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GDNF hyperalgesia is mediated by PLCγ, MAPK/ERK, PI3K, CDK5 and Src family kinase signaling and dependent on the IB4-binding protein versican

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Abstract

The function of the isolectin B4 ($IB4^+$)-binding and GDNF-dependent Ret (Ret^+)-expressing non-peptidergic subpopulation of nociceptors remain poorly understood. We demonstrate that acute administration of GDNF sensitizes nociceptors and produces mechanical hyperalgesia in the rat. Intrathecal IB4-saporin, a selective toxin for $IB4^+/Ret^+$-nociceptors, attenuates GDNF but not NGF hyperalgesia. Conversely, intrathecal antisense to Trk A attenuated NGF but not GDNF hyperalgesia. Intrathecal administration of antisense oligodeoxynucleotides targeting mRNA for versican, the molecule that renders the Ret expressing nociceptors IB4-positive (+), also attenuated GDNF but not NGF hyperalgesia, as did ADAMTS-4, a matrix metalloprotease known to degrade versican. Finally, inhibitors for all five signaling pathways known to be activated by GDNF at GFRα1/Ret; PLCγ, CDK5, PI3K, MAPK/ERK and Src family kinases, attenuated GDNF hyperalgesia. Our results demonstrate a role of the non-peptidergic nociceptors in pain produced by the neurotrophin GDNF and suggest that the IB4-binding protein versican functions in the expression of this phenotype.

Keywords

GDNF; IB4; versican; pain; sensory neurons

Introduction

C-fiber nociceptors have been divided into two classes based on their neurotrophic factor dependence (Molliver et al., 1997, Snider and McMahon, 1998). One expresses the tyrosine kinase A (Trk A) receptor, and depends on nerve growth factor (NGF) for trophic support (Verge *et al*., 1989b), the other expresses the receptor tyrosine kinase Ret, the common signaling component for the receptor of glial cell-line derived neurotrophic factor (GDNF) family ligands (Molliver et al., 1997, Bennett et al., 1998). Almost 50% of all Ret expressing nociceptors also express the GDNF family receptor α1 (GFRα1), which serves as the ligand binding component of the GDNF receptor complex (Bennett et al., 1998). GFRα1/Ret-expressing sensory neurons are dependent on GDNF (Moore et al., 1996, Matheson et al., 1997). Several studies have further characterized these subpopulations of nociceptors, based on the expression of immunocytochemical markers. Thus, the Trk A-positive subset contain the neuropeptides substance P and calcitonin gene-related peptide (CGRP) (Verge et al., 1989a, Averill et al., 1995), while the GFRα1/Ret expressing subset can be characterized by their binding to isolectin B4 (IB4) (Streit et al., 1985, Silverman and Kruger, 1990, Molliver

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et al., 1997), which has recently been shown to bind the extracellular matrix molecule versican (Bogen et al., 2005). These subsets of afferents project to distinct but overlapping areas of the spinal dorsal horn. The NGF-dependent, Trk A/neuropeptide-expressing subset, project to lamina 1 and outer lamina 2 (Molliver et al., 1995), whereas the GDNF-dependent, GFRα1/Ret expressing subset projects predominantly to the inner part of lamina 2 (Streit et al., 1985, Kitchener et al., 1993).

The phenotypic differences between the NGF- and GDNF- dependent nociceptors and the different termination areas of their central projections suggest that they represent different functional classes, which might sense and transmit different aspects of pain-related information to the spinal cord (Snider and McMahon, 1998, Stucky and Lewin, 1999). While signaling in the Trk A-positive nociceptors has been studied extensively (Stoeckel et al., 1975, Otten et al., 1980, Verge et al., 1995, Malik-Hall et al., 2005), the function of the Ret expressing subpopulation has only recently begun to be examined (Bennett et al., 1998, Hucho et al., 2005, Malin et al., 2006, Luo et al., 2007). Thus, while spinal intrathecal administration of GDNF produces analgesia (Boucher et al., 2000, Wang et al., 2003) it has recently been shown that cutaneous IB4-binding nociceptors chronically exposed to high levels of endogeneous GDNF, in transgenic mice overexpressing this neurotrophic factor in the skin, have lower mechanical thresholds than wildtype mice (Albers et al., 2006). However, the underlying cellular mechanisms of this sensitization remain to be elucidated. It is also unclear why mechanical hyperalgesia is not observed in the GDNF overexpressing animals (Albers et al., 2006). To address these questions we determined if *acute* peripheral administration of GDNF is able to induce nociceptor sensitization and mechanical hyperalgesia. Because the GDNF-dependent non-peptidergic nociceptors are also defined by their binding to IB4 (Silverman and Kruger, 1990, Molliver et al., 1997, Bennett et al., 1998), we additionally determined the function of versican, the isolectin B4 binding molecule associated with this subset of nociceptors (Bogen et al., 2005). Finally, we examined the effect of inhibitors of signaling pathways known to mediate effects of GDNF acting at GFRα1/Ret, as novel targets to the treatment of pain syndromes mediated by this subpopulation of primary afferent nociceptors.

Material and methods

Animals

All experiments were performed on male Sprague Dawley rats (250–350 g; Charles River Laboratories, Hollister, CA). The animals were housed in a controlled environment in the animal care facility of the University of California, San Francisco, under a 12 h light/dark cycle. Food and water were available *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee at UCSF and adhered to the guidelines of the American Association of Laboratory Animal Care, National Institutes of Health and the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Effort was made to minimize the number of animals used and their suffering.

Drugs and solutions

GDNF, Roscovitine, Bisindolylmaleimide 1 hydrochloride and PKA Inhibitor 6–22 amide were purchased from Calbiochem (EMD Biosciences Inc., La Jolla, CA, USA), NGF and SU 6656 from Sigma-Aldrich (Saint Louis, MO, USA), and U73122, Wortmannin, LY294002, PP2 and U0126 from Biomol (Plymouth Meeting, PA, USA). IB4-Saporin was obtained from Advanced Targeting Systems (San Diego, CA, USA).

Stock solutions of NGF and GDNF in sterile isotonic saline (Baxter Healthcare Corp., Deerfield, IL, USA) were prepared and stored at −20°C until use. Inhibitors were dissolved in 10 % DMSO containing H_2O and mixed with a stock solution of GDNF at the time of injection.

Each rat received 1μg of inhibitor and 10 ng GDNF in a 5 μl injection volume and mechanical nociceptive thresholds were tested 30 min after the injection.

IB4-saporin was dissolved and diluted in sterile filtered PBS ($conc_f$ = 8 ng/μl) prior of being used and intrathecally administered by using a 30-gauge needle inserted on the midline between the fifth and sixth lumbar vertebrae. This protocol has previously been shown to reduce the number of IB4-binding sensory neurons in the affected dorsal root ganglia and their central processes terminating in the dorsal horn of the spinal cord (Nishiguchi et al., 2004; Joseph et al., 2008).

