N/OFQ modulates orofacial pain induced by tooth movement through CGRP-dependent pathways

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Abstract

Background: Nociceptin/orphanin FQ (N/OFQ) has been revealed to play bidirectional roles in orofacial pain modulation. Calcitonin gene-related peptide (CGRP) is a well-known pro-nociceptive molecule that participates in the modulation of orofacial pain. We aimed to determine the effects of N/OFQ on the modulation of orofacial pain and on the release of CGRP.

Methods: Orofacial pain model was established by ligating springs between incisors and molars in rats for the simulation of tooth movement. The expression level of N/OFQ was determined and pain level scored in response to orofacial pain. Both agonist and antagonist of N/OFQ receptor were administered to examine their effects on pain and the expression of CGRP in trigeminal ganglia (TG). Moreover, gene therapy based on the overexpression of N/OFQ was delivered to validate the modulatory role of N/OFQ on pain and CGRP expression.

Results: Tooth movement elicited orofacial pain and an elevation in N/OFQ expression. N/OFQ exacerbated orofacial pain and upregulated CGRP expression in TG, while UFP-101 alleviated pain and downregulated CGRP expression. N/OFQ-based gene therapy was successful in overexpressing N/OFQ in TG, which resulted in pain exacerbation and elevation of CGRP expression in TG.

Conclusions: N/OFQ exacerbated orofacial pain possibly through upregulating CGRP.

1 Background

Orofacial pain induced by tooth movement, highly prevalent among orthodontic patients (1), is characterized by a cascade of inflammatory reactions in periodontal tissues that eventually elicits pain sensation (2). As is well documented, orofacial pain sensation is initiated at periodontal sensory terminals, modulated at trigeminal ganglia (TG), replayed at trigeminal nucleus and finally reaches sensory cortex via thalamus (2). Particularly, TG undergo dramatic adaptations in response to orofacial pain (3, 4). Specifically, abundant proteins are upregulated and downregulated in concert to modulate orofacial pain (5, 6).

N/OFQ is derived from a precursor, prepronociceptin (PNOC), the sequence of which shares similar structural features to precursors of classical opioid peptides (7). The receptor of Nociceptin/orphanin FQ (N/OFQ), also designated as ORL1, belongs to the opioid receptor family and is widely distributed in both central and peripheral nervous system, especially abundant in pain modulation regions (8-11). Unlike other members of opioid family, N/OFQ plays a crucial role in pain modulation in a bidirectional manner, depending on a series of complex factors including pain quality, administration routes and dosages (12-15). Our previous study elucidated the pro-nociceptive effect of N/OFQ in periodontal tissue for orofacial pain (16). However, it is still largely unknown the effects of N/OFQ on orofacial pain in TG.

Calcitonin gene-related peptide (CGRP), a 37-amino-acid neuropeptide, is widely distributed in central and peripheral nervous system (17, 18). It is well documented that CGRP is essential for the modulation of

orofacial pain, especially migraine (19, 20). Our previous study revealed that CGRP played an important role in the modulation of orofacial pain (21). A further study showed that CGRP was co-expressed with N/OFQ in TG, justifying their interactions in pain modulation (22).

Therefore, in this study, we aimed to explore the specific role of N/OFQ in the modulation of orofacial pain and its impacts on CGRP expression.

2 Methods

2.1 Animals

In total, 365 male Sprague–Dawley rats (200-250 g) were obtained from the Animal Experimental Center at Sichuan University. They were maintained in the animal facility and kept in an air-conditioned room at 21 °C with a 12 h light-dark cycle. Standard rat chow and water were provided ad libitum. Animal experiments were performed in accordance with protocols that were approved by the ethical committee of the State Key Laboratory of Oral Diseases, Sichuan University (WCCSIRB-D-2015-006). Following general anesthesia with intraperitoneal injection of sodium pentobarbital (30 mg·kg⁻¹), rats were placed in supine positions, and intraoral NiTi alloy closed-coil springs were ligated between left upper first molars and incisors to mimic orthodontic tooth movement. Four force levels were used in this study, i.e., 0 g, 20 g, 40 g and 80 g. Force magnitudes were determined through a force meter (Tiantian, Changsha, China). Rats were euthanized by decapitation following anesthesia with pentobarbital sodium (50 mg·kg⁻¹) six hours following drug administrations. Rats that did not receive any intervention were regarded as the baseline control for each group.

