/ml. The NPS level in the LH homogenates was measured with an ELISA kit. (Cat. No. CSB-EL016026MO, Cusabio, College Park, MD, USA) with a detection range of 4.69–300 pg/ml.

Chemicals

NPS and [tBu-D-Gly⁵] NPS were synthesised and purified as previously described [46]. N-(2-methyl-6-benzoxazolyl)-N-1,5-naphthyridin-4-yl-urea (SB-334867, a selective OX₁R antagonist), 6-methyl-2-(phenylethynyl)pyridine hydrochloride (MPEP, a selective mGlu₅R antagonist) and orexin-A were purchased from Tocris Bioscience (Bristol, UK). Substance P, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (AM251, a CB₁R antagonist), and *cis*-2-(Diphenylmethyl)-N-[(2-iodophenyl)methyl]-1-azabicyclo [2.2.2] octan-3-amine oxalate salt (L-703,606, a selective NK₁R antagonist) were purchased from Sigma-Aldrich. NPS and [tBu-D-Gly⁵] NPS were

dissolved in 0.9% normal saline. Substance P was dissolved in 0.1 M acetic acid. SB-334867, L-703,606, MPEP and AM251 were dissolved in dimethyl sulfoxide (DMSO). All drugs were prepared at the working concentration for the intended *i.pag.* or *i.c.v.* injection doses.

Statistical analysis

Data are expressed as the mean \pm S.E.M. and the “n” indicates the number of mice tested in each group. In the hotplate test, two-way ANOVA with post hoc Bonferroni test was used to analyse time courses of antinociceptive effects among different groups. The antinociceptive effect was also assessed by the area under the curve (AUC) of the time courses of quantification of the line graph from baseline to the last time-point of the experiment. Each AUC bar graph was calculated by one-way ANOVA followed by Tukey’s multiple comparison test. Student’s T-test was employed to analyse the results obtained in EIA and ELISA tests. Differences were considered significant if $p < 0.05$.

Results

NPS is antinociceptive when given by *i.c.v.* but not *i.pag.* microinjection in mice

NPS when given by *i.c.v.* injection at the doses 0.3 and 1.0 nmol, which did not affect the spontaneous locomotor activity of mice (Additional file 2: Figure S2), significantly prolonged the latency of nociceptive response in the hot-plate test (Fig. 2a and b) in a time- ($F_{6,38} = 5.696$, $p < 0.001$, two-way ANOVA, Fig. 2a) and treatment-dependent ($F_{4,23} = 10.25$, $p < 0.001$, two-way ANOVA, Fig. 2a) manner. However, when NPS was given by *i.pag.* microinjection, it did not produce significant antinociceptive effect at either 0.3 or 1.0 nmol (Fig. 2a and b). This suggests that the site of action for NPS-induced supraspinal antinociception is brain region(s) other than the vlPAG.

NPS (*i.c.v.*)-induced antinociception was antagonized by *i.c.v.* blockade of NPSRs

To investigate if the central antinociceptive effect of NPS is mediated via NPSR, we co-administered [tBu-D-Gly⁵] NPS (10 nmol, *i.c.v.*), a selective and potent NPSR antagonist [47], along with NPS (0.3 or 1.0 nmol, *i.c.v.*) to mice before the hot-plate test. [tBu-D-Gly⁵] NPS at 10 nmol (*i.c.v.*) did not affect the nociceptive response in naïve mice, but completely blocked the antinociceptive effect of *i.c.v.* NPS at the doses of 0.3 and 1.0 nmol (Fig. 2c and d) The overall comparison of the time course of the antinociceptive effect showed a significant difference between time and treatment ($F_{30,222} = 1.872$, $p = 0.0057$, two-way ANOVA, Fig. 2c). This suggests the central antinociceptive effect of NPS is mediated by NPSRs in the brain.

NPS (0.3 nmol, *i.c.v.*), substance P (5 nmol, *i.pag.*) and orexin-A (1 nmol, *i.pag.*) induced comparable antinociceptive effects in mice

To substantiate our hypothesis that a cascade mediated by NPS, orexins and substance P sequentially is involved in SIA, we evaluated equipotent doses of these three neuropeptides in a concurrent behavioural assay. As shown in Fig. 2e and f, NPS (0.3 nmol, *i.c.v.*) produced an antinociceptive effect in the mouse hot-plate test that was comparable to the effects induced by *i.pag.* microinjection of orexin-A at 1 nmol and *i.pag.* substance P at 5 nmol, respectively, with a significant difference between time and treatment ($F_{18,120} = 1.924$, $p = 0.0198$, two-way ANOVA, Fig. 2e).

Substance P (*i.pag.*)-induced antinociception was antagonized by *i.pag.* blockade of NK₁Rs, mGlu₅Rs or CB₁Rs

To ascertain if the NK₁R-mGlu₅R-CB₁R pathway revealed by the electrophysiological study of Drew et al. (2009) [36] is involved in *i.pag.* substance P-induced antinociceptive effect, we challenged the antinociceptive effect of substance P with selective antagonists of NK₁Rs (L-703,606), mGlu₅Rs (MPEP) and CB₁Rs (AM251), respectively, in a treatment-dependent manner ($F_{3,18} = 5.316$, $p = 0.0084$, two-way ANOVA, Fig. 3a; $F_{3,18} = 10.97$, $p = 0.0003$, two-way ANOVA, Fig. 3b; $F_{3,17} = 5.929$, $p = 0.0059$, two-way ANOVA, Fig. 3c). Indeed, *i.pag.* co-administration of L-703,606 (10 nmol), MPEP (30 nmol) or AM251 (30 nmol) with substance P (5 nmol) significantly antagonized the antinociceptive effect of *i.pag.* substance P (Fig. 3).

Orexin-A (*i.pag.*)-induced antinociception was antagonized by *i.pag.* blockade of NK₁Rs and mGlu₅Rs

Our previous findings that *i.pag.* orexin-A induced antinociception through an OX₁R-initiated eCB signalling [11], where the downstream mechanism was highly similar to *i.pag.* substance P induced antinociception as shown in the subsection above. In order to ascertain the interaction between orexin-A and substance P in the vlPAG, we challenged orexin-A-induced antinociception with *i.pag.* NK₁R and mGlu₅R antagonists, respectively. Co-administration of L-703,606 (10 nmol, *i.pag.*) or MPEP (30 nmol, *i.pag.*) significantly antagonized *i.pag.* orexin-A (1 nmol)-induced antinociception (Fig. 4). The overall comparison of time courses of the antinociceptive effect showed a significant difference between time and treatment ($F_{18,108} = 3.841$, $p < 0.001$, two-way ANOVA, Fig. 4a; $F_{18,108} = 4.597$, $p < 0.001$, two-way ANOVA, Fig. 4b). These results in combination with our previous findings suggest that orexin-A-induced antinociception is mediated by OX₁Rs, NK₁Rs, mGlu₅Rs and CB₁Rs sequentially in the vlPAG.

