Peripheral Synthesis of an Atypical Protein Kinase C Mediates the Enhancement of Excitability and the Development of Mechanical Hyperalgesia Produced by Nerve Growth Factor

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Running title: NGF-induced synthesis of PKMζ enhances excitability and sensitivity

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Abstract

Nerve growth factor plays a key role in the initiation as well as the prolonged heightened pain sensitivity of the inflammatory response. Previously, we showed that NGF rapidly augmented both the excitability of isolated rat sensory neurons and the mechanical sensitivity of the rat's hind paw. The increase in excitability and sensitivity were blocked by the myristolated pseudosubstrate inhibitor of atypical PKCs (mPSI), suggesting that an atypical PKC may play a key regulatory role in generating this heightened sensitivity. Our findings raised the question as to whether NGF directs changes in translational control, as suggested for long-lasting LTP, or whether NGF leads to the activation of an atypical PKC by other mechanisms. The current studies demonstrate that enhanced action potential firing produced by NGF was blocked by inhibitors of translation, but not transcription. In parallel, *in vitro* studies showed that NGF elevated the protein levels of PKMζ, which was also prevented by inhibitors of translation. Intraplantar injection of NGF in the rat hind paw produced a rapid and maintained increase in mechanical sensitivity whose onset was delayed by translation inhibitors. Established NGF-induced hypersensitivity could be transiently reversed by injection of rapamycin or mPSI. These results suggest that NGF produces a rapid increase in the synthesis of PKMζ protein in the paw that augments neuronal sensitivity and that the ongoing translational expression of PKMζ plays a critical role in generating as well as maintaining the heightened sensitivity produced by NGF.

Highlights:

- NGF produced a significant time-dependent increase in the number of APs, which was blocked by inhibitors of translation, but not transcription
- NGF increased the levels of PKMζ protein that was blocked by inhibitors of translation
- Injection of NGF produced a hindpaw mechanical hypersensitivity whose onset was delayed by inhibitors of translation
- Established mechanical hypersensitivity produced by NGF could be reversed by either rapamycin or mPSI

Keywords: sensory neuron; sensitization; neurotrophin; protein synthesis; excitability; hyperalgesia

Abbreviations: APs, action potentials; CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LTP, long-term potentiation; MPE, maximal possible effect; mPSI, myristolated pseudosubstrate inhibitor of atypical PKCs; NGF, nerve growth factor; RE, response efficiency; VFH, von Frey hair; ZIP, zeta inhibitory peptide

Introduction

Neurotrophins, such as nerve growth factor (NGF), play key roles in the initiation of the inflammatory response by their ability to activate or traffic a variety of immune cells to a site of injury (Levi-Montalcini et al., 1996; Skaper, 2001; Villoslada and Genain, 2004; Nockher and Renz, 2006; Linker et al., 2009; Seidel et al., 2010). An early study demonstrated that the levels of NGF were elevated in blister exudates obtained from the hindpaw skin of rats (Weskamp and Otten, 1987). Additionally, application of NGF was shown to lead to the release of histamine (Bruni et al., 1982; Mazurek et al., 1986) and serotonin (Horigome et al., 1993) from rat peritoneal mast cells. NGF was proven to be chemotactic for human (Gee et al., 1983) and mouse (Boyle et al., 1985) polymorphonuclear leukocytes. Human B cells express the TrkA receptor for NGF (Otten et al., 1989; Brodie and Gelfand, 1992) and their proliferation was augmented upon exposure to NGF (Otten et al., 1989; Brodie and Gelfand, 1992; Thorpe and Perez-Polo, 1987). Finally, immune-competent cells, such as mouse CD4+ and CD8+ T lymphocytes (Ehrhard et al., 1993; Santambrogio et al., 1994) and rat peritoneal mast cells (Leon et al., 1994), express the mRNA for NGF as well release biologically active NGF upon activation.

In this capacity, NGF can also enhance the sensitivity of nociceptive sensory neurons to different modalities of stimulation and thereby lead to heightened pain states (reviewed by McMahon, 1996; Woolf, 1996). Intraperitoneal injection of NGF was reported to greatly enhance the sensitivity to both mechanical and thermal stimulation of the hindpaw of a rat (Lewin et al., 1993). Intraplantar injection of complete Freund's adjuvant (CFA) also augments the hindpaw sensitivity to mechanical or thermal stimulation; this CFA-induced hypersensitivity

was blocked by injection of an antibody to NGF, indicating that elevated NGF directly mediates this inflammatory pain (Woolf et al., 1994; Lewin et al., 1994; Nicol and Vasko, 2007).

The hypersensitivity of nociceptive sensory neurons after exposure to inflammatory mediators has, in some ways, been likened to the effects of agonist- or high frequency-stimulation of nerve fibers in the hippocampus that result in long-term potentiation (LTP). The long-lasting or maintenance phase of LTP depends on the synthesis of new proteins (Stanton and Sarvey, 1984; Kelleher et al., 2004; Costa-Mattioli et al., 2009) wherein one key protein associated with LTP is the atypical PKC known as PKMζ (Sacktor et al., 1993; Sacktor, 2011). PKMζ can be expressed from an internal promoter within the full length PKCζ gene, resulting in a truncated product that lacks the regulatory domain, rendering this product constitutively active (Hernandez et al., 2003). Several different studies suggested that this variant plays a key role in the maintenance of long-term synaptic strength. For example, treatment with the myristolated pseudosubstrate inhibitor (mPSI) of atypical PKCs reversed established LTP (Ling et al., 2002). In an *in vivo* study of conditioned taste aversion (CTA) as an animal model of memory, infusion of mPSI into the insular cortex suppressed the CTA memory (Shema et al., 2007). Finally, lenti-viral over-expression of PKMζ enhanced CTA memory, whereas introduction of a dominant-negative PKMζ led to suppression (Shema et al., 2011).

Our previous studies demonstrated that treatment with NGF acutely enhanced the excitability of isolated rat sensory neurons (Zhang et al., 2002; Zhang et al., 2012) and that intraplantar injection of NGF produced a significant hypersensitivity to mechanical and thermal stimulation of the rat's hindpaw (Khodorova et al., 2013, 2017). The NGF-induced augmentation of excitability, mechanical, and thermal sensitivity were blocked by pretreatment with mPSI. In

addition, siRNA targeted to PKCζ significantly reduced the expression of PKMζ, but not that of either PKCζ or PKCλ/ι, and blocked the NGF-mediated increases in the excitability of sensory neurons (Zhang et al., 2012). These observations indicate that PKMζ plays a key regulatory role in generating the heightened sensitivity resulting from exposure to NGF. Our findings then suggest two possible explanations: NGF engages the translational control pathway, as has been suggested for long-lasting LTP in the central nervous system, or by some other mechanisms NGF leads to the activation of an atypical PKC in the peripheral nervous system.

