

Delta Opioid Receptors Presynaptically Regulate Cutaneous Mechanosensory Neuron Input to the Spinal Cord Dorsal Horn

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SUMMARY

Cutaneous mechanosensory neurons detect mechanical stimuli that generate touch and pain sensation. Although opioids are generally associated only with the control of pain, here we report that the opioid system in fact broadly regulates cutaneous mechanosensation, including touch. This function is predominantly subserved by the delta opioid receptor (DOR), which is expressed by myelinated mechanoreceptors that form Meissner corpuscles, Merkel cell-neurite complexes, and circumferential hair follicle endings. These afferents also include a small population of CGRP-expressing myelinated nociceptors that we now identify as the somatosensory neurons that coexpress mu and delta opioid receptors. We further demonstrate that DOR activation at the central terminals of myelinated mechanoreceptors depresses synaptic input to the spinal dorsal horn, via the inhibition of voltage-gated calcium channels. Collectively our results uncover a molecular mechanism by which opioids modulate cutaneous mechanosensation and provide a rationale for targeting DOR to alleviate injury-induced mechanical hypersensitivity.

INTRODUCTION

The cutaneous mechanosensory system is critical for the detection and discrimination of innocuous and noxious mechanical

stimuli that elicit sensations of touch and pain, respectively (Basbaum et al., 2009; Delmas et al., 2011; Lewin and Moshourab, 2004). However, innocuous stimuli in the setting of injury can also evoke pain. Indeed, skin hypersensitivity to light mechanical stimuli (also called mechanical allodynia or touch-evoked pain) is one of the most common and distressing symptoms of nerve injury-induced neuropathic pain (Costigan et al., 2009).

Of particular importance is the identification of the primary sensory neurons of the dorsal root ganglion (DRG) that mediate mechanical allodynia. We and others have shown that ablation or silencing of several populations of unmyelinated nociceptors (C fibers) does not alter nerve injury-induced mechanical hypersensitivity in rodents (Abrahamsen et al., 2008; Cavanaugh et al., 2009; Scherrer et al., 2010). By contrast, selective compression block of myelinated axons (A fibers), which eliminates the normal sense of touch while preserving C fiber function, abolishes touch-evoked neuropathic pain in humans (Campbell et al., 1988). Electrophysiological studies demonstrate that pharmacological disinhibition of spinal cord circuits or peripheral injuries that cause mechanical hypersensitivity strengthen A β and A δ fiber input to nociceptive lamina I spinal neurons, uncovering a mechanism by which activation of low-threshold A fibers by normally innocuous mechanical stimuli can cause pain (Torsney, 2011; Torsney and MacDermott, 2006). Together these results indicate that cutaneous mechanosensitive A fibers contribute to touch-evoked pain and that drugs that dampen the function of these neurons might be an effective treatment.

Delta, kappa, and mu opioid receptors (DOR, KOR, and MOR, respectively) are G protein-coupled receptors that regulate neurotransmission, including at the level of primary afferent DRG neurons (Williams et al., 2001). Opioids that preferentially

activate MORs (e.g., morphine, oxycodone, and fentanyl) are widely used to treat severe pain, but their efficacy in chronic neuropathic pain is subject to considerable uncertainty (McNicol et al., 2013). A better understanding of the neural circuits and molecular mechanisms underlying opioid analgesia is necessary for a more rational use of opioids in the clinic.

The expression pattern of DOR in DRG remains a subject of substantial controversy. We recently showed that DOR is predominantly expressed by DRG neurons with myelinated axons (Scherrer et al., 2009). Furthermore, DOR-selective agonists display anti-allodynic properties in murine models of touch-evoked neuropathic and inflammatory pain, and DOR null mice exhibit increased mechanical hypersensitivity after peripheral injury (reviewed in Gavériaux-Ruff and Kieffer, 2011; Ossipov et al., 2004). These findings suggested that DOR may be expressed by cutaneous mechanosensory A fibers and that DOR-mediated regulation of these afferents could counteract nerve injury-associated mechanical hypersensitivity.

With the recent discovery that functionally distinct classes of A fibers depend on distinct neurotrophins for their development and survival, it is now possible to test this hypothesis. Thus, cutaneous A β low-threshold mechanoreceptors (LTMRs) express the neurotrophin receptors TrkC and/or Ret (Bourane et al., 2009; Fünfschilling et al., 2004; Li et al., 2011; Luo et al., 2009; Senzaki et al., 2010), while A δ D-hair LTMRs express TrkB (Li et al., 2011; Stucky et al., 1998). This molecular characterization distinguishes touch-encoding cutaneous A fibers from myelinated nociceptors, which most often express TrkA (Fang et al., 2005) and the neuropeptide CGRP (Lawson et al., 2008), and from proprioceptors that coexpress parvalbumin, TrkC, and the transcription factor Runx3 (de Nooij et al., 2013).

Building on the ability to classify subpopulations of myelinated afferents, here we conducted a neuroanatomical and electrophysiological analysis to resolve the functional organization of opioid receptors in DRG.

RESULTS

DORGFP Knockin Mouse Faithfully Reports DOR Distribution in DRG and CNS

We previously used a DORGFP knockin mouse (Scherrer et al., 2006) to establish the expression pattern of DOR in DRG (Scherrer et al., 2009). Most DORGFP+ DRG neurons have large-diameter cell bodies and express the marker of myelinated afferents neurofilament 200 (NF200) (67% of DORGFP+ cells are NF200+) (Figures 1A and 1F; Table S1 available online). DORGFP is expressed by 27% of all NF200+ DRG neurons. The majority of DORGFP+ NF200– cells bind the isolectin B4 (IB4) (91% IB4+), and 19% of IB4+ cells express DORGFP (Figures 1F and S1A; Table S1), indicating that DOR+ small-diameter neurons comprise a subset of nonpeptidergic C nociceptors. Accordingly, very few DORGFP+ NF200– cells express substance P (SP) (~5%), and only about 2% of SP+ neurons express DORGFP (Figures 1F and S1B; Table S1).

This DORGFP expression pattern differs drastically from the immunoreactivity (ir) pattern generated in wild-type mice by an antibody that purportedly recognizes the N-terminal domain of the receptor (anti-DOR³⁻¹⁷). DOR-ir was observed in a larger cell

population (about half of DRG neurons), mainly of small diameter (Bao et al., 2003; He et al., 2011; Wang et al., 2010). Furthermore, DOR-ir was always found to colocalize with SP in peptidergic C nociceptors, an essential requirement for a SP-DOR direct interaction that was proposed to sort DOR in large dense-core vesicles and regulate its surface expression (Guan et al., 2005). In situ hybridization studies performed by the same group suggested that *Oprd1* mRNA, which encodes DOR, might be present in 71% of small-diameter DRG neurons.

To address this controversy, we took advantage of the recent development of an ultrasensitive and specific method to detect single mRNA molecules in tissues (QuantiGene ViewRNA, Panomics). We found that in DRG from wild-type mice, *Oprd1* mRNA is concentrated in large-diameter neurons that express NF200 (Figures 1B, 1F, S1C, and S1D). These DRG cells often contain hundreds of labeled dots in their cytoplasm. Thus, 62% of DRG neurons with *Oprd1* mRNA are NF200+. We then asked whether the small-diameter, NF200– DRG neurons that express *Oprd1* mRNA belong to the peptidergic or nonpeptidergic class of C nociceptors. We found that among 219 SP+ profiles examined, 204 did not contain a single labeled dot. Only 3 out of 219 cells displayed 5 or more labeled dots in their cytoplasm and were considered positive (~2%; Figures 1F and S1E), while 12 had only 1 or 2 dots, which was comparable to the background level. Only about 2% of NF200– DRG neurons with *Oprd1* mRNA express SP, while the great majority (~82%) of these cells bound IB4 (Figures 1D–1F).

We next compared the DORGFP fluorescence pattern in the CNS to the binding pattern of selective DOR radioligands, [³H]-Deltorphin II and [³H]-DPDPE, in wild-type mice. We observed that DORGFP distribution is strikingly similar to the binding pattern of both DOR radioligands throughout the CNS. Thus, DORGFP fluorescence and radioligand binding are particularly intense in basolateral amygdala, striatum, cortex, pontine nucleus, olfactory bulb, and olfactory tubercle (Figure 1G). In the spinal cord, both DORGFP fluorescence and radioligand binding are seen throughout the gray matter (Figure 1H). This distribution pattern differs profoundly from that generated by the anti-DOR³⁻¹⁷ antibody, which labels exclusively the terminals of peptidergic afferents in superficial dorsal horn (Guan et al., 2005). Finally, note that binding of radioligands is lost in tissues from *Oprd1* knockout mice, demonstrating the specificity of DOR labeling and localization (Figure 1G).

We conclude that the remarkably high correspondence between the DORGFP distribution pattern and that of *Oprd1* mRNA and DOR protein in wild-type mouse, which is consistent with previous studies (Cahill et al., 2001; Goody et al., 2002; Mansour et al., 1987, 1994; Mennicken et al., 2003; Minami et al., 1995), argues very strongly that DORGFP expression faithfully recapitulates that of native DOR.