Enzymes and antibodies

Hyaluronidase was purchased from Sigma-Aldrich and ADAMTS-4 from Calbiochem. The monoclonal anti-versican antibody 12C5, developed by RA Asher (Asher et al., 1991) was obtained from the Developmental Studies Hybridoma Bank founded under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA, USA). The monoclonal mouse anti-GAPDH antibody was obtained from Abcam (Cambridge, MA, USA).

Mechanical nociceptive threshold testing

The nociceptive flexion reflex was quantified using the Ugo Basile analgesymeter (Stoelting, Chicago, IL, USA), in which a linearly increasing mechanical force is applied to the dorsum of the rats hind paw. The nociceptive threshold was defined as the force in grams at which the rat withdrew its paw, and baseline paw-pressure threshold was defined as the mean of three readings before the test agents were injected. Each paw was treated as an independent measure and each experiment was performed on a separate group of rats.

Single fiber in vivo electrophysiology

In vivo single fiber electrophysiology experiments were performed as previously described (Chen and Levine, 2001). Briefly, rats were anesthetized with sodium pentobarbital (initially 50 mg/kg, i.p., with additional doses given throughout the experiment to maintain areflexia) and the saphenous nerve, which innervates the medial-dorsal surface of the hind paw, was exposed and dissected free from accompanying blood vessels. Bipolar stimulating electrodes were placed under the nerve, 2 to 3 cm distal to the recording site. The nerve was cut nearby the recording site and fine fascicles of axons dissected from the nerve were placed on a recording electrode. Single action potentials were first detected by electrical stimulation of the nerve. The conduction velocity of a fiber was calculated by dividing the distance between the stimulating and recording electrodes by the latency of the electrically-evoked action potential. Fibers that conducted slower than 2 m/s were classified as C-fibers (Willis, 1985). Receptive fields of individual C-fibers were located using a mechanical search stimulus, a blunt probe with a smooth tip. The fiber was determined as cutaneous if it was activated by lifting and stimulating the skin and/or if the receptive fields moved when the skin was moved relative to subcutaneous tissue. Non-cutaneous fibers were not further studied. The electrically-evoked action potential corresponding to the C-fiber whose receptive field had been identified was verified by the latency delay technique, in which electrically-evoked spikes resulted in longer latency when the receptive field of the same fiber was stimulated. Mechanical threshold was determined with calibrated von Frey hairs (VFHs) and defined as the lowest force that elicited ≥2 spikes within 1 s, in at least 50% of trials. The neural activity of C-fibers was captured and stored in an IBM compatible PC through Micro 1401 (CED, Cambridge, UK) interface. The offline analysis was performed by *Spike 2* software (CED).

Antisense and mismatch preparation

The 23-mer versican antisense and mismatch oligodeoxynucleotides (ODNs) were purchased from Invitrogen (Carlsbad, CA, USA). The antisense ODN sequence 5'-CAC ACA TAG GAA GTC TCA GTA GG-3' was directed against a unique rat sequence of exon 9 which is present in all known versican splice variants. The corresponding GenBank accession number and ODN position within the cDNA sequence are AF072892 and 1373–1395, respectively. The mismatch ODN sequence 5'-**A**A**A** ACA T**T**G G**T**A GT**A** TCA GT**C A**G-3' corresponds to the versican antisense sequence with seven bases mismatched (denoted in bold). A search of the NCBI database to *Rattus norvegicus* identified no other sequences homologous to that used in this experiment. Prior to being used, lyophilized ODNs were reconstituted in nuclease-free 0.9 % NaCl to a concentration of 12 μg/μl and stored at −20°C until use.

Antisense and mismatch ODNs for Trk A were prepared as we have described previously (Malik-Hall et al., 2005). The antisense ODN sequence 5'-CAT CAA CGA AGT CAC CAG ACC G-3' was directed against a unique sequence of the rat Trk A. The corresponding GenBank accession number and ODN position within the cDNA sequence are M85214 and 121–142, respectively. The mismatch ODN sequence 5'-CA**A** CA**T** CGA AGT **G**AC GAG ACC **G**-3'corresponds to the Trk A antisense with four bases mismatched (denoted in bold).

A dose of 40 μg of versican or Trk A antisense or mismatch ODN was intrathecally administered in a volume of 20 μl once daily over three days. It has been demonstrated previously that this protocol can be used to down regulate the expression of several different proteins in the dorsal root ganglia (Alessandri-Haber et al., 2003, Dina et al., 2004, Malik-Hall et al., 2005, Dina et al., 2005, Joseph et al., 2007, Dina et al., 2008). Prior to each injection rats were anaesthetized with 2.5% isofluorane containing oxygen. ODNs were injected by using a 30-gauge needle inserted intrathecally on the midline between the fifth and sixth lumbar vertebrae.

Protein extraction and Western blotting

L4 and L5 DRGs from rats that were treated with 40 μg of versican antisense or mismatch ODN once daily over three days were dissected out 24 h after the last injection. Each DRG was transferred into 50 μl 0.2% NP-40, 0.2% Tween 20, 100 mM NaCl and a cocktail of 4 times protease inhibitor (Roche Diagnostics Corp., Indianapolis, IN, USA) containing 10 mM $Na_xH_xPO_4$-buffer, pH 7 and vigorously shaken for 2 h at room temperature (RT). After adding an equal volume of 50 U hyaluronidase in 4 times protease inhibitor and 50 mM NaCl containing 140 mM NaOAc-buffer, pH 5.3, the DRGs were incubated under shaking for 4 h at 37°C. After adding 50 μl of 6% SDS and 4 times protease inhibitor containing 300 mM Tris-HCl, pH 7.8, DRGs were further incubated under shaking at 4°C overnight. All samples were sonicated for 30 sec and homogenized using an hand-held plastic pistell. Solubilized proteins were extracted by an 15 min centrifugation at 14000 rpm and 10°C. The protein concentration was determined with the micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with BSA as a standard. 50 μg protein of each sample were mixed with β-mercaptoethanol free sample buffer and electrophoresed on 4 to 15% precast polyacrylamide gels (Biorad, Hercules, CA, USA) in 25 mM Tris containing 192 mM glycine and 0.1% SDS. Proteins were subsequently blotted to a NC-membrane by using the semidry method (transfer time 2.5 h at 75 mA with 47.9 mM Tris, 38.9 mM glycine, 0.038% SDS and 20% methanol). Membranes were cut in half across the level of the 64 kDa marker bands. The lower part (< 64 kDa) of the membranes was blocked with 5% BSA and 0.1% Tween 20 containing TBS (TBST) and the upper part (> 64 kDa) with 5% dry milk containing TBST. Membranes were incubated at 4° overnight with either mouse anti-GAPDH (1:500) or the 12C5 antibody (1:250) in 5% BSA or dry milk containing TBST, washed with TBST (three times for 10 minutes each) and probed for 2 h at RT with the anti-mouse horseradish peroxidase conjugated secondary antibody (1:1000, Santa

Cruz Biotechnology, Santa Cruz, CA, USA) diluted in 5% BSA or dry milk containing TBST. After washing with TBST (three times, 10 min each) immunoreactivities were visualized using the enhanced ECL detection system (Pierce). Results were analyzed by computer-assisted densiometry and levels of versican immunorectivity were normalized with respect to the GAPDH control levels in each sample.

