Nociceptin/Orphanin FQ receptor expression in clinical pain disorders and functional effects in cultured neurons

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Running Title: NOP receptor in clinical pain disorders

Abstract

The Nociceptin/Orphanin FQ peptide receptor (NOP), activated by its endogenous peptide ligand Nociceptin/Orphanin FQ (N/OFQ), exerts several effects including modulation of pain signalling. We have examined, for the first time, the tissue distribution of the NOP receptor in clinical visceral and somatic pain disorders by immunohistochemistry, and assessed functional effects of NOP and μ opioid receptor activation in cultured human and rat dorsal root ganglion (DRG) neurons. Quantification of NOP-positive nerve fibres within the bladder sub-urothelium revealed a remarkable several-fold increase in Detrusor Overactivity ($p<0.0001$) and Painful Bladder Syndrome patient specimens ($p=0.0014$), compared to controls. In post-mortem control human DRGs, 75-80% of small/medium neurons (≤ 50 μm diameter) in the lumbar (somatic) and sacral (visceral) DRG were positive for NOP, and fewer large neurons; avulsion-injured cervical human DRG neurons showed similar numbers. NOP-immunoreactivity was significantly decreased in injured peripheral nerves ($p=0.0004$), and also in painful neuromas ($p=0.025$). Calcium imaging studies in cultured rat DRG neurons demonstrated dose-dependent inhibition of capsaicin responses in the presence of N/OFQ, with an IC50 of 8.6 pM. In cultured human DRG neurons, 32% inhibition of capsaicin responses was observed in the presence of 1 pM N/OFQ ($p<0.001$). The maximum inhibition of capsaicin responses was greater with N/OFQ than μ-opioid receptor agonist DAMGO. Our findings highlight the potential of NOP agonists, particularly in urinary bladder overactivity and pain syndromes. The regulation of NOP expression in visceral and somatic sensory neurons by target-derived neurotrophic factors deserves further study, and the efficacy of NOP selective agonists in clinical trials.

Keywords:

NOP receptor; Nociceptin/Orphanin FQ; pain; bladder.

Abbreviations:

DAMGO,	[D-Ala^2,NMe-Phe^4,Gly-ol^5]-enkephalin
DO,	detrusor overactivity
DRG,	dorsal root ganglion
MOP,	μ opioid peptide receptor
NOP,	Nociceptin/Orphanin FQ peptide receptor, ORL 1, opioid receptor-like 1
N/OFQ	Nociceptin/Orphanin FQ
NF,	neurofilament
PBS,	Painful Bladder Syndrome

1. Introduction

The Nociceptin / orphanin FQ peptide receptor (NOP; also called opioid receptor-like 1, ORL1 receptor) is a G-protein-coupled receptor (GPCR) sharing high sequence identity with the three classically recognised opioid receptor types, μ, δ, and κ, also termed MOP, DOP and KOP respectively [26; 39]. The endogenous ligand for the NOP receptor, known as Nociceptin [37] or Orphanin FQ [46], is a seventeen amino acid peptide whose N-terminal tetrapeptide sequence is related to that of the opioid peptides (FGGF or YGGF), and whose basic core is similar to Dynorphin, the endogenous peptide for the κ receptor. Inspite of these similarities, the NOP receptor does not recognize the majority of opioid ligands, and Nociceptin/Orphanin FQ (N/OFQ) itself has low affinity for the classic opioid receptors [46]. NOP receptor agonists are highly effective analgesics in a range of pre-clinical pain models, and provide the prospect of new treatments for chronic pain without abuse liability in humans (see [33]).

The NOP receptor couples to the same Gi/Go protein-mediated second-messenger systems as the opioid receptors and produces inhibition of adenylate cyclase, activation of an inwardly rectifying K+ conductance, and inhibition of voltage-sensitive Ca2+ channels [36]. These intra-cellular effectors generally act to inhibit cellular excitability, and suggest a role for the N/OFQ / NOP system in the modulation of neuronal activity and transmitter release [22, 41].

Activation of the NOP receptor is effective in several animal models of neuropathic pain and visceral hypersensitivity disorders. To illustrate, both peripheral [42] and spinal [15; 42; 59] NOP activation reduced mechanical allodynia in the rat chronic constriction injury model, and peripheral NOP receptor activation reduced capsaicin-induced thermal nociception in

non-human primates [24]. In rats receiving TNBS instillation, N/OFQ inhibited colonic hyperalgesia after intraperitoneal application [1]. Clinically, therapeutic effectiveness of N/OFQ instillation has been reported in patients with Detrusor Overactivity [24], as well as in patients with Painful Bladder Syndrome [30].

Most of the data on NOP receptor expression is derived from rodents, and using mRNA expression analysis (see [38]). NOP is expressed both in the central nervous system and in peripheral tissues. However, little is known about the localization of the NOP receptor in human tissues, and information about any changes in expression levels in human disease is virtually absent. Therefore, we investigated NOP receptor levels in tissues from humans with pain and hypersensitivity disorders; further, we investigated NOP receptor signalling in cultured rat and human dorsal root ganglion neurons using calcium imaging.

The aim of our studies was to validate the NOP receptor as a target in tissues from clinical pain disorders, and identify patient groups with chronic pain and hypersensitivity in which activation of NOP may yield a therapeutic benefit.

2. Materials and Methods

2.1. Clinical Tissues

A range of clinical tissues including sensory ganglia (dorsal root ganglia, DRG), peripheral nerve, and urinary bladder were studied. This study was done with approval from the Local Research Ethics Committees, and full informed consent was obtained from all tissue donors.

2.2. Urinary Bladder

Bladder tissue specimens were obtained from control subjects (n=20), patients with Overactive Bladder (Detrusor Overactivity; DO, n = 20), and Painful Bladder Syndrome (PBS, n = 8). All participants underwent clinical history and examination. The control subjects were under investigation for asymptomatic microscopic haematuria [60]. Subjects with Overactive Bladder demonstrated symptomatic urinary urgency and urge incontinence assessed using the ICIQ-LUTS QOL questionnaire, > 10 voids per day on a minimum 3 day bladder diary, and systolic detrusor overactivity with no cough stress leakage during cystometry. Urodynamic stress incontinence controls demonstrated symptomatic stress incontinence assessed using the ICIQ-LUTS QOL questionnaire, <6 voids per day on a minimum 3 day bladder diary, absence of systolic or provoked detrusor overactivity during cystometry, cystometric capacity >450ml, and positive cough stress leakage at video cystourethrography. Patients with Painful Bladder Syndrome met the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) research criteria for Interstitial Cystitis, as described by us previously [60]. Flexible or rigid cystoscopic bladder biopsies were obtained from a consistent site just above and lateral to the ureteric orifices. A urine specimen was sent for culture before each cystoscopy to confirm all patients had sterile urine cultures at the time of cystoscopy. A full thickness PBS bladder specimen was also obtained during surgery [60].

