

# Distribution of delta and mu opioid receptor mRNA in rodent dorsal root ganglia neurons

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## Abstract

Primary afferents are responsible for transmitting signals produced by noxious stimuli from the periphery to the spinal cord. Mu and delta opioid receptors (MOP and DOP) have analgesic properties and are highly expressed in dorsal root ganglia (DRG) neurons. In humans, spinal DOP is almost exclusively located on central terminals of DRG neurons, whereas in rodents, it is expressed both on presynaptic terminals and spinal neurons. In this study, we aimed to assess the distribution of MOP and DOP in the DRGs of mice and rats. Using *in situ* hybridization and immunofluorescence, we visualized MOP and DOP mRNA together with various neuronal markers. In rats and mice, we show that both receptors are expressed, albeit to different extents, in all types of neurons, namely, large and medium myelinated neurons (NF200-positive), small nonpeptidergic (IB4- or P2X3R-positive) and peptidergic C fibres (Tac1-positive). Overall, DOP mRNA was found to be mainly expressed in large and medium myelinated neurons, whereas MOP mRNA was mainly found in C fibres. The distribution of MOP and DOP, however, slightly differs between rats and mice, with a higher proportion of small non-peptidergic C fibres expressing DOP mRNA in mice than in rats. We further found that neither morphine nor inflammation affected the distribution of the receptor mRNA. Because of their location, our results confirm that MOP and DOP have the potential to alleviate similar types of pain and that this effect could slightly differ between species.

## KEYWORDS

delta opioid receptor, dorsal root ganglia, *in situ* hybridization, mu opioid receptor, primary afferent fibres, rodents

**Abbreviations:** CFA, complete Freund's adjuvant; DAPI, 4',6-diamidino-2-phenylindole; DRG, dorsal root ganglia; DOP, delta opioid peptide receptor; GPCR, G protein-coupled receptor; GRK2, GPCR-regulated kinase; IB4, isolectin B4; KOP, kappa opioid peptide receptor; MOP, mu opioid peptide receptor; NF200, neurofilament of 200 kDa; P2X3R, purinergic receptor P2X 3; Tac1, preprotachykinin-1.

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## 1 | INTRODUCTION

Noxious thermal, mechanical and chemical stimuli activate nociceptors innervating the skin, muscles and viscera. Activation of nociceptors by noxious stimuli produces action potentials that propagate along the axons of primary afferents to the central nervous system (Almeida et al., 2004). Lightly myelinated A $\delta$  and nonmyelinated, peptidergic and nonpeptidergic C fibres are the two principal subtypes of primary afferent fibres involved in the transmission of noxious stimuli (Garland, 2012; Julius & Basbaum, 2001; Woolf & Ma, 2007). Blocking transmission in primary sensory neurons therefore represents an efficient approach for the treatment of pain (Berta et al., 2017).

Currently, opioids remain the standard of care for the treatment of moderate to severe pain. Opioids mediate their effects through the activation of three receptors, namely, mu ( $\mu$ , MOP), delta ( $\delta$ , DOP) and kappa ( $\kappa$ , KOP), all belonging to the G<sub>i</sub> protein-coupled receptor (GPCR) family. All opioids currently used for the management of pain mainly target the MOP to provide analgesia. However, the activation of this receptor is also responsible for multiple undesired effects, including respiratory depression, constipation, tolerance and addiction. Both DOP and KOP have also been shown to produce some level of analgesia and therefore appear to be promising targets for the development of novel pain therapies (Berthiaume et al., 2020; Chavkin, 2011; Gaveriaux-Ruff & Kieffer, 2011; Kieffer & Gaveriaux-Ruff, 2002; Quirion et al., 2020; Van't Veer & Carlezon, 2013).

DOP and MOP receptors are both expressed in primary afferents, where they likely play a role in analgesia. Although the exact distribution of DOP and its coexpression with MOP in DRGs remain controversial, our group has shown previously that DOP agonists, under certain conditions, can efficiently inhibit nocifensive behaviour induced by chemical, thermal and mechanical stimuli (Beaudry et al., 2011; Normandin et al., 2013).

In the human spinal cord, binding experiments revealed that DOP is solely expressed in the superficial lamina (Mennicken et al., 2003). Most interestingly, the human spinal cord has been shown to be devoid of DOP mRNA, suggesting that the receptors are exclusively found on central terminals of sensory nerves and not in spinal cord neurons (Mennicken et al., 2003). This observation suggests that important interspecies differences exist between humans and rodents since DOP is found both on presynaptic and postsynaptic terminals in the spinal cord (Mennicken et al., 2003).

In the present study, we examined the distribution of DOP and MOP in DRG neurons by combining RNAscope, a highly sensitive and specific *in situ*

hybridization approach, with immunolabelling. The distribution of DOP and MOP receptor mRNA was studied in both rat and mouse DRGs. We further investigated whether chronic morphine treatment or inflammatory pain altered the distribution of the receptors.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

#### 2.1.1 | Rats

All experiments were conducted on adult male Sprague–Dawley rats (weighing 300–350 g) obtained from Charles River Laboratories, Quebec, Canada. They were maintained on a 12-h light/dark cycle, and food/water was available *ad libitum*. Experiments were approved by the animal care committee at the Université de Sherbrooke (protocol 2018-2046) and in accordance with policies and directives of the Canadian Council on Animal Care.

#### 2.1.2 | Mice

All experiments were conducted on adult male C57Bl6 mice (weighing 20–30 g) obtained from Charles River Laboratories, Quebec, Canada. They were maintained on a 12-h light/dark cycle, and food/water was available *ad libitum*. Experiments were approved by the animal care committee at the Université de Sherbrooke (protocol 2018-2046) and in accordance with policies and directives of the Canadian Council on Animal Care.

### 2.2 | Chronic morphine treatment

Morphine treatments were provided as previously described (Gendron et al., 2006). Briefly, the rats received subcutaneous injections of morphine sulphate (Medisca Canada, St-Laurent, Quebec, Canada; increasing doses of 5, 8, 10 and 15 mg/kg at 12 h apart). Control rats were injected with equivalent volumes of saline. L4-5 lumbar DRGs were collected 12 h after the last morphine (15 mg/kg) or saline injection, rapidly frozen on dry ice and kept at  $-80^{\circ}\text{C}$  until use.

### 2.3 | Chronic inflammation

Chronic inflammatory pain was induced by a 100- $\mu\text{l}$  intraplantar injection of a 1:1 saline: complete Freund's adjuvant (CFA; Sigma, St. Louis, MO, USA) emulsion in

the right hind paw of rats under isoflurane anaesthesia. Ipsilateral and contralateral L4-5 lumbar DRGs were collected 72 h after CFA injection, rapidly frozen on dry ice and kept at  $-80^{\circ}\text{C}$  until use.