TrkA, TrkC, and Ret Coexpression Reveals that DOR+ NF200+ Neurons Are Heterogeneous and Include Nociceptors and LTMRs

We next resolved the molecular identity of DORGFP+ NF200+ neurons using a host of markers that define DRG neuron subpopulations. Opioid receptor-expressing DRG neurons are

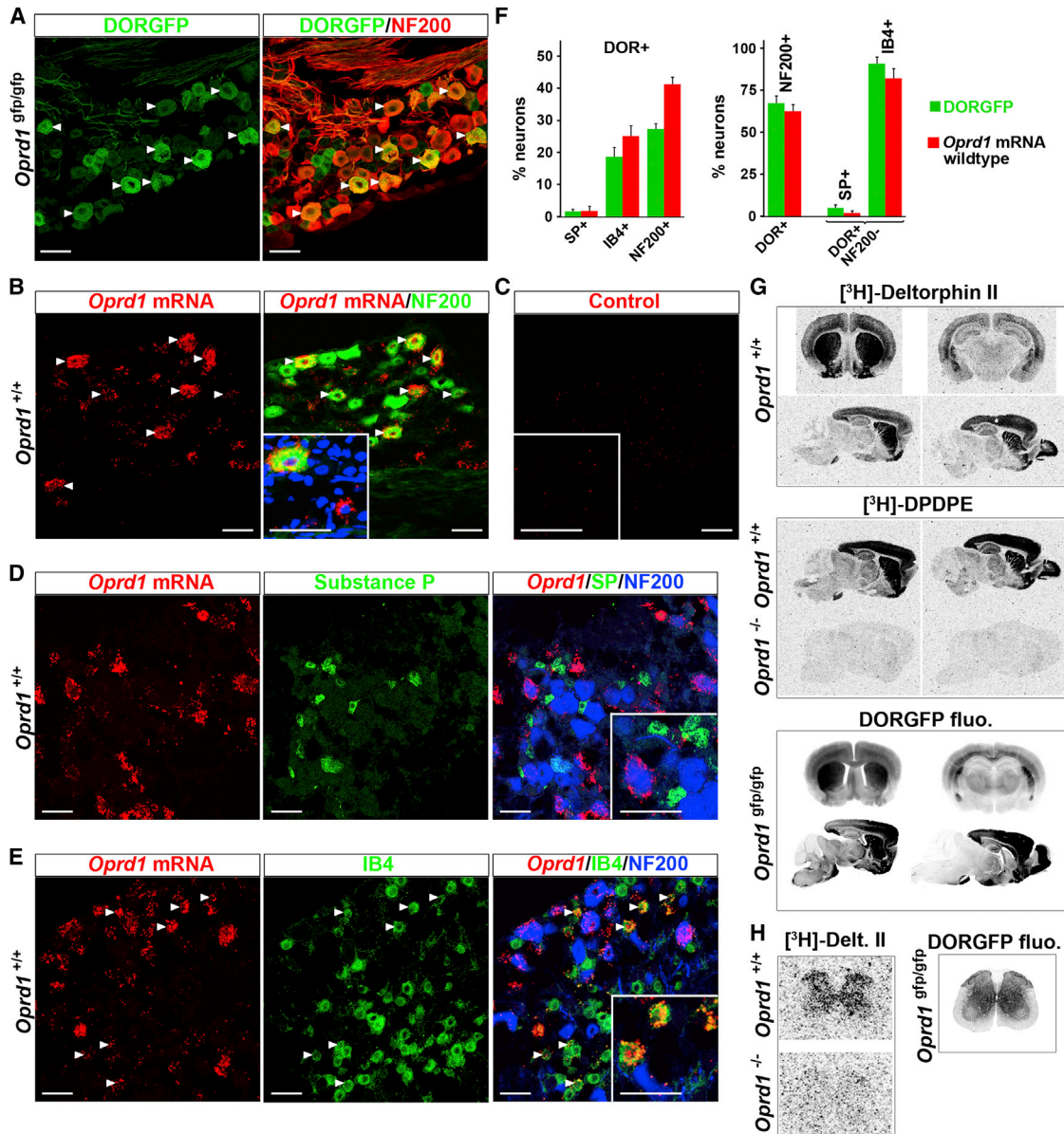


Figure 1. DORGFP Mouse Faithfully Reports DOR Expression in CNS and DRG, where DOR Is Predominantly Expressed by Large-Diameter, Myelinated Neurons

(A) Costaining with anti-GFP and -NF200 antibodies shows that 67% of DORGFP+ DRG neurons express the marker of myelinated neurons NF200. (B) *Opr1* mRNA is also predominantly present in NF200+ neurons in DRG from wild-type mice. (C) Control showing that in the absence of the specific probe against *Opr1* sequence, there is no in situ hybridization signal in DRG neurons. (D) Substance P+ (SP+) cells almost never contain *Opr1* mRNA in DRG neurons from wild-type mice. (E) The great majority of NF200- cells that contain *Opr1* mRNA bind the isolectin B4 (IB4). (F) Quantification of (A)–(E) and of Figures S1A and S1B. Left: neurons that coexpress DOR among those that express SP, or bind IB4, or express NF200. Right: DOR+ neurons that coexpress NF200, and DOR+ NF200- neurons that coexpress SP or bind IB4. (G) The distribution of fluorescence in brain slices from DORGFP mice is identical to the binding pattern of DOR radioligands [³H]-Deltorphin II and [³H]-DPDPE in wild-type mice. Radiolabeling is lost in *Opr1* null mice, demonstrating specificity. (H) In wild-type mouse spinal cord, [³H]-Deltorphin II binds to DORs that are present throughout the gray matter, consistent with DORGFP fluorescence pattern. Data are represented as means ± SEM. Scale bars represent 50 μm. See also Figure S1.

generally considered to be nociceptors, but given the anti-allodynic properties of DOR agonists, we hypothesized that DOR+ DRG neurons could include LTMRs. Consistently, we found

that most of the DORGFP+ NF200+ neurons (~62%) in fact express TrkC (Figures 2A and 2B; Table S1). To characterize these neurons further and identify proprioceptors, we crossed

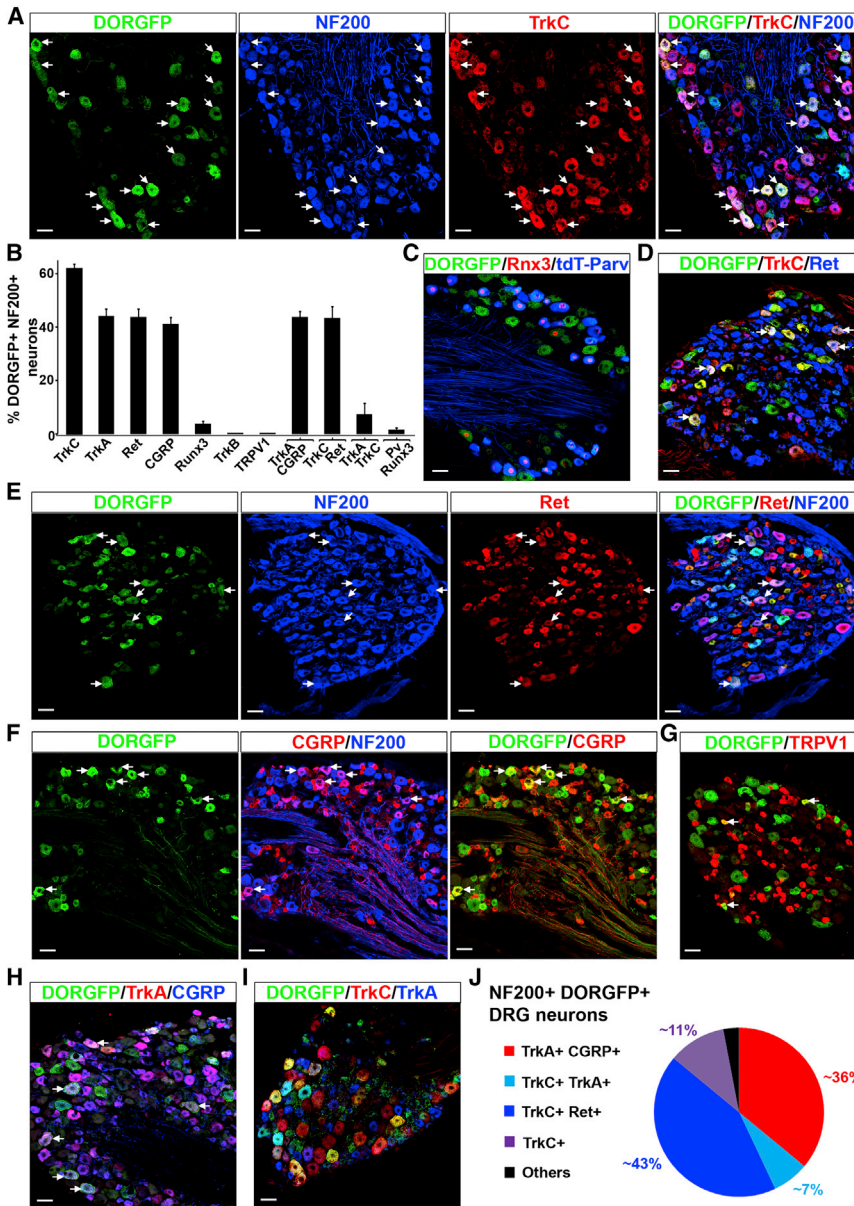


Figure 2. DOR-Expressing Myelinated DRG Neurons Express the Markers of A β Low-Threshold Mechanoreceptors TrkC and Ret

(A) TrkC is expressed by about 62% of NF200+ DORGFP+ DRG neurons. Arrows indicate cells where costaining occurs.

(B) Quantification of (A) and (C)–(I).

(C) Proprioceptors, identified by the coexpression of parvalbumin and Runx3, do not express DORGFP. Note that some DORGFP+ neurons individually express either parvalbumin or Runx3.

(D) Approximately 70% of DORGFP+ TrkC+ neurons coexpress Ret (arrows) indicating that these neurons are probably myelinated cutaneous mechanoreceptors, including A β LTMRs.

(E) About 40% of large-diameter myelinated (NF200+) DORGFP+ neurons (arrows) and almost all small-diameter DORGFP+ neurons express Ret.

(F) Approximately 36% of NF200+ DORGFP+ neurons express CGRP (arrows).

(G) Large-diameter DORGFP+ DRG neurons do not express the noxious heat sensor TRPV1. A very small number of small-diameter neurons coexpress DOR and TRPV1 (arrows).

(H) Large-diameter DORGFP+ neurons that express CGRP also immunostain for the nociceptor marker TrkA (arrows).

(I) Almost all large-diameter DORGFP+ neurons express either TrkC or TrkA.

(J) Molecular identity of myelinated (NF200+) DORGFP+ neuron subpopulations.

Data are represented as means \pm SEM. Scale bars represent 50 μ m.

DORGFP animals with reporter mice in which the tdTomato fluorescent protein is expressed by parvalbumin+ DRG neurons. Figure 2C shows that DORGFP+ neurons do not include proprioceptors, marked by the coexpression of parvalbumin and the transcription factor Runx3 (de Nooij et al., 2013) (Figures 2B and 2C; Table S1). Rather, we found that many DORGFP+ TrkC+ DRG neurons coexpress Ret, indicating that they are probably cutaneous mechanoreceptors, including A β LTMRs (Figures 2B and 2D–2E; Table S1) (Bourane et al., 2009; Fünfschilling et al., 2004; Luo et al., 2009; Senzaki et al., 2010). We also determined the identity of the remaining ~36% of DORGFP+ NF200+ neurons. This population coexpresses TrkA and the neuropeptide CGRP, but not TRPV1 (Figures 2B and 2F–2H; Table S1), indicating that they correspond to a particular class of capsaicin-insensitive A nociceptors. Finally, we did not

observe any DORGFP+ NF200+ neurons that express the D-hair marker TrkB (Figures 2B and S2B; Table S1).

