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Serotonin-gated inward currents are three times more frequent in rat hairy skin sensory afferents than in those innervating the skeletal muscle

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Abstract

Tight whole-cell patch clamp was performed in 191 DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate) retrogradely labeled rat sensory afferents from skin shoulders ($n = 93$) and biceps femoris muscles ($n = 98$). 5-HT-gated inward currents were evoked with 50-μM serotonin (5-HT; 5-hydroxytryptamine), and their frequency and current densities were compared between skin and skeletal muscle sensory afferents. To evaluate if 5-HT-gated inward currents coexist with other ligand-gated currents, the skin and skeletal muscle sensory afferents were also sequentially exposed to external solution at pH 6.8, ATP (50 μM), and capsaicin (1 μM). 5-HT evoked inward currents in 72% (67 of 93) of hairy skin sensory afferents and in only 24% (24 of 98) of skeletal muscle sensory afferents, and this difference was statistically significant ($p < 0.0000$, chi-square test). The current densities obtained in hairy skin and skeletal muscle sensory afferents were not significantly different. They were -45.8 ± 7.7 and -32.4 ± 10.5 pA/pF, respectively (mean ± SEM, $p < 0.30734$). These results indicate that 5-HT-gated inward currents are three times more frequently evoked in small- to medium-sized sensory afferents (25–40 μm) innervating the hairy skin than on those innervating the skeletal muscle. When cells were gathered in two clusters, the difference was four times larger in the small-sized cluster (25–32 μm) and two times larger in the medium-sized cluster (33–40 μm). The results can be explained if the superficial somatic (cutaneous) nociceptive system is more exposed than the deep somatic nociceptive system (musculoskeletal) to physical and chemical stimuli inducing 5-HT-mediated inflammatory pain.

Keywords

5-HT-gated inward currents, sensory neurons, nociception, inflammatory pain, skin, skeletal muscle, dorsal root ganglion cells, rat

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Introduction

Nociceptive pain has different characteristics depending on the affected tissue. Thus, superficial cutaneous pain is subjectively described as a well-localized sharp, pricking, or burning sensation; musculoskeletal deep somatic pain, as a diffuse, dull, or aching sensation; and visceral pain, as a deep cramping sensation that may be referred.[1] In general, it is considered that musculoskeletal pain is more diffuse and longer lasting than cutaneous pain,[2,3] and these differences in quality may rely on peripheral and central mechanisms.[4] Like that, muscle pain more strongly activates regions of the brain associated with emotional processing than skin pain,[5] and peripheral nociceptors are considered a heterogeneous population of neuronal afferents that are classified in several ways, such as by their expression of transducer molecules or peptidergic content.[6] The functional significance of this heterogeneity has been demonstrated, and various subtypes of nociceptors are thought to contribute in diverse forms to inflammatory and neuropathic chronic pain.[7] The study of these differences is helpful in guiding novel therapeutic strategies to deal with chronic pain.

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The differences between musculoskeletal and cutaneous afferents can also be observed in nociceptive activation. Hence, small muscle afferents express more acid-sensing ion channels 3 (51%) when compared to cutaneous afferents (28%).[8] In contrast, muscle afferents immunoreactive for the P2X3 receptor were only 3% when compared to cutaneous (37%) and visceral (31%) afferents.[9] No difference has been observed on transient receptor potential (TRP) V1 and TRPV2 expression in small cutaneous CGRP + afferents (53.5% TRPV1 + and 11.6% TRPV2+) and small muscle CGRP + afferents (53.1% TRPV1 + and 8.8% TRPV2+, respectively).[10] On the other hand, 5-HT is considered to play an important role in pain perception,[11] and the nociceptive role of 5-HT_3 receptors is relatively well established in several tissues,[12–15] particularly in the colon,[16] where antagonists of the 5-HT_3 receptors are used to treat the pain related to the irritable bowel syndrome.[17] Nevertheless, although the distribution of 5-HT_3 receptors has been relatively well assessed in the gastrointestinal tract,[18,19] it remains unclear in tissues, such as the skin and the skeletal muscle.

5-HT mediates inflammatory pain in the skin in conditions, such as contact dermatitis,[20] skin burning,[14] and itching;[14] and in skeletal muscle in conditions, such as chronic myalgia[12,13] and neuropathic pain.[6,7] In order to assess the differences in 5-HT_3 receptor activation from skeletal muscle and skin sensory afferents, in this study, the frequency of 5-HT-activated inward currents in DiI retrogradely labeled rat sensory neurons from skin shoulders and biceps femoris muscles was compared.

Materials and methods

Sensory afferents retrograde labeling

Forty-one Sprague-Dawley male rats from 250 to 300 g were anesthetized by intramuscular injection of 1 mL/kg rat anesthetic containing in mg/mL, Ketamine 80 and Xylazine 10. In all rats, the skin was shaved and opened by four incisions: two paraspinal to shoulder level and two on top of each of the biceps femoris muscles. Sensory afferents innervating hairy skin and skeletal muscle were labeled by retrograde transport of a lipophilic fluorescent dye DiI (Molecular Probes, Eugene, OR). DiI was injected intradermically in both shoulders and inside both biceps femoris muscles. Ten microliters of a suspension of 25 mg/mL of DiI (previously dissolved at 5% in DMSO) in external saline solution was injected per point, divided into 10 injections of 1 μL each. When no leakage of DiI or bleeding was observed, the four incisions were sutured with Nylon, which was removed one week later. Once DiI was injected, the rats were taken for two weeks to the housekeeping and animal care unit prior to their sacrifice for primary cell cultures preparation. The animals were treated in accordance with the principles of the Guide to the Use of Experimental Animals of the National Institute of Health (USA). All experimental protocols were revisited and approved by the Animal Care and Use Committee of Universidad del Valle.

Sensory afferents primary culture and identification

Rats injected with DiI weighting 300 to 350 g were deeply sedated with isoflurane (50%, Baxter) in a gas chamber and immediately decapitated. After death, the spinal column was removed and dissected. Dorsal root ganglia (DRG) were dissected free using fine forceps and microdissecting scissors. The right and left DRG from C_4 to T_2 for hairy skin afferents and from L_2 to L_6 for skeletal muscle afferents were collected. These ganglia were cut into pieces and underwent enzymatic dissociation successively in papain and collagenase/dispase solutions; this was followed by trituration in Hanks solution. The dissociated cells were then plated on polylysine- and laminin-coated glass coverslips in F12 medium (Gibco BRL) plus nerve growth factor (NGF; 50 ng/mL, Biomedical Technologies, Inc.) at 37 °C in 5% CO_2. After 2 h, the medium was changed to L15 (Gibco BRL) plus NGF, and the cells were maintained at 22 °C in humidified air. Petri dishes filled with external solution and one coverslip attached were mounted on a Nikon Diaphot inverted microscope with epifluorescence equipment. Afferent cells were identified by fluorescence microscopy (filter set XF102, Omega Optical). Only intensely fluorescent cells were assessed. Electrophysiological recordings were completed on 25- to 40-μm-sized labeled sensory afferents within 12 to 24 h after plating. Cells smaller than 25 μm were not studied since they were poorly labeled with DiI, and cells larger than 40 μm were not studied since most of them are considered as nonnociceptive sensory afferents. Cell soma diameter was estimated from the average of the longest and shortest axis as measured through an eyepiece micrometer scale. Only one cell was used per coverslip.

