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Src family kinases maintain latent sensitization, a model of inflammatory and neuropathic pain

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Abstract

Latent sensitization is a long-term model of chronic pain in which pain sensitivity is continuously suppressed by opioid receptors. This is demonstrated by the induction of mechanical allodynia by opioid antagonists, something that occurs in animals with latent sensitization but not in normal animals. Different intracellular signals may mediate the initiation, maintenance and expression of latent sensitization. As a criterion for the involvement of a signal in the maintenance of latent sensitization, we proposed that inhibitors of that signal should permanently eliminate the induction of allodynia by an opioid antagonist. Since latent sensitization requires NMDA receptors, which are potentiated by Src family kinases (SFKs), we hypothesized that SFKs maintain latent sensitization. We tested this hypothesis in rats with latent sensitization induced in one hindpaw by the inflammatory agent complete Freund's adjuvant (CFA) or by spared nerve injury. After von Frey measures of mechanical allodynia returned to baseline, vehicle or the SFK inhibitor PP2 were injected intrathecally. The opioid antagonist naltrexone injected intrathecally 15 min later produced allodynia in control rats but not in rats injected with PP2. PP2 or vehicle were injected daily for two more days and naltrexone was injected intrathecally five days later. Again, naltrexone induced allodynia in the control rats but not in the rats injected with PP2. Results were similar when latent sensitization was induced either with CFA and spared nerve injury. We concluded that a SFK, probably Fyn, maintains latent sensitization induced by inflammation or nerve injury.

Introduction

Chronic pain is a dysregulation of pain processing mechanisms that causes them to stay sensitized even after the healing of the initial injury. Evidence for this idea is provided by the latent sensitization model of chronic pain, which shows that certain injuries put the organism in a long-term state in which pain sensitivity is present but is continuously suppressed by opioid and α_{2A} adrenergic receptors (Taylor & Corder, 2014; Marvizon *et al.*, 2015). The simultaneous presence of pain sensitization and its suppression is evidenced by the ability of antagonists of μ, δ and κ opioid receptors and α_{2A} adrenergic receptors to induce episodes of mechanical allodynia and thermal hyperalgesia (Campillo *et al.*, 2011; Corder *et al.*, 2013; Walwyn *et al.*, 2016). Moreover, mice with genetic deletion of μ-opioid receptors, either global (Walwyn *et al.*, 2016) or restricted to nociceptive afferents (Severino *et al.*, 2018), are unable to fully recover from hyperalgesia and do not show induction of allodynia by an opioid antagonist.

There are different forms of latent sensitization depending on its triggering injury: inflammation (Corder *et al.*, 2013; Walwyn *et al.*, 2016), nerve injury (Solway *et al.*, 2011), plantar incision (Rivat *et al.*, 2009; Campillo *et al.*, 2011) or opioids (Rivat *et al.*, 2007; Rivat *et al.*, 2009). However, it remains to be determined whether they have different mechanisms.

We propose that different intracellular signals mediate the initiation, expression and maintenance of latent sensitization. Initiation signals would be those elicited by the triggering injury that lead to the establishment of latent sensitization. Maintenance signals would be those that ensure that the sensitized state endures over time. Expression signals would be those required for the occurrence of hyperalgesia when they are recruited by the maintenance signals. Drugs that inhibit the expression latent sensitization would eliminate the allodynia induced by opioid antagonists only while they are present, whereas drugs that inhibit its maintenance should eliminate antagonist-induced allodynia permanently. It is important to find drugs that inhibit the maintenance of latent sensitization because they may cure chronic pain by reversing pain mechanisms back to the normal state.

NMDA receptors (Celerier *et al.*, 2000; Corder *et al.*, 2013), adenylyl cyclase (Corder *et al.*, 2013) and protein kinase A (Fu *et al.*, 2019) have been found to mediate latent sensitization, but it is unclear whether they mediate its expression or its maintenance. In addition, a Src family kinase (SFK) acting in conjunction with mitogen-activated protein kinase kinases 1 and 2 (MEK1/2) has been proposed to mediate latent sensitization (Araldi *et al.*, 2017; Araldi *et al.*,

2018). Since NMDA receptors in the dorsal horn are potentiated by SFK phosphorylation (Guo *et al.*, 2002; Abe *et al.*, 2005; Xu *et al.*, 2006; Chen *et al.*, 2010; Geng *et al.*, 2010; Chen *et al.*, 2014), we hypothesized that SFKs mediate the maintenance of latent sensitization. We tested this hypothesis by determining whether the SFK inhibitor PP2 reverses the allodynia produced by the opioid antagonist naltrexone (NTX) in latent sensitization induced by inflammation and by nerve injury.

