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Selective inhibition of ERK1/2 blocks NGF to BDNF signaling and suppresses the development of and reverses already established pain behavior in rats

Yoshikazu Matsuoka and Jay Yang*

Department of Anesthesiology, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706, U.S.A

Abstract

Brain-derived neurotrophic factor (BDNF) plays a key role in the development of pathological pain. Although it is known that nerve growth factor (NGF) induces BDNF mRNA through extracellular signal-regulated kinases (ERK), whether ERK1/2 or ERK5, two closely related members of the ERK family, mediates this signal is still unclear because classical MEK inhibitors block both pathways. We studied the involvement of ERK-signaling in NGF induction of BDNF in PC12 cells, cultured dorsal root ganglia neurons, and in rats subjected to neuropathic pain models using ERK1/2 and ERK5 specific tools. Selective activation of ERK1/2 up-regulated BDNF mRNA in PC12 cells, whereas selective ERK5 activation did not. AZD6244, a potent selective inhibitor of ERK1/2 activation, blocked NGF induction of BDNF mRNA *in vitro* suggesting that NGF induction of BDNF is mediated by ERK1/2. siRNA experiments indicated that both ERK1 or ERK 2 can signal suggesting that both pathways must be blocked to prevent NGF-induced increase in BDNF mRNA. Intraperitoneal injection of AZD6244 prevented the development of pain in rats subjected to the chronic constriction injury and reversed already established pain in the spared nerve injury model. Immunohistochemical studies showed decreased phospho-ERK1/2-immunoreactivity in dorsal root ganglia and BDNF-immunoreactivity in ipsilateral spinal dorsal horn in the drug-treated rats. Our results suggest the possible use of AZD6244, already in human clinical trials as an anti-cancer agent, for the treatment of pathological pain.

Keywords

AZD6244; neuropathic pain; drug therapy; MEK inhibitor; NGF-BDNF signaling

Introduction

Nerve growth factor (NGF), a member of the neurotrophin family, is released from keratinocytes, fibroblasts, Schwann cells and mast cells in injured or inflamed tissues (Radtke et. al., 2010; Wu et. al., 2007, 2009). Released NGF binds to the trkA receptor on sensory nerve endings, and through induction of brain-derived neurotrophic factor (BDNF),

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*Address for correspondence: Dr. Jay Yang, Department of Anesthesiology, University of Wisconsin SMPH, SMI 301, 1300 University Avenue, Madison, WI 53706, U.S.A., jyang75@wisc.edu, 1-608-265-6710 (phone), 1-608-265-3542 (fax).

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plays a key role in the signaling mediating nerve injury leading to pathological pain. Systemic administration of NGF to rats induced BDNF mRNA in dorsal root ganglia (DRG) neurons expressing trkA receptors (Apfel et. al., 1996) and cultured DRG neurons stimulated with NGF showed up-regulation of BDNF mRNA (Matsuoka et. al., 2007). BDNF in turn enhances excitability of the spinal cord through several effector mechanisms including reduction of repolarizing A-type potassium current in the dorsal root ganglion neurons (Cao et. al., 2010), activation of NR2B-containing NMDA receptors (Geng et. al., 2010), and down regulation of the KCC2 chloride transporter with a consequent reversal of chloride gradient, rendering formerly inhibitory GABAergic neurons excitatory (Coull et. al., 2005). The BDNF-induced increased excitability in the superficial dorsal horn (SDH) appears to be cell-type-specific (Lu et. al., 2007, 2009). These reports support a key role of BDNF in SDH hyperexcitability leading to chronic pain.

Extracellular signal-regulated kinases (ERKs) transduce extracellular signals to intracellular events, and ERK1/2 are involved in the development of pain hypersensitivity after tissue and nerve injury (Ji et. al., 2009). Activation of ERK1/2 is observed in SDH and DRG in the rats subjected to several different acute and chronic pain models and the classical MEK1/2 inhibitors, PD98059 and U0126, suppressed pain behavior and BDNF expression in the spinal cord (Obata et. al., 2003a, 2004; Zhuang et. al., 2005). These compounds have been considered specific inhibitors of MEK1/2 due to its lack of effect on p38 and JNK MAPKs; however, these alleged specific MEK1/2 inhibitors can also inhibit ERK5, another member of the MAPK family closely related to ERK1/2 (Kamakura et. al., 1999). ERK5 activation in DRG and SDH has been implicated in mediating inflammatory (Xiao et. al., 2008) and pathological pain (Obata et. al., 2007).

Although several lines of evidence suggest that NGF induces BDNF in DRG neurons, the intracellular signaling that transduces this induction of BDNF is not clear. NGF phosphorylates ERK1/2 and ERK5 in PC12 cells (Kamakura et. al., 1999; Obara et. al., 2009) and U0126 blocks NGF induction of BDNF (Park et. al., 2006). *In vivo* intrathecal injection of U0126 suppresses BDNF induction in DRG neurons after peripheral inflammation (Obata et. al., 2003a). These studies support the role of ERKs in NGF-BDNF signaling. However, because of the lack of specificity of the chemical inhibitors utilized, whether ERK1/2 and/or ERK5 mediates NGF-BDNF signaling remains unclear. Gaining a detailed understanding of the signaling leading to BDNF induction is critical in order to develop a mechanism-based treatment of pathological pain. We studied the role of ERK1/2 and ERK5 in this signaling cascade and development of pathological pain using ERK1/2 and ERK5 specific tools.