Based on this anatomical analysis, we conclude that DORGFP+ myelinated DRG neurons are remarkably more heterogeneous than previously thought and segregate into two functionally distinct classes: a TrkA+ CGRP+ nociceptive population (~36%) and a TrkC+ and/or

Ret+ population of cutaneous mechanoreceptors, which probably includes A β LTMRs (~62%) (Figure 2J).

MOR Is Expressed by Myelinated Nociceptors and Colocalizes with DOR in CGRP+ NF200+ DRG Neurons

Although MOR agonists regulate neurotransmitter release from both C and A nociceptors (Heinke et al., 2011), the precise molecular identity of the myelinated afferents that express MOR has so far remained elusive. Here we used an anti-MOR antibody with demonstrated specificity in mice null for MOR expression (Scherrer et al., 2009) to characterize these cells. We found that almost all NF200+ MOR+ neurons express CGRP (~99%) but very rarely TrkC (~3%), Ret (~5%), or TrkB (0%) (Figures 3A–3G; Table S2). About 8% of NF200+ MOR+ neurons also express TRPV1. Because of antibody incompatibility, we could not

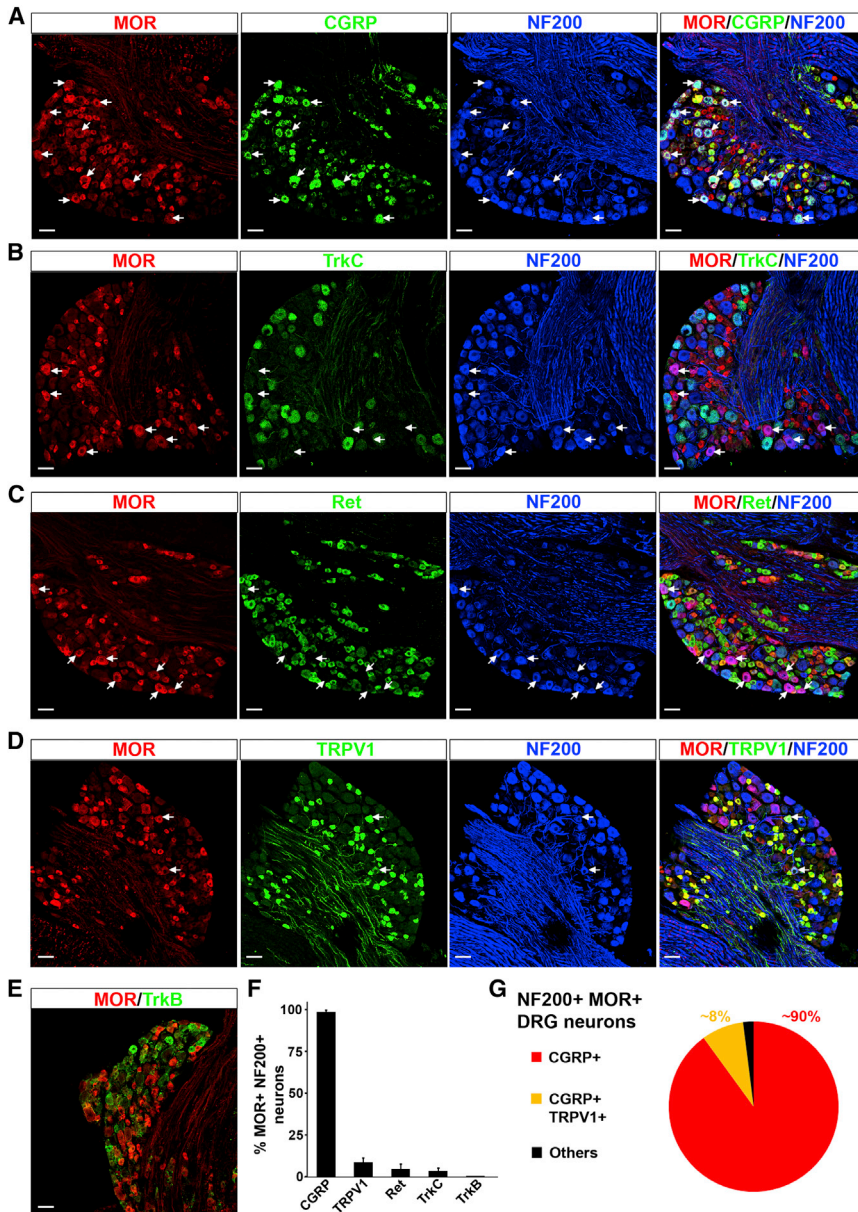


Figure 3. Myelinated DRG Neurons that Express MOR Coexpress CGRP, but Not Markers of A β Low-Threshold Mechanoreceptors

(A) Virtually all MOR+ myelinated neurons (NF200+) express CGRP (arrows indicate NF200+ MOR+ neurons), indicating that these neurons are TrkA+ nociceptors (see Figure S2C).

(B) NF200+ MOR+ neurons (arrows) rarely express TrkC.

(C) The great majority of NF200+ MOR+ neurons express neither Ret nor TrkC (see B), indicating that they are neither proprioceptors nor A β LTMRs.

(D) A small subpopulation of NF200+ MOR+ neurons also expresses TRPV1 (arrows).

(E) MOR+ neurons do not express the D-hair receptor marker TrkB.

(F) Quantification of (A)–(E).

(G) Molecular identity of myelinated (NF200+) MOR+ neuron subpopulations.

Data are represented as means \pm SEM. Scale bars represent 50 μ m.

MOR (Figure 4A). We found that among 294 CTB+ DRG neurons, 53 expressed DORGFP (~21%). By contrast, we never observed CTB+ MOR+ cells, which is consistent with our contention that only DOR, but not MOR, is expressed by A β LTMRs.

We next investigated the identity of the DRG cells that coexpress DOR and MOR. Previous studies provided biochemical and behavioral evidence that DOR-MOR functional interactions occur in neurons of the pain pathway (Costantino et al., 2012) but to date failed to identify the sensory neurons in which these interactions might take place (although see He et al., 2011; Wang et al., 2010). As we previously reported (Scherrer et al., 2009), the incidence of coexpression of these two opioid receptors in all DRG neurons

is, in fact, low (less than 5%). However, our analysis now establishes that most DORGFP+ MOR+ DRG neurons express NF200 (~88%), corresponding to ~6% of all myelinated DRG neurons (Figures 4A–4C). About one-third of NF200+ DORGFP+ or NF200+ MOR+ express the other opioid receptor, while coexpression in NF200– neurons is very rare (Tables S1 and S2). Moreover, all the NF200+ DORGFP+ MOR+ neurons belong to the CGRP+ class of A nociceptors, and the two receptors are colocalized at the plasma membrane in these cells (Figures 4D and 4E).

To complete our analysis, we next examined small-diameter unmyelinated (NF200–) DRG neurons and tested whether DOR or MOR are expressed by a population of C LTMRs that express tyrosine hydroxylase (TH) (Li et al., 2011). We found that DOR+ and MOR+ unmyelinated DRG neurons are predominantly

test directly whether NF200+ MOR+ neurons coexpress TrkA. Nevertheless, since ~95% of CGRP+ DRG neurons belong to the TrkA+ population of nociceptors in rodents (Figure S2C), we conclude that all NF200+ MOR+ DRG neurons are peptidergic A nociceptors, which distinguishes the MOR+ population of NF200+ DRG neurons from those that express DOR.

To test this hypothesis further, we took advantage of the characteristic anatomy of A β LTMRs. Some A β LTMRs, unlike any other DRG neurons, project not only to the spinal cord dorsal horn, but also to the dorsal column nuclei (DCN) (Giuffrida and Rustioni, 1992), brainstem regions critical for integration of touch information. We stereotaxically microinjected a retrograde tracer, the B fragment of cholera toxin (CTB), into the DCN of mice. This protocol labeled A β LTMR cell bodies in lumbar DRGs, which we then coimmunostained for DORGFP and

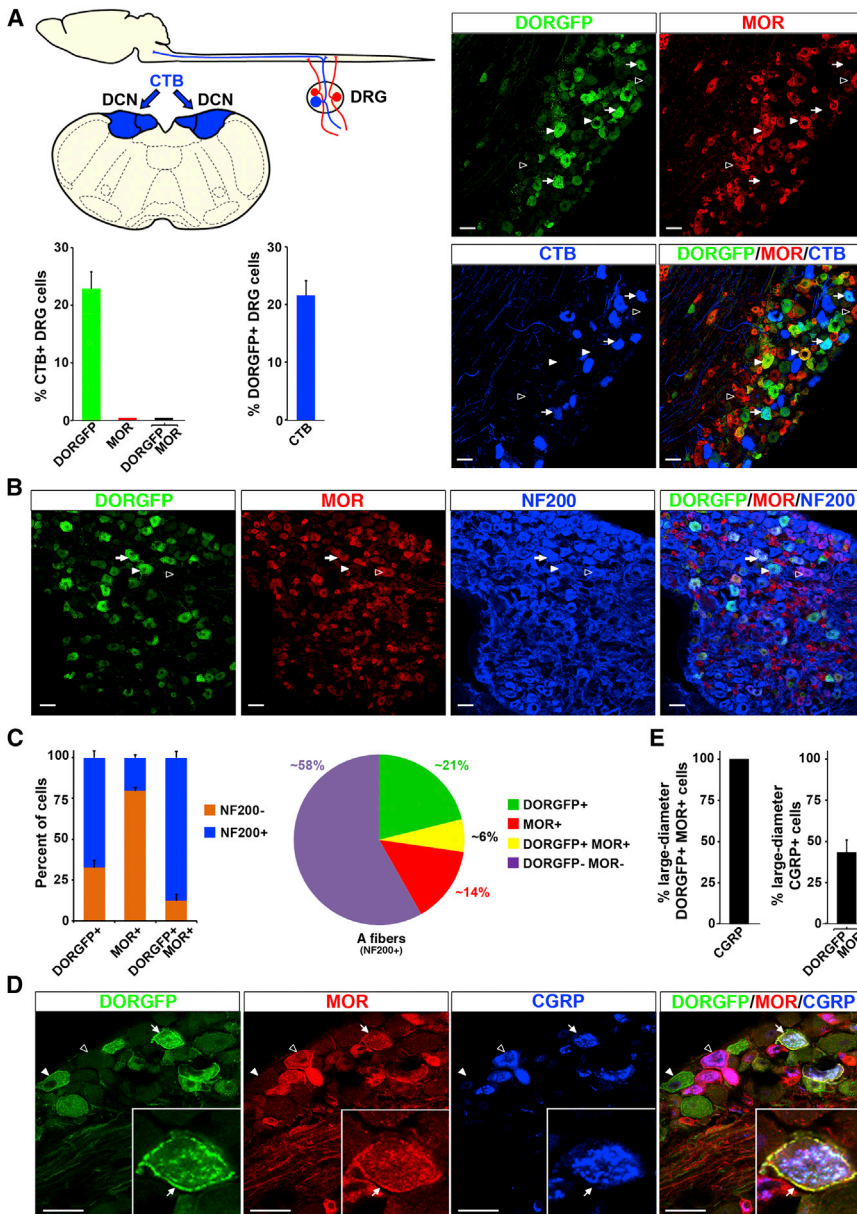


Figure 4. DOR and MOR Are Colocalized at the Plasma Membrane of CGRP+ A Nociceptors

(A) Stereotaxic injection of the B fragment of cholera toxin (CTB) into the dorsal column nuclei (DCN) labels A β LTMR cell bodies in lumbar DRGs. Only DORGFP+ DRG neurons display CTB immunoreactivity ($22.9\% \pm 2.9\%$), while MOR+ ($0.0\% \pm 0.0\%$) and DORGFP+ MOR+ cells do not ($n = 294$ CTB+ cells).