## 2.4 | Tissue collection

Rats and mice were anaesthetized under isoflurane and killed by decapitation. The lumbar DRGs (L4-L5-L6) were dissected and snap frozen on dry ice. They were covered with Tissue-Tek<sup>®</sup> Optimal cutting temperature compound (O.C.T.) and stored at  $-80^{\circ}\text{C}$  until sectioning. Fifteen micrometre-thick sections were cut on a cryostat and mounted on SuperFrost plus glass slides. Each section mounted on a slide was taken 105–110  $\mu\text{m}$  apart from the previous section to avoid sampling the same cells more than once. The slides were stored at  $-20^{\circ}\text{C}$  until in situ hybridization was performed.

## 2.5 | In situ hybridization on rat DRG sections: RNAscope<sup>™</sup>

The sections were postfixed in 4% paraformaldehyde (PFA) diluted in 0.1-M phosphate buffer (PB) at pH 7.4. After two washes in phosphate buffered saline (PBS), the sections were dehydrated in subsequent ethanol baths of 50%, 70% and two times 100% ethanol for 5 min. After drying, the slides were incubated for 30 min with protease IV and then washed twice in PBS. The probes were heated at  $40^{\circ}\text{C}$  for 10 min and then cooled to room temperature. Probes for rat DOP (Rn-Oprd1; cat 457011), rat MOP (Rn-Oprm1-C2; cat 410691-C2) and rat Tac1 (Rn-Tac1-C3; cat 450661-C3) were used. The slides were incubated for 2 h with the probes at  $40^{\circ}\text{C}$  in a Hybezen oven. After they were washed twice in RNAscope buffer, the slides were sequentially incubated in AMP-1 FL reagent for 30 min, AMP-2 FL for 15 min, AMP-3 FL for 30 min and AMP-4 FL ALT A, ALT B or ALT C for 15 min at  $40^{\circ}\text{C}$  in a Hybezen oven. Slides were finally washed twice for 2 min and processed for immunostaining or mounted in Prolong<sup>™</sup> Diamond Antifade Mountant with DAPI (Invitrogen, Eugene, OR, USA). Protease IV, probes, RNAscope reagents and the Hybezen oven were purchased from ACDBio (Newark, CA, USA).

## 2.6 | In situ hybridization of mouse DRG sections: RNAscope<sup>™</sup>

The sections were postfixed in 4% PFA diluted in 0.1-M PB at pH 7.4. After two washes in PBS, the sections were

dehydrated in subsequent ethanol baths of 50%, 70% and two times 100% ethanol for 5 min. After drying, the slides were incubated for 30 min with protease IV and then washed twice in PBS. Slides were then incubated with 0.5X TrueBlack Lipofuscin Autofluorescence Quencher for 30 s to reduce lipofuscin autofluorescence and then washed twice in PBS. The probes were heated at  $40^{\circ}\text{C}$  for 10 min and then cooled to room temperature. Probes for the mice DOP (Mm-Oprd1; cat 427371), mice MOP (Mm-Oprm1-C2; 315841-C2), mice P2rx3 (Mm-P2rx3-C3; cat 521611-C3) and mice Tac1 (Mm-Tac1-C3; cat 410351-C3) were used. The slides were incubated for 2 h with the probes at  $40^{\circ}\text{C}$  in the Hybezen oven. After they were washed twice in RNAscope buffer, the slides were sequentially incubated in AMP-1 FL reagent for 30 min, AMP-2 FL for 15 min, AMP-3 FL for 30 min and AMP-4 FL ALT A, ALT B or ALT C for 15 min at  $40^{\circ}\text{C}$  in a Hybezen oven. Slides were finally washed twice for 2 min and processed for immunostaining or mounted in Prolong<sup>™</sup> Diamond Antifade Mountant with DAPI (Invitrogen). Protease IV, probes, RNAscope reagents and the Hybezen oven were purchased from ACDBio.

## 2.7 | NF200 and IB4 immunostaining

The sections previously stained in in situ hybridization were washed twice for 2 min in Tris buffer saline (TBS; 50-mM Tris and 15-mM NaCl) at pH 7.6 containing 0.05% Tween 80 (TBS-T). Nonspecific sites were blocked with TBS supplemented with 1% bovine serum albumin (BSA) and 10% normal goat serum (NGS) overnight at  $4^{\circ}\text{C}$ . Sections were then incubated for 2 h with the primary antibody mouse anti-NF200 (Sigma Life Science; N0142) 1:1000 or isolectin B4 (Sigma-Aldrich, St. Louis, MO, USA; L-2140) 1:1000 diluted in TBS-1% BSA. After 3 5-min washes in TBS-T, sections were incubated in the secondary antibody Alexa Fluor 488 goat anti-mouse (Invitrogen; A11001) diluted 1:500 or streptavidin Alexa Fluor 488 (Invitrogen; S11223) diluted 1:500 in TBS-1% BSA for 30 min at room temperature. After they were washed in TBS-T 3 times for 5 min, the slides were mounted in Prolong<sup>™</sup> Diamond Antifade Mountant with DAPI (Invitrogen) and stored at  $4^{\circ}\text{C}$  until visualization under a Leica DM4000 microscope for quantification or Leica TCS SP8 STED for higher quality images.

## 2.8 | Quantification

The distribution of DOP and MOP mRNA in DRGs was assessed by analysing the DRGs of three rodents per

condition. For each animal, six different sections, each separated by 105–110  $\mu\text{m}$ , were analysed and quantified. Each DRG section was fully analysed using multiple images (without sampling the same cells twice). For each image, the number of DOP+ cells, MOP+ cells, DOP+ and the fibre marker (NF200, IB4, P2XR3 or Tac1), MOP+ and the fibre marker (NF200, IB4, P2XR3 or Tac1), DOP and MOP+ and the three together (DOP, MOP and the fibre marker) was determined. The cell was considered positive for a specific mRNA if there were at least five distinct fluorescent puncta. The images were processed using Photoshop version 5.2.1.441 (Adobe Systems) and quantified using ImageJ.

