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## The p75<sup>NTR</sup> SIGNALING CASCADE MEDIATES MECHANICAL HYPERALGESIA INDUCED BY NERVE GROWTH FACTOR INJECTED INTO THE RAT HIND PAW

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### Abstract

Nerve Growth Factor (NGF) augments excitability of isolated rat sensory neurons through activation of the p75 neurotrophin receptor (p75<sup>NTR</sup>) and its downstream sphingomyelin signaling cascade, wherein neutral sphingomyelinase(s) (nSMase), ceramide, and the atypical PKC (aPKC), PKM $\zeta$ , are key mediators. Here we examined these same receptor-pathways *in vivo* for their role in mechanical hyperalgesia from exogenous NGF. Mechanical sensitivity was tested by the number of paw withdrawals in response to 10 stimuli (PWF = n/10) by a 4g von Frey hair (VFH, testing "allodynia") and by 10g and 15g VFHs (testing "hyperalgesia"). NGF (500 ng/10  $\mu$ l) injected into the male rat's plantar hind paw induced long lasting ipsilateral mechanical hypersensitivity. Mechano-hypersensitivity, relative to baseline responses and to those of the contralateral paw, developed by 0.5–1.5h and remained elevated at least for 21–24h. Acute intraplantar pre-treatment with nSMase inhibitors, GSH or GW4869, prevented the acute hyperalgesia from NGF (at 1.5h) but not that at 24h. A single injection of N-acetyl sphingosine (C2-ceramide), simulating the ceramide produced by nSMase activity, induced ipsilateral allodynia that persisted for 24h, and transient hyperalgesia that resolved by 2h. Intraplantar injection of hydrolysis-resistant mPro-NGF, selective for the p75<sup>NTR</sup> over the TrkA receptor, gave very similar results to NGF and was susceptible to the same inhibitors. Hyperalgesia from both NGF and mPro-NGF was prevented by paw pre-injection with blocking antibodies to rat p75<sup>NTR</sup> receptor. Finally, intraplantar (1 day before NGF) injection of mPSI, the myristoylated pseudosubstrate inhibitor of PKC $\zeta$ /PKM $\zeta$ , decreased the hyperalgesia resulting from NGF or C2-ceramide, although scrambled mPSI was ineffective. The findings indicate that mechano-hypersensitivity from peripheral NGF involves the sphingomyelin signaling cascade activated via p75<sup>NTR</sup>, and that a peripheral aPKC is essential for this sensitization.

### Keywords

nerve growth factor; PKM $\zeta$ ; aPKCs; sphingomyelinase; hyperalgesia; pain

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## 1

Nerve growth factor (NGF) plays a critical role in development and growth of peripheral sensory neurons, and also induces thermal and mechanical sensitization of these neurons in adult mammals. Much evidence indicates that in adults NGF may still have some trophic role, but its main function is to initiate and maintain nociceptor hypersensitivity as a part of inflammatory and immune responses after tissue injury (see Pezet and McMahon, 2006). The trophic actions of NGF have been attributed to activation of a receptor tyrosine kinase (TrkA) that is expressed on peripheral and central neurons and distinguished by its high affinity for NGF (Meakin and Shooter, 1992; Barker and Murphy, 1992; Fundin et al., 1997). Nerve growth factor is also known to induce hypersensitivity and pain in humans (Dyck et al., 1997; Svensson et al., 2003; Rukwied et al., 2010) and to decrease nociceptive thresholds in rodent models of pain (Lewin et al., 1993; Woolf et al., 1994; Woolf 1996; McMahon et al., 1995; Hathway and Fitzgerald, 2006; Mills et al., 2013)

Results of a recent study exploring the capacity of NGF to directly and acutely modulate the excitability of isolated sensory neurons suggest that such actions follow activation of the low affinity NGF-binding receptor, p75 neurotrophin receptor (p75<sup>NTR</sup>), which can trigger activation of the downstream sphingomyelin signaling cascade (for review see Nicol and Vasko, 2007; Zhang et al., 2012). Neutral sphingomyelinase(s) (nSMase), ceramide and the atypical PKC (aPKC), PKM $\zeta$ , are important effector molecules of this intracellular pathway.

In the present work we aimed to determine the contribution of these mediators of the p75<sup>NTR</sup> signaling pathway to the nociceptive mechanical hypersensitivity produced by local NGF administration in rats *in vivo*. The results show that the p75<sup>NTR</sup> is essential for this response and that inhibition of nSMase, i.e., of ceramide liberation from sphingomyelin, and inhibition of peripheral aPKCs have preventive actions on the development of NGF-dependent mechanical hypersensitivity.

## 2.0 Experimental Procedures

Experiments were conducted in adult male Sprague-Dawley rats (235–330g). Rats were housed in groups of 2 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, 1996) as reviewed and approved by the Harvard Committee on Animals

### 2.1 Mechanical testing

Unrestrained rats were placed on an elevated plastic mesh floor (28 × 17.5 cm; 9.5 × 9.5mm openings) and allowed to habituate for 25–40 min before initial testing. Paw Withdrawal Frequency to mechanical stimulation was determined using calibrated von Frey hairs (VFH) applied perpendicular to the plantar surface of a hind paw through spacing in the mesh. Each VFH (4g, 10g and 15g) was applied 10 times for 3 sec, separated by a 3 sec interval. Testing with the next VFH started ca. 8–10 min after the beginning of the testing with a previous force. Testing started with a lowest force of 4g, and continued with increasing forces, with all three forces tested with 10 probings in each test period. To avoid stress and to obtain consistent responsiveness to the same force, the rats were habituated and tested on mesh racks over 5–6 days before each experiment (training period). Withdrawal responses were registered initially on the ipsilateral paw (IPSI) in 4 rats, then on the contralateral paw (CLP), for each VFH. The number of paw withdrawals, n, occurring in response to 10 stimuli (range: n=0–10) was used to assess mechanical sensitivity, and graphed as Paw Withdrawal Frequency (n) for each force.

## 2.2. Injection procedures

NGF, GSH, C2-ceramide, GW4869 or its vehicle, alone or with NGF, were injected subcutaneously (s.c.) in a 20  $\mu$ L volume into the mid-plantar hind paw, 1 cm distal from the heel. The non-selective atypical myristoylated pseudosubstrate inhibitor (mPSI; also known as ZIP, Eichholtz et al., 1993; Thiam et al., 1999) was injected s.c. into the plantar surface (40  $\mu$ g/20  $\mu$ L). Injections occurred 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 for intraplantar (i.pl.) injection; 5–10 min later “normal” nocifensive responses (thresholds, latencies) could be assessed.

## 2.3. Chemicals

NGF- $\beta$  (rat) (Sigma-Aldrich, St. Louis MO, USA) was made as a stock solution (100 ng/ $\mu$ L of phosphate buffered saline (PBS: pH7.4)) and stored in 40  $\mu$ L aliquots at  $-80^{\circ}\text{C}$ . L-Glutathione (GSH, Sigma-Aldrich) was dissolved in PBS immediately before each injection (fresh made solutions for pre- and co-injections). Prior to the injection, NGF stock aliquots were diluted (1:1) in PBS or mixed with GSH solutions (for co-injections) to the noted final concentration of 50 ng/ $\mu$ L. A non-hydrolyzable mutated homologue of Pro-NGF, mPro-NGF (Alomone Labs, Jerusalem, Israel) was reconstituted in PBS, then centrifuged at  $10,000\times g$  for 5 min to remove any undissolved particulates before paw injection. C2-ceramide (Enzo Life Sciences, Farmingdale, NY) was dissolved initially as a 25  $\mu$ g/ $\mu$ L DMSO stock solution, stored at  $-80^{\circ}\text{C}$  in aliquots, then diluted to 20  $\mu$ g/10  $\mu$ L DMSO before the injection. GW4869 (Sigma-Aldrich) was dissolved in DMSO as a 2 mM working solution, and aliquots were prepared under an inert gas (nitrogen) and stored at  $-80^{\circ}\text{C}$ . The atypical PKC pseudosubstrate inhibitor, mPSI (Calbiochem, USA; Enzo Life Sciences) was dissolved immediately before the experiment, first in  $\text{H}_2\text{O}$  to 5  $\mu$ g/ $\mu$ L and then diluted 1:10 in PBS.

A polyclonal blocking antibody to rat p75<sup>NTR</sup> was generously donated by Prof. Louis Reichardt (University of California, San Francisco); 20  $\mu$ L volumes were injected into the plantar paw surface 4 h before the injection of NGF or Pro-NGF. As a control for the p75<sup>NTR</sup> blocking antibody, a rabbit IgG solution containing approximately the same total protein was used ( rabbit polyclonal IgG; Abcam, MA USA).

