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Abstract

The aim of the present study was to investigate the cause of normal response behaviors to noxious heat despite the marked loss of small neurons in the dorsal root ganglion (DRG) as a result of neonatal capsaicin treatment. Capsaicin (50 mg/kg) was injected subcutaneously (s.c.) into mice on either day P2 or P15; control mice received a vehicle injection. Twenty days after the capsaicin injection, 2% carrageenan (20 µL) was injected into the right hind paw of each animal. Twenty-four hours after the carrageenan injection, behavioral tests using noxious heat stimuli (NHS; Hargreaves method) and noxious mechanical stimuli (NMS; von Frey method) were performed using the control and capsaicin-treated mice. Pre-carrageenan measurements were used as the baseline values for each group. After the experiments, the mice were perfused with a mixture of 4% paraformaldehyde and 0.2% picric acid in a 0.1-M phosphate buffer (pH 7.4). The L4 and L5 DRGs were extracted, sectioned using a cryostat, and immunostained using a TRPV1 antibody and reacted with isolectin IB4. The P2 capsaicin-injected mice exhibited a marked increase in their analgesic responses to NHS, compared with both their baseline values and the respective control mice. Both the capsaicin-treated and control animals exhibited significant hyperalgesia in response to NMS. Naturally, the control P15 mice exhibited a shorter response time to NHS than their baseline values, while the capsaicin-injected P15 mice exhibited increased algesia comparable to the baseline values of the control. The capsaicin-injected mice also exhibited algesia in response to NMS. The number of TRPV1-immunoreactive (ir) small neurons decreased by 90% in the P2 capsaicin-injected mice and 45% in the P15 capsaicin-injected mice, whereas an increase in IB4-positive neurons was seen in both the P2 and P15 capsaicin-injected mice. In association with the decrease in larger neurons, the numbers of smaller neurons were increased in both P2 and P15 capsaicin-injected mice. TRPV1-ir small neurons are closely correlated with inflammatory heat pain perception, suggesting the enhancement of an unknown acute noxious heat sensor in the present study. The present findings indicate that noxious mechanical stimuli can be sensed despite the presence or absence of TRPV1-ir or IB4-positive neurons.

Introduction

Previously, we reported that mice treated with capsaicin on day 2 of life behaved normally in response to noxious heat stimuli (hot plate: 55°C, radiant heat) [1, 2]. This finding is very odd because the capsaicin-treated animals behaved normally despite the profound loss of capsaicin-sensitive, small dorsal root ganglion neurons and unmyelinated C-fibers [3, 4]. On the other hand, mice pretreated with capsaicin on day 15 after birth behaved abnormally in response to noxious heat, exhibiting severe delayed time thresholds in the hind paw withdrawal response (analgesia) [2]. Interestingly, capsaicin-receptor, TRPV1 knockout mice also behaved normally on a hot plate at 55°C but not at 52.5°C [5] and at 50°C and 52.5°C [6]. These paradoxical phenomena suggested the existence of a TRPV1-independent mechanism for acute noxious heat sensation [7, 8]. Woodbury et al. [9] hypothesized that two pain transduction mechanisms for noxious heat may exist: a TRPV1/2-independent mechanism and a TRPV1-dependent mechanism. The former mechanism would detect noxious heat under normal conditions without the presence of functional TRPV1 or TRPV2, while the latter would require functional TRPV1 under pathological conditions, such as inflammation and tissue damage. Recently, Jankowski et al. [10] suggested that TRPV1-positive (+) but IB4-negative (-) and only heat-sensitive cutaneous C-fibers are silent under normal conditions but can become functional under pathophysiological conditions. Thus, TRPV1 is believed to transduce pain sensation under inflammatory conditions, rather than under normal physiological conditions. However, many authors have raised the issue that the heat-sensing

mechanism cannot be simply explained by the presence of TRPV1 [7, 11, 12].