Material and methods

Animals

Animals were male adult (2-4 months old) Sprague-Dawley rats (Envigo, Indianapolis, IN). A total of 30 rats were used in the study. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to NIH guidelines. Efforts were made to minimize the number of animals used and their suffering.

Chemicals

Naltrexone (NTX) and PP2 were purchased from Tocris Bioscience (Minneapolis, MN). CFA and other reagents were obtained from Sigma-Aldrich (St. Louis, MO). PP2 was dissolved at 10 mM in DMSO and then diluted to 30 µM in saline (final DMSO was 0.3%). NTX was dissolved in saline.

Complete Freund's Adjuvant (CFA) injections

Undiluted CFA was injected subcutaneously in a volume of 50 µl into one hindpaw using a 50 µl Hamilton syringe and a 26 gauge needle. The needle was inserted at an oblique angle from the heel in the middle of the paw, near the base of the third toe. It was held in place for 15 s and then gently withdrawn.

Spared nerve injury (SNI)

A variation of the SNI model was performed as described (Solway *et al.*, 2011). Rats were anaesthetized with isoflurane. At the dorsal upper thigh level, the skin was cut and the muscle separated to expose the trifurcation of the sciatic nerve. The common peroneal and sural nerves were ligated and then cut on both sides of the suture to remove 2-3 mm of each nerve, leaving

the tibial nerve intact. The muscle fascia were sutured in a simple continuous pattern. The cut in the skin was closed using a subcuticular intradermal pattern. Rats were given daily injections of the analgesic carprofen for 3 days. The presence of motor weakness or signs of paresis was established as criterion for immediate euthanasia, but this did not occur in any of the rats.

Intrathecal (i.th.) cannulation and injections

Rats were implanted with chronic intrathecal catheters under isoflurane (2–4%) anesthesia as described (Storkson *et al.*, 1996; Walwyn *et al.*, 2016). After cutting the skin and muscle, a 20G needle was inserted between the L5 and L6 vertebrae to puncture the dura mater. The needle was removed and the catheter (20 mm of PE-5 tube heat-fused to 150 mm of PE-10 tube) was inserted into the subdural space and pushed rostrally to terminate over L5-L6. The PE-10 end of the catheter was tunneled under the skin and externalized over the head. The skin was sutured and the catheter was flushed with 10 µl saline and sealed. Rats were housed separately and used 7 days after surgery. Intrathecal injection volume was 10 µl of injectate plus 10 µl saline flush. Solutions are preloaded into a PE-10 tube and delivered within 1 min. The position of the catheter was examined postmortem. Termination of the catheter inside the spinal cord or its occlusion were exclusion criteria, but this was not observed in any of the rats. Three rats lost the catheter after day 28 and were excluded from further measures.

Measures of mechanical allodynia

Mechanical allodynia was measured using von Frey filaments (Touch-Test) by the two-out-of-three method as described (Kingery *et al.*, 2000; Michot *et al.*, 2012; Jarahi *et al.*, 2014; Walwyn *et al.*, 2016; Chen *et al.*, 2018). Rats were habituated for periods of 30 min for 3 days to acrylic enclosures on an elevated metal grid (IITC Life Science Inc., CA). A series of von Frey filaments were applied in ascending order to the plantar surface of the hindpaw for a maximum of 3 s. A withdrawal response was counted only if the hindpaw was completely removed from the platform. Each filament was applied three times, and the minimal value that caused at least two responses was recorded as the paw withdrawal threshold (PWT). The 15 g filament was taken as cut-off threshold.

