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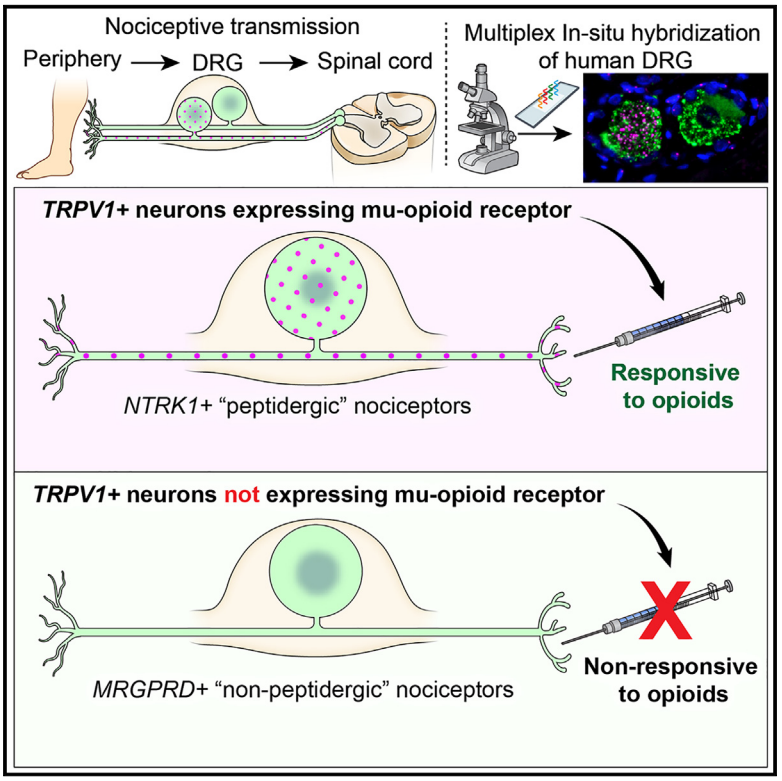
The μ-opioid receptor differentiates two distinct human nociceptive populations relevant to clinical pain

Graphical abstract



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In brief

Staedtler et al. describe a dichotomy of human nociceptors into *OPRM1*-expressing neurons that share molecular features with rodent peptidergic neurons associated with tissue damage pain and *OPRM1*-negative neurons that mostly resemble murine non-peptidergic neurons expressing the superficial skin marker *MRGPRD*. This division provides a cellular-molecular framework for human pain control.

Highlights

- *OPRM1* expression in the human DRG distinguishes two broad nociceptive populations
- *OPRM1*-positive nociceptors show molecular resemblance to rodent peptidergic neurons
- Most *OPRM1*-negative nociceptors express the murine superficial skin marker *MRGPRD*
- The κ-opioid receptor gene *OPRK1* is mainly expressed in satellite glial cells

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The μ-opioid receptor differentiates two distinct human nociceptive populations relevant to clinical pain

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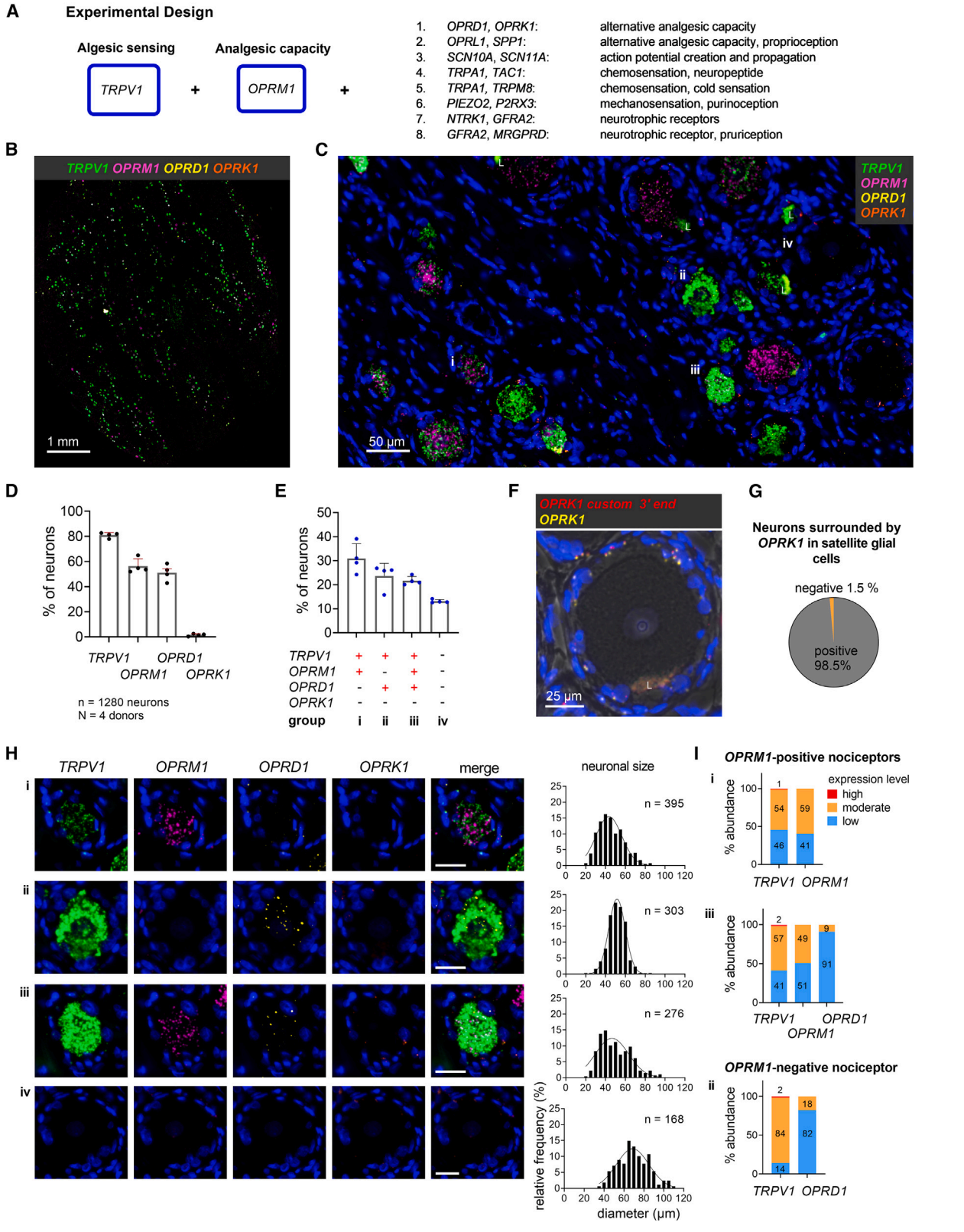
SUMMARY

The shortfall in new analgesic agents is a major impediment to reducing reliance on opioid medications for control of severe pain. In both animals and man, attenuating nociceptive transmission from primary afferent neurons with a μ-opioid receptor agonist yields highly effective analgesia. Consequently, deeper molecular characterization of human nociceptive afferents expressing *OPRM1*, the μ-opioid receptor gene, is a key component for advancing analgesic drug discovery and understanding clinical pain control. A co-expression matrix for the μ-opioid receptor and a variety of nociceptive channels as well as δ- and κ-opioid receptors is established by multiplex *in situ* hybridization. Our results indicate an *OPRM1*-positive population with strong molecular resemblance to rodent peptidergic C-nociceptors associated with tissue damage pain and an *OPRM1*-negative population sharing molecular characteristics of murine non-peptidergic C-nociceptors. The empirical identification of two distinct human nociceptive populations that differ profoundly in their presumed responsiveness to opioids provides an actionable translational framework for human pain control.

INTRODUCTION

Opioids acting at the μ-opioid receptor are mainstays of clinical management of severe tissue damage pain.[1–3] Their adverse side effect profile and the risk for addiction, however, impose limits on clinical use and drive the search for alternative analgesic targets.[4–7] A crucial element of opioid analgesia is the inhibition of transmission from nociceptive primary afferent neurons to second-order neurons in the dorsal spinal cord,[8–10] making these afferents critical targets for analgesic drug development. Understanding, identifying, and molecularly distinguishing the most relevant "pain control neuron" are essential steps for focusing analgesic drug development efforts. The idea that a clinically relevant opioid receptor-expressing population is present in the dorsal root ganglion (DRG) is supported by human experimental pain studies that model clinically relevant pain. These models frequently apply *sustained* experimental noxious stimulation to skin and deep tissues, and significant pain reduction can be achieved by systemic opioids in response to variety of exogenous stimuli including noxious heat, cold, pressure, pinch, and ischemia (Tables S1–S5).[11–14] The variety of stimuli suggests that, in humans, μ-opioid receptors are expressed by heterogeneous and/or multimodal nociceptive afferent populations.

Clinically relevant sustained pain from tissue damage is transmitted mainly by unmyelinated C-fibers,[15,16] supporting the idea that C-nociceptors are the major targets of μ-receptor agonists. Based on rodent studies, C-nociceptors have been divided into two major populations, with only one of them having the capacity to transmit sustained pain from tissue damage.[17–21] This population has been classically termed "peptidergic" nociceptors due to their production of algogenic peptides such as CGRP (calcitonin gene-related peptide) and substance P. They also express the heat- and inflammation-activated ion channel TRPV1 (transient receptor potential vanilloid receptor 1), the μ-opioid receptor, and the neurotrophic receptor TrkA (tropomyosin receptor kinase A)[17,22–25] and innervate both skin and deep tissues.[23,26–28] By contrast, the second murine population, termed "non-peptidergic" C-nociceptors, express low levels of -neuropeptides and TRPV1, the δ-opioid receptor, and the neurotrophic receptor GFRA2.[19,21,29–31] The most prevalent non-peptidergic population NP1 is marked by the expression of the itch-related receptor MRGPRD (Mas-related G-protein-coupled receptor D)[21] and innervates exclusively the murine superficial epidermis.[32] The functional relevance of this division is supported by mouse optogenetic studies that demonstrate guarding behaviors, which are indicative of a sustained pain-like experience, upon stimulation of peptidergic neurons. By



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contrast, stimulation of non-peptidergic *MRGPRD*+ neurons causes reflexive paw withdrawal[33,34] consistent with a proposal that these neurons form a "biowarning" system that mediates spinal reflex withdrawal prior to tissue damage.[35,36] Importantly, these neurons also contribute to pathological pain states such as neuropathic pain.[37–39]

Sequencing studies of human somatosensory afferent transcriptomes have revealed several nociceptive clusters that mostly follow organizational principles of murine DRG neurons, yet a precise delineation into the aforementioned main populations, including an unambiguous expression of low-expressed G-protein-coupled receptors, such as opioid receptors or MRGPRD, has not been achieved.[40–42] Observations in humans report a high degree of responsiveness to opioids in cases of severe sustained pain, but minimal responsiveness to opioids to short-lasting, threshold-level pain,[1] and reduced responsiveness to neuropathic pain (Table S6).[43–45] This suggests that the basic division of nociceptors is also functionally true in humans. Thus, the aim of the present investigation is to identify the population of DRG nociceptive neurons with the greatest relevance to clinical pain control. Specifically, we hypothesize that this population of human C-fiber neurons is represented by neurons that express the nociresponsive ion channel TRPV1 in conjunction with the μ-opioid receptor. Therefore, this population is sensitive to both opioid agonists and a variety of nociceptive stimuli, making it relevant to tissue damage pain and opioid analgesia. For the empirical identification of this population, we designed a comprehensive set of gene probes for multiplex fluorescence *in situ* hybridization. This investigation of human nociceptors provides insight into analgesic target validation which is a crucial component for achieving successful translation. Specifically, confirming the expression of putative analgesic targets in the most relevant nociceptive population expressing *TRPV1* and *OPRM1* is required for peripherally driven analgesia.