Experimental Procedures

Isolation and Maintenance of Sensory Neurons

Sensory neurons were harvested from young adult Sprague-Dawley rats (80-150 g) (Harlan Laboratories, Indianapolis, IN, USA). Briefly, male rats were killed by placing them in a chamber that was then filled with CO_2. Dorsal root ganglia (DRG) were isolated and collected in a conical tube with sterilized Puck's solution. The tube was centrifuged for 1 min at approximately 2000 x g and the pellet was resuspended in 1 ml Puck's solution containing 10 U of papain (Worthington, Lakewood, NJ, USA). After 15 min incubation at 37°C, the tube was centrifuged at 2000 x g for 1 min and the supernatant was replaced by 1 ml F-12 medium containing 1 mg collagenase IA and 2.5 mg dispase II (Roche Diagnostics, Indianapolis, IN, USA). The DRGs were resuspended and incubated at 37°C for 20 min. The suspension was centrifuged for 1 min at 2000 x g and the supernatant was removed. The pellet was resuspended in F-12 medium supplemented with 10% heat-inactivated horse serum and 30 ng/ml NGF (Harlan Bioproducts, Indianapolis, IN, USA) and mechanically dissociated with fire-polished glass pipette until all visible chunks of tissue disappeared. Isolated cells were plated onto either plastic coverslips (electrophysiology experiments) or 6-well tissue culture plates

(Western blotting experiments); both surfaces were previously coated with 100 µg/ml poly-D-lysine and 5 µg/ml laminin. Cells were then maintained in culture in an F-12 medium supplemented with 30 ng/ml NGF at 37°C and 3% CO_2 for either 18-24 h before electrophysiological recording or for 48 h before administering treatments and collecting cell lysates for Western blotting experiments. All procedures were approved by the Animal Use and Care Committee of the Indiana University School of Medicine.

Electrophysiology

Recordings were made using the whole-cell patch-clamp technique as previously described (Zhang et al., 2012). Briefly, a coverslip with sensory neurons was placed into a culture dish containing normal Ringer's solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, with pH adjusted to 7.4 using NaOH; after approximately 15 min, the cover slip was transferred to the recording chamber filled with Ringer's solution. Recording pipettes were pulled from borosilicate glass tubing (Model G85165T-4, Warner Instruments, Hamden, CT, USA). Recording pipettes had resistances of 2-5 MΩ when filled with the following solution (in mM): 140 KCl, 5 $MgCl_2$, 4 ATP, 0.3 GTP, 0.25 $CaCl_2$, 0.5 EGTA, (calculated free Ca^{2+} concentration of 100 nM, MaxChelator), and 10 HEPES, at pH 7.2 adjusted with KOH. Whole-cell voltages were recorded with an Axopatch 200 or Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were acquired and analyzed with pCLAMP 10 (Molecular Devices). All drugs were applied with a VC-8 bath perfusion system (Warner Instruments). NGF was used at a concentration of 100 ng/ml, which was based on the observation that this concentration produced a significant sensitization of the capsaicin-gated current in small-diameter rat sensory neurons (Shu and Mendell, 1999). In the current clamp experiments, the neurons were held at their resting potentials (between −45 and

−65 mV) and a depolarizing current ramp (1000 ms in duration) was applied. The amplitude of a ramp was adjusted to produce between 2 and 4 action potentials (APs) under control conditions for each individual neuron and then that same ramp was used throughout the recording period for that particular neuron. Voltages were filtered at 5 kHz and sampled at 2 kHz. At the end of each recording, the neuron was exposed to 400 nM capsaicin. This neurotoxin was used to distinguish capsaicin-sensitive sensory neurons as these neurons are believed to transmit nociceptive information (Holzer, 1991). However, the correlation between capsaicin sensitivity and neuronal identity as a nociceptor is not absolute; some nociceptive neurons are insensitive to capsaicin and some capsaicin-sensitive neurons are not nociceptors (Petruska et al., 2000). Therefore, this agent was used to define a population of small-diameter sensory neurons that could serve a nociceptive function. All results presented in this report were obtained from capsaicin-sensitive neurons, unless otherwise stated. All experiments were performed at room temperature, ~23°C.

Western Blot

After 48 h in culture, the medium was removed from the DRG cells, each well was washed with 1 ml PBS (37°C), and then 1 ml of F12 medium with no additional NGF was added to each well for 15 min at 37°C in 3% CO_2. Either vehicle or a final concentration of 50 µg/ml cycloheximide was then added to the culture medium and the cells were incubated for an additional 60 min. At this point, vehicle (100 µl F12 medium, no NGF) or NGF (100 µl of 1 ng/µl NGF in F12 medium) was added to the appropriate wells and the cells were returned to the incubator for 30 min. The culture medium was then removed from the cells, the plates were placed on ice, and each well was then washed twice with 1 ml ice-cold PBS. Cells were lysed with 150 µl per well of ice-cold RIPA lysis buffer (catalog #20-188, Millipore Corp., Billerica, MA) containing a 100-fold dilution

of Protease Inhibitor Cocktail Set III (catalog #539134, EMD Biosciences, San Diego, CA) and phosphatase inhibitors (1 mM sodium fluoride, 1 mM activated sodium orthovanidate). Cells were scraped from the wells, lysates were collected into 1.5 ml tubes, and the lysates were sonicated (Fisher Scientific Sonic Dismembrator 550, setting 3, 2 pulses, 1 sec each pulse). Cell debris was removed by centrifugation at 2500 x g for 5 min at 4°C. Supernatants were transferred to fresh tubes. The total protein concentrations in the lysates were measured using the Bradford Method (catalog #500-0006, Protein Assay Dye Reagent, Bio-Rad Laboratories, Hercules, CA).

Equivalent amounts of reduced, denatured protein (20 µg) were separated by electrophoresis in a NuPAGE® 4-12% Bis-Tris gel (catalog #NP0335BOX, Invitrogen, Carlsbad, CA) using NuPAGE® MES-SDS running buffer (catalog #NP0002, Invitrogen). The separated proteins were then transferred from the gel to an Invitrolon PVDF membrane (catalog #LC2005, Invitrogen). The membranes were blocked with 1X TBS containing 5% (w/v) non-fat dry milk for 1 h at room temperature, with agitation. To measure atypical PKC (aPKC) expression levels, a polyclonal rabbit antibody that recognizes PKCζ, PKMζ, and PKCλ/ι (sc-216, 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was incubated in 1X TBS, 0.1% (v/v)Tween 20 and 1% (w/v) non-fat dry milk for 18 hs at 4°C with agitation. These protein isoforms were distinguishable based on differences in their molecular weights. Reference protein expression was determined using rabbit polyclonal antibody to HPRT (sc-20975, 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mouse monoclonal antibody to actin (catalog #MA5-11869, ACTN05, C4, 1:2500, Thermo Scientific, Rockford, IL). After primary antibody incubation, membranes were washed (3 x 10 min) with 1X TBS containing 0.1% (v/v) Tween 20, at room temperature, with agitation. The membranes were then incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (sc-2004, 1:2500, Santa Cruz

Biotechnology Inc., Santa Cruz, CA) in TBS-Tween-milk for 1.5 h at room temperature, with agitation. After washing, immunoreactive bands on the membranes were developed by SuperSignal™ West Dura Extended Duration Substrate (catalog #34076, Thermo Scientific, Rockford, IL) and visualized by exposure to CL-X Posure™ film. The film was photographed with a Kodak DC290 CCD camera and the density of each band was measured using Kodak 1D 3.6 software (Kodak Scientific Imaging Systems, New Haven, CT). The summary data are expressed as the density of the experimental bands normalized to the density of their respective HPRT bands. Three independent experiments were conducted for statistical analysis.

