## Title

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## Authors

Hendrich, J
Alvarez, P
Chen, X
et al.

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# GDNF induces mechanical hyperalgesia in muscle by reducing $I_{BK}$ in IB4+ nociceptors

**Jan Hendrich**[a,c], **Pedro Alvarez**[a,c], **Xiaojie Chen**[a,c], and **Jon D Levine**[a,b,c]

[a]Department of Oral and Maxillofacial Surgery, University of California San Francisco

[b]Department of Medicine, University of California San Francisco

[c]Division of Neuroscience, University of California San Francisco

## Abstract

We have assessed the mechanism underlying glial cell-derived neurotrophic factor (GDNF)-induced mechanical hyperalgesia in the gastrocnemius muscle, using patch clamp electrophysiology, *in vivo* electrophysiology and behavioral studies. Cultured isolectin B4-positive (IB4+) dorsal root ganglion (DRG) neurons that innervated this muscle were held under current clamp; the majority developed an increase in action potential duration (a factor of increase of 2.29 ± 0.24, compared to 1.13 ± 0.17 in control, $P<0.01$) in response to GDNF (200 ng/ml) by 15 min after application. They also demonstrated a depolarization of resting membrane potential (RMP), but without significant changes in rheobase, action potential peak, or after-hyperpolarization. Large-conductance voltage- and calcium-activated potassium (BK) channels, which have recently been shown to play a role in the repolarization of IB4+ nociceptors, were inhibited under voltage clamp, as indicated by a significant reduction in the iberiotoxin-sensitive current. *In vivo* single-fibre recording from muscle afferents revealed that injection of iberiotoxin into their peripheral nociceptive field caused an increase in nociceptor firing in response to a 60 s suprathreshold stimulus (an increase from 392.2 ± 119.8 spikes to 596.1 ± 170.8 spikes, $P<0.05$). This was observed in the absence of changes in the mechanical threshold. Finally, injection of iberiotoxin into the gastrocnemius muscle produced dose-dependent mechanical hyperalgesia. These data support the suggestion that GDNF induces nociceptor sensitization and mechanical hyperalgesia, at least in part, by inhibiting BK current in IB4+ nociceptors.



Corresponding author: Jon Levine (jon.levine@ucsf.edu).
**Present / Permanent address:** Room C555, Campus Box 0440, 521 Parnassus Avenue, University of California at San Francisco, CA, USA, 94143-0440. Tel.: +1 415 476 5108; fax: +1 415 476 6305.

**Author contributions**

All experiments were performed in the laboratory of Jon Levine. The following aspects of the study were performed by the authors as stated:

1. Conception and design of the experiments: Jan Hendrich, Pedro Alvarez, Xiaojie Chen, Jon Levine.
2. Collection, analysis and interpretation of data: Jan Hendrich, Pedro Alvarez, Xiaojie Chen, Jon Levine.
3. Drafting the article or revising it critically for important intellectual content: Jan Hendrich, Jon Levine.

All authors approved the final version of the manuscript.

The authors declare no conflict of interest

**Keywords**

Pain; Potassium channel; Growth factor; Electrophysiology; Behavior

## 1. Introduction

Two functionally distinct classes of small-diameter nociceptors can be defined by their dependence on different neurotrophic factors, nerve growth factor (NGF) and GDNF (Snider and McMahon, 1998, Stucky and Lewin, 1999). GDNF-dependent nociceptors, which can be identified by their binding of isolectin B4 (IB4), terminate in the inner part of lamina II of the spinal dorsal horn. In contrast, IB4-negative (IB4-) neurons terminate in lamina I and the outer part of lamina II (Braz et al., 2005). There has been some controversy over the incidence of this nociceptor subset among muscle afferents, ranging from 15–40% (Molander et al., 1987, Pierce et al., 2006), in general a lower proportion than in the skin (Plenderleith and Snow, 1993). However, we have found that IB4+ muscle afferents play a major role in numerous models of inflammatory and ergonomic muscle pain (Alvarez et al., 2011, Alvarez et al., 2012a).

In addition to long-term support of target neurons, GDNF can have more short-term effects on ion channels in midbrain (Yang et al., 2001, Wang et al., 2003) and also sensory (Takeda *et al.*, 2010) neurons. The present study was designed to investigate the ionic basis of the mechanical hyperalgesia induced by GDNF application to nociceptors innervating the gastrocnemius muscle in the rat. Toward this end we have characterised action potentials evoked by electrical stimulation of IB4+ muscle afferents *in vitro*, examined BK (also termed $K_{Ca}$, SLO1, maxi K) current, and evaluated the role of this ion channel in mechanical hyperalgesia in the gastrocnemius muscle in the rat using behavioral and single-fibre *in vivo* electrophysiology studies. BK channels are present in more than 90% of small-diameter IB4+ sensory neurons, but only in a minority of IB4- neurons (Zhang *et al.*, 2010) They play an important role in regulating neuronal excitability (Fakler and Adelman, 2008), and regulate both the frequency and action potential duration in a variety of neurons (Lancaster and Nicoll, 1987, Pineda et al., 1992, Shao et al., 1999, Faber and Sah, 2003, Sausbier et al., 2006, Gu et al., 2007). Although acute modulation of BK channels by glucocorticoids (Lovell *et al.*, 2004) and ethanol (Gruss et al., 2001, Dopico, 2003), both of which affect nociceptive function (Ikeda et al., 2002, Liu et al., 2010b), has been reported, we are not aware of any reports of short-term modulation of BK channels by growth factors.

## 2. Experimental Procedures

### 2.1. Ethical Approval

Animal care and use conformed to National Institutes of Health guidelines. The University of California, San Francisco, Institutional Animal Care and Use Committee approved the experimental protocols employed in these experiments.