The presence of channels other than TRPV1 for sensing acute noxious heat has been repeatedly emphasized [13-15]. We hypothesized that the remaining capsaicin-insensitive, IB4 (+) nociceptive sensory neurons in mice treated with capsaicin on day 2 of life can sense acute physiological heat despite the severe loss of TRPV1 (+) neurons [14]. In contrast, a noxious heat-sensitive and IB4 (+) subgroup of small neurons may disappear in mice pretreated with capsaicin on day 15 of age, thereby leading to hypoalgesia (analgesia) to acute noxious heat. However, such animals would show thermal hyperalgesia because of the remaining TRPV1(+) neurons under inflammatory conditions. The focus of the present study was to confirm these ideas. Although the question of whether TRPV1(+) or IB4(+) neurons can also cause transduction in response to noxious mechanical stimuli is controversial [16, 17], mice treated with capsaicin on days 2 or 15 after birth were injected with carrageenan into the hind paw of each animal to investigate the effects of inflammation on the responses to noxious thermal and mechanical stimuli.

Materials And Methods

All the experiments performed in this study were conducted in accordance with the guidelines outlined in the “Legislation for the protection of animals used for scientific purposes” (Directive 2010/63/EU) and were approved by the Committee on Experimental Animals and the Ethical Treatment of Animals of the University of Tokushima.

Capsaicin (50 mg/kg) was injected subcutaneously (s.c.) into two groups of mice, i.e., 2 (n = 6) and 15 (n = 12) days after birth. Twenty days after the injection, 2% carrageenan (20 μL) was injected into the right plantar hind paw of each animal in both groups. The control mice were injected with the vehicle alone 2 (n = 8) and 15 (n = 12) days after birth. Twenty-four hours later, carrageenan was injected according to the time interval reported by Xu et al. [18]; behavioral tests using noxious heat stimuli (Hargreaves method, intensity: 20) and noxious mechanical stimuli (von Frey method) were then performed using control and capsaicin-treated animals from each experimental group. For each behavioral test, three measurements were performed at three-minute intervals. The results of tests performed before carrageenan injection were used as the baseline values. The mean threshold times for withdrawal latency and bending force in response to noxious heat and mechanical stimuli, respectively, were estimated. A P value less than 0.05 was regarded as significant (Student t-test).

At the end of the experiments, mice from each group were perfused with a mixture of 4% paraformaldehyde and 0.2% picric acid in a 0.1-M phosphate buffer. The lumbar dorsal root ganglia (L4 and L5 DRGs) were then excised, sectioned using a cryostat at a thickness of 40 μm, and stained using FITC-coupled plant isolectin B4 (IB4, 10 mg/mL; Vector Lab. Inc., Burlingame, USA) for 24 h at 4°C. The sections were then immunostained with rat polyclonal anti-TRPV1 antibody (diluted 1: 500; Alomone Labs Ltd., Israel) for 24 h at 4°C with gentle agitation, followed by staining with Texas Red conjugated goat anti-rabbit IgG (diluted 1: 400; Rockland Inc., Gilbertsville, USA). Specimens without the primary antibody and IB4 were used as controls. The precise staining procedures have been described elsewhere [19]. Immunofluorescence specimens were observed using a fluorescence microscope (DP70; Olympus, Tokyo, Japan). The number of TRPV1 (+) and IB4 (+) neurons was counted from 2-5 sections in each case. The long diameter of TRPV1 (+) and IB4 (+) neurons containing nuclei was measured directly on the monitor. Significant differences were estimated using the $\aleph^2$ test.

Results

Effects of carrageenan on the responses to noxious thermal and mechanical stimuli after treatment with capsaicin on day 2 after birth

As shown in Fig. 1, the baseline response to noxious heat was not significantly different between the control (2.7 ± 0.3 sec) and the capsaicin-injected P2 mice (3.2 ± 0.6 sec). To our surprise, the capsaicin-treated P2 mice exhibited a markedly increased analgesic response (6.2 ± 0.6 sec, $P < 0.01$) to noxious heat**,** compared with both the baseline value (3.2 ± 0.6 sec) and the respective control mice (2.7 ± 0.3 sec).