(B) DORGFP and MOR are expressed by largely nonoverlapping populations of DRG neurons. Some NF200+ neurons express only DOR (white arrowhead) or MOR (black arrowhead); a third population coexpresses both receptors (arrow).

(C) Quantification of (B) shows that $87.8\% \pm 3.9\%$ of the DRG neurons that coexpress DOR and MOR are NF200+ (bar graph) and that $6.2\% \pm 1.1\%$ of all NF200+ DRG neurons coexpress both receptors (pie chart) ($n = 1,091$). DOR predominates in NF200+ neurons ($67.3\% \pm 4.3\%$), MOR in NF200- neurons ($79.8\% \pm 1.9\%$) (bar graphs).

(D) DORGFP and MOR are coexpressed at the plasma membrane of large-diameter CGRP+ DRG neurons, which are most often mechanonociceptors (arrow) (Lawson et al., 2008). White arrowhead points to a large-diameter DORGFP+ CGRP- MOR- DRG neuron that probably belongs to the TrkC+ population described in Figure 1. Black arrowhead illustrates that all large-diameter MOR+ DRG neurons, and the vast majority of MOR+ small-diameter cells, coexpress CGRP.

(E) All DORGFP+ MOR+ large-diameter DRG neurons coexpress CGRP ($100.0\% \pm 0.0\%$) and this population corresponds to $43.3\% \pm 7.7\%$ of all CGRP+ large-diameter DRG neurons ($n = 229$). Data are represented as means \pm SEM. Scale bars represent 50 μ m.

IB4-binding MrgprD+ mechanonociceptors and peptidergic TRPV1+ thermonociceptors, respectively, but not TH⁺ C LTMRs (Figure S3).

DOR+ Myelinated DRG Neurons Innervate Merkel Cells, Hair Follicles, and Meissner Corpuscles, Mechanosensory Organs in the Skin

To clarify the identity of NF200+ DRG neurons that express DOR, we next analyzed the morphology of the peripheral terminals of DORGFP-expressing DRG neurons using a whole-mount cleared skin preparation.

We focused on three distinct skin structures that are major contributors to cutaneous mechanosensation: Merkel cells, Meissner corpuscles, and hair follicles. We first immunostained hairy skin of the back of DORGFP mice with an anti-

body against cytokeratin 8 (CK8) and observed the characteristic distribution of Merkel cells in touch domes associated with guard hairs. Furthermore, as illustrated in Figures 5A, 5C, and S4A, we found that the majority of Merkel cells are innervated by DORGFP+ NF200+ axons that terminate as flattened disks surrounding the base of Merkel cells. This anatomical organization is characteristic of the Merkel cell-neurite complex and establishes that DOR is expressed by slowly adapting I (SAI) A β LTMRs that detect light skin indentation.

We next examined cutaneous mechanosensory organs innervated by rapidly adapting (RA) A β LTMRs, which include Meissner corpuscle afferents, and hair follicle longitudinal lanceolate endings. Recent studies demonstrated that Ret expression in large-diameter DRG neurons early during development marks these RA A β LTMRs (Bourane et al., 2009; Luo et al., 2009). Consistent with our demonstration of Ret and DORGFP coexpression in large-diameter DRG neurons (Figure 2), we found that some DORGFP+ NF200+ axons innervate Meissner

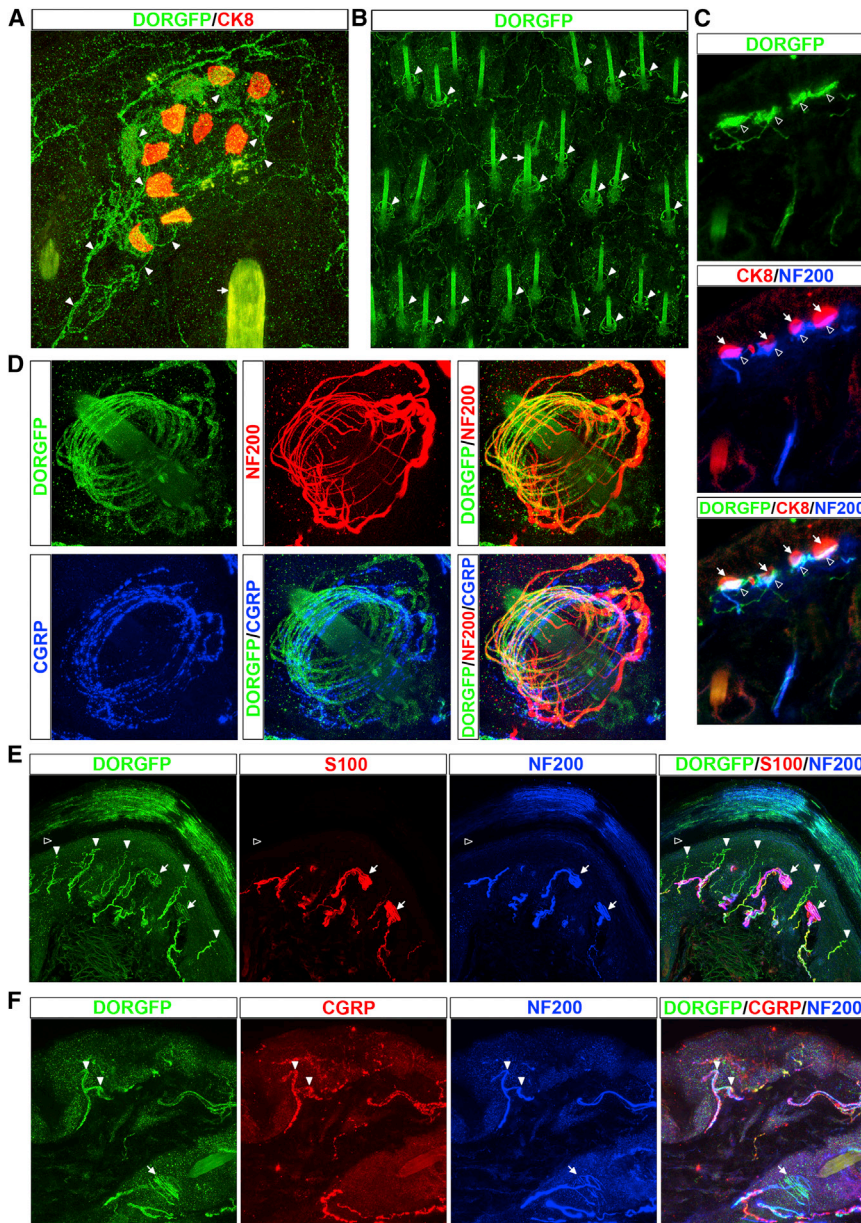


Figure 5. The Terminals of DORGFP-Expressing DRG Neurons Form Touch-Detecting Mechanosensory Organs in the Skin

(A) Peripheral axons of DORGFP+ DRG neurons (green, arrowheads) innervate cytokeratin 8 (CK8)-immunoreactive Merkel cells grouped in touch domes associated with guard hairs (autofluorescent hair shaft, white arrow). (B) DORGFP+ terminals form circumferential, but not longitudinal, endings (arrowheads) around follicles of most pelage hairs, including guard hairs (arrow, center of the image). (C) DORGFP+ axons end as NF200+ flattened disks (arrowheads) surrounding the base of Merkel cells (arrows). (D) DORGFP+ axons that form circumferential endings around follicles are myelinated (NF200+) and are distinct from CGRP+ terminals. Innervation of a guard hair is shown. (E) DORGFP+ NF200+ axons form Meissner corpuscles in the dermal papillae of the mouse footpad. The Meissner corpuscles are localized by immunoreactivity of the glial protein S100 (arrows). DORGFP+ NF200- axons penetrate further into the epidermis (white arrowheads), consistent with their nonpeptidergic MrgprD+ identity. Black arrowhead indicates the boundary between stratum corneum (autofluorescent dead cells) and stratum granulosum. (F) Myelinated DORGFP+ afferents with epidermal-free nerve endings often coexpress CGRP (arrowheads). Circumferential endings around the follicle of the small caliber hairs (arrow) express DORGFP and NF200, but not CGRP. Autofluorescence generates the fluorescence of the hair shafts (yellow in A, green in B and D).

corpuscles, visualized with S100 glial protein immunostaining in dermal papillae of the footpad (Figure 5E).

We then analyzed the innervation of hair follicles by DORGFP+ afferents. Strikingly, DORGFP+ NF200+ axons formed circumferential endings around follicles of the great majority of guard and smaller pelage hairs but very rarely longitudinal lanceolate endings (Figures 5B, 5D, and S4A–S4C). The physiological correlate of circumferential endings is still unclear; however, their association with hair follicles suggests a function in mechanosensation. Because DORGFP+ terminals that innervate hair follicles are distinct from CGRP+ circumferential endings (Figures 5D, 5F, and S4D), we propose that the DORGFP+ TrkC+ DRG neurons, which often coexpress Ret, form circumferential endings and that DOR represents a

marker of this poorly characterized population of DRG neurons.

Finally, we observed that numerous DORGFP+ axons terminate as free nerve endings in the skin, a morphology that is characteristic of nociceptors (Figures 5F and S4E). Based on the typical morphology of the DORGFP+ NF200- axons that extend to the stratum granulosum (Zylka et al., 2005), we suggest that these correspond to the peripheral terminals of DORGFP+ MrgprD+ C mechanoreceptors (Figures 5E, S4D, and S4E). We presume that the NF200+ DORGFP+ CGRP+ free nerve endings arise from the population of TrkA+ DORGFP+ myelinated nociceptors that coexpress MOR (Figures 5F and S4E).