RESULTS

We investigated human DRG neurons from four tissue donors for the expression of *TRPV1* and *OPRM1*. Data from a variety of probe pairs were integrated to obtain a comprehensive picture of the expression of potential analgesic targets (Figure 1A). If we include all experiments and all neurons into the counting analysis, 56.3% ± 2.1% of neurons were characterized as *TRPV1*+*OPRM1*+ (Figure S3). We identified a second population of TRPV1+ and OPRM1-negative neurons. Both populations express multiple algesic markers and neurotrophic receptors that provisionally characterize them as nociceptive. A third prominent population of large-diameter neurons did not express any of the algesic markers and was classified as non-nociceptive. These definitions based on transcription can be further substantiated by functional investigations. Additionally, according to their neuronal diameters, 88.7% of *TRPV1*+*OPRM1*+ nociceptors could be classified as small- to medium-diameter neurons (see STAR Methods, Figure S7), which is consistent with a nociceptive population.

OPRM1-positive and *OPRM1*-negative human nociceptors express *OPRD1* while *OPRK1* is expressed in satellite glial cells

Both the δ- and κ-opioid receptors (encoded by *OPRD1* and *OPRK1*, respectively) represent potential alternative analgesic targets due to inhibitory effects on neurotransmitter release at synapses in the dorsal horn.[2] Whether they are expressed by *TRPV1*+*OPRM1*+ nociceptors associated with rodent sustained pain had not been elucidated. We evaluated pooled data of 4 tissue donors (n = 1,280 neurons). *TRPV1* was expressed in 81% ± 2.1%, *OPRM1* in 56.3% ± 5.9%, *OPRD1* in 51.1% ± 6.5%, and *OPRK1* in 1.6% ± 1% of human DRG neurons (Figure 1D). The abundance of neurons expressing *TRPV1* in the human DRG is shown in the whole DRG section (Figure 1B). When considering the co-expression patterns of all four markers, we observed four prevalent populations (Figures 1E; Table S7), which we characterized for cell size and expression levels of transcripts. Two of them were *TRPV1*+*OPRM1*+ nociceptive populations, one was a *TRPV1*+*OPRM1*-negative nociceptive population, and one a non-nociceptive population. The most abundant *TRPV1*+*OPRM1*+ population (labeled i, detected in 30.9% ± 6.2% of the analyzed neurons) did not express transcripts for any additional opioid receptor subtype, while population iii (21.7% ± 1.8%) expressed *OPRD1* in addition to *TRPV1* and *OPRM1*. The *OPRM1*-negative population (ii) showed positivity for

Figure 1. *OPRM1*-positive and *OPRM1*-negative human nociceptors express *OPRD1* while *OPRK1* is expressed in satellite glial cells

(A) Overall schematic of experimental design for 4-Plex *in situ* hybridization studies. The major nociceptive ion channel TRPV1 is paired with the major analgesic receptor (μ-opioid, *OPRM1*) and a series of genes coding for algesic and analgesic mediators.

(B) Scanned image of a complete section from human L3 DRG hybridized for the heat- and inflammation-activated channel TRPV1 (green), the μ-opioid (*OPRM1*) (magenta), δ-opioid (*OPRD1*) (yellow), and κ-opioid receptor (*OPRK1*) (orange). Note the strong expression and high prevalence of neuronal *TRPV1* expression which tends to obscure the signal from the other genes at this magnification.

(C) Enlargement showing the multiple neuronal signals. Representative neurons are labeled i–iv and are characterized further in (H).

(D) Percentage of 1,280 DRG neurons expressing each individual transcript. Note the comparatively low neuronal expression of *OPRK1*.

(E) Percentage of DRG neurons expressing the most common transcript combinations, which defines populations i–iv. Bar graphs in (D) and (E) show mean, standard deviation (SD), and individual values from four independent tissue donors.

(F) Single-neuron example demonstrating the expression of *OPRK1* in satellite glial cells surrounding the neuron, as detected by the standard probe (yellow) and, as a technical replicate, the custom probe (red). The large fluorescent patch, "L," is lipofuscin. See also Figures S4 and S5.

(G) The preponderance of neurons that are surrounded by *OPRK1* (κ-opioid receptor) expressing satellite cells.

(H) Individual channel and multi-channel microscopy images of representative neurons for each population (i–iv, as in C) and the corresponding populations' cell size distribution. Scale bar, 25 μm.

(I) Percentages of nociceptors showing low, medium, or high expression levels for *TRPV1* and each opioid receptor transcript averaged across the 4 tissue donors.

TRPV1 and *OPRD1* (23.6% ± 5.3%). A presumably non-nociceptive population did not express any of the four transcripts (iv, 13.1% ± 0.7%). These four main populations represented 89.3% ± 1.5% of sampled neurons. Microscopic images of a representative neuron of each of the four major populations and the cell diameter distributions of each population are shown in Figure 1H. *TRPV1*+*OPRM1*+ (i) and *TRPV1*+*OPRM1*+*OPRD1*+ (iii) populations consisted of a heterogeneous group of mostly small- and medium-diameter neurons ($\bar{x}$ = 45.8 ± 12.7 μm [i], $\bar{x}$ = 50 ± 15.6 μm [iii]). In contrast, *OPRM1*-negative nociceptors were medium sized with a uniform, homogeneous cell size distribution ($\bar{x}$ = 51.2 ± 8.7 μm). Neurons that did not express any marker were medium to large in size ($\bar{x}$ = 69.7 ± 15.4 μm). In order to evaluate the potential of the δ-opioid receptor as a pharmaceutical target for pain relief, including the potential for μ-δ-heterodimers,[46] we evaluated the expression level of each transcript in a given population. For this aim, we determined thresholds for each marker in each donor section for low, moderate, and high expression levels. While *OPRM1* was expressed similarly both in a low and in a moderate fashion, *OPRD1* showed mostly low expression levels, especially in population iii (91%) (Figure 1I). To summarize, the gene encoding the δ-opioid receptor was expressed at low levels in a subpopulation of the relevant *TRPV1*+*OPRM1*+ population.

The κ-opioid receptor gene is mainly expressed in satellite glial cells

Transcripts for *OPRK1* within sensory neurons were a scarce observation (1.6% of sampled neurons, Figures 1D and S4). Instead, we observed ubiquitous expression of *OPRK1* in non-neuronal cells, mostly in subpopulations of satellite glial cells (SGCs) surrounding somatosensory neurons (Figures 1C, 1H, and S5). This was not an expected finding based on our previous investigations in rat[47] and the existing literature.[48–50] To validate our result, we designed a second probe against *OPRK1* targeting a different region of the transcript (see STAR Methods section). Co-staining with both probes showed overlapping or closely juxtaposed puncta (Figures 1F and S5). The quantitative results reported in this manuscript are based on the custom-made *OPRK1* probe. We quantified that 98.5% ± 0.9% of all characterized neurons (*n* = 1280) showed *OPRK1* transcripts in surrounding SGCs, indicating *OPRK1* is likely a ubiquitous transcript in SGCs (Figure 1G). These data indicate that *OPRK1* is primarily a non-neuronal receptor in the human DRG.

OPRL1 is expressed by proprioceptors and a subpopulation of *OPRM1*-positive nociceptors

The nociceptin opioid-like receptor (encoded by *OPRL1*) is a receptor with a wide anatomic distribution in the body, peripheral nervous system (PNS), and CNS that can support a broad spectrum of behavioral and physiological actions.[51,52] We previously demonstrated its expression in rat nociceptive and proprioceptive primary afferent neurons.[47] Its expression by nociceptive afferents relevant for human pain has not been evaluated. We analyzed human DRGs co-labeled for *TRPV1*, *OPRM1*, *OPRL1*, and the proprioceptive marker osteopontin (*SPP1*).[21,53] We analyzed 1,277 neurons and observed *TRPV1* in 87.5% ± 2.6%, *OPRM1* in 58.8% ± 3.1%, *OPRL1* in 48.9% ± 6.3%, and *SPP1* in 15.6% ± 3.1% of neurons (Figure 2B). Analysis of co-expression patterns of all transcripts indicated four prevalent populations (Figure 2C; Table S8) that were representative of 89.6% ± 3.02% of sampled neurons. These included two *TRPV1*+*OPRM1*+ nociceptive populations, a *TRPV1*+*OPRM1*-negative nociceptive population, and a non-nociceptive population. The largest population consisted of *TRPV1*+*OPRM1*+ *OPRL1*+ nociceptors (i, 31% ± 6.7%), which showed a broad cell size distribution ($\bar{x}$ = 51.8 ± 12.9 μm). The second group (ii, 25.1% ± 6.6%) consisted of small-diameter *TRPV1*+*OPRM1*+ neurons ($\bar{x}$ = 39.6 ± 10.2 μm) that did not express *OPRL1*. *OPRM1*-negative *TRPV1*+ neurons (iii, 23.8% ± 2.1%) did not express *OPRL1* and were characterized by a homogeneous medium-sized cell diameter distribution ($\bar{x}$ = 50.2% ± 8.5) as described before. A relatively small population expressed both *SPP1* and *OPRL1* (iv, 9.7% ± 1.5%) and consisted of medium- to large-diameter neurons ($\bar{x}$ = 73.5 ± 15.5 μm) (Figure 2D), indicating that the majority of the previously identified non-nociceptive population expresses both *SPP1* and *OPRL1*. Characterization of *OPRL1* expression levels revealed low levels in 95% of the *TRPV1*+*OPRM1*+*OPRL1*+ population (i). The low *OPRL1* expression was in contrast to *OPRM1* expression levels, which could be classified as moderate in 57% of the same population (Figure 2E). To summarize, *OPRL1* was expressed at low expression levels only in a subpopulation of the relevant *TRPV1*+*OPRM1*+ population.

The genes encoding $Na_V1.8$ and $Na_V1.9$ show different expression levels in *OPRM1*-positive and *OPRM1*-negative nociceptors

Voltage-gated sodium channels (VGSCs) play a crucial role in nociception as they are essential for the initiation and conduction of action potentials from peripheral to central nerve terminals.[54,55] The isoforms $Na_V1.8$ (*SCN10A*) and $Na_V1.9$ (*SCN11A*) are preferentially expressed in human nociceptive afferents.[40–42,56] In 1,310 analyzed neurons, we found *TRPV1* to be the most expressed of the four markers (87.7% ± 3.3%). More than half of the neurons expressed *OPRM1* (57.8% ± 4.1%), consistent with results of earlier probe sets. *SCN10A* and *SCN11A* were also expressed by a majority of DRG neurons (83.3% ± 3.5% and 85.5% ± 3.4%, respectively) (Figure 3B). Analysis of co-expression patterns of all four markers revealed three prevalent populations (Figure 3C; Table S9), two nociceptive and a non-nociceptive population. These three populations represented 93.2% ± 1.5% of sampled neurons. A representative cell of each of the three most common neuronal populations with the cell size distribution of that population is shown in Figure 3E. The most abundant population (i) was *TRPV1*+ *OPRM1*+ nociceptors that expressed transcripts for both VGSCs (53.6% ± 3.5%). This group contained a wide distribution of cell sizes consisting of mostly small- and medium-diameter neurons ($\bar{x}$ = 50 ± 13 μm), (Figure 3D). The second population (ii) consisted of *OPRM1*-negative *TRPV1*+ nociceptors that expressed both VGSCs (28.4% ± 0.9%) and showed a homogeneous cell size distribution ($\bar{x}$ = 52.5 ± 8.8 μm) as described before. VGSCs showed different expression levels between the two nociceptive populations. *SCN10A* ($Na_V1.8$) was more highly expressed in the *OPRM1*-positive population (median intensity