Statistical analysis

Group data are presented as mean +/− SEM and comparisons between groups were performed using Student's t-test, ANOVA followed by Scheffe multiple comparison *post-hoc* test, or Wilcoxon signed rank test, as appropriate. A probability of $p<0.05$ was considered as statistically significant.

Results

GDNF induces mechanical hyperalgesia

To determine the acute effect of GDNF on mechanical nociceptive threshold we injected recombinant rat GDNF intradermally on the dorsum of the rat hind paw. As shown in figure 1A, intradermal GDNF produced a dose-dependent mechanical hyperalgesia (100 pg - 100 ng, cumulative dosing protocol with each sequentially higher dose administered at 30 min intervals). To study the time course of GDNF hyperalgesia we injected a single 10 ng dose of GDNF. This dose produced a decrease in mechanical nociceptive threshold that was already significant 3 min post-injection, reached its maximum by 30 min and lasted for 3 weeks (Fig. 1B).

GDNF sensitizes nociceptors

To determine if acute GDNF-induced hyperalgesia is caused by sensitization of nociceptive afferents we next evaluated the effect of intradermal injection of GDNF on C-fiber function. The mechanical thresholds of cutaneous C-fibers were measured before and 15 min after the injection of GDNF into their mechanical receptive field. As shown in Figure 2 the mechanical threshold of C-fibers is significantly decreased by GDNF, compatible with the suggestion that the hyperalgesia induced by GDNF is due, at least in part, to sensitization of the peripheral terminals of the primary afferent nociceptor, and providing confirmation of the finding of Albers and colleagues that overexpression of GDNF in the skin is associated with lower mechanical thresholds in cutaneous nociceptors (Albers et al., 2006).

GDNF acts on (IB4+)-nociceptors

To test the hypothesis that GDNF induces hyperalgesia by acting at IB4(+)-nociceptors, animals received a single intrathecal injection of 40 ng IB4-saporin 24 h prior to intradermal treatment with 10 ng GDNF. As shown in figure 3A IB4-saporin significantly attenuated hyperalgesia induced by GDNF. In contrast the mechanical hyperalgesia induced by 1 μg NGF was not attenuated by pretreatment with IB4-saporin. NGF hyperalgesia was, however, blocked by the intrathecal injection of antisense oligodeoxynucleotides to Trk A, the high affinity receptor for NGF while GDNF-induced hyperalgesia was not affected (Fig. 3B).

Versican dependence of GDNF hyperalgesia

To evaluate for a role of versican in the function of IB4(+)-nociceptors, we treated rats intrathecally with oligodeoxynucleotides antisense or mismatch to versican. Western blot analysis showed that antisense ODNs significantly attenuate the expression level of three different versican variants with respect to mismatch ODN treated animals (Fig. 4A). When GDNF was injected into the paw of antisense treated animals the mechanical hyperalgesia it

normally induces was significantly attenuated, compared to mismatch treated animals (Fig. 4B). NGF-induced hyperalgesia was, however, not affected by the versican antisense (Fig. 4B).

Role of versican in GDNF hyperalgesia

Versican is an extracellular matrix proteoglycan composed of multifunctional globular N- and C- terminal regions that are joined by one or two stretched glycosaminoglycan (GAG) attachment domains (GAG α and GAG β) which are modified with chondroitine sulfate chains (Wight, 2002). In order to analyze which part of versican is neccessary to mediate GDNF hyperalgesia we intradermally administered the matrix metalloprotease ADAMTS-4 which has been shown to degrade versican by cleaving the glycosaminoglycan attachment domains at postion E55-Q56 (GAGα) or E93-A94 (GAGβ) thereby releasing most of the molecule including all chondroitin sulfate chains into the extracellular space (Sandy et al., 2001) (Westling et al., 2004). As shown in fig. 5A administration of ADAMTS-4 followed by GDNF totally blocked GDNF hyperalgesia suggesting that the N-terminus of versican is dispensable in mediating GDNF hyperalgesia. ADAMTS-4 alone had no effect on nociceptive threshold (Fig. 5A).

Signaling pathways for GDNF hyperalgesia

Five second messengers have been implicated in GDNF signaling via the GFRα1/Ret receptor complex: phospholipase Cγ (PLCγ), cyclin-dependent kinase 5 (CDK5), src family kinases (SFK), extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) (Trupp et al., 1999, Ledda et al., 2002, Paveliev et al., 2004, Tucker et al., 2006). We found that inhibitors of all five pathways - U73122 for PLCγ, Wortmannin and LY294002 for PI3K, PP2 and SU6656 for src family kinases (SFK), U0126 for MEK 1/2, and Roscovitine for CDK5 - significantly attenuated GDNF hyperalgesia whereas inhibitors targeting PKA or PKC had no effect (Fig. 6).

Discussion

While Albers and colleagues have shown that IB4-positive (+) nociceptors from transgenic mice that overexpress GDNF in the skin had lower mechanical nociceptive thresholds, these mice did not demonstrate mechanical hyperalgesia (Albers et al., 2006). The disparity between electrophysiological and behavioral studies and the cellular mechanisms mediating GDNF sensitization of nociceptors remained to be elucidated (Albers et al., 2006). To determine if GDNF can sensitize nociceptors and induce mechanical hyperalgesia we studied the acute effect of intradermally administered GDNF. We found a rapid onset mechanical hyperalgesia with a significant decrease in the paw-pressure threshold already detectable after 3 min, which lasted for more than 14 days. Failure to detect mechanical hyperalgesia in GDNF overexpressing mice might be due to a species difference (Price and Flores, 2007).