DORGFP+ Myelinated Afferents Respond to Innocuous and Noxious Mechanical Stimuli

Our neuroanatomical analysis demonstrating that Meissner corpuscle and Merkel cell afferents express DORGFP suggested that these DORGFP+ DRG neurons are Aβ LTMRs. However, neither the physiological properties nor the mechanosensitivity of other NF200+ DORGFP+ DRG neurons forming

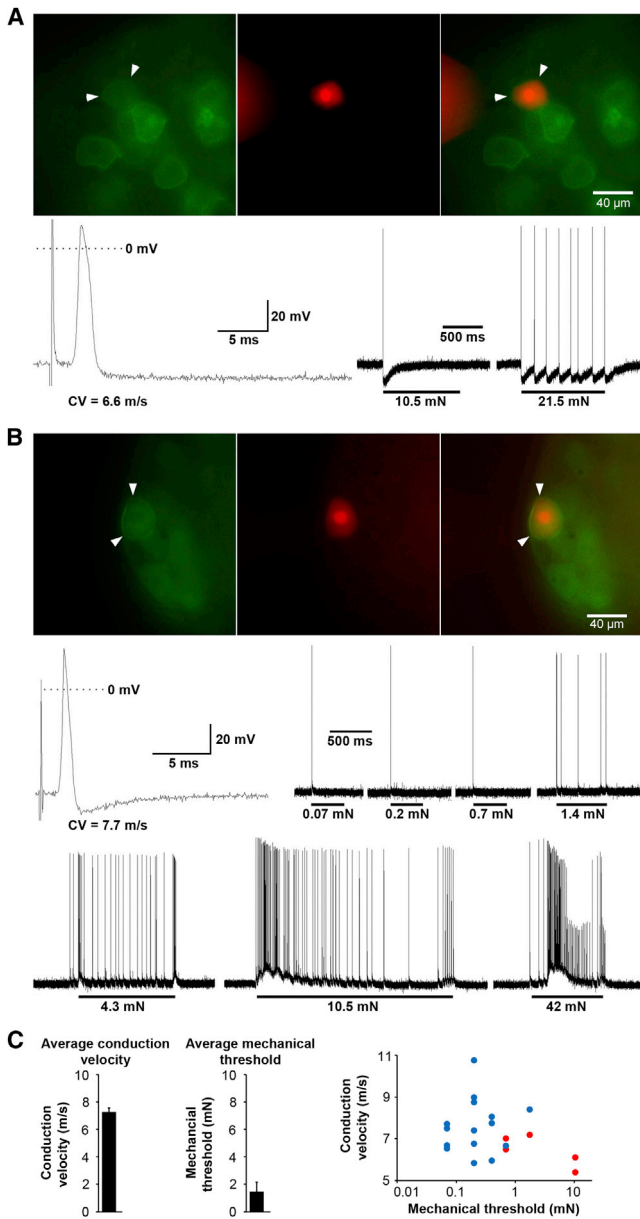


Figure 6. DORGF-Expressing DRG Neurons Are Mechanosensitive and Include High- and Low-Threshold A δ Mechanoreceptors

(A) The conduction velocity (CV, 6.6 m/s), broad action potential (bottom left), high mechanical threshold (10.5 mN, bottom right), and activity-induced hyperpolarization of the DORGF+ neuron (green; red: neurobiotin) are characteristics of high-threshold A δ mechanoreceptors.

(B) In comparison to (A), the DORGF+ A δ fiber recorded exhibits a relatively narrow action potential and low mechanical threshold (0.07 mN, similar to LTMRs), indicating that this myelinated nociceptor might also participate in touch sensation.

(C) Conduction velocity (7.3 m/s \pm 0.3 m/s) and mechanical threshold (1.5 mN \pm 0.7 mN) of 20 DORGF+ DRG neurons show that most neurons that project to the trunk skin are low-threshold A δ mechanoreceptors (see example in B). These cells (blue symbols in right panel) generally have large receptive fields composed of multiple isolated spots, while the other, comparatively high-threshold, population of DORGF+ mechanoreceptors (red; see example in A) tends to have smaller receptive fields composed of a single spot.

Data are represented as means \pm SEM.

circumferential or free nerve endings are established. To address this question, we used a largely intact somatosensory system preparation and characterized 20 randomly selected large-diameter DORGF+ DRG neurons. These neurons had conduction velocities (CVs) between 5.4 and 10.7 m/s and relatively broad somal action potentials, with inflections on the falling phase of the spike, a property of A δ nociceptors (Koerber and Woodbury, 2002; Lawson, 2002) (Figure 6). Importantly, all 20 of these DORGF+ neurons responded to mechanical stimulation. We further tested nine of these 20 cells for heat sensitivity and found that, in contrast, only one cell responded to noxious heat and did so weakly. DORGF+ A δ fibers are thus AM mechanoreceptors and probably do not contribute to thermosensation. Furthermore, the DORGF+ A δ nociceptors were not uniform, but rather consisted of at least two distinct populations. One population included cells with broad somal spikes, high mechanical threshold (including cells responding only to noxious stimuli >10 mN), single spot-like small receptive fields (RFs), and pronounced long-lasting activity-induced hyperpolarization (Figures 6A and 6C). These features are characteristic of myelinated high-threshold mechanoreceptors that project to lamina I-II and V of the dorsal horn (Woodbury and Koerber, 2003).

The second, larger population of DORGF+ A δ nociceptors comprised cells with relatively low mechanical thresholds, from 0.07 to 0.7 mN, narrower action potentials, and large RFs composed of multiple isolated spots (Figures 6B and 6C). These afferents probably correspond to a population of myelinated mechanoreceptors that project widely from spinal laminae I to V (Woodbury and Koerber, 2003). Thus, while the latter DORGF+ cells encode stimulus intensity and fire vigorously in response to noxious mechanical stimuli, which defines them as nociceptors, these afferents also respond to light touch and innocuous mechanical force (<5 mN), project to the deep dorsal horn, and thus might participate in both touch and pain sensation.

Note that a few DORGF+ neurons had A β CVs and very narrow, uninflected spikes indicating that they were putative low-threshold mechanoreceptors. We could not identify and characterize the physiological properties of these DORGF+ A β fibers because their axons did not travel out of the intact dorsal cutaneous nerves and instead were cut during preparation (see Supplemental Experimental Procedures), but we presume that they may correspond to DORGF+ SAI or hair follicle afferents (Figure 5).

Presynaptic DORs Depress Glutamate Release from Myelinated Mechanoreceptors onto Laminae III–V Neurons of the Spinal Dorsal Horn

The dominant action of opioid receptors in the nervous system is to depress neurotransmission by Gi/o protein $\beta\gamma$ subunit-mediated modulation of ion channel conductances (Williams et al., 2001). In sensory neurons, MOR agonists inhibit voltage-gated calcium channels (VGCCs) to reduce C fiber-derived glutamatergic neurotransmission to laminae I–II spinal neurons (Heinke et al., 2011; Taddese et al., 1995), consistent with MOR expression in peptidergic C nociceptors that project to superficial laminae of the dorsal horn. We reasoned that DOR function in myelinated mechanoreceptors might parallel that characteristic

action of MOR on C nociceptors. To test this hypothesis, we examined the effect of the DOR-selective agonist deltorphin II on glutamatergic neurotransmission between A fibers and spinal neurons in laminae III–V. Indeed the myelinated afferents that express DORGFP, including SAI A β afferents, RA Meissner corpuscles A β afferents (Luo et al., 2009), and myelinated mechanoreceptors (Woodbury and Koerber, 2003), project to this area of the spinal cord dorsal horn.

We used spinal cord slices from wild-type mice to test the presence of DOR at the terminals of myelinated mechanoreceptors. We first recorded miniature excitatory postsynaptic currents (mEPSCs), reflecting spontaneous release of glutamate from sensory neurons and excitatory interneurons, and found that deltorphin II (500 nM) altered neither the amplitude nor the frequency of mEPSCs (Figure 7A). We therefore took a different approach and recorded EPSCs evoked by stimulation of the dorsal root at an intensity that triggers glutamate release from A β fibers. We used a paired-pulse protocol to examine deltorphin II's presynaptic effects on monosynaptic eEPSCs. We observed that deltorphin II significantly reduced the peak amplitude of the first eEPSC by \sim 56% in 10 of the 17 recorded cells (i.e., 10 responsive neurons in which deltorphin II reduced peak amplitude by at least 10%; Figures 7B–7D, 7G, and 7H). This effect was reversible with wash (Figures 7B and 7C). The ratio of the peak amplitude of the second eEPSC over the first is generally related to the probability of neurotransmitter release. We found that deltorphin II significantly increased the paired-pulse ratio in responsive cells (\sim 32%; Figures 7D, 7G, and 7I), consistent with deltorphin II reducing glutamate release from A β fibers. To test for a possible contribution of DOR expressed by the postsynaptic neuron under study, we interrupted G protein activation in the recorded cells by adding GDP- β S to the pipette solution. We again observed a deltorphin II-induced decrease in first eEPSC peak amplitude and an increase in paired-pulse ratio in a similar proportion of spinal neurons (8 of the 14 recorded cells), indicating that deltorphin II's effect does not require postsynaptic activation of G proteins (Figures 7E and 7G–7I). Importantly, application of the selective DOR antagonist naltrindole blocked the deltorphin II effect. Thus, in the presence of naltrindole, deltorphin II altered neither the first peak amplitude nor the paired-pulse ratio in any of the 9 recorded cells (Figures 7F–7I), while naltrindole alone had no effect (Figure S5). Taken together, our findings support the conclusion that a presynaptic activation of DORs on the central terminals of A β fibers underlies deltorphin II's action and that DOR functions as a regulator of innocuous mechanical input to the spinal cord.

DOR Activation Inhibits VGCCs in Myelinated Mechanoreceptors

We next investigated the molecular mechanism by which DOR modulates glutamate release from myelinated mechanoreceptors using DRG primary culture from DORGFP mice. Figures 8A and S6 show that the neurochemistry of DORGFP+-cultured cells corresponds to what we observed in DRG sections: most large-diameter DORGFP+ neurons coexpressed NF200 and TrkC (Figure 8A), and the great majority of small-diameter NF200– DORGFP+ neurons bound IB4 (Figure S6).