Experimental procedures

Plasmids and virus production

Lentivirus vectors were created by a triple transfection of HEK293T cells (CRL-11268, ATCC, Manassas, VA, USA) with a shuttle vector with the gene of interest subcloned, pVSVG for pseudo-typing, and Δ 8.9 packaging-plasmid. For expression of gene-of-interest, an in house-modified pLL 3.7 shuttle vector (VRMC-39, ATCC) where the mouse CMV promoter drives both the expression of the gene and the neomycin selectable marker was used. Culture media containing virus particles were collected 48 and 72 h after transfection and concentrated by ultrafiltration (Amicon Ultra 100K, Millipore, Billerica, MA, USA) and the viral titer was estimated by transducing and counting fluorescence positive cells. The resulting lentivirus expressed the fluorescence reporter for easy identification of transduced cells and sorting by a cell sorter for further expansion of a mixture of transduced cell population. Molecular manipulations of cDNA to create various tagged constructs were achieved using standard methods. All clones created in house and those obtained from

outside vendors were sequenced and the identity and fidelity of the constructs confirmed. The C-terminal epitope tagged constitutively active CA-MEK1 and CA-MEK5 have been shown to specifically activate the downstream ERK1/2 or ERK5, respectively (Cameron et al., 2003).

Cell culture

The rat pheochromocytoma cell line PC12 was cultured in Dulbecco's modified Eagle's medium (4.5 g/L glucose) and supplemented with 5% fetal bovine serum, 10% horse serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Neonatal rat DRG neurons (Lonza, Walkersville, MD, USA) were cultured in serum-free NbActive4 medium (BrainBits, Springfield, IL, USA). PC12 cells and DRG neurons were plated on dishes coated with poly-D-lysine, in an incubator containing 5% CO₂ at 37°C. PC12 cells were serum-starved for 14–16 h before the stimulation with NGF.

For NGF stimulation, cells were incubated with 100 ng/mL of 2.5S NGF (BD Biosciences, Bedford, MA, USA) for 6 h. AZD6244 (Selleck Chemicals, Houston, TX, USA) was added to the medium 1 h before NGF stimulation. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin, in an incubator containing 5% CO₂ at 37°C. HEK cells were transiently transfected with CA-MEK1, CA-MEK5 and ERK5wt subcloned in pCI/neo eukaryotic expression vector (Promega, Madison, WI, USA) for 24 h. Cells were stimulated with 10 ng/mL of epidermal growth factor (Invitrogen, Carlsbad, CA, USA) for 5 min.

siRNA transfection

PC12 cells were transfected with siRNA targeting rat ERK1 and ERK2 (Ambion, Austin, TX, USA) for 48 h before NGF stimulation. Neon Transfection System (Invitrogen) was used according to manufacturer's instructions (1500Vx 10 ms × 3 pulses). Each siRNA was used at a concentration of 100 nM. Scramble siRNA (Ambion) was used as a negative control.

SDS-PAGE and Western Blotting

Cells were lysed 5–30 minutes after NGF stimulation and total protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins were denatured and reduced by boiling in SDS and β-mercaptoethanol containing buffers. Electrophoresis was performed on 10% polyacrylamide gel and the proteins were transferred from the gel onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked for 1 h with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature and incubated with primary antibodies in TBST containing 1% skim milk overnight at 4°C or for 2 h at room temperature. Anti-ERK1/2 (1:10000, Promega), anti-ERK5 (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-phospho-ERK (pERK) (1:1000, Cell Signaling Technology) were used. The blots were washed three times and incubated with HRP-conjugated secondary antibody in TBST containing 1% skim milk at room temperature for 1 h. After rinsing with TBST, proteins were detected by enhanced chemiluminescence method (Western lightning chemiluminescence reagent plus, Perkin Elmer, Waltham, MA, USA).

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with QIAzol (Qiagen, Germantown, MD, USA) per the manufacturer's instructions. cDNA was reverse transcribed from 1 µg of total RNA with a QuantiTect Reverse Transcription Kit (Qiagen). Genomic DNA was removed by the

genomic DNA wipeout buffer included in this kit. Templates were amplified in a 25 μ L reaction mixture containing 12.5 μ L SYBR Jump Start Taq (Sigma-Aldrich), 0.25 μ L ROX reference dye, 11 μ L DNase-free H₂O, 0.2 μ M each of forward and reverse primers, 0.25 μ L cDNA solution. Real-time PCR analysis was performed with a MX3000P (Stratagene, La Jolla, CA, USA) with the following amplification conditions: 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. PCR specificity was confirmed by BLAST search and melting curve analysis after the amplification. Relative expression was quantified using the $\Delta\Delta C_t$ method. 18S rRNA was used as an internal standard. Sequences of primers are following: rat 18S rRNA (GenBank ID: M11188) Forward; 5'-CCATTCGAACGTCTGCCCTAT-3', Reverse; 5'-GTCACCCGTGGTCACCATG-3'. Rat BDNF (GenBank ID: NM_012513) Forward; 5'-GCGGCAGATAAAAAGACTGC-3', Reverse; 5'-GCAGCCTTCCTTCGTGTAAAC-3'.

Animals

This study was approved by the Animal Care and Use Committee of University of Wisconsin. Animals were treated in accordance with the Ethical Guidelines for Investigation of Experimental Pain in Conscious Animals issued by the International Association for the Study of Pain (Zimmermann, 1983). Male Sprague-Dawley rats (250–280 g, Harlan, Madison, WI, USA) were used. The rats were housed with a 12 h light/dark cycle with food and water available *ad libitum*. Rats were subjected to a chronic constriction injury (CCI) of the sciatic nerve (Bennett and Xie, 1988), or a spared nerve injury (SNI) of the sciatic nerve (Decosterd and Woolf, 2000). In the CCI model, the rat was anesthetized with isoflurane and the left common sciatic nerve was exposed at the mid-thigh level. Four ligatures of 4–0 chromic gut were tied loosely around the nerve with 1 mm spacing between knots and the wound was closed in 2 layers. AZD6244 (1 mg/kg) or vehicle (10% DMSO in saline serving as a control) were administered intraperitoneally immediately after the surgery and once daily for 3 days. For the SNI model, the rats were anesthetized and the left sciatic nerve exposed as in the CCI model. The common peroneal and the tibial nerves were tightly ligated with 5-0 silk and the distal 2 mm of the nerves cut and removed; the sural nerve remained intact. Intraperitoneal injection of AZD6244 (1 mg/kg) or vehicle were started on day 3 after the mechanical allodynia was confirmed and continued daily for 3 days.