The threshold for noxious mechanical stimuli decreased significantly (hypersensitivity) at 24 hours after carrageenan injection in both the control (2.6 ± 0.5 g, $P < 0.01$) and the capsaicin-treated mice (2.0 ± 0.3 g, $P < 0.01$), compared with the baseline values (4.8 ± 0.4 g and 4.8 ± 0.6 g, respectively) (Fig. 1).

Effects of carrageenan on the responses to noxious thermal and mechanical stimuli after

treatment with capsaicin on day 15 after birth

Similar to our previous studies, capsaicin-injected P15 mice exhibited hypoalgesia (analgesia) (7.5 ± 0.6 sec, $P < 0.01$), compared with that in the control (4.2 ± 0.5 sec) (Fig. 2).

Naturally, the control P15 mice showed a significantly shorter response time (3.0 ± 0.3 sec, $P < 0.05$) to noxious heat stimuli than their baseline values (4.2 ± 0.5 sec), while the capsaicin-injected P15 mice showed a significantly increased algesia (4.9 ± 0.4 sec, $P < 0.01$) comparable to the baseline value in the control (4.2 ± 0.5 sec) (Fig. 2). The baseline value of the capsaicin-treated mice suggested hypoalgesia (5.8 ± 0.3 g, $P < 0.01$), compared with that of the control (4.4 ± 0.3 g), in response to noxious mechanical stimuli (Fig. 2). The capsaicin-injected mice showed significant algesia (4.2 ± 0.3 g, $P < 0.01$) in response to inflammatory noxious mechanical stimuli, compared with the baseline values (5.8 ± 0.3 g), and the value of the control at 24 h after carrageenan injection (4.8 ± 0.2 g) (Fig. 2).

Differences in the number of IB4-positive and TRPV1-positive DRG neurons between capsaicin-injected P2 and P15 mice

The number of TRPV1 (+) small DRG neurons (<30 μm) decreased by 90.8% in the capsaicin-injected P2 mice, while the number in mice that received a capsaicin injection at P15 decreased by 44.4% (Fig. 3, Table 1). The number of IB4 (+) neurons significantly increased in both the capsaicin-injected P2 (13.8%, $P < 0.05$) and P15 (19.6%, $P > 0.01$) mice, compared with that in the control P2 mice (11.1%). However, the number of IB4-positive small neurons (<30 μm) significantly increased, whereas the number of large neurons (>30 μm) significantly decreased in both P2 and P15 animals (Fig. 3, Table 2).

Discussion

Here, the cause of the paradoxical response to a noxious heat stimulus will mainly be discussed. First, the descriptions of TRPV1 (+) and IB4 (+) sensory neurons in recent reports may be useful when considering the generation of an acute (physiological) noxious thermal sense.

Noxious heat and TRPV1- and IB4-positive or -negative neurons

TRPV1 neurons are reportedly essential for sensing both acute (hot plate: 55°C, radiant heat) and inflammatory-induced noxious heat conditions, unlike the primary afferent neurons that sense noxious mechanical stimuli [20]. The destruction of selective nociceptive neurons and their nerve terminals may provide an important strategy for pain control [21]. However, the highly selective deletion of specific (TRPV1) nociceptive neurons does not completely eliminate the sensing of noxious thermal stimuli. Even higher doses of resiniferatoxin (RTX)-treatment did not destroy a population of heteromeric large diameter TRPV1/TRPA1-positive neurons, which believed to be capable of mediating residual thermal responses [22]. Importantly, neurons resistant to RTX or to capsaicin are able to sense noxious heat.