Behavioral Measurements of Mechanical Sensitivity

Experiments were conducted on a total of 65 adult male Sprague-Dawley rats (240-300 g). Rats were housed in groups of two per cage under a 12:12 h dark:light cycle and were provided with food and water *ad libitum*. Animals were experimentally treated and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (Guide, 2011), using protocols as reviewed and approved by the Harvard Committee on Animals. Unrestrained rats were placed on an elevated plastic mesh floor (28 x 17.5 cm; 9.5 x 9.5 mm openings) and allowed to habituate for 25-40 min before initial testing. Paw Withdrawal Frequency to mechanical stimulation was determined using a 10 or 15 g calibrated von Frey hair (VFH) applied perpendicularly to the plantar surface of the hind paw through the spacing in the mesh floor. The VFH was applied 10 times, each for 3 sec maximum, or until paw withdrawal. To minimize stress and to obtain consistent responsiveness to the force, the rats were habituated and tested on these same mesh racks over 5-6 days before any test substances were injected (training period). Four rats were tested together in any single test session. Each rat in any single session received the same treatment, and the tester was not blinded to the treatment.

Withdrawal responses were registered initially on the ipsilateral (NGF-injected) paw and then on the contralateral paw. The number of paw withdrawals, n, occurring in response to a total of 10 stimuli with any single VFH force was used to assess mechanical sensitivity, *and reported* as the "Response Efficiency" (RE).

In experiments designed to test the preventive activity of translational inhibitors, cycloheximide (n=11), rapamycin (n=12), or their respective vehicle (n=6, for each group, n=12 total), alone or with NGF, were injected subcutaneously (s.c.) in a 10 µl volume (with 4 µg NGF injected in 20 µl), into the mid-plantar hind paw, 1 cm distal from the heel. Injections were performed under brief general anesthesia from inhalation of the rapidly reversible agent sevoflurane (Abbott Labs, N. Chicago, IL, USA). After anesthesia was discontinued, the righting reflex recovered in <30 sec; 5-10 min later "normal" baseline nocifensive responses could be assessed. To test the ability of specific protein synthesis inhibitors to prevent the development of NGF-induced hyperalgesia, the following injection protocols were used. The first injection of cycloheximide occurred 1 h prior to the NGF injection, and the second injection was mixed with NGF. Rapamycin was injected once, 0.5 h prior to NGF with concentrations and doses indicated in the Results and figure legends. In control experiments, NGF was injected 1 h after the injection of the vehicle used for cycloheximide (PBS, 10 µl), or 0.5 h after the injection of the vehicle used for rapamycin (DMSO, 10 µl). In experiments intended to reverse an established, long-lasting hyperalgesia, rapamycin (10 µl, n=9) or vehicle, (DMSO, 10 µl, n=8) was injected 3 days after paw injection of 3-5 µg of NGF. The role of aPKCs in NGF-induced hypersensitivity was tested by intraplantar injection of mPSI (40 µg, 20 µl, n=11) or its inactive, scrambled analogue, (scr-mPSI, n=8) 3 days after 4 µg of NGF.

Data Analysis- In vitro experiments

Results are presented as the means ± standard error of the mean (SEM). Statistical differences between the controls and those obtained under various treatment conditions were determined by either an ANOVA or a repeated measures (RM) ANOVA when appropriate. When a significant difference was obtained, *post hoc* analyses were performed using a Holm-Sidak all-pairs test; the degrees of freedom (df) and the sum of squares (ss) are reported. If the data set failed the normality test, either a Kruskal-Wallis one-way ANOVA on ranks was performed, followed by a Tukey or a Dunn's all pairwise test yielding an H value and the df or a Friedman repeated measures ANOVA followed by a Tukey all pairwise test yielding a Chi square value X^2 and the df. The df and H or X^2 are reported. Results were considered statistically significant when $P \leq 0.05$ (SigmaStat 3.5 Software).

In vivo experiments

Behavioral results are graphically presented as medians ± 25^{th} and 75^{th} percentile (boxplot) and 10^{th} and 90^{th} percentiles (whiskers). Statistical analysis applied Kruskal-Wallis tests followed by Wilcoxon Rank Sum tests for pairwise comparisons. Analyses by Kruskal-Wallis are reported with the value of H, degrees of freedom (df), and probability of significance (P) values for all the groups being compared; pair-wise comparisons by the Wilcoxon Rank Sum test, are reported as the number of samples in the compared groups and the exact P values. Two comparisons were made: 1. Response Efficiency after injections of NGF compared to Response Efficiency during the Baseline, pre-injection period, for the different translational inhibitor tests of prevention. 2. Comparisons, at the same time point, of Response Efficiency in vehicle-injected rats with those of inhibitor-injected rats. The effectiveness of an inhibitor to reverse existing hypersensitivity was quantified as follows; the increase in paw response efficiency on post-NGF day 3 (D3)

compared to baseline was defined as Maximum Possible Effect (MPE) of NGF. Suppression of RE by the inhibitors on day X after NGF injection (DX) was expressed as %MPE, where %MPE = [(RE@DX – RE@D3) / (RE@D3 – baseline RE)] x 100.

Chemicals- In vitro experiments

F-12 Nutrient Mixture (catalog #21700-075, Gibco) was supplemented with the following per liter: 1.18 g $NaHCO_3$ (catalog #S6014, Sigma), 1X (2 mM) L-glutamine (catalog #25030-081, Gibco), 50 units penicillin-50 mg/ml streptomycin (catalog #15070-063, Gibco), 10% heat-inactivated horse serum (catalog #26050-088, Gibco), 9 µg/ml 5-fluoro-2'-deoyuridine (catalog #F-0503, Sigma), and 21 µg/ml uridine (catalog #U-3750, Sigma). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Actinomycin D and 4EGI-1 were dissolved in DMSO. Capsaicin, cycloheximide, and rapamycin were dissolved in 1-methyl-2-pyrrolidinone (MPL). The MPL stock solutions were then diluted with Ringer's solution to yield the appropriate concentrations. The vehicle, MPL was typically used at 1,000- to 5,000-fold dilutions. Our earlier studies demonstrated that MPL does not affect the potassium or sodium currents in the DRG sensory neurons (Zhang et al., 2002).