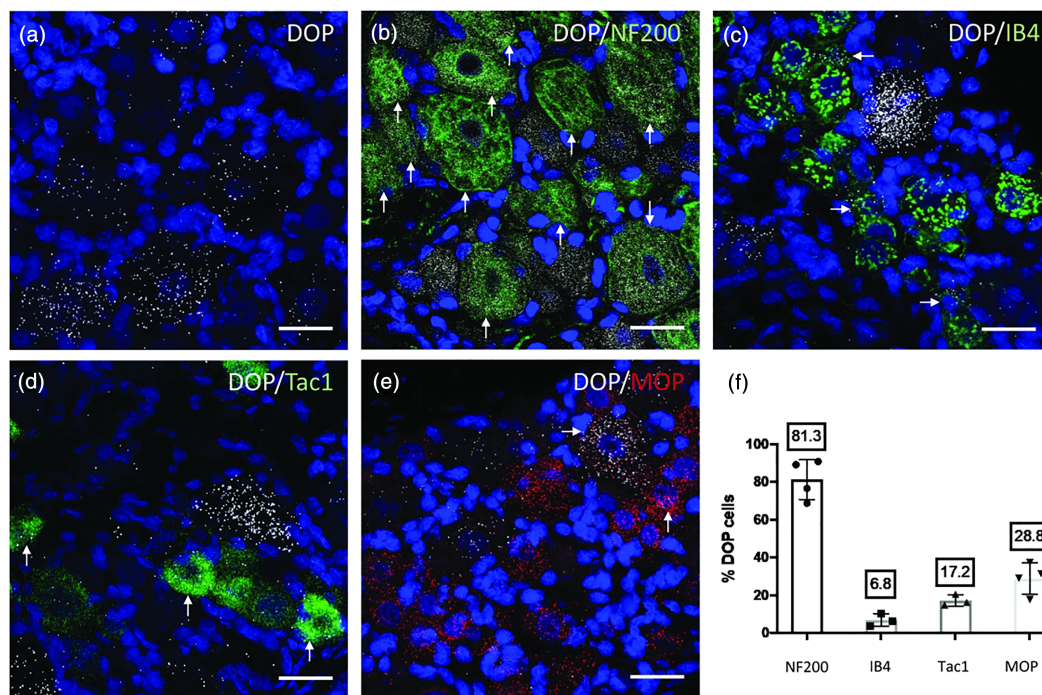
### 3 | RESULTS

#### 3.1 | DOP and MOP mRNA distribution in rat DRG neurons

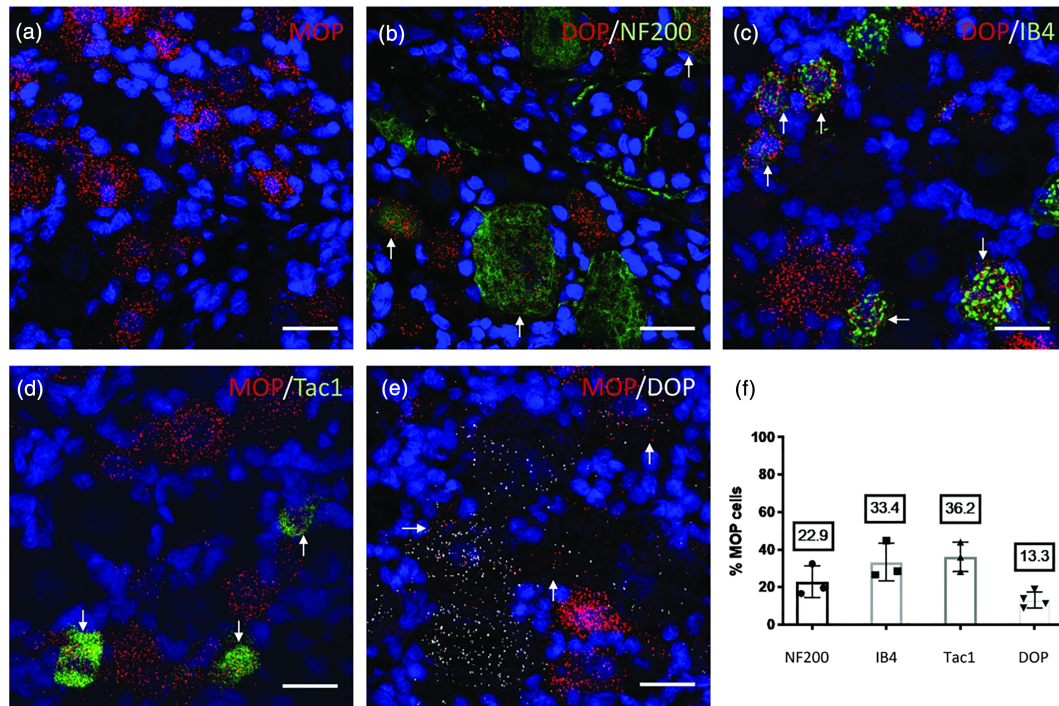
The RNAscope<sup>®</sup> Fluorescent Multiplex assay on fresh frozen tissue was used to identify DOP- and MOP-positive cells in rat dorsal root ganglia (DRG). Using specific probes for each of the receptors, we identified a proportion

of cells expressing either the delta opioid receptor (DOP; Figure 1, white labelling) or the mu opioid receptor (MOP; Figure 2, red labelling). As expected, punctate labelling in DRGs was found in cells of various sizes, resembling neurons (Figures 1a and 2a). Because the RNAscope<sup>®</sup> Fluorescent Multiplex assay allows the detection of up to three targets at the same time, we coincubated DOP and MOP probes and observed a number of cells coexpressing both receptors (Figures 1e and 2e, white arrows). No staining was observed when using a negative control probe (not shown).

To determine the distribution of DOP mRNA among rat DRG neuronal subpopulations, we employed 3 markers commonly used to label the different types of neurons. Examples of DOP-positive DRG neurons expressing either NF200, IB4, or Tac1 are shown in Figure 1b–d, respectively (white arrows). We found that  $81.3\% \pm 10.5\%$  of DOP-positive cells were positively labelled for NF200, a marker of large- and medium-diameter myelinated fibres (Figure 1b,f). A much lower proportion of DOP-positive cells were labelled with isolectin B4 (IB4;  $6.8\% \pm 3.4\%$ ), a marker of unmyelinated nonpeptidergic neurons (Figure 1c,f), whereas  $17.2\% \pm 3.0\%$  of DOP-positive cells expressed



**FIGURE 1** Distribution of DOP mRNA in rat DRG neurons. The distribution of DOP mRNA was investigated in rat DRG neurons by combining in situ hybridization and immunofluorescence. DOP mRNA expression was visualized using RNAscope technology (a–e; white punctate labelling). DOP mRNA was found in a proportion of NF200-positive, large and medium myelinated neurons (b), IB4-labelled unmyelinated nonpeptidergic C fibres (c), unmyelinated peptidergic C fibres expressing Tac1 mRNA (d), and neurons expressing MOP mRNA (red punctate labelling) (e). (f) Percentage of DOP-positive cells expressing each marker. Data are presented as the mean  $\pm$  S.E.M. White arrows indicate cells where costaining for DOP mRNA and the various neuronal markers can be observed. Nuclei were stained with DAPI (blue). Scale bars = 25  $\mu\text{m}$



**FIGURE 2** Distribution of MOP mRNA in rat DRG neurons. The distribution of MOP mRNA was investigated in rat DRG neurons by combining in situ hybridization and immunofluorescence. MOP mRNA expression was visualized using RNAscope technology (a–e; red punctate labelling). MOP mRNA was found in a proportion of NF200-positive, large and medium myelinated neurons (b), IB4-labelled unmyelinated nonpeptidergic C fibres (c), unmyelinated peptidergic C fibres expressing Tac1 mRNA (d), and neurons expressing DOP mRNA (white punctate labelling) (e). (f) Percentage of MOP-positive cells expressing each marker. Data are presented as the mean  $\pm$  S.E.M. White arrows indicate cells where coexpressing for MOP mRNA and the various neuronal markers can be observed. Nuclei were stained with DAPI (blue). Scale bars = 25  $\mu$ m

preprotachykinin-1 (Tac1) mRNA, the precursor for substance P, was used here as a marker for unmyelinated peptidergic neurons (Figure 1d,f). Finally, we also observed that  $28.8\% \pm 8.2\%$  of DOP-positive DRG neurons coexpressed MOP mRNA (Figure 1e,f).