To determine whether DOR activation by deltorphin II affects VGCC function in myelinated mechanoreceptors, we recorded voltage-activated calcium currents in large-diameter DORGFP+ neurons (Figure 8B). Figures 8C–8E illustrate that deltorphin II (500 nM) strongly inhibited VGCCs in all DORGFP+ cells recorded, with peak amplitude decreased by \sim 38% compared to control. Furthermore, current rise time was significantly increased by deltorphin II (\sim 116% of control) (Figures 8C–8E). In DORGFP-negative large-diameter neurons, deltorphin II had no effect on current amplitude or rise time. We next used a DRG primary culture from wild-type mice to test whether deltorphin II also inhibits VGCCs in large-diameter neurons in this preparation. We labeled cultured neurons with IB4-biotin and streptavidin-Alexa Fluor 555 and recorded deltorphin II effects in a total of 43 wild-type DRG neurons (Figure 8F). Deltorphin II (500 nM) inhibited VGCCs in 2 of 10 large-diameter DRG neurons, in 2 of 23 IB4+ small-diameter DRG neurons, and in 1 of 11 IB4– small-diameter DRG neurons (Figures 8G and 8H). When a high-resistance seal could be maintained, we applied the MOR agonist DAMGO (500 nM) after deltorphin II and wash. DAMGO inhibited VGCCs in 4 of 7 IB4– small-diameter DRG neurons, in 5 of 17 IB4+ small-diameter DRG neurons, and in none of the 5 large-diameter DRG neurons tested (Figures 8G and 8H). Thus, small-diameter neurons frequently responded to DAMGO (9/24, 38%) but rarely to deltorphin II (3/34, 9%). This distinction was even more pronounced when considering specifically the IB4– subpopulation that comprises peptidergic C nociceptors. Deltorphin II-responding neurons represent a discrete population of DRG neurons, most of which have large-diameter cell bodies, consistent with the idea that DOR regulates neurotransmitter release from myelinated mechanoreceptors. Only 1 cell out of 29, an IB4– small-diameter DRG neuron, responded to both drugs.

Taken together, our histological and electrophysiological results in DORGFP and wild-type mice support a model of the functional organization of the opioid system in primary afferent sensory neurons in which DOR and MOR engage common molecular mechanisms to regulate neurotransmitter release from functionally distinct primary afferent DRG neurons. The product of this segregated opioid receptor organization is a modality-specific regulation of somatosensation.

DISCUSSION

DOR Expression Pattern in DRG and CNS

Central to the understanding of how opioids influence somatosensation is an analysis of the distribution profile of opioid receptors in DRG neurons. Here we localized DOR in wild-type mouse tissues using *in situ* hybridization, radioligand binding, calcium current inhibition in DRG neurons, and inhibition of neurotransmitter release by A β fibers in spinal cord slices. The results clearly indicate that, as we proposed based on the analysis of DORGFP knockin mice (Scherrer et al., 2009), DOR is predominantly expressed by large-diameter myelinated DRG neurons, as well as by a subpopulation of IB4-binding nonpeptidergic unmyelinated nociceptors, but very rarely by SP+ unmyelinated nociceptors. Importantly, our conclusions are consistent with previous studies performed in wild-type mice and rats that

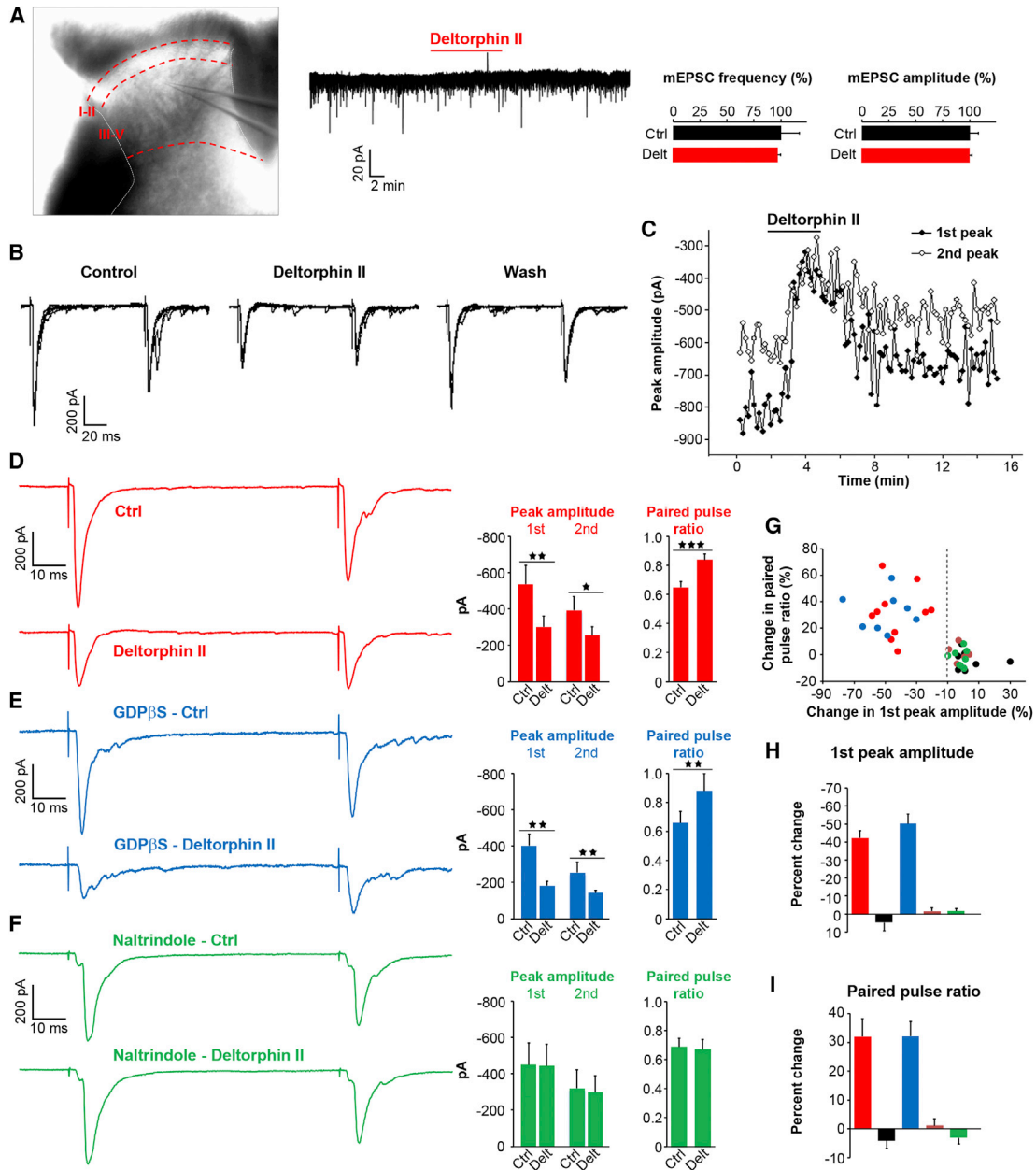


Figure 7. Presynaptic DORs Depress Aβ Fiber Input to Laminae III–V Spinal Neurons

(A) Voltage-clamp recordings of laminae III–V neurons in spinal cord slices from wild-type mice show that the DOR agonist deltorphin II (500 nM) did not significantly change miniature EPSC (mEPSC) frequency or amplitude (n = 15).

(B) Example of EPSCs evoked in a lamina III spinal neuron by stimulation of monosynaptically connected Aβ fibers using a paired-pulse protocol. Deltorphin II (500 nM) depressed EPSC peak amplitude and altered the ratio between the amplitudes of the second and first peaks.

(C) Time course of the deltorphin II inhibitory effect on EPSC peak amplitudes.

(D) Deltorphin II (500 nM) significantly reduced the first peak amplitude and increased the paired-pulse ratio in 10 of the 17 recorded cells (responsive neurons).

(E) GDPβS (1 mM in internal solution) did not block deltorphin II's effect (8/14 recorded neurons with GDPβS responded to deltorphin II, a proportion similar to D), which confirms a presynaptic mechanism of action.

(F) The DOR antagonist naltrindole (1 μM) prevented deltorphin II's effect in all recorded neurons (n = 9).

(G) Summary showing all recorded cells. Red, deltorphin II responsive neurons; black, nonresponsive neurons; blue, deltorphin II responsive neurons with GDPβS; brown, nonresponsive neurons with GDPβS; green, cells recorded in the presence of naltrindole. The dotted line represents the threshold value (–10%) of percent change of first peak amplitude at which cells were considered responsive.

(H) Average deltorphin II effect on first peak amplitude. Color code as in (G).

(I) Average deltorphin II effect on paired-pulse ratio. Color code as in (G).

Data are represented as means ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