To determine if acute GDNF-induced hyperalgesia is due to peripheral nociceptor sensitization we tested if the same protocol for intradermal administration of GDNF affects the mechanical threshold of C-fiber nociceptors; administration of GDNF into the mechanical receptive field lowered the mechanical threshold of half of all cutaneous C-fibers, which is twice the reported number of small caliber nociceptors that bind IB4 and express GFRα1/Ret (Bennett et al., 1998). However, that the number of cutaneous nociceptors that respond to GDNF exceeds the number of those expressing GFRα1/Ret might have been expected. More than 50% of all IB4-binding, Ret expressing small caliber fibers express GFRα2 the ligand binding component of the receptor for neuturin (NRTN) which has been shown to also bind GDNF in presence of Ret (Sanicola et al., 1997, Bennett et al., 1998). Moreover it has been demonstrated *in vivo* that GDNF rescues almost all axotomized IB4-binding, Ret expressing sensory neurons even though not all of them express GFRα1 (Bennett et al., 1998, Bennett et al., 2000, Wang et al.,

2003). Thus, our data support the suggestion that in the rat, GDNF induces mechanical hyperalgesia, an effect mediated, at least in part, by nociceptor sensitization.

To determine if GDNF hyperalgesia is mediated by the Ret expressing IB4(+)-subset of nociceptors, we studied the effect of IB4-saporin, that has been shown to be a specific neurotoxin for IB4-binding nociceptors (Vulchanova et al., 2001, Nishiguchi et al., 2004, Joseph et al., 2008). Pretreatment with IB4-saporin almost completely eliminated GDNF hyperalgesia, supporting the idea that GDNF is producing hyperalgesia by acting on the Ret expressing IB4(+)-subpopulation of sensory neurons. To confirm the specificity of IB4-saporin for Ret expressing nociceptors we additionally tested the effect of IB4-saporin on NGF hyperalgesia. IB4-saporin had no effect on NGF hyperalgesia. On the other hand, Trk A antisense attenuated NGF-induced hyperalgesia but had no effect on GDNF-induced hyperalgesia. These data support the suggestion that GDNF induces hyperalgesia by acting on Ret expressing, IB4-binding nociceptors.

One of the most enigmatic aspects of the Ret expressing sensory neurons is their unique ability to be labeled by the plant lectin IB4 (Molliver et al., 1997, Bennett et al., 1998). Based on the specificity of the IB4-labeling for the GDNF dependent subset of Ret(+)-positive nociceptors, we speculated that there might be a functional connection between GDNF signaling and versican, the molecule that renders this subset of nociceptors IB4-positive (Bogen et al., 2005). Indeed, spinal administration of ODN antisense targeting versican mRNA attenuates not only the detectable level of three different versican variants in DRG extracts, it also attenuates GDNF, but not NGF, hyperalgesia, supporting the idea of a functional connection between GDNF signalling and versican.

Versican is an extracellular matrix proteoglycan. Its association with the plasma membrane is mediated by its binding to hyaluronate (LeBaron et al., 1992, Bogen et al., 2005). Versican binds to hyaluronate with its N-terminal hyaluronate binding domain while the C-terminus projects away from the plasma membrane. Because of its broadly linear structure and perpendicular orientation to the plasma membrane, it seems unlikely that versican directly interacts with GFRα1/Ret, the receptor components for GDNF. One possible explanation for our finding that GDNF hyperalgesia can be blocked by a knockdown of the expression of versican is that versican's glycosaminoglycan side chains might act as a low-affinity receptor for GDNF or soluble GFRα1 produced and secreted by the surrounding tissue that is innervated by the peripheral nociceptive terminals. Of note in this regard, identification of GDNF was initially based on its high-affinity binding to heparin (Lin et al., 1993). Moreover, it has recently been reported that GDNF signaling requires heparan sulfate proteoglycans in addition to the GFRα1/Ret receptor complex (Barnett et al., 2002, Tanaka et al., 2002). Even though versican is a chondroitin sulfate proteoglycan it has already been shown that its chondroitin sulfate chains bind molecules that might prefer binding to heparin or heparan sulfate, such as the growth factor midkine, the chemokine RANTES or L- and P-selectin (Zou et al., 2000, Kawashima et al., 2000, Hirose et al., 2001).

In a first attempt to test our hypothesis we administered GDNF and the matrix metalloprotease ADAMTS-4 which cleaves, beside others, both of versican's GAG attachment domains thereby releasing most of the molecule including all chondroitin sulfate chains into the extracellular space (Sandy et al., 2001, Westling et al., 2004). Intradermal administration of ADAMTS-4 followed by GDNF totally blocked GDNF hyperalgesia. Even though versican might not be the only target of ADAMTS-4, this result strongly supports the idea that versican's glycosaminoglycan chains are necessary to mediate GDNF hyperalgesia.

To identify the second messenger pathway mediating GDNF hyperalgesia, we tested the effect of inhibitors for the five second messenger pathways known to be activated by GDNF acting

at GFRα1/Ret. It has been shown in neurons that GDNF acting at GFRα1/Ret receptor complex can signal by at least five different second messengers, i.e., SFK, PI3K, ERK/MAPK, CDK5, PLCγ (Trupp et al., 1999, Ledda et al., 2002, Paveliev et al., 2004, Tucker et al., 2006). In the present study we observed that inhibitors of all five second messengers significantly attenuated GDNF hyperalgesia. That PI3K, ERK/MAPK and PLCγ can mediate mechanical hyperalgesia is well established (Aley et al., 2001, Zhuang et al., 2004, Malik-Hall et al., 2005). Src family kinases (SFK) have also been implicated in nociceptor sensitization, mainly by their action on TRP channels of the vanilloid subfamily (Zhang et al., 2005, Alessandri-Haber et al., 2008). The phosphorylation of TRPV1 and/or TRPV4 by SFKs is induced by inflammatory mediators like NGF, and seems to be accompanied by the translocation of TRPV channels to the plasma membrane (Zhang et al., 2005, Alessandri-Haber et al., 2008).

CDK5 is a unique member of the cyclin-dependent serine/threonine kinases. The activity of CDK 5 is regulated by the association with p35 or p39, or their truncated variants p25 and p30. It has recently been shown that peripheral inflammation stimulates calpain, which then cleaves p35 to generate p25 a much stronger activator of CDK5. The elevated CDK 5 activity has been shown to sensitize nociceptors, at least in part by phosphorylation induced sensitization of TRPV1 (Pareek et al., 2006, Pareek et al., 2007). Future studies will address interaction between these signaling pathways in the generation of GDNF hyperalgesia.

GDNF appears to be critical for the survival of sensory fibers; axotomized sensory afferents can be rescued by GDNF injected into the peripheral nerve or spinal cord (Matheson et al., 1997; Bennett et al., 1998; Ramer et al., 2000). Moreover, intrathecal GDNF has been shown to be analgesic by maintaining the normal neuronal phenotype of injured sensory afferents (Boucher et al., 2000; Wang et al., 2003). Based on these results it was speculated that GDNF might be of use for the treatment of neuropathic pain induced by nerve injury. However, there are still some questions that have to be answered before one might be able to evaluate the true therapeutic potential of GDNF. For example, GDNF can activate several signaling pathways. GDNF can be internalized and retrogradely transported to the cell body where it is believed to regulate gene expression or activate several classical 2nd messenger signalling cascades via Ret-dependent or independent mechanisms (Matheson et al., 1997; Trupp et al., 1999; Sariola and Saarma, 2003). However, it is far from clear which pathways are activated under different conditions. Also, while it has been shown previously that GDNF requires tumor growth factor beta (TGFβ) to exert its neurotrophic action on sensory neurons, it is still not known how GDNF increases the peripheral or central TGFβ concentration (Kriegelstein et al., 1998; Peterziel et al., 2002).