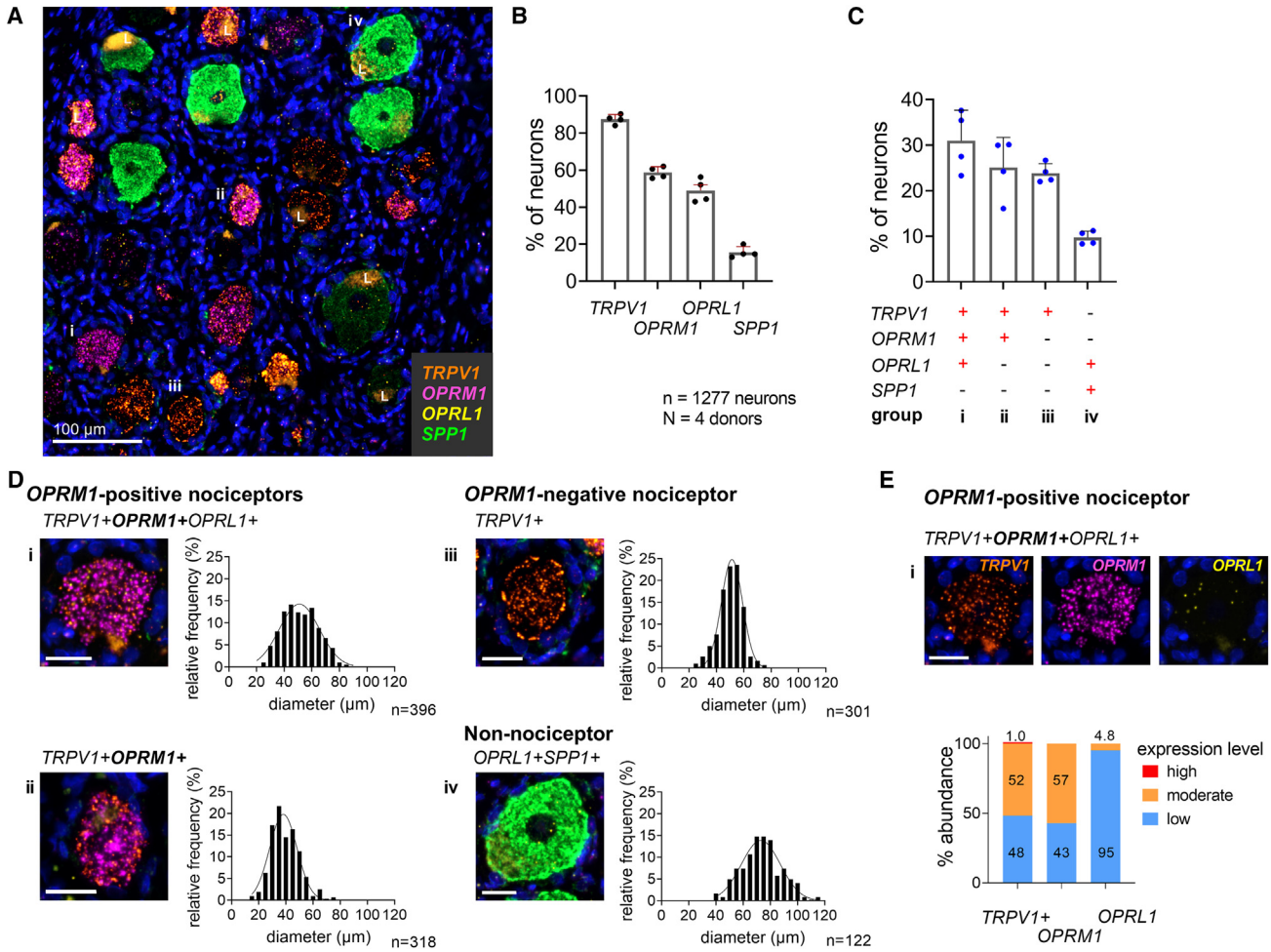


Figure 2. *OPRL1* is expressed by proprioceptors and a subpopulation of *OPRM1*-positive nociceptors

(A) Representative section of human DRG showing positive transcripts for TRPV1, the μ-opioid receptor (*OPRM1*), the opioid-related nociceptin receptor 1 (*OPRL1*), and osteopontin (*SPP1*), a marker for proprioceptive neurons. Lipofuscin is marked with an "L."

(B) Percentage of somatosensory neurons expressing each individual transcript.

(C) Percentage of 1,277 neurons expressing the most prevalent transcript combinations. Bar graphs in (B) and (C) show mean, SD, and individual values from four independent donors.

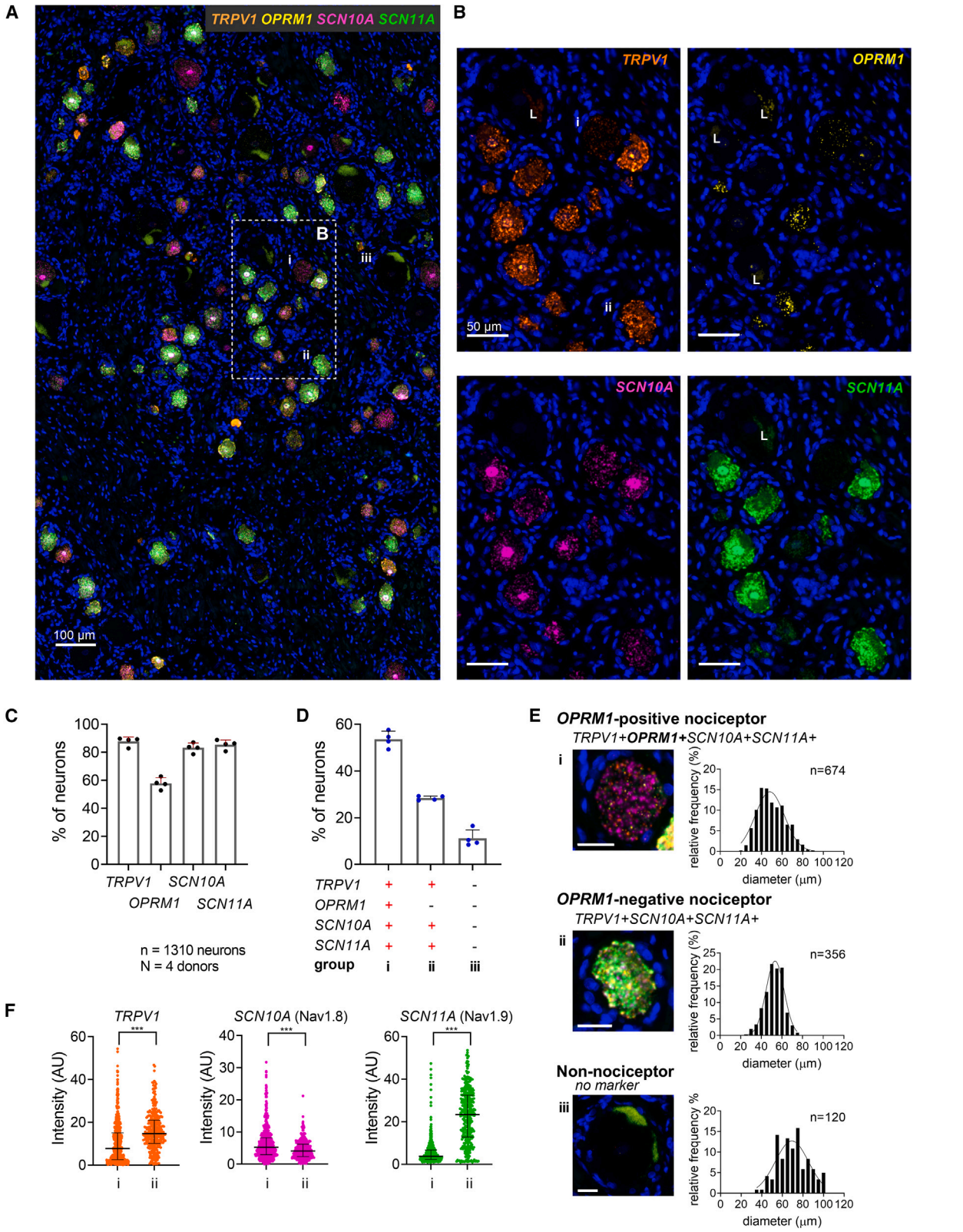
(D) Multi-channel microscopy images of a representative individual neuron from each population and the population's cell size distribution. *OPRL1* is expressed at a low level in the neurons illustrated in i and iv. The typical *OPRL1* hybridization signal can be seen in (E).

(E) Single-channel images of neuron shown in (Di). Categorized expression levels for each transcript of the *TRPV1+OPRM1+OPRL1+* population averaged across 4 independent tissue donors. Scale bars in (D) and (E) represent 25 μm.

5.3 arbitrary units [a.u.] versus median intensity 4.1 a.u.) (Figure 3F), while *SCN11A* ($Na_V1.9$) exhibited higher expression in the *OPRM1*-negative population (median intensity 23.4 a.u. versus median intensity 3.8 a.u.). This population also demonstrated a higher expression of *TRPV1* (median intensity 14.8 a.u. versus median intensity 7.8 a.u.). All differences were significant (Mann-Whitney U test, $p < 0.001$, respectively, after Bonferroni correction). The third population (iii, 11.2% ± 3.6%) expressed none of the four markers and consisted of medium-/large-diameter neurons ($\bar{x}$ = 70.4 ± 14.8 μm) (Figure 3E). Our results demonstrate that the genes encoding $Na_V1.8$ and $Na_V1.9$ are co-expressed in nociceptive neurons and that $Na_V1.8$ transcripts are enriched in the *OPRM1*-expressing population.

TAC1 (substance P) is selectively expressed in *OPRM1*-positive nociceptors

Substance P (encoded by *TAC1*) is a neuropeptide and a marker for peptidergic nociceptors transmitting sustained pain in rodents.[33,34,57] This peptide modulates nociceptive responsiveness of second-order spinal cord neurons,[58,59] especially during intense noxious stimulation[60] and can include activation of both TRPV1 and TRPA1.[61] To investigate the expression of these genes in human nociceptors, we analyzed DRG sections for expression levels of *TRPV1*, *OPRM1*, *TRPA1*, and *TAC1.* We analyzed 1,316 neurons and observed *TRPV1* in 83.3% ± 4.1%, *OPRM1* in 61.5% ± 4.6%, *TRPA1* in 37.2% ± 4.8%, and *TAC1* in 31.2% ± 6.7% of the analyzed neurons (Figure 4B). When we considered the



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co-expression patterns of all four markers, we detected six prevalent populations: three *TRPV1*+*OPRM1*+, two *TRPV1*+*OPRM1*-negative nociceptive, and one non-nociceptive population (Figure 4C; Table S10). These six populations represented 89.7% ± 2.8% of the analyzed neurons. *TRPV1*+*OPRM1*+ neurons that did not express *TRPA1* nor *TAC1* were the most common population (i, 22.9% ± 7.5%). They showed a broad cell size distribution of mostly small-/medium-sized neurons ($\bar{x}$ = 53.2 ± 14.5 μm). Within the *TRPV1*+*OPRM1*+ populations, two expressed *TAC1*: a small-diameter ($\bar{x}$ = 34.6 ± 7.6 μm) population that also expressed *TRPA1* (ii, 16.3% ± 4.0%) and a small-/medium-diameter population ($\bar{x}$ = 49.4 ± 10.3 μm) that did not express *TRPA1* (v, 13.4% ± 5.4%). We observed significantly higher expression levels for *TAC1* and *TRPV1* in the *TRPV1*+*OPRM1*+*TRPA1*+*TAC1*+ (i) population than in the *TRPV1*+*OPRM1*+*TAC1*+ (v) population (median intensity for *TRPV1* 8.9 a.u. versus 4.1 a.u., for *TAC1* 50.4 a.u. vs. 20 a.u., $p < 0.001$, Mann-Whitney U test; see Figure 4F). *OPRM1*-negative populations were characterized by expression of *TRPV1* and *TRPA1* (iii, 14.1% ± 1.6%) or only *TRPV1* (vi, 9.1% ± 1.4%). These neurons were medium sized with a homogeneous cell size distribution (47.2 ± 7.8 μm [iv], $\bar{x}$ = 50.7 ± 8.7 μm [vi]) as described before. Non-nociceptive neurons expressed none of the four markers (iv, 14.0% ± 3.7%) and had medium/large cell sizes ($\bar{x}$ = 66.4 ± 13.7 μm) (Figures 4C and 4E). In terms of nociception, the *OPRM1*+*TRPV1*+*TRPA1*+*TAC1*+ neurons are a subpopulation of the aforementioned analyzed *TRPV1*+*OPRM1*+*SCN10A*+*SCN11A*+ population and are likely associated with sustained tissue damage pain.

