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Nociceptin/Orphanin FQ in PAG modulates the release of amino acids, serotonin and norepinephrine in the rostral ventromedial medulla and spinal cord in rats

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ABSTRACT

High density Nociceptin/Orphanin FQ (N/OFQ) and its receptor (NOPr) have been found in the ventrolateral periaqueductal gray (vlPAG), a main output pathway involved in the descending pain-control system. Our previous study demonstrated that the microinjection of N/OFQ into the vlPAG markedly facilitated nociceptive responses of spinal dorsal horn neurons. The aim of the present work was to further provide evidence for the supraspinal mechanisms of action for N/OFQ-mediated nociceptive facilitation by examining the effect of N/OFQ in the vlPAG on neurotransmitter release in the descending pain-control system, including the nucleus raphe magnus (NRM), nucleus reticularis gigantocellularis (NGC) and dorsal horn of the spinal cord. The results showed that the microinjection of N/OFQ into the vlPAG produced robust decreases in 5-hydroxytryptamine (5-HT, serotonin), norepinephrine (NE), and γ-aminobutyric acid (GABA), and increase in glutamate (Glu) release in the spinal dorsal horn. Spinal application of 5-HT, 2-Me-5-HT (5-HT_3 receptor agonist), muscimol ($GABA_A$ receptor agonist), and baclofen ($GABA_B$ receptor agonist) significantly blocked intra-vlPAG-induced facilitation on nociceptive responses. However, the extracellular concentrations of these neurotransmitters in the NRM and NGC exhibited diversity following intra-vlPAG of N/OFQ. In the NRM, intra-vlPAG injection of N/OFQ significantly decreased 5-HT, NE, and Glu, but increased GABA release. Differently, in the NGC, both NE and GABA releases were attenuated by intra-vlPAG of N/OFQ, whereas the concentration of 5-HT and Glu exhibited a trend to increase. These findings provide direct support for the hypothesis that intra-PAG of N/OFQ-induced facilitation of nociceptive responses is associated with the release of 5-HT, NE, and amino acids.

1. Introduction

Nociceptin/Orphanin FQ (N/OFQ) [40,56] is a heptadecapeptide which is recognized as the endogenous ligand of the N/OFQ receptor (NOPr, initially called "opioid receptor-like 1", ORL1) [7,45]. N/OFQ has been implicated in many physiological and pathological functions, including pain regulation, stress response, feeding, learning, and memory [12,26,41]. Among these, the effects of N/OFQ on pain regulation were actively pursued. In analogy with opioid receptors, NOPr is widely distributed through the CNS in those regions involved in pain transmission processing, especially the ventrolateral periaqueductal gray (vlPAG) [25,49,57]. The roles of N/OFQ in pain modulation were shown to depend on the site of administration, and consensus opinion is that N/OFQ exhibits pro-nociceptive effects in the brain and antinociceptive effects in the spinal cord [12,20,44,61,70,71]. Tian et al. reported that morphine-induced analgesia was antagonized by intracerebroventricular N/OFQ and potentiated by intrathecal N/OFQ [64,65]. Intra-PAG N/OFQ was also shown attenuating the tail-flick inhibition produced by the microinjection of morphine or kainic acid into the PAG [47]. Our previous studies showed that the microinjection of N/OFQ into the PAG, especially vlPAG, markedly facilitated C-response and post-discharge of spinal dorsal horn WDR neurons evoked by noxious electrical stimulation, suggesting the involvement of the PAG in pro-nociceptive effects of N/OFQ at the supraspinal level [76]. Blockade of the rostral ventromedial medulla (RVM), including the nucleus raphe magnus (NRM) and nucleus reticularis gigantocellularis (NGC), by lidocaine significantly attenuated intra-vlPAG N/OFQ-induced facilitation on C-response and post-discharge of WDR neurons [75]. The PAG and RVM are generally believed to comprise a descending system of pain modulation. Anatomical and physiological studies have established that the vlPAG output neurons project to the RVM, which in turn project to the spinal dorsal horn to modulate nociception [35,36,43].

N/OFQ is demonstrated to be quite selective for NOP receptor with no appreciable affinity for the μ-, δ-, or κ-opioid receptors.

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Like opioid agonists, N/OFQ inhibits adenylate cyclase, increases inwardly rectifying potassium conductance and inhibits calcium channel currents [23,46]. Electrophysiological studies revealed a profound and indiscriminate inhibition of neuronal firing of the majority or even all neurons in most brain regions [24,25]. Thus, the pro-nociception of N/OFQ in the PAG might be explained that N/OFQ acts directly on NOP receptor on the PAG excitatory output neurons (such as glutamatergic, serotonergic, and neurotensinergic neurons) that may project to the RVM, so as to inhibit the activation of the descending inhibition system; or indirectly eliminate the inhibitory link from the PAG to RVM via inhibitory interneurons (such as opioidergic and GABAergic neurons), thereby facilitating the nociceptive responses of spinal dorsal horn neurons. However, the data concerning the influence of supraspinal N/OFQ upon extracellular concentrations of Glu, GABA, 5-HT, and NE in the NRM, NGC, and spinal cord has not, as yet, been reported. The present study, therefore, sought to provide evidence for the supraspinal mechanisms for N/OFQ-mediated facilitatory effect on nociceptive transmission. The working hypothesis for this study is that N/OFQ into the vlPAG may inhibit excitatory output from the PAG to the RVM, which in turn inhibits the descending serotonergic and noradrenergic projections to the spinal cord, leading to nociceptive facilitation via disinhibiting excitatory neurons in the spinal dorsal horn.

2. Methods

2.1. Animals

Experiments were performed on adult male Sprague–Dawley rats weighing 230–300 g. Rats were supplied by the Experimental Animal Center of Fudan University and were on a 12:12 light–dark cycle with a room temperature of 23 ± 1 °C, and received food and water *ad libitum*. The treatment of the animals was approved by the Animal Care and Use Committee of Shanghai, and confirmed to NIH guideline.

2.2. Surgery

Rats were initially anesthetized with sodium pentobarbital (45 mg/kg, i.p.) for surgery. After cannulation of the trachea and left jugular vein, the rat's head was positioned in a sterotaxic frame with the incisor bar 3 mm below the horizontal zero. Small craniotomies were performed. Three holes were drilled on the surface of the skull over the midbrain and medulla for inserting the guide cannulas into NRM and NGC for microdialysis and vlPAG for drugs administration. The positions of the NRM (11.3 mm caudal to Bregma, midline, and 9.0 mm below the cerebellum surface), NGC (11.3 mm caudal to Bregma, 0.8 mm lateral, and 7.5 mm below the cerebellum surface) and vlPAG (7.6 mm caudal to Bregma, 0.5 mm lateral, and 5.2 mm below the cortical surface) were determined according to the atlas of Paxinos and Watson [51]. In all cases, guide cannulas were lowered to 1 mm above the intended site. For spinal microdialysis or electrophysiological recording, a laminectomy was performed over the lumbar enlargement to expose the spinal cord. The vertebral column was rigidly fixed in the frame with clamps. The exposed spinal cord was covered by 1.5% warm agar (37 °C). A small well (10 × 5 mm) above the area of recording was cut carefully in the agar covering for topical drug administrations. An initial dose (45 mg/kg) of gallamine triethiodide was injected i.v. to paralyze the musculature and then the animal was artificially ventilated. Throughout the experiments of the microdialysis and electrophysiological recording, continuous anesthesia and paralysis were maintained (sodium pentobarbital: 4–5 mg/kg/h; gallamine triethiodide: 8–10 mg/kg/h). The physiological condition of the animals was monitored by recording the electrocardiogram, end-tidal CO_2 and rectal temperature to ensure that these parameters remained within 320 ± 30 beats/min, 3.5–5% and 37–38 °C, respectively.

