Inhibition of Ca^{2+} Currents by a μ -Opioid in a Defined Subset of Rat Sensory Neurons

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Activation of the endogenous opioid system can suppress pain without affecting other sensations, but the cellular mechanism of this selectivity is unclear. The analgesia might be due to inhibitory synapses arranged only on neurons whose activity leads to pain sensations. Alternatively, opioids might be released broadly, with neurons involved in pain sensation being especially sensitive. Therefore, we asked whether different subsets of rat dorsal root ganglion (DRG) sensory neurons vary in their sensitivity to opioids. Dissociated neurons were subdivided according to the spinal laminae to which they likely had projected, and whether they had innervated muscle. Using the patch-clamp method, we measured the inhibition of Ca^{2+} current by DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol), a peptide that selectively activates the μ (morphine) receptor. We also investigated the presence of different types of Ca^{2+} channels. In DRG neurons chosen at random, Ca^{2+} currents were inhibited by DAGO to widely varying degrees, with an average inhibition of 38%. Ca^{2+} currents in neurons in a subset that projects to laminae I and II had a lower average inhibition, and unlike the randomly selected cells, the responses were predictable and tightly distributed about the mean. This indicates that the variability of opioid sensitivity among DRG neurons reflects the presence of different subsets of cells. Since neurons projecting to laminae I and II, the projection site of nociceptive neurons, did not show high opioid sensitivity, there is no evidence that nociceptive neurons have stronger responses to opioids. But a firm conclusion is impossible because projection site does not strictly define sensory modality.

[Key words: opioids, sensory neurons, dorsal root ganglion, Ca^{2+} channels, μ -opioid receptor]

The selective suppression of pain while other sensations persist is the defining feature of analgesia and a property of the endogenous opioid system (Fields, 1987). Effects of opioids on pain pathways could occur at various levels from the periphery to the cortex, but opioids clearly inhibit neurotransmitter release

from primary sensory neurons due to pathways descending from brainstem nuclei (Willis et al., 1977; Basbaum and Fields, 1984). Does inhibition at the primary sensory neuron contribute to the specificity of opioids for pain? This would be the case either if opioid synapses formed specifically onto nociceptive neurons or if nociceptive neurons were especially sensitive to opioids that were released broadly onto all cells. Neuropeptides generally are considered to act over broad spaces; for example, leu-tinizing hormone-releasing hormone (LHRH) persists for at least 30 sec after release in sympathetic ganglia, during which time it can diffuse about 100 μm , and clearly affects cells remote from the presynaptic release site (Jan and Jan, 1983). Considering the possibility of nonsynaptic actions of opioids, we asked whether primary sensory neurons vary in their opioid sensitivity in any systematic manner.

Previous work shows that opioids acting at the μ (morphine) receptor inhibit Ca^{2+} channels in dissociated dorsal root ganglion (DRG) sensory neurons (Schroeder et al., 1991) and in a neuroblastoma cell line (Seward et al., 1991). The sensitivity of individual DRG neurons varied widely, with a fraction being insensitive, whereas all cells from the cell line were inhibited by μ -opioids. DRG neurons also vary in their responses to κ - and δ -opioids (Werz and MacDonald, 1985). Since the DRG neurons are a mixed population of cells that differ in the sensations that they transduce and encode, it is possible that the variable sensitivity to opioids correlates with this variety of sensory modalities.

The ideal way to address such questions would involve knowing the sensory modality of dissociated DRG cells. Unable to accomplish this, we have divided cultured DRG neurons into subsets based on the spinal laminae to which they had projected and the organ that they had innervated when they were in the rat. Spinal laminae were inferred using monoclonal antibodies that distinguish sensory neurons having different central projection patterns (Dodd et al., 1984; Dodd and Jessell, 1985). The peripheral projection was determined by retrograde transport of a lipid-soluble dye that was focally injected 2 d before the DRG were dissected. The spinal projection site gives the best information because of the map of sensory modality projecting across spinal laminae (Brown, 1981; Perl, 1983). In this article, identified subsets and randomly selected DRG neurons are compared with regard to the opioid sensitivity of their somal Ca^{2+} current and the expression of Ca^{2+} channel subtypes.