2.2 Drug administration into TG

The administration of drugs and lentivirus vectors into TG was conducted according to our previous study with modifications that shaving was not performed to avoid its impact on behavioral testing (23). Following general anesthesia with intraperitoneal injection of sodium pentobarbital (30 mg·kg⁻¹), the injected region was disinfected with 75% ethanol. The injected position is between tympanic bulla and the posterior edge of mandibular ramus. The injected direction is middle upper, perpendicular to the long axis of rat body and 15 degree to coronary plane. When the needle reached trigeminal fossa, the drug was slowly injected at a constant speed. The injection process should last 1 min, and remained in situ for 1 min before withdrawn. Specifically, 15 µl nociceptin (10 nM), 15 µl UFP-101 (10 nM) or 15 µl normal saline was administered right after spring placement (0h) and on 1st day, 3rd day, 5th day and 7th day to evaluate the effects of N/OFQ on pain and CGRP expression. UFP-101 ([Nphe¹, Arg¹⁴, Lys¹⁵] N/OFQ-NH₂) is a specific peptide antagonist of N/OFQ which has been reported to have high efficacy and durability in vivo (24). N/OFQ-overexpressing lentivirus vector suspension (10 µl), blank vector suspension (10 µl) or normal saline (10 µl) were administered into TG one week before spring placement to assess the effects of N/OFQ-based gene therapy on pain and CGRP expression.

2.3 Orofacial pain assessment (rat grimace scale, RGS)

The assessment of orofacial pain was performed through rat grimace scale (RGS) six hours following drug administrations, strictly according to our previous study (25). In brief, rats were placed in transparent cubicles and videotaped for 30 min for each testing session. For each session, 10 images of facial expressions for each rat were extracted for scoring. The RGS scoring was performed based on the facial expression changes in orbit, nose, ear and whisker. The surrogate pain levels were obtained by subtracting the baseline RGS scores from the ones for testing sessions.

2.4 Tissue processing and analysis

For immunostaining, V1/V2 of TG were placed in liquid nitrogen and cryosectioned at a thickness of 10 μm by using microtome (Thermo Fisher Scientific, USA). The tissue samples were immunostained with primary antibodies against CGRP (1:200, Abcam, Cambridge, UK) and FITC-labelled rabbit anti-goat secondary polyclonal antibody IgG (1:100, Abcam, Cambridge, UK). Image observation and acquisition were performed by using a fluorescence microscope (Leica, Germany).

For western blot, V1/V2 of TG tissue samples were homogenized through RIPA lysis buffer with PMSF (Beyotime Biotechnology, China). Following electrophoretic separation, proteins were transferred onto PVDF membranes and blocked with 5% skim milk in TBST solution. Afterwards, sealed PVDF membranes were incubated in the primary polyclonal antibody against FLAG (1:3000, Sigma, USA), N/OFQ or β -actin, then washed with TBST and incubated with secondary polyclonal antibody (1:4000, Santa-cruz, USA). The protein blot densities were analyzed using ImageJ Software (NIH, Bethesda).

Simple Western (WES) analysis was performed on a WES system (ProteinSimple, WS-2471). The V1/V2 of TG tissues were homogenized with RIPA lysis buffer plus PMSF (Beyotime Biotechnology, China). After dilution and degeneration, the extracted proteins, along with the primary polyclonal antibody against CGRP, N/OFQ (Abcam, Cambridge, UK) or β -actin, horseradish peroxidase (HRP)-conjugated secondary antibody and chemiluminescent substrate, were put into the microplate as instructed. The target band intensity of Western Blot results was analyzed using Compass Software.