2.3. Drugs administration

Heptadecapeptide OFQ was synthesized and purified by the Shanghai Institute of Biochemistry, Chinese Academy of Science. UFP-101 ([Nphe1, Arg14, Lys15]N/OFQ-NH2) was purchased from Tocris (Bristol, UK). Muscimol, baclofen, 5-HT, and 2-m-5-HT (2-methyl-5-hydroxytryptamine maleate) were purchased from Sigma (St. Louis, MO). All the reagents were dissolved in normal saline (NS). Arrowhead double-headed proteinase inhibitor (1 g/L, a product of the Shanghai Institute of Biochemistry), which has been reported to be able to inhibit trypsin, chymotrypsin and kallikrein [74], was added to the solution containing OFQ as well as control NS solution.

For intra-vlPAG injection, OFQ (0.1 μg) or UFP-101 (2 μg) in a volume of 0.25 μl was administered into the vlPAG via an injection cannula that extended 1 mm beyond the tip of the guide cannula. The injection was delivered over a 2-min period, and the injection cannula was left in place for an additional 2 min to minimize spread of the drug along the injection track.

For intrathecal (i.t.) injection, an intrathecal catheter (PE-10 tube) was inserted through the gap between the L4 and L5 vertebrae and extended to the subarachnoid space of the lumbar enlargement (L4 and L5 segments) under anesthesia. The catheter was filled with sterile NS (approximately 4 μl), and the outer end was plugged. The cannulated rats were allowed to recover for 3–4 days. Rats that showed any neurological deficits resulting from the surgical procedure were excluded from the behavioral experiment. Muscimol, baclofen, 5-HT or 2-m-5-HT, was injected over a period of 1 min via the catheter at a volume of 10 μl, followed by 5 μl NS for flushing.

In electrophysiological experiment, the drugs (muscimol and 5-HT) were dripped directly onto the exposed area of the spinal cord in a volume of 50 μl.

2.4. Microdialysis and HPLC

The microdialysis probe (CMA/12, 1 mm dialyzing membrane, Carnegie Medicine AB, Stockholm, Sweden) was, respectively, inserted into the unilateral NRM and NGC via the guide cannula to 1 mm beyond the tip of the guide cannula, as well as the ipsilateral spinal dorsal horn to a depth of 1.3 mm from the spinal surface using a micromanipulator. The dialysis probe was connected to a microinfusing pump (CMA/100, Stockholm, Sweden, Microdialysis Syringe 1.0 ml) and the outlet cannula was connected to the microfraction collector (CMA/200). The probe was perfused with artificial cerebrospinal fluid (ACSF) at a flow rate of 2 μl/min [78]. After dialysate levels stabilized (~60 min), and four sequential 30-min samples were collected as baseline values, NS (0.25 μl) was administered into the vlPAG ipsilateral to the dialyzed NRM, NGC, and spinal dorsal horn. Sequential 30-min samples were collected for an additional 90 min. Then N/OFQ (1 nmol/0.25 μl) was administered and the samples were collected. Aliquots were frozen at −80 °C for later analysis.

Extracellular concentrations of 5-HT and NE were determined by high performance liquid chromatography (HPLC) with an electrochemical detector (BAS, 200A). Standard solution or samples (20 μl) were injected into the column (3 μm phase II ODS column 100 × 3.2 mm, USA), separated with mobile phase, detected by chemical detector at oxidation potentials 700 mV against the Ag/AgCl electrode and quantified by BAS ChromGraph programs. The detection limit was ~0.5 pg/20 μl for monoamines.

Glu and GABA concentrations were measured by reversed HPLC coupled to fluorometric detection. A phase Sep C18 column (3 μm, 150 × 4.6 mm) was used. The mobile phase contained 0.1 M sodium acetate, 10% methanol, 2% tetrahydrofurance, pH 6.95, and was perfused at a flow rate of 1 ml/min (Beckman 110B pump; Beckman Instruments, Fullerton, CA, USA). A linear gradient system was used to clean the column after elution of Glu and GABA. This involved switching to 90% methanol for 2 min before switching back to the original acetate buffer. The excitation and emission wavelengths set at 370 and 450 nm, respectively. Acquisition and analysis of chromatograph were performed by means of a computer-controlled system (Beckman system Gold). The limit of detection was about 0.15 pmol/20 μl.

2.5. Extracellular recording

Single unit extracellular recordings were made at L4–5 segments, 400–1100 μm from the surface of the spinal cord with a glass micropipette filled with 0.5 M sodium acetate (impedance 8–10 MΩ at 1000 Hz). The micropipette was inserted perpendicularly to the spine into the dorsal horn from a point about mid-way between the midline and medial edge of the dorsal root entry zone. Electrical search stimulation (15 mA, 1 Hz, 1 ms pulse) was applied via two stainless steel needles inserted into the skin of the ipsilateral hindpaw to find neurons that responded to the stimulation during the advancement of microelectrode. When a single unit was isolated, the response characteristics of the neuron were identified with mechanical stimuli of a range of varying intensities, including brushing or touching the receptive field with a hair brush, pressing the skin with a blunt probe, and pinching a fold of skin with a toothed forceps. Wide dynamic range (WDR) neurons responded to all these stimuli with greater degree to pinch. After one WDR neuron was identified, electrical test stimulation was applied to the receptive field (RF) and the evoked discharges were recorded. Stimulus strength was two times the threshold for a C-response, and a train of 10 stimuli (0.3 Hz, 1 ms) was given with 10 min intervals. The total number of spikes per ten stimuli was calculated by the computer data collection system (SMUP-PC). As in previous studies [79], the evoked responses were separated, according to latency, into Aβ- (0–20 ms), Aδ- (20–90 ms), C-fiber response (90–300 ms) and post-discharge activity (300–800 ms). Since C-response and post-discharge activities were considered to be nociceptive-related, the effects of OFQ on the C-response and post-discharge activities of WDR neurons were used in the present study.

2.6. Immunohistochemistry

Rats were killed by overdose of chloral hydrate (80 mg/kg) and perfused transcardially with NS followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were then removed, post-fixed in the same fixative for 6 h at 4 °C, and immersed in 10–30% gradient sucrose in PB for 24–48 h at 4 °C for cryoprotection. Coronal sections (30 μm) were cut in a cryostat (Leica 1900, German) and processed for immunofluorescence. Briefly, the sections were blocked with 10% donkey serum in 0.01 M PB saline (PBS, pH 7.4) with 0.3% Triton X-100 for 1 h at RT and incubated overnight at 4 °C with a mixture of rabbit anti-NOPr (KOR-3, 1:1000, Santa Cruz Biotechnology,) and mouse anti-Glu (1:1000, Chemicon) or mouse anti-GABA (1:1000, Sigma) primary antibody in PBS with 1% normal donkey serum and 0.3% Triton X-100. Following 3 × 15-min rinses in PBS, the sections were incubated in rhodamine-conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch) or fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch) or for 2 h at 4 °C, then washed in PBS. All sections were coverslipped with a mixture of 50% glycerin in PBS, and then observed with a Leica SP2 confocal laser-scanning microscope (Mannheim, Germany). The specificity of immunostaining was verified by omitting the primary antibodies, and immunostaining signal disappeared after omitting primary antibodies. The specificity of primary antibodies was verified by the pre-absorption experiment. Brain sections were first incubated with a mixture of NOPr (KOR-3) primary antibody and the corresponding blocking peptide for KOR-3 (20 μg/ml; Neuromics) overnight, followed by secondary antibody incubation. NOPr (KOR-3) immunostaining signal was abolished after absorption (Fig. 4B).

2.7. Tail-flick latency measurement

The tail-flick latency (TFL) to radiant heat was examined for evidence of heat hyperalgesia in animals using the Model 336 Combination Unit (IITC/Life Science Instruments, USA). Rats were lightly restrained in a plastic cylinder with the head, back, and tail protruding. Radiant heat was applied to the blackened surface of the rat tail at a distance of 5–6 cm from its tip to evoke the tail-flick (TF) reflex. The current was adjusted in order that the baseline TFL was 4–5 s. Noxious heating of the tail was given repeatedly with a time interval of 10 min between applications. If the TF reflex was suppressed, a cut-off time of 7.0 s was adopted to minimize damage to the skin. Baseline TFL was defined as the mean of at least three determinations performed at 10-min interval before drug administration.