## 2.4. Experimental design

NGF was injected s.c. into the plantar surface of the rat’s hind paw, always at a dose of 500 ng in 10  $\mu$ L. To test GSH effectiveness against NGF-induced hyperalgesia, GSH was delivered in either of two injection protocols. 1) Acute pre-treatment: the first injection occurred at “15” (15–18) min prior to NGF and the second injection was delivered mixed with the NGF. Two different dosing levels were delivered, one in which GSH was applied at a low total dose (0.6  $\mu$ mol/paw) and another at a high total dose (1.8  $\mu$ mol/paw; the concentrations and doses are indicated in the Results and figure legends). 2) Prolonged pre-treatment: the first injection occurred 1.5h prior to the NGF injection, and the second, as in (1), above, mixed with NGF. For these experiments, GSH was delivered at the high total dose (1.8  $\mu$ mol/paw). In Control experiments NGF- $\beta$  (rat) was injected: 1) “15” (15–18) min after the injection of the vehicle for GSH (PBS, 10  $\mu$ L); 2) a single injection of GSH, without NGF, was administered at a dose intermediate (1.2  $\mu$ mol/paw) between the “high” and the “low” total dose condition, which resulted in the highest concentration (120 mM) of GSH used for either acute or prolonged pre-treatment.

To corroborate the effects of GSH, a structurally different nSMase inhibitor, GW4869 (2 mM, 10  $\mu$ L) (or its vehicle) was injected once, either 17 min before or 1h after NGF.

In another group of rats, membrane-permeant C2-ceramide alone, or its vehicle, was tested for its ability to affect mechanical sensitivity after either a single i.pl. injection (20 µg/10 µl) or following multiple injections of the same dose, spaced 75 min apart. This separation time was chosen to be long enough to allow the acute, transient effects from a single dose to fully reverse and therefore to minimize the role of a “sensitized state” in altering responses to subsequent doses of the allogen.

To establish the importance of atypical PKCs (e.g., PKM $\zeta$ ) for NGF-dependent hyperalgesia, a non-selective inhibitor of aPKCs, myristoylated pseudosubstrate inhibitor (mPSI, 40 µg/20µL), or its vehicle, were injected s.c. into the plantar surface of the rat’s hind paw. Such injection caused substantial edema, without accompanying changes in mechano-sensitivity, but requiring a delay in the subsequent injection of NGF. The mean value for each force was taken as the baseline paw withdrawal frequency at 1 day after mPSI injection, when the paw size had returned to normal, shortly after which NGF was injected into the paw.

## 2.5. Analysis

Data are presented as means  $\pm$  S.E.M and evaluated using GraphPad InStat version 3.0 (GraphPad Software, CA, USA). Since the hind paw withdrawal frequency data presented here do not follow a normal distribution, statistical analysis used nonparametric tests. Friedman test followed by Dunn’s *post hoc* test was applied to compare repeated measures of Paw Withdrawal Frequency with baseline values. An unpaired, two-tailed Mann-Whitney U-test was used to compare responses, to the same force at the same time point, of the ipsilateral and contralateral paws. Wilcoxon matched pairs (two-tailed) test, with repeated measures correction, was applied for comparison of responses at different times with the baseline response. P values <0.05 were considered to be significant.

## 3.0. RESULTS

### 3.1. NGF and C2-ceramide sensitize the paw to mechanical stimulation

Intraplantar injection of NGF (500 ng/10 µL) induced a long lasting ipsilateral mechanical hyperalgesia. Paw withdrawal frequency for the 10g and 15g VFH, but not the 4g VFH, had significantly increased by 0.5–1.5h after injection and remained elevated at 21–22h, compared to either the baseline responses or the unchanged responses of the contralateral paw (Fig. 1). (Responses to the 4g VFH varied among cohorts of rats and had the largest variance, such that the changes after NGF sometimes reached significance but other times did not. Changes in the response to the 15g VFH stimulation were consistent.) This general pattern of a rapidly developing and persistent mechano-sensitization was observed independently many times when NGF was injected into naïve paws.

A single injection of N-acetyl sphingosine (C2-ceramide, 20 µg/10 µL), a cell-permeant ceramide analog, caused acute allodynia and hyperalgesia that, unlike that caused by NGF, had usually resolved by 2h (Fig. 2A). In separate experiments, attempting to extend the hypersensitivity by increasing the duration of C2-ceramide’s presence in the paw, three sequential identical doses were injected, each 75 min apart, timed so that the effect from one single dose had resolved before the next injection. This also caused an acute, ipsilateral increase in mechanical responsiveness to stimulation with all three forces, one that was still present at 2h after the last injection (Fig 2B), but had resolved by 24h (data not shown).

### 3.2. Antihyperalgesic actions of GSH

To further evaluate the role of the sphingomyelin cascade, we first tested whether inhibition of nSMase, the enzyme catalyzing the liberation of ceramide, would suppress NGF-induced

hyperalgesia. Acute pre-treatment with a semi-selective inhibitor of nSMase activation, GSH (Liu and Hannun, 1997; Liu et al., 1998), pre-injected (120 mM, 10  $\mu$ l) 15 min before and then co-injected (60 mM, 10  $\mu$ l) with NGF (total GSH dose = 1.8  $\mu$ moles/paw), prevented significant acute mechano-sensitization at 1.5h but did not affect the increase in mechano-sensitivity measured the next day ( $n=8$ ) (Fig. 3A compare to Fig. 1A). Glutathione applied at a lower total dose of 0.6  $\mu$ mol (40 mM and 20 mM concentrations for pre- and co-injections, respectively) did not attenuate NGF-induced enhancement of ipsilateral mechanical responsiveness ( $n=8$ , data not shown). In contrast to the effects of immediate pretreatment with GSH, when the higher pre-treatment dose (120 mM, 10  $\mu$ l) was given 1.5h beforehand followed by a co-injection of GSH (60 mM, 10  $\mu$ l) with NGF, hyperalgesia was not reduced, despite the high total dose of the inhibitor ( $n=4$ ; Fig. 3B). In controls, injection of GSH into the naïve paw, at a total dose (1.2  $\mu$ mol; 120 mM, 10  $\mu$ l) that was intermediate between “high” and “low” doses, evoked swelling of the paw in 50% of rats by 3–4h, but did not change mechanical responsiveness in either ipsilateral, or contralateral paws, as compared to baseline values ( $n=4$ ). Similarly, no changes in the mechanical responsiveness were observed during the 1.5h pre-treatment of GSH, before the injection of NGF ( $n=8$ ).

### 3.3. Attenuation of NGF-induced hyperalgesia by GW4869

Since GSH was used at a high concentration and affects many other proteins we examined a more selective inhibitor of nSMase, GW4869 (Luberto et al., 2002; Marchesini et al., 2003). This cell-permeant, non-competitive, nSMase inhibitor was injected into the paw 17 min prior to NGF. The effect of this single application (2 mM, 10  $\mu$ L; dose 20 nmoles) was similar to the actions of the acute pre-treatment with high dose GSH; it prevented the hypersensitivity at 0.5–1.5h, but not at 23h after NGF injection ( $n=8$ ) (Fig. 4A). In the vehicle control group, wherein NGF was injected following pre-injection of DMSO, hyperalgesia developed as after NGF alone, both responses to 10g and 15g forces being augmented significantly at 0.5–23h ( $n=8$ ) (Fig. 4B). In neither group of rats were any changes in responsiveness of the contralateral paw observed (data not shown). Therefore the inhibitor of nSMase GW4869 prevented NGF-induced local mechanical hyperalgesia, although this effect lasted less than one day.

Pre-injection of the paw with GW4869 had no effect on the acute hyperalgesia caused by the C2-ceramide injection ( $n=8$ , data not shown). This result is consistent with an upstream inhibition of GW4869, at the nSMase location, and also shows that the inhibitor did not alter any reactions of the downstream pathway.

When GW4869 (2 mM, 10 $\mu$ L) was injected 1h after NGF it failed to reverse the already developed mechanical hyperalgesia ( $n=7$ ) (Fig. 4C). Some tendency to attenuate the hyperalgesia was noticed at 35–50 min after injection of GW4869; the NGF-induced increase in mechanical response to 10 and 15 g VFHs did not reach significance *vs.* baseline in a group test (Friedman+Dunn’s). These changes *vs.* ipsilateral baseline, however, were highly significant for 15 g VFH, when compared using the Mann-Whitney test ( $p=0.0006$ ).

### 3.4. Hypersensitivity from Pro-NGF

A non-hydrolyzable homologue of Pro-NGF (Pagadala et al., 2006, the larger precursor to mature NGF which has selectivity for the p75<sup>NTR</sup> over the TrkA receptor (Lee et al., 2001; Beattie et al., 2002; Ibañez, 2002; Teng et al., 2010), also caused hyperalgesia. Injections of this mPro-NGF (500ng/10 $\mu$ L) into the hindpaw increased responsiveness to 10 and 15 g VFH at 0.5 to 21 h, very similar to the time-course following NGF injection (Fig. 5; compare to Fig 1A). Responsiveness to the 4g VFH was not significantly changed.

### 3.5 A p75<sup>NTR</sup> blocking antibody abolishes NGF-induced hyperalgesia

To further confirm that the active receptor for NGF's actions is p75<sup>NTR</sup>, we used a receptor-specific antibody. Polyclonal rat anti-p75<sup>NTR</sup> antibodies (generously donated by Prof. Louis Reichardt, UCSF) were injected into the plantar hindpaw 4h before NGF or mPro-NGF injection. The antibody alone did not affect mechano-responsiveness (Fig. 6A). Subsequent injection of NGF (Fig. 6A) or mPro-NGF (in separate experiments, Fig. 6B), at the same s.c. location in the paw as the antibody injection, resulted in virtually no increase in paw mechano-sensitivity. By comparison, pre-injections of the same amount of naïve serum-derived IgG did not diminish the hypersensitivity from mPro-NGF (Fig. 6C) or from NGF (not shown).