Real-time PCR was conducted to determine the mRNA levels of N/OFQ (Pnoc gene) and CGRP (Calca gene) with GAPDH being the reference. PCR was performed using specific primers for rat CGRP (forward primer: GAAGAAGAAGCTCGCCTACTGG, reverse primer: CTGTCCAAGCTAGAGCCCTCA, expected size: 110 bp) and Pnoc (forward primer: GCTCACGTCCGCTGCTCTTTA, reverse primer: TCCACCTCATCGGCCTCATCT, expected size: 147bp). Total RNA was extracted from TG via Trizol RNA Extraction Kit (Pufei, Shanghai, China) and cDNA reversely transcribed through the M-MLV test kit (M1705, Promega). The expressions were quantified in a LightCycler480 (Roche, Switzerland) RT-PCR platform with the SYBR Premix Ex Taq (Takara, Dalian, China). The thermal profile was set at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and then at 60 °C for 30 s.

2.5 Lentivirus vector construction

The lentivirus vector was constructed as previously described (23). As shown in Figure 4A, a lentivirus vector GV320 (Shanghai Genechem, China) containing red fluorescence (Cherry) and vector marker (3FLAG) was recombined with rat Pnoc gene. Pnoc was expressed under ubiquitin promoter, and m-cherry under SV40 promoter. Thus, the two genes were under two independent promoters. The specific sequences retrieved from GenBank (NM_013007) are displayed in Additional file 1. The recombined sequence was amplified with PCR and DNA sequencing performed for sequence verification. Viral vectors were packaged and harvested by transfecting 293T cells, followed by visualization through fluorescence microscope and verification of Pnoc expression through WB. The viral titer was determined to be 2×10^8 TU/ml. All the procedures involving use of lentivirus vectors were performed in the laboratory with biosafety level 2 (BSL2) containment according to General Guidelines for Biosafety in Microbiology and Biomedical Laboratories (WS233-2002).

2.6 In vivo verification of TG transduction

Virus transduction into TG was verified using an in vivo fluorescence imaging system (In-Vivo Xtreme, Bruker, Germany) on 1st day, 3rd day, 5th day and 7th day. In-Vivo Xtreme is a broad-spectrum and highly sensitive imaging system for both fluorescence and bioluminescence imaging in vivo, including charge-coupled device (CCD) cameras (4872×3248 or 2048×2048 pixels), high-speed 500 μ A X-ray head, light-tight imaging chamber and complete automation and analysis capabilities, and the excitation and emission filter wavelength ranges are 410-760 nm and 535-830 nm, respectively. The fluorescence reporter gene was cherry with excitation/emission wavelengths of 587/610 nm.

2.7 Statistical analysis

Results are depicted as mean $\pm$ standard error of the mean (SEM). Two-way ANOVA with repeated measures were used to analyze the effects of force magnitudes (0 g, 20 g, 40 g and 80 g), time (0 d, 1 d, 3 d, 5d, 7 d and 14 d) and their interactions on RGS scores and the expression levels of N/OFQ. One-way ANOVA with Bonferroni post hoc test was employed to analyze the differences in PNOC expression, CGRP expression and orofacial pain among different time points in each group. All the statistical analyses were performed in SPSS 19.0 and Graphpad Prism 6.0, with a p value less than 0.05 being considered as statistical significance.

3 Results

3.1 Orofacial pain elevated N/OFQ expression in TG

We found that orofacial pain-like behaviors indicated by RGS scores was elicited following tooth movement and started to increase on 1st day, peaked on 3rd day, decreased on 5th day and returned to baseline level on 7th or 14th day (Figure 1A). The two-way ANOVA with repeated measures revealed that RGS scores was significantly influenced by time ($p < 0.001$), force magnitude ($p = 0.005$) and their interactions ($p < 0.001$). Areas under curve (AUCs) were similar between 0-g group and 20-g group ($p > 0.05$), while significantly higher in the 40-g group ($p < 0.05$) and 80-g group ($p = 0.00$) (Figure 1B).