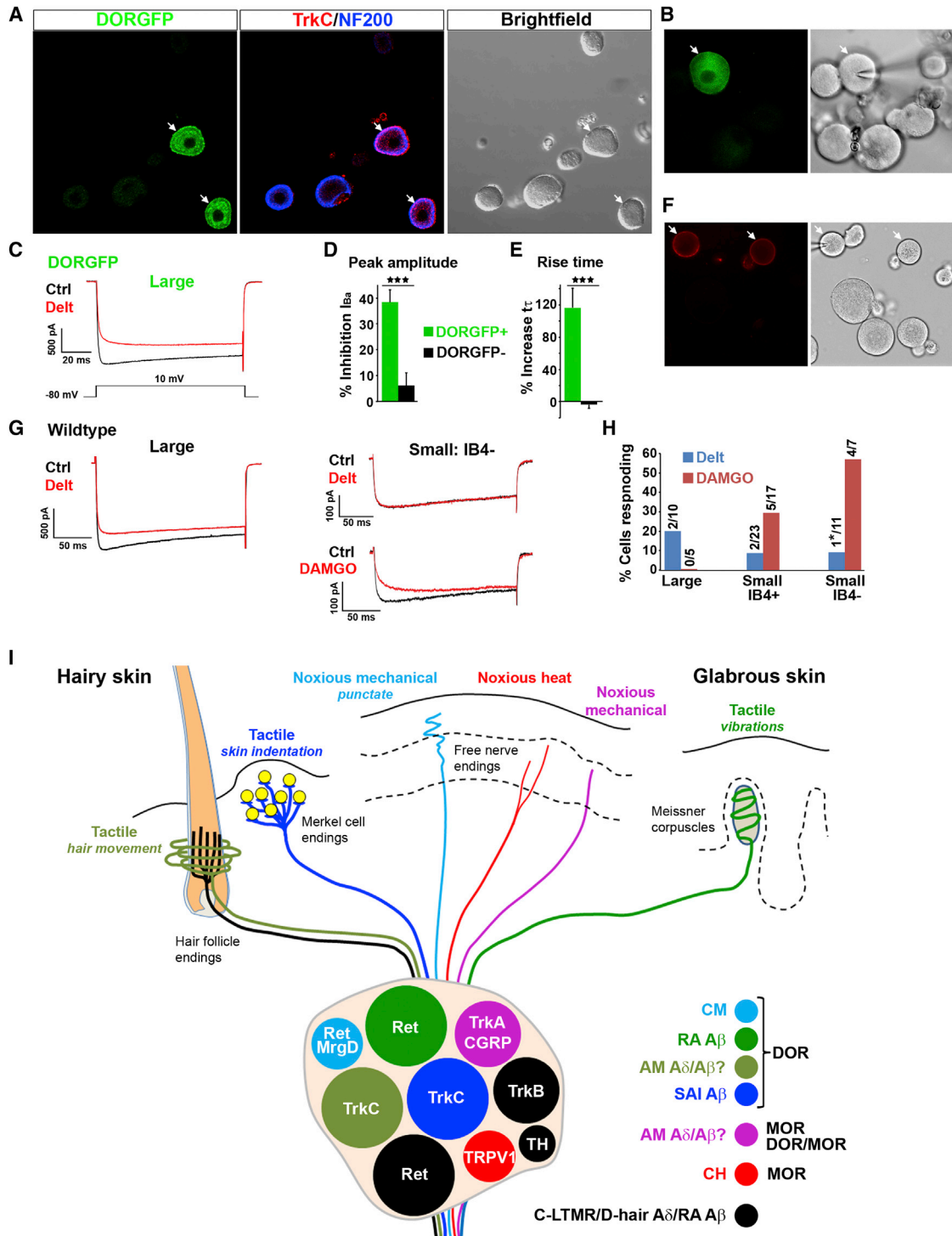


Figure 8. DOR-Mediated Inhibition of Voltage-Gated Calcium Channels in Cultured Large-Diameter DRG Neurons and Functional Organization of DOR and MOR in Cutaneous Somatosensory Neurons

(A) Large-diameter NF200+ DORGFP+ cells express TrkC in primary DRG culture (arrows).
 (B) DOR-expressing large-diameter DRG neurons (brightfield image at right) are identified by direct visualization of DORGFP fluorescence (arrow, left image).
 (C) Deltorphin II (500 nM) reduces amplitude and increases rise time of voltage-activated calcium currents in DORGFP+ DRG neurons, but not in DORGFP- DRG neurons.
 (D) Average effect of deltorphin II on current amplitude in DORGFP+ (n = 11) and DORGFP- large-diameter neurons (n = 7).

(legend continued on next page)

reported that “intense DOR mRNA hybridization was primarily observed over large ganglion cells immunopositive for neurofilament 200” (Mennicken et al., 2003) and that “among the neurons which highly expressed mu-, delta- or kappa-OPR mRNA, PPTA mRNA was not expressed in about 58%, 95%, or 24% of those neurons, respectively” (Minami et al., 1995).

On the other hand conclusions derived from use of the anti-DOR³⁻¹⁷ antibody disagree with these earlier findings, and with ours. In those studies, strong DOR³⁻¹⁷-ir is found in small-diameter DRG neurons and colocalized with SP (Bao et al., 2003; Guan et al., 2005). The mismatch is even more striking in the spinal cord, where DOR³⁻¹⁷-ir was found to be restricted to the terminals of peptidergic afferents in laminae I–II. By contrast, DOR ligand binding in wild-type mice, as well as DORGFP fluorescence, are observed throughout the spinal gray matter, consistent with in situ hybridization and electrophysiological recordings in spinal cord from rats that established the existence of DOR-expressing spinal neurons (Cahill et al., 2001; Eckert et al., 2001; Mennicken et al., 2003). Because DOR³⁻¹⁷-ir is intact in two strains of *Oprd1* knockout mice, we proposed that anti-DOR³⁻¹⁷ antibody recognizes a different molecule and misrepresents DOR distribution (Scherrer et al., 2009). This statement applies to several other commercial antibodies that we have evaluated (Scherrer et al., 2009), including an anti-DOR²⁻¹⁸ antibody (Wang et al., 2010) that recognizes an unidentified surface molecule expressed mostly in large-diameter, Runx3+ DORGFP- DRG neurons (Figure S7). To explain the discrepancy between the DOR expression pattern obtained with DORGFP mice and with the anti-DOR³⁻¹⁷ antibody, Wang et al. proposed that the GFP tag altered subcellular localization and trafficking of the receptor in DORGFP mouse. Note that here DORGFP knockin mice were used to identify DOR-expressing DRG neurons, not to study receptor distribution inside the cell. Because a variety of experimental conditions differ between DORGFP expression in the reporter mouse and the heterologous overexpression of distinct DOR-GFP fusion constructs in in vitro systems, it is unclear that differences in trafficking reported by Wang et al. are relevant to the DORGFP receptor in vivo. In fact, the agreement between the DORGFP fluorescence pattern and the DOR radioligand binding pattern in wild-type mice, both of which notably arise from labeling of receptors trafficked to

cellular compartments such as dendrites and axon terminals, together with the intact antinociceptive and prolocomotive properties of DOR agonists in DORGFP mice (Pradhan et al., 2009; Scherrer et al., 2006), strongly argues that the DORGFP traffics and functions as does the native DOR.

Surprisingly, in situ hybridization and electrophysiological experiments performed by Wang et al. also suggested that DOR was expressed by the majority of small-diameter neurons, including peptidergic C nociceptors (Wang et al., 2010). It is possible that inadequate in situ hybridization probes and/or excessive probe concentration or reaction time led to nonspecific labeling of DOR-negative DRG neurons. As for the electrophysiological experiments, seemingly excessive concentrations of DOR agonists (10 μM of deltorphin II or SNC80) were used for ligands with K_D in the nanomolar range. At these high concentrations, off-target effects cannot be excluded, in particular cross-activation of MOR by these ligands, a finding already documented using *Oprd1* and *Oprm1* knockout mice (Gendron et al., 2007; Scherrer et al., 2004). Note also that Wang et al. omitted to consider the possibility that convergent inputs from distinct DOR+ and MOR+ fibers can modulate neurotransmitter release onto a given spinal neuron, without requiring coexpression of the two receptors in the same primary afferent neurons. Similarly, data on the inhibition of spinal SP release by intrathecal DOR agonists (for example, Beaudry et al., 2011; Kouček et al., 2013) should be interpreted carefully, because both SP and DOR are expressed, and possibly coexpressed, by numerous spinal neurons, thus preventing definitive conclusions to be drawn regarding DOR and SP coexpression in DRG neurons. By contrast, CGRP is restricted to primary afferent terminals in the dorsal horn, and our data showing DOR and CGRP coexpression in large-diameter NF200+ DRG neurons, and DOR-mediated inhibition of VGCCs in these cells, are consistent with the idea that DOR agonists can reduce CGRP release (Overland et al., 2009) and further suggest this effect results mostly from an action on myelinated nociceptors.

Functional Organization of Opioid Receptors in Primary Afferent Somatosensory Neurons

We showed here that most DORGFP+ NF200+ DRG neurons coexpress TrkC and/or Ret, which mark several categories of

(E) Average effect of deltorphin II on current rise time in DORGFP+ (n = 11) and DORGFP– large-diameter neurons (n = 7). Results are expressed as mean ± SEM, ***p < 0.001.

(F) Labeling of IB4+ neurons in DRG culture from a wild-type mouse.

(G) In the DRG culture shown in (F), deltorphin II (500 nM) predominantly inhibited VGCCs in large-diameter neurons (>40 pF), while DAMGO (500 nM) predominantly inhibited VGCCs in small-diameter (<40 pF) IB4- neurons.

(H) Occurrence of deltorphin II- and DAMGO-mediated inhibition of VGCCs in 44 randomly selected neurons in DRG cultures from wild-type mice. Only one cell out of 29 tested for deltorphin II- and DAMGO-responsiveness (*) showed VGCC inhibition by both drugs.

(I) Schematic of the functional organization of DOR and MOR in cutaneous somatosensory neurons. DOR is expressed by populations of TrkC+ and/or Ret+ myelinated mechanoreceptors, which include SAI and RA Aβ LTMRs that form the Merkel cell-neurite complex (yellow) and Meissner corpuscles, respectively. DOR is also expressed by myelinated mechanonociceptors (AM), some of which are Aδ fibers, have low threshold, and respond to innocuous mechanical stimuli. The axons derived from DOR+ TrkC+ AM Aδ mechanonociceptors, which often coexpress Ret, may form circumferential endings around hair follicle, although this remains speculative. Additionally, DOR is expressed by Ret+ MrgprD+ C mechanonociceptors (CM) that innervate epidermal stratum granulosum. By contrast, MOR is predominantly expressed by capsaicin- and heat-sensitive TRPV1+ C nociceptors (CH) (many of which are visceral, not cutaneous, afferents) and regulates heat pain perception. Colocalization of DOR and MOR occurs in myelinated TrkA- and CGRP-expressing neurons, which are presumptive high-threshold Aδ or Aβ mechanonociceptors. Most TH+ C LTMRs, TrkB+ D-hair mechanoreceptors, and Ret+ Aβ LTMRs that form longitudinal endings around hair follicles do not appear to express DOR or MOR.

Data are represented as means ± SEM.

myelinated mechanoreceptors (Bourane et al., 2009; Fünfschilling et al., 2004; Luo et al., 2009; Senzaki et al., 2010) (Figure 8I). In retrograde labeling experiments, we found that many myelinated DORGFP+ neurons project to the dorsal column nuclei. Analysis of sensory neuron terminals in the skin demonstrated that DORGFP+ DRG neurons innervate hair follicles, Merkel cells, and Meissner corpuscles, forming mechanosensory organs essential for touch. Recordings from DORGFP+ DRG neurons further showed that DOR is expressed by myelinated mechanonociceptors, including some with mechanical thresholds in the innocuous range. Electrophysiological experiments in spinal cord slices also established that DOR is present on the central terminals of A β fibers. Altogether, these data indicate that DOR+ DRG neurons are mechanoreceptors and include populations of low-threshold afferents that generate touch sensation (Figure 8I).