2.3. Dorsal root ganglia (DRG)

Control DRG (n = 10, 5 lumbar and 5 sacral) were obtained from the Netherlands Brain Bank from human cadavers collection with consent and described methodology [45]. Cervical avulsed DRG were available from 2 young male patients who had surgical repair of traumatic brachial plexus injuries, as described [50].

2.4. Peripheral nerves

Specimens of injured limb nerves (n = 10) and painful neuromas (n = 7) were collected from patients during surgery for painful neuroma excision / relocation or peripheral nerve repair. Uninjured nerve trimmings (n = 12), used as for grafting in nerve repairs during surgery, served as controls.

2.5. Immunohistology

An antibody to the NOP receptor (sc-15309) was obtained from Santa Cruz Biotechnology (Santa Cruz, USA), and used initially at dilutions from 1:50 to 1:5000 (final dilution 1:100). The NOP receptor antibody (sc-15309) is a rabbit polyclonal antibody with an epitope corresponding to amino acids 161-245 mapping to an internal region of the NOP receptor (KOR-3) of human origin. In our studies, this antibody reacted with neuronal fibres and DRG neurons in human tissues (Figs. 1 - 3). Specificity controls for immunostaining were satisfactory: nerve structures were immunostained in a titre specific manner (Fig. 2A), and no immunostaining was observed when the primary antibody was omitted. Additionally, no staining was observed in human cerebellum (Fig. 2B), a tissue where absence or only very limited presence of both NOP receptor transcripts and N/OFQ binding has been demonstrated [11, 44, 57]. Similar immunoblotting results were reported with this antibody compared to other antibodies directed against the C or N terminus of the receptor [23]. This antibody has been previously shown to immunostain rat myenteric neurons [61], and both immunostain and Western blot cultured rat astrocytes [20] and spinal cord [21] with the expected band of ~40kDa, at dilutions similar to our studies. Western blotting studies with this antibody has also revealed that NOP receptor expression of an expected 40KDa band in distal colonic samples of TNBS treated rats [1]. An affinity-purified rabbit antibody to TRPV1 (C22,

unrestricted gift from GlaxoSmithKline, Stevenage, UK, final dilution 1:10,000) against a synthetic peptide sequence of human TRPV1 sequence was used in this study as previously described [50]. We have previously demonstrated the specificity of this TRPV1 antibody in the bowel [13] and bladder [10]. A cocktail of monoclonal antibodies to the phosphorylated and non-phosphorylated Neurofilaments of size 200 kDa (Clone N52, Sigma-Aldrich, Dorset, UK) and the 57 kDa type III filament, peripherin (Novocastra Laboratories, Newcastle, UK) were used at final titres of 1:20,000 and 1:500 respectively, as structural neuronal markers.

Tissues specimens were snap frozen in liquid nitrogen and stored at -70°C until use. Tissues were supported in optimum cutting tissue (OCT) medium (R A Lamb Ltd, Eastbourne, UK). Tissue sections (15µm thick) were collected onto poly-L-lysine (Sigma, Poole, UK) coated glass slides, and post-fixed in 4% w/v paraformaldehyde in 0.15M phosphate buffered saline (PBS) for 30 minutes. Endogenous peroxidase was blocked by incubation in industrial methylated spirits (IMS) containing 0.3% w/v hydrogen peroxide for 30 minutes. After rehydration with PBS buffer, sections were incubated overnight with primary antibody at dilutions listed above. Sites of primary antibody attachment were revealed using nickel-enhanced, avidin-biotin peroxidase (ABC - Vector Laboratories, Peterborough, UK) as described. Sections were counter-stained for nuclei in 0.1% w/v aqueous neutral red, dehydrated and mounted in xylene-based mountant (DPX; BDH/Merck, Poole, UK), prior to photomicrography.

2.6. Image Analysis

For urinary bladder studies, images were captured (x40 magnification) in the sub-urothelium of each tissue section. Five fields per tissue section were scanned, and the mean value used in subsequent statistical analysis. For DRG neurons, NOP receptor and TRPV1-

immunoreactive nucleated neurons were counted visually, their diameter measured using a calibrated microscope eyepiece graticule, and results expressed as % of total number of neurons. The peripheral nerve analyses were based on the density of nerve fibres, as described in our previous published studies [3; 13]. Immunoreactive fibres were quantified using computerised software, where analogue images were captured via video link to an Olympus BX50 microscope and converted into digital monochrome images, and analysed using analySIS (version 5.0) software. The grey-shade detection threshold was set at a constant level to allow detection of positive immunostaining and the area of highlighted immunoreactivity obtained as a percentage (% area) of the field scanned. The analyses were performed in blinded manner. The Mann-Whitney test was used for statistical analysis (P values < 0.05 were considered statistically significant).

2.7. Rat DRG neuron cultures

Bilateral DRG from all levels were harvested from 5 adult female Wistar rats (250 gms, Charles River, Margate, Kent), enzyme-digested in 0.5% dispase/0.2% collagenase for 3 hours, followed by 30 minutes in papain (0.1%, 12 U/ml, Sigma), and mechanically dissociated in BSF2 medium containing soybean trypsin inhibitor/DNAse to obtain a single cell suspension, as previously described [7; 8]. DRG neurons at a density of 5000 neurons/ml were incubated in BSF2 medium (containing 2% HIFCS, 0.1 mg/ml transferrin, 60 ng/ml progesterone, 0.16 μg/ml sodium selenite, 3 mg/ml bovine serum albumen (B.S.A.), penicillin /streptomycin at 100 μg/ml each, 16 μg/ml putrescine, 10 μg/ml insulin), and the neurotrophic factors Nerve growth factor (NGF 100 ng/ml), Glial cell-line derived neurotrophic factor (GDNF 50 ng/ml) and Neurotrophin 3 (NT3, 50 ng/ml), for 48 hours before being studied.