Electrophysiology

Patch-clamp experiments were conducted in the tight whole-cell configuration at room temperature. Ligand-gated currents were evoked sequentially by external solution at pH 6.8, 5-HT (50 μM), ATP (50 μM), and capsaicin (1 μM) on dissociated DiI labeled sensory afferents from the shoulders hairy skin and the biceps femoris muscles. This sequence remained always the same, taking into account that the main objective was to evaluate the responses to 5-HT and to avoid alterations in the

responses by modulation with 5-HT, ATP, or capsaicin. An extracellular pH 6.8 solution was used as a positive control of previously reported differences among sensory afferents from skin and skeletal muscle[8] and in order to avoid activation of capsaicin receptors. Holding potential was always −70 mV. The control external solution was continuously perfused during experiments and contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 MES, and 5 glucose, adjusted to pH 7.4 with N-Methyl-D-Glucamine. This solution was adjusted to pH 6.8 with HCl for the acid-evoking solution. Ligand-gated currents were evoked by perfusing the cells for 1 s with ATP, 2 s with external pH 6.8 or 5-HT, and 3 s with capsaicin, through an array of 10 μL capillary pipettes that were changed within 20 ms with a computer-driven solenoid valve system. 5-Hydroxytryptamine hydrochloride and ATP-Na_2 were added to the control external solution from a 50-mM stock prepared in water at pH 7.4 (stored at 4 °C and −20 °C, respectively). Capsaicin was prepared daily in ethanol (final concentration was 0.07%). Patch pipettes were pulled from borosilicate glass (Garner Glass, Claremont, CA) to 2.5–3 MΩ resistance. The standard internal solution contained (in mM): 135 methane sulfonic acid, 150 KOH, 10 KCl, 8 NaCl, 1 $MgCl_2$, 10 MOPS, 0.3 Na_3-GTP, 2 ATP-Mg, and 0.5 EGTA, adjusted to pH 7.0 with KOH. Data acquisition was performed with the pClamp 8.0 software (Axon Instruments, Inc.). All chemicals were from Sigma Co. unless otherwise stated.

Statistical analysis

Ligand-gated currents were analyzed with the Origin 7 (Microcal Software Inc.). Ligand-gated currents threshold was taken at 50 pA. Leak currents were subtracted off-line. Cell capacitance was calculated by integrating the capacitative transient (5 mV step, $V_h = -70$ mV). All data are presented as the mean ± SEM A two-sample *t* test was used to check if current densities were different among them (significance $p < 0.05$). The chi-square test (STATISCA 8.0, StatSoft, Inc.) was used to check the statistical significance of the differences in percentages found between skin and skeletal muscle afferents responding to the various stimuli (significance $p < 0.05$).

Results

5-HT-gated inward currents

Ligand-gated currents in small- to medium-sized (25–40 μm) sensory afferents labeled with DiI were examined. 5-HT (50 μM) evoked inward currents more frequently in rat sensory afferents innervating the hairy skin of shoulders than on those innervating the biceps femoris muscles. Indeed, 5-HT evoked inward currents in 72% (67 of 93) of hairy skin sensory afferents, but only 24% (24 of 98) of skeletal muscle sensory afferents were activated by 5-HT (Figure 1(c)). This difference was significant ($p < 0.0000$, chi-square test). The current densities evoked by 5-HT were not significantly different among hairy skin and skeletal muscle sensory afferents. They were -45.8 ± 7.7 and -32.4 ± 10.5 pA/pF, respectively (mean ± SEM, $p = 0.30734$; Figure 1(d)). Cell capacitance ranged from 27.5 ± 1.2 to 56.7 ± 1.3 pF in hairy skin afferents and from 26.8 ± 1.3 to 55.3 ± 1.1 pF in skeletal muscle afferents. Figure 1(a) and (b) shows typical currents evoked by 5-HT in the sensory afferents studied. Considering that this range of cell size is wide, cells were also grouped into two clusters and analyzed: one small-sized cell cluster from 25 to 32 μm and a medium-sized cell cluster from 33 to 40 μm.

In the two cell clusters, 5-HT-gated inward currents were more frequently evoked in sensory afferents innervating the hairy skin of shoulders than on those innervating the biceps femoris muscles. Certainly, in the 25–32 μm cell cluster, 5-HT evoked inward currents in 67% (44 of 66) of hairy skin sensory afferents, but only 16% (11 of 68) of skeletal muscle sensory afferents were activated by 5-HT (Figure 1(c)). This difference was significant ($p < 0.0000$, chi-square test). In this cluster (25–32 μm), current densities evoked by 5-HT were not significantly different amid hairy skin and skeletal muscle sensory afferents. They were -11.8 ± 1.1 and -7.7 ± 2.1 pA/pF, respectively (mean ± SEM, $p = 0.09906$; Figure 1(d)). Cell capacitance in this cluster ranged from 27.5 ± 1.2 to 31.4 ± 1.4 pF in hairy skin afferents and from 26.8 ± 1.3 to 32.6 ± 1.4 pF in skeletal muscle afferents. In the 33–40 μm cell cluster, 5-HT evoked inward currents in 85% (23 of 27) of hairy skin sensory afferents, but only 43% (13 of 30) of skeletal muscle sensory afferents were activated by 5-HT (Figure 1(c)). This difference was significant ($p < 0.0011$, chi-square test). In this cluster (33–40 μm), current densities evoked by 5-HT were not significantly different between hairy skin and skeletal muscle sensory afferents. They were -110.8 ± 10.4 and -53.3 ± 12.4 pA/pF, respectively (mean ± SEM, $p = 0.01827$; Figure 1(d)). Cell capacitance in this cell cluster ranged from 49.2 ± 1.1 to 56.7 ± 1.3 pF in hairy skin afferents and from 48.1 ± 0.9 to 55.3 ± 1.1 pF in skeletal muscle afferents. The current densities were significantly larger in the medium-sized cell cluster compared to the small-sized cell cluster ($p < 1.58086\text{E-}6$ for the skin afferents and $p < 0.02378$ for the skeletal muscle afferents).

Extracellular pH 6.8-gated inward currents

To assess if 5-HT-gated currents coexist with other ligand-gated currents in the hairy skin and skeletal muscle sensory afferents, the presence of low pH, ATP,

