Experimental Neurology 291 (2017) 62–73

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Activation of NPFFR2 leads to hyperalgesia through the spinal inflammatory mediator CGRP in mice

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ARTICLE INFO

Article history:
Received 11 October 2016
Received in revised form 25 January 2017
Accepted 1 February 2017
Available online 5 February 2017

Keywords:
CGRP
Hyperalgesia
Neuropeptide FF (NPFF)
NPFFR2
DRG
Pain transmission

ABSTRACT

Neuropeptide FF (NPFF) is recognized as an opioid modulating peptide that regulates morphine-induced analgesia. The aim of this study was to delineate the role of NPFFR2 in pain transmission. We found the expression levels of NPFF and NPFFR2 were increased in the lumbar dorsal horn of animals with CFA- and carrageenan-induced inflammation and both NPFFR2 over-expressing transgenic (NPFFR2-Tg) and NPFFR2 agonist-treated mice displayed hyperalgesia. BOLD signals from functional MRI showed that NPFFR2-Tg mice exhibited increased activation of pain-related brain regions after painful stimulation when compared to WT mice. Inflammatory mediators within the spinal cord, calcitonin gene-related peptide (CGRP) and substance P (SP), were up-regulated in NPFFR2-Tg and chronic NPFFR2 agonist-treated mice. In DRG cultures, treatment with an NPFFR2 agonist induced the expression and release of CGRP, an action which was blocked by NPFFR2 siRNA. Furthermore, treatment with a CGRP antagonist ameliorated the pain hyperalgesia in NPFFR2-Tg mice, returning the pain threshold to a control level. However, treatment with a SP antagonist reduced the pain responses in both WT and NPFFR2-Tg mice and did not suppress pain hypersensitivity in NPFFR2-Tg mice. Together, these results demonstrate that NPFFR2 activation modulates pain transmission by up-regulating the pain mediator CGRP, leading to hyperalgesia.

1. Introduction

Neuropeptide FF (NPFF; with the sequence FLFQPQRF) belongs to the RF-amide peptide family and was first isolated from bovine brain by virtue of the shared N-terminal sequence, RF-NH_2 (Yang et al., 1985; Yang et al., 2008). There are five subgroups of the RF-amide family that include NPFF, prolactin releasing peptide (PrRP), RF-amide related peptide (RFRP), kisspeptin, and pyroglutamylated RF-amide peptide (QRFP) (Jhamandas and Goncharuk, 2013). NPFF is ubiquitously expressed in the central nervous system with highest expression in the hypothalamus, posterior pituitary and spinal cord (Yang et al., 2008). Two Gi protein-coupled receptors have been identified as NPFF cognate receptors, NPFFR1 (GRP147) and NPFFR2 (GPR74) (Bonini et al., 2000; Elshourbagy et al., 2000). In addition to bind NPFF, NPFFR1 is further recognized as the receptor for RFRP (also known as NPVF) (Hinuma et al., 2000; Liu et al., 2001). NPFFR2 is highly expressed in pain-processing regions such as spinal dorsal horn, thalamus and dorsal raphe nucleus (Liu et al., 2001; Zeng et al., 2003). In DRG neurons, it is synthesized in cell bodies and trans-located to the afferent sensory nerve terminals in the spinal dorsal horn (Gouarderes et al., 2000). The NPFF-NPFFR system is primarily known to function in nociception and opiate analgesia (Panula et al., 1999; Yang et al., 2008). In the spinal cord, these effects are modulated through NPFFR2, since NPFFR1 expression has not been detected (Yang et al., 2008).