2.8. Human DRG neuron cultures

Avulsed cervical DRG were obtained from 4 patients with brachial plexus avulsion injury undergoing reconstruction surgery at the RNOH Stanmore, UK, with patient consent and approval of the local ethics committee. Avulsed DRG were collected in Ham's F12 containing penicillin and streptomycin (100 μg/ml each), minced, enzyme-digested in Ham's F12 containing 0.2% collagenase/0.5% dispase for 3 hours, followed by 30 minutes in papain (0.1%, 12 U/ml, Sigma UK), and mechanically dissociated to obtain a single cell suspension [7]. Neurons were plated on collagen/laminin coated MatTek dishes (MatTek Corp., USA), in Ham's F12 nutrient medium containing 10% heat inactivated fetal calf serum, and antibiotics, and incubated at 37°C in a humid environment.

2.9. Calcium imaging

Functional effects of acute N/OFQ (Orphanin FQ, Merck Millipore, San Diego, CA, USA) and DAMGO ([D-Ala^2,NMe-Phe^4,Gly-ol^5]-enkephalin, Tocris Bioscience, U.K.) treatment to capsaicin responses were determined in Fura2 AM (Molecular Probes) loaded neurons as previously described [6; 7; 9]. Responses to paired capsaicin stimuli, with and without N/OFQ or DAMGO, were measured as a change from the baseline 340/380 nm excitation ratio. Experiments were conducted at 37°C in a humidified environment on an inverted Nikon microscope (Diaphot 300), and alternately excited at 340 and 380 nm wavelengths. Images were captured every 2 s in each of the three channels (phase, 340 nm, and 380 nm), and recordings of intracellular changes in bound and unbound Ca^{2+} ratio were obtained before, during and after the addition of test compounds. This provided baseline recordings as well as intracellular changes in Ca^{2+} levels in response to added compounds. Cells were uniformly loaded with the dye and no intracellular compartmentalisation of the loaded dye was observed. Images were acquired with a Hamamatsu Orca CCD Camera and analysed

with AQM Advance Kinetic imaging software. Individual cells under study were highlighted as regions of interest for calculating the mean ratios of bound to unbound calcium. In each experiment, neurons were exposed to capsaicin for a maximum of two applications only, first to identify capsaicin sensitivity and second to test the effect of the added drugs after the washout period. As capsaicin stimulation is known to cause desensitisation, we employed a published protocol where a minimum concentration (200 nM) and brief period of application (15 s) was used to identify capsaicin-sensitive neurons (demonstrating a rapid increase in 340/380 ratio and sustained response), which was followed by washout of medium and a rest period of 30 min before the second challenge [9]. Responses were measured as the difference between baseline and peak ratio change and the second response was normalised to the first response; subtracting the percent response from 100 gave the value for percent inhibition. Average % inhibition was compared between groups. Calculation of percent inhibition: for each experiment, response to 200 nM capsaicin = R1, response to 1 μM capsaicin (with or without added compounds) = R2, % response = R2/R1 X 100, % inhibition = 100 - % response.

3. RESULTS

3.1 Urinary Bladder

Many intensely stained nerve fibres were seen in the urothelium and sub-urothelium in bladder tissue from patients with PBS and DO, fewer in controls (Figs. 1A-C). Image analysis of NOP-positive nerve fibre staining within the sub-urothelium showed a significant increase in tissues both from patients with DO ($p<0.0001$) and PBS ($p = 0.0014$), compared to controls (Fig.1D).
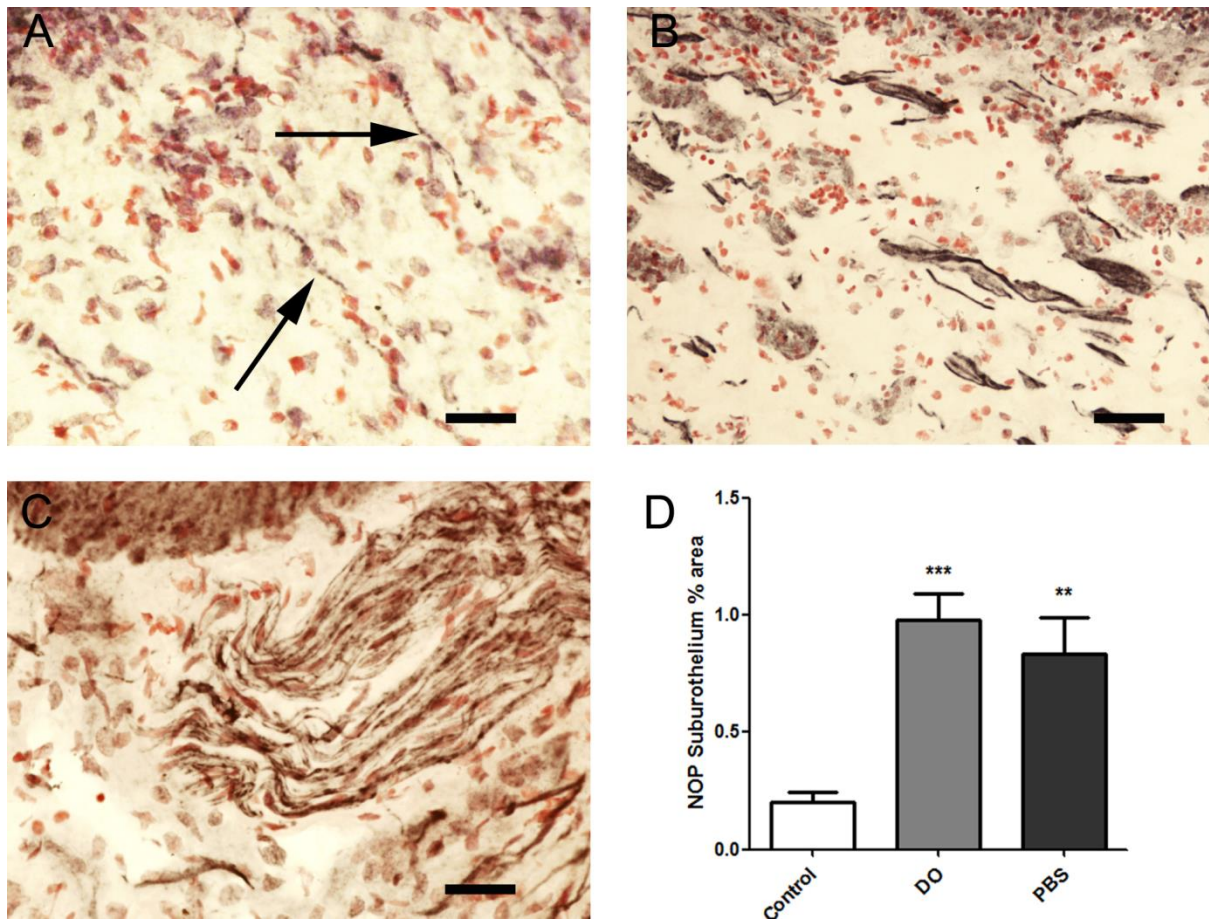
Figure 1. NOP-immunoreactive nerve fibres in human urinary bladder. A, NOP nerve fibre staining (arrows) in sub-urothelium of a control specimen, Scale bar =50µm. B, NOP staining in sub-urothelium from a patient with PBS, scale bar = 100 µm and C, NOP staining in sub-urothelium from a patient with IDO, Scale bar =50µm. D, Image analysis of NOP in urinary bladder (mean ± SEM), ***p<0.0001 and **p = 0.0014.