As displayed in Figure 1C & 1D, our results revealed that the expression levels of N/OFQ started to increase on 1st day and gradually decreased after 5th day following orthodontic tooth movement. Areas under curve (AUCs) were similar between 0-g group and 20-g group ($p>0.05$), while significantly higher in the 40-g group ($p<0.001$) and 80-g group ($p=0.001$) (Figure 1E). Thus, a force magnitude of 40 g was used for the following experiments.

3.2 The effects of N/OFQ agonist and antagonist on pain modulation and CGRP expression

As depicted in Figure 2, two-way ANOVA with repeated measures indicated that the RGS scores were significantly influenced by different groups ($P<0.05$) and time ($P<0.05$). The RGS scores were significantly higher in the N/OFQ agonist group than in the normal saline (NS) group on 3rd day, 5th day and 7th day ($p<0.01$). Moreover, the RGS scores were significantly lower in the antagonist UFP-101 group than in the NS group on 3rd day, 5th day and 7th day ($p<0.01$).

As displayed in Figure 3A and 3B, more CGRP-positive neurons were detected in the N/OFQ group and fewer CGRP-positive neurons were observed in the UFP-101 group. Two-way ANOVA with repeated measures indicated that CGRP expression was significantly influenced by different groups ($P<0.05$) and time ($P<0.05$). The expression levels of CGRP were significantly higher in the N/OFQ group than in the NS group on 3rd day, 5th day and 7th day (all $p<0.05$), and significantly lower in the UFP-101 group than in the NS group on 1st day, 3rd day, 5th day and 7th day (all $p<0.05$) (Figure 3C).

3.3 Successful transduction of Pnoc-overexpressing lentivirus vectors into TG

As displayed in Figure 4B, the Pnoc-overexpressing lentivirus vectors were able to transduce 293T cells, as evidenced by the cherry fluorescence. This overexpression was functionally successful as the N/OFQ-3FLAG fusion protein was detected (Figure 4C). Moreover, immunofluorescence staining and in vivo fluorescence imaging showed that lentivirus vectors were transduced at trigeminal ganglia (Figure 4D and 4E).

Two-way ANOVA with repeated measures indicated that the expression level of both PNOC gene and N/OFQ protein were significantly influenced by different groups ($P<0.05$) and time ($P<0.05$). The real-time PCR revealed that the expression level of PNOC gene was significantly higher in the PNOC-lenti-OE group (denoting Pnoc-overexpressing lentivirus transduction) than in the Ctrl-lenti group (blank lentivirus vector) on 3rd day, 5th day, 7th day and 14th day (all $p<0.05$) (Figure 5A). The western blot showed that the level of N/OFQ protein was significantly higher in the PNOC-lenti-OE group than in the Ctrl-lenti group and the NS group on 3rd day, 5th day and 7th day (all $p<0.05$) (Figure 5B & 5C).

3.4 The effects of N/OFQ overexpression on CGRP expression and pain modulation

As displayed in Figure 6A, two-way ANOVA with repeated measures indicated that the CGRP expression was significantly influenced by different groups ($P<0.05$) and time ($P<0.05$). The expression level of CGRP was significantly higher in the PNOC-lenti-OE group than in the Ctrl-lenti group on 3rd day, 5th day, 7th day

and 14th day (all $p < 0.05$). Moreover, as depicted in Figure 6B, the trend of the changes in expressions of CGRP and N/OFQ were highly correlated ($r = 0.925$).