Behavioral assessment

Before the behavioral assessment, the rats were allowed to acclimate for 30 minutes. Thermal withdrawal latency was measured with the Hargreaves apparatus (Ugo Basile, Collegeville, PA, USA) before the surgery (baseline) and on indicated days after nerve injury. The rats were placed individually on an elevated glass floor and radiant heat was aimed at the plantar surface of the hind paws. The latencies were measured three times at 5 min intervals and averaged. The values were expressed as differences from the baseline for each individual rat. Mechanical threshold was measured with the dynamic plantar anesthesiometer (Ugo Basile). Rats were placed individually on an elevated plastic mesh. The metal filament was applied to the plantar surface of hind paws. The upward force was increased from 1 to 50 gram and the force at withdrawal was recorded. Measurements were performed three times at 5 min interval and averaged. The behavioral assessments were done by a single investigator blinded to the drug treatment.

Flow cytometry

Rat blood samples were collected in tubes containing EDTA. We labeled pERK1/2 positive cells according to the methods described previously (Chow et. al., 2005) with some modifications. Briefly, 50 μ L of whole blood was stimulated with 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 10 min at 37°C. Erythrocytes were lysed by addition of 1 mL deionized H₂O for 30 sec and followed immediately by the addition of 117

μ L 10 $\times$ PBS. Unlysed cells were fixed by 4% paraformaldehyde for 10 min. After centrifugation for 5 min at 1000 $\times$ g, cells were washed with PBS containing 4% fetal bovine serum and permeabilized with 50% methanol for 15 min at 4°C. Cells were incubated with rabbit anti-pERK1/2 antibody (1:500, Cell Signaling Technology) for 30 min at room temperature. After washing with PBS containing 4% fetal bovine serum, cells were incubated with anti-rabbit-AlexaFluor 488 (1:1000, Invitrogen) for 30 min at room temperature. For *in vitro* experiment, whole blood was incubated with AZD6244 for 30 min before PMA stimulation. For *in vivo* experiment, rats were injected AZD6244 (1 mg/kg) intraperitoneally. Blood samples were collected before, 30 min and 24 h after the drug injection. Flow cytometry was performed by Guava Easy Cyte Plus (Millipore) and analyzed by CytoSoft software (Millipore).

Immunohistochemistry

Under deep anesthesia, the rats were perfused with 50 mL saline followed by 500 mL 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) transcardially on day 3 after CCI. Bilateral L4 DRGs and L4–L5 spinal cords, identified by the lumbar enlargement and the emergence of the nerve roots, were dissected and post-fixed with the same fixative for 30 min and then soaked in phosphate-buffered 30% sucrose overnight. Tissue sections were cut (25 μ m for spinal cord and 15 μ m for DRG) on a cryostat and thaw-mounted on glass slides. Sections were incubated with anti-BDNF (chicken, 5 μ g/mL, R&D Systems, Minneapolis, MN, USA), anti-pERK1/2 (rabbit, 1:3000, Cell Signaling technology) for 24 h at room temperature followed by anti-chicken-HRP (1:100, Promega), anti-rabbit-AlexaFluor 594 (1:1000, Invitrogen) respectively for 2 h at room temperature. For BDNF staining, sections were incubated with AlexaFluor 488-conjugated tyramide (1:200, Invitrogen) for 10 min. Coverslips were placed on the slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were captured by fluorescent microscopy and analyzed by Image J software (National Institute of Health, Bethesda, MD, USA). For DRG images, a threshold was determined from the all-pixel histogram of images without the primary antibody. In SDH images, to distinguish BDNF-immunoreactivity (IR) from the background fluorescence, histogram in laminae I, II and histogram in laminae I, II and surrounding area were drawn. The number of pixels with low intensity was increased in the histogram including the surrounding area. These intensities were considered background level; the threshold was determined by the crossing point of these two histograms, and pixels above the threshold were counted. The pixel count was multiplied by the pixel intensity to arrive at the first-moment of IR which was quantified. This measure accounted for both the increase in the number and intensity of IR. For BDNF-IR positive DRG neuron counting, the images were all captured with the same exposure and neurons with intensities exceeding a threshold value displayed and directly counted (see Figure 8 legend for further details). Three measurements per animal were averaged. For double immunostaining, BDNF were double-labeled with anti-synaptophysin (1:1000, Chemicon, Temecula, CA, USA), OX-42 (1:800, Abcam, Cambridge, MA), NeuN (1:1000, Chemicon), CGRP (1:200, Abcam) or GFAP (1:200, Abcam) (all mouse monoclonal) for 24 h at room temperature. Goat anti-mouse AlexaFluor 594 (1:1000, Invitrogen) was used as the secondary antibody. Confocal images were captured with Nikon A1R (Nikon Instruments, Melville, NY, USA) equipped with Plan Apo 20 $\times$ (numerical aperture 0.75) and Plan Apo VC 60 $\times$ Oil (numerical aperture 1.4) objective lenses. The optical slice thickness was 1.0 μ m.

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Dunnett's post test, two-way repeated measure ANOVA followed by Bonferroni's post test or Student's t-test. $P < 0.05$ was considered statistically significant. Data are shown as mean $\pm$ standard errors of the mean.