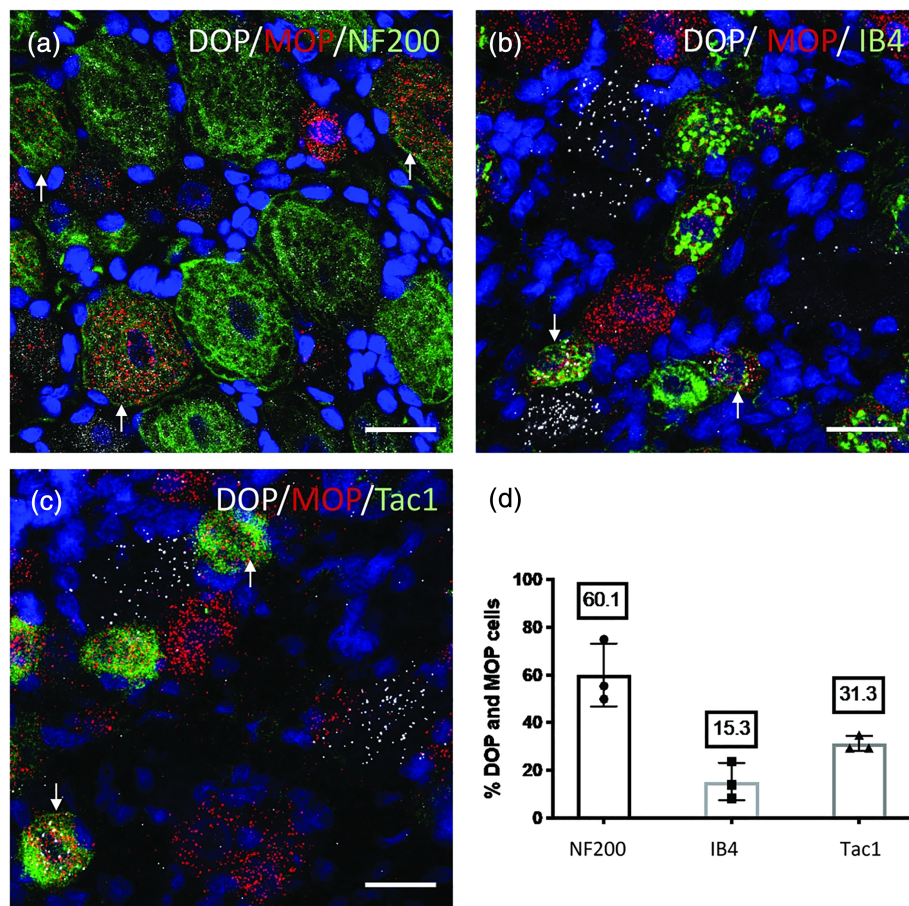
Similarly, the distribution of MOP mRNA in rat DRG neurons was investigated (Figure 2). We found that MOP mRNA was equally present in peptidergic and nonpeptidergic small-diameter neurons. Indeed,  $33.4\% \pm 10.0\%$  of MOP-positive cells coexpressed IB4 (Figure 2c,f) and  $36.2\% \pm 7.9\%$  expressed Tac1 (Figure 2d,f). Interestingly, a significant proportion of MOP-positive neurons were labelled for NF200 ( $22.9\% \pm 8.4\%$ ; Figure 2b,f). Only  $13.3\% \pm 4.3\%$  of MOP-positive DRG neurons also expressed DOP mRNA (Figure 2e,f).

Because cells coexpressing DOP and MOP are of particular interest for the development of new pain therapies targeting the DOP-MOP heteromer (Costantino et al., 2012; Fujita et al., 2014, 2015), we identified which DRG neurons express both receptors. We found that  $60.1\% \pm 13.2\%$  of cells expressing both receptors were of medium and large diameter and labelled with the marker

for myelinated neurons NF200 (Figure 3a,d; white arrows). The proportion of DOP- and MOP-positive cells coexpressing the marker for unmyelinated nonpeptidergic neurons, IB4, was much lower ( $15.3\% \pm 7.8\%$ ; Figure 3b,d; white arrows). Interestingly,  $31.3\% \pm 3.2\%$  of the neurons coexpressing DOP- and MOP-mRNA were peptidergic and labelled for Tac1 (Figure 3c,d; white arrows).

### 3.2 | DOP and MOP mRNA distribution in mouse DRG neurons

To compare the pattern of DOP and MOP expression between species, DOP and MOP mRNA were also detected in mouse DRG neurons using the RNAscope<sup>®</sup> Fluorescent Multiplex assay as described above. Using this approach, mouse DRG neurons expressing either one of the receptors (Figures 4a and 5a) or both receptors together (Figures 4e and 5e) could be visualized. The different types of DRG neurons were identified using an antibody raised against NF200 (large and medium myelinated fibres) and RNAscope probes specific for P2XR3



**FIGURE 3** Coexpression and distribution of DOP and MOP mRNA in rat DRG neurons. The distribution of cells expressing both DOP (white punctate labelling) and MOP mRNA (red punctate labelling) was studied. A proportion of cells expressing both DOP and MOP mRNA were also labelled with NF200 (a), IB4 (b), or Tac1 mRNA (c). (d) Percentage of DOP- and MOP-positive cells expressing each marker. Data are presented as the mean  $\pm$  S.E. M. White arrows indicate cells coexpressing DOP and MOP mRNA together with the indicated marker. Nuclei were stained with DAPI (blue). Scale bars = 25  $\mu$ m

(a marker for nonpeptidergic C fibres) and Tac1 (a marker for peptidergic C fibres).