NPFF-NPFFR signaling results in either pro-nociceptive or anti-nociceptive effects (Roumy and Zajac, 1998). These opposing outcomes can be specifically stimulated by different routes of NPFF administration (intra-thecal (i.t.) vs. intra-cerebroventricular (i.c.v.)), or regulation via two distinct NPFF receptors (Ayachi and Simonin, 2014; Lameh et al.,

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http://dx.doi.org/10.1016/j.expneurol.2017.02.003
0014-4886/

2010; Yang et al., 2008). For example, reduced pain threshold in the tail-flick test (Yang et al., 1985) and decreased morphine-induced analgesia (Dupouy and Zajac, 1997) was observed in rats after i.c.v. administration of NPFF. I.c.v. injection of an NPFFR2 selective agonist reversed morphine-induced analgesia (Roussin et al., 2005). Neutralization of endogenous NPFF was found to potentiate morphine analgesia (Kavaliers and Innes, 1992; Kavaliers and Yang, 1989). However, i.t. injection with NPFF or its analog produced analgesia or potentiated the opioid analgesic effect (Gouarderes et al., 1996; Kontinen and Kalso, 1995).

Nociceptors in the peripheral sensory nerve terminals can be activated by different inflammatory mediators that include cytokines and neurotrophins (Coutaux et al., 2005; Marchand et al., 2005). The ascending nociceptive pathway relies on a cascade of pain mediators, including glutamate, calcitonin gene-related peptide (CGRP) and substance P (SP). CGRP and SP deliver the nociceptive message from DRG to spinal cord neurons and the enhancement of these signals triggers nociceptive 'central sensitization' along the pain pathway (Basbaum et al., 2009; Seybold, 2009). These two neurotransmitters are synthesized in the DRG, stored together in the dense-core vehicles of sensory nerve terminals and co-released upon stimulation (Matteoli et al., 1988; Seybold, 2009; Snijdelaar et al., 2000). Two forms of CGRP, αCGRP and βCGRP, which share >90% sequence homology, bind to calcitonin receptor-like receptor (CLR) (Russell et al., 2014). αCGRP is the predominant form, with expression throughout the nervous system, while βCGRP expression is mostly restricted to the enteric nervous system (Mulderry et al., 1985; Russell et al., 2014). During pain transduction, CGRP is up-regulated by neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) to modulate the expression of pro-inflammatory cytokines (Lin et al., 2011; Russell et al., 2014). SP is mainly expressed in unmyelinated sensory fibers and like CGRP, its expression is also up-regulated by NGF (Snijdelaar et al., 2000). SP produces a slow excitatory postsynaptic potential and is involved in the transmission of delayed pain signals by binding to neurokinin 1 receptor (Snijdelaar et al., 2000; Zubrzycka and Janecka, 2000).

This study focuses on describing the function of NPFFR2 in the spinal cord to delineate its role in nociception. By using genetic and pharmacological tools, we explored a novel mechanism that involved the CGRP-leaded pain transmission in NPFFR2-mediated hyperalgesia.

2. Methods

2.1. Animals

Male C57BL/6 mice (age 8–10 weeks) were purchased from National Laboratory Animal Center (Taipei, Taiwan) and randomly housed 5–6 per cage in Specific pathogen free (SPF) animal room. Mice were acclimatized to the room with a controlled temperature, air humidity and 12 h day-night cycle (light on at 7:00 AM). Food (Western Lab 7001, Orange, CA, USA) and water were available ad libitum. All the behavioral tests took place during the light cycle. Animal handling and drug treatments were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Animal Care Committee of Chang-Gung University (CGU 08-06 and CGU 13-014).

2.2. NPFFR2 transgenic (Tg) mice

The generation of NPFFR2-Tg mice was described previously (Lin et al., 2015). In brief, C57BL/6 NPFFR2 cDNA was cloned into the pWHERE plasmid (InvivoGen, CA, USA) under a 5′-adjacent neuron-specific enolase (NSE) promoter. NPFFR2-Tg mice were generated on a C57BL/6 mice background and maintained as heterozygotes. The formal nomenclature of NPFFR2-Tg mice is C57BL/6C-Tg(Npffr2)^{Dyj2}. Littermate wild-type (WT) mice served as controls. Only male mice age 8–12 weeks were used.