### 3.6. The myristoylated pseudosubstrate inhibitor of aPKCs (mPSI) suppresses NGF-induced hyperalgesia

Previously published electrophysiological studies of isolated sensory neurons have shown that NGF-induced hyperexcitability depends on the nSMase > ceramide > S-1-P pathways, with an essential downstream role of aPKCs, specifically PKM $\zeta$  (Zhang et al., 2002, 2006, 2012; Nicol and Vasko, 2007). To determine whether aPKCs were involved in the local effects of NGF, we pre-treated rats peripherally with mPSI, a cell permeant inhibitor acting non-selectively on all aPKCs (Eicholtz et al., 1993; Thiam et al., 1999; Harishchandran and Nagaraj, 2005; Moscat et al., 2006). Edema of the paw followed acutely after mPSI injection, possibly due to its actions on vascular targets unrelated to aPKCs (Krotova et al., 2006), and this necessitated a delay before the injection of NGF. Therefore, this inhibitor was injected subcutaneously (40  $\mu$ g/20  $\mu$ l) into the plantar hind paw 24h before the injection of NGF. Pre-injection of mPSI alone did not increase the responsiveness to mechanical stimulation tested 24h later, the small changes being the same as from vehicle injection (Fig. 7A), and by then there was no edema. Locally administered mPSI strongly suppressed the development of mechanical hyperalgesia from NGF injected into the same plantar area ( $n=16$ , Fig. 7B, compare to Figures 1, Fig. 7D). A scrambled version of mPSI did not affect the hypersensitivity from NGF (Fig. 7C), showing the selectivity of this inhibitor for its aPKC inhibitory function (Krotova et al., 2006). In cohort-matched control experiments, a robust hyper-responsiveness was observed at 0.5–24h after NGF injection in the control group of rats, which had been pre-injected with intraplantar vehicle (PBS) 24h before NGF ( $n=13$ , Fig 7D).

As with NGF, intraplantar mPSI blocked the acute hyperalgesia resulting from injection of C2-ceramide into the paw (Fig. 7E). From 0.5 to 3h after C2-ceramide (20 $\mu$ g/10  $\mu$ L) injection into the paw, the responsiveness was unchanged from the baseline values ( $n=12$ ), contrasting with the enhanced sensitivity caused by this dose of C2-ceramide in naïve rats (cf. Fig. 2).

## 4.0. DISCUSSION

The mechanism of NGF-induced mechanical hyperalgesia is not completely known. NGF initiates a variety of intracellular signals through activation of its two receptors, TrkA, with a higher NGF affinity and slower dissociation (respectively,  $K_d \sim 10^{-11}$ M,  $t_{1/2} \sim 10$  min), and a broadly selective neurotrophin receptor, p75<sup>NTR</sup>, having a lower affinity and faster dissociation for NGF ( $K_d \sim 10^{-9}$ M,  $t_{1/2} \sim 3$  sec) (Meakin and Shooter, 1992). Both receptors are expressed on sensory neurons and are assumed to be activated by relatively high concentrations of NGF either released endogenously during inflammation, or administered exogenously (Woolf, 1996; Woolf et al., 1996; Nicol and Vasko, 2007). We tested the hypothesis that activation of the p75<sup>NTR</sup> signaling cascade that has been shown to sensitize isolated peripheral nociceptive neurons (Zhang et al., 2002; Zhang and Nicol, 2004; Zhang

et al., 2012) contributes to the sensitization of mechanical responsiveness by NGF in adult rats *in vivo*. Our findings show a rapidly developing tactile hypersensitivity, present at 1 h and persisting for at least 24h after hindpaw injection of 500 ng of NGF. Injection of the p75<sup>NTR</sup>-selective homologue Pro-NGF, which had been mutated to mPro-NGF in order to prevent the hydrolytic cleavage that frees mature NGF (Pagadala et al., 2006), produced an identical hypersensitivity. Direct binding studies show that Pro-NGF binds with ~5-fold higher affinity to p75<sup>NTR</sup> than to TrkA receptors (Lee et al., 2001; Nykjaer et al., 2004), although the predominant pathway in any action will depend on the relative levels of expression of the two receptors (Masoudi et al., 2009) and early evidence suggests that interactions between the two receptors may alter their activities (Benedetti et al., 1993).

Importantly, paw injection of an antibody directed against the rat p75<sup>NTR</sup> (Weskamp and Reichardt, 1991) prevented tactile hypersensitivity from either NGF or mutant Pro-NGF. This antibody showed a large selectivity for the p75<sup>NTR</sup> based on direct NGF binding studies and on their ineffectiveness in suppressing neuron survival and neurite outgrowth that are known to be mediated by Trk receptors (Fariñas et al., 1998). Inhibition of NGF- and mPro-NGF induced hyperalgesia by this antibody is consistent with an earlier report wherein treatment with the same antibody prevented the NGF-induced increase in excitability in isolated sensory neurons (Zhang and Nicol, 2004).

In addition, our results demonstrate that NGF/p75<sup>NTR</sup> – mediated hypersensitivity results from activation of a neutral sphingomyelinase, can be recapitulated by a membrane-permeant ceramide analogue, albeit only briefly compared to that of NGF, and, lastly, a key downstream effector is an atypical PKC in the periphery NGF-induced mechanical hypersensitivity in adult rats develops after a single systemic injection (1 µg/gm, s.c.) with a rapid (0–7h) and a slow (8–30h) phase (Lewin et al., 1993). The authors suggested that the delayed mechanical hypersensitivity in adult rats results from central changes, perhaps due to up-regulation of peptides in the peripheral

NGF-responsive sensory neurons, where the internalized NGF-TrkA complex in the periphery could be transported to central terminals within hours. Thermal hypersensitivity was attributed to both peripheral and central neuron sensitization. NGF's involvement in prolonged inflammatory hyperalgesia, after injection of Complete Freund's Adjuvant, appears to require both sensory neurons and mast cells (Woolf et al., 1996), both of which express TrkA receptors. Consistent with our results described above, intradermal injection of NGF (1µg) into the dorsum of the paw after just 10 min, resulted in tactile hypersensitivity that reached its peak change by 3h and lasted for several days (Malik-Hall et al., 2005). Most recently it has been shown that single intraplantar injections of NGF (0.3–5 µg/paw) reduced the mechanical withdrawal threshold maximally by 50–70 % after 1hr, which only slowly reversed over several weeks (Mills et al., 2013).

Previous publications present contradictory findings about the roles of TrkA vs. p75<sup>NTR</sup> in pain sensitization. Most publications support an essential role of the TrkA receptor (Mantyh et al., 2011). Mutated mice lacking p75<sup>NTR</sup> still develop mechanical and thermal hyperalgesia after systemic NGF delivery (Bergmann et al., 1998), suggesting that TrkA plays the dominant role. However, these knock-out mice have higher thresholds for acute nocifensive responses to cutaneous thermal and mechanical stimuli and show significant reductions in the density of epidermal neurites (Bergmann et al., 1997). Differences in the distribution of DRG cell sizes between p75<sup>NTR</sup> <sup>-/-</sup> mice and the wild-type <sup>+/+</sup> controls were modest, and there was no difference in their neurochemical profiles (Bergmann et al., 1997). Others, however, have reported a marked reduction of both peptide-containing neurites in the skin and thermal sensitivity in p75<sup>NTR</sup> knock-out mice (Lee et al., 1992). Also in support of TrkA involvement, intrathecal delivery of anti-sense oligodeoxynucleotides targeted to

TrkA decreased TrkA protein in peripheral nerve and prevented NGF-induced mechanical hyperalgesia (Malik-Hall et al., 2005).

In contrast, other studies suggest that the p75<sup>NTR</sup> plays a key role in pain. Inflammation resulting from CFA injection into the rat wrist joint induced an upregulation of p75<sup>NTR</sup> immunoreactivity, and local injections of blocking antibodies against the p75<sup>NTR</sup> reduced CFA-induced mechano-hyperalgesia (Iwakura et al., 2010). Similarly, intraplantar injections of CFA, NGF or Pro-NGF caused thermal hypersensitivity, and also increased the expression of CGRP in primary sensory neurons, both changes being prevented by the anti-p75<sup>NTR</sup> blocking antibody (Watanabe et al., 2008). Signaling through the sphingomyelin pathway is known to be activated by the low-affinity p75<sup>NTR</sup>, resulting in the liberation of ceramide (Dobrowsky et al., 1994). Consistent with this idea, C2-ceramide injected into the paw induces mechano-hyperalgesia that is mimicked by injection of TNF- $\alpha$  (Woolf et al., 1997), an effect that was blocked by the nSMase inhibitor GW4869 (Joseph and Levine, 2004). Recent findings further show that intraplantar injection of C2-ceramide produced thermal hyperalgesia that peaked 1h post-injection, as well as increased levels of TNF- $\alpha$  (Doyle et al., 2011a,b).