Two aspects of the approach are imperfect and limit the interpretation of results. First, identification of the spinal projection site does not strictly define sensory modality since several sensations can be represented in any lamina (Perl, 1991); thus, our method cannot clearly examine the opioid sensitivity of nociceptive neurons because we do not identify them explicitly.

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The second problem is that we measure inhibition of Ca^{2+} current at the cell soma; only if the synaptic terminals are similarly sensitive does this indicate suppression of synaptic transmission.

Materials and Methods

Tissue culture and recordings. The methods used for preparing adult rat DRG neurons were identical to those described previously (Schroeder et al., 1990a). Cells were studied within the first 2 d in culture. Cells were plated on collagen-coated etched grid coverslips (Bellco, Vineland, NJ) for experiments that required recording from an identified cell before and after immunohistochemistry.

Currents were recorded at room temperature using the whole-cell patch-clamp technique (Hamill et al., 1981). Leak and capacity currents were eliminated by digitally scaling and subtracting records obtained with subthreshold voltage pulses unless otherwise noted. Cell capacitance was calculated from the amount of charge that entered the cell during the charging transient produced by a step from -70 mV to -90 mV.

The extracellular solution contained (in mM) 10 CaCl_2 , 135 tetraethylammonium-Cl (TEA-Cl), and 10 HEPES, pH 7.4 adjusted with TEA-OH. DAGO (Peninsula Laboratories, Belmont, CA) was stored in vacuum-dried aliquots and dissolved in extracellular solution on the day of the experiment. Different extracellular solutions were perfused onto the cell through 1 μl pipettes as previously described (Schroeder et al., 1991).

The intracellular (pipette) solution contained (in mM) 100 CsCl, 1 Na₂ATP (equine; Sigma, St. Louis, MO), 0.3 Na₂GTP (Aldrich Chemical Co., Milwaukee, WI), 2.5 MgCl_2 , 10 EGTA, 2.9 CaCl_2 (pCa = 7.0), and 40 HEPES, pH 7.0 adjusted with TEA-OH.

Immunohistochemistry. Monoclonal antibodies, provided by Dr. Jane Dodd (College of Physicians and Surgeons of Columbia University, New York), were used to label subsets of cultured DRG neurons by a procedure modified from Dodd and Jessell (1985). Cultures were washed twice with L15 medium (L15 supplemented with 0.5 mg/ml BSA and 8 mM glucose) before and after incubations of 30–60 min duration in antibody-containing solutions. The primary antibodies were in tissue culture supernatant diluted 1:1 in L15 medium. The fluorescent secondary antibodies were diluted 1:100 in L15 medium. Goat anti-mouse IgM was from TAGO Immunologicals (Burlingame, CA), and goat anti-mouse IgG were from Boehringer Mannheim (Indianapolis, IN). Incubations were done at room temperature.

Dye injections. Adult Sprague–Dawley rats (90–275 gm) were anesthetized with pentobarbital (40 mg/kg) or with “rodent cocktail” (0.5 ml/kg; 100 mg/ml ketamine hydrochloride, 20 mg/ml xylazine hydrochloride, 10 mg/ml acepromazine), and 0.05 ml atropine (atropine sulfate, 1:120; AmVet Pharmaceuticals, Fort Collins, CO) was injected to reduce bronchial secretions. The lateral surface of the upper portion of the hindlimb was shaved and cleaned before making an incision to expose the underlying muscles. Four or five 1 μl injections of DiI (DiI₁₈; 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR; 5% w/v in dimethylformamide) were made into the muscles and the incision was closed with surgical clips. Animals were killed 2 d after the injection and the DRG were harvested.

Statistical treatment of data. We used two independent statistical tests to ask if labeled subsets of cells differed from randomly selected cells in their opioid sensitivity. First, we used χ^2 analysis to test whether the random and labeled cells came from the same population. Second, we used the central limit theorem (Daniel, 1991), which assumes that the randomly selected cells provide the “true” distribution. Then, the probability of observing the distribution obtained with the labeled cells was calculated.