### 2.2. Animals

Experiments were performed on adult male Sprague-Dawley rats weighing 180–240g for electrophysiology and 220–240g for behavioral testing (Charles River, Hollister, CA). Animals were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12-hour light / dark cycle and environmentally controlled conditions (7:00 AM–7:00 PM light cycles; 21–23°C) with food and water available *ad libitum*.

### 2. 3. Drugs

Unless specifically stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

### 2.4. Retrograde dye injection

DRG neurons innervating the gastrocnemius muscle were identified by their uptake of the retrograde tracer 1,1′-dioctadecyl-3,3,3′,3′-tertamethyllindocarbocyanine perchlorate (DiI). Rats were subjected to gaseous anesthesia (isofluorane, 3%), and an incision made to expose a section of biceps femoris muscle. A 30-g needle was passed through the biceps femoris muscle and into the belly of the gastrocnemius muscle; then 2 μl DMSO containing 2% DiI was slowly injected into each of 3 sites within the muscle. Each injection was performed over 2 min, followed by observation for 1 min to observe for any leakage. In a separate experiment, the overlap in fluorescence between DiI (injected into the muscle) and water containing 2% Fast Blue dye (applied to the surface of the muscle) was examined. No overlap in fluorescence was observed.

### 2.5. DRG cell-culture

DRGs were surgically removed from rats 5–8 days after DiI injection. Rats were decapitated and the vertebral column excised. The column was then opened up on the ventral side, and L4-L5 DRGs were removed and de-sheathed. Ganglia were treated with collagenase (0.125% in Neurobasal-A medium, Invitrogen, Carslbad, CA) for 90 min at 37°C, and then treated with 0.25% trypsin in $Ca^{2}$- and $Mg^{2+}$-free PBS (Invitrogen) for 10 min, followed by trituration (in Neurobasal-A) to produce a single-cell suspension. The suspension was centrifuged at 1000 RPM and re-suspended in Neurobasal-A medium supplemented with 50 ng/ml nerve growth factor, 100 U/ml penicillin/streptomycinand B-27 (all Invitrogen). Cells were then plated on coverslips coated with poly-DL-ornithine (0.1 mg/ml) and laminin (5 μg/ml; Invitrogen), and incubated at 37°C in 5% $CO_2$.

### 2.6. Imaging and cell counting

Cells were stained with fluoresecin-conjugated IB4 (Invitrogen), at a concentration 10 μg/ml (in water), 10 min before use. DiI-labeled cells were identified under epifluorescence illumination. The proportion of IB4+ cells was established from 20 coverslips, across 3 separate cultures. Cell counting occurred within 30 hr of cell dissociation to minimize the impact of culture conditions on the survival of different subpopulations of neurons. Neurons were identified by morphology and phase-brightness.

### *2.7. In vitro* electrophysiology

Electrophysiology was performed using an Axopatch 200A amplifier and pCLamp 8.2 spftware (Molecular Devices, Sunnyvale, CA). DRG neurons were subjected to current- or voltage-clamp after 2–36 hr in culture. For current-clamp, the external solution contained (in mM): NaCl (140), KCl (3), $MgCl_2$ (1), $CaCl_2$ (1), glucose (10), HEPES (10), adjusted to pH 7.3 and 320 mOsm. The pipette solution contained: KCl (140), EGTA (1); NaCl (10); MgATP (2); HEPES (10), adjusted to pH 7.3 and 310 mOsm.

Cells were held in the whole-cell configuration at −60 mV, following seal formation (seal resistance > 1GΩ). Whole-cell capacitance and series resistance were compensated (80%) using the amplifier circuitry. Cells with a series resistance >10 MΩ were not used for experimentation. Following the switch to current clamp, current steps of 50 pA were applied to find the minimum stimulus intensity required to elicit an action potential stimulation (rheobase). 800 ms steps were then applied every 2 min for the duration of the experiment. After the determination of rheobase, GDNF (200 ng/ml) or vehicle (external solution) was applied directly to the bath.

BK current-voltage (I-V) relationships were obtained by applying test pulses (from −90 to +60 mV in 10 mV increments) for 500 ms, from the holding potential of −60 mV. This was repeated until the currents had stabilized. At this point, the BK channel blocker iberiotoxin (100 nM) was added to the bath. A test pulse to +60 mV was elicited at 5 s intervals, and a corresponding reduction in the current response occurred with the onset of channel block. Once channel block had reached its maximal level, the I-V protocol was run again. For voltage-clamp experiments the external solution contained (in mM): choline-Cl (130); KCl (5); $CaCl_2$ (2.5); $MgCl_2$ (0.6); HEPES (5); glucose (10), adjusted to pH 7.4 and 320 mOsm. The pipette solution contained: KCl (30); K-methanesulfonate (110); $MgCl_2$ (1); HEPES (10); EGTA (0.1), adjusted to pH 7.2 and 310 mOsm.

**Data analysis—**Various characteristics of the rheobase action potentials were analyzed. Action potential duration was defined as the width at the membrane potential halfway between resting membrane potential (RMP) and action potential peak. Action potential peak refers to the maximal depolarization that occurred during the action potential. The afterhyperpolarization (AHP) refers to the maximal repolarization following the action potential. Offline analysis of I-V curves involved subtraction of the iberiotoxin-resistant current from the total current; this was defined as the BK channel component of the current. Currents were normalized according to cell capacitance, as determined by the amplifier compensation circuitry. Data were analyzed and plotted using Origin 6.1 software (OriginLab, Northampton, MA).