There is compelling evidence that NGF acts directly on sensory neurons. Capsaicin-evoked currents through TRPV1 receptors (Shu and Mendell, 1999, 2001), were enhanced via activation of TrkA (Galoyan et al., 2003; Zhu and Oxford, 2007). NGF-induced activation and up-regulation of TRPV1 (Winston et al., 2001; Zhang et al., 2005), and this receptor/channel's transport to peripheral C-fiber terminals of adult rat DRG neurons contributes to the maintenance of inflammatory heat hypersensitivity (Ji et al., 2002). In capsaicin-sensitive, small-diameter DRG neurons isolated from adult rats, both NGF and C2-ceramide acutely increased firing of action potentials (AP) as a result of the rapid augmentation of a tetrodotoxin-resistant sodium current and suppression of a delayed rectifier-type potassium current (Zhang et al., 2002); both actions appear to involve the p75<sup>NTR</sup> receptor. This effect from NGF, but not the identical one from C2-ceramide, was completely prevented by pretreatment with the same p75<sup>NTR</sup> blocking antibody used in the current studies (Zhang and Nicol, 2004). Furthermore, inhibitors of tyrosine kinase receptors failed to affect the increased AP firing produced by either NGF or BDNF (Zhang et al., 2008). Involvement of the sphingomyelin signaling pathway is evident in the NGF-induced augmentation of AP firing by its strong inhibition by glutathione, a cell-permeant inhibitor of nSMase, as also shown in our present *in vivo* study. This increase in excitability could be mimicked by intracellular perfusion with bacterial SMase, simulating the release of native ceramide by endogenous enzymes. Exogenous C2-ceramide enhanced the number of evoked APs, despite the presence of glutathione and NGF, indicating that ceramide's sensitizing actions lay downstream of NGF (Zhang et al., 2002), identical to the interactions shown here *in vivo*. Moreover, sphingosine-1-phosphate, a product of intracellular metabolism of ceramide by sphingosine kinase, acted as a second messenger in augmenting neuronal excitability (Zhang et al., 2006).

In the current work both intraplantar NGF and C2-ceramide induced mechanical hypersensitivity. However, NGF - evoked hyperalgesia persisted at least for 24h (also see Mills et al., 2013) whereas that from a single C2-ceramide injection had resolved by 2h. Multiple sequential injections of this dose of C2-ceramide extended the hypersensitivity after the last dose, but not for more than a few hours, still very brief compared to NGF. This difference in the duration of hypersensitivity may be explained by the different abilities of the two agents to persistently activate the common pathway. In isolated cells the two compounds cause essentially identical changes in excitability and its underlying ion currents (Zhang et al., 2002). But the C2-analogue of ceramide might be quite differently disposed of in the rat than the longer fatty acyl chain ceramides that are liberated *in vivo* by nSMase;



these ceramides may be bound to hydrophobic regions of membranes or proteins and thus less mobile for diffusion and less amenable to degradative enzymes (Liu and Hannun, 1997; Liu et al., 1998). Furthermore, other cells near the cutaneous injection site, e.g., keratinocytes, might also respond to NGF and to ceramide, leading to activation of pathways that are absent from isolated sensory neurons.

Two different nSMase inhibitors appeared effective against the acute hyperalgesic actions of NGF. Brief (15 min) pre-treatment with GSH, a selective inhibitor of nSMase activation (Liu and Hannun, 1997; Liu et al., 1998), prevented the acute NGF-induced hyperalgesia, measured 1.5h later, but did not affect mechanical hypersensitivity measured the next day. This finding suggests that there is a limited time during which GSH's inhibitory actions are effective, implying that the period of p75<sup>NTR</sup>-induced nSMase activation continues after the readily oxidized GSH has been exhausted or removed from the paw. To test the hypothesis of limited time of protection we injected a high concentration of GSH 1.5h before NGF (which was also co-injected with GSH). This earlier delivery of GSH failed to suppress the NGF-induced tactile hyperalgesia, showing that protection by GSH is indeed a short-lived phenomenon.

The cell-permeant non-competitive nSmase inhibitor GW4869, injected once shortly before NGF, also prevented the NGF-induced hyperalgesia at 0.5–1.5h, but not on the next day. Here, using a more stable inhibitor than GSH (Luberto et al., 2002), a preventive effect of nSMase inhibition also lasted only a short time. It is plausible that the inhibitor could have been removed from the paw by the next day, which may explain the lack of GW4869 anti-hyperalgesic actions at 23h post NGF. However, GW4869 when injected 1h after NGF also appeared to be completely ineffective against the previously developed hyperalgesia. These results imply that the liberation of ceramide by nSMase contributes to the induction rather than the maintenance of mechanical hypersensitivity in rat plantar skin, wherein the downstream effect of ceramide, once initiated, leads to continuous functioning of intracellular machinery to provide a hyperalgesic state. The short term preventive actions of peripheral nSMase inhibitors against NGF-induced hypersensitivity along with the acute hyperalgesic actions of exogenous C2-ceramide suggest an involvement of different signaling events, possibly through different sensory neuron sub-types innervating different spinal targets, to provide a long-lasting hyper-responsiveness from NGF (Kobayashi et al., 2012).

PKCs are profoundly important for cellular responses to extracellular stimulation (Gallegos and Newton, 2008; Gould and Newton, 2008; Newton, 2009) where the atypical PKCs seem to have a specialized role in mediating long-term changes (Moscat and Diaz-Meco, 2000; Hirai and Chida, 2003). Recent studies, either using a pseudosubstrate inhibitor targeting all aPKCs, mPSI (ZIP), or specifically knocking down PKM $\zeta$  by siRNA, identified this isozyme of PKC as the critical kinase in the NGF-p75<sup>NTR</sup> pathway driving sensitization of isolated sensory neurons (Zhang et al., 2012). Although the current results do not identify the specific aPKC that is involved, our experiments show that one of these isozymes appears to be essential in the periphery for hypersensitivity mediated through the p75<sup>NTR</sup>.

Atypical PKCs are claimed by some to be essential contributors supporting chronic hyperalgesia after nerve injury (King et al. 2012; see Price and Ghosh, 2013). Recruited by NGF to enhance the excitability of isolated rat sensory neurons (Zhang et al., 2012), PKM $\zeta$  also appears to be essential for the development and persistence of a sensitized state in the dorsal horn (Laferrriere et al., 2011; Marchand et al., 2011; Asiedu et al., 2011). These papers report an elevation of activated and total aPKCs, especially PKM $\zeta$ , in the dorsal horn after inflammation, and a reduction of inflammation-induced hyperalgesia by chemical inhibition of spinal PKM $\zeta$ . The current paper, and related previous *in vitro* studies (Zhang et

al., 2012), suggest that peripheral aPKCs may also contribute to the relatively long lasting changes in hyper-excitability that subserve prolonged hyperalgesia after NGF.

Figure 8 presents a scheme that summarizes this work. Key mediators of the p75<sup>NTR</sup> signaling pathway contribute to mechanical hyperalgesia induced by NGF, which is consistent with their sensitizing effects demonstrated in the isolated nociceptive DRG neurons. However, accumulating evidence on potential cross-regulation and signaling interactions between p75<sup>NTR</sup> and TrkA pathways in nociceptive sensory neurons, e.g., common activation of phosphatidylinositol 3-kinase (PI3K) (reviewed by Huang and Reichardt, 2003), or common up-regulation of substance P synthesis by both pathways (Skoff and Adler, 2006), indicate that signaling from both NGF-binding receptors could be involved in mechanical sensitization. The results reported here do not eliminate a role for the TrkA receptor in acute pain processing, but our findings do establish a greater role for p75<sup>NTR</sup> and its downstream signaling cascade than was previously recognized.

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## Abbreviations

<b>p75<sup>NTR</sup></b>	p75 neurotrophin receptor
<b>nSMase</b>	neutral sphingomyelinase
<b>aPKC</b>	atypical PKC
<b>DMSO</b>	dimethyl sulfoxide
<b>DRG</b>	dorsal root ganglia
<b>MAPK</b>	Mitogen activated protein kinase
<b>COX2</b>	Cyclo-oxygenase 2
<b>PLC</b>	phospholipase C
<b>PGE<sub>2</sub></b>	prostaglandin E2
<b>VFH</b>	von Frey hair
<b>NGF</b>	nerve growth factor
<b>C2-ceramide</b>	N-acetyl sphingosine
<b>GSH</b>	glutathione
<b>PKC<math>\zeta</math>/PKM<math>\zeta</math></b>	protein-kinase C zeta or protein-kinase M zeta
<b>TrkA</b>	tyrosine kinase receptor A
<b>IPSI</b>	ipsilateral paw
<b>CLP</b>	contralateral paw
<b>s.c.</b>	subcutaneously
<b>mPSI</b>	myristoylated pseudosubstrate inhibitor
<b>PBS</b>	phosphate buffered saline
<b>ODN</b>	oligodeoxyribonucleotides

<b>TRPV1</b>	transient receptor potential cation channel subfamily V member 1
<b>AP</b>	action potential
<b>siRNA</b>	small interfering RNA
<b>PI3K</b>	phosphatidylinositol 3-kinase
<b>BDNF</b>	brain derived neurotrophic factor