RGS scoring revealed that pain levels were significantly higher in the PNOC-lenti-OE group than in the Ctrl-lenti group and the NS group on 3rd day, 5th day, 7th day and 14th day (all $p < 0.01$) (Figure 6C). Then, we plotted the difference values between PNOC-lenti-OE and Ctrl-lenti groups against time for RGS scores and N/OFQ expression level. We found that the difference values for both groups were most prominent on 7th day. Pearson's correlation test showed that the trend of the difference values for RGS scores and N/OFQ expression level were highly correlated ($r = 0.994$) (Figure 6D).

4 Discussion

In this study, we found that N/OFQ expression was elevated in response to orofacial pain elicited by tooth movement. N/OFQ could exacerbate orofacial pain and elevate CGRP expressions while UFP-101 alleviated orofacial pain and downregulate CGRP expressions. Furthermore, overexpression of PNOC gene that upregulated N/OFQ in trigeminal ganglia was able to exacerbate pain level and increase CGRP expression level in trigeminal ganglia.

Orofacial pain, a constellation of various painful conditions in orofacial regions, includes migraine, trigeminal neuralgia, headaches, dental pain and tooth-movement pain (26, 27). Of particular, orofacial pain induced by tooth movement is a type of inflammatory pain at periodontal tissues due to force application (25). It is well accepted that tooth movement elicits orofacial pain by obstructing periodontal blood vessels that in turn initiate a cascade of inflammatory response (28). Our previous study revealed that tooth-movement-induced orofacial pain was initiated by a force above 20 g and that pain level differed among rats receiving different force magnitudes (2). But it is still largely unknown whether this paradigm works similarly between force magnitude and the expression level of N/OFQ in TG. Notably, we found that pain level and N/OFQ expression level were upregulated in the 0-g group, which could be attributed to the painful stimulus induced by bulky intraoral springs (25). Furthermore, the AUCs of pain and N/OFQ were similar between the 0-g group and the 20-g group, while significantly higher in the 40-g group and 80-g group, indicating that the threshold force that could incite the tooth-movement-dependent upregulation of pain level and N/OFQ lies between 20 g and 40 g, which is consistent with our previous study (25). Therefore, we suggest that 40-g force could be sufficient to elicit orofacial pain and an elevation of N/OFQ expression in trigeminal ganglia.

N/OFQ and its receptor are the fourth opioid family member discovered so far (29). Despite high sequence similarity between N/OFQ and other opioid ligands, the lacking of an N-terminal tyrosine renders N/OFQ to have a negligible affinity for the three classical opioid receptors, thereby making it functionally distinct from the classical opioid ligands (e.g., morphine) (30). Though NOP receptor inhibits voltage-gated calcium channels and activates inward potassium channels coupled to pertussis toxin-sensitive Gi/o proteins, thereby impacting on the neurotransmitter release and neuronal excitability like other opioid receptors, the pain-modulatory effects mediated by N/OFQ-NOP are more complicated (29).

N/OFQ has been revealed to exhibit either pro- or anti-nociceptive effects, depending on a series of complex factors such as pain quality, doses and administration routes (12-15). In terms of rodents, N/OFQ system exhibits antinociceptive effects when peripherally and spinally activated, while pronociceptive effects after supraspinal activation. Therefore, the net effect of systemically administered NOP agonists on nociception is dependent on relative contribution of peripheral, spinal and supraspinal sites of action, which varies between rodents and non-human primates (31). The sensation of orofacial pain induced by tooth movement is initially received by the sensory terminals at periodontium, transmitted to TG, relayed to trigeminal nucleus at medulla oblongata and projected to sensory cortex via thalamus (2). Our previous studies have reported that N/OFQ participates in pain modulation at periodontium and trigeminal nucleus caudalis (16, 32). Although previous findings have demonstrated that N/OFQ and its receptor are actively expressed in neuronal cells of trigeminal ganglia and our previous study found an indirect evidence that N/OFQ could modulate the expression of a key nociceptor (P2X3) on trigeminal neurons (22, 33), its pain-modulatory role at TG is still largely unknown. Our results revealed that intra-ganglionic administration of N/OFQ exacerbated pain while that of UFP-101 alleviated pain, suggesting that N/OFQ plays a pro-nociceptive role in orofacial pain induced by tooth movement. This finding was consistent with our previous studies where we found that periodontal administration of N/OFQ antagonist UFP-101 was able to alleviate pain in rats and that N/OFQ could upregulate the expression of a nociceptor P2X3 (16, 33). However, Borgland et al. revealed that N/OFQ inhibited calcium currents of trigeminal neurons, supporting its antinociceptive role in TG (34). We attribute this disagreement to the fact that N/OFQ was able to inhibit calcium currents in only a subpopulation of trigeminal neurons in the study by Borgland et al. (34).