Results

NGF phosphorylates ERK1/2 and ERK5, and increases BDNF mRNA

PC12 cells express the trkA receptor and these cells have been used as a model for studying many biological effects of NGF (Vaudry et. al., 2002). Western blot analyses with phospho-specific antibodies confirmed NGF phosphorylation of both ERK1/2 and ERK5 in a similar temporal manner both largely gone by 30 minutes after the initial stimulation (Fig. 1A). NGF (100 ng/ml) stimulation of PC12 cells resulted in an increase in the BDNF mRNA quantified by real-time RT-PCR and peaked at 6 h after the initial stimulation (Fig. 1B). This BDNF mRNA expression pattern was consistent with our earlier report on cultured DRG neurons (Matsuoka et. al., 2007). To dissect the role of ERKs in the NGF-induced increase in BDNF mRNA further, we used molecular reagents with documented specificity.

Selective activation of ERK1/2 but not ERK5 induces BDNF mRNA

CA-MEK1 and CA-MEK5 activates ERK1/2 and ERK5, respectively (Cameron et. al., 2003). We confirmed the specificity of the C-terminal epitope-tagged version of these constructs by transiently transfecting HEK293T cells and performing a Western blot with phospho-specific anti-ERKs antibodies. Co-transfection with CA-MEK1-DsRED selectively induced pERK1/2 whereas CA-MEK5-EGFP induced pERK5 (Fig. 2A). Next, we created lentivirus vectors expressing CA-MEK1-DsRED or CA-MEK5-EGFP to allow efficient transduction of the difficult-to-transfect PC12 cells. Lentiviral transduction of PC12 cells resulted in about 50% of the cells demonstrating fluorescence. The vectors co-expressed a neomycin selectable antibiotic resistance from an IRES construct, allowing further selection and amplification of PC12 cells transduced with the virus. Control cells were transduced with an EGFP-expressing lentivirus. CA-MEK1-DsRED, CA-MEK5-EGFP, or EGFP expressing PC12 cells were grown to homogeneity and the level of BDNF mRNA assayed. The BDNF mRNA level was increased in CA-MEK1-DsRED expressing cells without NGF stimulation to a level comparable to EGFP-expressing cells stimulated with NGF. The CA-MEK5-EGFP expressing cells without NGF stimulation only expressed control levels of BDNF mRNA (Fig. 2B) despite the fact that basal ERK5 is present in PC12 cells (data not shown). These results demonstrated that activation of ERK1/2 was sufficient for the induction of BDNF mRNA. A converse experiment attempting to selectively inhibit the ERK1/2 or ERK5 pathways using dominant-negative MEK1 or MEK5 constructs did not work since PC12 cells transduced with these reagents died within days after lentivirus transduction.

AZD6244, a selective MEK1/2 inhibitor, suppresses NGF-induced expression of BDNF mRNA

To selectively block the ERK1/2 pathway, we used AZD6244, a recently introduced selective MEK1/2 inhibitor. This non-ATP competitive MEK1/2 inhibitor exhibited preferred *in vivo* profile allowing it to enter a human clinical trial for its potential use as an anti-cancer drug (Adjei et. al., 2008; Bennouna et. al., 2011). We confirmed the ERK1/2 specific inhibition in PC12 cells treated with NGF. AZD6244 suppressed NGF-induced activation of ERK1/2 at 0.1 μ M, whereas AZD6244 did not suppress the activation of ERK5 even at 10 μ M (Fig. 3A, B). AZD6244 at 3 μ M was sufficient to completely inhibit ERK1/2 with no effect on ERK5, which completely blocked NGF induction of BDNF mRNA in PC12 cells (Fig. 3C). Although PC12 cells are widely used as a surrogate neuronal cell line, we confirmed induction of BDNF mRNA after NGF stimulation in cultured DRG neurons as reported previously (Apfel et. al, 1996; Matsuoka et. al., 2007) and demonstrated that AZD6244 blocked this induction identical to our results in PC12 cells (Fig. 3D). These results, with a selective chemical inhibitor, indicated that activation of ERK1/2 was necessary for NGF induction of BDNF mRNA.

Either ERK1 or ERK2 can transduce NGF

ERK1 and ERK2 are MAPKs with mostly interchangeable roles; however, some pathways are mediated selectively by one or the other (Sturgill, 2008). To investigate the involvement of ERK1 and ERK2 separately, PC12 cells were transfected with siRNA targeting ERK1 or ERK2 mRNA. Western blots confirmed selective knock-down of ERK1 or ERK2 48 h after transfection (Fig. 4A). Knocking-down of either ERK1 or 2 alone failed to suppress the NGF-induced up-regulation of BDNF mRNA. Simultaneous knock-down of both ERK1 and 2 inhibited NGF induction of BDNF mRNA (Fig. 4B) indicating a redundancy of this signaling pathway.

Systemic administration of AZD6244 reduces thermal hyperalgesia and mechanical allodynia in rats subjected to the chronic constriction injury

We assessed the effects of AZD6244 on the development of pathological pain in rats subjected to CCI and SNI models. Prior to implementing the *in vivo* pain models, we explored whether a once daily injection of AZD6244 at 1 mg/kg/day resulted in sufficient systemic concentration to inhibit ERK1/2 activation. Peripheral blood mononuclear cells (PBMC) were *in vitro* stimulated with 200 nM PMA. Forward and side scatter gated anti-pERK1/2 reactive signal corresponding to the PBMC with activated ERK1/2 was evaluated with a flow cytometer. Fig. 5A–E show representative scatter plots of peripheral blood cells and histograms of pERK1/2 labeled PBMC. Approximately 25% of PBMC treated with PMA were pERK1/2 positive with an AZD6244 concentration-dependent decrease in this percentage (Fig. 5F). PBMC isolated from rats injected with AZD6244 demonstrated a significant reduction in pERK1/2 (Fig. 5G) confirming that this drug dosage was sufficient to suppress ERK1/2 activation of PBMC *in vivo*.