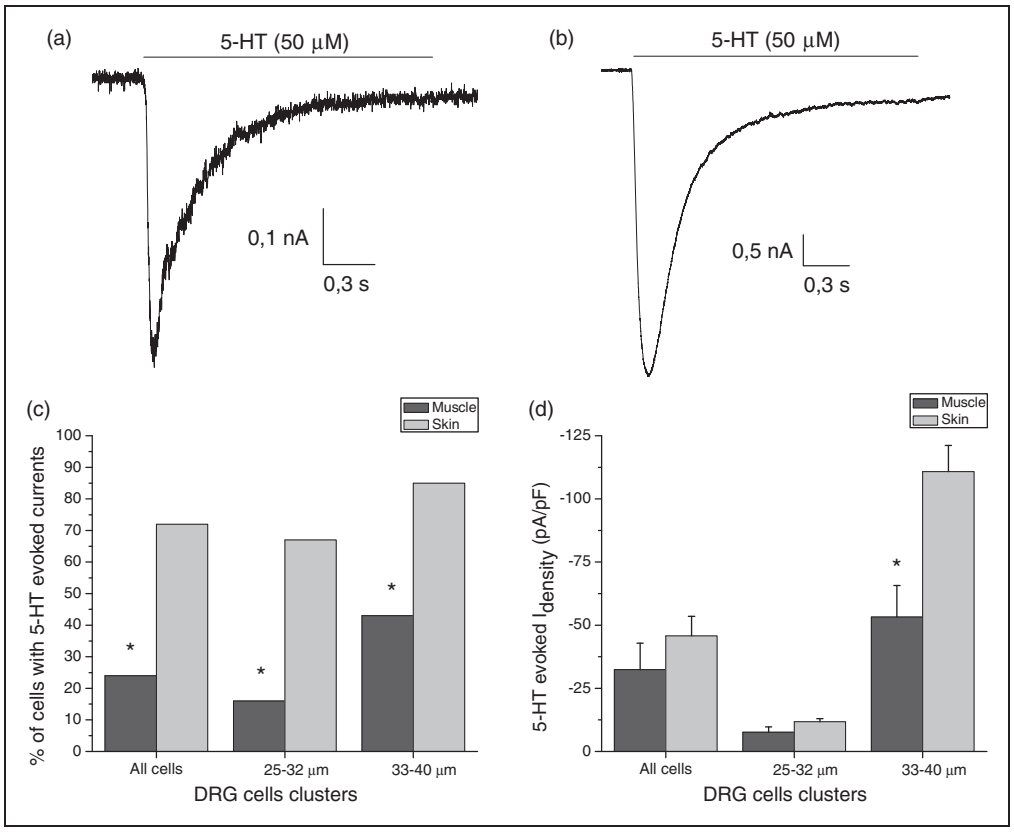

Figure 1. 5-HT evoked currents in the skin and the skeletal muscle rat sensory afferents. Typical current evoked by applying 5-HT (50 μM) to a 25–32 μm skeletal muscle sensory afferent (a). Typical current evoked by applying 5-HT (50 μM) to a 33–40 μm skin sensory afferent (b). Percentage of cells with 5-HT-gated inward currents in the skin and the skeletal muscle small- to medium-sized sensory afferents (c). Current density of 5-HT-gated inward currents in the skin and the skeletal muscle small- to medium-sized sensory afferents (d). For analysis purposes, the DRG cells studied were clustered all together or divided in two different groups (small-sized = 25–32 μm or medium-sized = 33–40 μM cell soma diameter). Data presented are mean ± SEM. The asterisks in (c) indicate that the frequency of 5-HT-gated inward currents in the skin and the skeletal muscle is significantly different ($p < 0.0000$ for all cells and the 25–32 μm group; $p < 0.0011$ for the 33–40 μM group). The asterisk in (d) indicates current densities that are statistically different between the skin and the skeletal muscle sensory afferents ($p < 0.01827$).
DRG: dorsal root ganglion.

and capsaicin-gated inward currents was also analyzed in the same cells exposed to 5-HT. An extracellular pH 6.8 solution evoked three types of inward currents in both cell clusters: one fast desensitizing, one slow desensitizing, and one fast–slow desensitizing (Figure 2(a)). In the 25–32 μm cell cluster, extracellular pH 6.8 evoked a fast desensitizing inward current in 23% (15 of 66) of hairy skin sensory afferents and in 68% (46 of 68) of skeletal muscle sensory afferents (marked as pH 6.8 (f) in Figure 2(b)). This difference was significant ($p < 0.0000$, chi-square test). The current densities evoked by the extracellular pH 6.8 solution were significantly different among the hairy skin and the skeletal muscle sensory afferents. They were -366.4 ± 52.1 and -211 ± 29.3 pA/pF, respectively (mean ± SEM, $p = 0.01592$; marked as pH 6.8 (f) in Figure 2(c)). In the 33–40 μm cell cluster, extracellular pH 6.8 evoked a fast desensitizing inward current in 81% (22 of 27) of the hairy skin sensory afferents and in 90% (27 of 30) of the skeletal muscle sensory afferents (marked as pH 6.8 (f) in Figure 2(d)). This difference was not significant ($p < 0.3552$, chi-square test). The current densities evoked by an extracellular pH 6.8 solution were not significantly different among the hairy skin and the skeletal muscle sensory afferents. They were -190.9 ± 20.8 and -127.4 ± 15.1 pA/pF, respectively (mean ± SEM, $p = 0.08786$; marked as pH 6.8 (f) in Figure 2(e)).

In the 25–32 μm cluster, the extracellular pH 6.8 solution gated also a slow desensitizing inward current in 32% (21 of 66) of hairy skin sensory afferents and in 38% (25 of 68) of skeletal muscle sensory afferents (marked as pH 6.8 (s) in Figure 2(b)). This difference was not significant ($p < 0.5466$, chi-square test). The current densities were not significantly different among the hairy skin and the skeletal muscle sensory afferents. They were -243.6 ± 51.5 and -280.3 ± 41.1 pA/pF,