Timeline

The timeline of the experiments is illustrated in Fig. 1. After habituating the rats to the von

Frey enclosures for 3 days, i.th. catheters were surgically implanted on day -7. Baseline von Frey measures were taken on days -4 and 0. After the measure on day 0, rats received CFA or SNI in one hindpaw. The mechanical allodynia produced by CFA or SNI was followed with von Frey measures on days 3 and 21. On day 28, a baseline von Frey measure was taken and the rats received i.th. injections of vehicle or PP2, and i.th. NTX 15 min later. For the following 2 h, von Frey measures were taken at 15, 30, 60, 120 min after NTX. The i.th. injections of vehicle or PP2 were repeated on days 29 and 30. On day 35, a baseline von Frey measure was taken, NTX was injected i.th., and von Frey measures were taken for 2h.

Sample size, randomization and blinding

The target sample size was n = 7-8 rats per group based on a power analysis, but 3 rats in the SNI-control group were excluded after day 28 because they had lost the i.th. catheter. Rats were randomly assigned to treatment and drug. For blinding, solutions of vehicle (for controls) and PP2 were prepared and randomly coded by JCM. WC injected the coded drugs i.th. and performed the von Frey measures. JCM analyzed the data and matched the codes with the drugs.

Data analysis

Data were analyzed using Prism 8.3 (GraphPad Software, San Diego, CA) and expressed as mean ± standard error of the mean. Statistical significance was set at 0.05. Statistical analyses consisted of repeated measures (by both factors) two-way ANOVA followed by Holm-Sidak's post-hoc tests.

Results

Latent sensitization induced by CFA

To determine if SFKs mediate the expression and maintenance of latent sensitization, we studied the short-term and long-term effects of the SFK inhibitor PP2 on the mechanical allodynia induced by NTX in rats with latent sensitization. One form of latent sensitization was induced by injecting 50 µl CFA in one hindpaw, a model of inflammatory chronic pain. CFA produced mechanical allodynia in the ipsilateral but no the contralateral hindpaw. Ipsilateral allodynia was strong at day 3 and decreased to near baseline values by day 28 (Fig. 2A, D). On day 28, rats were injected i.th. with vehicle (0.3% DMSO in saline) or PP2 (0.3 nmol) followed 15 min later by i.th. NTX (2.6 nmol). Vehicle or PP2 were injected daily for two more days. On

day 35, rats received a second i.th. injection of NTX. In the control group, the injections of NTX on day 28 (Fig. 2B) and on day 35 (Fig. 2C) induced bilateral mechanical allodynia in the hindpaws, which peaked at 15 min and lasted 2 h or more. In the rats injected with PP2, NTX-induced allodynia was eliminated both on day 28 (Fig. 2E) and on day 35 (Fig. 2F).

Results were analyzed by 2-way ANOVA with repeated measures for the two variables: time and hindpaw side (Table 1). Note that the variable 'time' indicates whether there was a significant effect of CFA (rows A and D) or NTX. The effect of NTX over time was significant in control rats (rows B, C) but not in rats injected with PP2 (rows E, F). The interaction of the variables 'time' and 'side' ('time x side') was significant after the injection of CFA but not after the injections of NTX, indicating that CFA produced allodynia ipsilaterally while NTX produced allodynia bilaterally.

Latent sensitization induced by SNI

We also studied the effect of PP2 on the latent sensitization induced by SNI, a model of neuropathic pain. SNI was performed by cutting the common peroneal and sural branches of the sciatic nerve (Solway *et al.*, 2011) instead of the common peroneal and tibial nerves (Marvizon *et al.*, 2019), because this allows a faster return to baseline responses. Indeed, mechanical allodynia produced by this SNI was strong at day 3 but had returned to baseline by day 28 (Fig. 3A, D). Like in the experiment in Fig. 2, on day 28 rats were injected i.th. with vehicle (0.3% DMSO in saline) or PP2 (0.3 nmol) and 15 min later with NTX (2.6 nmol). Vehicle or PP2 were injected daily for two more days, and on day 35 rats received a second injection of NTX. Both injections of NTX produced strong allodynia in the control rats (Fig. 3B, C) but no allodynia in the rats injected with PP2 (Fig. 3E, F).