2.8. Histology

At the end of the experiment, the dialysis membrane was perfused with 2% pontamine sky blue for 30 min for verifying the location of microdialysis probe. Also, intra-vlPAG microinjection sites were marked by injection of the same volume as N/OFQ (0.25 μl) of pontamine sky blue, and the location of the distal end of the intrathecal (i.t.) catheter was verified by the injection of 10 μl pontamine sky blue via the i.t. catheter.

After receiving an overdose of anesthesia, the animal was perfused transcardially with NS followed by 10% formalin. The brain and spinal cord were then removed and fixed in formalin for 6 h. Sixty micro sections were cut with a freezing microtome and mounted and stained with neutral red, so that the position of dialysis probe and microinjection could be verified histologically. Animals with incorrect probe and microinjection position were not included in the present analysis.

2.9. Data analysis

In the case of microdialysis coupled with HPLC experiments, data were expressed as a percentage of the baseline concentrations. Baseline value was calculated as the mean of four sequential samples. In the case of electrophysiological experiments, the neuronal responses were expressed as a percentage of the control responses. The differences between groups were compared using ANOVA followed by post hoc Dunnett's test or using Student's *t*-test if only two groups were applied. All data were expressed as means ± SEM, and the accepted level of statistical significance for all experiments was $p < 0.05$.

3. Results

3.1. Effects of intra-vlPAG N/OFQ on the release of 5-HT, NE, Glu, and GABA in the NRM and NGC

In the NRM and NGC, the basal dialysate concentrations of 5-HT, NE, Glu, and GABA were relatively stable. All the animals had basal

concentration of >0.5 pg/20 μl for monoamines, and of >0.15 pmol/20 μl for amino acids. Microinjection of NS (0.25 μl) into the unilateral vlPAG had no effect on extracellular concentrations of these neurotransmitters in the ipsilateral NRM and NGC throughout the 90-min observation period.

Microinjection of N/OFQ (0.1/0.25 μl) into the unilateral vlPAG significantly decreased the extracellular concentrations of 5-HT in the ipsilateral NRM (Fig. 1A). Two-way ANOVA analysis showed a significant effect of treatment ($p < 0.01$, $n = 5$; intra-vlPAG N/OFQ and NS), a significant effect of time ($p < 0.01$, before and after intra-vlPAG N/OFQ), and a nonsignificant effect of treatment × time interaction ($p > 0.05$). Similar to 5-HT, the extracellular NE and Glu concentrations in the ipsilateral NRM were also attenuated by N/OFQ into the unilateral vlPAG. There were significant differences in the release of NE and Glu between before and after intra-vlPAG N/OFQ, or N/OFQ and NS treatments (two-way ANOVA, $p < 0.05$, $n = 5$) (Fig. 1B and 1C). In contrary, intra-vlPAG N/OFQ produced a significant increase in extracellular GABA level in the NRM, compared with either before N/OFQ or those obtained from injecting NS into vlPAG (two-way ANOVA, $p < 0.05$, $n = 5$) (Fig. 1D). The microinjection sites in the vlPAG and the positions of dialysis probe tip in the NRM are shown in Fig. 1E.

In the NGC, the concentrations of extracellular 5-HT and Glu exhibited a trend to increase, although that did not reach statistical significance, following intra-vlPAG N/OFQ (Fig. 2A and C). However, the extracellular concentrations of NE and GABA in the NGC were significantly declined within 0–90 min after intra-vlPAG N/OFQ (two-way ANOVA, before and after intra-vlPAG N/OFQ, or N/OFQ and NS treatments, $p < 0.01$, $n = 6$) (Fig. 2B and D). The microinjection sites in the vlPAG and the positions of dialysis probe tip in the NGC are shown in Fig. 2E.

3.2. Effects of intra-vlPAG N/OFQ on the release of 5-HT, NE, Glu, and GABA in the spinal cord

There was a large variability between animals in the base dialysate concentrations of 5-HT in the spinal dorsal horn. Extracellular 5-HT ranged from less than the level of detection (<0.5 pg/20 μl) to 5 pg/ 20 μl. About 70% of the animals had basal 5-HT concentration (>0.5 pg/20 μl). The basal concentrations of NE, Glu, and GABA were relatively stable in the spinal cord. Microinjection of N/OFQ into the unilateral vlPAG markedly attenuated the extracellular concentrations of 5-HT, NE, and GABA in the ipsilateral spinal dorsal horn (two-way ANOVA, before and after intra-vlPAG N/OFQ, or N/OFQ and NS treatments, $p < 0.01$, $n = 7$) (Fig. 3A, B, and D). However, the extracellular Glu concentration in the spinal dorsal horn only exhibited a trend to increase following intra-vlPAG N/OFQ, but did not reach statistical significance (Fig. 3C). The micro-

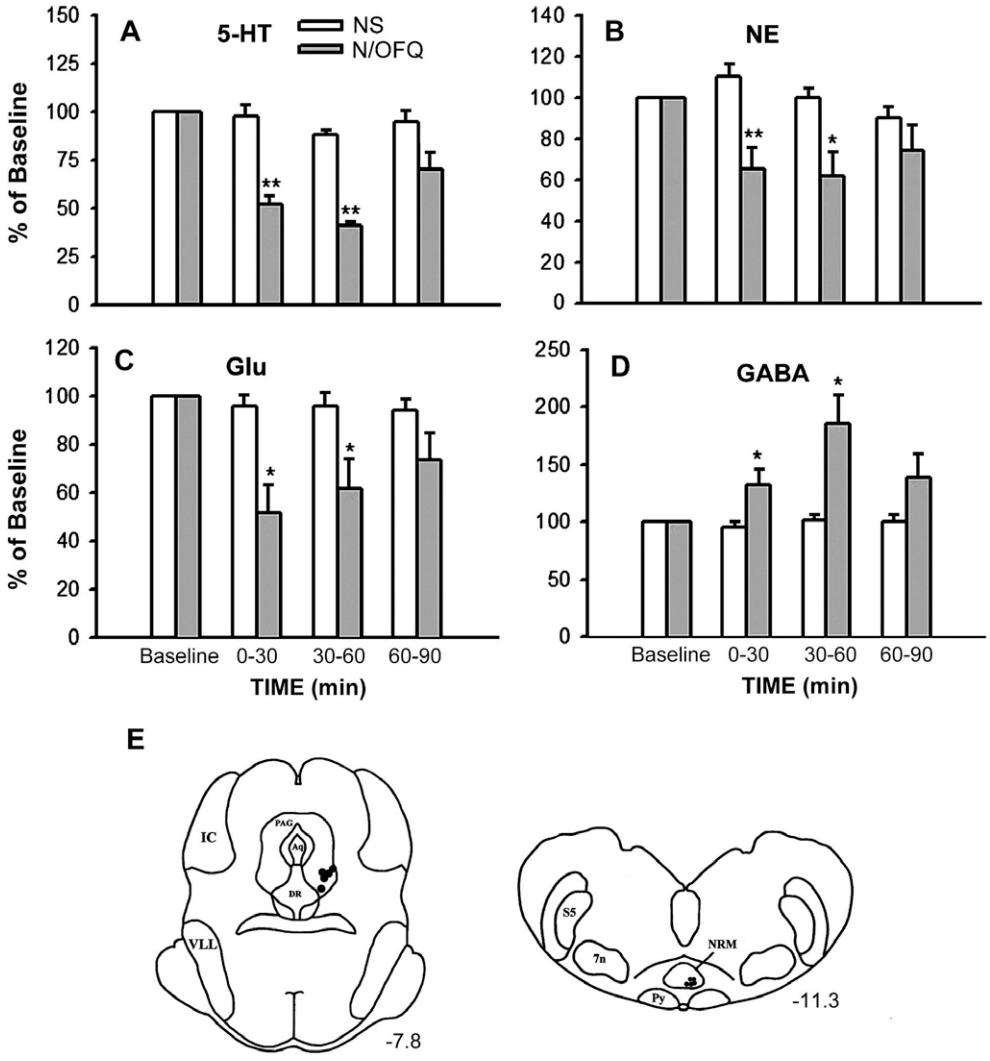

Fig. 1. Extracellular 5-HT (A), NE (B), Glu (C), and GABA (D) concentrations in the NRM after intra-vlPAG injection of N/OFQ (0.1 μg) or vehicle (NS). Data are means ± SEM, $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. NS control. *Abbreviations:* NRM, nucleus raphe magnus; vlPAG, ventrolateral periaqueductal gray.