In vivo experiments

NGF-β (rat) (Sigma-Aldrich, St. Louis MO, USA, or [556-NG-100/CF] R&D Systems, Minneapolis MN, USA) was made as a stock solution (100 ng/µl or 200 ng/µl in Ca^{2+} + Mg^{2+} free PBS, pH 7.4) and stored in 40 µl aliquots at –80°C. Prior to the injection, NGF stock aliquots were diluted (1:1) in PBS or mixed with the cycloheximide stock solution (for co-injections) to the indicated final concentration of 50-200 ng/µl. Cycloheximide (Sigma-Aldrich, St. Louis MO,

USA) was dissolved in PBS as a 20 mM stock solution and stored in 40 µl aliquots at –80°C; stock aliquots of cycloheximide were then diluted (1:1) in PBS. Rapamycin (Enzo Life Sciences, Farmingdale, NY) was dissolved in DMSO as a 20 mM stock solution, stored in aliquots at –80° C, and then diluted to 10 mM in DMSO before hind paw injection. The atypical PKC pseudosubstrate inhibitor, mPSI (Enzo Life Sciences) was dissolved first in dH_2O to 10 µg/µl, then diluted 1:5 in PBS and stored in 100 µl aliquots at −80°C. The same procedure was used for the scrambled pseudosubstrate inhibitor, a control substance for mPSI (Tocris/R&D Systems, Minneapolis, MN).

RESULTS

Inhibitors of translation, but not transcription, block the NGF-induced increase in excitability

Our previous studies demonstrated that both the NGF-mediated increase in excitability of sensory neurons and behaviorally assessed mechano- and thermal hypersensitivity were linked to the activity of the atypical PKC variant, PKMζ (Zhang et al., 2012; Khodorova et al., 2013, 2017). To determine whether this increased excitability was produced by protein synthesis, inhibitors of transcription or translation were used. As illustrated in Fig. 1A, in either untreated or vehicle-treated isolated sensory neurons (combined data), exposure to 100 ng/ml NGF produced a significant time-dependent increase in the number of action potentials (APs) evoked by a depolarizing ramp of current ($df=9$, $ss=402.9$, $n=10$, $P\leq0.001$). The untreated and vehicle-treated groups have been combined into a single control group since the effects of NGF were not different (Mann Whitney $U(4,6)=14.0$, $n=10$, $P\geq0.76$). In this combined data, there were no differences between the 6, 10, and 20 min treatments with NGF. In a separate series of experiments, a 30 min pretreatment with 10 µM actinomycin-D, an inhibitor of transcription (Kersten et al., 1960; Goldberg et al., 1962), failed to alter the capacity of NGF to increase

neuronal excitability (Fig. 1B, H=19.2, df=5, n=7-9, P≤0.05), producing a sensitization of AP firing that was similar to that found in untreated/vehicle-treated neurons. There were no differences between the 6, 10, and 20 min treatments with NGF. By contrast, pretreatment with inhibitors of translation, either 60 μM cycloheximide (30 min, panel C, df=6, ss=17.5, n=7, P=0.07) or 500 nM rapamycin (60 min, panel D, df=7, ss=62.6, n=8, P=0.12), blocked the increase in excitability produced by NGF. To corroborate the intended effects of rapamycin and cycloheximide, as they are known to have other potential effects, the small molecule inhibitor 4EGI-1 was used. This agent specifically blocks the interaction between the factors eIF4E and eIF4G and thereby inhibits cap-dependent translation; in Jurkat cells the IC_{50} for this blockage was 25-50 μM (Moerke et al., 2007). As shown in Fig. 3E, a 30 min pretreatment with 30 μM 4EGI-1 blocked the NGF-induced increase in excitability (df=6, ss=36.3, n=7, P=0.35).

A recent report demonstrated that high-frequency stimulation of the CA3 Schaffer collateral-CA1 synapse in hippocampal slices produced a rapid dephosphorylation of serine 406 in eIF4B. This dephosphorylation was associated with increased levels of PKMζ protein; both the dephosphorylation and the increased protein were sensitive to okadaic acid, an inhibitor of protein phosphatase 1 and 2A (Eom et al., 2014). Based on this finding, we measured the effects of okadaic acid on the capacity of NGF to enhance the excitability of sensory neurons. As shown in Fig. 1F, a 30 min pretreatment with 1 μM okadaic acid completely blocked the increase in AP firing produced by NGF (df=4, ss=3.1, n=7-9, P=0.75). In parallel recordings from sensory neurons isolated in the same tissue harvests, NGF significantly increased AP firing by about 3-fold (control 3.2 ± 0.2 APs vs. 10 min NGF 9.6 ± 0.7 APs, df=4, ss=5.3, n=5, P≤0.001, data not shown). These results are consistent with the observations in hippocampal slices. Collectively, these results demonstrate that the ability of NGF to augment the firing of APs in sensory neurons depends on translational but not transcriptional activity.

NGF augments the levels of PKMζ protein

The above results indicate that the sensitization produced by NGF requires protein synthesis. Such a finding raises the question as to whether this results in increased levels of PKMζ protein. Western blotting and densitometry of extracts from isolated sensory neurons were used to measure the changes in PKMζ protein in the absence or presence of NGF. We previously identified a band at ~49 kDa as PKMζ (Zhang et al., 2012) and this molecular weight is similar to that reported for PKMζ by Sacktor's laboratory (Sacktor et al., 1993; Hernandez et al., 2003). Film exposures of 1 s yielded measurable bands for the other isoforms of atypical PKCs, however this failed to yield a detectable band for PKMζ (data not shown). Therefore, longer exposures were used (typically 20-30 s) to assess the levels of PKMζ. A representative Western blot using the longer exposure time (see Fig. 2A) demonstrates that a 30 min treatment with 100 ng/ml NGF increased the density of the band at ~49 kDa. This finding is consistent with the electrophysiological results reported above. Pretreatment for 60 min with 50 µg/ml (178 µM) cycloheximide, an inhibitor of the elongation phase of translation, reduced the amount of PKMζ protein (lane CHX+ NGF+) compared to NGF alone (lane CHX− NGF+). The results from three separate Western blots are summarized in Fig. 2B wherein the measured density values were normalized to the values of their respective untreated controls. A 30 min exposure to 100 ng/ml NGF produced a significant increase (1.86 ± 0.30 fold, df=2, ss=0.5, n=3 tissue harvests, $P \leq 0.05$) in the amount of PKMζ protein compared to the untreated controls. Treatment with cycloheximide blocked the NGF-induced increase wherein the measured values for PKMζ protein were reduced to 1.30 ± 0.26. Cycloheximide by itself had no effect on the levels of PKMζ. Our previous work demonstrated that full length PKCζ and PKCλ/ι proteins have molecular weights of approximately 75 and 62 kDa, respectively (Zhang et al., 2012). Although PKMζ could not be detected at the 1 s exposures, band densities of the other atypical PKCs

were measured. In the current studies, NGF had no significant effect on the expression levels of PKCζ (1.39 ± 0.16 normalized to their respective untreated controls 1.0, df=2, ss=0.3, n=3 tissue harvests, P=0.12) or PKCλ/ι (1.19 ± 0.11, H=5.2, df=2, n=3 tissue harvests, P=0.16). Thus, these results indicate that NGF selectively increases the translational expression of PKMζ in isolated sensory neurons and that this increase is blocked by the protein synthesis inhibitor cycloheximide.