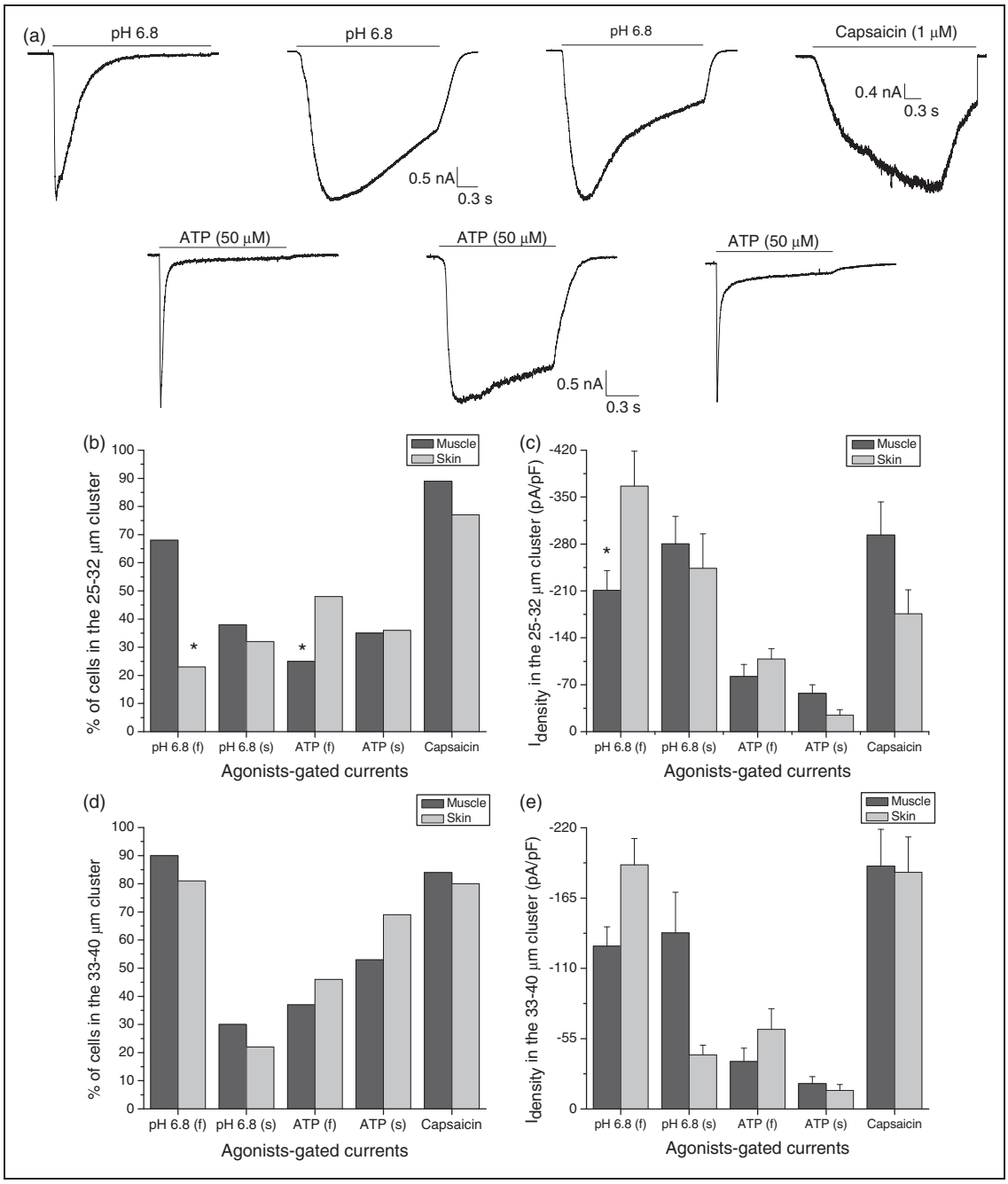

Figure 2. pH 6.8 external solution, capsaicin, and ATP evoked currents in the skin and the skeletal muscle rat sensory afferents. Typical currents evoked by pH 6.8 external solution and by applying capsaicin (1 μM) and ATP (50 μM) to a small- to medium-sized skin and skeletal muscle sensory afferents. The upper panel shows from left to right a pH 6.8-gated fast (f) desensitizing current, a pH 6.8-gated slow (s) desensitizing current, and a pH 6.8-gated fast–slow desensitizing current. Next, a capsaicin-gated current is shown. The lower panel shows from left to right an ATP-gated fast (f) desensitizing current, an ATP-gated slow (s) desensitizing current, and an ATP-gated fast–slow desensitizing current. (a) Percentage of cells with pH 6.8- or capsaicin- or ATP-gated currents in the skin and the skeletal muscle small-sized (25–32 μm) sensory afferents (b). Current densities of the pH 6.8- or capsaicin- or ATP-gated currents in the skin and the skeletal muscle small-sized (25–32 μm) sensory afferents (c). Percentage of cells with pH 6.8- or capsaicin- or ATP-gated currents in the skin and the skeletal muscle medium-sized (33–40 μm) sensory afferents (d). Current densities of the pH 6.8- or capsaicin- or ATP-gated currents in the skin and the skeletal muscle medium-sized (33–40 μm) sensory afferents (e). Data presented are mean ± SEM. The asterisks in (b) indicate that the frequency of these ligand-gated inward currents in the skin and the skeletal muscle is significantly different ($p < 0.0000$ for pH 6.8 evoked fast desensitizing inward currents; $p < 0.0048$ for pH ATP evoked fast desensitizing inward currents). The asterisk in (c) indicates current densities that are statistically different between skin and muscle sensory afferents ($p < 0.01592$).

respectively (mean ± SEM, $p = 0.58085$; marked as pH 6.8 (s) in Figure 2(c)). In the 33–40 μm cluster, the extracellular pH 6.8 solution evoked a slow desensitizing inward current in 22% (6 of 27) of hairy skin sensory afferents and in 30% (9 of 30) of skeletal muscle sensory afferents (marked as pH 6.8 (s) in Figure 2(d)). This difference was not significant ($p < 0.5055$, chi-square test). The current densities were not significantly different amid the hairy skin and the skeletal muscle sensory afferents. They were -42.2 ± 7.6 and -137.7 ± 32 pA/pF, respectively (mean ± SEM, $p = 0.07002$; marked as pH 6.8 (s) in Figure 2(e)). It should be noted that in the 25–32 μm skin afferents, 30% (9 of 30) of the extracellular pH 6.8-gated currents were fast desensitizing, 50% (15 of 30) slow desensitizing, and 20% (6 of 30) fast–slow desensitizing. In the 25–32 μm skeletal muscle afferents, 58% (35 of 60) of the extracellular pH 6.8-gated currents were fast desensitizing, 24% (14 of 60) slow desensitizing, and 18% (11 of 60) fast–slow desensitizing. In the 33–40 μm skin afferents, 74% (17 of 23) of the extracellular pH 6.8-gated currents were fast desensitizing, 4% (1 of 23) slow desensitizing, and 22% (5 of 23) fast–slow desensitizing. Finally, in the 33–40 μm skeletal muscle afferents, 69% (20 of 29) of the extracellular pH 6.8-gated currents were fast desensitizing, 7% (2 of 29) slow desensitizing, and 24% (7 of 29) fast–slow desensitizing. When the extracellular pH 6.8-gated currents were fast–slow desensitizing, both components were counted to calculate the percentage of positive cells, but for the current density, only the peak current of the fast component was taken into account.

Extracellular ATP-gated inward currents

ATP (50 μM) evoked three types of currents in both clusters of cells: one fast desensitizing, one slow desensitizing, and one fast–slow desensitizing (Figure 2(a)). In the 25–32 μm cluster, ATP evoked a fast desensitizing inward current in 48% (32 of 66) of hairy skin sensory afferents and in 25% (17 of 68) of skeletal muscle sensory afferents (marked as ATP (f) in Figure 2(b)). This difference was significant ($p < 0.0048$, chi-square test). The current densities were not significantly different amid the hairy skin and the skeletal muscle sensory afferents. They were -108.2 ± 15.6 and -82.3 ± 17.9 pA/pF, respectively (mean ± SEM, $p = 0.28137$; marked as ATP (f) in Figure 2(c)). In the 33–40 μm cluster, ATP evoked a fast desensitizing inward current in 46% (12 of 26) of hairy skin sensory afferents and in 37% (11 of 30) of skeletal muscle sensory afferents (marked as ATP (f) in Figure 2(d)). This difference was not significant ($p < 0.4717$, chi-square test). The current densities were not significantly different among the hairy skin and the skeletal muscle sensory afferents. They were -62.3 ± 16.1 and -37.2 ± 10.4 pA/pF, respectively (mean ± SEM, $p = 0.36648$; marked as ATP (f) in Figure 2(e)).

In the 25–32 μm cluster, ATP also gated a slow desensitizing inward current in 36% (24 of 66) of hairy skin sensory afferents and in 35% (24 of 68) of skeletal muscle sensory afferents (marked as ATP (s) in Figure 2(b)). This difference was not significant ($p < 0.8973$, chi-square test). The current densities were not significantly different amid the hairy skin and the skeletal muscle sensory afferents. They were -24.5 ± 8.2 and -57.2 ± 12.7 pA/pF, respectively (mean ± SEM, $p = 0.05709$; marked as ATP (s) in Figure 2(c)). In the 33–40 μm cluster, ATP evoked a slow desensitizing inward current in 69% (18 of 26) of hairy skin sensory afferents and in 53% (16 of 30) of skeletal muscle sensory afferents (marked as ATP (s) in Figure 2(d)). This difference was not significant ($p < 0.2244$, chi-square test). The current densities were not significantly different amid the hairy skin and the skeletal muscle sensory afferents. They were -14.5 ± 4.6 and -19.9 ± 5.4 pA/pF, respectively (mean ± SEM, $p = 0.59313$; marked as ATP (s) in Figure 2(e)). It should be noted that in the 25–32 μm skin afferents, 46% (20 of 44) of the extracellular ATP-gated currents were fast desensitizing, 27% (12 of 44) slow desensitizing, and 27% (12 of 44) fast–slow desensitizing. In the 25–32 μm skeletal muscle afferents, 32% (11 of 35) of the extracellular ATP-gated currents were fast desensitizing, 51% (18 of 35) slow desensitizing, and 17% (6 of 35) fast–slow desensitizing. In the 33–40 μm skin afferents, 18% (4 of 22) of the extracellular ATP-gated currents were fast desensitizing, 46% (10 of 22) slow desensitizing, and 22% (36 of 22) fast–slow desensitizing. Finally, in the 33–40 μm skeletal muscle afferents, 16% (3 of 19) of the extracellular ATP-gated currents were fast desensitizing, 42% (8 of 19) slow desensitizing, and 42% (8 of 19) fast–slow desensitizing. When the extracellular ATP-gated currents were fast–slow desensitizing, both components were counted to calculate the percentage of positive cells, but for the current density only the peak current of the fast component was taken into account.