OPRM1-positive nociceptors express *TRPM8*

Agonists of the μ-opioid receptor are known to inhibit cold pain induced by sustained stimulation,[62–64] implicating expression of cold-sensitive channels in *OPRM1*-expressing nociceptors. The transient receptor potential cation channel subfamily M (melastatin) member 8 (encoded by *TRPM8*) is activated by compounds such as menthol, mediates cold sensations into the noxious range, and is implicated in cold allodynia.[65,66] *TRPA1* has been reported to be expressed in human cold-sensing neurons,[42] and we examine the colocalization of these two transcripts in this experiment. We analyzed 1,310 DRG neurons for the expression of *TRPV1*, *OPRM1*, *TRPA1*, and *TRPM8*. We detected *TRPV1* in 82.3% ± 4.4%, *OPRM1* in 58.3% ± 7.3%, *TRPA1* in 44.2% ± 4.6%, and *TRPM8* in 39.7% ± 8.0% of neurons (Figure S6B). When we considered the co-expression patterns of all four markers, six prevalent populations were detected (Figure S6C; Table S11), of which three were *TRPV1*+*OPRM1*+ nociceptive, two *TRPV1*+*OPRM1*-negative nociceptive, and one non-nociceptive population. Neurons of these six populations represented 86.9% ± 3.2% of the analyzed neurons. The three *TRPV1*+*OPRM1*+ populations consisted of neurons that also co-expressed *TRPA1* and *TRPM8* (i, 20.1% ± 4.9%), only *TRPM8* (iii, 15.5% ± 3.3%), or neither *TRPA1* nor *TRPM8* (v, 12.2% ± 3.1%). The *TRPV1*+*OPRM1*+*TRPA1*+*TRPM8*+ population consisted of small-diameter neurons ($\bar{x}$ = 39.8 ± 9.2 μm), while the two latter populations showed a broad cell size distribution including mostly small- and medium-diameter neurons ($\bar{x}$ = 57.0 ± 12.7 μm [iii], $\bar{x}$ = 54.9 ± 13.9 μm [v]). *OPRM1*-negative nociceptors were either *TRPV1*+*TRPA1*+ (ii) (16.8% ± 2.6%) or only *TRPV1*+ (vi, 9.8% ± 4%). These two prevalent *OPRM1*-negative nociceptive populations did not express *TRPM8*. Both groups consisted of medium-sized neurons with homogeneous cell size distributions ($\bar{x}$ = 53.2 ± 9.5 μm [ii], $\bar{x}$ = 52.5 ± 9.6 μm [vi]) as described in previous paragraphs. Neurons expressing none of the four markers (iv, 12.5% ± 2.4%) were medium- to large-diameter neurons (71.3 ± 14 μm) (Figure S6D). Though we observed a high degree of co-expression of *TRPV1*, *TRPA1*, and *TRPM8*, pairwise analysis of linear correlations between those markers in a pooled sample of all *TRPV1*+/*OPRM1*+/*TRPA1*+/*TRPM8*+ neurons expressing these markers revealed mostly anticorrelated gene expression of *TRPV1* and *TRPM8* and *TRPM8* and *TRPA1*, respectively (Figure S6E). A subset of neurons showed significant expression of *TRPV1* and *TRPM8*, indicating potential sensitivity to both heat and cold (Figure S6E). Expression levels of *TRPV1* and *TRPA1* showed a more complex relationship with a subpopulation of neurons showing high expression levels for both transcripts. Our data demonstrate mostly anticorrelated expression of genes coding for heat- and cold-sensing receptors in *TRPV1*+*OPRM1*+ nociceptors, which indicates primarily distinct sensory encoding of noxious heat and cold. Our anatomic evidence supports that *TRPM8* is expressed in the *TRPV1*+*OPRM1*+ population.

Expression levels of *P2RX3* differ between *OPRM1*-positive and *OPRM1*-negative nociceptors

To address the polymodality of human nociceptors including mechanosensation and sensing of indicators of tissue damage such as ATP, we performed an *in situ* experiment including probes for transcripts of *PIEZO2* and *P2RX3* (encoding P2X3). PIEZO2 in the somatosensory system plays an essential role in sensing gentle touch, tactile pain, and proprioception.[67–69] We detected transcripts for *TRPV1* in 80.4% ± 3.3%, *OPRM1* in 57.6% ± 4.1%, *PIEZO2* in 75.4% ± 2.9%, and *P2RX3* in 77.3% ± 3.3% of the analyzed neurons (n = 1,264 neurons) (Figure 5B). All molecular markers showed a high degree of co-expression. With this

Figure 3. The genes encoding $Na_V1.8$ and $Na_V1.9$ show different expression levels in *OPRM1*-positive and *OPRM1*-negative nociceptors

(A) Representative section of human DRG showing neurons positive for TRPV1, the μ-opioid receptor (*OPRM1*), and voltage-gated sodium channels $Na_V1.8$ (*SCN10A*) and $Na_V1.9$ (*SCN11A*) transcripts. Representative neurons characterized further in (E) and (F) are labeled with small Roman numerals.

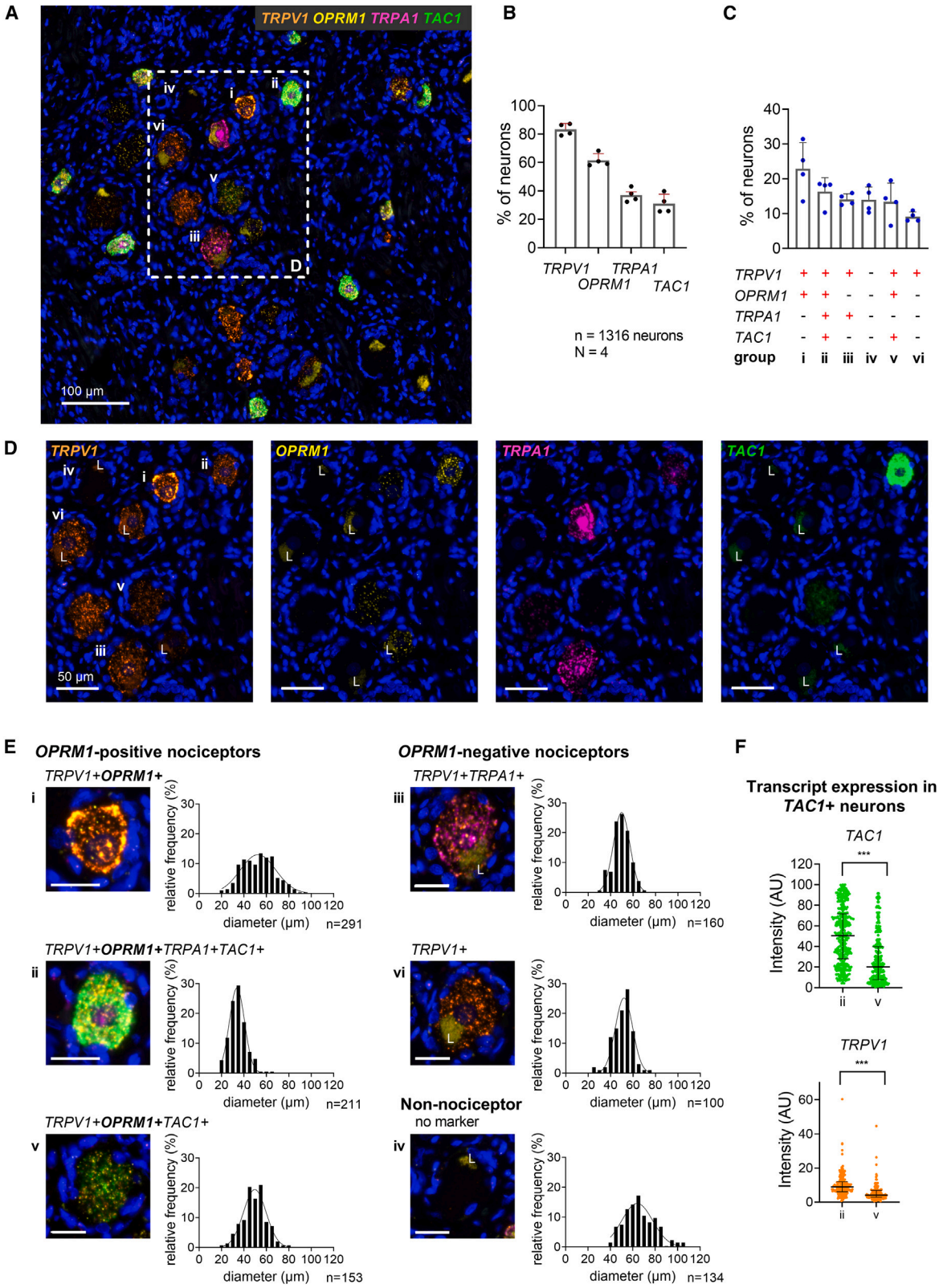
(B) Enlarged field outlined in (A) showing each individual transcript. Overlap of all four transcripts occurs in a substantial subpopulation (i). Lipofuscin is marked with an "L."

(C) Percentage of 1,310 neurons expressing each individual transcript.

(D) Percentage of neurons expressing the most common transcript combinations. Bar graphs in (C) and (D) show mean, SD, and individual values from four independent donors.

(E) Multi-channel microscopy images of a representative individual neuron of each population and the population's cell size distribution. Scale bars, 25 μm.

(F) Expression intensity of individual transcripts in *OPRM1*-positive (i) as compared to *OPRM1*-negative (ii) nociceptors. Transcript levels for *TRPV1* and *SCN11A* were significantly higher in the *OPRM1*-negative population. Median and interquartile range indicated. $p < 0.001$, Mann-Whitney U test.



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probe set we detected five major populations (Figure 5C; Table S12): three *TRPV1*+*OPRM1*+, one *TRPV1*+*OPRM1*-negative nociceptive, and a non-nociceptive population. Combined, these five groups represented 87.7% ± 2.7% of the analyzed neurons. Among *OPRM1*-positive nociceptors, *TRPV1*+*OPRM1*+*PIEZO2*+*P2RX3*+ neurons (i) were the most common (31.7% ± 7.2%) (Figure 5C), showing a broad cell size distribution ($\bar{x}$ = 51.3 ± 12.8 μm). A more homogeneous *TRPV1*+*OPRM1*+*P2RX3*+ population (iii, 18.1% ± 2.3%) consisted of small-diameter neurons ($\bar{x}$ = 38.1 ± 8.6 μm). *TRPV1*+*OPRM1*+ nociceptors that did not express *PIEZO2* nor *P2RX3* represented only a small population of small-diameter cells (v, 4.7% ± 2.4%, $\bar{x}$ = 34.6 ± 9.4 μm). *OPRM1*-negative *TRPV1*+ nociceptors expressed both *PIEZO2* and *P2RX3* (ii) (23.3% ± 2.9%) and showed again a homogeneous cell size distribution peaking at a medium cell diameter ($\bar{x}$ = 50.1 ± 8.6 μm). In this stain, we found only a minority of cells to not express any of the markers (n = 10, Table S12); instead, we observed a non-nociceptive population of medium-/large-diameter neurons ($\bar{x}$ = 70.0 ± 14.3 μm) that expressed *PIEZO2* (iv, 14.7% ± 2.2%), and presumably the proprioceptive marker *SPP1* in a previously described experiment (Figure 2D), which is consistent with the role of PIEZO2 in human proprioception[68] (Figure 5E). P2X3 is a purinergic ATP-sensitive receptor selectively expressed in nociceptive afferents[70,71] and a marker for rodent non-peptidergic C-fibers.[72,73] We noticed a differential expression across neuronal populations. Specifically, *P2RX3* showed highest expression (median intensity 21.7 a.u.) in *OPRM1*-negative nociceptors (ii) (Figure 5F). *TRPV1*+*OPRM1*+*PIEZO2*+*P2RX3*+ nociceptors (i) showed significantly less *P2RX3* expression (median intensity 8.8 a.u.), and *TRPV1*+*OPRM1*+*P2RX3*+ nociceptors (iii) showed the lowest *P2RX3* expression level (median intensity 3.9 a.u.). All differences were significant (Mann-Whitney U test, $p < 0.001$, respectively, after Bonferroni correction). *PIEZO2*, on the other hand, did not show differences in expression levels between *OPRM1*-positive and *OPRM1*-negative nociceptors (median intensity 4.8 a.u. [i], median intensity 5.3 a.u. [ii], p = 0.08, Mann-Whitney U test) (Figure 5F). These data underscore the prevalence of polymodal nociceptors in the human DRG and the high expression of the non-peptidergic marker *P2RX3* in *OPRM1*-negative nociceptors.