Taken together, our results demonstrate that GDNF sensitizes IB4^{+}/Ret^{+} C-fiber nociceptors and causes mechanical hyperalgesia, in the rat. The action of GDNF is dependent on the presence of the extracellular matrix molecule versican and mediated by signalling pathways known to be activated via GFRα1/Ret.

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Abbreviations

ADAMTS-4, A disintegrin and metalloproteinase with thrombospondin motivs 4
BSA, bovine serum albumin
CDK5, cyclin-dependent kinase 5
cDNA, copy of messenger RNA
CGRP, calcitonin gene-related peptide

DMSO, dimethylsulfoxide
DRG, dorsal root ganglia
GAG, glycosaminoglycan
GAPDH, glycerinaldehyde 3 phosphate dehydrogenase
GDNF, glial-derived neurotrophic factor
GFRα1, GDNF family receptor α 1
IB4, isolectin B4
LY 294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase
NaCl, sodium chloride
NaOAc, sodium acetate
NGF, nerve growth factor
NP-40, nonident P-40
ODN, oligodeoxynucleotide
p25/30/35/39, CDK5 activator proteins with an apparent molecular weight of 25, 30, 35 or 39 kDa
PBS, phosphate-buffered saline
PI3K, phosphatidylinositol 3-kinase
PKA, protein kinase A
PLCγ, phospholipase C gamma
Rantes, regulated upon activation normal T-cell expressed and secreted
Ret, rearranged during transfection
mRNA, messenger ribonucleic acid
PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyramidine
RT, room temperature
SDS, sodium dodecylsulfate
SFK, src family kinases
src, sarcoma
SU 6656, 2,3 dihydro-N,N-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl) methylene]-1H-indole-5-sulfonamide
TBS, Tris-buffered saline
TBS-T, Tris-buffered saline containing Tween 20
TGFβ, tumor growth factor beta
Trk A, tyrosine kinase A receptor
TRPV 1/4, transient receptor potential channel of the vanilloid subfamily 5 members 1 and 4
U 0126, bis[amino[(2-aminophenyl)thio]methylene]butanedinitrile
U-73122, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17yl)amino]hexyl]-1H-pyrrole-2,5-dione.

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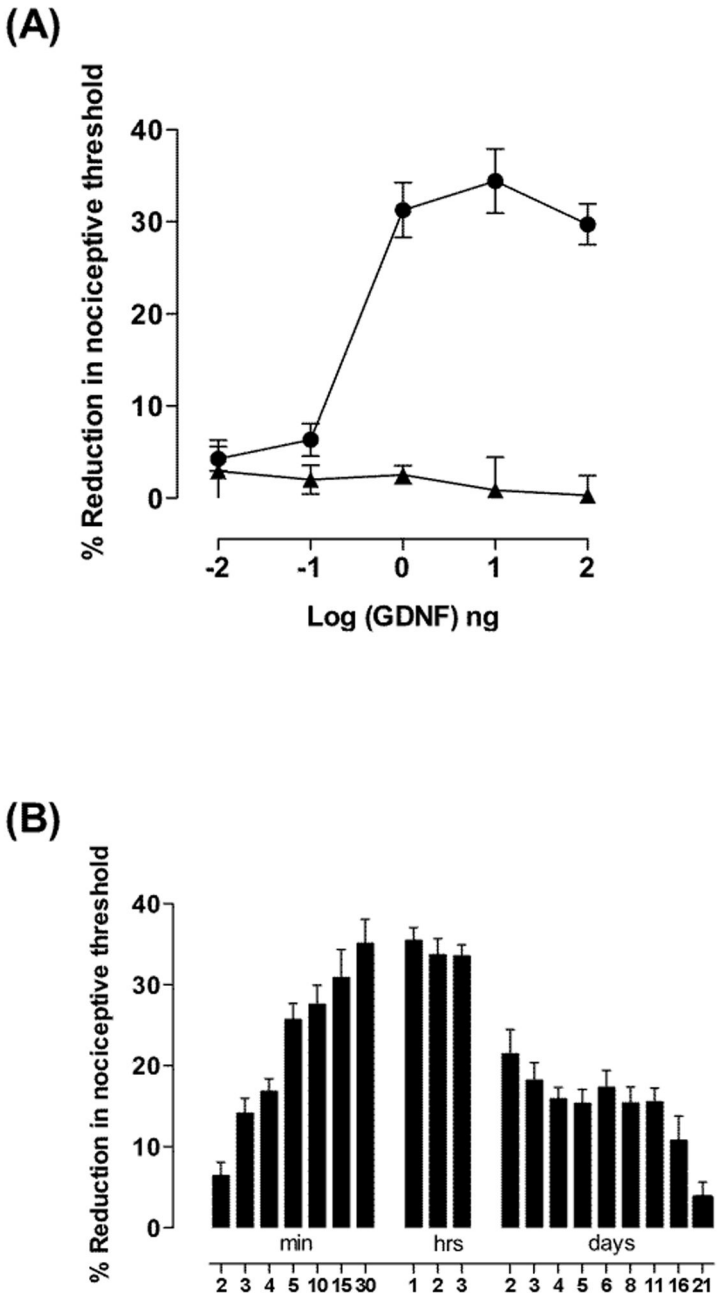

Figure 1. GDNF induces mechanical hyperalgesia

(A) Dose-response curve for the effect of intradermal GDNF on mechanical nociceptive threshold following administration into the dorsum of the rat hind paw. Incremental doses from 10 pg to 100 ng were administered at 30 min intervals. A two way ANOVA with one within subject factor (dose with 5 levels) and one between subject factor (treatment with two levels) showed a significant dose × treatment interaction ($F(1,10)= 22.129$, $p<0.001$), a significant main effect of treatment ($F(1,10)= 108.204$, $p<0.001$) and a significant overall effect of dose ($F(4,40)= 19.056$, $p<0.001$). A maximal decrease of the nociceptive threshold was obtained at 10 ng GDNF ($n = 6$, $p<0.001$).

(B) The time-response curve for hyperalgesia induced by 10 ng GDNF was investigated from 2 min to 21 days (n = 6). Hyperalgesia was first detectable at 3 min, reached a maximum 30 min post-injection, and lasted for 3 weeks.