Capsaicin-gated inward currents

Capsaicin (1 μM) evoked inward currents with variable kinetics in both cell clusters. For analyses purposes, they were reunited as capsaicin positive cells. Figure 2(a) shows a typical capsaicin-gated current recorded during this study. In the 25–32 μm cluster, capsaicin evoked inward currents in 77% (51 of 66) of hairy skin sensory afferents and in 89% (61 of 68) of skeletal muscle sensory afferents (marked as capsaicin in Figure 2(b)). This difference was not significant ($p < 0.0521$, chi-square test). The current densities were not significantly different among the hairy skin and the skeletal muscle sensory

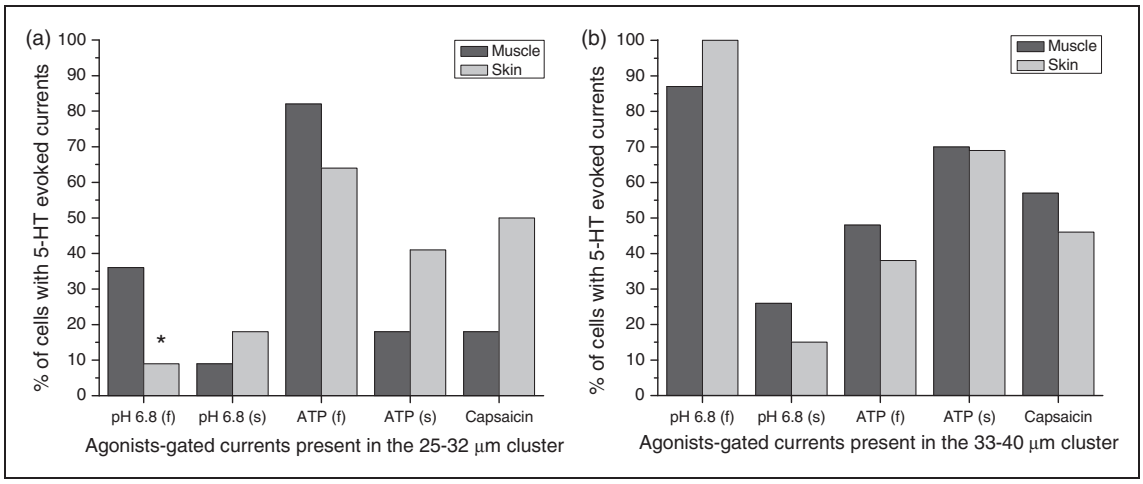

Figure 3. Percentage of skin and skeletal muscle small- to medium-sized sensory afferents with 5-HT-gated inward currents that also have pH 6.8- or capsaicin- or ATP-gated currents. Percentage of cells with 5-HT-gated inward currents that also have pH 6.8- or capsaicin- or ATP-gated currents in the skin and the skeletal muscle small-sized (25–32 μm) sensory afferents (a). Percentage of cells with 5-HT-gated inward currents that also have pH 6.8- or capsaicin- or ATP-gated currents in the skin and the skeletal muscle medium-sized (33–40 μm) sensory afferents (b). (f) means fast desensitizing and (s) means slow desensitizing (s). The asterisks in (a) indicate that the frequency of these ligand-gated inward currents in the skin and the skeletal muscle is significantly different ($p < 0.0218$ for pH 6.8 evoked fast desensitizing inward currents).

afferents. They were -175.9 ± 35.8 and -293.6 ± 49.1 pA/pF, respectively (mean ± SEM, $p = 0.05824$; marked as capsaicin in Figure 2(c)). In the 33–40 μm cluster, capsaicin evoked inward currents in 80% (21 of 26) of hairy skin sensory afferents and in 83% (25 of 30) of skeletal muscle sensory afferents (marked as capsaicin in Figure 2(d)). This difference was not significant ($p < 0.8027$, chi-square test). The current densities were not significantly different among the hairy skin and the skeletal muscle sensory afferents. They were -185.2 ± 27.6 and -190 ± 28.8 pA/pF, respectively (mean ± SEM, $p = 0.93159$; marked as capsaicin in Figure 2(e)).

Sensory afferents with 5-HT-gated inward currents are endowed with other ligand-gated inward currents

Considering that around 80% of the cells studied were sensitive to capsaicin, it was analyzed if sensory afferents with 5-HT-gated currents also have capsaicin or extracellular ATP or extracellular pH 6.8-gated currents. In the 25–32 μm cluster, 9% (4 of 44) of hairy skin and 36% (4 of 11) of skeletal muscle sensory afferents also had an extracellular pH 6.8-gated fast desensitizing inward current (marked as pH 6.8 (f) in Figure 3(a)), and this difference was significant ($p < 0.0218$, chi-square test). Eighteen percent (8 of 44) of hairy skin and 9% (1 of 11) of skeletal muscle sensory afferents also had and extracellular pH 6.8-gated slow desensitizing inward current (marked as pH 6.8 (s) in Figure 3(a)), and this difference was not significant ($p < 0.4660$, chi-square test). Sixty-four percent (28 of 44) of hairy skin and 82% (9 of 11) of skeletal muscle sensory afferents also had an ATP-gated fast desensitizing inward current (marked as ATP (f) in Figure 3(a)), and this difference was not significant ($p < 0.2504$, chi-square test). Forty-one percent (18 of 44) of hairy skin and 18% (2 of 11) of skeletal muscle sensory afferents also had an ATP-gated slow desensitizing inward current (marked as ATP (s) in Figure 3(a)), and this difference was not significant ($p < 0.1611$, chi-square test). Fifty percent (22 of 44) of hairy skin and 18% (2 of 11) of skeletal muscle sensory afferents also had a capsaicin-gated inward current (marked as capsaicin in Figure 3(a)), and this difference was not significant ($p < 0.0570$, chi-square test).

In the 33–40 μm cluster, 87% (20 of 23) of hairy skin and 100% (13 of 13) of skeletal muscle sensory afferents also had an extracellular pH 6.8-gated fast desensitizing inward current (marked as pH 6.8 (f) in Figure 3(b)), and this difference was not significant ($p < 0.1738$, chi-square test). Twenty-six percent (6 of 23) of hairy skin and 15% (2 of 13) of skeletal muscle sensory afferents also had an extracellular pH 6.8-gated slow desensitizing inward current (marked as pH 6.8 (s) in Figure 3(b)), and this difference was not significant ($p < 0.4582$, chi-square test). Forty-eight percent (11 of 23) of hairy skin and 38% (5 of 13) of skeletal muscle sensory afferents also had an ATP-gated fast desensitizing inward current (marked as ATP (f) in Figure 3(b)), and this difference was not significant ($p < 0.5870$, chi-square test). Seventy percent (16 of 23) of hairy skin and 69% (9 of 13) of skeletal muscle sensory afferents also had an ATP-gated slow desensitizing inward current (marked as ATP (s) in Figure 3(b)), and this difference was not significant

($p < 0.9833$, chi-square test). Fifty-seven percent (13 of 23) of hairy skin and 46% (6 of 13) of skeletal muscle sensory afferents also had a capsaicin-gated inward current (marked as capsaicin in Figure 3(b)), and this difference was not significant ($p < 0.5495$, chi-square test).