### 2.8. Mechanical nociceptive threshold in gastrocnemius muscle

Mechanical nociceptive threshold in the gastrocnemius muscle was measured as previously described (Alvarez *et al.*, 2011). Briefly, a digital force transducer equipped with a custom-made 7mm-diameter probe (Chatillon DFI2; Amtek, Inc., Largo, FL) was applied to the gastrocnemius muscle. Increasing force was then delivered until a nociceptive flexion reflex of the limb was observed. Rats were lightly restrained in an acrylic holder that allows for easy access to the hind limb and application of the transducer probe to the belly of the gastrocnemius muscle. The nociceptive threshold was defined as the force (in Newtons) required to produce a flexion reflex in the hind leg. Baseline limb-withdrawal threshold was defined as the mean of 3 readings taken at 5-minute intervals. Each hind limb was treated as an independent measure and each experiment performed on a separate group of rats.

Iberiotoxin was purchased from TOCRIS bioscience (Ellisville, MO, USA). Iberiotoxin was solubilized in sterile filtered phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (PBS-BSA). Stock solutions of Iberiotoxin (1 μg/10 μl dissolved in PBS-BSA and stored at −20°C) or GDNF (1 μg/μl dissolved in 0.9% NaCl containing 0.5% BSA and stored at −20°C) were further diluted in PBS-BSA immediately before injection. The $BK_{Ca}$ channel opener 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619) was dissolved in 100% ethanol and then diluted in PBS immediately before injection. The injection site was previously shaved and scrubbed with alcohol. Immediately after injections the skin puncture site was marked with a fine-tip indelible ink pen, so that the mechanical nociceptive threshold of the underlying injection site in the muscle could be repeatedly tested.

### *2.9. In vivo* electrophysiology

The *in vivo* single fiber electrophysiology technique employed was similar to that used previously in our recordings from cutaneous afferents (Chen *et al.*, 2010). Rats were anaesthetized with sodium pentobarbital (initially 50 mg/kg, i.p., with additional doses given throughout the experiment to maintain areflexia), their trachea cannulated, and heart rate

monitored. The body temperature (≥36.7°C) of animals were monitored and controlled by a TR-200 temperature controller (Fine Science Tools, Foster City, CA)

Animals were positioned on their right side and an incision made between the mid-thigh and shank on the dorsal skin of the left leg. The biceps femoris muscle was partially removed to expose the sciatic nerve and gastrocnemius muscle. The edges of the incised skin, fixed by a metal loop, provided a pool that was filled with warm mineral oil, immersing the sciatic nerve and gastrocnemius muscle. Neural activity was recorded from single fiber in the sciatic nerve, cut proximal to the recording site to prevent flexor reflexes during electrical stimulation of the muscle. Fine fascicles of axons were then dissected from the distal stump, and placed on a recording electrode. Single units were first detected by mechanical stimulation of the gastrocnemius muscle with a small blunt-tipped glass bar. Bipolar-stimulating electrodes were placed and held on the center of the receptive field, of the muscle afferent, by a micromanipulator (MM-3, Narishige, Japan). Conduction velocity of each fiber was calculated by dividing the distance between the stimulating and recording electrodes by the latency of the electrically evoked action potential. All recorded muscle afferents have conduction velocities in the range of type III (12%) or type IV (88%) fibers. Mechanical threshold was determined with calibrated von Frey hairs (VFH; Ainsworth, London, UK) and defined as the lowest force that elicited at least 2 spikes within 1 s, in at least 50% of trials. Sustained (60 s) suprathreshold (10 g) mechanical stimulation was accomplished by the use of a mechanical stimulator that consisted of a force-measuring transducer (Entran, Fairfield, NJ, USA), which was held by a micromanipulator (BC-3 and BE-8, Narishige) on the center of the receptive field for 60 s. Iberiotoxin (500 ng / 10 μl) was injected into the receptive field. Mechanical threshold was measured at 15 min after iberiotoxin injection, and the response to 10 g stimulus was recorded at 20 min after the injection. Neural activity and stimulus onset and termination were monitored and stored on a Windows OS computer with Micro 1401 interface (CED, Cambridge, UK) and analyzed off-line with Spike2 software (CED).

### 2.10. Statistics

For current-clamp experiments, action potential characteristics were compared 30 min after GDNF / vehicle application using an independent two-tailed Student's t-test. For voltage-clamp experiments, peak (normalized) BK currents were compared using an independent two-tailed Student's t-test. For behavioral experiments, one-way or two-way repeated measures analysis of variance (ANOVAs) with appropriate post hoc analyses to determine if significant differences between experimental groups were performed. If there was a significant group × time interaction, multivariate analyses (i.e., 1-way ANOVAs) were performed for all time points in order to determine which points accounted for the interaction. In these cases, a Bonferroni correction was applied in order to account for multiple comparisons. For within-subjects effects the Mauchly criterion was used to determine if the assumption of sphericity was met; if not, Greenhouse-Geisser $p$ values are presented. Statistical analyses were performed using Prism software (Graphpad). For *in vivo* electrophysiology studies, paired Student's t-tests were performed on recordings made before and after iberiotoxin injection. Statistical significance was set at $p<0.05$.

## 3. Results

### 3.1. GDNF effects on muscle afferents *in vitro*

To examine the effects of GDNF on the action potential of IB4+ muscle afferents, DRG neurons were held under current clamp (see Fig. 1A for representative current traces). Analysis of action potentials (at rheobase) revealed that GDNF produced an increase in action potential duration (Fig. 1B, factor increase of 2.29 ± 0.24; n = 8) compared to control

(factor increase of 1.13 ± 0.13; n = 7; significant to P<0.01 at 30 min, Student's two-tailed t-test). This was accompanied by a significant change in the RMP, which depolarized 4.35 ± 1.64 mV in control cells compared to 10.31 ± 2.19 mV (P<0.05, Student's two-tailed t-test) in GDNF-treated cells (Fig. 1B). Of the other electrophysiological characteristics that were analyzed, action potential peak amplitude and AHP amplitude showed variation over the course of the experiment, but were not significantly different between control and GDNF conditions (Fig. 1B).