Reverse RTX treatment after inflammation results in a profound loss of thermal sensation without any effect on mechanosensation [22]. For this reason, the authors speculated that RTX-resistant large TRPV1 (+) neurons survived but that their processes degenerated, i.e., that deafferentation occurred; consequently, analgesia to heat was produced despite the carrageenan-induced inflammation. TRPV1-diphteria toxin (DTA) mutant mice devoid of all sensory neurons in the TRPV1 lineage (including TRPA1 and TRPM8) were completely insensitive to a noxious heat stimulus (hot plate: 55?) or to cold even after inflammation induced by the injection of carrageenan into the hind paw [23]. The authors concluded that some redundancy in the large number of TRP- or other potential thermosensors might account for the residual thermal responses [23].

The chemical-genetic elimination of DRG neurons expressing the diphtheria toxin receptor (DTR), (i.e., DTR was inserted in the Mrgprd locus in murine embryonic stem cells, and the nearly complete loss of Mrgprd-positive DRG cells was induced by DTR), which comprise the vast majority of TRPV1(-)/IB4(+) neurons, results in selective noxious mechanical deficits; polymodal IB4(+) neurons are not sensitive to noxious heat stimuli but are sensitive to noxious mechanical stimuli [20]. A large population of DRG neurons expressing TRPV1 during development is transient and does not die, but rather survives throughout adulthood, largely forming the TRPV1(-)/IB4(+) population [24]. The majority (>85%) of IB4(+) cells, a sodium channel Nav 1.8-expressing nociceptive sensory neuron, were killed by diphtheria toxin A (DTA) knock-in mice [16]. IB4 (+) sensory neurons with Nav1.8 expression participated in inflammatory thermal and mechanical stimuli-induced hyperalgesia, but not in the high thermal sensation

and pain induced by neuropathy [16]. Furthermore, IB4 (+) DRG neurons ipsilateral to an area of spinal nerve ligation (experimental neuropathy) [25] or inflammation [26] began to express functional TRPV1, resulting in hyperalgesia to noxious heat or chemical stimuli in parallel with the hyperalgesic behavior of experimental animals [25]. Thus, the role of IB4 (+) neurons in the induction of inflammatory hyperalgesia was emphasized. These results support the hypothesis that IB4 (+) neurons are unrelated to the perception of noxious heat.

On one hand, the percentage of IB4 (+) small DRG neurons that responded to a noxious heat stimulus (42°C~47°C) was only 7.4%, whereas most of the IB4 (-) neurons (65.8%) responded to heat in a study using patch clamp recordings [25]. TRPV1-immunoreactivity was found only in CH (cutaneous-heat) neurons, and all the TRPV1 (+) CH fibers were IB4 (-) [26]. Since CH fibers, which are only sensitive to heat, were absent in the transgenic TRPV1-/- mice, CH fibers were suggested to play an essential role in the generation of inflammatory-induced hyperalgesia [26]. Actually, no difference was found in the normal heat response behaviors between WT (41.5 ± 0.41°C) and TRPV1-deficient mice (41.2 ± 0.68°C) [26]. In short, the role of IB4 (+) or (-) neurons in noxious heat sensation remains controversial.

A tentative explanation of the persistent response to noxious heat in the present study

Similar to the results of our previous studies [1, 2]), the capsaicin-treated P2 mice exhibited a normal response to acute noxious heat, while capsaicin-treated P15 mice exhibited severe hypoalgesia to noxious heat, in the present study. These results indicate that capsaicin-sensitive DRG neurons (TRPV1-positive) are not necessary for the sensation of acute noxious heat, since the remaining capsaicin-insensitive DRG neurons (TRPV1-negative) were capable of sensing the noxious heat. Irrespective of the inflammatory condition induced by carrageenan injection, the capsaicin-treated P2 mice exhibited hypoalgesia, probably because of the marked loss of TRPV1 (+) neurons. The defect in noxious thermal sensation was thought to be unaffected by anatomical changes (reductions) in the DRG neurons, but rather by alterations in gene expression encoding the trophic factor artemin (Artn, one of the GDNF family) and elderly animal receptors [27]. The polymodal nociceptors of IB4 (+) neurons [26] can sense acute noxious heat but are unlikely to be involved in the sensation of noxious heat under inflammatory conditions in the capsaicin-treated P2 mice in the present study.