Results

Identification of neuronal subsets

We identified subsets of cultured DRG neurons in two ways: (1) retrograde transport of a lipid-soluble fluorescent dye (DiI) from peripheral targets to the DRG, and (2) labeling with monoclonal antibodies directed against oligosaccharide structures that are selectively expressed on subsets of DRG neurons (Dodd et al., 1984; Dodd and Jessell, 1985). The central terminals of neurons expressing particular oligosaccharides are restricted to discrete areas of the dorsal horn of the spinal cord. Because the

central processes of DRG neurons segregate in the dorsal horn according to their sensory modality (Brown, 1981; Perl, 1983), the Dodd/Jessell antibodies might label functionally distinct subsets of DRG neurons.

Figure 1 shows DRG cells that have been in culture for 2 d. The three spherically shaped neuronal cell bodies are uniformly phase bright in the phase-contrast photograph (*a*); any fibroblasts are dark and have irregularly shaped cell bodies. Two days prior to dissection of the DRG, hindlimb muscles of the rat were injected with 5 μl of DiI solution. Two of the neurons contained DiI within their somas (*b*), indicating that they had innervated the injected muscle. One of the two neurons also expressed the 1B2 antigen on its surface (*c*).

A survey of 305 cells on one dish showed that 23% were 1B2 positive, 13% were muscle projecting, and 5% were members of both subsets (double labeled). Dodd and Jessell (1985) reported that a similar percentage of neurons were 1B2 positive in neonatal cultures.

Cells expressing the 1B2 antigen terminate in the spinal cord primarily in lamina I and the superficial two-thirds of lamina II, with a few processes terminating in the deeper one-third of lamina II (Dodd and Jessell, 1985). The central terminals of nociceptive and thermoceptive primary afferents are located predominantly within lamina I and lamina II (Christensen and Perl, 1970; Kumazawa and Perl, 1978; Light and Perl, 1979; Sugiura et al., 1986). Thus, a population of DRG neurons expressing the 1B2 antigen might be enriched in nociceptive and thermoceptive neurons. In contrast, a population of B2 antigen-expressing cells, which principally project to laminae III and IV, might be enriched in low-threshold mechanoreceptors, in agreement with the central projections of such neurons (Brown et al., 1977, 1978, 1980, 1981). The two other antibodies that we have used are LA4 and SSEA3. LA4 neurons project primarily to the lower third of lamina II; SSEA3 neurons project to laminae III and IV, with a light projection to lamina I (Dodd and Jessell, 1985).

Different types of Ca^{2+} currents in neuronal subsets

We previously reported heterogeneous expression of T-type Ca^{2+} channels in rat sensory neurons (Schroeder et al., 1990b). Particularly interesting was the small fraction of cells that had giant T-currents exceeding a nanoampere in amplitude. Therefore, we hoped to find an identified subset of sensory neurons with these giant T-currents.

The densities of T-current in 68 randomly selected DRG neurons are indicated in the histogram in Figure 2. The inset shows records from three of the cells, with T-currents evoked by steps to -30 mV and high-threshold Ca^{2+} currents measured with steps to $+10$ mV. Inset *a* shows records from a cell with no T-current, corresponding to the first column in the histogram. Inset *b* shows a typical T-current in that its amplitude is substantially less than the high-threshold current. Inset *c* shows a giant T-current with an amplitude greater than the high-threshold current; all such cells had T-current densities of 35 pA/pF or greater. These giant T-currents should dramatically promote burst firing of action potentials (White et al., 1989).

Table 1 shows average membrane capacitance, T-current density, and measurements of the high-threshold current from cells labeled with four different antibodies and cells shown to innervate muscle. The average T-current densities (column 2) and the SD are similar among the antibody-labeled populations. Not one of the many labeled cells had a giant T-current. The average

Figure 2. The number of DRG cells chosen at random that exhibit different T-type Ca^{2+} current densities (pA/pF). *Inset*, records of T-currents and high-threshold Ca^{2+} currents from three cells with very different T-current amplitudes; *numbers* indicate the test voltages (mV). T-current amplitudes were measured as peak current evoked by steps from -70 mV to either -30 mV or -20 mV. Bin width is 5 pA/pF and terminates at the value indicated.

high-threshold current densities (column 3) are also similar among the subsets, as are the ratios of total:sustained high-threshold currents. Cell size, as measured by membrane capacitance (column 1), was clearly different between some subsets: cells with the SSEA3 and B2 antigens were smaller than 1B2 cells, for example. In short, the labeled subsets may differ in cell size but are similar in their expression of different types of Ca^{2+} channels. Moreover, the giant T-currents apparently exist on a subpopulation of DRG cells that are not labeled by any of the five indicators used here.