CCI caused thermal hyperalgesia and mechanical allodynia in the ipsilateral hind paw of vehicle-treated rats evaluated on Day 3 after surgery. Daily *i.p.* injection of AZD6244 (1 mg/kg/day) after surgery suppressed the development of both thermal hyperalgesia and mechanical allodynia (Fig. 6A, B). We did not find any adverse events such as weight loss, diarrhea, or obvious motor deficiency from the daily AZD6244 administration consistent with the reports of lack of gross behavioral side effects from this drug (Huynh et al., 2007).

Next, we wanted to evaluate whether MEK1/2 inhibition can reverse already established pain behavior. For this experiment, we used the SNI pain model since this model results in a more consistent and prolonged pain behavior (Decosterd and Woolf, 2000). SNI caused mechanical allodynia in all rats by Day 3 after surgery and AZD6244 injections reversed the decreased withdrawal threshold. The rats once again developed mechanical allodynia upon stopping the drug administration attaining withdrawal thresholds identical to the vehicle treated group (Fig 6C). There was no change in the thermal withdrawal latency (data not shown) as expected for this model.

Immunohistochemical study of L4 DRG and SDH

pERK1/2-IR was found in DRG neurons and the surrounding satellite cells ipsilateral to the CCI consistent with previous reports (Obata et. al., 2003a; Zhuang et. al., 2005). Unlike the previously reported small diameter neurons preferentially activated by inflammatory stimulus or the medium to large diameter neurons enhanced after axotomy (Obata et. al., 2003a), CCI which exhibits both inflammatory and neuropathic components resulted in increased pERK1/2-IR in all neurons with no predilection for a specific subpopulation. A quantitative assessment of the IR taking into consideration the background IR, number of pixels exceeding the threshold, and the fluorescence intensity in those pixels, demonstrated a greater anti-pERK1/2-IR in the ipsilateral L4 DRG from rats treated with vehicle. This difference was not observed in L4 DRGs isolated from rats treated with AZD6244 (Fig. 7).

A small number of BDNF-immunoreactive L4 DRG neurons were observed at baseline and this number greatly increased at Day 3 after CCI consistent with earlier reports using the inflammatory or nerve transection models (reviewed in Obata & Noguchi, 2006). Rats treated with AZD6244 demonstrated a greatly reduced number of BDNF-immunoreactive DRG neurons (Figure 8) as expected if pERK1/2 were involved in the BDNF expression in the post-injury neurons.

To study whether the inhibition of pERK1/2 by AZD6244 altered BDNF expression in the SDH, the L4–5 spinal cord was immunostained with an anti-BDNF antibody. The BDNF-IR was exceedingly weak requiring tyramide amplification for visualization. Preliminary experiments showed that the exact level of the spinal cord within L4 and L5 from which the sections were obtained did not affect the quantitative measurement of BDNF-IR (data not shown). The BDNF-IR was mainly found in laminae I and II in the spinal cord, and CCI increased this IR in the ipsilateral SDH (Fig. 9A) at Day 3. The ipsilateral BDNF-IR was indistinguishable from the contralateral side by Day 8 coincident with the recovery of pain behavior (data not shown). Daily injection of AZD6244 suppressed the increase in ipsilateral BDNF-IR in the SDH (Fig. 9B, D).

While there is general agreement on the role of BDNF in the signaling mediating injury to neuropathic pain, the anatomic source of BDNF remains controversial- some suggesting DRG and others microglia or SDH neurons (Michael et al., 1997; Ha et al, 2001; Coull et al, 2005; Li et al, 2008; Geng et al., 2010). Double immunostaining showed co-localization of the BDNF-IR with anti-synaptophysin- and anti-CGRP-IR in the SDH but not with the neuronal marker NeuN, microglial marker OX-42, or the astrocytic marker GFAP (Fig. 10), demonstrating that, in our hands, the increased BDNF-IR in the injured animal was largely confined to the sensory afferent terminals. Together with the observed increase in BDNF-IR in the DRG neurons, our data is consistent with a pERK1/2-dependent increase in BDNF production in the DRG neurons, an anterograde transport of BDNF by the primary afferent fibers, and a likely release of BDNF in the spinal cord.

Discussion

We investigated the involvement of ERK pathways in NGF-induced BDNF up-regulation *in vitro* and the effect of an ERK inhibitor on the development of pathological pain *in vivo*. Using highly selective constitutively-active MEK1 or 5 molecular reagents, we demonstrated that NGF-induced up-regulation of BDNF mRNA was mediated by ERK1/2, but not by ERK5 in cultured PC12 cells and primary DRG neurons. Selective siRNA knockdown of ERK1 vs. ERK2 indicated either ERK1 or 2 can signal and inhibition of both ERK1 and 2 was necessary to interrupt the NGF induction of BDNF mRNA. The MEK1/2 specific inhibitor, AZD 6244, attenuated the development of thermal hyperalgesia and mechanical allodynia in rats subjected to the CCI model and reversed already established mechanical allodynia in rats subjected to the SNI model. Immunohistochemical studies confirmed that AZD6244 decreased phosphorylation of ERK1/2 in ipsilateral L4 DRG and reduced BDNF in the SDH. This is the first study demonstrating AZD6244 inhibition of pathological pain.