3.2 Dorsal root ganglia

The NOP receptor antibody immunostained sub-populations of small (≤ 50µm) and large (> 50µm) diameter neuronal cells in avulsed hDRG; increasing dilutions of antibody diminished immunostaining (Fig 2A). Similar immunostaining was observed in control post-mortem sacral and lumbar DRG (Fig. 2C).
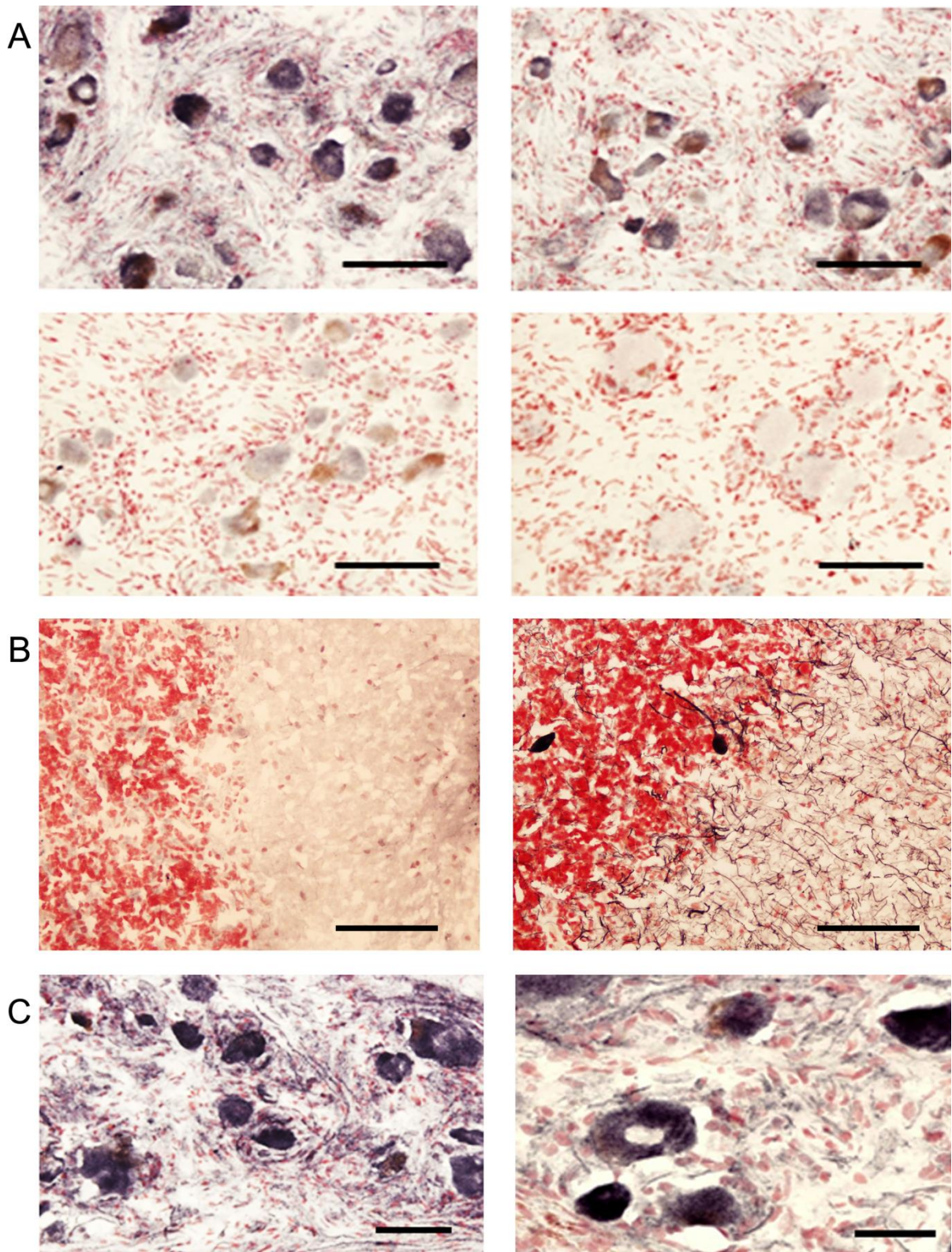
Figure 2 NOP-immunoreactive neurons in human DRG and cerebellum as control. A, Serial dilution of NOP antibody immunostaining in human avulsed cervical DRG, top left at antibody dilution 1:100, top right at 1:200, bottom left 1:400, bottom right no antibody.

Scale Bar = 120µm (same magnification for all panels); B, Serial sections of cerebellum with NOP antibody (left panel), and Neurofilament cocktail antibodies (right panel), scale bar = 100µm. C, NOP antibody staining in post-mortem sacral (left panel) and lumbar (right panel) human DRG, scale bar = 100µm and 50µm respectively.