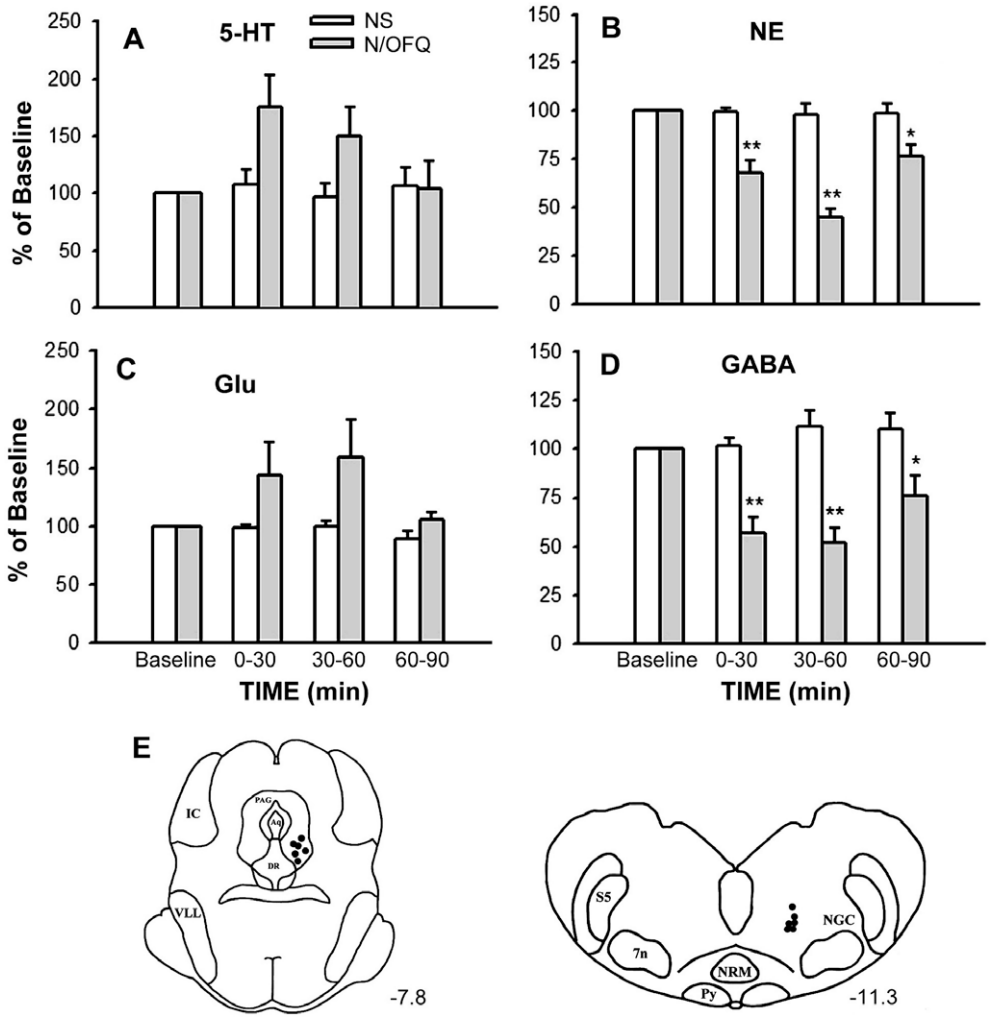

Fig. 2. Extracellular 5-HT (A), NE (B), Glu (C), and GABA (D) concentrations in the NGC after intra-vlPAG injection of N/OFQ (0.1 μg) or vehicle (NS). Data are means ± SEM, $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. NS control. *Abbreviation:* NGC, nucleus reticularis gigantocellularis.

injection sites in the vlPAG and the positions of dialysis probe tip in the spinal dorsal horn are shown in Fig. 3E.

3.3. Expression of NOPr in the vlPAG

A specific anti-NOP receptor (KOR-3) antibody was used to examine the distribution of NOPr in the vlPAG. Immunohistochemistry results showed that NOPr-immunoreactive (NOPr-IR) signals were diffusely distributed in the vlPAG with a moderate to high density. Most of the NOPr-immunoreactive (NOPr-IR) signals appear to be localized at the periphery of the soma and processes of cells (Fig. 4A). In some cases, the labeling is continuous and outlines the whole cell, appear as empty ellipsoid bags, covered by thin dense shells. In a few cases, lower density of immunoreactivity can be encountered in the cytoplasm of some of the labeled cells (Fig. 4A). Glu- and GABA-immunoreactive products were seen in the soma, proximal dendrites, and some axon terminals or dendritic endings in vlPAG neurons (Fig.4C and D). Either NOPr-IR with Glu-IR or NOPr-IR with GABA-IR double-labeled cells was observed in the vlPAG (Fig. 4C and D).

3.4. Involvement of spinal 5-HT and 5-HT3 receptor in nociceptive facilitation produced by intra-vlPAG of N/OFQ

To determine whether the decrease in spinal 5-HT is associated with intra-vlPAG N/OFQ-induced nociceptive facilitation, we examined the effects of spinal administration of 5-HT and selective 5-HT3 receptor agonist 2-m-5-HT on nociceptive responses of WDR neurons in the spinal dorsal horn.

A total of 46 WDR neurons were recorded in 24 rats. All the neurons responded to both brush and noxious pinch, with a more pronounced response to the latter, and exhibited two types of discharges, i.e. early discharges (A-fiber response) and late discharges (C-fiber response), when intense electrical current was delivered to the receptive fields (RFs). As reported previously [75], microinjection of N/OFQ (0.1 μg), but not NS into the vlPAG markedly facilitated the C-response and post-discharge activities. The maximal effect was usually observed within 20–40 min after administration. The N/OFQ-induced facilitation on C-responses and post-discharges of WDR neurons was prevented by UFP-101 (2 μg), a NOPr antagonist, given 3 min prior to OFQ (Fig. 5A). Two-way ANOVA revealed significant effect of treatments ($F_{2,17} = 112.175$, $p < 0.01$), and interaction between intra-vlPAG treatment and time ($F_{10,85} = 18.705$, $p < 0.01$). At the dose of 2 μg/0.25 μl, UFP-101 alone into the vlPAG did not modify the basal C-responses and post-discharges.