Expression of transcripts for neurotrophic and MRGPRD receptors differentiates *OPRM1*-positive and *OPRM1*-negative human nociceptors

By labeling for growth factor receptors, we tested the hypothesis that our results, which are indicative of a human nociceptor classification into *OPRM1*-positive and *OPRM1*-negative cells, follow the developmental principles of murine DRG neurons. These studies describe a division among nociceptors according to the expression of the neurotrophic receptors TrkA (encoded by *NTRK1*) for large-diameter A-fiber and peptidergic C-fiber nociceptors, and neurotrophic receptors such as GFRA2 for non-peptidergic C-fiber nociceptors.[19,20,74] We analyzed 1,298 neurons and detected *TRPV1* in 82.7% ± 4.5%, *OPRM1* in 59.6% ± 6.5%, *NTRK1* in 50.5% ± 3.5%, and *GFRA2* in 30.8% ± 2.2% of neurons (Figure 6C). Classification of neurons according to the co-expression of all markers confirmed our hypothesis: we detected a prevalent *TRPV1*+*OPRM1*+*NTRK1*+ population (i, 41% ± 5.1%) and an *OPRM1*-negative *TRPV1*+*GFRA2*+ population (ii, 21.2% ± 2.6%). Only a small *TRPV1*+*OPRM1*+ population did not express *NTRK1* (iii, 10% ± 2.2%) (Figure 6D; Table S13). The *TRPV1*+*OPRM1*+*NTRK1*+ population consisted of mostly small- and medium-diameter neurons ($\bar{x}$ = 46.6 ± 13 μm), while the *TRPV1*+*OPRM1*+*NTRK1*-negative population consisted mainly of small-diameter neurons ($\bar{x}$ = 42.6 ± 10.5 μm) (Figure 6E). The *OPRM1*-negative *TRPV1*+*GFRA2*+ population, as described for all other experiments, consisted of medium-sized neurons ($\bar{x}$ = 52.4 ± 8.6 μm). A non-nociceptive population (9.9% ± 3.8%) that did not express any of the markers of this experiment consisted of medium- to large-diameter neurons ($\bar{x}$ = 70 ± 12.7 μm). These four main populations represented 82.1% ± 5.5% of the analyzed neurons. Only a small fraction of neurons co-expressed both neurotrophic receptors (n = 61, Table S13), which confirms a basic distinction of human nociceptors into NTRK1-expressing "peptidergic" C-nociceptors associated with sustained pain in rodents and GFRA2-expressing "non-peptidergic" nociceptors. These data reinforce our observed dichotomy of the nociceptive neuronal population.

The largest group within the murine non-peptidergic *GFRA2*+ population consists of nociceptors that express the itch-related receptor MRGPRD.[21] In rodents these fibers do not innervate deep tissues but do terminate selectively in the most superficial skin layers.[32] Since the human *OPRM1*-negative population observed in our experiments shares many molecular features with murine non-peptidergic neurons such as high expression levels for *P2RX3* and *SCN11A*,[72,73,75–77] we hypothesized a human analog to the proposed skin threat detector molecularly defined by co-expression of *GFRA2* and *MRGPRD*.[35] In this experiment we found *TRPV1* expressed in 86.7% ± 6.5%, *OPRM1* in 61.3% ± 6.1%, *GFRA2* in 35.8% ± 7.7%, and *MRGPRD* in 22.6% ± 7.2% of neurons (n = 1,271 neurons) (Figure 6G). We observed a division into two main nociceptive populations: a large *TRPV1*+*OPRM1*+ population (i, 53.1% ± 5.8%), encompassing a wide range of mostly small- to medium-sized

Figure 4. *TAC1* (substance P) is expressed in subpopulations of *OPRM1*-positive nociceptors

(A) Representative section of human DRG showing neurons expressing transcripts for TRPV1, the μ-opioid receptor (*OPRM1*), the chemo-sensitive receptor TRPA1, and substance P precursor (*TAC1*).

(B) Percentage of 1,316 neurons expressing each individual transcript.

(C) Percentage of neurons expressing the most common transcript combinations. Bar graphs in (B) and (C) show mean, SD, and individual values from four independent donors.

(D) Enlarged field shown in (A) for each individual transcript.

(E) Multi-channel microscopy images of a representative individual neuron of each population and the population's cell size distribution. Scale bars, 25 μm. Lipofuscin is marked with an "L."

(F) Expression intensity for *TAC1* and *TRPV1* in populations ii and v. The quad+ population ii shows significantly higher expression of *TAC1* and *TRPV1* and is polyresponsive to algesic mediators. Median and interquartile range indicated. $p < 0.001$, Mann-Whitney U test.

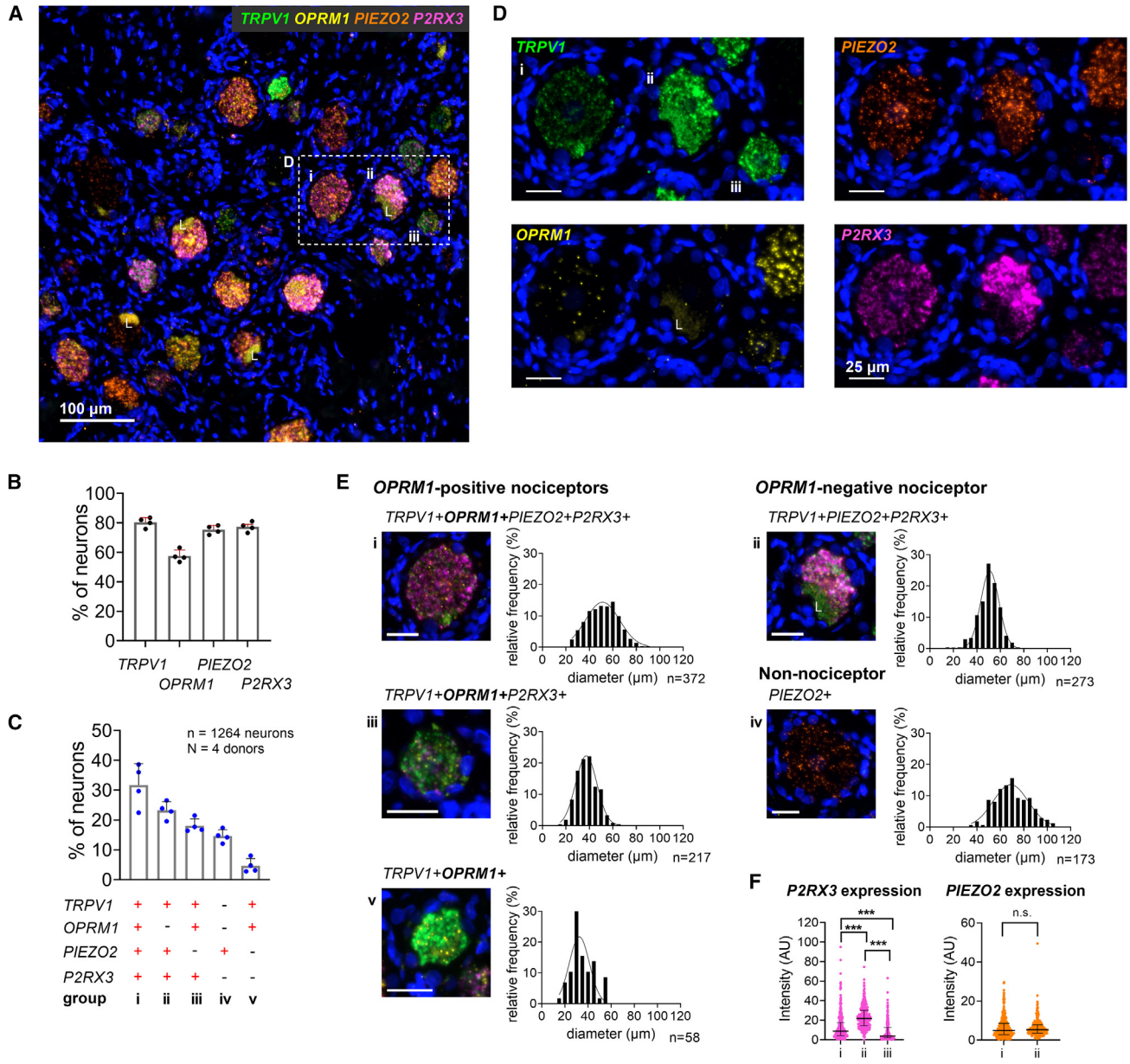

Figure 5. Expression levels of *P2RX3* differ between *OPRM1*-positive and *OPRM1*-negative nociceptors

(A) Representative section of human DRG showing neurons expressing transcripts for TRPV1, the μ-opioid receptor (*OPRM1*), the mechano-sensitive receptor PIEZO2, and the purinergic ATP receptor P2X3 (*P2RX3*).

(B) Percentage of 1,264 neurons expressing each individual transcript.

(C) Percentage of neurons expressing the most common transcript combinations. Group iv expresses *PIEZO2* only and very likely represents a population of proprioceptors as shown in Figure 2Div. Bar graphs in (B) and (C) show mean, SD, and individual values from four independent donors.

(D) Enlarged field shown in (A) for each individual transcript.

(E) Multi-channel microscopy images of a representative individual neuron of each population and the corresponding population's cell size distribution. Scale bars, 25 μm. Lipofuscin is marked with an "L."