Translation inhibitors block the NGF-induced mechanical hypersensitivity

The systemic injection of NGF (Lewin et al., 1993) or its intraplantar injection into the hindpaw of a rat produces a hypersensitivity to both thermal and mechanical stimulation (Woolf et al., 1994; Andreev et al., 1995, respectively). Our previous studies demonstrated that pretreatment with a blocking antibody to the p75 neurotrophin receptor prevented the NGF-induced increase in neuronal excitability (Zhang and Nicol, 2004) as well as the mechanical hypersensitivity resulting from the intraplantar injection of either NGF or proNGF (Khodorova et al., 2013). These results indicate that NGF can enhance neuronal sensitivity *in vivo*, an effect that may depend on translational control. To explore this possibility, the inhibitors of translation, cycloheximide or rapamycin, were injected into the intraplantar surface of the rat's hindpaw prior to the injection of NGF. As illustrated in Fig. 3, both cycloheximide and rapamycin delayed the development of NGF-induced mechanical hypersensitivity. (There being no difference among the baseline, pre-injection Response Efficiency in the 3 treatment groups (H=0.72, df=2, P=0.70), these were merged to give a single baseline value; n=29). In vehicle control animals (n=6), where DMSO was injected 0.5 h before NGF (500 ng/10 µl), a significant increase in the responsiveness to the 15 g force over the baseline values (n=29) was detected at all test times after NGF (H=27.5, df=4, P≤0.0001); at 0.5 h P=0.006, at 1 h P=0.006, at 3.5 h P=0.001, and at

22 h P=0.001. In the untreated control animals, the single injection of 500 ng of NGF induced a mechanical hypersensitivity that slowly declined between days 1 and 4, and achieved a full recovery on day 5 (data not shown). Injection of the vehicle alone, whether it be aqueous buffer (PBS) or DMSO, had no effect on paw responsiveness over one day, in the current experiments (data not shown), and in many previously published studies (e.g., Khodorova et al., 2013).

In contrast to the robust increase in R.E. for the vehicle control group, for the group where cycloheximide was injected 1 h before and then co-injected with NGF (total dose 200 nmol/paw, each injection 10 mM, n=11), the increase in mechanical sensitivity (H=17.7, df=4, P=0.001) was blocked at both 0.5 h (P=0.98 vs. baseline, n=29) and 1 h (P=0.82 vs. baseline) but was significant at 3.5 h (P=0.03 vs. baseline) and 22 h (P=0.0002) after NGF. Similarly, when rapamycin (100 nmol/paw, n=12) was injected 0.5 h prior to NGF (H=22.01, df=4, P=0.0002), no significant increase in mechanical sensitivity occurred at 0.5 h (P=0.41), 1 h (P=0.08), or 3.5 h (P=0.16, all vs. baseline, n=29). In a parallel analysis, comparing the responses at each time between the vehicle control group and the two different Inhibitor treatment groups, at baseline there was no difference (as noted above). At 0.5 h, (H=10.31, df=2, P=0.006); cycloheximide (n=11) vs. vehicle (n=6), P=0.006; rapamycin (n=12) vs. vehicle (n=6), P=0.005. At 1 h, (H=6.52, df=2, P=0.039), cycloheximide vs. vehicle P=0.02, rapamycin vs. vehicle P=0.04. For both cycloheximide and rapamycin treatments, the mechanical sensitivities had reached the vehicle control values when measured at 22 h (H=0.70, df=2, P=0.70), for cycloheximide vs. vehicle P=0.46 and for rapamycin vs. vehicle P=0.49). These results demonstrate that inhibition of translation can delay the increase in mechanical sensitivity produced by NGF, although the suppressive effects are short-lived. The time course of this suppression may depend on the metabolic stability of these compounds. For example, in an *in vitro* study, the half-life of rapamycin in rat plasma was approximately 2.2 h (Ferron and Jusko, 1998).

Inhibitors of translation or atypical PKCs reverse the sustained hypersensitivity produced by NGF

Intraplantar injection of higher doses of NGF (1-5 μg) produced a mechanical hypersensitivity that persisted for at least 4 days after the initial injection. As shown in Fig. 4A, injection of NGF (4 μg/20 μl) produced a sustained hypersensitivity to the 15 g VFH stimulus over this period, with a slight fade of effect on D5 48 h after DMSO vehicle injection ($H=26.9$, $df=5$, $n=8$, $P\leq0.0001$). There were no significant differences in the levels of NGF-induced mechanical hypersensitivity between the 1, 3, and 4 μg/20 μl injections (a X^2 analysis comparing the REs from these three doses of NGF at D3 (72 h) gave $P=0.543$); therefore, these results have been combined in Fig. 4A. With this same protocol for producing sustained mechanical hypersensitivity, another group of rats (n=9) received a single injection of rapamycin (10 mM, 10 μl) 3 days after an injection of 4 μg NGF (for all test times in this group $H=25.8$, $df=5$, $P\leq0.001$). Mechanical hypersensitivity was reduced at 24 h after rapamycin compared to the value one day previously ($P=0.01$, D4 vs. D3, 4 h after rapamycin). The rapamycin-diminished response, at 24h, was still above the pre-NGF baseline value ($P=0.01$), showing that a complete reversal of NGF-induced hypersensitivity was not achieved with this dose of rapamycin. Furthermore, the response had not changed at 1.5 h after rapamycin ($P=0.53$ vs. D3, before rapamycin) nor at 4.5 h after rapamycin ($P=0.07$ vs. D3, before rapamycin); it appears that the effects of the inhibitor were not significant over the first 4 h after its injection, but were so by 1 day. By 48 h after rapamycin (D5), mechanical sensitivity had returned to its pre-rapamycin level (see Fig. 4B, $P=0.20$, D5 vs. D3 before rapamycin).

These changes in mechanical hypersensitivity after the injection of rapamycin were then normalized to their maximal possible effect (MPE), measured at D3 (see Procedures section for details). As shown in Fig. 4C, at 24 h after the injection of rapamycin there was a significant reduction (approximately 40% MPE) in normalized hypersensitivity ($H=12.2$, $df=2$, $P=0.002$ for all 3 times, $P=0.002$ for D4 24 h vs. D3, 4 h after rapamycin, all $n=9$). By 48 h (D5) after rapamycin the mechanical hypersensitivity had returned to the value measured at D3 ($P=0.57$). Although these effects were transient, the results suggest that continued mRNA translation is important in maintaining the hypersensitivity after exposure to NGF.