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### Highlights

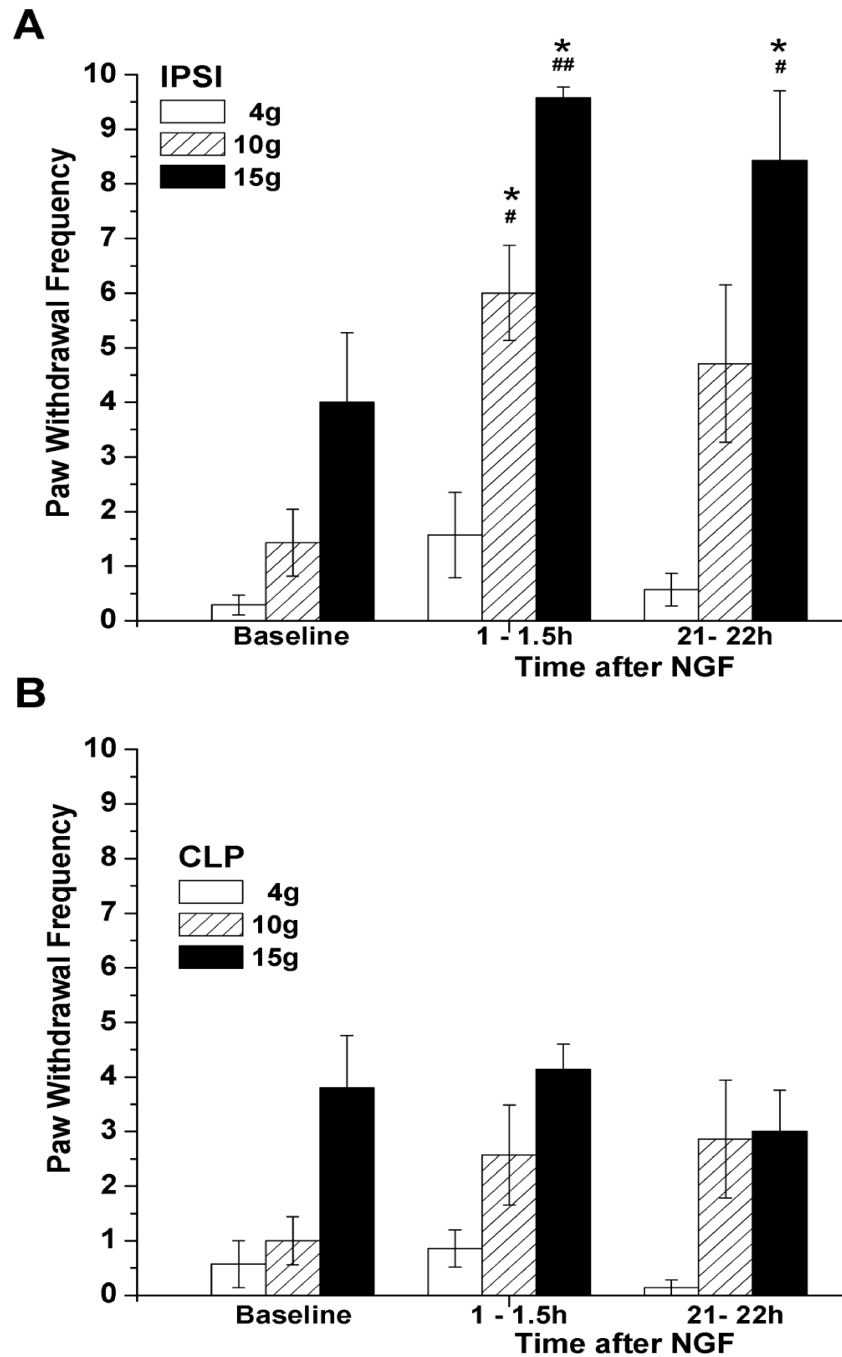
Subcutaneously injected NGF induces tactile hyperalgesia for more than 24 hours.

A p75<sup>NTR</sup> – selective agonist, Pro-NGF, causes the same effect.

A blocking antibody of the p75<sup>NTR</sup> prevents the hyperalgesia from NGF and Pro-NGF.

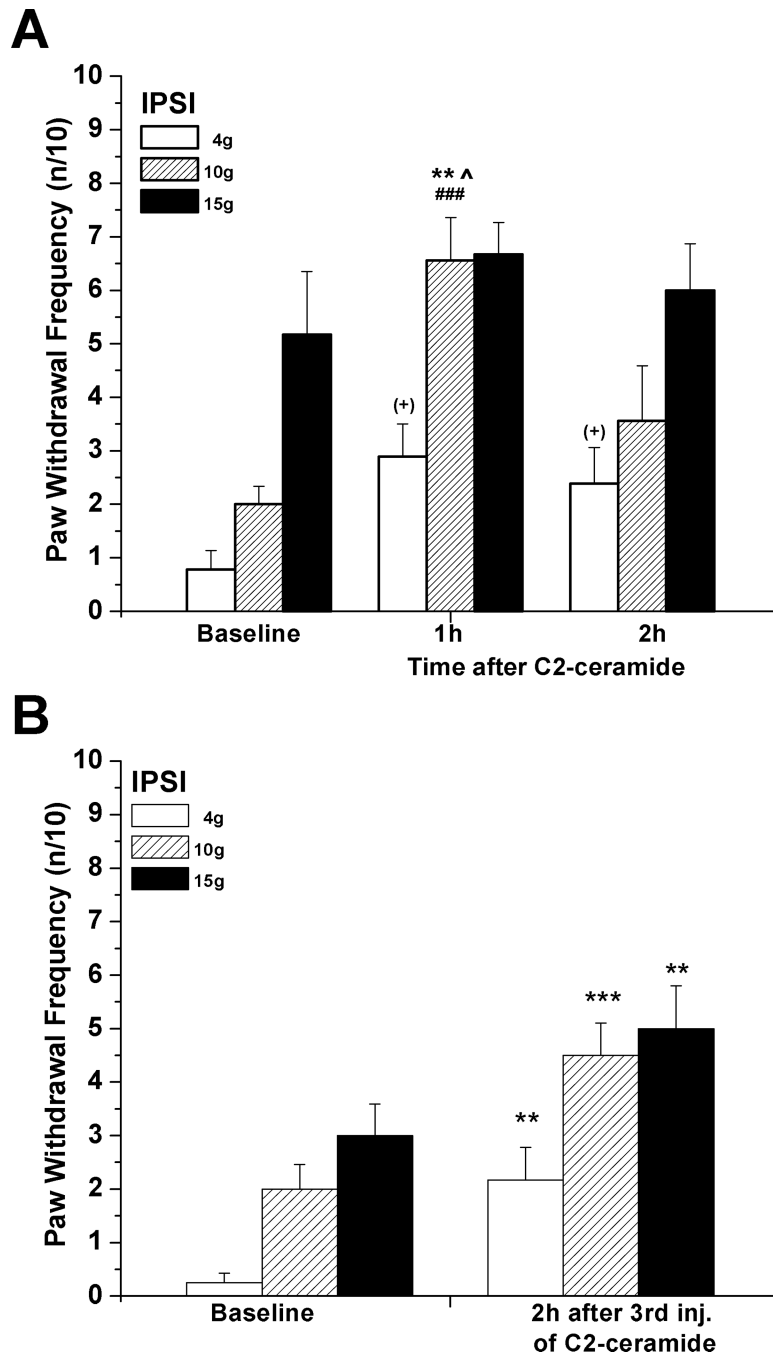
Intermediates of the neutral sphingomyelinase pathway simulate this effect of NGF.

An inhibitor of aPKCs injected beforehand in the same paw suppresses NGF's effects.



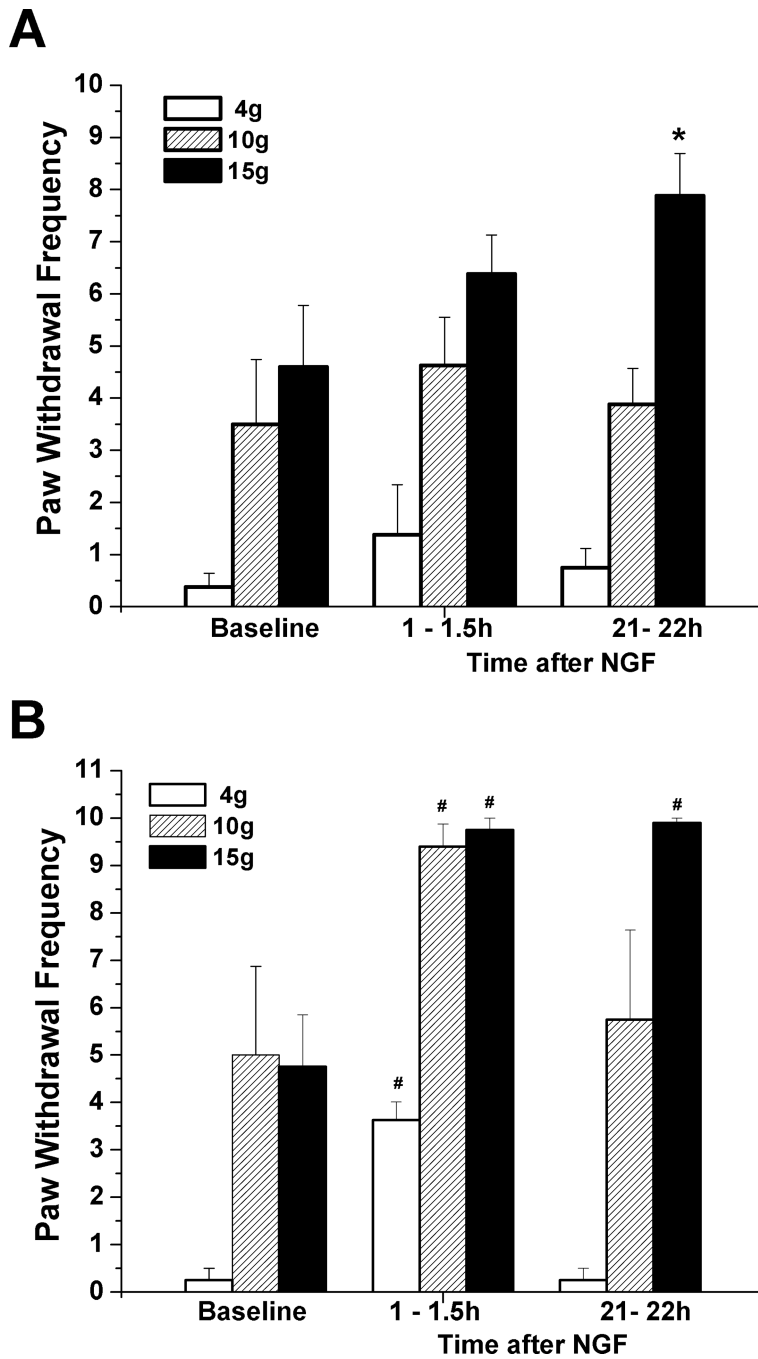
**Figure 1.** Mechanical hyperalgesia induced by NGF. (A) Paw withdrawal frequency in response to stimulation with 10g and 15g VFH was enhanced following subcutaneous injection of 500 ng/10  $\mu$ L NGF into the rat plantar hind paw ( $n=7$ ). \* $P<0.05$  vs. baseline values (Friedman test followed by Dunn's *post hoc* test), # $P<0.05$ , ## $P<0.001$  vs. CLP (two-tailed Mann-Whitney test). (B) No changes in mechanical responsiveness occurred in the contralateral plantar hind paw following local administration of NGF.