Serial section co-localisation studies at optimal antibody dilutions showed that the NOP receptor was expressed in most of the TRPV1-positive small DRG cells (arrowed, Fig. 3A, NOP left panel, TRPV1 right panel). NOP and TRPV1-positive cell counts in DRGs are shown in Fig. 3B and 3C. The total number of nucleated neurons counted positive for NOP ≤50µm and >50µm respectively were 636 and 45 for lumbar, 542 and 54 for sacral, 402 and 55 for avulsed cervical human DRG.
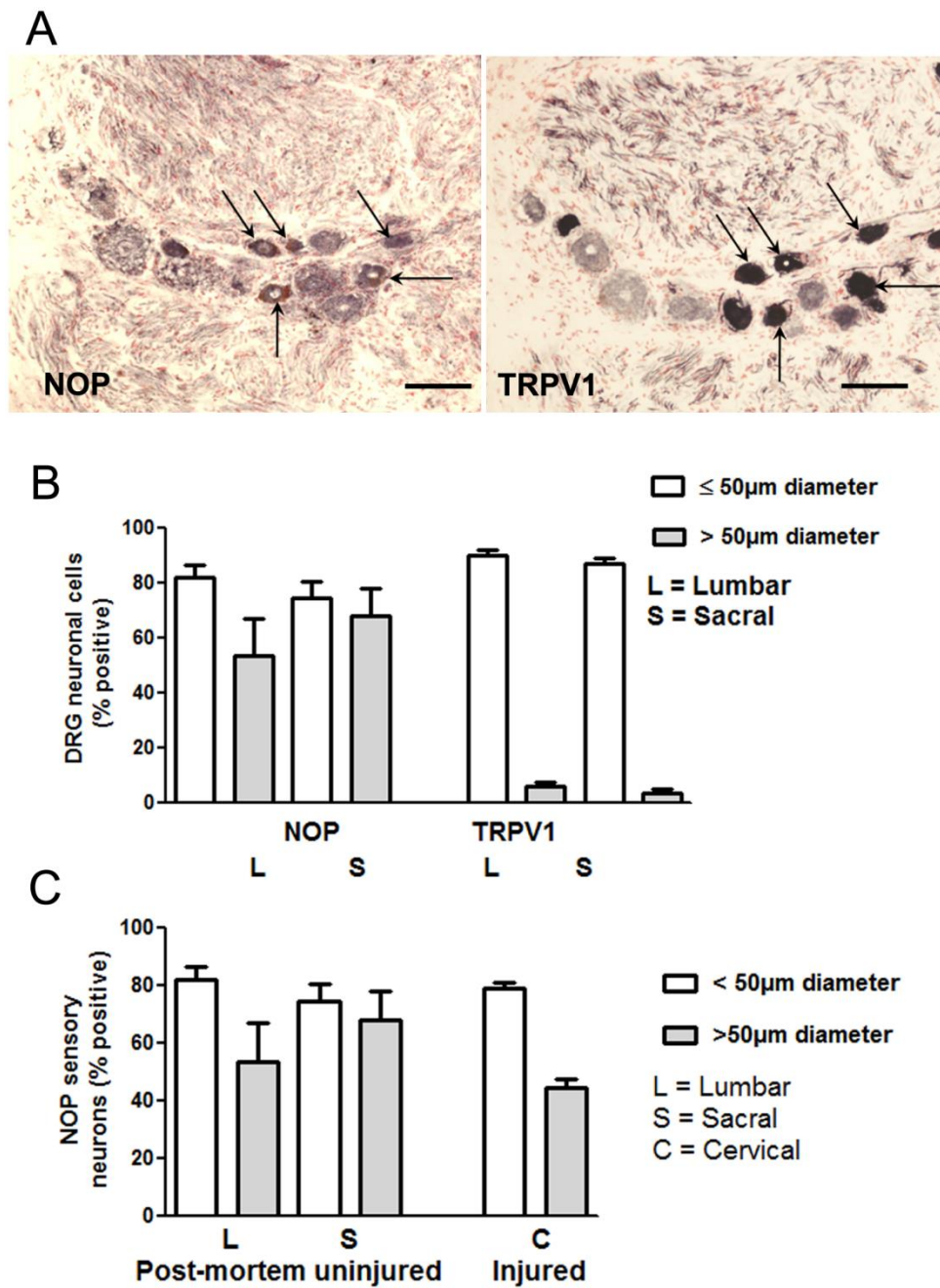
Figure 3 NOP and TRPV1 in human DRG neurons. A, Co-localisation (arrowed) of NOP (left) and TRPV1(right) in serial sections of human DRG, scale bar = 100µm. B, Bar charts showing DRG counts of small and large diameter neurons for NOP and TRPV1 in control post-mortem lumbar and sacral DRG. C, Bar charts showing DRG counts of small and large diameter neurons for NOP in control lumbar and sacral DRG in comparison with avulsed cervical DRG.

3.3. Peripheral nerves

NOP receptor nerve fibre staining was seen in all control (Fig. 4A) and injured limb nerves, and painful neuroma specimens (Fig. 4C, E); there were fewer nerve fibres immunostained than with Neurofilament cocktail antibodies in the same specimens (Fig. 4B, D and F). Image analysis of NOP receptor-positive nerve fibres showed a significant decrease in the injured nerve group compared to control nerves (Fig. 5 top, $p = 0.0004$), and also versus painful neuroma specimens ($p = 0.025$).
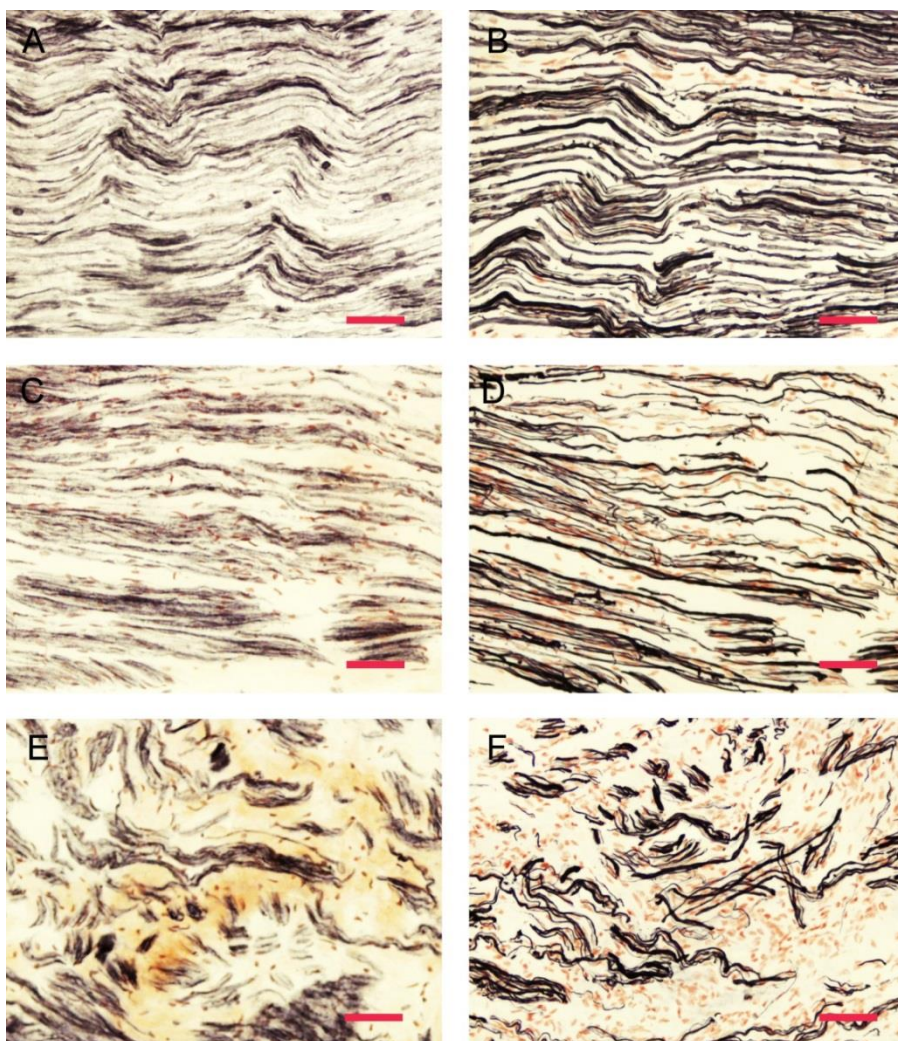