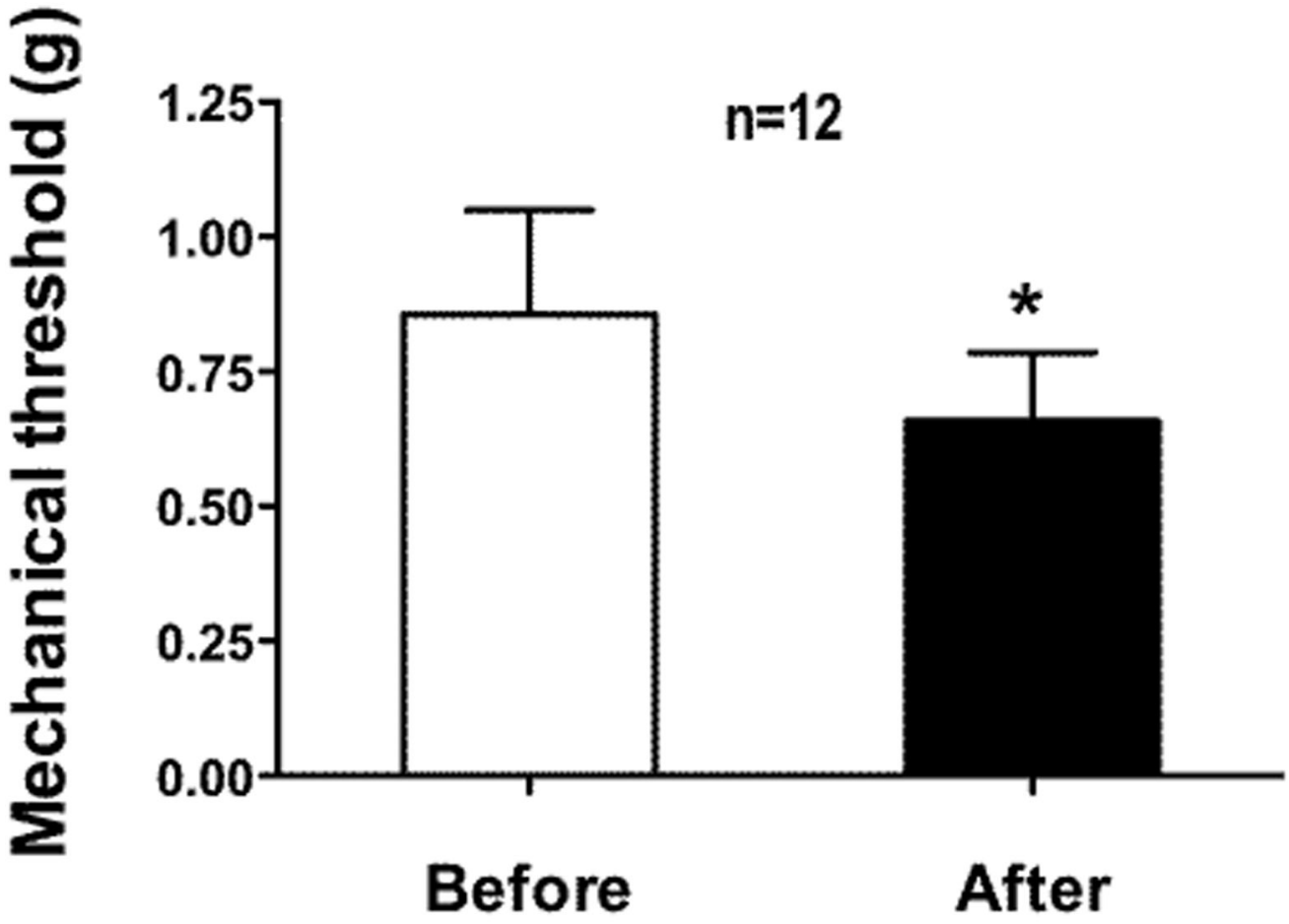

Figure 2. GDNF sensitizes C-fiber nociceptors

Mechanical threshold in C-fiber nociceptors before and 15 min after the injection of GDNF into the receptive field of identified nociceptive C-fibers. The average mechanical threshold of the cutaneous C-fibers whose receptive field were injected with GDNF is decreased by approximately 20 % (n = 12, $p<0.05$; Wilcoxon signed rank test).

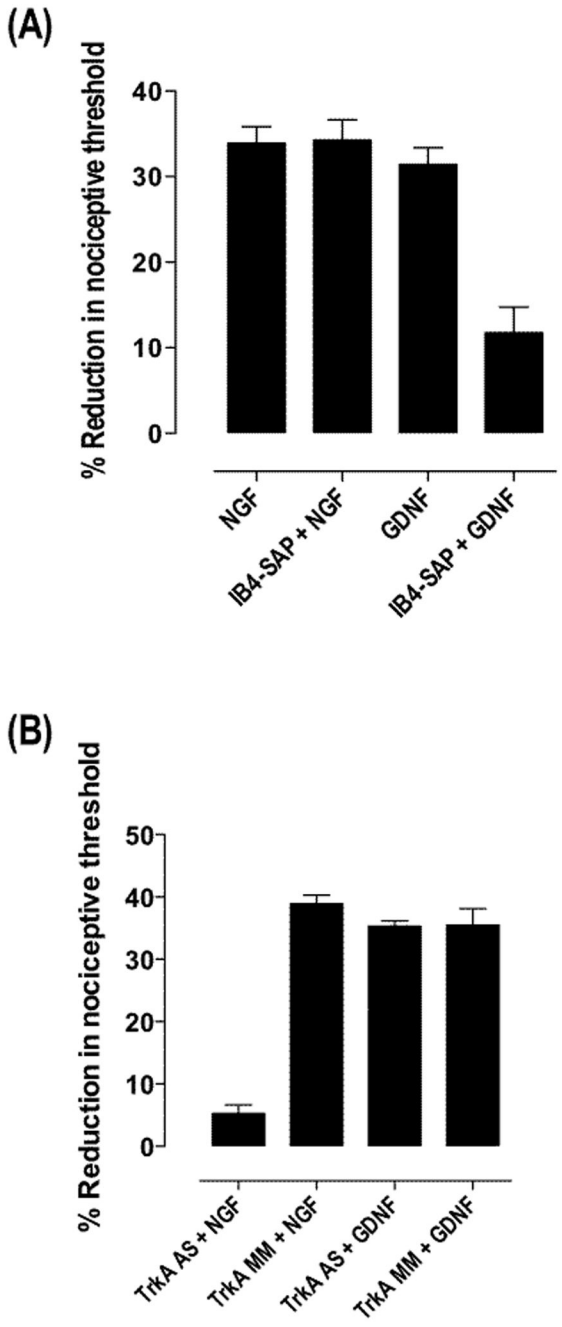

Figure 3. GDNF hyperalgesia is dependent on IB4 (+)- nociceptors

(A) GDNF but not NGF hyperalgesia is inhibited by intrathecal administration of IB4-saporin. One way ANOVA with one between subjects factor (treatment) showed a significant effect of treatment ($F(3,26) = 13.202$; n = 4 to 8, $p<0.001$). Scheffe *post-hoc* analysis showed that the group of rats treated with IB4-saporin and GDNF differ significantly from the other three groups ($p<0.001$ in each case) but there were no differences between the other three groups. (B) NGF but not GDNF hyperalgesia can be blocked by intrathecal administration of oligodeoxynucleotides antisense to Trk A. One way ANOVA with one between subjects factor (treatment) showed a significant effect of treatment ($F(3,20) = 133.95$; n = 4 to 8, $p<0.001$). Scheffe *post-hoc* analysis showed that the group of animals that received antisense