A subset that responds predictably to μ -opioids

DRG neurons selected at random displayed a wide range of inhibition by μ -opioids. Figure 3 shows the effect of DAGO, an enkephalin analog (Tyr-D-Ala-Gly-MePhe-Gly-ol) that is a specific μ -receptor agonist, on Ca^{2+} currents from three different sensory neurons. The traces are Ca^{2+} currents evoked by steps from -70 mV to $+20$ mV in the absence (larger traces) and presence (smaller traces) of $1 \mu\text{M}$ DAGO, a saturating concentration. The graphs show peak Ca^{2+} current versus experimental time for many such steps, with DAGO application indicated by bars. Is this wide variation of μ -opioid responses due to functionally distinct subtypes of DRG neurons? To address this, we compared opioid responses of randomly selected DRG neurons to those that innervate muscle or are labeled by the 1B2 monoclonal antibody. Because the 1B2 antibody labels cells projecting to superficial laminae, the cell population might be enriched in high-threshold sensory neurons.

Before surveying μ -opioid inhibition of Ca^{2+} currents in antibody-positive subsets, we asked if the antibody labeling procedure altered DAGO inhibition of Ca^{2+} currents. Figure 4 shows

Figure 1. Three views of the same field of cells 2 d in culture: phase optics (a), rhodamine fluorescence (b), and fluorescein fluorescence (c). Two days prior to dissection, hindlimb muscles of the rat had been injected with DiI, which emits red fluorescence. The culture dish had been treated in the previous hour with the 1B2 antibody and an FITC-conjugated secondary antibody, which emits green fluorescence. Scale bar: $50 \mu\text{m}$.

Table 1. Ca²⁺ current components in subsets of DRG neurons

Subset	Capacitance	T-current density	High-threshold current density	Total : Sustained high-threshold current ratio
1B2 positive	52.8 ± 21.47 (n = 94)	3.8 ± 3.5 (n = 76) (range, 0–20.8)	35.7 ± 21.7 (n = 76)	5.1 ± 2.92 (n = 42)
Muscle projecting	68.6 ± 46 (n = 56)	2.1 ± 4.0 (n = 56) (range, 0–28.7)	37.6 ± 28.6 (n = 56)	4.7 ± 2.8 (n = 46)
LA4 positive	45.5 ± 40.5 (n = 13)	3.6 ± 5.4 (n = 13) (range, 0–21.3)	25.4 ± 15.3 (n = 13)	
SSEA3 positive	28.3 ± 8.9 (n = 15)	2.8 ± 2.0 (n = 15) (range, 0–7.3)	37.1 ± 23.2 (n = 15)	3.1 ± 0.4 (n = 7)
B2 positive	29.4 ± 13.7 (n = 9)	3.5 ± 3.6 (n = 9) (range, 0–11.0)	44.9 ± 24.9 (n = 9)	
LA4 negative ^a	101.4 ± 74.2 (n = 13)	11.8 ± 29.2 (n = 13) (range, 0–112)	25.4 ± 25.7 (n = 11)	
1B2 negative ^a	81.3 ± 72.3 (n = 11)	6.9 ± 14.5 (n = 11) (range, 0.9–52.5)	23.2 ± 21.4 (n = 10)	

Measurements are given as the average ± SEM (number of cells in parentheses). T-current density is the peak current measured at –30 or –20 mV divided by the cell's membrane capacitance. High-threshold current density is the peak current measured at +10 mV divided by membrane capacitance. Total:sustained ratio is the peak current measured at +20 mV divided by the amplitude remaining after 1 sec of depolarization. Holding potentials were –70 mV or –80 mV. Note the broader range of sizes and the greater average T-current densities in the unlabeled cells. The preferred spinal projection sites for the various labeled cells are as follows: 1B2, laminae I and superficial two-thirds of laminae II; LA4, lower third of laminae II; SSEA3, laminae I (light projection), III, and IV; B2, laminae III and IV (Dodd and Jessell, 1985).