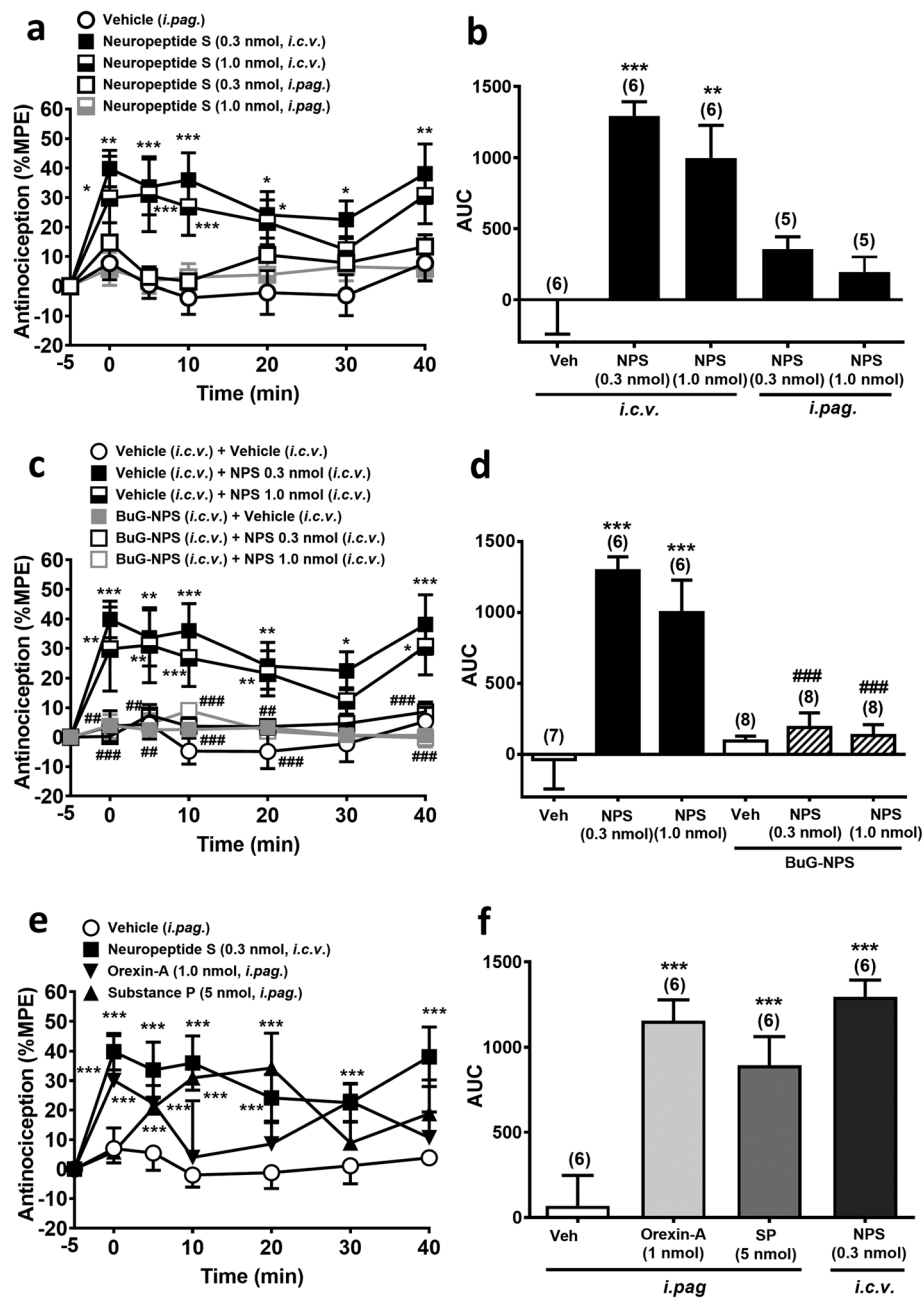


Fig. 2 Antinociceptive effects induced by NPS, orexin-A and substance P in the mouse hot-plate test. **a-b**: Antinociceptive effects of NPS (0.3 & 1.0 nmol) by *i.c.v.* or *i.pag.* microinjection. **c-d**: Antinociceptive effects of *i.c.v.* NPS challenged by an NPSR antagonist, [tBu-D-Gly²] NPS (10 nmol, *i.c.v.*). **e-f**: A comparison of antinociceptive effects of orexin-A (1 nmol, *i.pag.*), substance P (5 nmol, *i.pag.*), and NPS (0.3 nmol, *i.c.v.*). **a, c** and **e**: The time course of the antinociceptive effect expressed as the percentage of the maximal possible effect (MPE) (two-way ANOVA /post hoc Bonferoni test). **b, d** and **f**: The area under the curve (AUC) of the % MPE measured within 40 min in each treatment group (one-way ANOVA /post hoc Tukey test). The number denoted in the parentheses above each bar is the *n* number of mice tested in each group. Data are mean ± S.E.M. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the vehicle control group, ###*p* < 0.001 vs. NPS 0.3 or 1.0 group

NPS (*i.c.v.*)-induced antinociception was antagonized by *i.pag.* blockade of OX₁Rs, NK₁Rs, mGlu₅Rs or CB₁Rs

Next, we investigated whether the now established OX₁R-NK₁R-mGlu₅R-CB₁R cascade in the vIPAG is involved in the supraspinal antinociceptive effect of NPS.

Co-administration of the respective antagonists of OX₁Rs (SB-334867, 15 nmol, *i.pag.*), NK₁Rs (L-703,606, 10 nmol, *i.pag.*), mGlu₅Rs (MPEP, 30 nmol, *i.pag.*) or CB₁Rs (AM251, 30 nmol, *i.pag.*), significantly suppressed the antinociceptive effect of *i.c.v.* NPS (0.3 nmol) (Fig. 5),

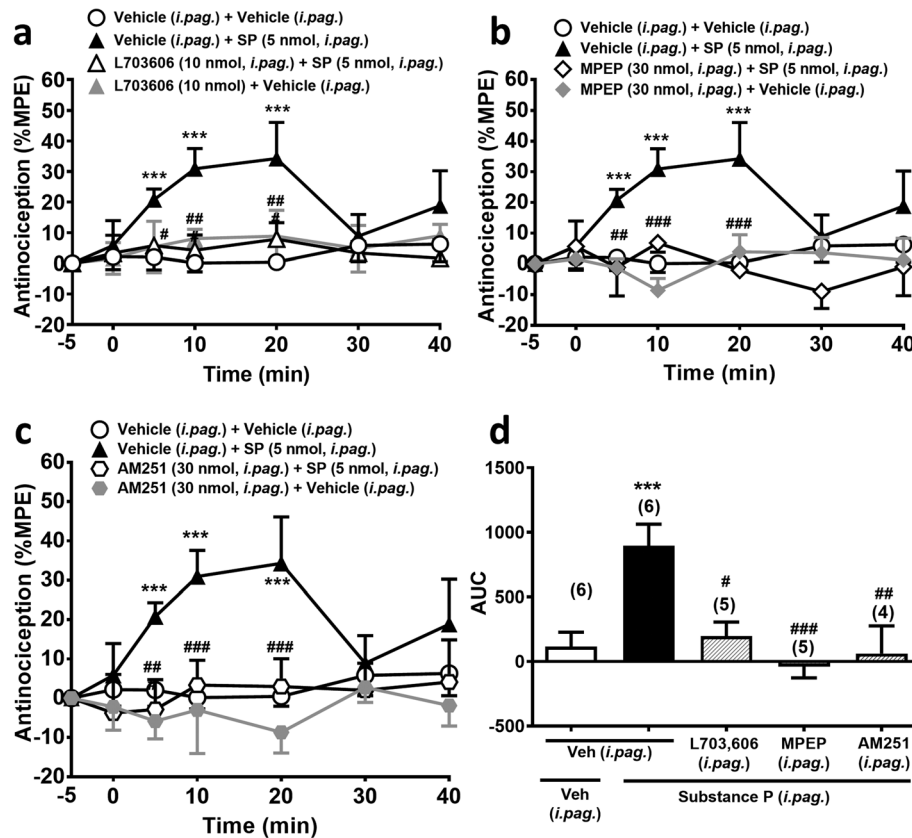


Fig. 3 Substance P (*i.pag.*)-induced antinociception is antagonized by *i.pag.* blockade of NK₁Rs, mGlu₅Rs or CB₁Rs. **a-c.** Time courses of antinociceptive effects (expressed as % MPE) induced by substance P (5 nmol, *i.pag.*) in combination with the vehicle or the antagonist of NK₁Rs (L-703,606, 10 nmol, *i.pag.*, **a**), mGlu₅Rs (MPEP, 30 nmol, *i.pag.*, **b**), and CB₁Rs (AM251, 30 nmol, *i.pag.*, **c**) in the mouse hot-plate test. (two-way ANOVA /post hoc Bonferroni test). **d:** The AUC of the antinociceptive effect in each treatment group (one-way ANOVA /post hoc Tukey test). The antagonist was *i.pag.* co-administered with *i.pag.* substance P. The data presentation and statistics are the same as in Fig. 2. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the vehicle control group; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. the Substance P group

in a time- ($F_{6,114} = 3.252$, $p = 0.0055$, two-way ANOVA, Fig. 5a; $F_{6,114} = 2.936$, $p = 0.0106$, two-way ANOVA, Fig. 5b; $F_{6,114} = 2.603$, $p = 0.211$, two-way ANOVA, Fig. 5c; $F_{6,114} = 2.2$, $p = 0.0479$, two-way ANOVA, Fig. 5d) and treatment- ($F_{3,19} = 36.96$, $p < 0.001$, two-way ANOVA, Fig. 5a; $F_{3,19} = 28.58$, $p < 0.001$, two-way ANOVA, Fig. 5b; $F_{3,19} = 67.33$, $p < 0.001$, two-way ANOVA, Fig. 5c; $F_{3,19} = 23.44$, $p < 0.001$, two-way ANOVA, Fig. 5d) dependent manner. Thus, *i.c.v.* NPS-induced analgesia is mediated by the OX₁Rs, NK₁Rs, mGlu₅Rs and CB₁Rs in the vlPAG.