Spinal application of NS had no effect on intra-vlPAG N/OFQ-induced facilitation on C-response and post-discharge activities of WDR neurons. Three minutes before Intra-vlPAG of N/OFQ, spinal application of 5-HT (0.5 μg), or 2-m-5-HT (1 μg, a selective 5-HT3 receptor agonist) significantly blocked intra-vlPAG N/OFQ-induced facilitation on nociceptive responses of the WDR neurons (two-

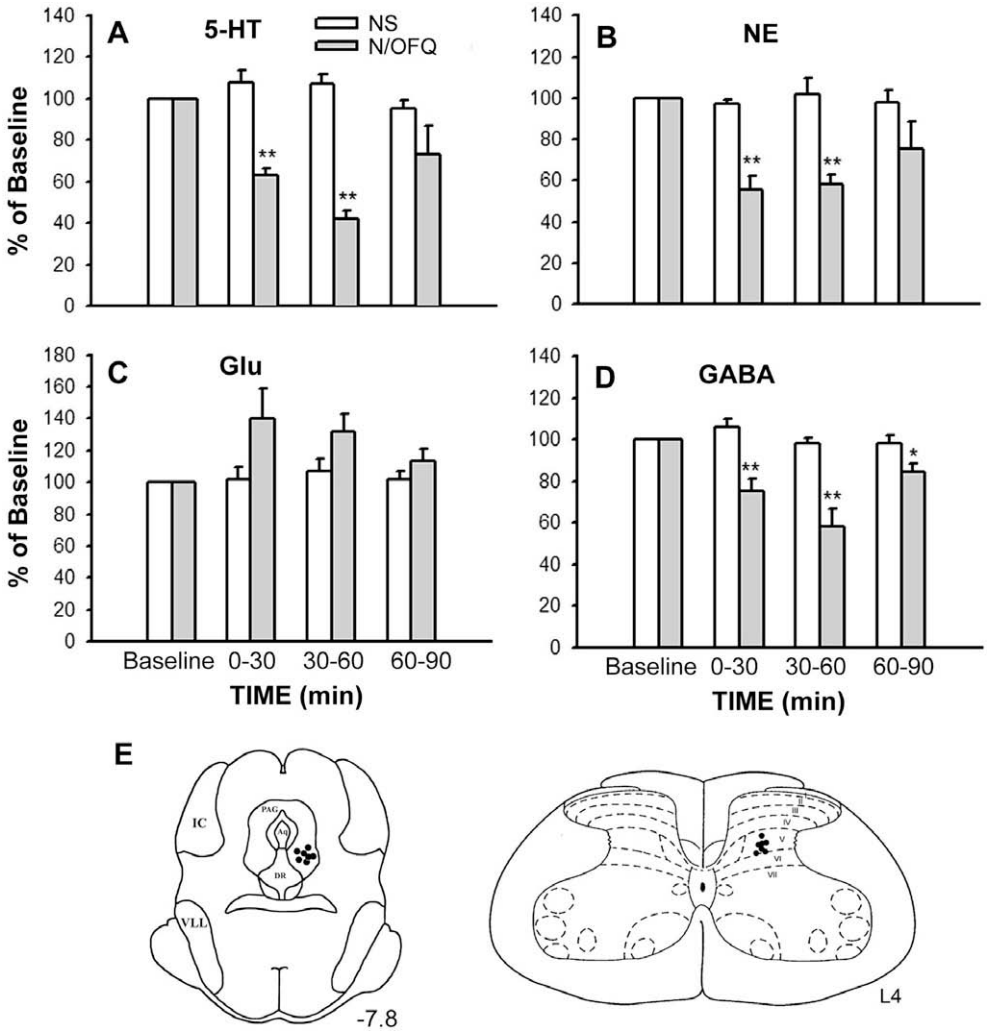

Fig. 3. Extracellular 5-HT (A), NE (B), Glu (C), and GABA (D) concentrations in the dorsal horn of the spinal cord after intra-vlPAG injection of N/OFQ (0.1 μg) or vehicle (NS). Data are means ± SEM, $n = 7$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. NS control.

way ANOVA, $p < 0.01$; $n = 6–8$) (Fig. 5B and Fig. 6). At the doses of 0.5 μg (5-HT) and 1 μg (2-m-5-HT), respectively, 5-HT and 2-m-5-HT alone had no effect on basal C-responses and post-discharges of WDR neurons in the spinal dorsal horn.

3.5. Involvement of spinal $GABA_A$ and $GABA_B$ receptors in nociceptive facilitation produced by intra-vlPAG of N/OFQ

To further determine if the decrease in spinal GABA is associated with intra-vlPAG N/OFQ-induced nociceptive facilitation, we simultaneously examined the effects of spinal administrations of $GABA_A$ receptors agonist muscimol (MUS, 0.03 μg) and $GABA_B$ receptors agonist baclofen (BAC, 0.03 μg) on radiant heat-evoked TF reflex.

The stability of the radiant heat-evoked TF reflex was first tested. The baseline of TFL was stable for a period of 80 min examined. As reported previously [69], intra-vlPAG of 0.1 μg N/OFQ produced marked facilitation of TF reflex, which peaked at 20 min after F/OFQ injection. The N/OFQ-induced facilitation on TF reflex was prevented by UFP-101 (2 μg), given 3 min prior to OFQ (Fig. 7A). Neither intra-vlPAG of NS nor UFP-101 alone into the vlPAG modified the basal TFL.

When MUS (0.03 μg) or BAC (0.03 μg) was intrathecally injection 3 min prior to intra-vlPAG of N/OFQ, the intra-vlPAG N/OFQ-induced facilitation on TF reflex was significantly blocked (Fig. 7B). At the dose of 0.03 μg, either MUS or BAC alone had no effect on basal TFL. Two-way ANOVA revealed significant effect of treatments ($F_{4,31} = 112.175$, $p < 0.01$), and interaction between treatment and time ($F_{24,196} = 18.705$, $p < 0.01$).

4. Discussion

4.1. Intra-vlPAG of N/OFQ regulates the release of neurotransmitters in the NRM

The present results showed that intro-vlPAG of N/OFQ significantly attenuated 5-HT, NE, and Glu, but increased GABA release in the NRM, reflecting a decrease in excitatory and an increase in inhibitory connections from the PAG to the NRM.

The excitatory link between the PAG and the NRM primarily includes glutamatergic, serotonergic, and neurotensinergic (NT) neurons [6,18,32]. The attenuation of 5-HT, NE, and Glu extracellular concentrations in the NRM is likely to be mediated directly by neurons expressing NOPr [17]. The inhibition of 5-HT and NE release in forebrain structures provided functional support for such an inhibitory influence of N/OFQ on noradrenergic and serotonergic transmission [59,60]. Also, A7 noradrenergic pathway is probably involved in the attenuation of NE release in the NRM. Anatomical