2.3. Induction of inflammation in mice

Mice were anesthetized with 1.5–2% isoflurane. Complete Freund's adjuvant (CFA, with 0.05 mg heat-killed and dried *Mycobacterium tuberculosis*, Sigma, St. Louis, MO, USA) was mixed 1:1 with saline to become a water-in-oil emulsion. 30 μl of saline, CFA mixture or 1% carrageenan (Sigma) were injected into both hindpaws of the corresponding groups and lumbar spinal dorsal horn was collected 4, 10, 20, 30 or 72 h after administration for the subsequent PCR quantification. In a different set of experiment, thermal hyperalgesia were measured by the Hargraves' plantar test at time 0 (before administration) and 24, 48, 72 h after the treatment.

2.4. Real-time PCR

Total RNA was isolated from tissues using TRIzol® reagent according to the manufacturer's protocol. The mRNA was transcribed into cDNA via reverse transcription PCR (HT BioTechnology, England UK). The cDNA levels for corresponding targets were measured by real-time PCR using the Bio-Rad iCycler PCR Thermal Cycler (Bio-Rad, Hercules, CA, USA) and SYBR (Bio-Rad). The PCR protocol was as follows: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 30 s for 40 cycles. The primer sequences are Rpl35a-forward 5′ GCT GTG GTC CAA GGC CAT TTT 3′; Rpl35a-reverse, 5′ CCG AGT TAC TTT TCC CCA GAT GAC 3′; NPFFR2-forward, 5′ ACA TCT ACC CTT TCG CCC AC 3′; NPFFR2-reverse, 5′ GCT TCT CCC ATT TCC TCT ATC AA 3′; NPFF-forward, 5′ GTA TGC CCA CAT TCC AGA CA 3′; NPFF-reverse, 5′ TGG AGC AGA ACA CGA AAG AG 3′. Threshold cycle, Ct, which correlates inversely with the target mRNA levels, was measured and relative expression levels were calculated. After normalization to Rpl35a, data were expressed as a percentage of the corresponding control.

2.5. Nociceptive tests

Mechanical hyperalgesia was measured by the von Frey method. Mice were placed in a clear plexiglass box (L5 × W5 × H10 cm) on an elevated mesh screen. A calibrated von Frey rigid tip (Electronic von Frey Anesthesiometer, IITC Life Science, CA, USA) was applied to the plantar surface of each hindpaw in a series of logarithmically ascending forces. The responses were recorded in grams of paw withdrawal and the average of five applications was referred to as the mechanical hyperalgesia threshold. The interval of each application was 5 min. Thermal hyperalgesia was measured by the hot plate or the Hargraves' plantar test. For the hot plate test, mice were placed on a 50 °C hot plate chamber (Ugo Basile, Varese, Italy) and the first nociceptive response, including lick, raised hindpaw or jump was recorded. For the Hargraves' plantar test, a commercial plantar test instrument was used (Ugo Basile). Mice were placed in the animal enclosure (L10 × W10 × H14 cm) to adapt for 1 h. Infrared intensity was set at 15 and was randomly applied to the mouse plantar surface of each hindpaw. The responses were recorded in seconds of paw withdrawal latency and the average of five applications was referred to as the thermal hyperalgesia threshold. The interval of each application was 5 min.

2.6. Functional MRI (fMRI)

Adult male mice (age 10–11 weeks) were initially anesthetized with 1.5% isoflurane. A pair of needle electrodes was inserted subcutaneously into the left forepaw with a distance of 2–3 mm between the two needles for electrical stimulation. A PE10 tube was then placed subcutaneously underneath the back skin for further infusion of dexmeditomidine (Dexdomitor®, Pfizer, New York, NY, USA). Mice then received a subcutaneous injection in the back of 0.05 mg/kg dexmeditomidine. Isoflurane was slowly reduced and stopped within 10 min after the dexmeditomidine injection. The mice were then continuously infused with dexmeditomidine 0.1 mg/kg/h via a syringe