### 3.2. GDNF inhibits $I_{BK}$

$I_{BK}$ was isolated by subtraction of the iberiotoxin-sensitive current from the total current in IB4+ neurons (Fig. 2A). Iberiotoxin is a selective blocker of BK channels (Galvez *et al.*, 1990). A 15 – 25 min pre-incubation in GDNF produced a significant (P<0.05 at +60 mV, Students two-tailed t-test) reduction in mean peak iberiotoxin-sensitive current (normalised to cell membrane capacitance; see Fig. 2B); the peak current in response to a +60 mV step was 0.77 ± 0.2 nA/pF (n = 8), compared to 2.2 ± 0.5 nA/pF in the control condition (n = 10). There was no significant difference in mean cell body diameter between control (24.8 ± 1.1 μm) and GDNF-treated (22.9 ± 1.1 μm) neurons. Representative current traces for both conditions are shown in Figure 2C.

### 3.3. IB4+ small-diameter muscle afferents

The percentage of IB4+ DiI-labeled neurons *in vitro* was assessed as a means of determining the proportion of muscle afferents that might contribute to the onset of mechanical hyperalgesia after GDNF injection (Alvarez and Levine, in manuscript). IB4+ neurons were found to make up 70.6% of small diameter (<30 μm) L4 and L5 DRG muscle afferents (from a total of 252 DiI-labeled neurons, observed across 3 separate cultures / animals). Figure 2D shows representative images of IB4+ neurons *in vitro*.

### 3.4 Effects of $BK_{Ca}$ modulators and GDNF on muscle mechanical nociceptive threshold

To assess the involvement BK channels on muscle mechanical nociception, the selective blocker iberiotoxin was injected i.m. (1 ng-1 μg, administered in a cumulative dosing protocol with sequentially higher doses administered at 45 min intervals) into the gastrocnemius muscle. This produced a dose-dependent mechanical hyperalgesia (Fig. 3A), which was significant at doses of 10 ng or higher of iberiotoxin (Fig. 3A). No significant reduction in mechanical nociceptive threshold was observed with injection of vehicle (PBS-BSA).

To confirm whether the BK channels are involved in GDNF-induced muscle hyperalgesia, GDNF was injected in the gastrocnemius muscle previously injected with the BK channel opener NS1619 (20 μg/20 μl) or its vehicle (10% ethanol-PBS). Fifteen minutes after the intramuscular injection of NS1619 or its vehicle, no significant changes in muscle mechanical nociceptive threshold were noticed (Fig. 3B). However, NS1619, but not its vehicle, significantly inhibited the decrease in mechanical nociceptive threshold induced by GDNF (10 ng/20 μl) as revealed in readings taken 30 and 60 min after GDNF injection (Fig. 3B).

### 3.5. Iberiotoxin produces an increase in firing rate in muscle nociceptors

Single fiber recordings were made from nociceptors innervating the gastrocnemius muscle. The injection of iberiotoxin into the peripheral receptive field caused an increase in action potential firing in response to a suprathreshold mechanical stimulus (10 g) applied for 60 s (Fig. 4A); the number of spikes measured over 60 s increased from 392.2 ± 119.8 before iberiotoxin injection, to 596.1 ± 170.8 after (P<0.05, n = 12, paired t-test). However, there

was no clear difference in the time-course of the response to iberiotoxin injection. In addition, iberiotoxin did not induce any significant change in the mechanical threshold (Fig. 4C) of the fibers recorded.

## 4. Discussion

In this study we have demonstrated that GDNF acts to prolong action potential duration and inhibit BK currents in small-diameter IB4+ muscle DRG neurons, and confirmed that this block of BK channels can cause mechanical hyperalgesia and increased spike firing (in response to mechanical stimulation) *in vivo*. Action potential broadening occurred within 10 min of applying GDNF to the bath, and increased throughout the 30 min testing period. Inhibition of BK current was observed within the same period after GDNF application. These actions of GDNF have a latency too short to be explained by alterations of gene expression, but too long to be explained by a direct blocking effect on the channels.

GDNF is one of a family of 4 ligands (the others being neurturin, artemin and persepherin), all of which signal through a specific GDNF-family receptor to activate receptor tyrosine kinase (RET) signaling (Airaksinen and Saarma, 2002). RET-null mice die soon after birth (Schuchardt *et al.*, 1994), but selective knockout of nociceptors that express both RET and $Na_v1.8$ has demonstrated that RET signaling is essential for the survival of and peripheral innervation by these nociceptive neurons (Golden *et al.*, 2010).

It has been shown that intrathecal delivery of GDNF can reduce pain in certain neuropathic states (Bennett et al., 1998, Boucher et al., 2000), and also that restoration of GDNF in muscle tissue can reduce mechanical allodynia and thermal sensitivity following chronic-constriction injury (Shi *et al.*, 2011). However it has also been shown that GDNF can have pro-algesic effects: mice over-expressing GDNF had decreased mechanical (but not thermal) thresholds in skin (Albers *et al.*, 2006); an antibody to GDNF reduced the hyperalgesia associated with complete Freund's adjuvant injection (Fang *et al.*, 2003); injection of GDNF in the hindpaw also produced an acute thermal hyperalgesia within 1 hr (Malin et al., 2006, Bogen et al., 2008); and inflammation increases GDNF production in the dorsal root ganglion (Amaya *et al.*, 2004). GDNF has also been shown to increase the proportion of cells responsive to the TRPA1 agonist allylisothiocyanate (Ciobanu *et al.*, 2009). Thus, while it has been suggested that GDNF has analgesic effects in multiple models of neuropathic pain (Bennett et al., 1998, Boucher et al., 2000, Adler et al., 2009), it may produce pronociceptive effects (Albers et al., 2006, Shi et al., 2011) and contribute to pain in the setting of inflammation (Fang *et al.*, 2003).