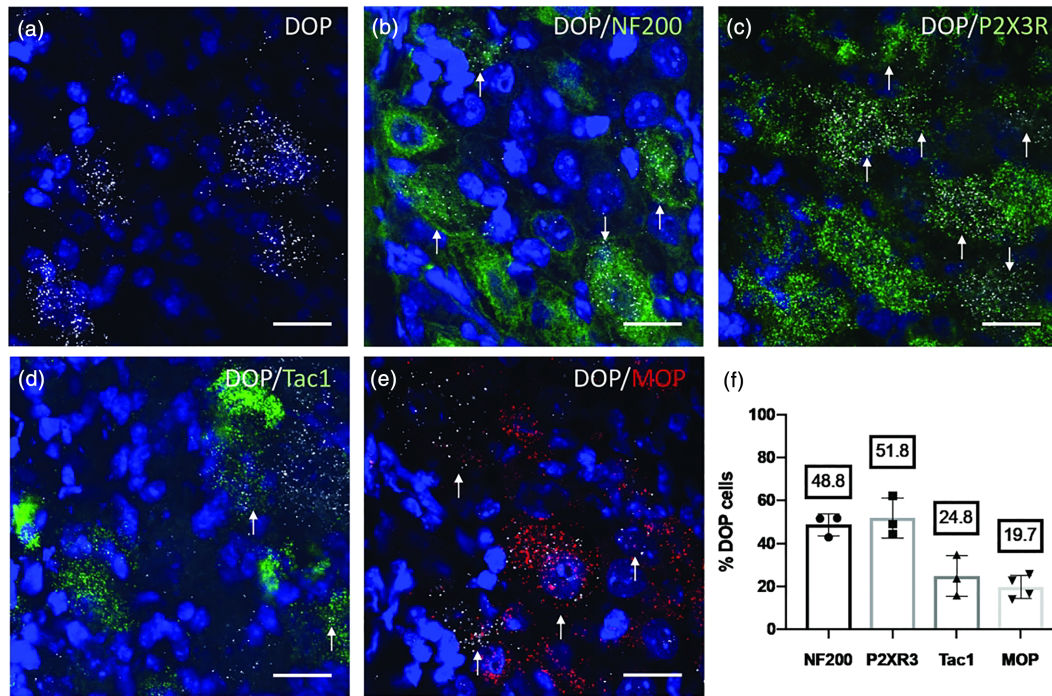
When we assessed the distribution of DOP in DRGs, we found that  $48.8\% \pm 5.1\%$  of DOP-positive neurons coexpressed NF200 (Figure 4b,f; white arrows) and  $51.8\% \pm 9.3\%$  coexpressed P2XR3 mRNA (Figure 4c,f; white arrows). In addition, we observed that  $24.8\% \pm 9.5\%$  of DOP-positive cells expressed Tac1 mRNA (Figure 4d,f; white arrows). Finally, we determined that  $19.7\% \pm 5.3\%$  of DOP-positive cells coexpressed MOP mRNA in mouse DRGs (Figure 6e,f; white arrows).

Similarly, we studied the distribution of MOP mRNA in mouse DRG neurons. Interestingly, we found that MOP mRNA was distributed almost equally in all types of DRG neurons. Indeed,  $23.2\% \pm 9.7\%$  of MOP-positive cells coexpressed NF200 (Figure 5b,f; white arrows),  $36.3\% \pm 10.8\%$  coexpressed P2XR3 mRNA (Figure 5c,f; white arrows) and  $40.3\% \pm 2.2\%$  coexpressed Tac1 mRNA (Figure 5d,f; white arrows). Finally,  $17.4\% \pm 4.5\%$  of MOP-positive neurons were found to coexpress DOP mRNA (Figure 5e,f; white arrows).

We then focused on cells coexpressing both DOP and MOP mRNA. As expected, in mouse DRGs, most cells expressing both receptors were found to be medium- to

large-diameter myelinated neurons. More specifically,  $58.7\% \pm 16.0\%$  of cells coexpressing DOP and MOP mRNA were positive for the marker NF200 (Figure 6a,d; white arrows). Interestingly, a large proportion of DOP- and MOP-positive cells were found to be nonpeptidergic fibres. Indeed,  $38.2\% \pm 8.3\%$  of DOP-MOP cells expressed P2XR3 mRNA (Figure 6b,d; white arrow). Finally,  $25.1\% \pm 12.2\%$  of DOP-MOP cells were found to express Tac1, a marker for small unmyelinated peptidergic neurons (Figure 6c,d; white arrow).

We further compared the distribution of the receptors between species. We observed that the distribution of DOP mRNA in medium- to large-diameter myelinated neurons and nonpeptidergic C fibres significantly differed between rats and mice ( $p = 0.004$  and  $p = 0.0076$ , respectively; unpaired *T* test with Welch's correction). Indeed, the proportion of DOP-expressing DRG neurons labelled with NF200 was higher in rats than in mice, whereas IB4 labelling was less frequent in rats than was P2XR3 labelling in mice. On the other hand, the proportion of DOP-positive cells coexpressing Tac1 or MOP mRNA was not significantly different between the species. The distribution of MOP mRNA was, however, similar between rats and mice for all the different neuronal markers. Similarly, no difference was observed between species for the



**FIGURE 4** Distribution of DOP mRNA in mouse DRG neurons. The distribution of DOP mRNA was investigated in mouse DRG neurons by combining in situ hybridization and immunofluorescence. DOP mRNA expression was visualized using RNAscope technology (a–e; white punctate labelling). DOP mRNA was found in a proportion of NF200-positive, large and medium myelinated neurons (b), in unmyelinated nonpeptidergic C fibres expressing P2X3 (c), in unmyelinated peptidergic C fibres expressing Tac1 mRNA (d), and in neurons expressing MOP mRNA (red punctate labelling) (e). (f) Percentage of DOP-positive cells expressing each marker. Data are presented as the mean  $\pm$  S.E.M. White arrows indicate cells where costaining for DOP mRNA and the various neuronal markers can be observed. Nuclei were stained with DAPI (blue). Scale bars = 20  $\mu$ m

coexpression of DOP and MOP mRNA. Interestingly, although the frequency of DOP and MOP mRNA coexpression in medium- to large-diameter and peptidergic fibres did not significantly differ between species, their presence in nonpeptidergic C fibres was more frequent in mice than in rats ( $p = 0.01$ ).