Restraint stress-induced analgesia was prevented by *i.c.v.* blockade of NPSR or *i.pag.* blockade of OX₁Rs, NK₁Rs or mGlu₅Rs

We have previously demonstrated that the vlPAG OX₁R-CB₁R pathway is involved in the SIA induced by an acute restraint stress in mice [12]. We subsequently examined whether the now established NPSR-OX₁R-NK₁R-mGlu₅R-CB₁R cascade is involved in the

SIA induced by the same restraint stress protocol. Mice receiving an acute restraint stress for 30 min exhibited significant reduced paw withdrawal response in the hot-plate test. This SIA diminished within 20 min (Fig. 6a) [12] and was significantly prevented in mice *i.c.v.* pre-treated with an NPSR antagonist ([tBu-D-Gly⁵] NPS, 10 nmol) (Fig. 6a and d) or with *i.pag.* Pretreated with NK₁R (L-703,606, 10 nmol) (Fig. 6b and d) and mGlu₅R (MPEP, 30 nmol) antagonists (Fig. 6c and d), respectively. The overall comparison of the time course of the antinociceptive effect showed a significant difference between time and treatment ($F_{18,114} = 4.317$, $p < 0.001$, two-way ANOVA, Fig. 6a; $F_{18,108} = 3.780$, $p < 0.001$, two-way ANOVA, Fig. 6b; $F_{18,108} = 3.501$, $p < 0.001$, two-way ANOVA, Fig. 6c). Take together with our previous findings that SB-334867 (15 nmol, *i.pag.*) and AM251 (30 nmol, *i.pag.*) prevented SIA [12], it is suggested that SIA is mediated via the NPSR-evoked OX₁R-NK₁R-mGlu₅R-CB₁R cascade in the vlPAG.

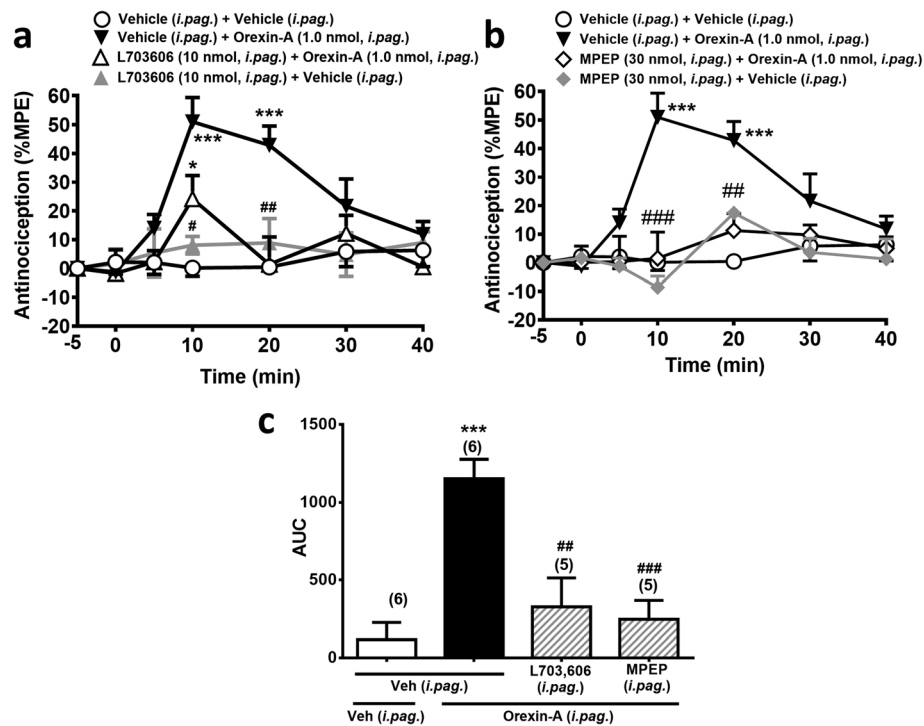


Fig. 4 Orexin-A (*i.pag.*)-induced antinociception is antagonized by *i.pag.* blockade of NK₁Rs or mGlu₅Rs. **a-b:** Time courses of antinociceptive effects (expressed as % MPE) induced by orexin-A (1.0 nmol, *i.pag.*) in combination with the vehicle or the antagonist of NK₁Rs (L-703,606, 10 nmol, *i.pag.*, **a**) or mGlu₅Rs (MPEP, 30 nmol, *i.pag.*, **b**) in the mouse hot-plate test. (two-way ANOVA /post hoc Bonferroni test). **c:** The AUC of the antinociceptive effect in each treatment group (one-way ANOVA /post hoc Tukey test). The antagonist was *i.pag.* co-administered with *i.pag.* orexin-A. The data presentation and statistics are the same as in Fig. 2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the vehicle control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the orexin-A group

All the tested antagonists at the administered doses that attenuated SIA had no effect on the number of crossing and rearing, including the NPSR antagonist ([tBu-D-Gly⁵] NPS, 10 nmol, *i.c.v.*) (Additional file 3: Figure S3a and b), the NK₁R antagonist (L-703,606, 10 nmol, *i.pag.*) (Additional file 3: Figure S3, C and D) and the mGlu₅R antagonist (MPEP, 30 nmol, *i.pag.*) (Additional file 3: Figure S3, E and F), similar to the OX₁R antagonist (SB-334867, 15 nmol, *i.pag.*) and the CB₁R antagonist (AM251, 30 nmol, *i.pag.*) which we have reported previously [12]. This supports that these antagonists attenuate SIA by blocking their respective endogenous ligands.

Restraint stress elevated NPS level in LH and substance P level in vIPAG

Measurement of the neuropeptide content in the brain homogenate revealed that restraint stress significantly elevated the NPS level in the LH (df = 12, $t = 2.987$, $p < 0.05$, Student's *t*-test, Fig. 7a) and the substance P level in the vIPAG (df = 9, $t = 2.72$, $p < 0.05$, Student's *t*-test, Fig. 7b). Similar elevations in orexin-A levels were observed in the vIPAG of restrained mice as we previously reported [12].

Discussion

In this study, we found that the antinociceptive effect of *i.c.v.* NPS was blocked by *i.c.v.* injection of an NPSR antagonist and *i.pag.* injection of antagonists for OX₁Rs, NK₁Rs, mGlu₅Rs and CB₁Rs, respectively. These results suggest that orexins, substance P, glutamate and eCBs in the vIPAG are involved in supraspinal NPS-induced antinociception. In addition, blockade of either NPSRs, OX₁Rs, NK₁Rs, mGlu₅Rs or CB₁Rs suppressed the antinociception induced by a 30 min-restraint stress that increased the NPS level in the LH as well as the substance P level in the vIPAG. This suggests that NPS plays a role in SIA by activating the OX₁R-NK₁R-mGlu₅R-CB₁R-mediated sequential cascade that leads to antinociception through a disinhibition mechanism (i.e. inhibition of GABA release) mediated by GqPCR-PLC-DAGL-2-AG-CB₁R signalling in the vIPAG [11, 12] (Fig. 8). Our results also suggest that restraint stress suppresses pain sensitivity *in vivo* by engaging the NPS-orexin-A-substance P-glutamate signalosome to initiate the eCB-mediated retrograde disinhibition mechanism in the vIPAG. Integrating with the existing literature, the findings from the present study may fill in the gaps,