GDNF has been shown to inhibit potassium channels in nociceptors (Yang et al., 2001, Takeda et al., 2010). BK channels play an important regulatory role in nociceptors; blockade using charybdotoxin, paxilline or iberiotoxin in these neurons produces action potentials with longer durations, and a reduced amplitude of AHP (Scholz et al., 1998, Zhang et al., 2003, Li et al., 2007, Zhang et al., 2010). In our experiments, the finding of broadening of action potentials is compatible with the suggestion that BK currents might be reduced by application of GDNF. Although iberiotoxin has also been reported to increase firing frequency, we found that in cultured muscle sensory neurons GDNF produced no increase in the frequency of action potentials elicited in response to the rheobase depolarizing step; the vast majority of neurons fired only a single action potential over the course of the 500 ms step (data not shown) throughout the duration of the experiment. However, this contrasted with our *in vivo* data, which demonstrated a rise in the firing frequency of muscle nociceptors in response to intramuscular iberiotoxin injection.

The significant reduction in the iberiotoxin-sensitive component of the I-V curve confirmed an inhibition of BK current by GDNF application. While there was a small reduction in the

iberiotoxin-resistant potassium current, this was not statistically significant. Thus, the iberiotoxin-sensitive potassium current in IB4+ nociceptors appears to be the main (if not sole) potassium current affected by GDNF application. Consistent with this view, preventive treatment with the BK channel opener NS1619 (Olesen et al., 1994) markedly inhibited the mechanical hyperalgesia induced by GDNF. Furthermore, injection of iberiotoxin into the gastrocnemius muscle of rats produced a significant reduction in mechanical nociceptive threshold (i.e. mechanical hyperalgesia), confirming the importance of BK channels in muscle nociceptors *in vivo*. Of note, the hyperalgesia induced by iberiotoxin was of similar magnitude to that produced by GDNF (Alvarez *et al.*, 2012b). Although further investigation is required to confirm the precise proportion of IB4+ nociceptors that innervate muscle, the finding that roughly 70% of the DiI-positive small-diameter DRG neurons *in vitro* were IB4+ supports the idea that they can make a significant contribution in muscle pain syndromes (Alvarez et al., 2011, Alvarez et al., 2012a).

The mechanism of action of GDNF inhibition of BK channel currents involves intracellular processes yet to be described. We have previously shown that mechanical cutaneous hyperalgesia caused by GDNF can be significantly attenuated by blocking any of the five second messengers (phospholipase Cγ (PLCγ), cyclin-dependent kinase 5(CDK5), sarcoma (Src) family kinases (SFK), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)) activated by GDNF signaling via the GFRα1/Ret receptor complex (Bogen *et al.*, 2008). Whilst this has not yet been confirmed for mechanical hyperalgesia in muscle, there remains plenty of scope for GDNF to inhibit BK channel function. Indeed, several of the second messenger pathways activated by GDNF have been shown to alter BK channel function, for example inhibition by CDK5 (Bai *et al.*, 2012) as well as potentiation by Src tyrosine kinase (Ling *et al.*, 2000) and PI3K (Shanley *et al.*, 2002).

The identification of BK channels inhibition as an effect of GDNF provides an additional role for GDNF beyond its classical role as a survival factor for neurons. BK channels play a role in a variety of tissues, including kidney and smooth muscle in addition to its neuronal distribution (Wu and Marx, 2010). The increased release of GDNF from skeletal muscle in pathological conditions (Suzuki *et al.*, 1998) correlates with reported pain in large proportions of these individuals (Zebracki and Drotar, 2008, Engel et al., 2009), and this pain indicates that BK channels might represent a relevant molecular target for future treatment of clinical conditions characterized by muscle pain. The BK channel opener NS1619 has recently been shown to attenuate mechanical allodynia and hyperalgesia (Chen et al., 2009, Liu et al., 2010a), further highlighting the role of BK channels in mechanical pain.

## 5. Conclusions

The role of GDNF in nociception is certainly complex, having seemingly contradictory effects in different pain states. Such complex effects echo the opposing effects of GDNF in stroke (Arvidsson *et al.*, 2003), and suggest that GDNF plays more than a simple retrograde survival role according to the classical view of neurotrophic factors (Levi-Montalcini, 1987). We add to the accumulating evidence for additional roles of GDNF in pain by demonstrating an acute down-regulation of BK channel function in sensory neurons, and the resulting mechanical hyperalgesia it can produce in the musculoskeletal system.