(F) Expression intensities of *P2RX3* and *PIEZO2* in nociceptive populations. Both transcripts are expressed in *OPRM1*-positive and -negative nociceptors. While the expression level of *PIEZO2* is similar between both populations, *P2RX3* shows the highest expression in *OPRM1*-negative nociceptors. Median and interquartile range indicated. $p < 0.001$, Mann-Whitney U test, after Bonferroni correction.

neurons ($\bar{x}$ = 47.1 ± 13 μm), and an *OPRM1*-negative *TRPV1*+ population co-expressing *GFRA2* and *MRGPRD* (ii, 18.3% ± 7.4%) that consisted of medium-sized neurons with a homogeneous cell size distribution ($\bar{x}$ = 53.9 ± 6.1 μm) (Figures 6H and 6I; Table S14). Of all *OPRM1*-negative neurons co-expressing *TRPV1* and *GFRA2*, 74.5% ± 17.2% % also expressed

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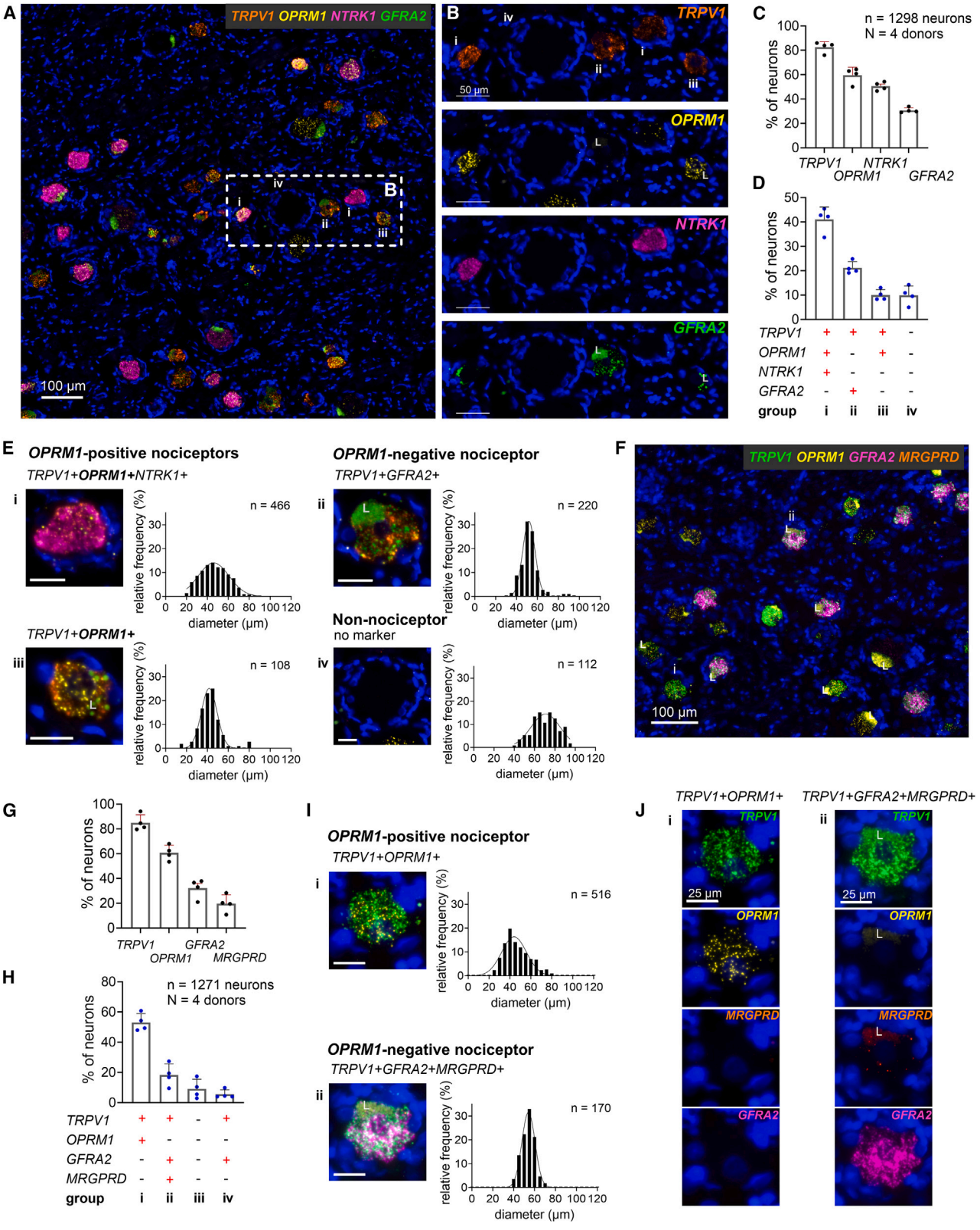

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MRGPRD. To summarize, our data support the hypothesis of a human "non-peptidergic" population expressing *MRGPRD* and define further the molecular distinction between *OPRM1*-positive and *OPRM1*-negative nociceptive populations that co-exist in the human DRG.

DISCUSSION

The present study investigates human somatosensory afferent neuronal populations relevant to nociception and opioid analgesia. Based on multiplex combinatorial *in situ* hybridization experiments, we were able to detect and define two main populations of C-nociceptors. The discriminator between these populations is the expression or lack of expression of *OPRM1*. They are further delineated by the expression of growth factor receptor genes, which follows the development of murine C-nociceptors. The first population expresses *OPRM1* and the gene coding for the nociresponsive channel TRPV1 and shares molecular attributes of murine peptidergic C-nociceptors mediating sustained pain. The second population expresses *TRPV1* and other algogenic receptors but not *OPRM1*. These neurons resemble murine *non*-peptidergic C-nociceptors. Our observations support the hypothesis of a human "tissue damage" nociceptor that is responsive to clinically used opioids and would be most relevant to analgesic drug development. Multiple experimental opioid administration studies, plus decades of experience with intrathecally administered opioids in human patients, indicate that the first, "peptidergic" population is critical for transmitting clinically relevant nociceptive pain and that this transmission can be controlled by opioids (Table S6). The second, "non-peptidergic" population comprises mainly *MRGPRD*-positive neurons that are hypothesized and has been shown in mice to terminate superficially in the epidermis and act as a "threat detector."[32] This population does not express *OPRM1* and therefore is unlikely to be responsive to opioids. Importantly, *MRGPRD*+ neurons contribute to pathological pain states including neuropathic pain in rodents.[38,39] In humans, neuropathic pain is less responsive to intrathecal opioids than nociceptive pain (Table S6) and less manageable with systemic opioids,[43,45] which supports our transcriptionally based findings.

OPRM1-positive nociceptors consist of a heterogeneous group of mostly multimodal neurons expressing markers for cold sensation (*TRPM8*), chemical sense (*TRPA1*), inflammation and tissue damage (*P2RX3*), mechanosensation (*PIEZO2*), neuropeptides (*TAC1*), and opioid receptors other than the μ-opioid receptor (*OPRD1*, *OPRL1*). *OPRM1*-negative nociceptors are multimodal neurons expressing transcripts for TRPV1 and PIEZO2, as well as the neurotrophic receptor GFRA2, the itch-related receptor MRGPRD, and the δ-opioid receptor (*OPRD1*), as well as high expression levels of transcripts for P2X3 and $Na_V1.9$ (Figure 7). An additional finding in this study is that the κ-opioid receptor in humans is expressed in *non-neuronal* SGCs.

In situ hybridization is a high-fidelity technique that allows for precise identification and localization of gene transcripts expressed in somatosensory neuronal perikarya over a range of expression levels and captures genes with low level transcription, such as opioid receptors and *MRGPRD*. An unambiguous assignment of these transcripts to human nociceptive populations could not be achieved by sequencing methods due to reasons of sensitivity[40–42] or spatial resolution.[41] Though our results confirm basic organizational principles of human nociceptive afferents of these studies, they formulate some significant differences (Figures S8–S16).

The feasibility of alternative opioid receptors as targets to relieve sustained tissue damage pain

Preclinical data suggest that all opioid receptors including the nociceptin receptor regulate transmission of nociceptive input into the spinal cord,[78–80] making them potential pharmacological targets for peripheral pain control. Additionally, such efforts were aimed at avoiding adverse side effects of μ-opioid receptor agonists. These considerations generated ongoing efforts to develop agonists to opioid receptors other than the μ-opioid receptor.[81–84] Subsequently, the peripheral κ-opioid agonist difelikefalin was approved for itch, but to date positive results in advanced clinical trials have not been forthcoming for pain (Table S15). Our current results provide a molecular-biological explanation for failures of past efforts and a pathway for future endeavors. The critical parameters are adequate expression of the gene in the correct cell population and that this population is represented by a sufficient number of cells to have a

Figure 6. Expression of transcripts for neurotrophic and MRGPRD receptors differentiates *OPRM1*-positive and *OPRM1*-negative human nociceptors

(A) Representative section of human DRG showing neurons expressing transcripts for TRPV1, the μ-opioid receptor (*OPRM1*), and the neurotrophic receptors TrkA (*NTRK1*) and GFRA2.

(B) Enlarged window as shown in (A) for each marker individually.

(C) Percentage of 1,298 neurons expressing each individual transcript.

(D) Percentage of neurons expressing the most common transcript combinations. *NTRK1* and *GFRA2* differentiate *OPRM1*-positive and -negative nociceptors. Bar graphs in (C) and (D) show mean, SD, and individual values from four independent donors.

(E) Multi-channel microscopy images of a representative individual neuron of each population and the population's cell size distribution. Scale bars, 25 μm.

(F) Representative section of human DRG showing positive transcripts for TRPV1, the μ-opioid receptor (*OPRM1*), the neurotrophic receptor GFRA2, and the pruritogenic receptor MRGPRD.

(G) Percentage of neurons showing transcripts for each marker individually.

(H) Percentage of neurons expressing the most common molecular marker combinations. Bar graphs in (G) and (H) show mean, SD, and individual values from each donor.

(I) Multi-channel microscopy images of a representative individual neuron of populations (i) and (ii) and the corresponding cell size distributions. Scale bars, 25 μm.

(J) Individual transcripts of representative neurons shown in (I). Lipofuscin is marked with an "L."Most *OPRM1*-positive nociceptors are characterized by expression of *NTRK1* (TrkA), while *OPRM1*-negative nociceptors express transcripts for the neurotrophic receptor GFRA2 and mostly the itch-related receptor MRGPRD, suggesting distinct populations of *OPRM1*+ "peptidergic" and *OPRM1*− "non-peptidergic" neurons.

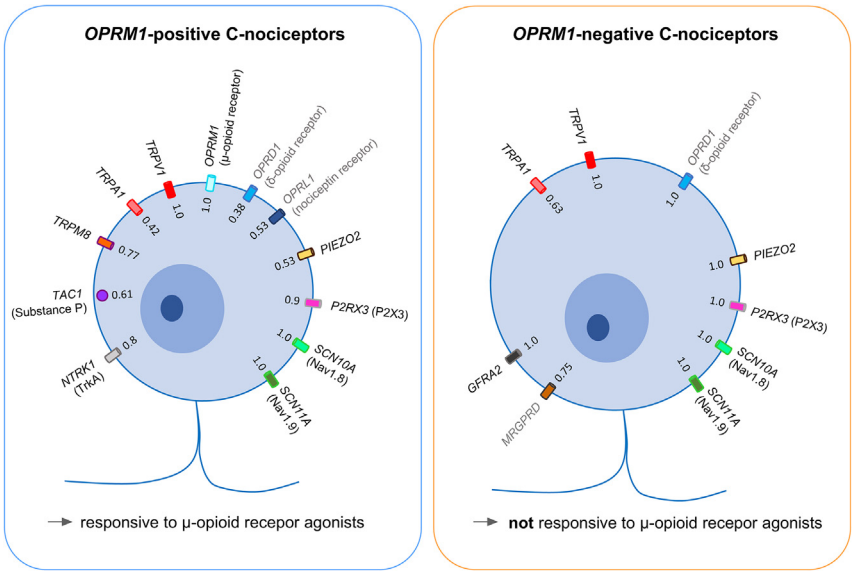

Figure 7. Expression of transcripts for ion channels, neuropeptide, and receptors in *OPRM1*-positive and *OPRM1*-negative C-nociceptors

Transcripts expressed in *OPRM1*-positive (left) and *OPRM1*-negative (right) C-nociceptors. Numbers indicate fraction of nociceptors of main populations that do express the individual transcript. Receptors/transcripts in gray indicate genes with low expression levels in the two populations as determined by *in situ* hybridization. *OPRM1*-positive nociceptors (left) are highly polymodal and likely consist of several subpopulations. In these neurons the μ-opioid receptor is the main opioid receptor with little contribution from δ-opioid or nociception receptors and nearly no contribution from the κ-opioid receptor (which we show in humans is expressed in satellite glial cells, see Figure 1F). *OPRM1*-negative neurons are polymodal and typically express *TRPV1* and PIEZO2, indicating potential responsiveness to thermal and mechanical stimulation. Most of them express the murine superficial skin marker *MRGPRD*. In this population the only opioid receptor is the δ-opioid receptor, which is expressed in low levels.

pharmacological impact. Sustained tissue damage pain involves a broad population of nociceptors that support complex transduction mechanisms.[85,86] From this frame of reference, *OPRD1* and *OPRL1* show low amounts of transcript in about half of neurons relevant for analgesia, which implies that peripheral agonist monotherapy would have a marginal analgesic effect and would require a combinatorial approach to fully inhibit relevant primary afferent populations. The fraction of human DRG neurons expressing transcripts for opioid receptors approximately matched previous *in situ* hybridization (ISH) studies for *OPRM1*,[87] and previous functional studies in human DRG neurons for the μ-opioid (MOR), δ-opioid (DOR), and nociception receptor (NOR) proteins.[50] This was different for the κ-opioid receptor (KOR, encoded by *OPRK1*), which we detected ubiquitously in SGCs and only marginally in neurons. In contrast to this finding, functional studies implied neuronal KOR expression. Though their signal could have been influenced by satellite cell κ-opioid receptors, this discrepancy cannot be resolved without further investigation.[49,50] Our data suggest that the potential contribution of KOR to modification of nociception cannot be directly mediated by afferent neurons. To summarize, the low expression levels and small fractions of relevant nociceptors expressing DOR or NOR, plus non-neuronal expression of KOR, make these receptors unlikely candidates for successful peripheral analgesic monotherapy in the context of sustained tissue damage pain.