^a The LA4-negative and 1B2-negative rows refer to unlabeled cells in cultures exposed to these antibodies.

the effect of DAGO (1 μM) on the Ca²⁺ current in a 1B2-positive neuron before (*a*) and after (*b*) labeling with the 1B2 antibody and the fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgM. DAGO inhibited the Ca²⁺ current by about 50% both before and after the labeling procedure. Thus, the antibody labeling procedure neither diminished nor enhanced the DAGO inhibition of the Ca²⁺ current.

The histograms in Figure 5 show (top to bottom, respectively) the distribution of responses to the μ-opioid DAGO (1 μM, a saturating concentration) in 73 randomly selected DRG neurons, 44 muscle-projecting DRG neurons, 46 1B2-positive DRG neurons, and 18 doubled-labeled neurons. Randomly selected cells had a broad response profile. Nine cells (12%) lacked opioid sensitivity, and 19 (26%) were hypersensitive in that the Ca²⁺ current was inhibited by greater than 55%. Muscle-projecting cells exhibited a similar broad response profile. The degree to which Ca²⁺ currents were inhibited by DAGO did not correlate with either the transient to sustained high-threshold Ca²⁺ current ratio or the presence of TTX-insensitive Na⁺ current.

Cells labeled with the 1B2 antibody had a different profile of responses than randomly selected cells: there were few opioid-insensitive cells, and no cells were hypersensitive. The average inhibition of 1B2-labeled cells was somewhat less than that for randomly selected cells (25% vs. 38%), but the more dramatic difference was the SD (13% for 1B2 cells vs. 25% for randomly

selected cells). We used two statistical tests to explore the possibility that 1B2 cells have a specific opioid response compared to the broad range of responses of the randomly selected cells. First, χ² analysis finds a probability less than 0.005 that the 1B2 data and the random data come from cells that are in the same population with regard to opioid sensitivity of their Ca²⁺ current. Second, using the central limit theorem (Daniel, 1991), we found the probability of obtaining the tight 1B2 distribution if the “true” distribution was that of the randomly selected cells. The probability of having only the two insensitive cells is 0.047, and the probability that no cells would be inhibited by more than 55% is less than 0.0001; the product of these gives the probability of losing both extremes. The statistics confirm what was evident during the course of experiments: 1B2-labeled cells differ from randomly selected cells in that their responses to DAGO are predictable.

Discussion

Limits of interpretation

We found that Ca²⁺ currents in different DRG cell bodies vary dramatically in their sensitivity to μ-opioids. Such variability might be related to differences in sensory modality among different DRG neurons or might be an artifact of the cell dissociation, culture, or recording conditions. When we studied opioid sensitivity of a subset of DRG neurons that preferentially project

Figure 3. Heterogeneity of responses to DAGO among three different DRG neurons. Ca^{2+} currents were evoked by steps from -70 mV to -20 mV. Graphs display peak current versus experimental time for each cell, and arrows point to data corresponding to the traces above. Bars indicate application of DAGO. The use of different DAGO concentrations in the middle and right panels demonstrates that $1 \mu\text{M}$ is a saturating concentration.

to spinal laminae I and II, the responses were tightly distributed about the mean, which was less than the mean of randomly selected cells. Thus, cells in this identified subset respond predictably to opioids whereas randomly selected DRG cells vary widely in their responses. This implies that the variability among randomly selected DRG neurons reflects the presence of various subsets that differ in opioid sensitivity.

Considering our starting hypothesis to be that nociceptive neurons should be more sensitive to opioids than non-nociceptive neurons, the results provide two surprises: (1) cells that preferentially project to laminae I and II, the projection site for nociceptive neurons (Christensen and Perl, 1970; Kumazawa and Perl, 1978; Sugiura et al., 1986; Perl, 1991), were not especially sensitive to opioids; (2) about 90% of randomly chosen neurons are sensitive to opioids. The simplest interpretation is that most sensory neurons are opioid sensitive and nociceptive neurons are not more sensitive than others. If these results at the cell soma reflect the sensitivity of the presynaptic terminals, they suggest that analgesic effects of opioids on sensory neurons are due to anatomy (the specific arrangement of opioid synapses onto nociceptive neurons rather than nociceptive neurons being hypersensitive to opioids). But any such interpretation must be tentative because non-nociceptive neurons, including thermoreceptors and C-mechanoreceptors, also project to laminae I and II (Perl, 1991). The possibility remains that the antibodies label a subset of cells that are not nociceptive.