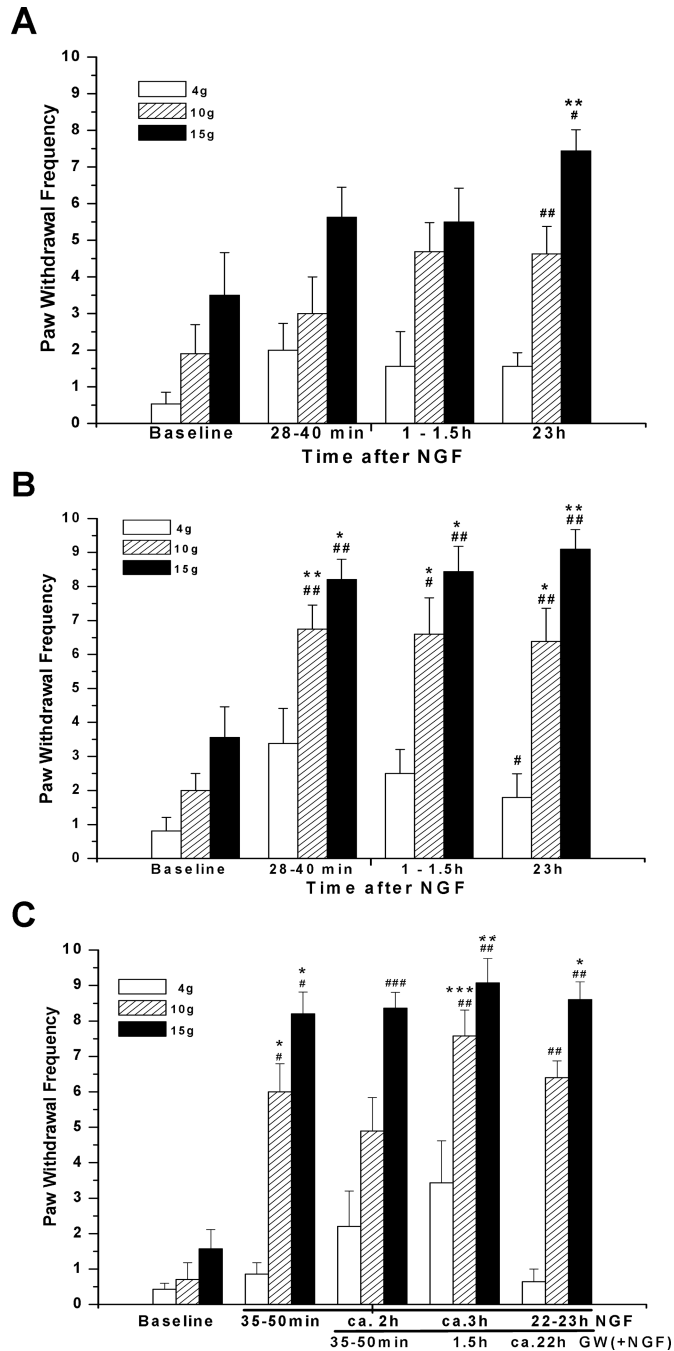
**Figure 2.** Mechanical hyperalgesia induced by local C2-ceramide. **(A)** C2-ceramide (20  $\mu$ g/10  $\mu$ L) injected s.c. into the rat plantar hind paw caused an acute increase in responsiveness to stimulation with low and medium forces (4g, 10g) ( $n=9$ ).  $**P<0.01$  vs. baseline,  $^{\wedge}P<0.05$  vs. responses at 2h (Friedman test followed by Dunn's *post hoc* test),  $###P<0.005$  compared to the contralateral values (two-tailed Mann-Whitney test); for allodynia: (+)  $p=0.0450$  (Friedman test), without significant difference between rank sum means (Dunn's *post hoc* test). **(B)** In comparison, significantly elevated ipsilateral responsiveness to stimulation by all 3 forces was observed at 2h after the 3<sup>rd</sup> of 3 consecutive s.c. injections of C2-ceramide

(20  $\mu\text{g}/10 \mu\text{l}$ ) given every 75min into the same plantar spot ( $n=12$ ).  $**P < 0.01$ ,  $**P < 0.005$  (Wilcoxon matched pairs test, two-tailed).



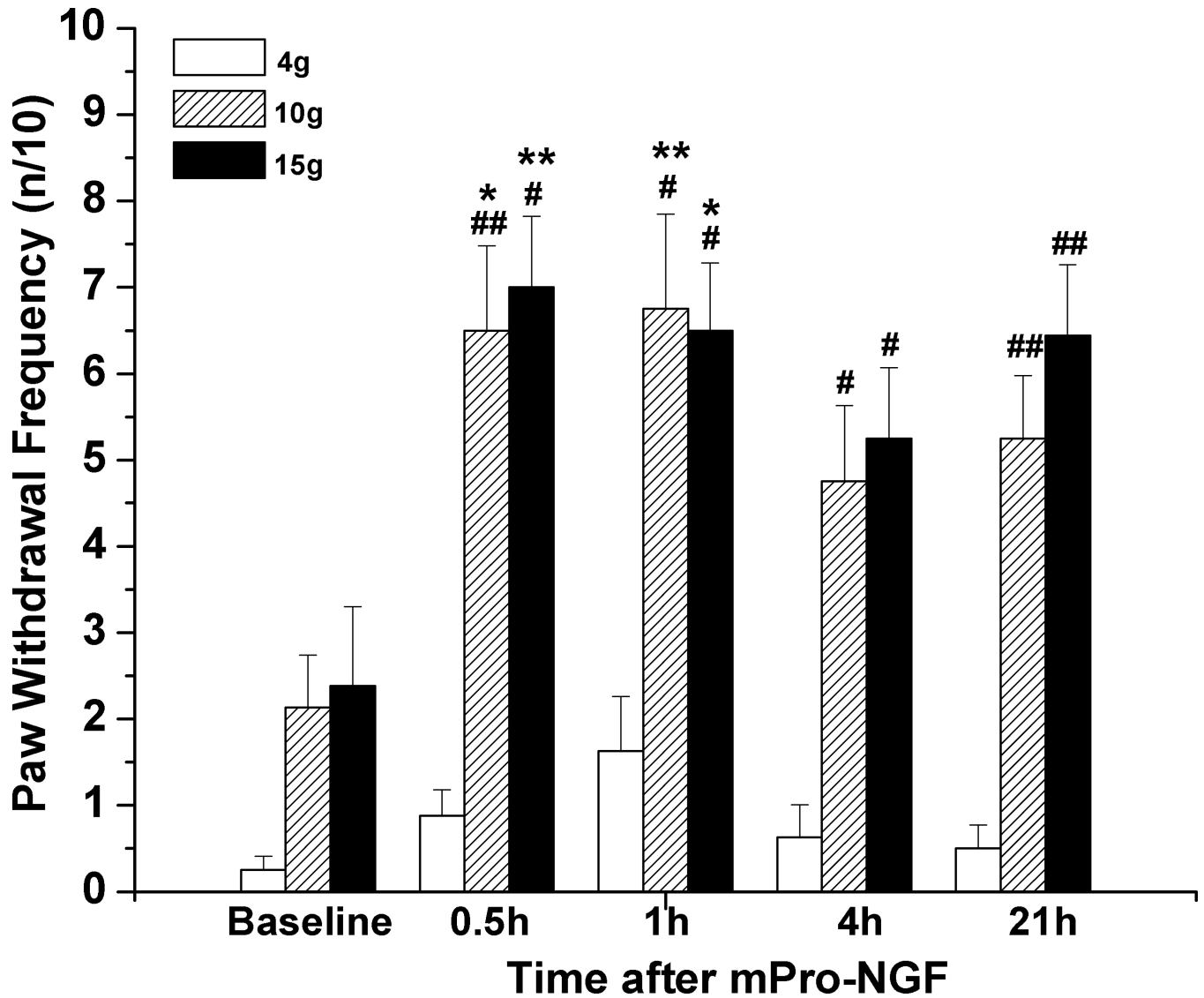
**Figure 3.** Glutathione (GSH), an inhibitor of nSMase, prevented the acute hyperalgesia induced by NGF. **(A)** GSH (1.8  $\mu$ moles/paw total dose), when pre-injected (120 mM, 10  $\mu$ L) 15 min before and then co-injected (60 mM, 10  $\mu$ L) with NGF (500 ng/10  $\mu$ L), prevented hyperalgesia measured at 1–1.5h, but did not that measured on the next day ( $n=8$ ). \* $P<0.05$  vs. baseline IPSI (Friedman test followed by Dunn's *post hoc* test). **(B)** When glutathione (1.8  $\mu$ moles/ paw total dose) was injected (120 mM, 10  $\mu$ L) s.c. intraplantar 1.5h before and then co-injected (60 mM, 10  $\mu$ L) with NGF (500 ng/10  $\mu$ L) the acute stage of hyperalgesia