Discussion

Serotonin (5-HT) is considered a mediator of inflammatory pain in skin[14] and skeletal muscle.[12] In the periphery, 5-HT can be released from platelets, mast cells, or basophils that infiltrate an area of tissue damage. In this study, it was assessed if small- to medium-sized sensory afferents innervating the hairy skin are endowed with 5-HT-gated inward currents as frequently as sensory afferents innervating the skeletal muscle. It was found that (1) 5-HT-gated inward currents are three times more frequently evoked in small- to medium-sized sensory afferents innervating the hairy skin than on those innervating the skeletal muscle, and this difference was significant. (2) This difference is larger in small-sized (four times in 25–32 μm) than in medium-sized (two times in 33–40 μm) sensory afferents. (3) The density of the currents evoked by 5-HT tended to be larger in the hairy skin than on the skeletal muscle sensory afferents, but they were not significantly different. (4) The density of the currents evoked by 5-HT tended to be larger in medium-sized than in small-sized neurons, in both the hairy skin and the skeletal muscle sensory afferents. (5) Although the sensory afferents endowed with 5-HT-gated currents are endowed with various other types of ligand-gated currents, in the small-sized neurons, they were observed more frequently associated with an ATP-gated fast desensitizing current, and in the medium-sized neurons, they were more commonly associated with an extracellular pH 6.8-gated fast desensitizing current, and an ATP-gated slow desensitizing current.

In sensory neurons, 5-HT-gated inward currents are mediated by 5-HT_3 receptors, which are ligand-gated channels of the pentameric receptor superfamily that are permeable to Na^+, K^+, and Ca^{2+}.[21,22] The 5-HT_3 receptor family comprises the homomeric 5-HT_{3A} receptor and heteromeric receptors including the combination of the A subunit with B, C, D, or E subunits.[22] The best characterized are 5-HT_{3A} and 5-HT_{3AB} receptors.[23] The presence of the 5-HT_3 receptor mRNA has been demonstrated in rat DRGs.[24,25] Using in situ hybridization histochemistry, it was determined that 5-HT_{3B} subunit mRNA is expressed in 43% of rat DRG neurons, and the 5-HT_{3A} subunit is expressed in 70% of rat DRG neurons.[26] The expression of both subunits was similar (around 40%) in small- to medium-sized (26 to 40 μm) DRG neurons.[26,27] However, another study indicates that only 20% of small- to medium-sized (25 to 45 μm) DRG neurons express the 5-HT_{3B} subunit.[28] Using electrophysiology, it was previously observed that 5-HT evoked inward currents in around 50% of DRG cells randomly assessed, and no difference was noticed with heart sensory afferents.[29] These results indicate that 5-HT_3 receptors may be expressed in 50%–70% of rat sensory afferents, being 5-HT_{3A} the dominant subunit present in those receptors. In the present study, 5-HT evoked inward currents in 48% of the small- to medium-sized (25–40 μm) sensory afferents.

The presence of 5-HT_3 receptors in DRG neurons of different sizes allows to suggest that they may convey nociceptive and mechanosensitive information.[26] Here, it was observed that an average of 53% of the DRG cells that were positive to 5-HT were also positive to extracellular pH 6.8, an association that was robust in medium-sized (33–40 μm) sensory afferents. Medium-sized (33–40 μm) sensory afferents positive to extracellular pH 6.8-gated fast desensitizing currents have been classified as nociceptors and mechanoreceptors.[30,31] However, it has been observed that sensory nerve terminals in the Merkel cell-nerve endings showed strong positive immunoreactions of 5-HT_{1A} and 5-HT_{1B} receptors but not of 5-HT_3 receptors,[32] indicating that 5-HT-gated inward currents would not be mechanosensitive in the skin.

On the other hand, the nociceptive role of 5-HT_3 receptors is relatively well established.[11–14] In the present study, it was observed that an average of 80% of the DRG cells that were positive to 5-HT were also positive to ATP. The association with ATP-gated fast desensitizing currents was stronger in small (25–32 μm) sensory afferents, and the association with ATP-gated slow desensitizing currents was stronger in medium (33–40 μm) sensory afferents. Small-sized (25–32 μm) sensory afferents with ATP-gated fast desensitizing currents have been classified as nociceptors.[30,31] These ATP-gated currents have been associated in sensory neurons to $P2X_1$ and $P2X_3$ receptors.[33] It has been demonstrated that most of the small sensory neurons have $P2X_3$ mRNA and that this receptor is mainly expressed in C-fiber neurons.[34] Thus, regarding the present results, it can be argued that at least 64% of the sensory afferents activated by 5-HT in the hairy skin and 82% from the skeletal muscle may be C-type nociceptors. On the other hand, sensory neurons with ATP-gated slow desensitizing currents, associated to $P2X_2$ and $P2X_{2/3}$ receptors, have been classified as nociceptors.[35] Therefore, it can be proposed that from the medium sensory afferents activated by 5-HT, at least 70% from the hairy skin and 69% from the skeletal muscle may be Aδ-type nociceptors.

Subsequently, considering their strong association with ATP-gated currents, it can be suggested that the role of 5-HT evoked currents would be mainly

nociceptive, and they would be mediating inflammatory pain, just like the one that happens in contact dermatitis,[20] skin burning,[14] itching,[14] and chronic myalgia.[12,13] Accordingly, after functional elimination of the receptor, it has been demonstrated that 5-HT_3 receptors are not required for acute nociception in response to physiological stimuli.[15] Instead, the 5-HT_3 receptor seems to contribute to persistent nociceptive processing without inducing a concomitant edema in the setting of tissue injury, and the peripheral 5-HT_3 receptor contribution would be via an action on both myelinated (Aδ afferents) and unmyelinated (nopeptidergic C-fibers) nociceptors.[15] It should be noticed that in that study, it was also found that most of nociceptors that express the 5-HT_3 receptors are capsaicin insensitive (only 13% were positive).[15] In the present study, an average of 47% of the DRG cells that were positive to 5-HT were also positive to capsaicin. The reason for the discrepancy is not clear but could be related to the use in the current study of acutely dissociated primary afferents cultured in the presence of NGF, a condition that may be upregulating the expression of ligand-gated ion channels.[36–39]