Figure 4. NOP immunostaining in human peripheral nerves. NOP (A) and Neurofilaments cocktail (B) in control human nerve, NOP (C) and Neurofilaments cocktail (D) in injured nerve, and NOP (E) and Neurofilaments cocktail (F) in painful neuroma, scale bar =100μm.

Neurofilament cocktail staining was similar in these groups (Fig. 5 middle). The ratio of the % area NOP receptor to Neurofilament cocktail antibody immunostaining was significantly decreased in the injured group (Fig. 5 bottom, p = 0.0004), and in the painful neuroma group (p = 0.011), compared to controls.
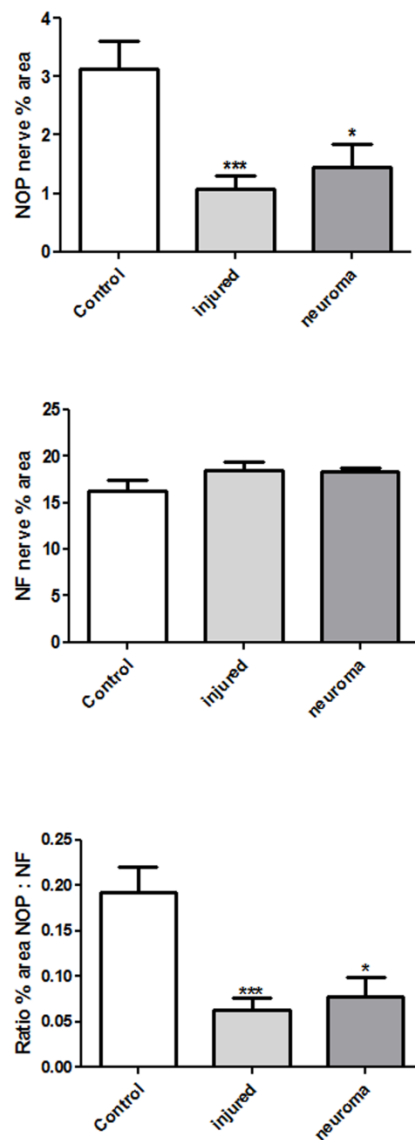

Figure 5. NOP image analyses in human peripheral nerves (mean ± SEM). Top ***p =0.0004, *p = 0.025; Middle, Neurofilaments cocktail in nerve fibres; Bottom NOP : NF ratio, ***p = 0.0004, *p =0.011.

3.4. Effect of N/OFQ on capsaicin responses in cultured rat DRG neurons

Individual neurons were tested for capsaicin sensitivity with a 200 nM test dose of capsaicin. This was followed by washout and change of medium and a rest period of 30 minutes, after which the same neurons were stimulated with 1 μM capsaicin. Responses were measured as the difference in amplitude from baseline to peak response. In the absence of added drugs (control), the average value of the second response was reduced due to tachyphylaxis to 83 +/- 5% of the first response, as expected in rat DRG neurons and published previously (Fig. 6) [5-7; 9]. After the rest period, when cells were incubated with N/OFQ for 10 minutes, a concentration-dependent inhibition of the second capsaicin challenge responses was observed (Fig. 6). A robust calcium signal following ionomycin addition at the end of the experiment confirmed that the neurons were viable.

Pre-incubation with 0.01 nM N/OFQ (10 minutes incubation) significantly reduced the response to 1 μM capsaicin, while a subsequent response to 2 μM ionomycin was still present indicating cell viability. Pre-incubation with 0.1 nM N/OFQ led to an almost complete abolition of capsaicin-induced calcium flux. In rDRG neurons, capsaicin responses were concentration-dependently inhibited in the presence of N/OFQ, with an IC_{50} of 8.6 pM (95% CI 4.4 - 17 pM) (Fig. 6). Dose dependent inhibition of capsaicin responses in rDRG neurons were also observed in the presence of DAMGO, with maximum inhibition at 0.1 nM DAMGO, and an IC_{50} of 1.2 pM (95% CI 0.26 - 5.2 pM) (Fig. 6).

While the maximum inhibition of capsaicin responses was observed with 0.1 nM of either N/OFQ or DAMGO, the inhibition due to N/OFQ was greater (100%; 95% CI 90 - 111 %) than that due to DAMGO (55%; 95% CI 50 - 60 %), suggesting a much higher efficacy for the NOP receptor system in inhibiting TRPV1 receptor signalling. The maximum inhibition by morphine, a prototypical non-peptide MOP agonist, was previously shown to be similar

($67.22 \pm 4.3\%$ at a saturating concentration of 10 µM; [5]) to that of DAMGO, suggesting that the effect in this system may be independent of the agonist and a property of the receptor, or downstream receptor coupling.