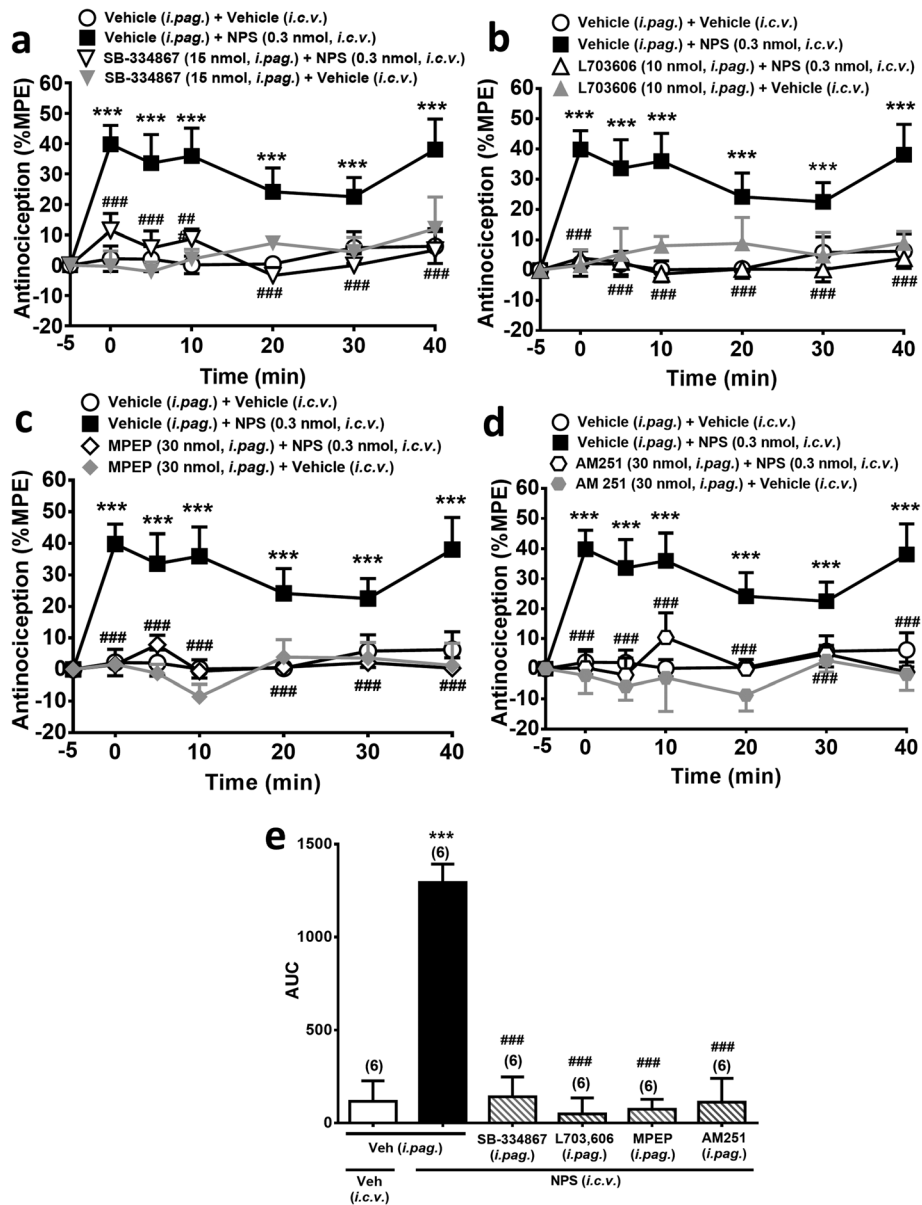


Fig. 5 NPS (*i.c.v.*)-induced antinociception is antagonized by *i.pag.* blockade of OX₁Rs, NK₁Rs, mGlu₅Rs or CB₁Rs. **a-d**: Time courses of antinociceptive effects (expressed as % MPE) induced by NPS (0.3 nmol, *i.c.v.*) in combination with the vehicle or the antagonists of OX₁Rs (SB-334867, 15 nmol, *i.pag.*), NK₁Rs (L-703,606, 10 nmol, *i.pag.*), mGlu₅Rs (MPEP, 30 nmol, *i.pag.*) or CB₁Rs (AM251, 30 nmol, *i.pag.*) in the mouse hot-plate test. (two-way ANOVA /post hoc Bonferroni test). **e**: The AUC of the antinociceptive effect in each treatment group (one-way ANOVA /post hoc Tukey test). The antagonist was *i.pag.* Administered immediately before *i.c.v.* injection of NPS. The data presentation and statistics are the same as in Fig. 2. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the vehicle control group; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. the NPS group

denoted as [★], among the signalling pathways of SIA as demonstrated by several research groups, as illustrated in Fig. 1.

Substance P exerted antinociceptive effect via NK₁Rs, mGlu₅Rs and CB₁Rs in the vlPAG

Drew et al. [36], using an electrophysiological approach, has shown that, in the vlPAG, substance P can facilitate the release of glutamate that

subsequently activates postsynaptic mGlu₅Rs located at perisynaptic sites, leading to the synthesis of 2-AG that retrogradely inhibits presynaptic GABA release via CB₁Rs. Gregg et al. [40], using a behavioural approach, also demonstrated that activating mGlu₅Rs in the PAG can induce an antinociceptive effect mediated by 2-AG and CB₁Rs. Here, we further demonstrated that this substance P-initiated and mGlu₅R-mediated eCB retrograde signalling contributes to the

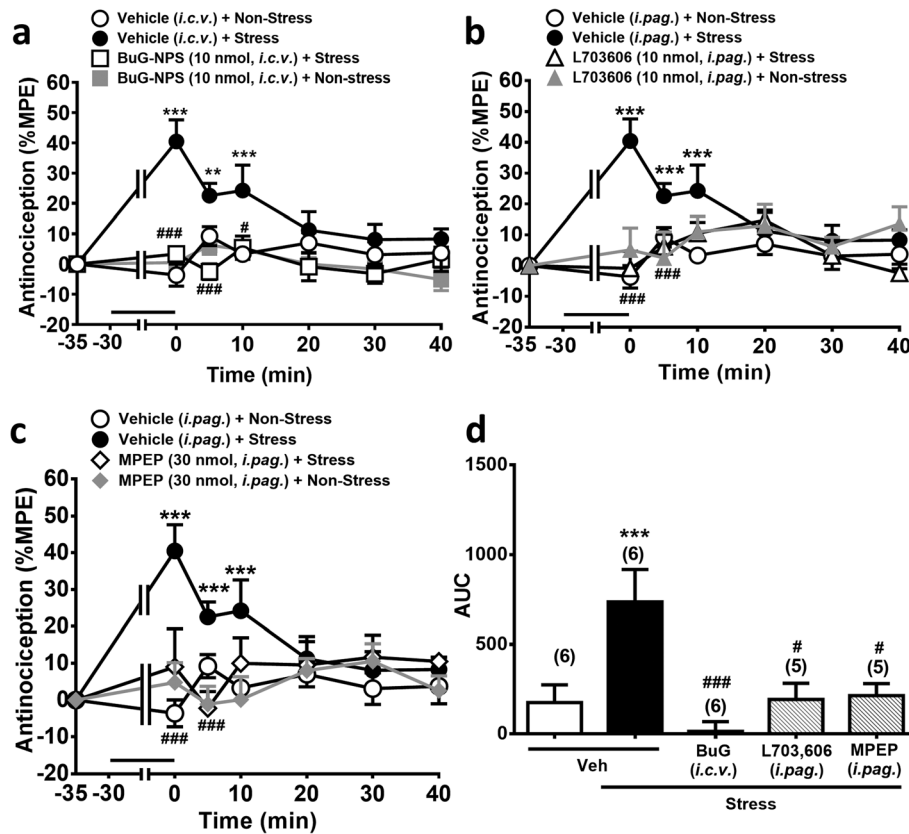


Fig. 6 Restraint stress-induced antinociception (SIA) is prevented by *i.c.v.* blockade of NPSR or by *i.pag.* blockade of NK₁Rs or mGlu₅Rs. **a-c**: Time courses of antinociceptive effects (expressed as % MPE) induced by a 30 min-restraint stress (horizontal bars) in mice pre-treated with vehicle or the antagonist of NPSRs ([tBu-D-Gly⁵] NPS, 10 nmol, *i.c.v.*), NK₁Rs (L-703,606, 10 nmol, *i.pag.*) or mGlu₅Rs (MPEP, 30 nmol, *i.pag.*) in the hot-plate test. (two-way ANOVA /post hoc Bonferroni test). **d**: The AUC of the antinociceptive effect in each treatment group (one-way ANOVA /post hoc Tukey test). The antagonist was *i.c.v.* or *i.pag.* administered immediately before restraint stress. The data presentation and statistics are the same as in Fig. 2. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the vehicle control group; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. the Stress group