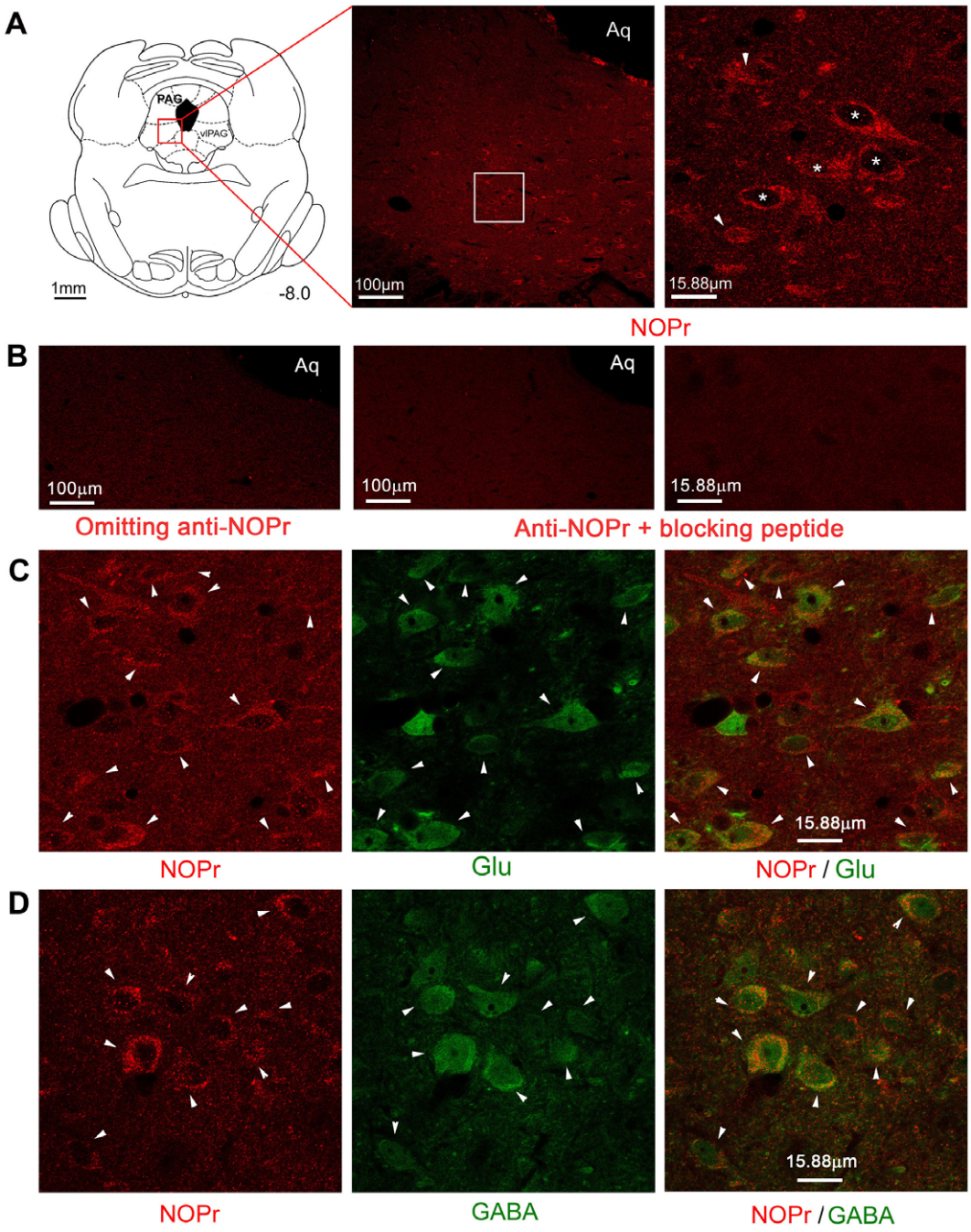

Fig. 4. (A) Distribution of NOPr-immunoreactivity in the vlPAG. Right, a high magnification showing the strong NOPr-immunoreactivity around the soma as an almost continuous covering of the neuronal membrane (asterisks). In some of the cells the cytoplasm exhibits a low density of immunoreactivity (arrowheads). (B) Negative control. Omitting NOPr antibody and pre-absorption of NOPr antibody with the NOPr blocking peptide abolished immunostaining. (C) Double immunofluorescence showing the colocalization of NOPr- and glutamate (Glu)-immunoreactivity in the vlPAG neurons. Arrowheads indicate double-labeled cells. (D) Double immunofluorescence showing the colocalization of NOPr-and GABA-immunoreactivity in the vlPAG neurons. Arrowheads indicate double-labeled cells. *Abbreviation:* Aq, aqueduct.

studies have established that the vlPAG output neurons project to the A7 or A5 noradrenergic nuclei, which in turn innervate the RVM [2,3,10,16,30,39].

In the present study, we observed that NOPr colocalized with Glu in the vlPAG by double immunofluorescence (Fig. 4). However, excitatory pathways projecting from the PAG to the NRM neurons initiating descending inhibition are subject to tonic, inhibitory control by GABA inhibitory interneuron in the PAG. The relief of GABA control by, for example, μ-opioid, contributes to the induction of analgesia from the PAG [9,15,27]. N/OFQ may also prevent the opioid-mediated increase in the PAG excitatory output via NOPr on opioidergic neurons in the vlPAG [47,67,69].

Considering that only few GABA neurons project from the PAG to the NRM (approximately 1.5% of all GABA-immunoreactive neurons in the PAG) [55], the effect of N/OFQ in the PAG on GABA release in the NRM is unlikely to be directly mediated by GABAergic output in the PAG. The interaction of various types of neurons and local neuronal circuit in the NRM may play important roles in modulating GABA concentration in the NRM following intro-

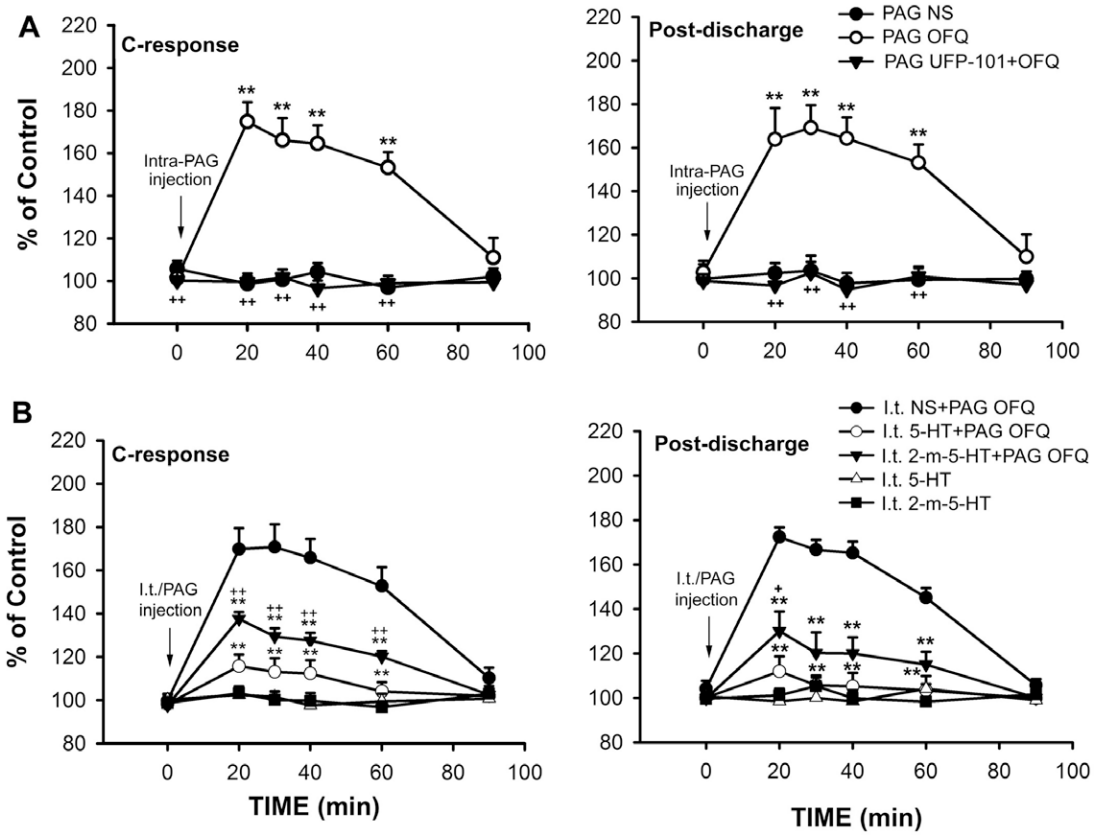

Fig. 5. Activation of spinal 5-HT receptors antagonized intra-vlPAG N/OFQ-induced facilitatory effects on C-response and post-discharge activities of WDR neurons in the spinal dorsal horn. (A) Intra-vlPAG injection of N/OFQ (0.1 μg) significantly facilitated C-response and post-discharge activities of WDR neurons. Pre-administration of NOPr antagonist UFP-101 (2 μg) completely blocked the N/OFQ-induced facilitatory effects. Intra-vlPAG of UFP-101 (2 μg) was given 3 min prior to OFQ. Data are expressed as the mean percentage of control ± SEM, $n = 6–8$. $^{**}p < 0.01$ vs. intra-vlPAG NS; $^{++}p < 0.01$ vs. intra-vlPAG N/OFQ. (B) Intrathecal injection of 5-HT (0.5 μg) or 5-HT3 receptor agonist 2-m-5-HT (1 μg) significantly blocked intra-vlPAG N/OFQ-induced facilitation on C-response and post-discharge activities of WDR neurons. Intrathecal injection (i.t) of 5-HT or 2-m-5-HT was given 3 min prior to intra-vlPAG injection of N/OFQ. Data are expressed as the mean percentage of control ± SEM, $n = 6–8$. $^{**}p < 0.01$ vs. i.t. NS + intra-vlPAG N/OFQ; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. i.t. 2-m-5-HT alone.

vlPAG of N/OFQ. The significance of N/OFQ-induced increase in GABA release in the NRM may be to suppress the activity of descending antinociceptive outflow thus leading to pro-nociceptive effect.