In the present study, it was observed that 5-HT-gated inward currents are three times more frequently evoked in small- to medium-sized sensory afferents innervating the hairy skin than on those innervating the skeletal muscle. This strongly suggests that small to medium (25–40 μm) rat sensory neurons innervating hairy skin are more likely to express 5-HT-gated ion channels than those that innervate skeletal muscle. This observation can be explained if 5-HT_3 receptors are part of a nociceptive system that is activated when tissues are exposed to physical and chemical stimuli that can be allergic and irritant by means of their nature and their concentration. In that sense, as the superficial somatic and the visceral nociceptive systems are more exposed to the external environment, they will be more exposed to those allergic and irritant stimuli than the deep somatic nociceptive system, therefore, due to that 5-HT_3 receptors will be highly expressed in the superficial somatic and in the visceral nociceptive systems than in the deep somatic nociceptive system. Indeed, the superficial somatic (cutaneous) nociceptive system mediates a sharp pricking or burning pain associated with ongoing activation of nociceptors in the skin, subcutaneous tissue, or mucous membranes, and it is produced by external mechanical, chemical, or thermal events and dermatologic disorders.[1] On the other side, the deep somatic (musculoskeletal) system mediates a dull or aching pain associated with ongoing activation of nociceptors in muscles, tendons, joint capsules, fasciae, or bones, and it is produced by overuse strain, mechanical injury, cramping, ischemia, or inflammation.[2,3]

Several studies indicate that sensory afferents innervating skin and skeletal muscle are diverse.[8,40–43] Certainly, nociceptors innervating muscle express more substance P and CGRP, and less isolectin B4 and somatostatin, when compared to nociceptors innervating skin.[41,42] Muscle afferents on the other side had the highest proportions of carbonic anhydrase, but the lowest proportions of profiles with lectin binding.[42] Likewise, using immunocytochemistry, retrograde labeling, and electrophysiology, it was previously reported that small (<25 μm) muscle sensory afferents are more likely to express acid-sensing ion channels 3 than those that innervate skin (51% of small muscle afferents vs. 28% of small skin afferents).[8] Besides, it has been shown in mice that TRPV1-positive afferents would comprise approximately 16% of all cutaneous c-fibers and approximately 40% of the WGA-labeled skeletal muscle sensory afferents.[40] Finally, it has been reported that small and medium diameter skin nociceptors express either TRPV1 or TRPV2 in isolation; meanwhile skeletal muscle nociceptors coexpressed both TRPs.[43]

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References

1. Berry PH, Chapman CR, Covington EC, et al. *Pain: current understanding of assessment, management, and treatments*. Reston, VA: National Pharmaceutical Council and the Joint Commission for the Accreditation of Healthcare Organizations, 2001. http://americanpainsociety.org/uploads/education/npc.pdf (accessed 16 August 2017).
2. Gregory NS and Sluka KA. Anatomical and physiological factors contributing to chronic muscle pain. In: Taylor BK and Finn D (eds) *Behavioral neurobiology of chronic pain*. Berlin Heidelberg: Springer, 2014, pp.327–348. https://link.springer.com/chapter/10.1007%2F7854_2014_294 (accessed 16 August 2017).
3. Sikandar S, Aasvang EK and Dickenson AH. Scratching the surface: the processing of pain from deep tissues. *Pain* 2016; 6: 95–102. https://www.futuremedicine.com/doi/abs/10.2217/pmt.15.50?journalCode=pmt (accessed 16 August 2017).

4. Arendt-Nielsen L and Yarnitsky D. Experimental and clinical applications of quantitative sensory testing applied to skin, muscles and viscera. *J Pain* 2009; 10: 556–572. http://www.sciencedirect.com/science/article/pii/S152659000900371X (accessed 16 August 2017).
5. Takahashi K, Taguchi T, Tanaka S, et al. Painful muscle stimulation preferentially activates emotion-related brain regions compared to painful skin stimulation. *Neurosci Res* 2011; 70: 285–293. http://www.sciencedirect.com/science/article/pii/S0168010211001027 (accessed 16 August 2017).
6. Gold MS and Gebhart GF. Nociceptor sensitization in pain pathogenesis. *Nat Med* 2010; 16: 1248–1257. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5022111/ (accessed 16 August 2017).
7. Cavanaugh DJ, Lee H, Lo L, et al. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc Natl Acad Sci U S A* 2009; 106: 9075–9080. http://www.pnas.org/content/106/22/9075.short (accessed 16 August 2017).
8. Molliver DC, Immke DC, Fierro L, et al. ASIC3, an acid-sensing ion channel, is expressed in metaboreceptive sensory neurons. *Mol Pain* 2005; 1: 35. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1308857/ (accessed 16 August 2017).
9. Bradbury EJ, Burnstock G and McMahon SB. The expression of P2X 3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Mol Cell Neurosci* 1998; 12: 256–268. http://www.sciencedirect.com/science/article/pii/S1044743198907199 (accessed 16 August 2017).
10. Tsukagoshi M, Goris RC and Funakoshi K. Differential distribution of vanilloid receptors in the primary sensory neurons projecting to the dorsal skin and muscles. *Histochem Cell Biol* 2006; 126: 343–352. https://link.springer.com/article/10.1007/s00418-006-0167-4 (accessed 16 August 2017).
11. Gerdle B, Ghafouri B, Ernberg M, et al. Chronic musculoskeletal pain: review of mechanisms and biochemical biomarkers as assessed by the microdialysis technique. *J Pain Res* 2014; 7: 313–326. http://thejournalofheadacheandpain.springeropen.com/articles/10.1186/1129-2377-15-63 (accessed 16 August 2017).
12. Gershon MD and Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology* 2007; 132: 397–414. http://www.sciencedirect.com/science/article/pii/S001650850602436X (accessed 16 August 2017).
13. Hoffman JM, Tyler K, MacEachern SJ, et al. Activation of colonic mucosal 5-HT(4) receptors accelerates propulsive motility and inhibits visceral hypersensitivity. *Gastroenterology* 2012; 142: 844–854. http://www.sciencedirect.com/science/article/pii/S0016508512000030 (accessed 16 August 2017).
14. Camilleri M, Chey WY, Mayer EA, et al. A randomized controlled clinical trial of the serotonin type 3 receptor antagonist alosetron in women with diarrhea-predominant irritable bowel syndrome. *Arch Intern Med* 2001; 161: 1733–1740. http://jamanetwork.com/journals/jamainternalmedicine/fullarticle/648702 (accessed 16 August 2017).
15. Christidis N, Kang I, Cairns BE, et al. Expression of 5-HT3 receptors and TTX resistant sodium channels (Na(V)1.8) on muscle nerve fibers in pain-free humans and patients with chronic myofascial temporomandibular disorders. *J Headache Pain* 2014; 15: 63. http://thejournalofheadacheandpain.springeropen.com/articles/10.1186/1129-2377-15-63 (accessed 16 August 2017).
16. Nordlind K, Azmitia EC and Slominski A. The skin as a mirror of the soul: exploring the possible roles of serotonin. *Exp Dermatol* 2008; 17: 301–311. http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0625.2007.00670.x/abstract (accessed 16 August 2017).
17. Zeitz KP, Guy N, Malmberg AB, et al. The 5-HT3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors. *J Neurosci* 2002; 22: 1010–1019. http://www.jneurosci.org/content/22/3/1010.long (accessed 16 August 2017).
18. Kirkup AJ, Brunsden AM and Grundy D. Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. *Am J Physiol Gastrointest Liver Physiol* 2001; 280: G787–G794. http://ajpgi.physiology.org/content/280/5/G787.long (accessed 16 August 2017).
19. Yaakob NS, Chinkwo KA, Chetty N, et al. Distribution of 5-HT3, 5-HT4, and 5-HT7 receptors along the human colon. *J Neurogastroenterol Motil* 2015; 21: 361–369. http://www.jnmjournal.org/journal/view.html?doi=10.5056/jnm14157 (accessed 16 August 2017).
20. El-Nour H, Lundeberg L, Boman A, et al. Study of innervation, sensory neuropeptides, and serotonin in murine contact allergic skin. *Immunopharmacol Immunotoxicol* 2005; 27: 67–76. http://www.tandfonline.com/doi/abs/10.1081/IPH-51617?journalCode=iipi20 (accessed 16 August 2017).
21. Loyd DR, Henry MA and Hargreaves KM. Serotonergic neuromodulation of peripheral nociceptors. *Semin Cell Dev Biol* 2013; 24: 51–57. http://www.sciencedirect.com/science/article/pii/S1084952112001620 (accessed 16 August 2017).
22. Niesler B, Walstab J, Combrink S, et al. Characterization of the novel human serotonin receptor subunits 5-HT3C, 5-HT3D, and 5-HT3E. *Mol Pharmacol* 2007; 72: 8–17. http://molpharm.aspetjournals.org/content/72/1/8.long (accessed 16 August 2017).
23. Miles TF, Lester HA and Dougherty DA. Allosteric activation of the 5-HT3AB receptor by mCPBG. *Neuropharmacology* 2015; 91: 103–108. http://www.sciencedirect.com/science/article/pii/S0028390814004699 (accessed 16 August 2017).
24. Chen JJ, Vasko MR, Wu X, et al. Multiple subtypes of serotonin receptors are expressed in rat sensory neurons in culture. *J Pharmacol Exp Ther* 1998; 287: 1119–1127. http://jpet.aspetjournals.org/content/287/3/1119.long (accessed 16 August 2017).
25. Pierce PA, Xie GX, Levine JD, et al. 5-Hydroxytryptamine receptor subtype messenger RNAs in rat peripheral sensory and sympathetic ganglia: a polymerase chain reaction study. *Neuroscience* 1996; 70: 553–559.