oligodeoxynucleotides to Trk A mRNA and NGF was significantly different from the other groups ($p<0.001$ in each case).

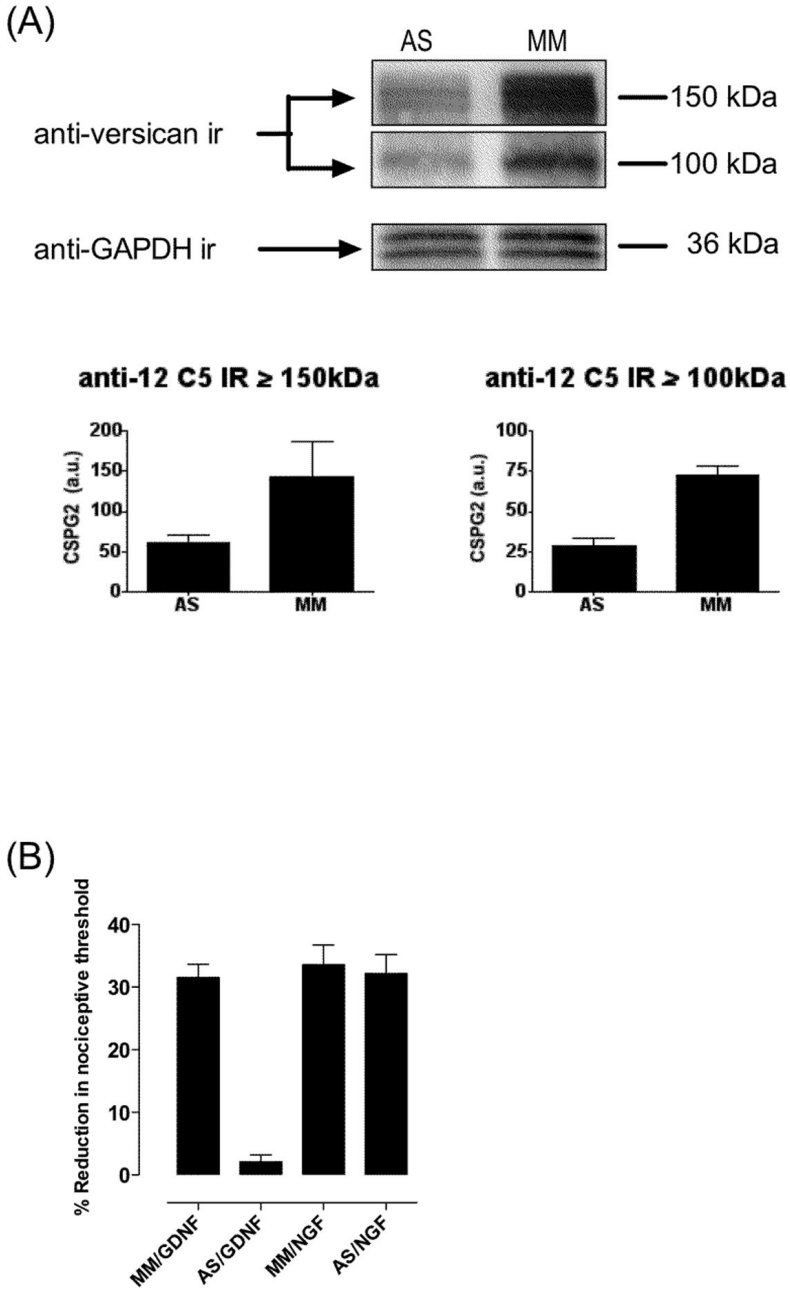

Figure 4. GDNF but not NGF hyperalgesia can be blocked by a versican knockdown

(A) Down regulation of versican expression. Analyzing the protein expression by Western blot demonstrated a significant knockdown of versican in the DRG from rats treated with antisense-ODN to versican mRNA (upper bands, ≥150 kDa = 62 +/− 2 arbitary units; lower band, ≥100 kDa = 26 +/− 2 arbitary units) compared to mismatch-ODN treated rats (upper bands, ≥150 kDa = 143 +/− 3 arbitary units; lower band, ≥100 kDa = 63 +/− 3 arbitary units, normalized to the reference protein GAPDH; $P < 0.05$ (unpaired Student's t-test), a 57 +/− 1% (≥150kDa) and 59 +/−3% (≥100 kDa) decrease in versican immunoreactivity).

(B) GDNF hyperalgesia can be blocked by a down regulation of versican expression. One way ANOVA with one between subject factor (treatment) showed a significant main effect of

treatment ($F(3,16) = 42.892$; n=4 to 6; $p<0.001$). Scheffe post-hoc analysis showed that the group of animals that were treated with antisense to versican is significantly different form all other groups ($p<0.001$ in each case).