Capsaicin-treated P15 mice with severe hypoalgesia (hyperanalgesia) to noxious heat exhibited a decreased threshold time (algesia) after carrageenan injection comparable to the baseline values of the control, indicating the effects of inflammation. This finding suggests that a subset of DRG neurons, probably TRPV1 (+) neurons, respond to noxious heat during the presence of inflammation in capsaicin-treated P15 mice.

Previously, we speculated that approximately 30% of the remaining capsaicin-resistant, IB4 (+) but TRPV1 (-) small neurons might play a pivotal role in pain transduction against acute noxious heat stimuli, despite the absence of TRPV1-expressing neurons, in capsaicin-treated P2 mice [15]. However, a putative potential loss (25%-50%) of IB4 (+) neurons in capsaicin-treated P15 mice resulted in hyposensitivity to noxious heat [2, present study]. Unexpectedly, despite the increase in IB4 (+) neurons after capsaicin injection, P15 capsaicin-injected mice exhibited hypoalgesia to noxious heat. IB4 (+) neurons are probably not functional under the effect of capsaicin in these animals.

The role of IB4 (+) nociceptors in the detection of acute noxious heat stimuli under normal conditions has been emphasized [9, 28, 29]. Quite recently, the upregulation of T-type calcium channels (Cav3.2) in IB4 (+), nonpeptidergic thermal C-fiber nociceptive neurons was shown to be responsible for normal thermal algesia in streptozotocin-induced diabetic rats [30]. Thus, IB4 (+) small neurons may play a central role in the sensing of acute noxious heat.

A few reports have indicated that TRPV2, an enigmatic TRPV channel, is not a mediator of the remaining heat response in TRPV1-deficient [11, 31] and in ex vivo somatosensory preparations in mice [26]. Thus, the participation of TRPV2-positive neurons in the remaining noxious heat sensation experienced by capsaicin-treated P2 mice can be reasonably ignored.

In contrast to the decrease in larger IB 4 (+) neurons, IB4 (+) small neurons increased in both P2 and P15 capsaicin-treated DRGs. Capsaicin might inhibit the growth of the IB4 (+) small neurons during development.

Noxious mechanical nociception

The capsaicin-treated P2 mice exhibited algesia to noxious mechanical stimuli in a manner similar to the control animals, suggesting that a subset of neurons other than TRPV1(+) neurons respond to noxious mechanical stimuli in the presence of inflammation. The capsaicin-treated P15 mice exhibited hypoalgesia at baseline, but exhibited algesia after carrageenan injection to the noxious mechanical stimulation. This finding indicates that the number of neurons sensing acute noxious mechanical stimulation decreased or became non-functional as a result of the effect of capsaicin, but that some neurons continued to function under inflammatory condition. Either acute noxious mechanical or inflammatory mechanical noxious stimuli were sensed irrespective of the decrease in TRPV1 (+) neurons or were not sensed irrespective of the increase in IB4 (+) neurons. Thus, both acute noxious and inflammatory noxious mechanical sensations seem to be unrelated to the presence of TRPV1 (+) or IB4 (+) neurons.

Collectively, these findings suggest three possible mechanisms for the perception of acute noxious heat stimuli. First, the TRPV1 channel may sense acute noxious heat as well as inflammatory heat hyperalgesia. Second, the equivocal TRPA1 channel may be a candidate noxious heat sensor. However, the probability of this option is rather low because TRPA1 channels are essential for the thermotaxis behavior responsible for the movement of flies or mosquitoes to favorable environmental temperatures or host animals but do not act as high-temperature nocisensors [32 - 34]. Third, we believe that a subgroup of undefined IB4 (+) and TRPV1 (-) small neurons possessing some ion channels may be capable of sensing acute noxious heat stimuli.