4.2. Intra-vlPAG of N/OFQ regulates the release of neurotransmitters in the NGC

Our previous study revealed that the microinjection of N/OFQ into the NRM and NGC exhibited opposite effects: analgesia in the NRM and hyperalgesia in the NGC [77]. Accordingly, the present results showed diverse changes in the excitatory and inhibitory neurotransmitters release in the NGC following N/OFQ into the vlPAG.

In the NGC, the concentrations of extracellular GABA and NE were significantly decreased, and 5-HT and Glu were slightly increased following the microinjection of N/OFQ into the vlPAG. It has been demonstrated that GABAergic output from the PAG targets RVM reticulospinal neurons [48]. Compared with that in the NRM, there is a much higher proportion (approximately 18%) of GABA neurons projecting from the PAG to the NGC [55]. It is possible that N/OFQ acts directly on NOPr on the vlPAG GABAergic neurons projecting to the NGC, thereby attenuating GABA release in the NGC. The present study has shown double staining neurons of NOPr- and GABA-IR in the vlPAG (Fig. 4). Intra-PAG N/OF-induced reduction of NE concentration in the NGC may share a similar mechanism to that in the NRM. As for the light increases in 5-HT and Glu in the NGC, it is likely to be a complex integrated effect. It has been reported that both serotonergic and glutamatergic neurons are controlled by a large population of GABAergic and inhibitory α_2-adrenoceptors (α-AR) [28,50,58]. Relief of GABAergic control may contribute to the increases in 5-HT and Glu in the NGC.

Both the NRM and NGC are important as the bulbar relays for the descending pain modulation from the PAG [5,8,13,14,19]. Our previous study revealed that selective blockade of either the NRM or NGC partially attenuated the intra-vlPAG N/OFQ-induced facilitation of nociceptive responses of WDR neurons. Moreover, when the NRM and NGC were simultaneously blocked, the intra-vlPAG N/OFQ-induced facilitation was completely eliminated, suggesting that both the NRM and NGC mediate pro-nociceptive effect of N/OFQ in the vlPAG [75]. The present study showed that intra-vlPAG N/OFQ produced different changes in the levels of GABA, Glu, and 5-HT release in the NRM and NGC, suggesting that the NRM and NGC are differentially involved in the facilitation induced by N/OFQ in the vlPAG. Interestingly, there appear to be coincident descending inhibitory and facilitatory pathway from the RVM, especially the NGC [21,37,38,80,81]. Low intensity electrical stimulation or microinjection of low dose of glutamate or high dose of baclofen, a $GABA_B$ receptor agonist, produce the facilitation of spinal behavioral and dorsal horn neuronal responses to noxious stimulation [63,66,80,81]. However, high

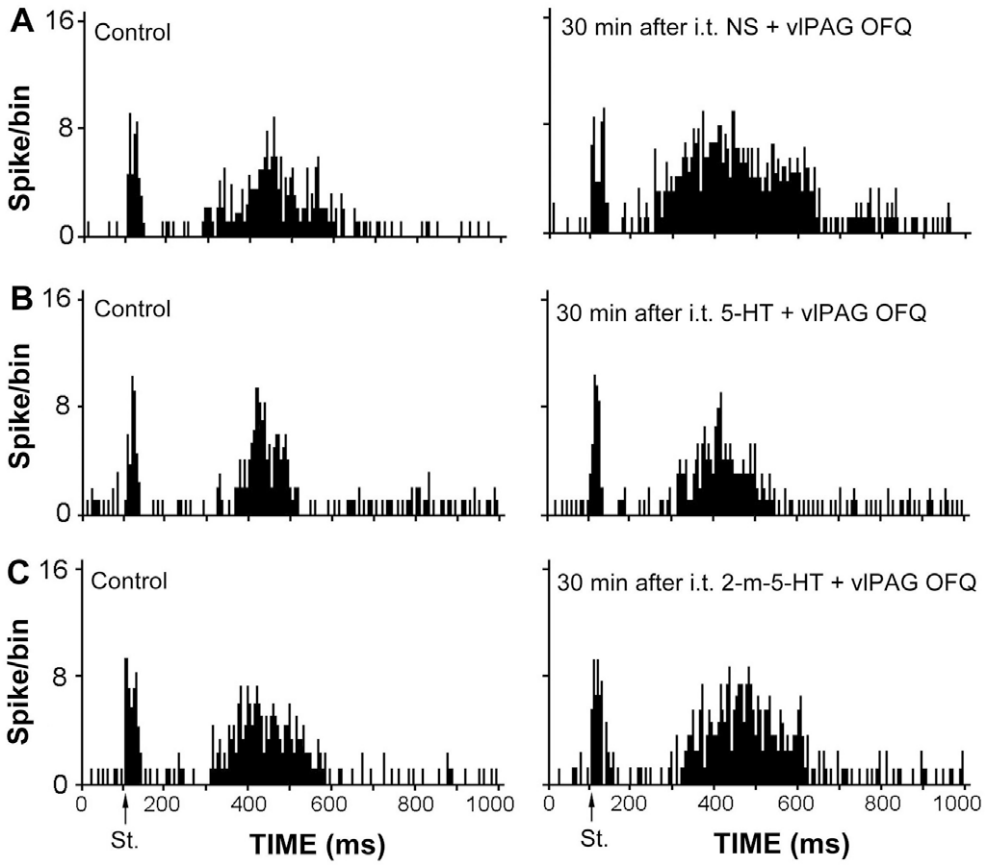

Fig. 6. Peristimulus histograms (10 trials) showing C-response and post-discharge activities of spinal WDR neurons to noxious subcutaneous electrical stimulation. (A) Intrathecal (i.t.) injection of NS had no effect on intra-vlPAG N/OFQ-induced facilitation of C-response and post-discharge activities of WDR neurons. (B and C) Intrathecal (i.t.) injection of 5-HT (0.5 μg, B) or 5-HT3 receptor agonist 2-m-5-HT (1 μg, C) prevented intra-vlPAG N/OFQ-induced facilitation of C-response and post-discharge activities of WDR neurons.

intensity electrical or chemical stimulation, or a low concentration of baclofen in the NGC inhibits spinal nociceptive neuronal activity and produces behavioral antinociception [63,80,81]. Furthermore, Wei et al. reported that selective chemical lesions of the NRM significantly increased thermal hyperalgesia and spinal Fos expression after hindpaw inflammation. In contrast, the selective bilateral destruction of the NGC led to an attenuation of hyperalgesia spinal Fos expression, suggesting that there is a potent descending facilitatory system involving the NGC [72]. It should be appreciated that N/OFQ in vlPAG-induced increase in GABA and decrease in 5-HT and Glu levels in the NRM may weaken descending inhibitory pathway, whereas the decrease in GABA and increases in 5-HT and Glu levels in the NGC may reinforce descending facilitatory pathway. Both of them contribute together to vlPAG N/OFQ-induced nociceptive facilitation (Fig. 8).