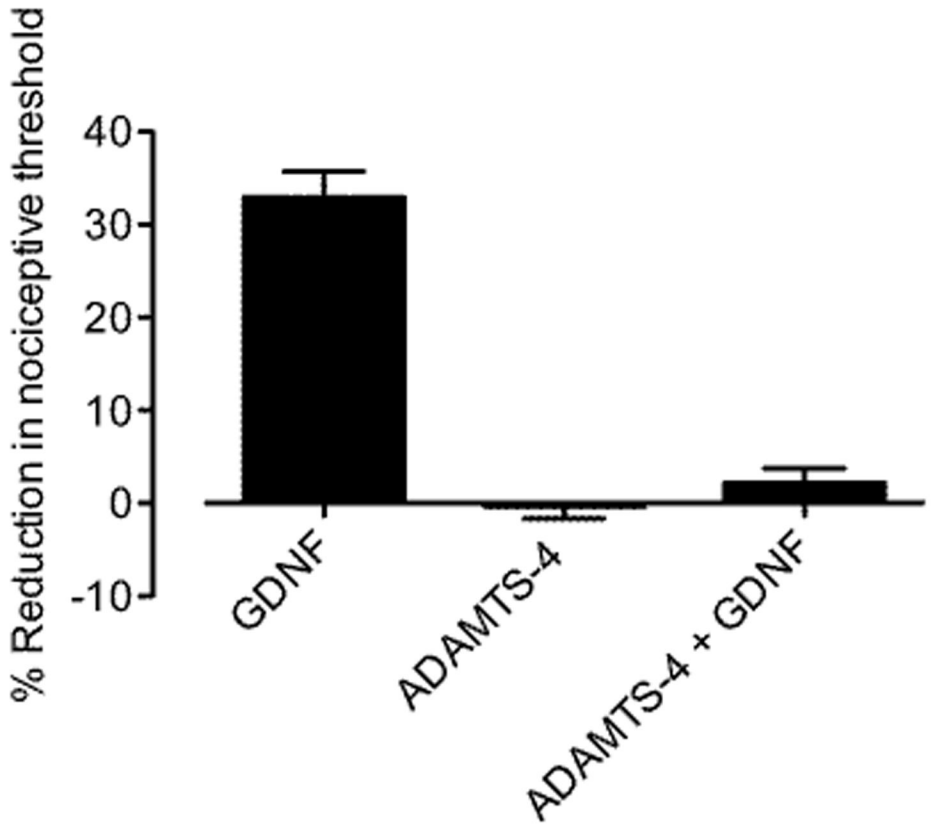

Figure 5. GDNF hyperalgesia can be attenuated by the degradation of versican

ADAMTS-4 blocks GDNF hyperalgesia. One way ANOVA with one between subjects factor (treatment) showed a significant effect of treatment ($F(2,15) = 87.457$; $n = 6$, $p<0.001$). Scheffe *pos-thoc* analysis showed that the group of animals that received just GDNF differ significantly from the other two groups ($p<0.001$).

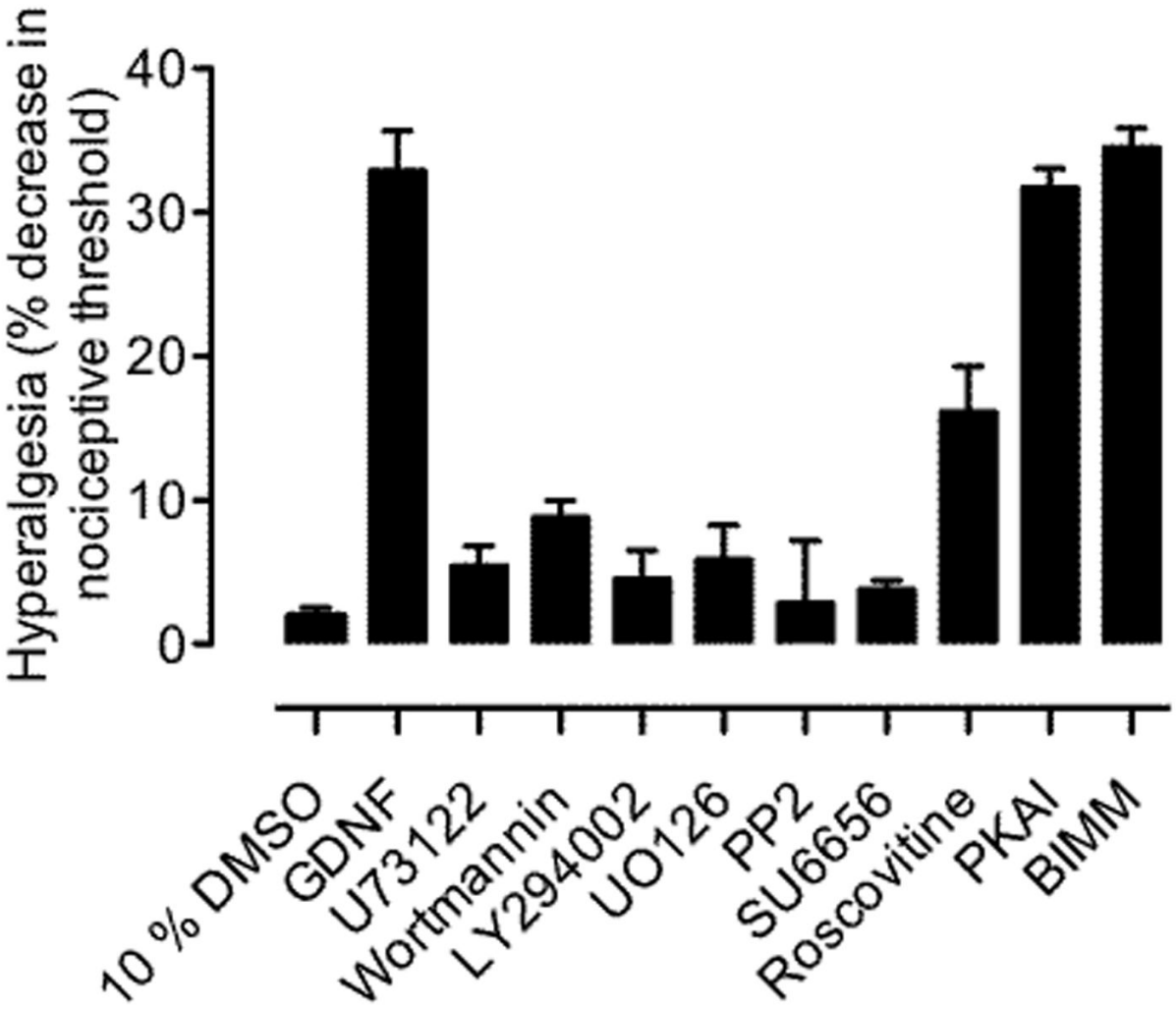

Figure 6. Signaling pathways mediating GDNF hyperalgesia

GDNF-induced mechanical hyperalgesia is mediated by five different signaling pathways. Rats received a single intradermal injection of 10 ng GDNF and 1 μg inhibitor for each signaling pathway. Readings of the mechanical nociceptive threshold were taken 30 min post-injection. As shown inhibitors of PLCγ (U73122, *n* = 6), PI3K (Wortmannin and LY294002, both n = 4), MEK 1/2 (U0126, n = 6), SFK (PP2 and SU6656, both n = 4) and CDK5 (Roscovitine, n = 6) all significantly attenuated GDNF hyperalgesia (all $p<0.001$) whereas inhibitors to PKA (PKA inhibitor 6–22 amide, n=6) and PKC (Bisindolylmaleimide 1 hydrochloride, n=6) had no significant effect on GDNF hyperalgesia (p=1.000, in both cases). Note that 10% DMSO alone did not change paw-pressure threshold.