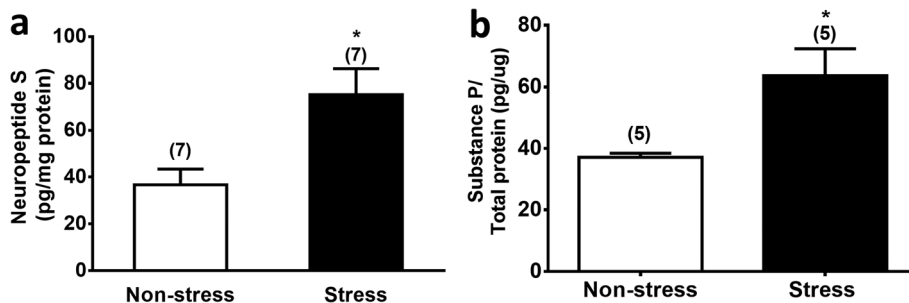


Fig. 7 Restraint stress increases the NPS level in the LH (**a**) and the substance P level in the vIPAG (**b**). Brain tissues containing the LH or vIPAG were punched and homogenized from restrained mice immediately after a 30 min-restraint stress (stress group) or from unrestrained control mice (non-stress group). NPS levels in LH homogenates were measured by an ELISA kit (Cusabio, College Park, MD, USA), whereas substance P level in vIPAG homogenates were measured by an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). **p* < 0.05, ***p* < 0.01 vs. the Non-stress control group (Student's t-test)

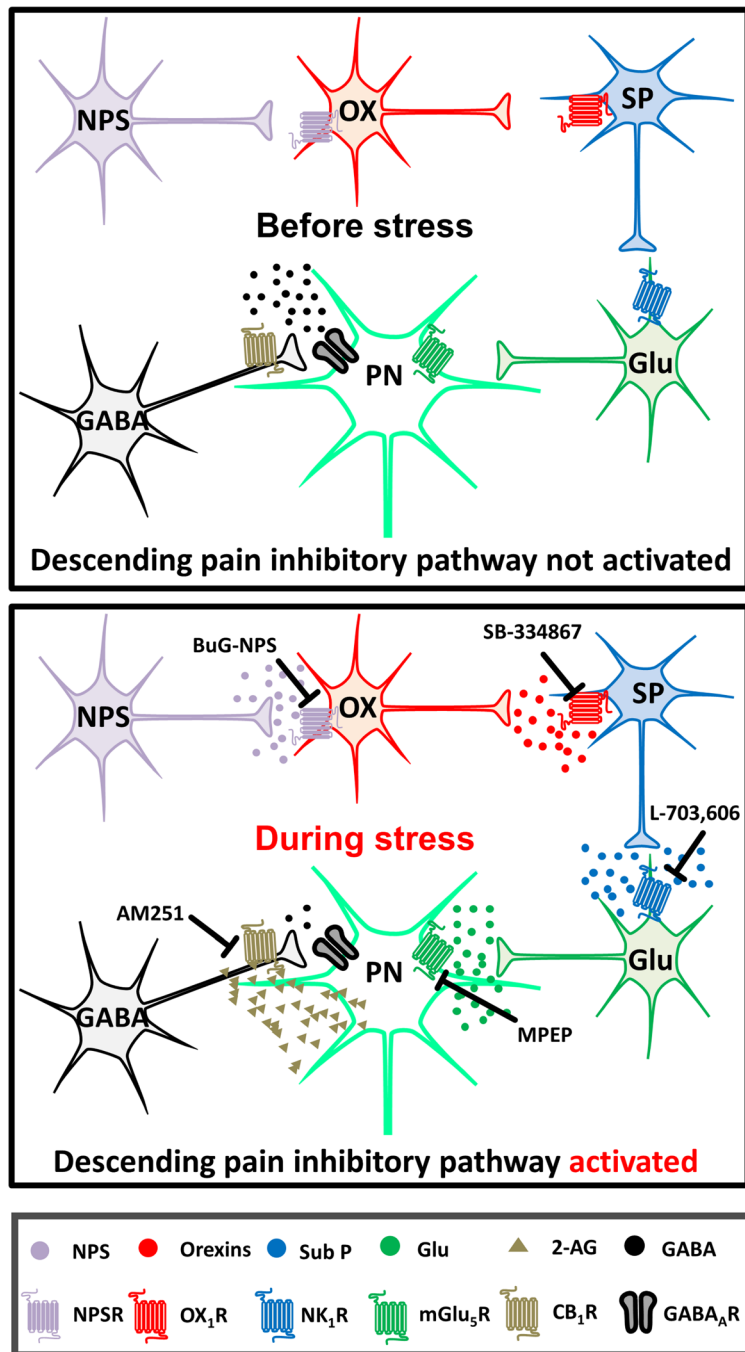


Fig. 8 A proposed schema illustrating how NPS, orexins, substance P, mGlu₅R and endocannabinoid (2-AG) may be involved in SIA. Before stress, the projection neurons in the vIPAG is under GABAergic inhibitory control. During stress, hypothalamic orexin neurons (OX) are activated by NPS, which is released possibly from the NPS neurons in peri-LC and/or the KF-PBN in mice [24], releasing orexins that activate the OX₁R on neurokinin (SubP) neurons and release substance P in the vIPAG. Then, substance P activates the NK₁R-containing glutamate (Glu) neurons, yielding massive glutamate that in turn activates perisynaptic mGlu₅R to initiate the GqPCR signalling and generate 2-AG. This endocannabinoid then retrogradely activates presynaptic CB₁R to inhibit GABA release in the vIPAG, ultimately leading to analgesia. The points of pharmacological intervention performed in this study are marked with blunt arrows, labelled with the respective antagonists. The images of neurons are adapted from Illustration Toolkit Neuroscience by Motifolio. PN: projection neuron. GABA_AR: GABA_A receptor

antinociceptive effect of substance P in the vPAG since *i.pag.* substance P-induced antinociception was antagonized by *i.pag.* blockade of NK₁Rs, mGlu₅Rs or CB₁Rs. The present study also supports that substance P is antinociceptive at the supraspinal level and the vPAG is one of the sites of action.

The substance P-NK₁R-glutamate-mGlu₅R cascade acts downstream of orexin-induced antinociception in the vPAG

The finding that the NK₁R antagonist attenuated orexin-induced antinociception in the vPAG (Fig. 4) suggests that substance P acts downstream of orexin-induced antinociception. This finding is in agreement with a recent study that reported that the level of substance P in the vPAG was increased following *i.pag.* orexin-A administration in rats [48]. Previously, we have shown that orexin via OX₁Rs induces analgesia through a GqPCR-PLC-DAGL-2-AG-CB₁R retrograde disinhibition mechanism in the vPAG [11]. Given that mGlu₅R, a GPCR, is also coupled to Gq proteins and mediates the antinociceptive effect via the same 2-AG-dependent disinhibition mechanism in the PAG [40] as the OX₁R [11], it is reasonable to suggest that the mGlu₅R is a downstream target after OX₁R-NK₁R activation. That is, orexin may induce analgesia via a cascade mediated by the OX₁R-substance P-NK₁R-glutamate-mGlu₅R-PLC-DAGL-2-AG-CB₁R signalling sequentially in the PAG (Fig. 8).

This sequential cascade may be able to explain the previous finding that *i.pag.* blockade of NK₁Rs attenuated LH stimulation-induced antinociception [49]. It is likely that orexin is the mediator released from the LH to induce antinociception indirectly via the NK₁R in the PAG. Additionally, involvement of substance P in the antinociceptive effect of orexin may also explain our previous electrophysiological finding that, in certain recorded vPAG neurons, orexin-A did not induce postsynaptic depolarization but attenuated GABA release via presynaptic CB₁Rs [11]. In addition to the 2-AG spill-over hypothesis, orexin-A may activate neurokinin neurons to release substance P that indirectly inhibits GABA release via mGlu₅R-eCB signalling in those neurons that were not depolarized by orexin-A.