3.5. N/OFQ inhibition of capsaicin mediated calcium influx in hDRG neurons

In human DRG (hDRG) neurons, 32% inhibition of capsaicin responses was observed in the presence of 1 pM N/OFQ ($P<0.001$, n = 3 neurons), which is in the same order of magnitude as the inhibition observed with rat DRGs. In the absence of N/OFQ, inhibition observed by the second stimulus due to tachyphylaxis was 8.7% (n= 5 neurons).
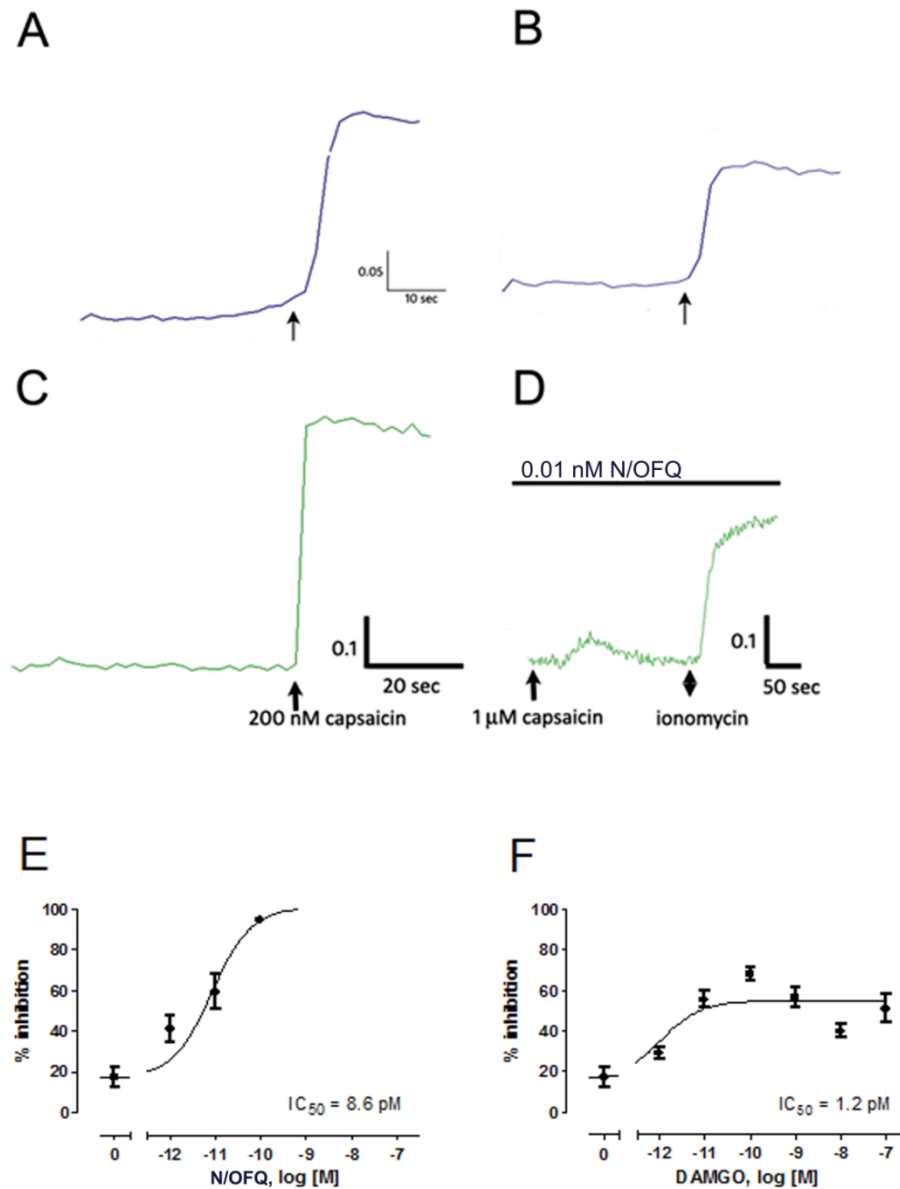
Figure 6. Effect of N/OFQ on capsaicin responses in rat DRG neurons. In rDRG neurons, sample trace of a response to 200 nM capsaicin (arrow, A). Trace of response to 1 μM capsaicin applied after washout and 30 minutes rest period in control neurons (B). Response to 200 nM capsaicin (C) significantly attenuated in the presence of 0.01 nM N/OFQ, though ionomycin response was robust (D). Inhibition of capsaicin responses with increasing dose of N/OFQ. Control n=7 neurons, 0.001 nM n=3 neurons, 0.01 nM n= 6 neurons, 0.1 nM n=14 neurons. (E). Inhibition of capsaicin responses in the presence of increasing concentrations of

DAMGO. Control n=7 neurons, 0.001 nM n = 4 neurons, 0.01 nM n= 11 neurons, 0.1 nM, n = 8 neurons, 1 nM, n = 5 neurons, 10 nM, n = 9 neurons, 100 nM, n = 4 neurons. (F).

4. Discussion

The NOP receptor is involved in a range of physiological systems, including pain pathways [26]. As previous studies of receptor distribution and function were mainly pre-clinical [38], and NOP mRNA splice variants were described in human and rat DRG [58], we set out to determine the presence of the NOP receptor protein in tissues from patients with chronic pain and hypersensitivity disorders. We focused on peripheral nerve and DRG tissue, and innervation of visceral organs. Importantly, we also examined the functional effects of the endogenous NOP receptor ligand N/OFQ in cultured rat and human dorsal root ganglion neurons.