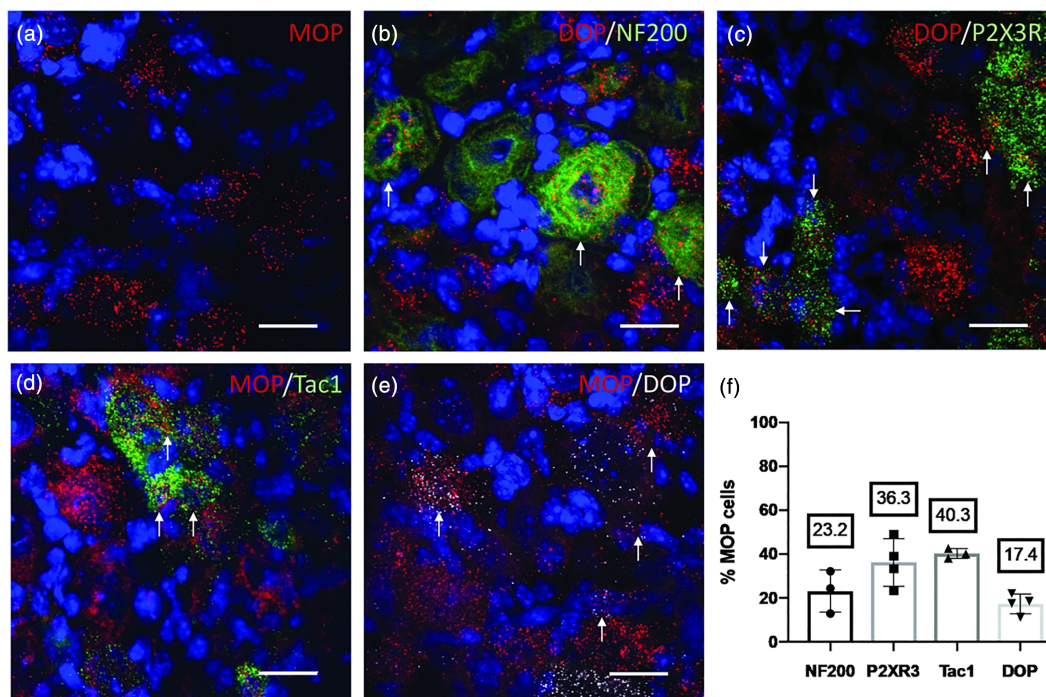
### 3.3 | Effect of chronic morphine injections on the distribution of DOP and MOP mRNA in rat DRG neurons

In rats and mice, we have previously shown that morphine treatment can increase the level of DOP at the cell surface of cells (Cahill et al., 2001), including DRGs (Gendron et al., 2006), and that this effect was paralleled with an increased analgesic effect of DOP-selective agonists (Cahill et al., 2001; Gendron, Esdaile, et al., 2007). To determine whether this could be due to a change in the expression patterns of the receptors, we further investigated the distribution of DOP and MOP mRNA in DRG neurons of rats treated with saline or with escalating doses of morphine (5, 8, 10, 15 mg/kg) given over 24 h. As shown in Figure 7a, no significant difference was

found in the distribution of DOP mRNA within DRG neurons when tissue from morphine-treated animals was compared to saline-treated rats. Similarly, the distribution of MOP mRNA in DRG neurons was not affected by the 24-h morphine treatment (Figure 7b). Unsurprisingly, the identity of cells coexpressing both receptors also remained unchanged in morphine-treated animals (Figure 7c).

### 3.4 | Effect of CFA-induced inflammation in the hind paw on the distribution of DOP and MOP mRNA in rat DRG neurons

Our previous studies showed that inflammation induced by a subcutaneous injection of complete Freund's adjuvant (CFA) in the hind paw also produced an increase in cell surface DOP and in the analgesic effects of DOP agonists (Gendron et al., 2006; Gendron, Esdaile, et al., 2007; Gendron, Pintar, & Chavkin, 2007). We therefore assessed the potential impact of a 72-h CFA treatment on the distribution of DOP and MOP mRNA in rat DRG neurons. When the distribution of DOP (Figure 8a) and



**FIGURE 5** Distribution of MOP mRNA in mouse DRG neurons. The distribution of MOP mRNA was investigated in rat DRG neurons by combining in situ hybridization and immunofluorescence. MOP mRNA expression was visualized using RNAscope technology (a–e; red punctate labelling). MOP mRNA was found in a proportion of NF200-positive, large and medium myelinated neurons (b), in unmyelinated nonpeptidergic C fibres expressing P2X3 (c), in unmyelinated peptidergic C fibres expressing Tac1 mRNA (d), and in neurons expressing DOP mRNA (white punctate labelling) (e). (f) Percentage of MOP-positive cells expressing each marker. Data are presented as the mean  $\pm$  S.E.M. White arrows indicate cells where costaining for MOP mRNA and the various neuronal markers can be observed. Nuclei were stained with DAPI (blue). Scale bars = 20  $\mu$ m

MOP mRNA (Figure 8b) in lumbar (L3–L5) DRGs located ipsilateral or contralateral to the inflammation were compared, no difference was observed in their distribution. Similarly, paw inflammation did not affect the identity of neurons coexpressing both receptors (Figure 8c).

#### 4 | DISCUSSION

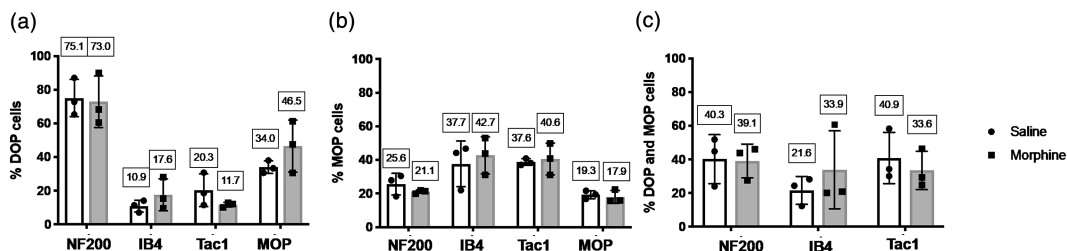
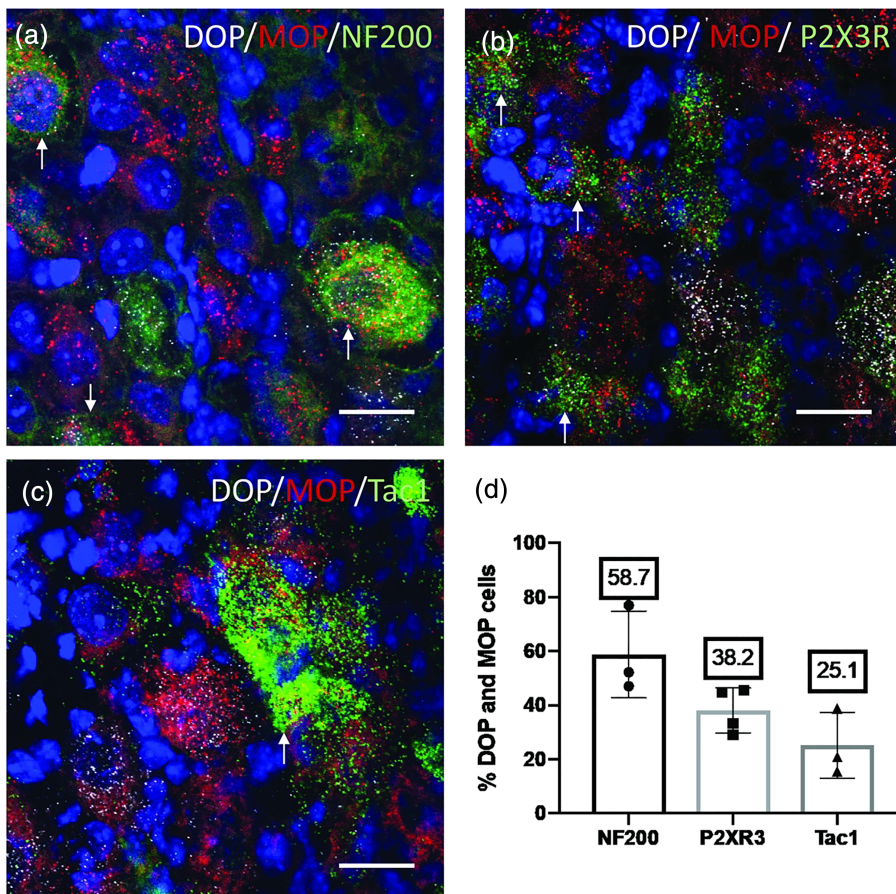
The distribution of delta (DOP) and mu (MOP) opioid receptors in the nervous system of rats and mice has previously been described (Mansour et al., 1994). Using RT-PCR, in situ hybridization and immunofluorescence (Gendron et al., 2006; Ji et al., 1995; Mennicken et al., 2003; Minami et al., 1995; Rau et al., 2005; Wang & Wessendorf, 2001; Zhang, Bao, Arvidsson, et al., 1998; Zhang, Bao, Shi, et al., 1998), it was observed that DOP and MOP are both expressed in different types of primary sensory neurons. Most interestingly, important interspecies differences were suggested. In the current study, we investigated the expression of MOP and DOP in DRG neurons of mice and rats using RNAscope, a recently developed in situ hybridization approach