ERK signaling in pathological pain

Our results showed that NGF induction of BDNF mRNA signals through ERK1/2 *in vitro* and a selective inhibition of ERK1/2 activation *in vivo* normalized pain behavior in rats subjected to the CCI model. The suggestion that ERK1/2 is involved in pathological pain is of course not new. Phosphorylation of ERK1/2 was increased in L4/5 DRG after sciatic nerve transection and continuous intrathecal administration of U0126 suppressed pERK- and BDNF-IR in DRG with a normalization of the pain behavior (Obata et. al., 2003a). Similar

results were found in rats subjected to the L5 spinal nerve ligation (Zhuang et. al., 2005) and CCI (Obata et. al., 2004). The classical MEK inhibitors, such as U0126 and PD98059, considered to be specific inhibitors of MEK1/2 and frequently used for assessing the role of ERK1/2 in pain research are now known to suppress activation of ERK5 (Kamakura et. al., 1999) as well. Our *in vivo* study showed that selective ERK1/2 inhibition with systemic AZD6244 administration normalized the painful behavior unambiguously demonstrating the role of ERK1/2 in the CCI model. However, phosphorylation of ERK5 was increased in L4/5 DRG after L5 spinal nerve ligation (Obata et. al., 2007), and intrathecal injection of ERK5 antisense oligonucleotide suppressed painful behaviors. Signals mediating pathological pain are multi-modal and both ERK1/2 and ERK5, and other MAPKs (Obata et. al., 2004), are likely to play a role in the development of pain in different pain models. Further studies utilizing gene targeted mice with selective deletion of ERK5 in the DRG neurons that can be created by crossing the already reported NaV1.8 promoter-driven Cre recombinase mice with the loxP-ERK5 mice (Stirling et. al., 2005; Kimura et. al., 2010) should provide an unambiguous answer on the role of ERK5 in pathological pain.

Our results in PC12 cells suggested a redundant role of ERK1 and 2 in NGF-BDNF signaling. A recent study investigated the pain-phenotype in ERK1 knock out mice subjected to a variety of inflammatory and chronic pain models (Alter et. al., 2010) concluding that ERK1 was not required but rather ERK2 played a predominant role in murine pain models. We speculate that ERK1 and 2 play redundant roles and a mechanism-based therapy for pathological pain targeting ERK signaling must inhibit both ERK1 and 2.

Cellular localization of spinal BDNF

Direct subarachnoid injection of BDNF elicits pain behavior, and sequestration of BDNF by a trkB-Fc chimeric antibody or anti-BDNF antibody abrogates pain in several animal models (Yajima et. al., 2005; Coull et. al., 2005). It is also clear that BDNF mRNA and protein in the spinal cord increases (Miletic and Miletic, 2002; Geng et. al., 2010; Ha et. al., 2001) and the obligatory role of BDNF in painful behavior is well-established, at least in inflammatory pain models (reviewed in Merighi et. al., 2008). The CCI model we employed has both inflammatory and neuropathic components (Cui et. al., 2000; Kleinschnitz et. al., 2006) and we expect BDNF to play a key role in mediating painful behavior in this model. However, the cellular source of BDNF is controversial. If NGF-BDNF signaling in the DRG is the basis for increased BDNF expression in the spinal cord, it is reasonable to assume that the increased BDNF production in the DRG is transported to the afferent terminal, resulting in the increased expression of BDNF, mainly in laminae I and II. Our immunohistochemical data from rats subjected to CCI demonstrated an increase in BDNF-IR in the DRG neurons and the spinal cord BDNF-IR mainly localized to laminae I and II co-localizing with synaptophysin- and CGRP-IR in agreement with Michael et. al. (1997), but not with glial, astrocytic, or neuronal markers. On the other hand, activated microglia cells can be a source of BDNF leading to painful behavior (Coull et. al., 2005), and suppression of microglial activation with minocycline attenuates neuropathic pain (reviewed in Suter et. al., 2007). We cannot exclude the possible effect of systemically-administered AZD6244 on spinal microglia-derived BDNF in our observed suppression of painful behavior despite our immunohistochemical data suggesting otherwise.

Duration of BDNF increase after injury

Literature indicates a wide range of timing and duration of BDNF increase depending on the pain model and assay techniques used. For the CCI model, spinal cord BDNF assayed by ELISA peaked on day 7, returning to normal levels by day 28 (Miletic and Miletic, 2002) in agreement with elevated BDNF-IR in the spinal cord at one and two weeks assayed by immunohistochemistry (Ha et. al., 2001). The paw incision model resulted in peak BDNF-

IR on day 1, normalizing by day 3 (Li et. al., 2008), and the CFA (Cho et. al., 1997) and L5 spinal ligation (Geng et. al., 2010) models similarly induced a transient increase in BDNF expression over couple of days but normalized by 2 weeks after injury. The persistence of painful behavior and spinal cord BDNF expression in rodents may vary depending on the degree of neuronal injury induced by the model (Obata et. al., 2003a). ATF-3 immunostaining, as a marker for neuronal injury, revealed only about 10% injury in our rats (data not shown) and may have contributed to the normalization of thermal allodynia and spinal cord BDNF-IR by day 8 after injury. Nevertheless, TrkB-BDNF plays an obligatory role not only in the induction phase but also in the maintenance phase of chronic pain (Wang et. al., 2009), and targeting this signaling could be an attractive approach to preventing the development of chronic pain after injury and reversing the already established painful state as suggested by our data confirming the reversal of mechanical allodynia by AZD6244.