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## Abbreviations

| | |
|---|---|
| **AHP** | after-hyperpolarization |
| **BK channel** | Large-conductance voltage- and calcium-activated potassium channel |
| **DRG** | dorsal root ganglion |
| **GDNF** | glial cell-derived neurotrophic factor |
| **IB4** | isolectin B4 |
| **IB4+** | isolectin B4-positive |
| **IB4-** | isolectin B4-negative |
| **I-V** | current-voltage |
| **NGF** | nerve growth factor |
| **RMP** | resting membrane potential |

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- Glial cell-derived neurotrophic factor (GDNF) causes mechanical hyperalgesia in muscle.
- We used behavioral techniques and electrophysiology to study rat muscle nociceptive neurons
- GDNF (200 ng/ml) altered action potential duration and reduced BK channel currents.
- A BK channel blocker induced mechanical hyperalgesia, and increased nociceptor firing *in vivo.*
- GDNF inhibits BK channel currents to cause mechanical hyperalgesia in muscle

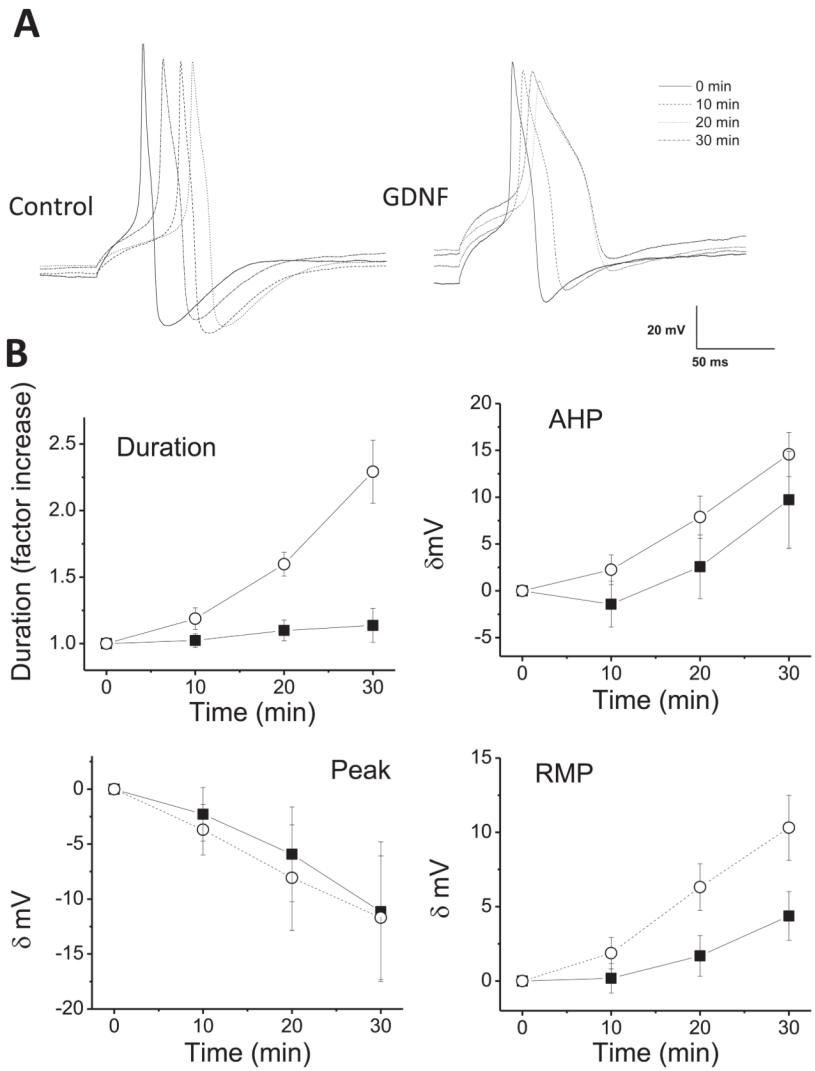


**Figure 1. Effect of GDNF on action potentials recorded from IB4+ sensory neurons innervating the gastrocnemius muscle in the rat**

**A**. Representative action potentials elicited over time in two small-diameter IB4+ neurons that innervated the gastrocnemius muscle, exposed to either 200 ng/ml GDNF or vehicle. The action potential at rheobase is shown before, and 10, 20 and 30 min after GDNF or vehicle application to the bath.

**B.** Mean action potential characteristics (duration, AHP amplitude, action potential peak and RMP) are shown for 7 cells in control (squares) and 8 cells in GDNF (circles) conditions. The factor of increase in action potential duration was significantly greater in GDNF-treated cells (2.29 ± 0.24) than in control (1.13 ± 0.13; $P<0.01$) at 30 min. Also, the depolarization of RMP was significantly greater in GDNF-treated cells (10.31 ± 2.19 mV) compared to control (4.35 ± 1.64 mV; $P<0.05$) at 30 min. No significant changes were observed in AHP amplitude or action potential peak.