still occurred ( $n=4$ ,  $p=0.0324$  for both 4g and 15g forces (Friedman test);  $\#P<0.05$  compared to the CLP responses (two-tailed Mann-Whitney test)



**Figure 4.** A short-lasting preventive effect of GW4869 against NGF-induced hyperalgesia. **(A)** A cell-permeant non-competitive, nSMase inhibitor, GW4869 (2 mM, 10  $\mu$ L; dose 20 nmoles/plantar paw), injected 17 min prior to NGF (500 ng/10  $\mu$ L), prevented acute hyperalgesia, at 0.5–1.5h, but not the later hyperalgesia, at 23h after injection ( $n=8$ ). **(B)** Hyperalgesic actions of NGF in controls, where vehicle (DMSO, 10  $\mu$ L) injection preceded NGF ( $n=8$ ). **(C)** GW4869 (2 mM, 10  $\mu$ L; dose 20 nmoles/plantar paw) was ineffective against NGF-induced hyperalgesia when it was injected 1h after NGF (500 ng/10  $\mu$ L) ( $n=7$ ). Note significantly increased responses at 35–50min after NGF (injection into naïve paw), at 2h

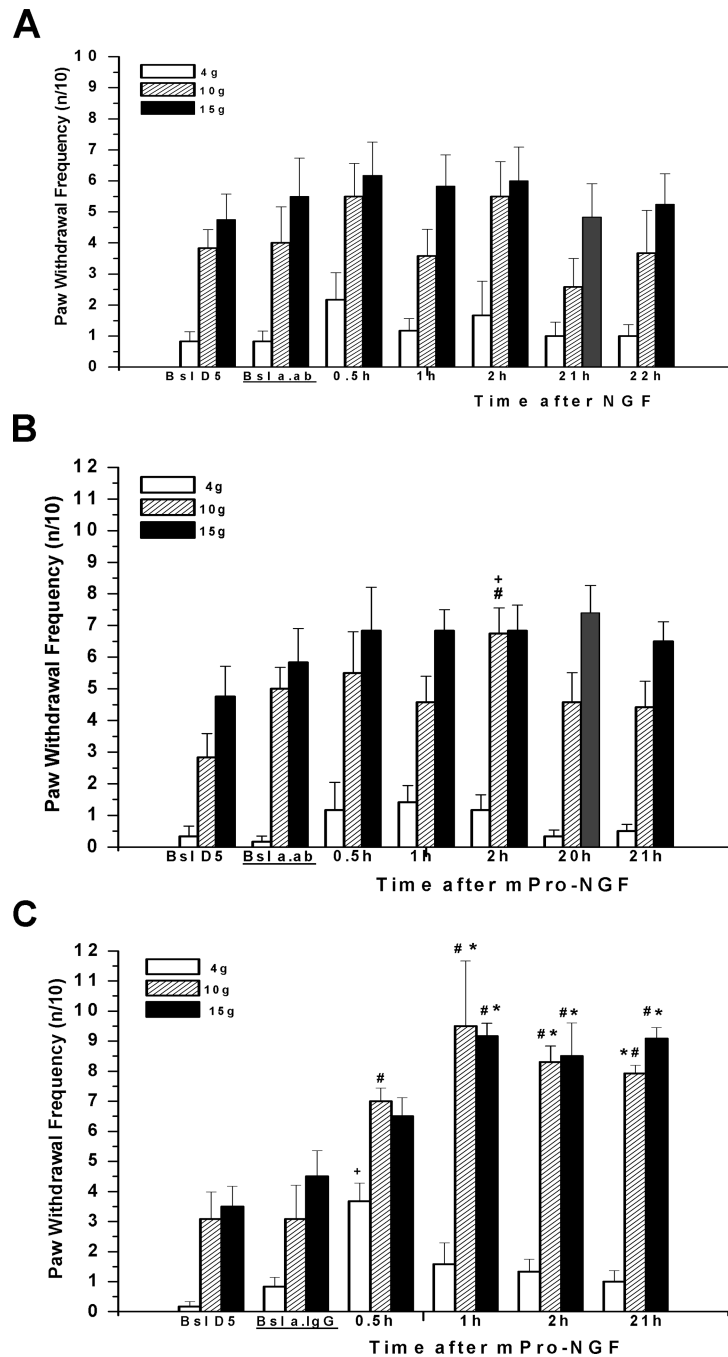
(=35–50min post GW4869; for 15g as compared with CLP, Mann-Whitney test), at 3h (=1.5h post GW4869), and at 22–23h (=ca. 22h post GW4869). \* $P < 0.05$ , \*\* $P < 0.01$  vs. baseline (Friedman test followed by Dunn's *post hoc* test), # $P < 0.05$ , ## $P < 0.005$ , ### $P < 0.001$  compared to the CLP values (two-tailed Mann-Whitney test).



**Figure 5.**

Mechanical ipsilateral hyperalgesia is induced by mutated, protease-resistant Pro-NGF. Paw withdrawal frequency in response to stimulation with 10g and 15g VFH was enhanced following s.c. injection of mPro-NGF ( 500 ng/10  $\mu$ L) into the rat plantar hind paw ( $n=8$ ).

\* $P<0.05$ , \*\* $P<0.01$  vs. baseline values (Friedman test followed by Dunn's *post hoc* test), # $P<0.05$ , ## $P<0.01$  vs. baseline values (Wilcoxon matched pairs test, two-tailed).