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Illustrations

Illustration 1

Fig. 1. Behavioral responses to noxious heat and mechanical stimuli in P2 mice at 24 h after carrageenan injection. Capsaicin-treated mice showed normal response behaviors before carrageenan injection (baseline value) but exhibited hypoalgesia (analgesia) at 24 h after injection. Both the control and capsaicin-treated mice exhibited hyperalgesia to noxious mechanical stimuli. *P < 0.05, **P < 0.01

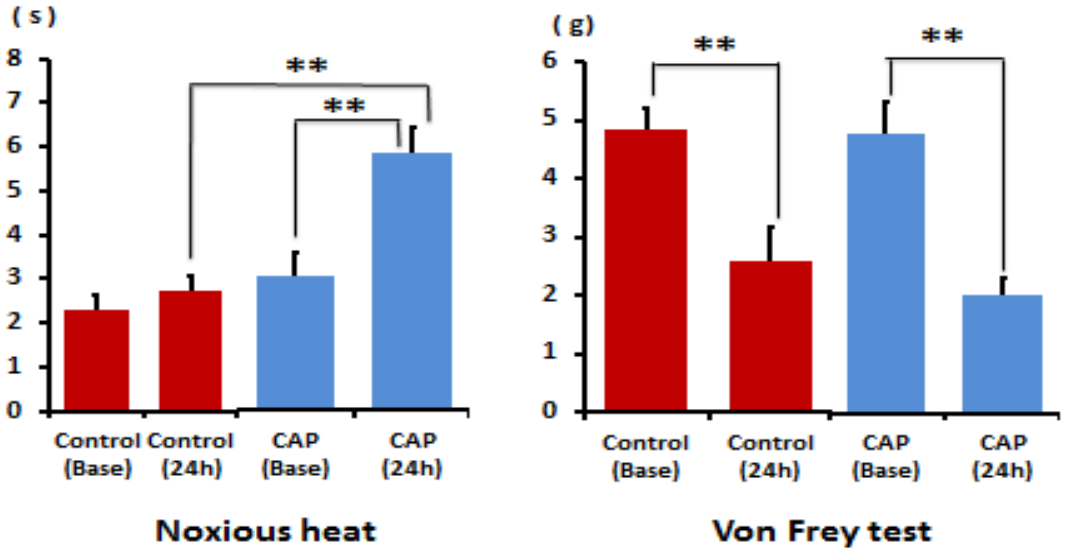

Illustration 2

Fig. 2. Behavioral responses to noxious heat and mechanical stimuli in P15 mice at 24 h after carrageenan injection. Capsaicin-treated mice exhibited hypoalgesia (analgesia) to noxious heat stimuli, compared with the responses of the control animals. However, capsaicin-treated mice exhibited algesia in response to noxious heat stimuli after carrageenan injection, similar to the results observed in control mice. Capsaicin-treated mice exhibited analgesia in response to mechanical noxious stimuli, but exhibited algesia after carrageenan injection. *P < 0.05, **P < 0.01