Subdividing cultured sensory neurons

Is there a best strategy for identifying sensory modality in neurons that have been excised from the animal? The simplest is

measurement of cell body diameter; this is of interest because small-diameter axons conduct signals slowly and tend to encode high-threshold stimuli (Hursh, 1939; Gasser, 1955). Scroggs and Fox (1992) have discovered that giant T-type Ca^{2+} currents

Figure 4. Antibody binding does not affect the amplitude of the DAGO response. *a*, Ca^{2+} currents recorded with and without DAGO before the cell was labeled. *b*, The response of the same cell after labeling with the 1B2 antibody and the FITC-conjugated secondary antibody. Voltage steps are from -70 mV to $+20$ mV. Many cells on the gridded coverslip were tested before the labeling procedure. After the antibody incubations, the previously recorded cells were located and this cell was found to be labeled; it was then patch clamped again and tested with DAGO. The large, slow-capacity transient (upward deflection) is due to the presence of processes extending from the cell's soma; such cells were selected for this control experiment because they better withstood the abuse of multiple recordings and antibody washings. No capacity subtraction has been done. Note that the overall Ca^{2+} current is less in *b*, but the percentage of decline with DAGO is about the same.

Figure 5. Histograms of the number of cells showing various degrees of inhibition by DAGO. *Top*, randomly selected cells; *upper middle*, cells that had innervated hindlimb muscles; *lower middle*, cells that bound the 1B2 antibody; *bottom*, cells that both bound the 1B2 antibody and had innervated hindlimb muscles. Percentage inhibition was calculated as the fractional decrease in peak Ca^{2+} current amplitude upon exposure to $1 \mu\text{M}$ DAGO (as in Fig. 3).

consistently appear in rat DRG neurons that have a specific range of soma diameters ($33\text{--}38 \mu\text{m}$). In this case, simply observing soma diameters led to a subset that we did not find using four different monoclonal antibodies (Table 1). The result may mean that some sensory modalities involve cells of uniform soma diameters. (We found no correlation between T-type Ca^{2+} current density and cell capacitance in our neurons. Since most of our recordings were on cells kept 1 d in culture, extending processes may have increased capacitance to a variable degree in different cells, thereby blurring correlations between current and capacitance.)

Although the Scroggs and Fox result demonstrates a meaningful correlation between soma diameter and ion channel expression, soma diameter cannot generally be expected to indicate sensory modality. Whereas very small and very large cell

somas have axons with slow and fast conduction velocities, respectively, no correlation exists between soma size and conduction velocity for cells in the broad, intermediate size range ($35\text{--}50 \mu\text{m}$ soma diameter; Lee et al., 1986). Moreover, different unmyelinated axons with low conduction velocities ($<1.5 \text{ m/sec}$) encode a variety of different sensations including innocuous touch and temperature (Perl, 1991).

Use of the Dodd/Jessel monoclonal antibodies is also imperfect. The antibodies label cells that project to particular laminae; thus, they can reflect sensory modality with no more certainty than does laminar projection site. As mentioned above, nociceptors project to laminae I and II, but not all cells projecting to laminae I and II are nociceptors. Perl and colleagues have attempted to correlate further the antibodies to sensory modality by labeling cells after their sensory modality has been determined *in vivo* (Perl, 1991). It is a heroic experiment because only one cell per DRG can be investigated and the cell must not suffer damage from the recording if it is to be successfully labeled with antibody. They have tested the SSEA3 and SSEA4 antibodies, finding that (1) SSEA4 labels cells encoding a variety of different cutaneous sensations, (2) only high-threshold mechanoreceptors have been double labeled with both SSEA3 and SSEA4, and (3) muscle spindle cells and myelinated fibers innervating hair follicles were never labeled with either antibody. The results indicate that the correct antibody, or combination of antibodies, might identify sensory modality, but such identification is not possible without extensive prior work. It's unfortunate that our results cannot be correlated with certainty to any sensory modality. Nevertheless, differences in ion channel expression and modulation among identified DRG subsets, as seen in our opioid work and the Scroggs and Fox work on T-channels, clearly imply functional differences among neurons that transduce different sensory modalities.

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