We previously showed that treatment with mPSI, an inhibitor of the atypical PKCs, prevented the development of both the NGF-induced increase in neuronal excitability *in vitro* (Zhang et al., 2012) and the mechano-hypersensitivity *in vivo* (Khodorova et al., 2013). These findings raise the question of whether exposure to mPSI could reduce mechano-hypersensitivity during the sustained period after higher NGF. Following a similar protocol as described above for tests of rapamycin on sustained NGF-induced mechanical hypersensitivity, we found that intraplantar injection of mPSI on D3 (40 µg) after NGF resulted in a transient reduction in R.E. ($H=18.4$, $df=4$, $P=0.001$), with a significant decrease in the hypersensitivity measured 24 h later ($n=11$, $P=0.016$, D3 vs. D4), falling to levels that were not different from baseline ($n=11$, $P=0.10$) (see Fig. 5A). Like the findings for rapamycin, the sensitivity returned to the pre-mPSI values 24 h later ($P=0.73$, D5 vs, D3). In contrast, injection of the same dose of a scrambled analogue of mPSI (scr-mPSI) that has no inhibitory effect on aPKCs, had no effect at D4 ($P=0.65$, D4 vs. D3; Fig. 5A). After scr-mPSI, responsiveness remained significantly above baseline for the next 3 days ($H=25.5$, $df=4$, $P\leq0.0001$) (Fig. 5A). These results were then normalized by comparing the responsiveness after injected substances to the hypersensitivity measured at D3, before substance injection but after NGF, in each respective animal. As shown in Fig. 5B, mPSI

caused a significant drop in the normalized %MPE responsiveness (H=7.8, df=2, P=0.02). At 24 h after the injection of mPSI the hypersensitivity was significantly reduced, by ~66% of the MPE (P=0.005), whereas after the injection of scr-mPSI there was no change in the hypersensitivity relative to D3 (P=0.31). At 48 h (D5) after inhibitor injection, the mechanical hypersensitivities for those rats receiving the mPSI injection returned to their pre-injection values (P=0.73). Taken together, these findings demonstrate that the NGF-induced mechanical hypersensitivity *in vivo* can be delayed by pretreatment with rapamycin or cycloheximide and that established hypersensitivity can be transiently reversed by either rapamycin or mPSI. These results indicate that translational synthesis in the periphery plays a critical role in generating as well as maintaining the heightened mechanical sensitivity produced by NGF, and suggest that PKMζ is a likely candidate for the synthesized critical mediator.

DISCUSSION

In this report we demonstrate that synthesis of a signaling protein is essential for the acute mechanical hypersensitivity caused by NGF, and for the changes in sensory neuron excitability that underlay that hypersensitivity. Similarly, the protein levels of PKMζ *in vitro* were elevated after NGF treatment, which was blocked by pretreatment with an inhibitor of translation, cycloheximide. The enhanced AP firing that resulted after NGF exposure was also blocked by inhibitors of translation, but not by an inhibitor of transcription. Intraplantar injection of NGF produced a rapid increase in the local mechanical sensitivity that was maintained for 4-5 days. The onset of this NGF-induced hypersensitivity was delayed by pretreatment with either rapamycin or cycloheximide. Interestingly, the maintained mechanical hypersensitivity could be transiently reversed by injection of either rapamycin or an inhibitor (mPSI) of atypical PKCs. Although our results indicate that NGF leads to the persistent on-going synthesis of a mediator

that promotes hypersensitivity (which we believe to be PKMζ), the transient action of the translation inhibitors might suggest that the half-life or stability of this mediator is limited, whereas the coding mRNA is present before NGF stimulation and then becomes persistently active. Thus, the *in vitro* results demonstrate that NGF produces a rapid increase in the translational synthesis of PKMζ. Elevated levels of this atypical PKC result in the enhanced AP firing of isolated small diameter sensory neurons as well as the heightened mechanical sensitivity of the hindpaw. These observations would suggest that NGF when applied to either the neuronal cell bodies or the distal terminals of sensory (nociceptive) afferents leads to the acute translation of PKMζ.

As noted above, the NGF-mediated *in vivo* mechanical sensitivity is significantly increased after just 30 min of exposure. Changes in mechanical sensitivity in this amount of time are much too rapid to be accounted for by the retrograde transport of an NGF-induced signal back to the DRG. Consistent with this idea, Stoeckel et al. (1975) showed that upon injection of ^{125}I-NGF into the forepaw of a rat, it took at least 6 h to detect a significant increase in labeled NGF in the cervical (C6/7) DRG. Maximal levels were attained between 11 and 16 h. Therefore, these results for the slow retrograde movement of NGF to the DRG are incompatible with the rapid increase in mechanical hypersensitivity observed in our study. Thus, the rapid enhancement of mechanical sensitivity is consistent with the idea of localized translation in those mechano-sensitive fibers innervating the hind paw skin.

The key findings of this study are also consistent with our previous studies. In isolated sensory neurons, pretreatment with mPSI, but not bisindolylmaleimide I (an inhibitor of conventional and novel PKC isoforms), completely blocked the NGF-induced increase in evoked AP firing,

suggesting that an atypical PKC played a key role in augmenting the excitability (Zhang et al., 2012). Treatment with siRNAs targeted to the atypical PKCs decreased both the mRNA and protein levels for the PKMζ subtype, but not the full length PKCζ or PKCλ/ι isoforms of the atypical PKCs. Functionally, these siRNAs also blocked the enhanced AP firing produced by NGF. Internal perfusion of neurons with either recombinant PKCζ or PKCλ/ι via the recording pipette produced a significant increase in AP firing, which likely results from the high degree of homology in the catalytic domains for the atypical PKCs. In support of these *in vitro* findings, pre-injection of mPSI into the intraplantar surface significantly reduced the mechanical hypersensitivity resulting from a subsequent intraplantar injection of NGF (Khodorova et al., 2013).

PKMζ may play a causal role in regulating the level of neuronal sensitivity. Previous studies have used the pseudosubstrate inhibitor to explore the role of the atypical PKCs. It is important to note that this pseudosubstrate inhibitor (sometimes referred to as ZIP, zeta inhibitory peptide) lacks the selectivity to discriminate between the different isoforms of the atypical PKCs because of the high degree of homology in the pseudosubstrate domain (Selbie et al., 1993; Akimoto et al., 1994; Hirai and Chida, 2003). Using an interleukin-6-induced model of persistent sensitization, Asiedu et al. (2011) showed that intrathecal injection of ZIP greatly accelerated the recovery of the resulting mechanical allodynia for both the acute IL-6-mediated allodynia as well as the long-lasting allodynia produced by PGE_2. To further examine the role of atypical PKCs in promoting this persistent sensitization, a lenti-viral expression of a constitutively active form of PKCζ in the dorsal horn of the spinal cord produced a sensitization that lasted for approximately 6 days (Asiedu et al., 2011). Two additional studies demonstrated that intrathecal injection of ZIP suppressed the second phase of the pain response evoked by intraplantar injection of formalin (Marchand et al., 2011; Laferrière et al., 2011). These results

indicate that an atypical PKC (although the specific isoform was not resolved) plays a critical role in the heightened sensitivity that results from a variety of inflammatory insults. However, since substances injected intrathecally can diffuse down the spinal roots to the DRG as well as enter the spinal cord, it is unclear if the critical atypical PKC is expressed in primary afferents or in spinal neurons (or glia).

Prior studies established that a long-lasting form of synaptic plasticity depends on the synthesis of new protein (late-phase long-term potentiation, L-LTP)(reviewed in Kelleher et al., 2004; Costa-Mattioli et al., 2009). In addition, L-LTP can be produced by exposure to a variety of agonists, in particular, brain-derived neurotrophic factor (BDNF). In recordings from the Schaffer collaterall-CA1 pyramidal cell synapse of the adult rat hippocampus, exposure to BDNF elicited a dramatic increase in synaptic transmission/strength that was blocked by translational inhibitors, such as cycloheximide or rapamycin (Kang and Schuman, 1996; Tang et al., 2002). In cortical neurons isolated from E18/19 rat embryos, exposure to BDNF generated a rapid increase (within 30 min) in protein synthesis as measured by the incorporation of ^{35}S-methionine (Takei et al., 2001), which was blocked by treatment with either the PI3K inhibitor LY249002 or rapamycin, but not the transcriptional inhibitor actinomycin D. These pharmacological results are similar to our previous findings that demonstrated that the NGF-induced sensitization of AP firing was prevented by the inhibitor of PI3K, but not that of MEK (Zhang et al., 2012), which suggested that a NGF-PI3K pathway is essential for enhancing neuronal excitability. Taken together with our current observations, these results would indicate that NGF augments neuronal excitability through a PI3K-mediated pathway that depends on the translation of newly synthesized protein.