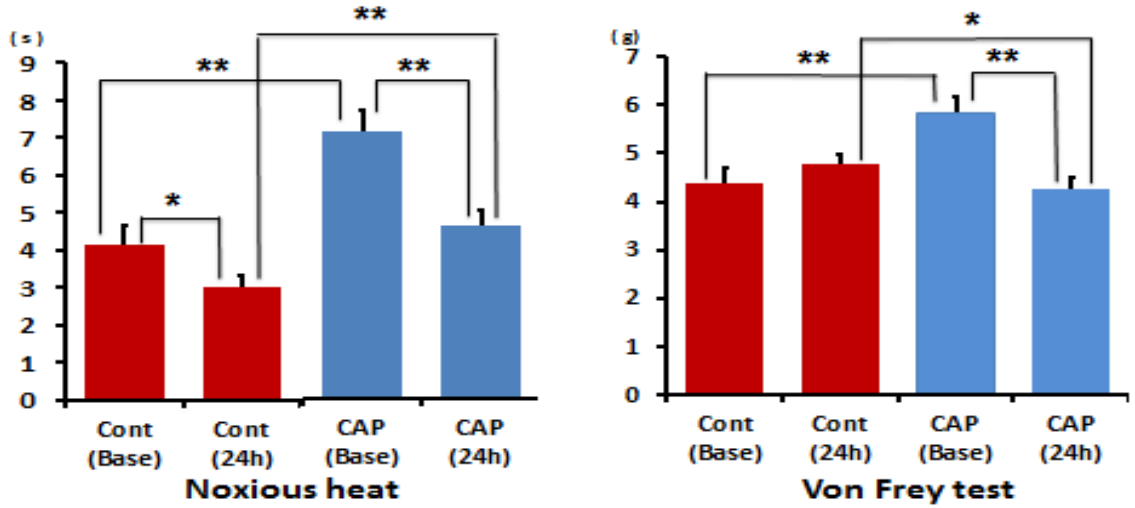

Illustration 3

Fig 3

TRPV1-ir and IB4-positive small neurons in the DRG of either P2 or P15 mice. The numerous small TRPV1-ir neurons in the control DRG (b, arrows) were scarcer in the P2 (d) and P15 (f) mice treated with capsaicin. Small- to medium-sized IB4 (+) neurons are visible in the control DRG (a, arrows). The number of IB4-positive neurons increased after capsaicin treatment in the P2 (c) and P15 (e) mice. Bar: 50 μm

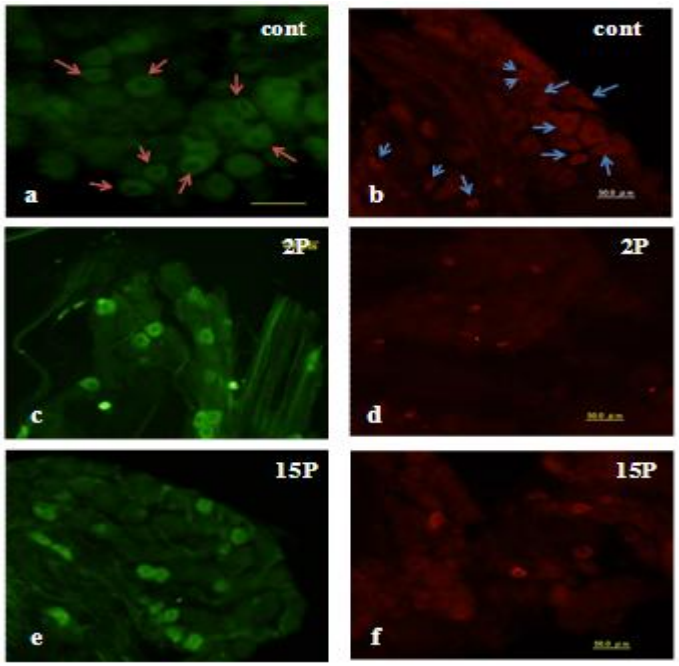

Illustration 4

Table 1

Table 1. Number of TRPV1-positive neurons

	P2		P15	
dia. (μm)	Control	CAP	Control	CAP
10 - 15	27	1	26	6
16 - 20	29	1	18	10
21 - 25	27	1	8	8
26 - 30	4	5	1	5
31 - 35	0	9	2	6
36 - 40	0	0	0	3
41 - 45	0	0	0	1
TRPV1/Total %	87/886 9.8	17/841 2.0	55/656 8.4	40/700 5.7

Illustration 5

Table 2

Table 2. Number of IB4-positive neurons

dia. (μm)	Control	CAP (P2)	CAP (P15)
10 - 15	4	1	2
16 - 20	0	10	16
21 - 25	12	31	58
26 - 30	14	36	45
31 - 35	16	8	13
36 - 40	23	5	3
41 - 45	12	0	0
46 - 50	7	0	1
IB4/Total %	88/886 11.1	91/841 13.8	138/700 19.6

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