4.3. Intra-vlPAG of N/OFQ regulates the release of neurotransmitters in the spinal cord

Descending serotonergic pathway from the RVM and descending noradrenergic pathway from the locus ceruleus (LC), A7 and A5 groups to the spinal dorsal horn mediates at least in part of the antinociception produced by the PAG [1,43]. Electrical or chemical stimulation of the RVM or PAG induces a significant increase in the release of 5-HT and NE in the spinal cord, and this is accompanied by antinociception [11,22,62]. Iontophoretic application of 5-HT or α_2 receptor agonists inhibit nociceptive-evoked activity of dorsal horn neurons [73], and intrathecal injection of 5-HT or α_2 receptor antagonists block the antinociception produced by PAG or NRM stimulation [1,34,52,53]. Therefore, intra-vlPAG N/OFQ-induced decrease in the release of 5-HT and NE in the spinal cord in the present study might be resulted from suppressing excitatory connections from the PAG to RVM, or LC, A7, and A5 (directly or via the RVM), which project through the dorsolateral funiculus to the dorsal horn of the spinal cord [4,31,43]. Moreover, intra-vlPAG N/OFQ-induced the elevation of GABA concentration in the NRM probably contributes the decreases in 5-HT and NE release in the spinal cord. Virtually, all monoaminergic cell groups are subject to inhibition by local GABAergic and glycinergic neurons in the NRM [2,15,68]. The inhibitory influence of N/OFQ on the descending serotonergic and noradrenergic pathway was further supported by the present results that spinal administration of 5-HT and 5-HT_3 receptor agonist blocked vlPAG N/OFQ-induced facilitation of nociceptive dorsal horn neurons.

The origins from supraspinal structures and spinal cord inhibitory interneurons are two major sources of spinal GABA [43]. Stimulation of the PAG could directly activate GABAergic neurons in the NRM and cause a release of GABA in the spinal cord to inhibit nociceptive dorsal horn neurons [11], and descending 5-HT and NE could also activate inhibitory interneurons in the superficial layers of the dorsal horn through 5-HT_3 receptors and α_1 adrenoceptors, which in turn inhibits dorsal horn projection neurons by releasing inhibitory neurotransmitters, such as GABA [33,34,53,54]. Correspondingly, it is easily understood that N/

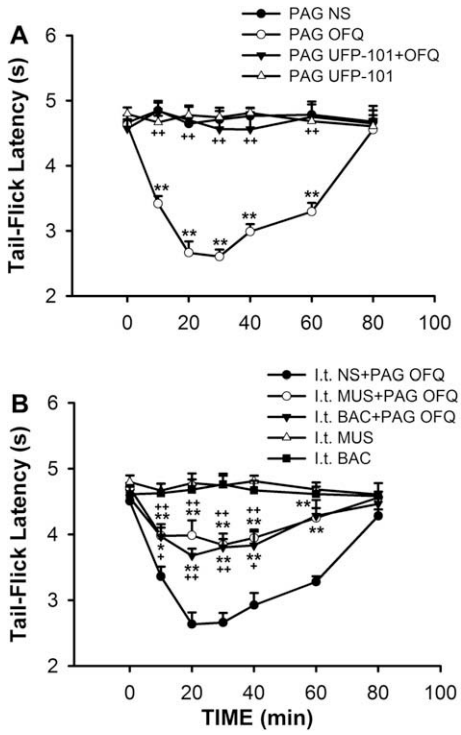

Fig. 7. Activation of spinal $GABA_A$ and $GABA_B$ receptors antagonized intra-vlPAG N/OFQ-induced facilitatory effects on rat tail-flick (TF) reflex. (A) Intra-vlPAG injection of N/OFQ significantly facilitated TF reflex. Pre-administration of NOPr antagonist UFP-101 (2 μg) completely blocked the N/OFQ-induced facilitation. Intra-vlPAG of UFP-101 (2 μg) was given 3 min prior to OFQ. Data are means ± SEM, $n = 8$. $^{**}p < 0.01$ vs. intra-vlPAG NS; $^{++}p < 0.01$ vs. intra-vlPAG N/OFQ. (B) Intrathecal injection of $GABA_A$ receptor agonist muscimol (MUS, 0.03 μg) or $GABA_B$ receptors agonist baclofen (BAC, 0.03 μg) significantly suppressed intra-vlPAG N/OFQ-induced facilitation on TF reflex. Intrathecal injection (i.t) of muscimol or baclofen was given 3 min prior to intra-vlPAG injection of N/OFQ. Data are means ± SEM, $n = 7–8$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. i.t. NS + intra-vlPAG N/OFQ; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. i.t. muscimol or baclofen alone.

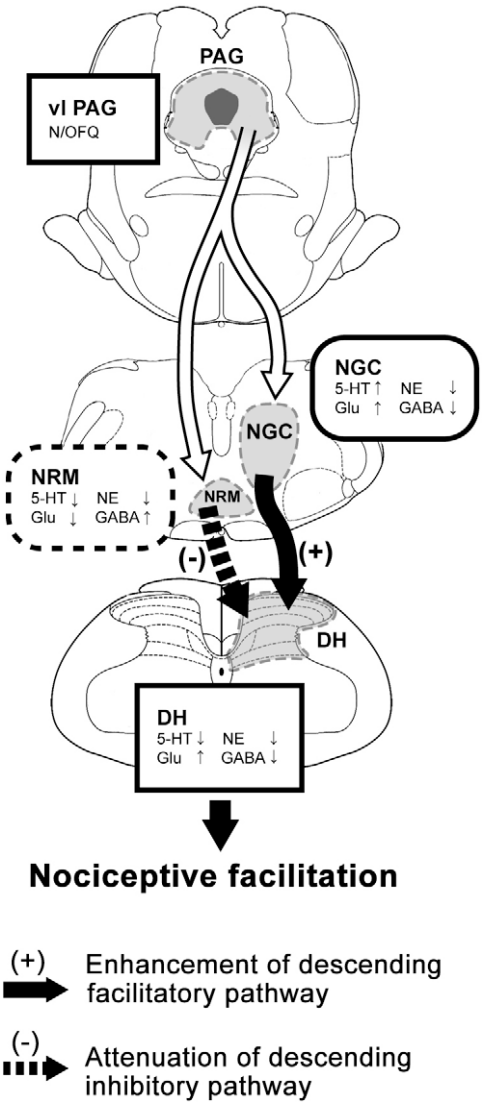

Fig. 8. Schematic illustration of the proposed mechanisms for effects of N/OFQ in the vlPAG on nociceptive facilitation. N/OFQ in the vlPAG may directly or indirectly inhibit excitatory or inhibitory pathways projecting from the PAG to the NRM and NGC by activating the NOP receptor expressed in PAG output neurons, which results in the complex changes of various neurotransmitters release in the NRM and NGC. The increase in GABA and decrease in 5-HT, NE, and Glu levels in the NRM may weaken descending inhibitory pathway, whereas the decrease in GABA and increase in 5-HT and Glu levels in the NGC may reinforce descending facilitatory pathway. These two effects synergically facilitate nociceptive transmission at the level of the spinal dorsal horn.

OFQ in the PAG may relieve an excitatory link to the RVM and A7, A5 nuclei, leading to a decrease in the release of GABA in the spinal cord via both direct supraspinal origin and indirect spinal local interneurons. Similar to 5-HT and NE, the decrease in GABA release in the spinal cord may also contribute to the mechanisms of intra-vlPAG N/OFQ-induced pro-nociception. The present study demonstrated that spinal application of $GABA_A$ and $GABA_B$ agonist antagonized the intra-vlPAG N/OFQ-induced facilitation on nociceptive TF reflex.

The release of Glu from primary afferent fibers to the spinal dorsal horn is a major transmitter of nociceptive transmission, where in it exerts excitatory, sensitizing actions upon both projection neurons and excitatory interneurons. While descending Glu exerts an excitatory influence upon GABAergic and glycinergic inhibitory interneurons in the spinal cord, in theory, may be involved in mechanisms of descending inhibition [29,42,43]. The present study showed that the extracellular Glu concentration in the spinal dorsal horn exhibited a trend to increase following intra-vlPAG N/OFQ, but did not reach statistical significance. It seems to be inconsistent with the report from Cui et al., in which activation, rather than inhibition, of the PAG produced Glu release in the spinal cord, and the release could serve as mediators that activate GABAergic and glycinergic neurons in the spinal cord [11]. In the present study, a light increase in Glu release may due to the interaction of various neurotransmitters release in the spinal cord by N/OFQ in the vlPAG, such as decrease in GABA release results in disinhibition on Glu release.

Conflict of interest

The authors declare that they have no conflict of interest.

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