In sharp contrast with DOR+ DRG neurons, we found that the majority of MOR+ DRG neurons display molecular and anatomical features characteristic of NGF-dependent peptidergic nociceptors (lack of expression of Ret, TrkC, and TrkB; no projection to the DCN; coexpression of CGRP and TRPV1). Our analysis in DRG agrees with the fact that MOR agonists reduce synaptic transmission between C and A δ nociceptors and superficial dorsal horn neurons (Heinke et al., 2011). Furthermore, morphine alters neither responses of gracile nucleus neurons evoked by A β fiber stimulation or brushing of the receptive field (Suzuki and Dickenson, 2002), touch-evoked Fos expression in the dorsal horn (Catheline et al., 2001), nor dynamic allodynia induced by strychnine-mediated disinhibition of dorsal horn circuits (Mirau-court et al., 2009). We propose, therefore, that MOR regulates neurotransmitter release from cutaneous nociceptive peptidergic TRPV1+ afferents that are heat sensitive and mechanically insensitive (Lawson et al., 2008) and, perhaps of particular clinical relevance, from TRPV1+ visceral afferents that signal injuries to deep tissues (e.g., postsurgical pain) (Figure 8I). Note, however, that MOR+ NF200+ DRG neurons might include some A β nociceptive fibers (Lawson, 2002) or A β LTMRs that do not project to the DCN. In our electrophysiological experiments in cultured DRG neurons from wild-type mouse, we found that DAMGO had no effect on VGCCs in the five large-diameter DRG neurons tested (Figure 8). This result is consistent with the idea that MOR is expressed by a relatively small population of NF200+ neurons (about 20%) (Figures 3 and 4) and suggests that blind recordings from many more DRG neurons would be necessary to determine precisely the proportion of myelinated neurons that express MOR. Note also that the DRG culture might not be fully representative of the neuronal diversity and proportions found in vivo because some neurons might be more sensitive than others to the dissociation procedure. Such factors might explain the subtle differences between electrophysiological results in DRG culture and those from our histological analysis.

Since DOR inhibits VGCCs in large-diameter NF200+ neurons, as does MOR in C fibers, our results suggest that in DRG the specific function of DOR and MOR in the control of somatosensation originates mostly from expression in distinct neuronal populations. Of course this arrangement does not exclude the existence of additional cell- or receptor-specific signaling mechanisms, such as those that regulate DOR surface expression and

functional competence (Hack et al., 2005; Patwardhan et al., 2005; van Rijn et al., 2012). Note that it is possible to isolate the main action of intrathecal DOR and MOR agonists against mechanical and heat pain, respectively, when using low doses of SNC80 and DAMGO (Scherrer et al., 2009). On the other hand, it is well established that DOR and MOR agonists can reduce both mechanical and heat pain (reviewed in Gavériaux-Ruff and Kieffer, 2011; Ossipov et al., 2004), especially when higher doses are used. Future studies will clarify whether this antinociceptive action across pain modalities results from the corecruitment of rarer populations of afferents (i.e., heat-sensitive DOR+ and mechanosensitive MOR+), polymodal spinal and descending control systems, or the combined activation of multiple opioid receptor types, possibly including multimers.

It is significant that we have identified discrete populations of neurons that do coexpress both receptors. First, as we reported previously (Scherrer et al., 2009), about 10% of DOR+ NF200–small-diameter DRG neurons are TRPV1+ peptidergic C nociceptors, all of which express MOR. Second, we now establish that the vast majority of DOR/MOR-coexpressing DRG neurons are in fact DRG neurons with myelinated axons (NF200+) that express CGRP and TrkA. Analogous to the MrgprA3-expressing DRG cells that only represent about 4% of DRG neurons but are entirely responsible for chloroquine-induced pruritus (Liu et al., 2009), the DOR/MOR-coexpressing cells probably have a significant and selective function in somatosensation despite their small number (<5% of all DRG neurons). Because CGRP+ cutaneous A fibers are mostly high-threshold mechanonociceptors (AM class; Koerber and Woodbury, 2002; Lawson et al., 2008), our results suggest that studies examining the functional relevance of interactions between DOR and MOR should include behavioral assays of A δ or A β fiber-mediated mechanical pain. Our model (Figure 8I) thus proposes that opportunities for DOR/MOR interaction in DRG, including DOR/MOR heterodimerization, is probably restricted to discrete cell populations, with the corollary that agents modulating these interactions may be useful to treat pain disorders resulting from dysfunction of these particular neurons, with limited side effects.

DOR-Mediated Control of Cutaneous Mechanosensation

Numerous studies have demonstrated that DOR-selective agonists reduce mechanical hypersensitivity induced by nerve or tissue injury in rodents (Gavériaux-Ruff and Kieffer, 2011). Because selective activation of DOR has limited utility against acute heat pain, these results were considered to reflect the prime function of DOR in the control of chronic, but not acute, pain. In light of accumulating evidence supporting the existence of peripheral labeled lines for distinct pain modalities (Basbaum et al., 2009), we rather propose that it is the preferential association of DOR with myelinated mechanonociceptors and LTMRs that underlies the particular analgesic profile of DOR agonists against acute mechanical pain and chronic mechanical hypersensitivity. A previous study using mice with a conditional deletion of *Oprd1* in DRG neurons expressing the sodium channel Nav1.8 suggested that DOR+ unmyelinated nociceptors, rather than NF200+ neurons, were essential for the anti-allodynic effect of DOR agonists (Gavériaux-Ruff et al., 2011). However, a fate map analysis has

since demonstrated that Cre expression in DRG from Nav1.8-Cre mice is, in fact, not restricted to nociceptors but rather is also observed in several categories of C and A LTMRs (Shields et al., 2012). As a result, the precise population of DRG neurons that underlies DOR-mediated anti-allodynia remains to be identified.

Our demonstration that DOR+ NF200- DRG neurons belong to the MrgprD+ subset of nonpeptidergic, IB4-binding, Ret+ C nociceptors (Figures S3E and 8I) agrees with a recent quantitative PCR study that showed that Ret is essential for the normal expression of *Oprd1* in the DRG (Franck et al., 2011) and the observation that a DOR agonist inhibits GDNF-induced hyperalgesia (Joseph and Levine, 2010). MrgprD+ DRG neurons respond to punctate noxious mechanical stimulation and are essential for acute mechanical pain, but not persistent injury-induced mechanical hypersensitivity (Cavanaugh et al., 2009). Conceivably, these DOR+ C nociceptors underlie the effect of DOR agonists on basal mechanical thresholds but are unlikely to mediate their anti-allodynic effects in models of inflammatory or neuropathic pain.

By contrast, DOR+ myelinated mechanoreceptors emerge as strong candidate neurons to signal mechanical allodynia. First, sensory testing in neuropathic pain patients indicates that large-diameter myelinated fibers, not C fibers, are essential for mechanical allodynia (Campbell et al., 1988). Second, the sensory modalities encoded by DOR+ myelinated mechanoreceptors precisely correspond to the innocuous stimuli that evoke pain in allodynic patients. Thus, movement of objects across the skin or light pressure that typically generate pain (dynamic or static allodynia, respectively), such as is caused by clothing, are expected to engage DOR+ hair follicle, Meissner corpuscle, and/or SAI afferents (Figure 8I). These DOR+ afferents could fire more vigorously in response to light touch after injury, causing pain. Alternatively, their normal response to innocuous mechanical stimulation could cause pain after injury if central disinhibition mechanisms are in effect. These two scenarios are not mutually exclusive, and future studies will investigate the mechanisms by which DORs present on the peripheral terminals of mechanoreceptors may regulate threshold and firing pattern to contribute to opioid analgesia (Stein and Machelska, 2011).

In summary, our results provide a conceptual framework for the functional organization of opioid receptors in primary afferent neurons and the inhibitory mechanisms by which opioids control mechanosensation. This model provides a cellular basis for a more rational use of MOR agonists in the clinic and will foster the design of alternative analgesic strategies, including DOR targeting, based on the identity of the primary afferents engaged and the nature of the stimulus causing pain, particularly for touch-evoked neuropathic pain and movement/pressure-evoked pain, two major clinical problems.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for further details.

Animals

All procedures were approved by the Stanford, Columbia, or UCSF Institutional Animal Care and Use Committees. See Supplemental Experimental Procedures for a description of the mice used.

Spinal Cord Slice Preparation and Electrophysiology

The lumbar spinal cord of postnatal days 15–22 (P15–P22) C57BL/6 mice was isolated, and transverse slices with dorsal roots attached were made using a vibrating microtome. Patch-clamp recording in whole-cell configuration and voltage-clamp mode at a holding potential of -70 mV was performed at room temperature (RT) on laminae III–V neurons. EPSCs were evoked (eEPSCs) by stimulating the dorsal root with a suction electrode (25 to 50 μ A intensity, 0.1 ms duration). eEPSCs with constant latency and no failures during a train of 20 stimuli at 50 Hz were considered monosynaptic responses mediated by A β fibers. Paired-pulse recordings were obtained by stimulating the dorsal root continuously at 0.1 Hz.

Primary DRG Culture and Electrophysiology

Lumbar and thoracic DRGs from P21–P42 DORGFP or C57BL/6 wild-type mice were collected, digested, centrifuged, resuspended in MEM complete medium, and plated onto Poly-D,L-ornithine hydrobromide-coated coverslips. Patch-clamp recording in whole-cell configuration was performed 2 to 6 hr after plating. Cells were classified as small or large based on capacitance (small < 40 pF < large). Recordings were performed in voltage-clamp mode with -80 mV holding potential and a 100 or 200 ms depolarization to 10 mV was used to activate VGCCs before and after perfusion of deltorphin II (500 nM) or DAMGO (500 nM). Current amplitude and rise time (20%–80%) were determined using Clampfit software.

Histology

Immunostaining was performed as described previously (Li et al., 2011; Scherrer et al., 2009). Briefly, mice were anesthetized and perfused transcardially with PBS followed by 10% formalin solution. Lumbar DRGs were dissected and cryoprotected in sucrose solution. Skin was dissected, postfixed in 10% formalin, cryoprotected, and cut at 20 μ m or processed for whole-mount staining. Tissue sections were incubated in blocking solution for 1 hr at RT, primary antibody overnight at 4°C, and secondary antibody for 2 hr at RT. Whole-mount staining was performed as described by Li et al. (2011). Images were acquired with a confocal microscope. Methods for in situ hybridization are described in detail in Supplemental Experimental Procedures. Radioligand binding experiments have been performed as previously described (Scherrer et al., 2009).

Largely Intact Somatosensory System Preparation and Electrophysiology

This preparation has been described previously (Koerber and Woodbury, 2002). Briefly, mice were anesthetized and perfused transcardially with oxygenated artificial cerebrospinal fluid (aCSF). The spinal cord, thoracic DRGs, dorsal cutaneous nerve, and trunk skin were dissected in continuity and pinned out in a recording chamber. DORGFP+ somata were impaled with micropipettes, and electrical search stimuli were delivered to the intact dorsal cutaneous nerve through an en passant suction electrode to identify cells with intact peripheral axons. Peripheral RFs were located with a fine sable paint brush or blunt glass stylus, and mechanical thresholds were determined with calibrated von Frey filaments. Thermal sensitivity was tested by applying heated (52°C) aCSF to the RF. Conduction velocity was calculated from spike latency and the distance between stimulating and recording electrodes.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2014.01.044>.

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