The PAG is not the site of action for NPS-induced supraspinal antinociception

In agreement with previous studies that *i.c.v.* NPS was antinociceptive in swiss mice [19–21], we also found *i.c.v.* NPS reduced the hot-plate nociceptive response in C57BL/6JNarl mice. Peng et al. [20] suggested that the PAG is likely the site of action of NPS since *i.c.v.* NPS increased the c-Fos expression in the PAG where the NPSR mRNA is abundant [23]. However, our findings that direct *i.pag.* microinjection of NPS failed to induce

antinociception and that *i.c.v.*, but not *i.pag.*, blockade of NPSRs antagonized *i.c.v.* NPS-induced antinociception indicate that *i.c.v.* NPS may act in brain regions other than the PAG to exert its antinociceptive effect.

NPS-induced antinociception is mediated via OX₁R-NK₁R-mGlu₅R-CB₁R sequentially in the vPAG

The findings that *i.pag.* blockade of OX₁Rs, NK₁Rs, mGlu₅Rs and CB₁Rs prevented *i.c.v.* NPS-induced antinociception suggest the involvement of the OX₁R-NK₁R-mGlu₅R-CB₁R signalling in the vPAG in the supraspinal antinociceptive action of NPS. The site of action is likely in the hypothalamic areas where orexin neurons are located, especially the LH that is involved in pain regulation. Ideally, it would be more precise to study the action of NPS and its antagonist on orexin neurons in the LH via intra-LH microinjection. However, due to the difficulty of performing both intra-LH and *i.pag.* cannulations in mice, *i.c.v.* and *i.pag.* microinjections were employed (Figs. 5 and 6). Nevertheless, several studies have suggested an interaction between NPS and orexin systems. Anatomical and functional studies suggest that NPS can activate orexin neurons and may modulate biological functions indirectly via released orexins. First, the hypothalamic regions where orexin neurons are located, including the LH, PFA, and DMH, are enriched with NPSRs [23]. Second, after *i.c.v.* injection of NPS in rats, fos-immunoreactive cells in the hypothalamus, especially in the LH, were orexin-A-positive [27, 28]. Third, NPS has been reported to be an upstream activator of the orexin system in feeding [27] and addiction [28] behaviours acting in the hypothalamus. Therefore, it is likely that NPS activates the orexin neurons at the LH, releasing orexins in the vPAG to induce antinociception.

SIA is mediated by endogenous NPS-initiated hypothalamic orexins via the OX₁R-NK₁R-mGlu₅R-CB₁R-mediated sequential cascade in the vPAG

Previously, we have demonstrated that SIA is mediated by orexins released from the LH, an important region for SIA [13], via an OX₁R-initiated 2-AG-dependent disinhibition mechanism in the vPAG [12]. Here, we extend the findings in this study to suggest that NPS activates hypothalamic orexin neurons and add substance P as downstream of vPAG OX₁R activation in this SIA mechanism. That is, during stress, hypothalamic orexin neurons are activated by NPS, which is released possibly from the peri-LC and/or the KF-PBN in mice [24], releasing orexins that activate the OX₁Rs on neurokinin neurons in the vPAG. Then, substance P is released and activates the NK₁R-containing glutamate neurons, yielding massive glutamate release that in turn activates perisynaptic mGlu₅Rs to initiate GqPCR

signalling and generation of 2-AG. This eCB then retrogradely activates presynaptic CB₁Rs to inhibit GABA release in the vPAG, ultimately leading to analgesia (Figs. 1 and 8). This conclusion is based on the following findings, which may fill the gaps [★] in the schema depicted in Fig. 1, that (1) stress increased NPS levels in the LH (Fig. 7a) and SIA was reduced by blocking NPSRs (Fig. 6a); (2) stress increased orexin levels in the vPAG and SIA was reduced by blocking OX₁Rs in the vPAG [12]; (3) stress increased substance P levels (Fig. 7b) and SIA was reduced by blocking NK₁Rs in the vPAG (Fig. 6b); (4) SIA was reduced by blocking either mGlu₅Rs (Fig. 6c), CB₁Rs or DAGL in the vPAG [12]. The antagonist/inhibitor of NPSRs (BuG-NPS, Fig. 6a), OX₁Rs (SB-334867) [12], NK₁Rs (L-703,606, Fig. 6b), mGlu₅Rs (MPEP, Fig. 6c), CB₁Rs (AM251) or DAGL (tetrahydrolipstatin) [12] employed at the dose blocking SIA, per se, did not affect nociceptive threshold in unrestrained normal mice, suggesting no non-specific effects of these antagonists employed at the concentrations used in this study.

Since 1990s, substance P has been reported to play a role in SIA while the site(s) of action remain unidentified. Rosen et al. [38] reported that substance P was released from the PAG of animals in response to a behavioural stress, suggesting that endogenous substance P contributes to SIA originated from the PAG-mediated descending pain inhibition. The finding that the antinociceptive effect induced by stimulating the LH was abolished by *i.pag.* L-703,606 [39], suggesting that stimulating the LH can release substance P to induce antinociception via the NK₁Rs in the PAG. Here, we provided direct evidence supporting that SIA is mediated by elevated substance P in the PAG.

Several lines of evidence have indicated the involvement of NPS in stress-induced responses. NPSRs are enriched in the amygdala and hypothalamus [22], stress-related brain regions. The number of *c-fos*-containing NPS neurons in the peri-LC and KF-PBN was increased after a short-term forced swim stress or restraint stress [24]. The current finding that the acute restraint stress that induces analgesia can increase the NPS level in the LH directly supports that NPS is released during stress and contributes to SIA.

Several reports have indicated a cross-modulatory relationship between NPS and the corticotrophin releasing factor (CRF) system in stress-related responses. Paneda et al. [50] reported that CRF₁ receptor may mediate NPS-induced cocaine reinstatement in mice. Conversely, Jungling et al. [4], demonstrated that CRF can modulate NPS neurons in the LC of mice following acute stress. It remains to be elucidated if interactions between the CRF system and the NPSR-OX₁R-NK₁R-mGlu₅R-CB₁R-mediated sequential cascade in SIA occur.

Limitations of the current study

In the current study, we found that NPS (*i.c.v.*) at 0.3 and 1 nmol in C57BL/6 did not induce significant hyperlocomotion (Additional file 2: Figure S2). This is different with previous studies, where *i.c.v.* NPS at doses of 0.1 and 1.0 nmol induced hyperlocomotion in C57BL/6 [50] and Swiss mice [4]. However, Rizzi et al. [51], Castro et al. [52] and Boeck et al. [53] consistently demonstrated that *i.c.v.* NPS, only at the dose of 0.1 nmol, but not 0.01 and 1.0 nmol, exhibited significant hyperlocomotion in CF-1 mice. Furthermore, Holanda et al. [21] reported that *i.c.v.* NPS at 0.1 nmol did not increase locomotor activity in CF-1 mice. The discrepancy among studies is unclear. It may be that the *i.c.v.* NPS doses employed under the conditions (mouse strain and the motor activity assessment) in the present study fall outside the optimal dose for inducing hyperlocomotion.

Conclusions

During stress, NPS is released to activate hypothalamic orexin neurons, releasing orexins that activate OX₁Rs on neurokinin neurons in the vPAG, releasing substance P that activates NK₁Rs on glutamate neurons, yielding massive glutamate that in turn activates perisynaptic mGlu₅Rs to initiate the G_qPCR signalling and then generate 2-AG, which then retrogradely activates presynaptic CB₁Rs to inhibit GABA release in the vPAG, ultimately leading to analgesia (Fig. 8).

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12929-019-0590-1>.

Additional file 1: Figure S1. The representative diagram of *i.pag.* (A) and *i.c.v.* (B) microinjections in mice. The diagrams were adapted from mouse brain atlas [44]. The black dots (A) and white dots (B) represent the microinjection sites.