Selective MEK1 inhibition as a mechanism-directed therapy for chronic pain

Our results support the importance of ERK1/2 signaling in pathological pain in agreement with others (Obata et. al., 2003a, 2004; Zhuang et. al., 2005). How then can we use this knowledge to translate into potential clinical care of patients with chronic pain? Experimentally, frequently-utilized MEK1 inhibitors PD98059 and U0126 are not approved for human usage. In contrast, AZD6244 has demonstrated clinical acceptability and currently with more than twenty ongoing phase II clinical trials (www.clinicaltrials.gov). Although we chose to administer this drug by *i.p.* injection in the rats in our present study, this drug is bioactive after oral administration in humans with a plasma $T_{1/2}$ of 6.1–14.5 h (Adjei et. al., 2008). One hundred milligram bid of oral administration was well-tolerated in the phase I study and C_{max} of AZD6244 was 486–895 ng/mL (*i.e.* 1.06–1.96 μ M) at this dose (Adjei et. al., 2008). *In vitro* study showed that IC_{50} for inhibition of pERK1/2 was 10.3 nM, whereas IC_{50} for inhibition of cell growth in cell lines were 59–473 nM (Yeh et. al., 2007) inhibiting ERK1/2 phosphorylation at a lower concentration than inhibition of tumor growth. There appears to be a long-lasting pharmacological effect of AZD6244 inhibiting phosphorylation of ERK1/2 for 24 h even as the tumor concentration of the drug decreased to a negligible level (Denton and Gustafson, 2010). Despite the well-tolerated once a day AZD6244 dosing used in the current study, the *in vivo* efficacy of ERK1/2 inhibition was confirmed by the analysis of PBMC, and most importantly, the ability of this treatment to block the development of painful behavior in rats subjected to pain models was confirmed. We speculate that this therapeutic benefit was due to the inhibition of NGF-BDNF signaling in DRG neurons but systemic administration of AZD6244 will clearly affect other cells such as microglia and immune cells known to play a role in pain signaling. Furthermore, it remains a formal possibility that AZD6244 may act on the BDNF signaling itself instead of its presumed action via inhibition of pERK1/2. Reagents with greater anatomic specificity such as AAV8 (Storek et. al., 2008) expressing a dominant-negative MEK1 construct capable of selectively inhibiting the NGF-BDNF signaling in the DRG neurons must be utilized to unambiguously confirm the anatomic site and mechanism of action. Nevertheless, the use of AZD6244 for treatment of pain in humans appears reasonable and further studies are indicated.

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Abbreviations

BDNF	brain-derived neurotrophic factor
CA-MEK1	constitutively active-MEK1
CCI	chronic constriction injury
DRG	dorsal root ganglion
ERK	extracellular signal-regulated kinases
IR	immunoreactivity
NGF	nerve growth factor
PBMC	peripheral blood mononuclear cells
pERK	phospho-ERK
PMA	phorbol 12-myristate 13-acetate
SDH	superficial dorsal horn
SNI	spared nerve injury
TBST	Tris-buffered saline containing 0.1% Tween 20

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Highlights

- ERK1/2, but not ERK5, mediated NGF-induced BDNF mRNA expression.
- Both ERK1 and 2 can mediate the NGF to BDNF signaling.
- Inhibition of both ERK1 and 2 are necessary to disrupt this pain signaling cascade.
- The MEK1/2 inhibitor AZD6244 blocked pERK1/2 and BDNF increase in DRG.
- AZD6244 prevented the development and reversed already established pain in rats.

Figure 1. NGF stimulation increases BDNF mRNA and activates both ERK5 and ERK1/2

A. Phosphorylation of ERKs after NGF stimulation. Cultured PC12 cells were stimulated with 100 ng/mL of NGF for indicated durations. The cell lysate was harvested at each time points and equal amount of proteins were loaded onto gels. Phosphorylated and total ERKs were detected by immunoblotting. **B.** Changes in expression of BDNF mRNA after stimulation with NGF. Cultured PC12 cells were stimulated with 100 ng/mL of NGF for indicated durations. Expression levels of BDNF mRNA were quantified by real-time RT-PCR. Each value was expressed relative to the baseline. $n = 4$ in each group. * $p < 0.05$ vs. baseline.

Figure 2. Selective activation of ERK1/2 increases BDNF mRNA

A. Phosphorylation of ERKs by constitutively-active (CA) MEK1 and MEK5. HEK293T cells were transiently transfected with CA-MEK1 or MEK5 along with wild-type ERK5 (ERK5wt). Epidermal growth factor (EGF) stimulation was done at 10 ng/mL for 5 min. Phosphorylated and total ERK were detected by immunoblotting. **B.** Effects of CA-MEK1 and MEK5 on expression of BDNF mRNA. PC12 cells were infected with CA-MEK1 or MEK5 lentivirus for 4 days. NGF stimulation was done at 100 ng/mL for 6 h. Expression of BDNF mRNA were quantified by real-time RT-PCR. Each value was expressed relative to the baseline. $n = 6$ in each group. * $p < 0.05$ vs. control. † $p < 0.05$ vs. CA-MEK5.

Figure 3. AZD6244 is a selective inhibitor of ERK1/2 and blocks NGF-induced increase in BDNF mRNA

A. Inhibitory effect of AZD6244 on phosphorylation of ERKs. PC12 cells were treated with AZD6244 at indicated concentrations for 1 h before NGF stimulation. Cells were stimulated with NGF at 100 ng/mL for 5 min. Phosphorylated form and total ERK were detected by immunoblotting. **B.** Densitometry was performed on pERKs. Each value was normalized to total ERK and expressed relative to baseline. $n = 3$ in each group. $*p < 0.0001$ vs. control. **C.** Effect of AZD6244 on expression of BDNF mRNA in PC12 cells. PC12 cells were pre-treated with 3 μ M of AZD6244 or vehicle for 1 h before NGF stimulation. NGF stimulation was done at 100 ng/mL for 6 h. Expression of BDNF mRNA were quantified by real-time RT-PCR. Each value was expressed relative to baseline. $n = 4$ in each group. $*p < 0.01$ vs. control. **D.** Effect of AZD6244 on expression of BDNF mRNA in cultured primary DRG neurons. The neurons were pre-treated with 3 μ M of AZD6244 or vehicle for 1 h before NGF stimulation. NGF stimulation was done at 100 ng/mL for 6 h. Expression of BDNF mRNA were quantified by real-time RT-PCR. Each value was expressed relative to baseline. $n = 3$ in each group. $*p < 0.05$ vs. control.