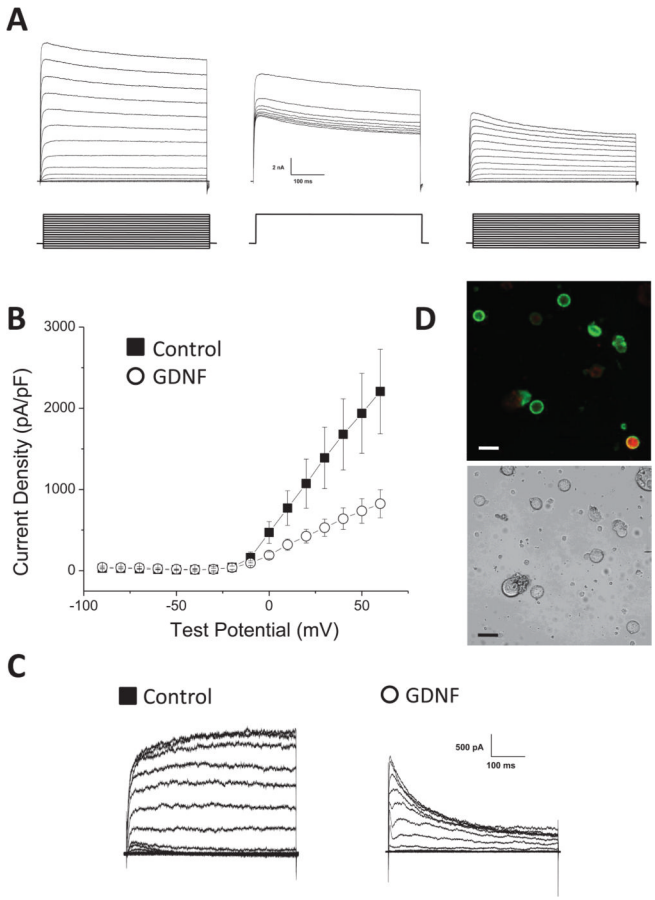


**Figure 2. GDNF inhibits BK current in IB4+ muscle afferents *in vitro***

**A.** A control I-V trace (left panel), onset of iberiotoxin block in response to a +60 mV pulse (middle panel), and iberiotoxin-resistant component of the current (right panel) are shown. Subtraction of the iberiotoxin-resistant component from the control I-V trace produced a trace denoted as BK current.

**B.** Mean iberiotoxin-sensitive I-V relationships of control (filled squares; n=10) and GDNF pre-incubated (open circles; n=8) neurons are shown. Mean current density at +60 mV was significantly reduced ($P<0.05$) in GDNF-treated cells (0.77 ± 0.2 nA/pF) compared to control (2.2 ± 0.5 nA/pF).

**C.** Representative current traces of control (filled square) and GDNF (open circle) pre-incubated neurons. BK currents showed variation both in current size and kinetics within the same conditions.

**D.** Fluorescent micrograph showing IB4 (green) and DiI (red) immunoreactivity in small-to-medium diameter DRG neurons (top panel). The corresponding brightfield micrograph is shown in the bottom panel. Images were taken <4 hours after dissociation. Scale bars represent 50 μm.