Nociceptor-selective VGSCs and analgesic efficacy

Our experiments confirm the preferential expression of transcripts for $Na_V1.8$ (*SCN10A*) and $Na_V1.9$ (*SCN11A*) in human nociceptors.[40–42,56] $Na_V1.8$ has gained attention as a most likely source for sustained firing related to tissue injury,[88] and conditional knockout of genes on $Na_V1.8$-positive nociceptors has become a surrogate for nociceptor-specific gene modification.[89,90] Additionally, interest in these channels comes from human mutations leading to insensitivity to pain.[91–93] $Na_V1.8$ inhibitors are being currently pursued as analgesics, with VX-548 having entered phase 3 clinical trials for post-surgical pain and painful diabetic neuropathy.[94] We detected significantly higher amounts of *SCN10A* ($Na_V1.8$) in *OPRM1*-positive than in *OPRM1*-negative nociceptors. The other channel included in our studies, $Na_V1.9$, is a threshold channel that provides a "window current" which contributes to action potential initiation in response to subthreshold stimuli.[95–97] The most evident difference in expression among the two sodium channel transcripts was the high expression of *SCN11A* ($Na_V1.9$) in the *OPRM1*-negative population, consistent with rodent non-peptidergic C-fibers,[75–77] and human transcriptomic studies.[41,42] A high level of excitability, potentially driven by high $Na_V1.9$ expression in *OPRM1*-negative nociceptors, supports their hypothesized role as threat detectors and may support altered excitability in pathological states, such as neuropathic pain.[37,38] The development of selective $Na_V1.9$ antagonists is at its beginnings[98] but seems to be an attractive avenue in controlling pain which is known to be poorly responsive to opioids, such as neuropathic pain (Table S6).[44,45]

Substance P precursor (*TAC1*) expression in *OPRM1*-positive nociceptors and implications for analgesic efficacy

The neuropeptides CGRP and substance P are synthesized by DRG neurons and are modulators of nociceptive transmission at the afferent synapse in the spinal cord.[58,59] These neuropeptides also represent molecular markers that identify murine peptidergic C-nociceptors.[21,33,34,99,100] In human DRG neurons, CGRP is widely expressed,[56] while substance P (encoded by *TAC1*) displays a more restricted profile in a subpopulation of small-diameter DRG neurons.[101,102] Substance P is released during sustained noxious stimulation.[60] In line with our hypothesis of a C-nociceptor population that mediates sustained pain

and is responsive to μ-opioid receptor agonists, *TAC1* expression was selectively detected in two subpopulations of *OPRM1*-positive nociceptors. One population is of particular interest due to its high expression of *TAC1* and co-expression with *TRPA1*. The presence of *TRPA1* in these cells is important because this channel responds to inflammatory conditions, tissue injury, and a wide spectrum of noxious chemicals,[103,104] further reinforcing the suggested role of this subpopulation in the transmission of tissue damage pain. Distinguishing the combinatorial expression of nociresponsive genes within distinct cell populations provides key information for evaluating peripheral analgesic strategies and their potential performance in various clinical pain indications. In this regard, nociceptive input of *TAC1*-expressing neurons is likely sufficient to cause pain; however, blocking transmission from only this population is apparently not sufficient to achieve effective analgesia.[105,106] Our data show the presence of an additional population that provides insight into the underlying translational problem. This population (i.e., *TAC1*-negative, *TRPV1*+*OPRM1*+, Figure 4E) is large and highly nociresponsive but transmits nociceptive information in a substance P-independent fashion. The lack of analgesic efficacy of substance P receptor antagonists is consistent with our formulation of incomplete blockade of nociceptive transmission.[105,106]

Transduction of hot and cold thermosensation

Electrophysiological studies classify most cold-sensitive neurons as C-fiber neurons.[107,108] Accordingly, we detected the gene encoding the cold-responsive channel TRPM8 mainly in small-diameter nociceptors, and specifically in *OPRM1*-positive nociceptors, consistent with human experimental pain studies demonstrating the effect of μ-opioid receptor agonists on sustained noxious cold stimulation.[13,63,64] *OPRM1*-negative C-nociceptors did not express this transcript. Human DRG neurons have been molecularly and electrophysiologically grouped into mostly distinct cold- or heat-sensitive populations.[40–42,109] We detected a high degree of co-expression between transcripts for TRPV1 and TRPM8. Further analysis revealed that expression levels of transcripts for these two receptors are mostly anticorrelated, as has been shown for rat DRG neurons.[47] We also observed a fraction of cells that show moderate/high expression levels of both *TRPV1* and *TRPM8*, implying that they can be activated by both heat and cold stimuli. This is supported by microelectrode recordings in humans that identified heat-cold units with an average heat activation threshold typical for TRPV1.[110]

The hypothesized cutaneous threat detector

A combinatorial evaluation of all experiments demonstrates two major C-nociceptive populations: the first is a heterogeneous *TRPV1*+*OPRM1*+ polymodal population. This population exists alongside a relatively homogeneous *TRPV1*+*OPRM1*− population that expressed *GFRA2*, *MRGPRD*, and high levels of both *SCN11A* ($Na_V1.9$) and *P2RX3* (P2X3). Based on the molecular profile of the *TRPV1*+*OPRM1*− population, we hypothesize a role in first-line cutaneous threat detection. Expression of *MRGPRD* in non-peptidergic rodent neurons marks nociceptors that exclusively innervate the superficial epidermis.[32] In humans, the specific topographical peripheral termination of these neurons is unknown, but experimental pain studies using intradermal injection of the MRGPRD-receptor agonist β-alanine, which causes itch and burning pain, indicate peripheral nerve endings in the skin.[111] In contrast to *OPRM1*-positive nociceptors, *TRPV1*+*OPRM1*− nociceptors consistently express *PIEZO2*, implying responsivity to heat and mechanical stimuli. Many human mechano-heat polymodal skin C-nociceptors start responding early in the stimulus-response function to both heat and mechanical stimulation,[112] often with a rapid brief response even to sustained noxious stimulation.[113,114] This brief neuronal response triggers withdrawal and escape behaviors that terminate the stimulus suggesting that this population is likely the major population for responding to brief painful stimulation. By contrast, sustained stimulation evokes activity of a second, slow-onset C-population in primates.[115] The lack of effect of μ-opioid agonists on threshold-level "sudden and fleeting" skin stimulation is consistent with the absence of *OPRM1* in the population we hypothesize to be a threat detector.[1,116] Recent studies revealed a role of ATP released from keratinocytes[117,118] in response to mechanical stimulation that excites peripheral nociceptive terminals.[119] Thus, this purine release stimulus may be quite superficial. The high expression of the ATP-sensing receptor P2X3 in the *TRPV1*+*OPRM1*− population is consistent with our hypothesis that this population represents multimodal skin threat detectors.

Implications for analgesic drug development

Beyond providing a combinatorial picture of nociceptive processes, the present dataset leads to several incisive formulations for advancing developmental efforts for new analgesic agents. Our objective is to provide a constructive critique and a framework for progress to determine candidate targets that exhibit more translational potential than others. Additionally, the results highlight the need to query human DRG or spinal cord early in the drug development process to better place animal studies into a stronger translational framework. The present study delineates the most relevant DRG neuron for human clinical analgesia, which we term the tissue damage nociceptor. In particular, peripherally acting analgesics should be directed at these critical cells. Another consideration that can affect peripherally acting analgesics is redundancy. These neurons contain multiple transducers of algesic stimuli,[120,121] and antagonism of a single channel is unlikely to result in significant block of nociceptive transmission. Indeed, redundancy was one of the major factors undermining the analgesic actions of TRPV1 antagonists despite clear evidence of target engagement.[122] Going forward, it may be a challenge to identify a simplified, single-molecule approach to fully effective peripheral analgesia that provides safety and specificity. However, the approaches outlined provide a template for first-stage evaluation.

Limitations of the study

A limitation of the current interpretation is that we rely on mRNA message to predict functional or pharmacological activity. This implies a correspondence between mRNA and functional receptor protein. In the present study, we have not performed electrophysiological studies in primary cultures of human DRG to elicit

responses to TRPV1 stimulation that are differentially responsive to opioids. Such a study implies that opioids would differentially modulate TRPV1 responses in DRG neurons, although opioids have been demonstrated to modulate depolarization elicited by KCl in neurons in mouse in DRG primary cultures.[50] In considering communication with post-synaptic spinal cord neurons, we have not conducted recordings of Ca imaging in co-cultures of human DRG and spinal cord to ascertain whether differential actions can be measured on post-synaptic neurons. Such experiments present technical difficulties with respect to sourcing of viable human tissue.[123] Nonetheless, the conclusion of distinct functions of the two main *OPRM1*+ and *OPRM1*− populations is supported by human clinical trials of opioids in tissue damage and neuropathic pain conditions (see Table S6). Additional functional pharmacologic evidence potentially with *in vivo* microneurography[124,125] or imaging of spinal cord[126–128] may further validate these predictions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michael Iadarola (michael.iadarola@nih.gov).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this study will be shared by the lead contact upon request. This includes multiplex fluorescence microscopic images and region-of-interest files. This paper does not report original code. Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.S.S. and M.J.I.; methodology, M.R.S., D.M., and M.J.I.; investigation, E.S.S. and D.M.K.; formal analysis, E.S.S.; visualization, E.S.S.; resources, A.J.M., D.M., and A.G.; writing – original draft, E.S.; writing – review and editing, E.S.S., M.R.S., and M.J.I.; funding acquisition, A.J.M.; supervision, D.M., A.J.M., and M.J.I.