Additional file 2: Figure S2. Effects of NPS on locomotor activity. Locomotor activity in the open field test was measured before and 10 min after *i.c.v.* administration of 0.3 nmol (A-B) or 1 nmol (C-D) of NPS. Locomotor activity was assessed by the number of crossing (A & C) and rearing (B & D) in the open field test for 5 min. Data are expressed as the mean ± S.E.M. (Unpaired t-test)

Additional file 3: Figure S3. Effects of [tBu-D-Gly⁵] NPS, L-703,606 or MPEP on locomotor activity. Locomotor activity in the open field test was measured before and 10 min after administration of [tBu-D-Gly⁵] NPS (10 nmol, *i.c.v.*) (A-B), L-703,606 (10 nmol, *i.pag.*) (C-D), or MPEP (30 nmol, *i.pag.*) (E-F). Locomotor activity was assessed by the number of crossing (A, C & E) and rearing (B, D & F) in the open field test for 5 min. Data are expressed as the mean ± S.E.M. (Unpaired t-test)

Abbreviations

2-AG: 2-arachidonoylglycerol; AM251: 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide; CB₁R, CB₂R: cannabinoid receptor; CRF: Corticotrophin releasing factor; DAGL: Diacylglycerol lipase; DMH: Dorsomedial hypothalamus; eCB: endocannabinoid; ELA: Enzyme immunoassay; ELISA: Enzyme-linked immunosorbent assay; GqPCR: Gq-protein coupled receptor; HPA: Hypothalamus-pituitary axis; *i.c.v.*: intracerebroventricular; *i.pag.*: intra-ventrolateral periaqueductal gray; L-703,606: *cis*-2-(Diphenylmethyl)-N-[(2-iodophenyl)methyl]-1-azabicyclo [2.2.2]

octan-3-amine oxalate salt; LC: Locus coeruleus; LH: Lateral hypothalamus; mGlu₅R: mGlu₅ receptor; MPE: Maximal possible effect; MPEP: 2-methyl-6-(phenylethynyl) pyridine hydrochloride; NK₁R: NK₁ receptor; NPS: Neuropeptide S; NPSR: Neuropeptide S receptor; OX₁R: OX₁ receptor; OX₂R: OX₂ receptor; PBN: Parabrachial nucleus; PFA: Perifornical area; PLC: Phospholipase C; PVN: Paraventricular nucleus; SB-334867: N-(2-Methyl-6-benzoxazolyl)-N'-1,5-naphthyridin-4-yl urea; SIA: Stress-induced analgesia; vIPAG: ventrolateral periaqueductal gray

Acknowledgements

We appreciate the contribution from Prof. Ken Mackie (Indiana University Bloomington, USA) for providing critical comments and suggestions in improving this manuscript.

Authors' contributions

MTL contributed to data interpretation, data analysis, results discussion and paper writing; YTC and YCC conducted experiments, analyzed data and paper writing; CCH and HJL conducted the experiments; RG and GC synthesized and provided compounds as well as contributed to results discussion; LCC designed experiments, analyzed data and wrote the paper. All authors read and approved the final manuscript.

Funding

This study was supported by grants from the Ministry of Science and Technology, Taiwan (MOST104-2314-B-002-053-MY3, MOST106-2321-B-002-019, MOST106-2811-B-002-124, MOST107-2321-B-002-010, MOST107-2811-B-002-008, MOST 108-2321-B-002-005 and MOST 108-2320-B-002-029-MY3), National Health Research Institutes, Taiwan (NHRI-EX102-10251NI, NHRI-EX107-10733NI) and Ministry of Education, Taiwan (107 M4022-3).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University following ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Graduate Institute of Brain and Mind Sciences, College of Medicine, National Taiwan University, Taipei 10051, Taiwan. ²Graduate Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan. ³Faculty of Pharmaceutical Sciences, UCSI University, 56000 Kuala Lumpur, Malaysia. ⁴Department of Pharmacology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan. ⁵Department of Chemical and Pharmaceutical Sciences, Laboratorio per le Tecnologie delle Terapie Avanzate (LTTA), Ferrara, Italy. ⁶Department of Medical Sciences and National Institute of Neurosciences, Section of Pharmacology, University of Ferrara, 44121 Ferrara, Italy. ⁷Graduate Institute of Acupuncture Science, China Medical University, Taichung 40402, Taiwan.

Received: 21 August 2019 Accepted: 18 November 2019

Published online: 09 January 2020

References

- Lewis JW, Cannon JT, Stapleton JM, Liebeskind JC. Stress activates endogenous pain-inhibitory systems: opioid and non-opioid mechanisms. *Proc West Pharmacol Soc*. 1980;23:85–8.
- Watanabe S, Kuwaki T, Yanagisawa M, Fukuda Y, Shimoyama M. Persistent pain and stress activate pain-inhibitory orexin pathways. *Neuroreport*. 2005;16:5–8.
- Xie X, Wisor JP, Hara J, Crowder TL, LeWinter R, Khroyan TV, Yamanaka A, Diano S, Horvath TL, Sakurai T, et al. Hypocretin/orexin and nociceptin/orphanin FQ coordinately regulate analgesia in a mouse model of stress-induced analgesia. *J Clin Invest*. 2008;118:2471–81.
- Jungling K, Liu X, Lesting J, Coulon P, Sosulina L, Reinscheid RK, Pape HC. Activation of neuropeptide S-expressing neurons in the locus coeruleus by corticotropin-releasing factor. *J Physiol*. 2012;590:3701–17.
- Ebner K, Muigg P, Singewald G, Singewald N. Substance P in stress and anxiety: NK-1 receptor antagonism interacts with key brain areas of the stress circuitry. *Ann N Y Acad Sci*. 2008;1144:61–73.
- Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell*. 1998;92:573–85.
- de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS 2nd, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A*. 1998;95:322–7.
- Tsujino N, Sakurai T. Orexin/hypocretin: a neuropeptide at the interface of sleep, energy homeostasis, and reward system. *Pharmacol Rev*. 2009;61:162–76.
- Harris GC, Aston-Jones G. Arousal and reward: a dichotomy in orexin function. *Trends Neurosci*. 2006;29:571–7.
- Chiou LC, Lee HJ, Ho YC, Chen SP, Liao YY, Ma CH, Fan PC, Fuh JL, Wang SJ. Orexins/hypocretins: pain regulation and cellular actions. *Curr Pharm Des*. 2010;16:3089–100.
- Ho YC, Lee HJ, Tung LW, Liao YY, Fu SY, Teng SF, Liao HT, Mackie K, Chiou LC. Activation of orexin 1 receptors in the periaqueductal gray of male rats leads to antinociception via retrograde endocannabinoid (2-arachidonoylglycerol)-induced disinhibition. *J Neurosci*. 2011;31:14600–10.
- Lee HJ, Chang LY, Ho YC, Teng SF, Hwang LL, Mackie K, Chiou LC. Stress induces analgesia via orexin 1 receptor-initiated endocannabinoid/CB1 signaling in the mouse periaqueductal gray. *Neuropharmacology*. 2016;105:577–86.
- Gerashchenko D, Horvath TL, Xie XS. Direct inhibition of hypocretin/orexin neurons in the lateral hypothalamus by nociceptin/orphanin FQ blocks stress-induced analgesia in rats. *Neuropharmacology*. 2011;60:543–9.
- Porreca F, Ossipov MH, Gebhart GF. Chronic pain and medullary descending facilitation. *Trends Neurosci*. 2002;25:319–25.
- Bannister K, Dickenson AH. The plasticity of descending controls in pain: translational probing. *J Physiol*. 2017;595:4159–66.
- Turunen PM, Jantti MH, Kukkonen JP. OX1 orexin/hypocretin receptor signaling through arachidonic acid and endocannabinoid release. *Mol Pharmacol*. 2012;82:156–67.
- Cristino L, Luongo L, Imperatore R, Boccia S, Becker T, Morello G, Piscitelli F, Busetto G, Maione S, Di Marzo V. Orexin-A and Endocannabinoid activation of the descending Antinociceptive pathway underlies altered pain perception in Leptin signaling deficiency. *Neuropsychopharmacology*. 2016;41:508–20.
- Kukkonen JP. G-protein-dependency of orexin/hypocretin receptor signalling in recombinant Chinese hamster ovary cells. *Biochem Biophys Res Commun*. 2016;476:379–85.
- Li W, Chang M, Peng YL, Gao YH, Zhang JN, Han RW, Wang R. Neuropeptide S produces antinociceptive effects at the supraspinal level in mice. *Regul Pept*. 2009;156:90–5.
- Peng YL, Zhang JN, Chang M, Li W, Han RW, Wang R. Effects of central neuropeptide S in the mouse formalin test. *Peptides*. 2010;31:1878–83.
- Holanda AD, Asth L, Santos AR, Guerrini R, de PS-RV CG, Andre E, Gavioli EC. Central adenosine A1 and A2A receptors mediate the antinociceptive effects of neuropeptide S in the mouse formalin test. *Life Sci*. 2015;120:8–12.
- 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.
- Clark SD, Duangdao DM, Schulz S, Zhang L, Liu X, Xu YL, Reinscheid RK. Anatomical characterization of the neuropeptide S system in the mouse brain by in situ hybridization and immunohistochemistry. *J Comp Neurol*. 2011;519:1867–93.
- Liu X, Zeng J, Zhou A, Theodorsson E, Fahrenkrug J, Reinscheid RK. Molecular fingerprint of neuropeptide S-producing neurons in the mouse brain. *J Comp Neurol*. 2011;519:1847–66.
- Smith KL, Patterson M, Dhillon 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;147:3510–8.