(Wang et al., 2012). When compared with other assays, this approach appears to be highly sensitive, specific and compatible with immunofluorescence for multiple labelling.

Over the years, numerous studies have shown DOP and MOP receptors to be both expressed in myelinated large- and medium-diameter neurons as well as in small peptidergic and nonpeptidergic C fibres (Gendron et al., 2006; Ji et al., 1995; Mansour et al., 1994; Minami et al., 1995; Rau et al., 2005; Wang & Wessendorf, 2001; Zhang, Bao, Arvidsson, et al., 1998; Zhang, Bao, Shi, et al., 1998). Based on conventional in situ hybridization and immunological approaches, the reported proportion of neurons expressing DOP is, however, highly variable. In some cases, DOP was found to be equally expressed in large- and small-diameter neurons (Gendron et al., 2006; Mansour et al., 1994; Wang & Wessendorf, 2001), whereas in other studies, DOP was found mostly in small-diameter neurons expressing substance P (Ji et al., 1995; Riedl et al., 2009; Wang et al., 2010; Zhang, Bao, Arvidsson, et al., 1998). The idea that DOP is expressed in all DRG neurons was, however, challenged by a study using knockin mice expressing an



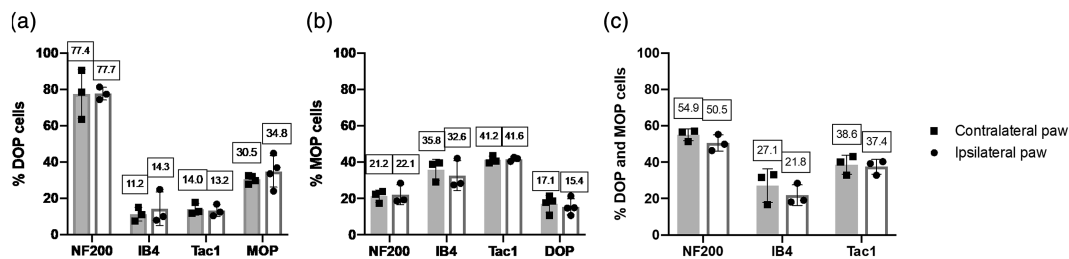
**FIGURE 6** Coexpression and distribution of DOP and MOP mRNA in DRG neurons. The distribution of cells expressing both DOP (white punctate labelling) and MOP mRNA (red punctate labelling) was studied. A proportion of cells expressing both DOP and MOP mRNA were also labelled with NF200 (a), P2X3 (b) or Tac1 mRNA (c). (d) Percentage of DOP- and MOP-positive cells expressing each marker. Data are presented as the mean  $\pm$  S.E. M. White arrows indicate cells coexpressing DOP and MOP mRNA together with the indicated marker. Nuclei were stained with DAPI (blue). Scale bars = 20  $\mu$ m



**FIGURE 7** Effect of chronic morphine treatment (48 h) on the distribution of DOP and MOP mRNA in rat DRG neurons. Rats were treated with saline or with increasing doses of morphine (5, 8, 10 and 15 mg/kg) over a period of 48 h. Twelve hours after the last injection, the animals were euthanized, and the tissue was processed for RNAscope in situ hybridization. (a) Percentage of cells expressing DOP mRNA in saline- and morphine-treated rats expressing the different neuronal markers. (b) Percentage of cells expressing MOP mRNA in saline- and morphine-treated rats expressing the different neuronal markers. (c) Percentage of cells coexpressing DOP and MOP mRNA in saline- and morphine-treated rats expressing the different neuronal markers. For each marker, the data obtained were compared between morphine- and saline-treated animals using the unpaired T test with Welch's correction. No significant differences were observed ( $p > 0.05$ )

eGFP-tagged version of DOP. In these mice, it was observed that the DOP receptor was predominantly expressed in large, myelinated DRG neurons as well as in a small proportion of nonpeptidergic C fibres (Scherrer et al., 2009). As opposed to what has been shown by others (Ji et al., 1995; Riedl et al., 2009; Wang et al., 2010; Zhang, Bao, Arvidsson, et al., 1998), DOP was only rarely found to be expressed in peptidergic C fibres, which are findings also supported by single-cell RNA sequencing

(Usoskin et al., 2015). In contrast, the immunolabelling of MOP revealed that the majority of cells expressing this receptor were peptidergic C fibres (Scherrer et al., 2009). These differences in distribution of the receptors can be attributed to the different techniques being used, the tissue processing, ligand sensitivity, the use of animal models and/or the use of different animal species. Another problem shared by most techniques and approaches is the threshold used to determine whether a