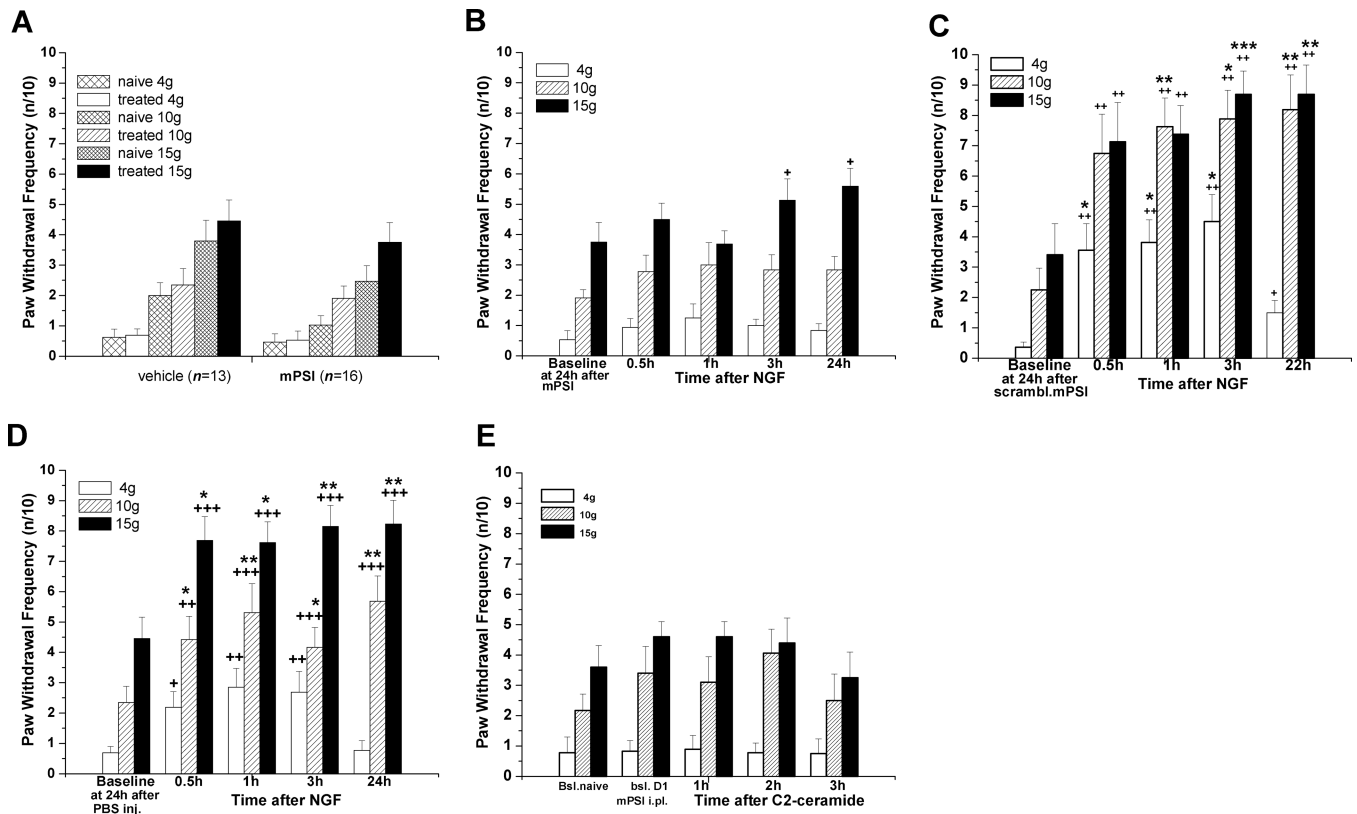


**Figure 6.**

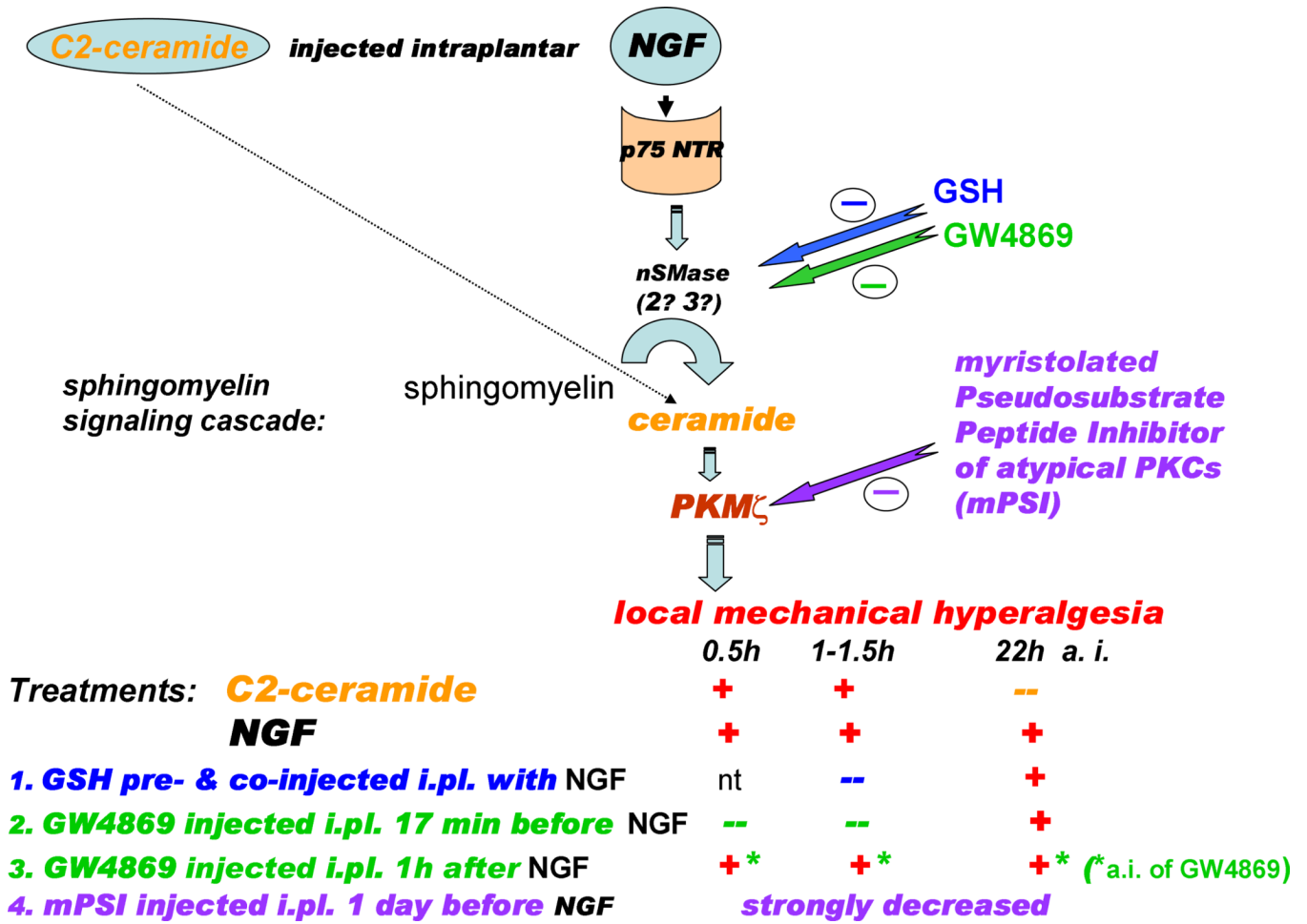
A polyclonal antibody to p75<sup>NTR</sup> prevented mechanical hyperalgesia induced by NGF and mProNGF. (A) When pre-injected (20  $\mu$ L) into the paw 4h before NGF (500 ng/10  $\mu$ L), the antibody prevented any significant hyperalgesia at 0.5h-21h ( $n=6$ ). (B) A similar inhibition occurred when the antibody was injected 4h before mPro-NGF (500 ng/10  $\mu$ L) ( $n=6$ ). Significant elevation of response was found only for the 10g force at 2h after mPro-NGF injection. # $P<0.05$  vs. baseline-after-antibody values (Wilcoxon matched pairs test, two-tailed), and + $P<0.05$  vs. baseline-after-antibody values and vs. 21h values for 3 groups comparison: Bsl a.a.b., 2h, 21h (Friedman test followed by Dunn's *post hoc* test). Bsl a.a.b. =



2.5h after ab.p75 injection. (C) In controls, where IgG (20  $\mu$ L) from naïve serum was injected 4h prior to mPro-NGF, hyperalgesia was fully present( $n=6$ ; compare with Fig 5). Bsl a.IgG=2.5h after IgG injection.

**Figure 7.**

Local pre-treatment with myristoylated pseudosubstrate inhibitor of atypical PKCs decreased hyperalgesia from NGF and C2-ceramide. For all panels the inhibitor (40  $\mu$ g /20  $\mu$ l) was injected subcutaneously into the plantar hind paw 24h before the intraplantar injection of the algogens. (A) mPSI alone did not change the plantar hind paw responsiveness to mechanical stimulation, when tested at 24h after injection into naïve rats. (B) NGF injected into the plantar area after mPSI induced a much smaller than usual hyperalgesia ( $n=16$ ): significant changes in responses were found only for 15g force ( $p=0.0489$ , Friedman Test for 5 groups; no significance by Dunn's *post hoc* test). However, even for 15g,  $P>0.05$  was found when the test was applied to 4 same day-groups, *excluding* 24h time point responses. Analysis by Mann Whitney test revealed a significant difference for comparisons with corresponding baseline values only for 4g and 15g, at 3h and 24h time points, respectively. (C) Injection into the plantar hind paw of scrambled mPSI (40  $\mu$ g /20  $\mu$ l), which lacks inhibitory action on aPKCs, 24h before the intraplantar injection of NGF, neither prevented, nor decreased hyperalgesia from NGF (500 ng/10  $\mu$ l) ( $n=8$ ). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  vs. baseline (Friedman test, 5 groups, followed by Dunn's *post hoc* test); # $P<0.05$ , ## $P<0.01$  vs. baseline (two-tailed Wilcoxon test). (D) Vehicle control: pronounced hyperalgesia at 0.5–24h after NGF injection was observed in rats pre-injected with vehicle (PBS) into the same plantar area 24h before ( $n=13$ ). \* $P<0.05$ , \*\* $P<0.01$  vs. baseline (Friedman test followed by Dunn's *post hoc* test). Some enhancement was revealed for responses to 4 g  $p=0.0032$  (Friedman test, 5 groups), however, no significance was found by Dunn's *post hoc* test. + $P<0.05$ , ++ $P<0.01$  compared to baseline values (two-tailed Wilcoxon test). (E) mPSI (40ug/20uL) injected i.pl. prevented mechanical hyperalgesia induced by C2-ceramide (20ug/10uL) ( $n=9$ ).



**Figure 8.**

The intracellular signaling pathway downstream of p75<sup>NTR</sup> which leads to mechanical hyperalgesia from NGF in rat plantar hind paw, and a summary of the antihyperalgesic effects of the treatments used in the current work. NGF induced a long-lasting hyperalgesia (up to 24h), while the effect from a single injection of C2-ceramide was brief (<2h). Both inhibitors of nSMase, GSH (at a high total dose) and GW4869, prevented the acute hyperalgesia from NGF. A *post facto* application of GW4869, however, failed to reverse NGF-induced hyperalgesia. Intraplantar pre-treatment with myristoylated pseudosubstrate inhibitor of aPKCs, mPSI, (1 day before) strongly decreased the hyperalgesic actions of NGF. Key: (+) - hyperalgesia was observed; (--) - hyperalgesia was not observed; nt – not tested.