CGRP is a well-known pronociceptive molecule for orofacial pain (35). The upregulation and release of CGRP are the hallmarks during pain episodes of orofacial pain (36). The release of CGRP to peripheral tissues could induce neurogenic inflammation that exacerbates pain (37, 38). And we found CGRP was upregulated and released to periodontal tissues in response to orofacial pain in our previous studies (21, 39). However, current evidence on the interaction between CGRP and N/OFQ is generally lacking, especially in the aspect of pain modulation. Previous studies have shown that N/OFQ were abundantly expressed with a high degree of coexpression with CGRP both in dorsal root ganglion and trigeminal ganglion, suggesting that N/OFQ modulated primary sensory nociception through interacting with CGRP (22, 40). In trigeminal neurons, N/OFQ-NOP system has been reported to impact on stimulated CGRP release, whereas how N/OFQ interacts with CGRP in response to pain stimuli is largely unknown (41, 42). In our present study, we found that N/OFQ upregulated while UFP-101 downregulated the expression level of CGRP in trigeminal ganglia, supporting the notion that N/OFQ has a pronociceptive role in trigeminal ganglia via promoting CGRP expression and release, thus facilitating transmission of pain signals. Noteworthy, UFP-101 actively decreased the amount of CGRP protein by approximately 60% on 1st day, which was attributed to the accumulative effect of UFP-101 administered respectively at baseline and 1st day.

In terms of methodology of this study, facial expressions have been widely used to evaluate pain level of non-verbal experimental animals, among which RGS is a standardized behavioral coding system demonstrated to have high accuracy and reliability (43). Interestingly, the RGS scores were greater for the NS group in Figure 2I than that in Figure 1B, which can be mainly attributed to the fact that the rats of Figure 2I received drug injection that aggravated their pain level, thus leading to generally higher RGS scores. Simple Western (WES) analysis is a revolutionized automated western blotting technique, the high accuracy and reproducibility in quantification of which has been validated by plenty of previous studies (44-46). The results of WES are actually simulated according to signal intensity of samples in different lanes, thus presented as bands with blurred margins. There may be slight differences in the band position of the same protein in different lanes due to different concentration of salty ions in each sample, while the difference is within an acceptable range. Lentivirus vector, a single-strand RNA with a length of 8 kb (49), is most suitable for the transduction of non-dividing cells, especially neurons and neural glial cells (50, 51). The integration of viral gene into host genome on one hand is beneficial for stable expression, which makes it a promising gene therapeutic tool for a variety of pain conditions, especially for chronic pain (47, 48, 52). Our previous study revealed that administration of lentivirus containing TRPV1 shRNA aiming at knocking down TRPV1 was effective in alleviating orofacial pain (53). In our present study, transduction of was successful in overexpressing N/OFQ in trigeminal ganglia and the overexpression was stable on 3rd day, 5th day and 7th day, but not on 14th day. We attribute this decrease of N/OFQ expression on 14th day to built-in analgesic pathways that were activated at the late stage of orofacial pain. Notably, the increase in N/OFQ did not reach statistical significance on 1st day, which might be significantly different if the virus was injected earlier (e.g., two weeks before spring placement). We showed that the overexpression of N/OFQ was able to upregulate CGRP expression and exacerbate orofacial pain induced by tooth movement. Moreover, the N/OFQ expression levels and pain levels were highly correlated with each other, further reinforcing the aforementioned results. Notably in Figure 3C, the expression level of CGRP in NS group was decreased, suggesting that trigeminal injection inhibited CGRP expression in TG. In contrast, the expression level of CGRP was significantly higher in N/OFQ group, which revealed that N/OFQ upregulated CGRP expression in TG.