**FIGURE 8** Effect of chronic inflammatory pain on the distribution of DOP and MOP mRNA in rat DRG neurons. Rats were injected in one hind paw with 100  $\mu$ l of complete Freund's adjuvant (CFA). Seventy-two hours after injection, the animals were euthanized, and the tissue was processed for RNAscope in situ hybridization. (a) Percentage of cells expressing DOP mRNA in ipsilateral and contralateral paws of CFA-treated rats expressing the different neuronal markers. (b) Percentage of cells expressing MOP mRNA in ipsilateral and contralateral paws of CFA-treated rats expressing the different neuronal markers. (c) Percentage of cells coexpressing DOP and MOP mRNA in ipsilateral and contralateral paws of CFA-treated rats expressing the different neuronal markers. For each marker, the data obtained were compared between ipsilateral and contralateral paws of CFA-treated animals using the unpaired T test with Welch's correction. No significant differences were observed ( $p > 0.05$ )

cell should be considered positive or negative for a specific marker, including receptors. Indeed, in most cases, a threshold must be applied to distinguish the background signal level from the specific labelling. With immunofluorescence, this step remains highly subjective and arbitrary. Using the in situ hybridization approach RNAscope, we demonstrate that in naïve rats, DOP and MOP mRNA are both expressed, at different levels, in all three types of DRG neurons. Indeed, we observed that DOP is mainly expressed in myelinated neurons, whereas MOP is mostly found in small peptidergic and non-peptidergic cells. Although found in a smaller proportion of small cells, DOP mRNA was also present in peptidergic and, to a lesser extent, in nonpeptidergic C fibres. On the other hand, MOP mRNA was found to be almost equally distributed among the small nonpeptidergic and peptidergic neurons. In mice, we also observed that DOP mRNA and MOP mRNA are distributed in large and medium myelinated neurons as well as in unmyelinated nonpeptidergic and peptidergic C fibres. Interestingly, in this species, DOP mRNA was predominantly expressed in small nonpeptidergic C fibres, whereas MOP mRNA was equally expressed in both types of C fibres.

As previously mentioned, interspecies differences exist in the expression of opioid receptors (Mennicken et al., 2003; Sharif & Hughes, 1989). Here, although no significant differences between mice and rats were found for the distribution pattern of MOP mRNA, a higher proportion of DOP-expressing DRG neurons was labelled with NF200 in rats than in mice. However, most interestingly, the rats had a smaller proportion of DOP-positive small nonpeptidergic neurons than mice. Admittedly, because of technical challenges, we used different markers to identify small nonpeptidergic neurons in rats and mice. Although the overlap might not be perfect,

both markers are commonly used to identify the subpopulation of small nonpeptidergic neurons. Furthermore, in rats and monkeys, P2X3 was shown to be almost exclusively present in IB4-positive neurons (Vulchanova et al., 1997, 1998). Nonetheless, our observations support the idea that there are differences in the distribution of opioid receptors among species and therefore differences in their respective roles.

The coexpression of DOP and MOP receptors in the DRG is also a matter of controversy. Of greater importance for this study is our previous observation that the activation of MOP with various agonists increases the analgesic effect of the DOP agonist deltorphin II (Cahill et al., 2001; Gendron, Esdaile, et al., 2007; Morinville et al., 2003), an effect potentially mediated by an increase in DOP at the cell surface (Cahill et al., 2001; Gendron et al., 2006; Morinville et al., 2003, 2004). Similarly, we previously showed that MOP is essential for increased DOP-mediated analgesia in a complete Freund's adjuvant inflammatory pain model (Gendron, Pintar, & Chavkin, 2007). Together, these findings suggest that MOP and DOP may interact to promote the cell surface targeting of DOP. Here, we show that in both species, a number of DOP-expressing DRG neurons also express MOP mRNA. Although approximately 60% of these cells were NF200-positive, a significant proportion of MOP- and DOP-positive neurons were small peptidergic and nonpeptidergic neurons, supporting a common role for these receptors in inhibiting different types of pain. We previously showed that both DOP and MOP agonists, when injected intrathecally, were able to alleviate chemical, thermal and mechanical pain (Beaudry et al., 2011; Normandin et al., 2013). Coexpression of DOP and MOP in small neurons not only supports similar antinociceptive properties of these receptors but also a possible physical interaction between them to form a distinct

pharmacological entity (Fujita et al., 2015; George et al., 2000; Levac et al., 2002). Functional studies also revealed an analgesic synergy between MOP and DOP in central and peripheral terminals of nociceptors (Bruce et al., 2019), an effect that was later localized to epidermal nerve fibre terminals of C-fibre nociceptors (Uhelski et al., 2020).

As mentioned above, repeated morphine treatment and chronic inflammatory pain have been shown to increase the antinociceptive effect of DOP agonists (for reviews, see Gendron et al., 2015, 2016). Although we previously suggested that this effect is due to the increased cell surface expression of DOP that was observed under these conditions (Cahill et al., 2007; Gendron et al., 2015, 2016), another possibility is that morphine treatment and inflammation alter the distribution of the receptors among the different neuronal subpopulations. In the current study, we found no significant change in the distribution of the receptors in rat and mouse DRG neurons, suggesting that the increase in DOP-mediated antinociceptive effects observed following morphine treatment or under inflammatory pain conditions is due to the membrane translocation of DOP rather than a change in expression among neuronal populations. Similarly, the identity of cells coexpressing DOP and MOP mRNA remained unchanged. Another possibility is that morphine treatment and inflammation produce modifications to the total level of expression of MOP and/or DOP. In rats, we previously showed that neither morphine nor inflammation produced a significant change in the level of DOP mRNA in the DRGs (Gendron et al., 2006). However, using antibodies others have shown that carrageenan-induced inflammation produced a slight increase in the percentage of MOP-expressing rat DRG neurons whereas the percentage of cells expressing DOP decreased. No effect on the distribution among the size of cells expressing these receptors was however observed (Ji et al., 1995).

In the peripheral endings of primary afferents, DOP was recently shown to be associated with GRK2, preventing its association with G proteins. Interestingly, bradykinin release induced by inflammation has been shown to provoke the sequestration of GRK2, which in turn restores DOP activity (Brackley et al., 2016, 2017). Whether this effect is also observed on central terminals remains to be determined but might represent another putative mechanism for the regulation of DOP. Using electrophysiological approaches, another study showed that inflammatory pain increased the functionality of DOP in DRG neurons by increasing the inhibition of  $Ca^{2+}$  channels, an effect accompanied by an increased mechanical antinociceptive effect (Pradhan et al., 2013). More recently, we used mass spectrometry to identify a

number of protein partners for DOP, both in transfected cells and in the mouse brain (Degrandmaison et al., 2020; St-Louis et al., 2017). Further studies are necessary to investigate whether the identified partners are involved in regulating the cellular routing/trafficking of DOP.

## 5 | CONCLUSION

Altogether, our results confirmed that DOP and MOP mRNA are expressed in all types of DRG neurons, both in mice and in rats. Although DOP mRNA is predominantly found to be mainly expressed in large and medium myelinated neurons, MOP mRNA is mainly expressed in C fibres. Interestingly, the distribution of MOP and DOP slightly differs among the studied species, with a higher proportion of small nonpeptidergic C fibres expressing DOP mRNA in mice than in rats. Further studies will be needed to investigate the distribution of these receptors in higher species, such as monkeys and humans.

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## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTIONS

BQ, CB and LC performed the experiments. BQ, CB, LC, JLP and LG analysed the results. BQ, CB, LC and LG designed the research and wrote the manuscript. All authors reviewed and edited the final version of the manuscript.

## PEER REVIEW

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## DATA AVAILABILITY STATEMENT

The authors declare that all data, protocols, and materials are detailed in the manuscript.

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