26. Okamura N, Reinscheid RK. Neuropeptide S: a novel modulator of stress and arousal. *Stress*. 2007;10:221–6.
27. Niimi M. Centrally administered neuropeptide S activates orexin-containing neurons in the hypothalamus and stimulates feeding in rats. *Endocrine*. 2006;30:75–9.
28. Kallupi M, Cannella N, Economidou D, Ubaldi M, Ruggeri B, Weiss F, Massi M, Marugan J, Heilig M, Bonnavion P, et al. Neuropeptide S facilitates cue-induced relapse to cocaine seeking through activation of the hypothalamic hypocretin system. *Proc Natl Acad Sci U S A*. 2010;107:19567–72.
29. Ubaldi M, Giordano A, Severi I, Li H, Kallupi M, de Guglielmo G, Ruggeri B, Stopponi S, Ciccocioppo R, Cannella N. Activation of Hypocretin-1/Orexin-A neurons projecting to the bed nucleus of the Stria Terminalis and Paraventricular nucleus is critical for reinstatement of alcohol seeking by neuropeptide S. *Biol Psychiatry*. 2016;79:452–62.
30. Hokfelt T, Pernow B, Wahren J. Substance P: a pioneer amongst neuropeptides. *J Intern Med*. 2001;249:27–40.
31. Mantyh PW. Neurobiology of substance P and the NK1 receptor. *J Clin Psychiatry*. 2002;63(Suppl 11):6–10.
32. Minami M, Kuraishi Y, Kawamura M, Yamaguchi T, Masu Y, Nakanishi S, Satoh M. Enhancement of preprotachykinin a gene expression by adjuvant-induced inflammation in the rat spinal cord: possible involvement of substance P-containing spinal neurons in nociception. *Neurosci Lett*. 1989;98:105–10.
33. Stewart JM, Getto CJ, Neldner K, Reeve EB, Krivoy WA, Zimmermann E. Substance P and analgesia. *Nature*. 1976;262:784–5.
34. Rosen A, Zhang YX, Lund I, Lundeberg T, Yu LC. Substance P microinjected into the periaqueductal gray matter induces antinociception and is released following morphine administration. *Brain Res*. 2004;1001:87–94.
35. Quirion R, Shults CW, Moody TW, Pert CB, Chase TN, O'Donohue TL. Autoradiographic distribution of substance P receptors in rat central nervous system. *Nature*. 1983;303:714–6.
36. Drew GM, Lau BK, Vaughan CW. Substance P drives endocannabinoid-mediated disinhibition in a midbrain descending analgesic pathway. *J Neurosci*. 2009;29:7220–9.
37. Drew GM, Mitchell VA, Vaughan CW. Glutamate spillover modulates GABAergic synaptic transmission in the rat midbrain periaqueductal grey via metabotropic glutamate receptors and endocannabinoid signaling. *J Neurosci*. 2008;28:808–15.
38. Rosen A, Brodin K, Eneroth P, Brodin E. Short-term restraint stress and s.c. saline injection alter the tissue levels of substance P and cholecystokinin in the peri-aqueductal grey and limbic regions of rat brain. *Acta Physiol Scand*. 1992;146:341–8.
39. Holden JE, Pizzi JA, Jeong Y. An NK1 receptor antagonist microinjected into the periaqueductal gray blocks lateral hypothalamic-induced antinociception in rats. *Neurosci Lett*. 2009;453:115–9.
40. Gregg LC, Jung KM, Spradley JM, Nyilas R, Suplita RL 2nd, Zimmer A, Watanabe M, Mackie K, Katona I, Piomelli D, Hohmann AG. Activation of type 5 metabotropic glutamate receptors and diacylglycerol lipase- α initiates 2-arachidonoylglycerol formation and endocannabinoid-mediated analgesia. *J Neurosci*. 2012;32:9457–68.
41. Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, Krey JF, Walker JM, Holmes PV, Crystal JD, et al. An endocannabinoid mechanism for stress-induced analgesia. *Nature*. 2005;435:1108–12.
42. Suplita RL 2nd, Farthing JN, Gutierrez T, Hohmann AG. Inhibition of fatty-acid amide hydrolase enhances cannabinoid stress-induced analgesia: sites of action in the dorsolateral periaqueductal gray and rostral ventromedial medulla. *Neuropharmacology*. 2005;49:1201–9.
43. Hwang LL, Wang CH, Li TL, Chang SD, Lin LC, Chen CP, Chen CT, Liang KC, Ho IK, Yang WS, Chiou LC. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. *Obesity (Silver Spring)*. 2010;18:463–9.
44. Franklin KBJ, Paxinos G. The mouse brain in stereotaxic coordinates: Academic. San Diego; 1997.
45. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–54.
46. Guerrini R, Camarda V, Trapella C, Calo 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;52:524–9.
47. Ruzza C, Rizzi A, Camarda V, Pulga A, Marzola G, Filaferro M, Novi C, Ruggieri V, Marzola E, Vitale G, et al. [tBu-D-Gly5] NPS, a pure and potent antagonist of the neuropeptide S receptor: in vitro and in vivo studies. *Peptides*. 2012;34:404–11.
48. Raoof M, Soofiabadi S, Abbasnejad M, Kooshki R, Esmaeili-Mahani S, Mansoori M. Activation of orexin-1 receptors in the ventrolateral periaqueductal grey matter (vIPAG) modulates pulpal nociception and the induction of substance P in vIPAG and trigeminal nucleus caudalis. *Int Endod J*. 2019;52:318–28.
49. Holden JE, Pizzi JA. Lateral hypothalamic-induced antinociception may be mediated by a substance P connection with the rostral ventromedial medulla. *Brain Res*. 2008;1214:40–9.
50. Paneda C, Huitron-Resendiz S, Frago LM, Chowen JA, Picetti R, de Lecea L, Roberts AJ. Neuropeptide S reinstates cocaine-seeking behavior and increases locomotor activity through corticotropin-releasing factor receptor 1 in mice. *J Neurosci*. 2009;29:4155–61.
51. 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;154:471–9.
52. Castro AA, Moretti M, Casagrande TS, Martinello C, Petronilho F, Steckert AV, Guerrini R, Calo G, Dal Pizzol F, Quevedo J, Gavioli EC. Neuropeptide S produces hyperlocomotion and prevents oxidative stress damage in the mouse brain: a comparative study with amphetamine and diazepam. *Pharmacol Biochem Behav*. 2009;91:636–42.
53. Boeck CR, Martinello C, de Castro AA, Moretti M, Dos Santos CT, Guerrini R, Calo G, Gavioli EC. Blockade of adenosine A2A receptor counteracts neuropeptide-S-induced hyperlocomotion in mice. *Naunyn Schmiedeberg's Arch Pharmacol*. 2010;381:153–60.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