http://www.sciencedirect.com/science/article/pii/0306452295003290 (accessed 16 August 2017).
26. Morales M, McCollum N and Kirkness EF. 5-HT(3)-receptor subunits A and B are co-expressed in neurons of the dorsal root ganglion. *J Comp Neurol* 2001; 438: 163–172. http://onlinelibrary.wiley.com/doi/10.1002/cne.1307/abstract (accessed 16 August 2017).
27. Morales M and Wang SD. Differential composition of 5-hydroxytryptamine3 receptors synthesized in the rat CNS and peripheral nervous system. *J Neurosci* 2002; 22: 6732–6741. http://www.jneurosci.org/content/22/15/6732.long (accessed 16 August 2017).
28. Nicholson R, Small J, Dixon AK, et al. Serotonin receptor mRNA expression in rat dorsal root ganglion neurons. *Neurosci Lett* 2003; 337: 119–122. http://www.sciencedirect.com/science/article/pii/S0304394002012569 (accessed 16 August 2017).
29. Benson CJ, Eckert SP and McCleskey EW. Acid-evoked currents in cardiac sensory neurons a possible mediator of myocardial ischemic sensation. *Circ Res* 1999; 84: 921–928. http://circres.ahajournals.org/content/84/8/921.long (accessed 16 August 2017).
30. Petruska JC, Napaporn J, Johnson RD, et al. Subclassified acutely dissociated cells of rat DRG: histochemistry and patterns of capsaicin-, proton-, and ATP-activated currents. *J Neurophysiol* 2000; 84: 2365–2379. http://jn.physiology.org/content/84/5/2365.long (accessed 16 August 2017).
31. Petruska JC, Napaporn J, Johnson RD, et al. Chemical responsiveness and histochemical phenotype of electrophysiologically classified cells of the adult rat dorsal root ganglion. *Neuroscience* 2002; 115: 15–30. http://www.sciencedirect.com/science/article/pii/S0306452202004098 (accessed 16 August 2017).
32. Tachibana T, Endoh M, Fujiwara N, et al. Receptors and transporter for serotonin in Merkel cell-nerve endings in the rat sinus hair follicle. An immunohistochemical study. *Arch Histol Cytol* 2005; 68: 19–28. https://www.jstage.jst.go.jp/article/aohc/68/1/68_1_19/_article (accessed 16 August 2017).
33. Petruska JC, Cooper BY, Gu JG, et al. Distribution of P2X1, P2X2, and P2X3 receptor subunits in rat primary afferents: relation to population markers and specific cell types. *J Chem Neuroanat* 2000; 20: 141–162. http://www.sciencedirect.com/science/article/pii/S0891061800000806 (accessed 16 August 2017).
34. Kobayashi K, Fukuoka T, Yamanaka H, et al. Differential expression patterns of mRNAs for P2X receptor subunits in neurochemically characterized dorsal root ganglion neurons in the rat. *J Comp Neurol* 2005; 481: 377–390. http://onlinelibrary.wiley.com/doi/10.1002/cne.20393/abstract (accessed 16 August 2017).
35. Cockayne DA, Dunn PM, Zhong Y, et al. P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. *J Physiol* 2005; 567: 621–639. http://onlinelibrary.wiley.com/doi/10.1113/jphysiol.2005.088435/abstract;jsessionid=F1A3A9F820CA133A7E46B8CD3D62EF20.f02t02 (accessed 16 August 2017).
36. Furukawa K, Akaike N, Onodera H, et al. Expression of 5-HT3 receptors in PC12 cells treated with NGF and 8-Br-cAMP. *J Neurophysiol* 1992; 67: 812–819. http://jn.physiology.org/content/67/4/812.short (accessed 16 August 2017).
37. Isenberg KE, Ukhun IA, Holstad SG, et al. Partial cDNA cloning and NGF regulation of a rat 5-HT3 receptor subunit. *Neuroreport* 1993; 5: 121–124. http://journals.lww.com/neuroreport/abstract/1993/11180/partial_cdna_cloning_and_ngf_regulation_of_a_rat.6.aspx (accessed 16 August 2017).
38. Zur KB, Oh Y, Waxman SG, et al. Differential up-regulation of sodium channel α-and β1-subunit mRNAs in cultured embryonic DRG neurons following exposure to NGF. *Mol Brain Res* 1995; 30: 97–105. http://www.sciencedirect.com/science/article/pii/0169328X9400283K (accessed 16 August 2017).
39. Eskander MA, Ruparel S, Green DP, et al. Persistent nociception triggered by nerve growth factor (NGF) is mediated by TRPV1 and oxidative mechanisms. *J Neurosci* 2015; 35: 8593–8603. http://www.jneurosci.org/content/35/22/8593.short (accessed 16 August 2017).
40. Christianson JA, McIlwrath SL, Koerber HR, et al. Transient receptor potential vanilloid 1-immunopositive neurons in the mouse are more prevalent within colon afferents compared to skin and muscle afferents. *Neuroscience* 2006; 140: 247–257. http://www.sciencedirect.com/science/article/pii/S0306452206001928 (accessed 16 August 2017).
41. O'Brien C, Woolf CJ, Fitzgerald M, et al. Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint. *Neuroscience* 1989; 32: 493–502. http://www.sciencedirect.com/science/article/pii/0306452289900961 (accessed 16 August 2017).
42. Perry MJ and Lawson SN. Differences in expression of oligosaccharides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. *Neuroscience* 1998; 85: 293–310. http://www.sciencedirect.com/science/article/pii/S0306452297006295 (accessed 16 August 2017).
43. Rau KK, Jiang N, Johnson RD, et al. Heat sensitization in skin and muscle nociceptors expressing distinct combinations of TRPV1 and TRPV2 protein. *J Neurophysiol* 2007; 97: 2651–2662. http://jn.physiology.org/content/97/4/2651.long (accessed 16 August 2017).