Earlier reports indicated that translation plays a key role in regulating the extent of the pain response after either inflammatory insults or neuropathic nerve injury. The formalin test is a commonly used model of inflammatory pain wherein the secondary response, which is believed to be mediated by sensitization of nociceptive spinal cord pathways, was suppressed in a dose-dependent manner by intraperitoneal injection of cycloheximide (Hou et al., 1997). Later studies showed that application of rapamycin by either intrathecal or intraplantar injection also suppressed this secondary response, implying important regulation by the mTOR pathway (Price et al., 2007). In addition, components of the mTOR pathway were localized to A-type nerve fibers found in the skin, many of which co-expressed N52, a marker for myelinated fibers (Jiménez-Díaz et al., 2008); in electromyographic recordings these same authors reported that rapamycin suppressed the secondary mechanical hyperalgesia (thought to be mediated by A-type fibers) resulting from the cutaneous injection of capsaicin. *In vivo* electrophysiological recordings from wide dynamic range spinal neurons showed that increased neuronal activity associated with the secondary response to formalin was blocked by pretreatment with rapamycin (Asante et al., 2009). This same group detected phosphorylated S6 kinase, a key kinase in mTOR-mediated translation, in the dorsal root ganglia (Asante et al., 2010). In support of our findings, NGF exposure (15 min duration) of dorsal root ganglia neurons isolated from adult mice resulted in the phosphorylation of serine-2448 of mTOR, 4EBP, eIF4E, eIF4G, and AKT (Melemedjian et al., 2010). Together, IL-6 and NGF resulted in increased protein synthesis that was blocked by a 30 min pretreatment with 500 nM rapamycin or the inhibitor of eIF4F complex formation, 4EGI-1 (Melemedjian et al., 2010).

In neurons, a key tenet of activity-dependent modulation of translation is that the specific mRNAs are properly localized in the dendritic fields. Using in situ hybridization, the mRNA for PKMζ was localized in both the cell bodies and the dendrites of hippocampal and sympathetic

neurons grown in culture (Muslimov et al., 2004); these authors found that two targeting elements were required to traffic PKMζ mRNA to the dendritic compartment. Interestingly, BC1 RNA, a repressor of translation initiation, can become co-localized with PKMζ mRNA, suggesting that there are trans-acting factors that can modulate localization of the PKMζ transcript. In addition, it was suggested that heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) also plays a key role since an antisense oligonucleotide targeted to hnRNP A2 significantly reduced the amount of PKMζ mRNA delivery to the neuronal dendrites (Muslimov et al., 2011). Recent work demonstrated that the ability of BC1 RNA to repress translation depends on the phosphorylation state of serine 406 of eIF4B (Eom et al., 2014). In hippocampal slices, high-frequency stimulation of the CA3 Schaffer collateral-CA1 synapse resulted in a rapid dephosphorylation of serine 406 in eIF4B that was associated with increased levels of PKMζ protein; both events were sensitive to okadaic acid (Eom et al., 2014). These findings suggest that that phosphorylation of eIF4B represses translation whereas dephosphorylation of this serine initiates translation, leading to an “on-demand” synthesis of key neuronal proteins. It is unknown whether this type of translational control is involved in the sensitization of sensory neurons resulting from exposure to pro-inflammatory mediators, such as NGF.

Conclusions

We demonstrate that NGF leads to the synthesis of the atypical PKC, PKMζ, and that this molecule plays a critical role in regulating the excitability of isolated sensory neurons as well as the behavioral sensitivity to mechanical stimulation. The maintained mechanical hypersensitivity produced by NGF could be transiently reversed by inhibition of either translation or atypical PKCs. These findings suggest that the ongoing translational synthesis of PKMζ

plays a critical role in the generation as well as the maintenance of the heightened mechano-sensitivity produced by NGF.

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Author contributions

JK helped design and performed/analyzed/ interpreted the biochemical/molecular studies; assisted in writing the manuscript. YHZ performed the electrophysiology studies. AK performed and analyzed the behavioral studies. GS designed, analyzed, interpreted, and wrote the results for the behavioral studies. GDN designed the study, analyzed and interpreted results, and wrote the manuscript. All authors approved the final version of the manuscript.

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Figure Legends

Figure 1. NGF produces sensitization of isolated sensory neurons that is blocked by inhibitors of translation, but not transcription. Panel A. Treatment with 100 ng/ml NGF significantly increases the number of evoked APs in either untreated sensory neurons or in neurons exposed to the vehicle DMSO alone. Asterisks represent a significant difference between the control and those treated with NGF. The ordinate and axis labels in A apply to panels B, C, D, and E. Panel B. A 30 min pretreatment with 10 μM actinomycin D (a transcription inhibitor) does not affect the sensitization produced by NGF. Asterisks represent a significant difference between the control and those treated with NGF. Panel C. A 30 min pretreatment with 60 μM cycloheximide (a translation inhibitor) blocks the sensitization produced by NGF. Panel D. A 60 min pretreatment with 500 nM rapamycin (a translation inhibitor) blocks the sensitization produced by NGF. Panel E. A 30 min pretreatment with 30 μM 4EGI-1 (a translation inhibitor) blocks the sensitization produced by NGF. Panel F. A 30 min pretreatment with 1 μM okadaic acid (an inhibitor of protein phosphatase 1/2A) blocked the sensitization produced by NGF.

Figure 2. NGF significantly increases the amount of PKMζ, protein, which is blocked by cycloheximide. Panel A illustrates a representative Western blot wherein exposure to 100 ng/ml NGF for 30 min increases the level of PKMζ protein (the 49 kDa band). In contrast, no NGF–induced increase in PKMζ occurs after pretreatment with 50 μg/ml cycloheximide for 1 h. The bottom panel indicates the amounts of HPRT protein as a loading control. Panel B summarizes the results obtained from three different Western blots obtained from three different tissue harvests. Exposure to 100 ng/ml NGF for 30 min significantly increased the amount of PKMζ protein normalized to their respective HPRT levels. The amounts of PKMζ after these different treatments were then normalized to their corresponding levels of PKMζ protein obtained for the