DECLARATION OF INTERESTS

A.G. is an employee and shareholder of AnaBios Corp.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human DRG Lumbar 3	AnaBios	210218DHA
Human DRG Lumbar 3	AnaBios	210221DHA
Human DRG Lumbar 3	AnaBios	210325DHA
Human DRG Lumbar 3	AnaBios	210405DHA
Critical commercial assays		
RNAscope® 4-Plex Ancillary kit	Advanced Cell Diagnostics	Cat#323120
RNAscope® Wash Buffer Reagents	Advanced Cell Diagnostics	Cat#310091
RNAscope® Probe Diluent	Advanced Cell Diagnostics	Cat#300041
RNAscope® H2O2 and Protease Reagents	Advanced Cell Diagnostics	Cat#322381
RNAscope® Target Retrieval Reagents	Advanced Cell Diagnostics	Cat#322000
RNAscope® Multiplex Fluorescent Detection Reagents V2	Advanced Cell Diagnostics	Cat#323110
RNAscope® Multiplex TSA Buffer	Advanced Cell Diagnostics	CAT#322810
Opal 520 Reagent Pack	Akoya Biosciences	SKU: FP1487001KT
Opal 570 Reagent Pack	Akoya Biosciences	SKU: FP1488001KT
Opal 620 Reagent Pack	Akoya Biosciences	SKU: FP1495001KT
Opal 690 Reagent Pack	Akoya Biosciences	SKU: FP1497001KT
Oligonucleotides		
RNAscope™ Probe- Hs-*GFRA2* (GDNF Family Receptor Alpha 2)	Advanced Cell Diagnostics	Cat#463011
RNAscope™ Probe- Hs-*MRGPRD* (MAS Related GPR Family Member D)	Advanced Cell Diagnostics	Cat#524871
RNAscope™ Probe- Hs-*NTRK1* (Neurotrophic Receptor Tyrosine Kinase 1)	Advanced Cell Diagnostics	Cat#402631
RNAscope™ Probe- Hs-*OPRD1* (Opioid Receptor Delta 1)	Advanced Cell Diagnostics	Cat#536061
RNAscope™ Probe- Hs-*OPRK1* (Opioid Receptor Kappa 1)	Advanced Cell Diagnostics	Cat#1148211
RNAscope™ Probe- Hs-*OPRK1-O1* (Opioid Receptor Kappa 1)	Advanced Cell Diagnostics	Custom made (13 ZZ targeting 1276–2137 bp of NM_000912.5)
RNAscope™ Probe- Hs-*OPRL1* (Opioid Related Nociceptin Receptor 1)	Advanced Cell Diagnostics	Cat#536071
RNAscope™ Probe- Hs-*OPRM1* (Opioid Receptor Mu 1)	Advanced Cell Diagnostics	Cat#410681
RNAscope™ Probe- Hs-*PIEZO2* (Piezo Type Mechanosensitive Ion Channel Component 2)	Advanced Cell Diagnostics	Cat#449951
RNAscope™ Probe- Hs-*P2RX3* (Purinergic Receptor P2X3)	Advanced Cell Diagnostics	Cat#406301
RNAscope™ Probe- Hs-*SCN10A* (Sodium Voltage-gated Channel Alpha Subunit 10)	Advanced Cell Diagnostics	Cat#406291
RNAscope™ Probe- Hs-*SCN11A* (Sodium Voltage-gated Channel Alpha Subunit 11)	Advanced Cell Diagnostics	Cat#404791
RNAscope™ Probe- Hs-*SPP1* (Secreted Phosphoprotein 1)	Advanced Cell Diagnostics	Cat#420101

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNAscope™ Probe- Hs-*TAC1* (Tachykinin Precursor 1)	Advanced Cell Diagnostics	Cat#310711
RNAscope™ Probe- Hs-*TRPA1* (Transient Receptor Potential Cation Channel Subfamily A Member 1)	Advanced Cell Diagnostics	Cat#503741
RNAscope™ Probe- Hs-*TRPM8* (Transient Receptor Potential Cation Channel Subfamily M Member 8)	Advanced Cell Diagnostics	Cat#543121
RNAscope™ Probe- Hs-*TRPV1* (Transient Receptor Potential Cation Channel Subfamily V Member 1)	Advanced Cell Diagnostics	Cat#415381
Software and algorithms		
Photoshop	Adobe	V25.0.0
Fiji	ImageJ	14.0/1.54f
Prism9	Graphpad	V9.4.1./9.5.1
Other		
Axio Imager.Z2 microscope	Zeiss	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Patients and ethics statements

Dorsal root ganglia (DRGs) were obtained from organ donors by AnaBios Corporation (San Diego, CA) in partnership with US organ procurement organizations. Legal consent for tissue retrieval and use of that tissue for research in a commercial setting according to US laws and regulations was warranted. The distribution of donor medical information complied with HIPAA regulations regarding donor privacy. All transfers of donor organs to AnaBios are fully traceable and periodically reviewed by US Federal authorities. Upon arriving at AnaBios, each set of DRGs was assigned a unique identifier number that was reproduced on all relevant medical history files, data entry forms, and electronic records. We received only anonymized and coded donor tissue and demographic information with no way to link back to original identifiers. This study did not meet the regulatory definition of human subjects research at NIH and hence did not require IRB approval.

L3 lumbar DRGs from four tissue donors (2 Females, 2 Males, gender as provided by AnaBios Corporation, mean age 22.5 ± 3.1 years, all Caucasian) were used for all analyses in the study. None of the donors suffered from a chronic pain condition or had indications of peripheral nerve damage. Detailed demographic information, cause of death, and tissue retrieval times are available in Table S16.

Due to the small sample size and differences in the causes of death between Female and Male donors, the influence of gender on the results of this study was not systematically assessed (but see Figure S3 for comparison of percentages of *TRPV1*+*OPRM1*+ nociceptors between Females and Males). This is a limitation to our research's generalizability.

METHOD DETAILS

Patients and dorsal root ganglia samples

Human dorsal root ganglia (DRG) were collected from four tissue donors and provided by AnaBios Corporation (San Diego, CA). At the time of tissue harvest, DRGs were flash frozen and stored at −80°C until processing. Immersion fixation was performed by submerging whole DRGs in room temperature 10% neutral buffered formalin, and then refrigerated for 16–24 h for fixation before embedding in paraffin blocks at Histoserv, Inc. (Germantown, MD) and sectioning at 6 μm. For each *in situ* hybridization experiment, we used one section per individual donor DRG and included all four sections in the analysis.

Fluorescent multiplex *in situ* hybridization and microscopic imaging

We performed 4-Plex fluorescent RNA *in situ* hybridizations using the RNAScope Multiplex Fluorescent V2 Assay (Advanced Cell Diagnostics, Newark, CA) following the manufacturer's instructions for formalin-fixed paraffin-embedded tissue. Target retrieval was performed for 20 min at 100°C. The catalog numbers of the probes used in these experiments are listed in Table S17. After hybridization, slides were imaged using an Axio Imager.Z2 scanning fluorescence microscope (Zeiss, Oberkochen, Germany) as described previously. Filter sets (Semrock, Rochester NY) for detecting DAPI, Opal520, Opal570, Opal620, and Opal690 fluorescent dyes (Opal Reagent Pack; Akoya Biosciences, Marlborough MA) were custom furnished as described previously[47,129,130] (Table S18).

Due to the unexpected staining results for *OPRK1*, a second *in situ* probe was designed to validate the results. In particular, the original probe was designed against the 3′ end of the transcript. In our redesign, we selected a non-overlapping region 5′ to the original location (base pairs 1276–2137 of NM_000912.5).

QUANTIFICATION AND STATISTICAL ANALYSIS

Visualization of merged composite images were constructed in Photoshop (v25.0.0, Adobe, San Jose, CA) and Fiji (ImageJ2.14.0/1.54f) in order to analyze the co-expression of transcripts. Cells were identified using a combination of DAPI-labeling of nuclear DNA and differential interference contrast (DIC) imaging. For quantification, cells were counted manually from one section per human tissue donor. In order to capture a representative subset of neurons, multiple windows (1 mm × 1 mm) located in different areas of the DRG were sampled to reach a minimum of 300 neurons per section (range 309–349, mean 323.2 ± 13). Lipofuscin autofluorescence was apparent in the 488 nm, 546 nm, and 594 nm channels, and was excluded from our analyses. This autofluorescence was identified by its simultaneous emission in multiple channels, including the 430 nm channel, which was included to capture autofluorescence. In the representative images, lipofuscin is marked with a capital "L" to distinguish it from real signal. We used the following inclusion criteria for neurons in the quantification. Neurons used for quantification were intact, and in cases where there was substantial lipofuscin, this tissue artifact occupied less than 50% of the cytoplasm. Cells were considered positive for expression of a molecular marker if they showed at least three cytoplasmic puncta. We estimated that three puncta per neuron would be a reasonable threshold to determine whether a neuron actively transcribes the gene of interest. Other groups use a threshold of four puncta,[49] but we found that we would miss out on some very small diameter cells (20–30 μm diameter) with low expression levels of some nociceptive markers. In general, neurons with an expression level of 3–5 puncta (mRNA) represented a small percentage of total quantified neurons (see Figure S1), and a change of threshold would have little effect on quantitative measures and qualitative results. In addition, the existence of non-specific signal in DRG tissue sections of all donors was excluded by performing *in situ* hybridization with negative control probes. Our inclusion criteria of three puncta was definitely above background which allowed to be more inclusive. *TRPV1* was usually co-expressed with typical markers for nociception, such as *P2RX3*, or with the analgesic marker *OPRM1* (see Tables S7–S14), which confirms its predominant expression in nociceptive neurons. Given that the sum of all *TRPV1*+*OPRM1*+ and *TRPV1*+*OPRM1*-neurons for each ISH experiment generally matched known percentages of nociceptive neurons in human[56] and mouse[19] dorsal root ganglia (Figures 1, 2, 3, 4, 5, and 6), we feel confident that we chose an adequate threshold for positive gene expression.

For each mRNA target and each donor, we determined the percentage of neurons positive for a molecular marker by assessing each neuron as positive or negative for the four mRNAs assessed in each 4-plex combination. This co-expression pattern was used to establish neuronal populations. Each individual (human donor) was assessed for differences before pooling, although no individuals showed notable unique differences in expression patterns. Complete counts of neuronal populations for each experiment can be found in Tables S7–S14. For each of the prevalent neuronal populations (>9% of all counted neurons) we analyzed cell size alongside expression levels of transcripts. We focused on populations comprising 9% or more as this analysis is prone to identifying multiple small subpopulations, and the less prevalent populations can be less reproducible or less biologically relevant.[47] One exception to this general rule was that we did characterize some *TRPV1*+*OPRM1*+ subpopulations below 9% prevalence as this was a major focus of the study. For cell size analysis, we included only cells that were sectioned through the center of the perikarya to achieve a more accurate circumference.[47,56] For calculation of cell size the neuronal cell borders were drawn based on the merged composite of all of the fluorescence channels and DIC using the Fiji freehand selection tool. The neuronal diameter was extrapolated from the area of the drawn region of interest (ROI_{size}) using the formula for the diameter of a circle (diameter = $2\sqrt{(\text{area}/\pi)}$). Based on existing human DRG literature and our results regarding the cell diameter distribution of *TRPV1*+*GFRA2*+*MRGPRD*+ nociceptors, which represent a molecularly defined C-fiber population[21] (see Figure S7), neurons with a diameter smaller than 50 μm were considered small-diameter neurons, and those with a diameter larger than 65 μm were considered as large-diameter cells that likely represent myelinated A-fibers (Figure 7).[131,132] For quantification of signal intensity inside individual DRG neurons, ROIs were drawn in the same manner as for ROI_{size}, but were altered to exclude areas of artifactual autofluorescence, such as that from lipofuscin. This prevented accidental quantification of artifactual signal. We measured the mean gray scale of unmanipulated signal using Fiji (ImageJ2.14.0/1.54f). Due to the TSA amplification, mRNA marked by fluorophore dye visible as puncta can vary in brightness. We found that the mean gray scale as provided by Fiji correlated well with the number of puncta, even when bright and dim puncta were included in the counts (Figure S1). For quantitative graphs, each channel was checked visually for non-specific, "bleed" signal coming from neighboring channels. Signal bleed was detected in some neurons (n = 10) from *TRPV1* (488 nm) to *OPRD1* (546 nm) in experiment 1 (Figure 1). We corrected for this by subtracting the signal intensity of the 488 nm channel of a region of interest capturing isolated background signal (ROI_{bleed}) from the 546 nm channel in that individual neuron (Figure S2). In order to compare signal intensities of different target genes (and/or detection channels), we determined threshold values for low, moderate, and high expression levels (see Figures 1I and 2E). High expression levels were defined as values larger than 3 standard deviations of the sample mean. For the distinction of low and moderate expression levels we found that a visually based determination of a threshold value was most reliable (manual scoring). Statistical testing was conducted using Prism GraphPad (Version 9.4.1. and 9.5.1.). Representative images were adjusted for brightness and contrast for visibility. Bar graphs in all figures show percentages of neurons expressing individual transcripts or combinations of transcripts for each human subject (mean ± standard deviation) (N = Human subjects; n = cells).