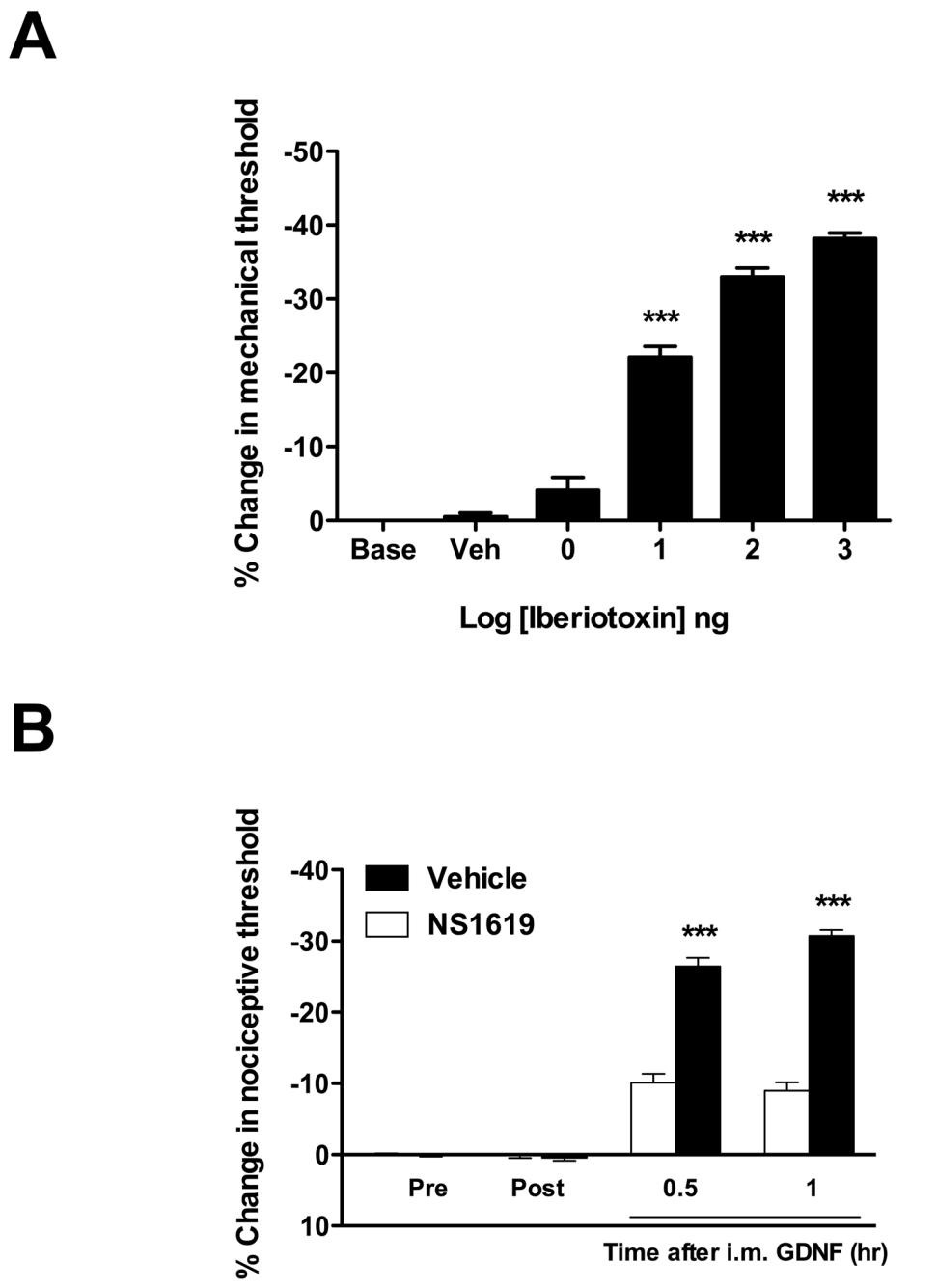


**Figure 3.**

Intra-muscular injection of iberiotoxin into the gastrocnemius muscle produces a dose-dependent reduction in mechanical nociceptive threshold. Mechanical threshold was significantly reduced ($P<0.001$) by the injection of 10 ng (22.1 ± 1.5%), 100 ng (33.1 ± 1.2%) and 1000 ng (38.2 ± 0.8%) iberiotoxin (n = 6 for each group). There was no significant reduction after injection of either 1 ng iberiotoxin (4.1 ± 1.8%) or vehicle (PBS-BSA 0.5±0.5%). **B.** NS1619 prevents GDNF-induced muscle mechanical hyperalgesia. While muscle mechanical threshold (Pre) is not modified by the treatment (Post) with either NS1619 (n=6, open bars) or its vehicle (n=6, solid bars), NS1619 significantly inhibited the decrease of mechanical threshold induced by GDNF compared to vehicle, at 30 (10.1 ±

1.3% versus 26.5 ± 1.2, respectively) and 60 (9 ± 1.2 versus 30.7 ± 0.9, respectively) min after GDNF injection. ***P<0.001

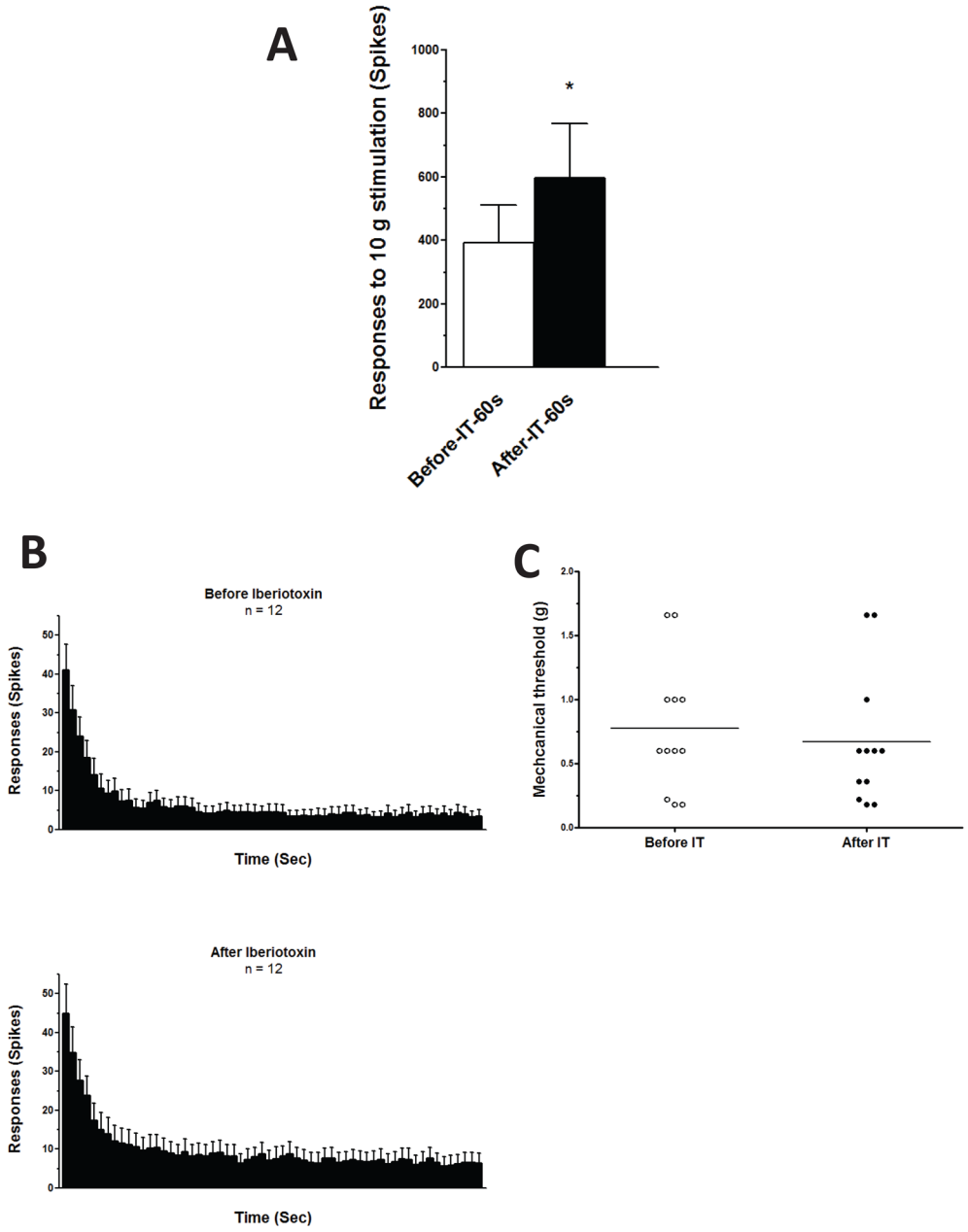


**Figure 4. Iberiotoxin produces increased firing in muscle nociceptors after iberiotoxin injection**

**A.** Bar chart showing the mean response of 12 recorded muscle nociceptors to sustained (60 s) 10 g stimuli, before and after intramuscular injection of iberiotoxin. The mean number of spikes fired increased from 392.2 ± 119.8 before iberiotoxin injection, to 596.1 ± 170.8 after ($P<0.05$).

**B.** Time course of the mean responses of muscle nociceptors to a suprathreshold 60 s stimulus, before (top panel) and after (bottom panel) iberiotoxin injection (n = 12). Bin width is 1 s.

**C.** The mechanical threshold of muscle nociceptors was not significantly altered by the application of iberiotoxin.