Though our study firstly revealed the modulatory role of N/OFQ system in tooth-movement-induced orofacial pain which is dependent on CGRP, there are some limitations in this study. Although CGRP has been well-documented as a pronociceptive factor in TG neural system, and our previous study has validated the pain-modulatory effect of CGRP on tooth-movement-induced pain, direct evidence on the regulatory role of CGRP on tooth-movement-induced pain in our present study was not validated. Besides, while gain-of-function study conducted using PNOX overexpression lentivirus suggested the pronociceptive effect of N/OFQ in trigeminal ganglia, the knockdown of PNOX may be more important to validate the therapeutic value of N/OFQ. Moreover, the role of NOP receptor and its relationship with orofacial pain and CGRP expression need specific investigations. Therefore, based on above limitations, further studies digging into more thorough pain-modulatory mechanisms of N/OFQ-NOP system are expected.

5 Conclusions

Taken together, we suggest that N/OFQ modulate orofacial pain induced by tooth movement possibly through CGRP-dependent pathways.

6 Declarations

Ethics approval and consent to participants

Animal experiments were approved by the ethical committee of the State Key Laboratory of Oral Diseases, Sichuan University (WCCSIRB-D-2015-006).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

In this study, LW and LH were mainly responsible for topic proposal and theoretical guidance, YX and HH were in charge of experiment conduction, ZS and LY contributed to data collection and statistical analysis, RL and TY were responsible for article writing and proofreading, LX and WY took charge of graphic production.

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Additional File

Additional file 1. Rat Pnoc overexpression sequence. The specific sequences were retrieved from GenBank (NM_013007), with red indicating PNOC mRNA sequence, and ACCGGT is AgeI enzyme cutting site.

Figures

Figure 1

The effect of tooth movement on orofacial pain level and N/OFQ expression. Two-way ANOVA with repeated measures were used to analyze the effects of force magnitudes (0 g, 20 g, 40 g, 80 g, n=30 for each group), time (0 day, 1st day, 3rd day, 5th day, 7th day, 14th day, n=5 for each group each day) and their interactions on the expression levels of N/OFQ and RGS scores. (A) Temporal changes of RGS scores in all the force groups. (B) One-way ANOVA with Bonferroni post hoc test was used to analyze the

area under curve (AUC) of RGS scores. (C) Western blot analysis for the quantification of N/OFQ expression in trigeminal ganglia, with β -tubulin being the internal reference. (D) Temporal changes of N/OFQ expression in all the force groups. (E) One-way ANOVA with Bonferroni post hoc test was used to analyze the AUC of the expression level of N/OFQ. (* P <0.05, 40 g group vs. 0 g group, 80 g group vs. 0 g group).

Figure 2

The effects of N/OFQ receptor agonist and antagonist on pain modulation. (A)-(D) Facial expression changes in the context of no pain. (E)-(H) Facial expression changes in the context of pain. (I) Temporal changes of RGS scores in NS group, N/OFQ group and UFP-101 group ($n=8$ for each group). One-way ANOVA with Bonferroni post hoc test was employed to analyze the effects of N/OFQ receptor agonist (N/OFQ), antagonist (UFP-101) and normal saline (NS) on RGS scores. (** P <0.01, NS group vs. N/OFQ group, NS group vs. UFP-101 group, N/OFQ group vs. UFP-101 group).