Figure 4. Either ERK1 or ERK2 can mediate the NGF-induced increase in BDNF mRNA

A. Selective knockdown of ERK1 and ERK2 confirmed by western blotting. PC12 cells were harvested 48 h after siRNA transfection and protein contents for ERK1, ERK2, ERK5, and GAPDH probed in a Western blot. Note the selective knock down of ERK1/2 by their respective siRNA without effect on ERK5. **B.** Effect of siRNA targeting ERK1 or ERK2 on expression of BDNF mRNA. PC12 cells were transfected with siRNA and 48 h later stimulated with 100 ng/mL of NGF for 6 h. Expression of BDNF mRNA were quantified by real-time RT-PCR. Each value was expressed relative to baseline. $n = 3$ in each group. $*p < 0.05$ vs. control.

Figure 5. Effects of AZD6244 on the phosphorylation of ERK1/2 in peripheral blood mononuclear cells (PBMC) assessed by flow cytometry

A. Osmotically-shocked partially lysed methanol fixed peripheral blood was gated by forward and side scatter to define the population corresponding to PBMCs. **B–E.** Representative histograms of anti-pERK1/2 antibody labeled PBMCs. Baseline phosphorylation in unstimulated PBMCs (**B**), after *ex vivo* stimulation with 200 nM PMA for 10 min (**C**), as in C but pre-treated with 1 or 5 μM AZD6244 before PMA stimulation (**D, E**). **F.** Summary bar graph of the percentage of pERK1/2 positive PBMCs demonstrating the inhibition of PMA-stimulated pERK1/2 by AZD6244. *n* = 3 in each group. **G.** *In vivo* effect of AZD6244 on phosphorylation of ERK1/2 in PBMC. Blood was harvested before the intraperitoneal injection of AZD6244, 15 minutes or 24 hrs after a single injection and subjected to the *ex vivo* PMA stimulation assay. *n* = 2 for each group.

Figure 6. The effects of AZD6244 on painful behaviors in rats subjected to CCI and SNI
A. The thermal stimulation withdrawal latencies were measured before subjecting the rats to CCI, and at Days 3. AZD6244 (1 mg/kg, *i.p.*) was administered daily for 3 days after surgery until the behavioral assessment. The values were expressed as differences from the baseline. $n = 10-11$ in each group. ** $p < 0.01$ vs. baseline. **B.** The mechanical withdrawal thresholds were measured before and at Day 3 after the CCI. $n = 5$ in each group. ** $p < 0.01$ vs. baseline. **C.** The mechanical withdrawal thresholds were measured before and at Day 3, 4, 6, 7, 8 after the SNI. $n = 6$ in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. baseline. Arrows indicate AZD6244 administration.

Figure 7. AZD6244 inhibits phosphorylation of ERK1/2 in the ipsilateral DRGs in rats subjected to CCI

A–D. Photomicrographs showing pERK1/2-immunoreactivity in ipsilateral (**A, C**) and contralateral (**B, D**) L4 DRG from rats with CCI. The rats were treated with vehicle (**A, B**) and AZD6244 (**C, D**). Scale bar in **D** = 100 μm . **E.** Representative all-points pixel intensity histograms in control, contralateral and ipsilateral L4 DRG neurons. **F.** Weighted intensity count ($\sum \text{pixel} \times \text{intensity}$) of pERK1/2-immunoreactivity above the threshold. The DRGs were harvested at 3 days after CCI. $n = 3$ in each group.

Figure 8. AZD6244 reduces the number of BDNF-immunoreactive DRG neurons

A. Photomicrographs of BDNF-IR neurons in L4 DRGs from DMSO or AZD6244 treated rats. Scale bar = 100 μ m. **B.** A fluorescent image of a DRG section (Day 3 after CCI ipsilateral to the injury) (top), pixel intensity distribution plot (middle) where the vertical line indicates the threshold chosen, and the resulting processed image used for cell counting (bottom). **C.** Summary bar plot of BDNF-IR neuron counts (mean percentage $\pm$ S.E.M.) for the stated conditions. * $P < 0.001$. At least 100 neurons from random fields captured from 3 different sections were analyzed for the cell counting. All slides were immuno-stained in parallel and the images captured in an identical manner with identical exposure times. The threshold chosen based on the most BDNF-IR section (i.e. ipsilateral side in DMSO treated rats) was stringent and may have resulted in an underestimation of the total BDNF-IR neurons counted.

Figure 9. AZD6244 decreases BDNF-immunoreactivity (IR) in ipsilateral spinal dorsal horn
A, B. Photomicrographs showing BDNF-IR in L4–5 spinal dorsal horn in rats with CCI. The rats were treated with vehicle (**A**) and AZD6244 (**B**). Scale bar in **B** = 200 μm. **C.** All-points-pixel histogram of laminae I, II and a slightly larger area including background. The threshold was determined as the intersection of the two histograms. Pixels above the threshold were counted as specific BDNF-IR. **D.** Histograms of the weighted intensity of BDNF-IR in laminae I and II ($\Sigma \text{ pixel} \times \text{intensity}$). The calculated weighted intensity measures were normalized to the contralateral spinal dorsal horn. The spinal cords were harvested at 3 days after CCI. $n = 3$ in each group. $*p < 0.05$ vs. contralateral.

Figure 10. Double-immunostaining of BDNF and other selected cellular markers in spinal dorsal horn

Distribution of BDNF (green) and marker proteins (red) in the ipsilateral spinal dorsal horn in rats subjected to CCI. High-powered images were taken by confocal microscopy (**B–F**). The red fluorescence labels CGRP (**A, B**), Synaptophysin (**C**), NeuN (**D**), OX-42 (**E**) and GFAP (**F**) immunoreactivity. Scale bar in **A** = 200 μm and **B** = 20 μm .