The NOP receptors were present in the majority of both small and large diameter neurons in DRG, at the cervical, lumbar, and sacral levels. This finding is supported by Knock-in mice studies with fluorescent-tagged NOP receptors in brain, spinal cord, and DRG neurons [43]. While NOP co-localised with TRPV1 in small diameter neurons, its presence (unlike TRPV1) in large diameter neurons suggests a potential role in addition to nociception. Further detailed quantitative studies of NOP receptor expression in somatic and visceral DRG neurons are required, including co-localisation of NOP receptors with TRPV1 and other pain targets. Overall, NOP receptor expression was clearly reduced in injured limb nerves and painful neuromas, suggesting retrogradely transported neurotrophic factors may be involved in regulating its expression, as for a number of nociceptor-related neuropeptides, ion channels and receptors. However, in a rodent study of partial sciatic nerve transection, and an

inflammatory pain model of complete Freund's adjuvant (CFA) injection into the hindpaw, a modest increase of both N/OFQ and its receptor immunoreactivity was observed in neurons after 7 days; the time course of changes and species differences thus deserve further study [14]. Residual or spouting nerve fibres in injured nerves may mediate hypersensitivity and which may be ameliorated by NOP receptors. In accord, peripheral [42] and spinal [15; 42; 59] NOP activation reduced mechanical allodynia in the rat chronic constriction injury model, and activation of peripheral NOP and MOP receptors exerted anti-hypersensitivity effects in a rodent pain model of diabetic polyneuropathy [48]. The mechanism of action of N/OFQ is likely to include inhibition of neurotransmitter release from peripheral nerve terminals [22, 41]. The down-regulation of NOP should be interpreted with caution as it does not necessarily support a causal role for this receptor in the pathogenesis of neuropathic pain, nor does it suggest a potential therapeutic target for clinical treatment. Nevertheless, the fact that receptor expression is regulated on a protein level may suggest an involvement of this pathway in the pathologies examined; further studies are needed to address this in a mechanistical manner.

Unlike injured limb nerves, a marked and significant increase in NOP receptor immunoreactive nerve fibres was observed in bladder specimens from patients with Overactive Bladder, and with Bladder Pain Syndrome. In rats, NOP receptors are present at several sites for the integration of the micturition reflex, and their activation has both excitatory or inhibitory effects, depending on the route of administration and the experimental conditions [31]. In humans, it has been shown that bladder instillation of N/OFQ in patients with a neurogenic/Overactive Bladder disorder alleviates the symptoms, increases bladder capacity, and elicits a robust, acute inhibitory effect on the micturition reflex [27, 28, 29]; effectiveness in Bladder Pain Syndrome has been reported as well [30],

supporting the use of NOP agonists as a novel drug for the treatment of painful bladder disorders. Pre-clinical models have provided further evidence that NOP receptor activation may be antinociceptive in visceral pain [31, 32]. Small-molecule NOP receptor agonists are effective in a mouse model of TNBS-induced colitis [51], of mustard-oil induced IBS with diarrhea [19], and rat colonic hyperalgesia [1]. BU08070, a mixed NOP/MOP receptor agonist, significantly reduced the severity of colitis in TNBS-treated mice [63]. Thus the N/OFQ system is an attractive target for novel drugs, which may be effective in the treatment of inflammatory or functional visceral disorders [2; 52; 63].

Previous studies have shown the sensitizing effects of neurotrophic factors on neuronal sensitivity in rodents [23] and humans [5-8], including TRPV1. As the tissue studies above showed co-localization of NOP and TRPV1 in DRG neurons, we investigated the effects of NOP receptor ligand activation on TRPV1 receptor-mediated Ca^{2+} signaling in primary human and rat DRG neurons cultured in the presence of neurotrophic factors, an *in vitro* model of neuronal sensitization [7]. The endogenous ligand N/OFQ was used to activate NOP receptors, which in both rat and human DRG neurons potently inhibited capsaicin-induced Ca^{2+} signaling with an IC_{50} of around 0.01nM, which is in the order of magnitude observed for N/OFQ binding to rat brain membranes, Kd ~0.02nM [4]. The MOP receptor peptide agonist DAMGO inhibited TRPV1 receptor-mediated Ca^{2+} signaling with a similar potency; however, N/OFQ proved to be much more efficacious than DAMGO or morphine [5] for maximum inhibition. As the NOP receptor couples to the same Gi/Go protein-mediated, second-messenger systems as the opioid receptors to inhibit adenylate cyclase, this mechanism is likely to affect TRPV1 signalling by dephosphorylation, leading to its desensitization [12; 18]. Similarly, the MOP agonist morphine has been demonstrated to act via inhibition of adenylate cyclase to inhibit PKA-potentiated TRPV1 responses [55]. Our

study utilized a previously described model of neurotrophic factor mediated neuronal hypersensitivity to capsaicin for analysing the effects of N/OFQ. Our results indicate that N/OFQ inhibits TRPV1 signaling with a high potency, compared with the nanomolar values generally obtained in biochemical studies performed in cells, and may be due to the sensitizing effects of the neurotrophic factors. However, this could be the result of efficient signal amplification in native cells used by us compared with recombinant systems. Future studies with specific NOP antagonists will be useful to confirm specificity of action.

The diverse potential of NOP receptor agonists for acute and chronic pain treatment in rodent and non-human primates has been recently reviewed [16]. AT-200, a high affinity N/OFQ receptor agonist, with low efficacy at MOP, ameliorated chronic hypoxia-induced mechanical, thermal and deep tissue/musculoskeletal hyperalgesia in HbSS-BERK sickle mice [52]. While previous preclinical models have shown opposing effects of NOP activation in the CNS [40; 62], reviewed by [49], recent studies using spinally administered bifunctional NOP/MOP ligands have shown attenuation of neuropathic and inflammatory pain, suggesting a promising profile as spinal analgesics [53; 56]. Spinally administered PWT2-N/OFQ, a tetrabranched derivative of N/OFQ, inhibited nociceptive and neuropathic pain in mice and non-human primate models; the PWT derivative was more potent than the natural peptide, and elicited long lasting effects in non-human primates [47]. Intracisternal N/OFQ and morphine also produced antinociceptive effects [17]. Peripherally mediated effects show promise, including a recent study describing efficacy of NOP receptor agonist SCH 486757 in suppressing capsaicin induced cough in a guinea pig model [34], currently being evaluated in a clinical trial [35].

In summary, we have demonstrated the expression of the NOP receptor in human peripheral nerve and visceral tissues, with a marked increase of NOP-positive nerve fibres in urinary bladder syndromes. N/OFQ and DAMGO produced dose-dependent inhibition of capsaicin responses in human and rat DRG neurons, in an *in vitro* model of neuronal sensitization. NOP receptor activation is thus a promising strategy for therapeutic intervention in clinical pain conditions, especially visceral hypersensitivity and overactivity disorders.

Authors' contributions: PA and PH conceived the original study, its design and coordination, and helped write the manuscript. YY participated in immunohistology studies and helped draft the manuscript. UA performed the in vitro experiments, to which YEK contributed, and helped draft the manuscript. GM, MS, MF, AM and TQ collected the clinical tissues, helped with the human tissue studies, and edited the manuscript. All authors read and approved the final manuscript.

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