Figure 3

The effects of N/OFQ receptor agonist and antagonist on CGRP expressions. (A) CGRP immunostaining in trigeminal ganglion of rats in NS group, N/OFQ group and UFP-101 group (3rd day). (B) Western blot analysis of CGRP expression in trigeminal ganglion of rats in NS group, N/OFQ group and UFP-101 group using WES. (C) Temporal changes of CGRP expression level in NS group, N/OFQ group and UFP-101 group (n=18 for each group, n=3 for each group each day). One-way ANOVA with Bonferroni post hoc test was employed to analyze the effects of N/OFQ receptor agonist (N/OFQ), antagonist (UFP-101) and normal saline (NS) on CGRP expressions. (* $P < 0.05$, NS group vs. N/OFQ group, NS group vs. UFP-101 group, N/OFQ group vs. UFP-101 group).

Figure 4

Construction of PNOC overexpression lentivirus vector. (A) The map of GV320 vector. (B) Successful transduction of lentiviral vector into 293T cells as shown by fluorescence microscopy that most cells were marked by enhanced red fluorescence protein (Cherry). (C) Western blot analysis of 293T cells transfected with positive reference (SURVIVIN-3FLAG-GFP, 48KD) (1), not transfected (2), and transfected with PNOC lentiviral vectors (N/OFQ-3FLAG, 23KD) (3). (D) Immunofluorescence images of TG in rat stained with cherry, which indicates successful transfection with PNOC lentivirus. (E) In vivo biofluorescence signals of TG in rat successfully transfected with PNOC lentivirus, which is consistent with immunofluorescence staining images.

Figure 5

The effects of PNOC overexpression lentivirus vector on PNOC and N/OFQ expression. (A) Temporal changes of PNOC mRNA expression level in trigeminal ganglia of rats in PNOC lentivirus group and control lentivirus group (n=15 for each group, n=3 for each group each day). One-way ANOVA with Bonferroni post hoc test was employed to analyze the effects of control lentivirus and PNOC overexpression lentivirus on PNOC mRNA expression. (* $P < 0.05$, ** $P < 0.01$, ctrl-lenti group vs. PNOC-lenti-OE group). (B) Temporal changes of N/OFQ expression in trigeminal ganglia of rats in NS group, control lentivirus group and PNOC lentivirus group (n=15 for each group, n=3 for each group each day). One-way ANOVA with Bonferroni post hoc test was employed to analyze the effects of normal saline, control lentivirus and PNOC overexpression lentivirus on N/OFQ expression. (* $P < 0.05$, PNOC-lenti-OE group vs. NS group, PNOC-lenti-OE group vs. ctrl-lenti group). (C) Western blot analysis of N/OFQ expression in trigeminal ganglion of rats in NS group, control lentivirus group and PNOC lentivirus group using WES.

Figure 6

The effects of PNOc overexpression on CGRP expressions and pain modulation. (A) Temporal changes of CGRP mRNA expression in trigeminal ganglia in control lentivirus group and PNOc lentivirus group ($n=15$, $n=3$ for each group each day). One-way ANOVA with Bonferroni post hoc test was employed to analyze the effects of control lentivirus and PNOc overexpression lentivirus on CGRP mRNA expression. ($*P<0.05$, $**P<0.01$, ctrl-lenti group vs. PNOc-lenti-OE group). (B) Changes in PNOc expression and CGRP expression in PNOc lentivirus group exhibited high correlation ($r=0.925$). (C) Temporal changes of RGS scores in NS group, control lentivirus group and PNOc lentivirus group ($n=8$ for each group). One-way ANOVA with Bonferroni post hoc test was employed to analyze the effects of normal saline, control lentivirus and PNOc overexpression lentivirus on RGS scores. ($**P<0.01$, PNOc-lenti-OE group vs. NS group, PNOc-lenti-OE group vs. ctrl-lenti group). (D) Difference value of orofacial pain level and N/OFQ expression level between PNOc lentivirus group and control lentivirus group. The trends of the two curves are similar and highly correlated ($r=0.994$).

Supplementary Files

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