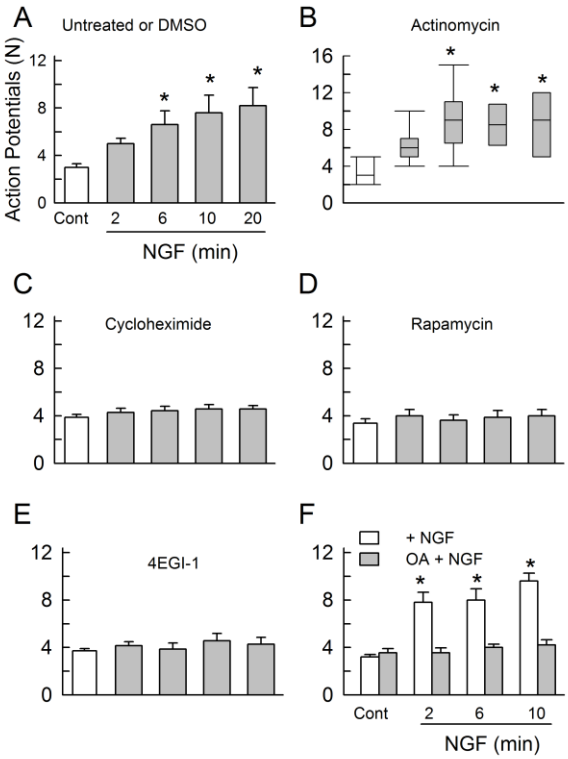
untreated condition (-NGF, -CHX) (data not shown). The asterisk represents a significant difference between the NGF+ CHX− and all other samples.

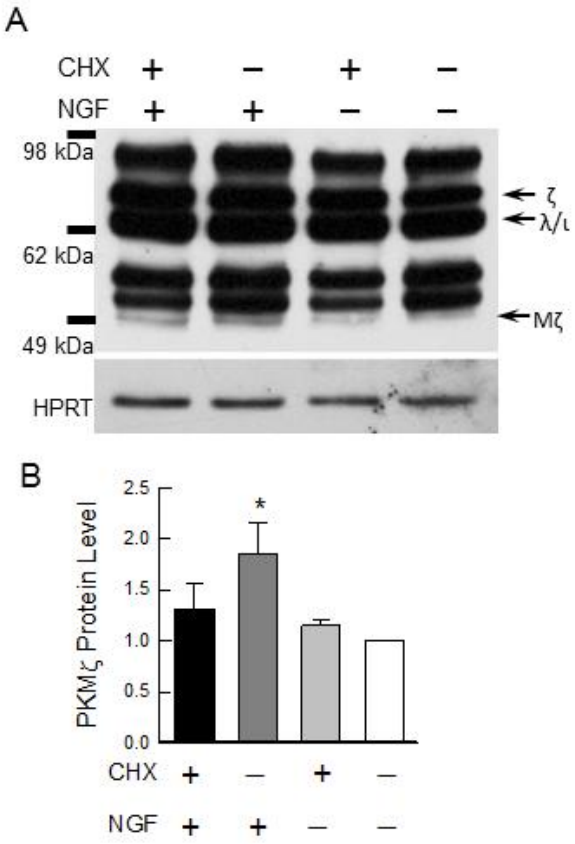
Figure 3. NGF produces mechanical hyperalgesia that is suppressed by either cycloheximide or rapamycin. Response Efficiency after NGF when paw is pre-treated by Vehicle (Veh), Cycloheximide (CHX) or Rapamycin (Rapa). When compared to the (merged) baseline value, responses to NGF (all for 15g VFH) after Vehicle injections (n= 6) are significantly different at 0.5, 1, 3.5 and 22 h, whereas those after CHX (n=11) or Rapa (n=12) only reach significance at 22 h (*** P≤0.005, ** P≤0.01, Kruskal-Wallis, Wilcoxon Rank sum tests). In comparison to the Responses after Vehicle, those after CHX or Rapa are significantly different at 0.5, 1 and 3.5 h (### P≤0.005, ## P≤0.01, # P≤0.05, Kruskal-Wallis/Wilcoxon Rank Sum tests).

Figure 4. Established NGF-induced mechanical hyperalgesia is transiently reversed by local injection of rapamycin. Panel A: local injection of the vehicle, DMSO, did not affect the pre-existing NGF-induced hypersensitivity, measured acutely or 24 h and 48 h after DMSO injection (n=8). Friedman's test for 6 groups comparing the baseline values with the responses at other time points: P=0.0004, *P≤0.05, **P≤0.01 vs. baseline with Dunn's post hoc pair-wise test. Panel B: the NGF-induced hypersensitivity was significantly reduced 24 h after the injection of rapamycin (10 mM; 10 µl) on D3 (n=9). Established hypersensitivity shown by Friedman's test for 6 groups comparing baseline value with the responses at other time points: P≤0.0001, then *P≤0.05, **P≤0.01, ***P≤0.001 vs. baseline (Dunn's post hoc pair-wise test). Effect of rapamycin shown by comparing the D4 (24 h of rapamycin) value vs. the 4 h D3 value or the D3 value before rapamycin, $^{\#\#}$P≤0.005, two-tailed Wilcoxon test Panel C: Injection of rapamycin produces a significant decrease (at D4), 24 h after rapamycin injection, in the maximal possible

hypersensitivity produced by NGF, as measured at D3 (% MPE) (P=0.002, Kruskal-Wallis test, **P≤0.01 vs. the 4 h D3 value and *P≤0.05 vs.48 h D5 value, Dunn's post hoc pair-wise test).

Figure 5. Established NGF-induced mechanical hyperalgesia is transiently reversed by local injection of mPSI. Panel A: Hindpaw injection of NGF (1 μg/10 μl) produced a significant increase in mechanical sensitivity on D3 (n=3), at which time mPSI (40 μg/20 μl) was injected into the same hindpaw. Measurements on D4 showed a reversal of the mechanical hypersensitivity back towards the baseline response value. These three data sets for each rat (n=3 rats), were taken at 24, 48 and 72 h after mPSI, respectively, and were then compared by Friedman's test, which showed a significant difference among all three with P=0.0015. Application of Dunn's test post-hoc for pair-wise comparison showed that D4 (24 h) and D5 (48 h) values differed significantly, **P≤0.01, but none of the other pairs were different. Panel B: Injection of mPSI produces a significant decrease in the percentage of the maximal possible hypersensitivity produced by NGF relative to that measured at D3 (%MPE). Rats receiving a local injection of scrambled mPSI (40 μg/20 μl, n=3) exhibited no significant change in mechanical sensitivity on D4 and D5. Friedman's test for 3 groups comparing value at 24 h with the responses at other time points: P=0.0015, **P≤0.01 vs. 48 h after mPSI (Dunn's post hoc test).

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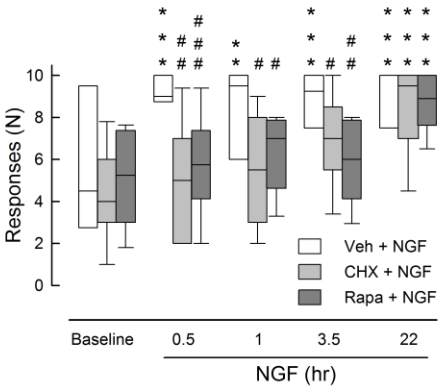
Responses (N)
10
8
6
4
2
0
Veh + NGF
CHX + NGF
Rapa + NGF
Baseline
0.5
1
3.5
22
NGF (hr)