Table 2 shows the results of a 2-way ANOVA, repeated measures by both variables, of the data in Fig. 3. SNI produced a significant effect over time (rows A, D) and a significant interaction 'time x side', showing that SNI produced ipsilateral allodynia. NTX produced a significant effect over time in the control rats (rows B, C). In the rats injected with PP2, the effect of NTX was also significant on day 28 (row E), but this was due to a decrease in allodynia at the 120 min time point (Fig. 3E) and not to the expected increase in allodynia at 15-30 min. On day 35 in the PP2-injected rats, the effect of NTX was marginally significant (row F) probably due to a small increase in allodynia in the ipsilateral paw at 60 min and a small decrease in allodynia at 120 min in the contralateral paw (Fig. 3F). However, these effects were

not significant in the Holm-Sidak's post-hoc test. Overall, the allodynia produced by NTX in rats with SNI-induced latent sensitization was eliminated by PP2.

Discussion

This study shows that the SFK inhibitor PP2 eliminated the mechanical allodynia produced by NTX in rats with latent sensitization induced either by CFA or SNI. A single i.th. injection of PP2 eliminated the effect of NTX given shortly afterwards, whereas two more daily injections of PP2 eliminated the allodynia induced by NTX given five days after the last injection. This indicates that a SFK is involved in the maintenance of latent sensitization, so that when the SFK is inhibited latent sensitization disappears.

One possible mechanism by which a SFK could maintain latent sensitization is through the activating phosphorylation of NMDA receptors, which are required for latent sensitization (Celerier *et al.*, 2000; Laulin *et al.*, 2002; Corder *et al.*, 2013). Central sensitization, the main spinal mechanism of chronic pain, is mediated by NMDA receptors present in dorsal horn neurons (Aicher *et al.*, 1997; Nagy *et al.*, 2004) and in primary afferent terminals (Marvizon *et al.*, 2002; Li *et al.*, 2004; McRoberts *et al.*, 2007; McRoberts *et al.*, 2011). These NMDA receptors are potentiated by tyrosine phosphorylation of their NR2B subunit (Guo *et al.*, 2002; Zhang *et al.*, 2008; Katano *et al.*, 2011; Li *et al.*, 2011) by the SFK Fyn (Abe *et al.*, 2005). Upstream, Fyn can be activated by BDNF and its trkB receptor through two pathways (Xu *et al.*, 2006; Geng *et al.*, 2010; Hildebrand *et al.*, 2016; Marcos *et al.*, 2017):

1) trkB → phosphoinositide 3-kinase → protein kinase B → Fyn
2) trkB → Raf → MEK1/2 → ERK1 → Fyn

The NMDA receptors in primary afferent terminals that induce substance P release also require SKF phosphorylation, which is driven by BDNF (Chen *et al.*, 2010; Chen *et al.*, 2014). Substance P activates neurokinin 1 receptors in lamina I neurons, which project to the parabrachial nucleus (Todd *et al.*, 2000; Todd *et al.*, 2002) and are required for latent sensitization induced by surgical incision and opioids (Rivat *et al.*, 2009; Arora *et al.*, 2016).

Our results with PP2 are consistent with those of Araldi et al. (2017; 2018), who found that in rats with CFA-induced latent sensitization the SFK inhibitor SU-6656 (27 nmol i.th.) and the MEK1/2 inhibitor U0126 (24 nmol i.th.) given together eliminated the NTX-induced allodynia. However, they reported that SU-6656 alone did not have an effect, while we found that PP2

produced an effect at a dose 100 times lower than SU-6656. We attribute this discrepancy to the higher affinity of PP2 for the SFK Fyn. If Fyn gets activated by pathway 2, the complete inhibition of Fyn by PP2 may make unnecessary the additional inhibition of MEK1/2 by U0126.

It is widely recognized that BDNF mediates neuropathic pain by downregulating the KCC2 cotransporter, which increases intracellular Cl^- and thus cancels the inhibition by $GABA_A$ and glycine receptors of excitatory interneurons (Coull *et al.*, 2005; Gagnon *et al.*, 2013). Inflammatory chronic pain appears to also be mediated by BDNF (Lin *et al.*, 2011; Lalisse *et al.*, 2018), although it is still unclear if different mechanisms mediate inflammatory and neuropathic pain (Peirs *et al.*, 2015; Peirs & Seal, 2016). Our results show that a SFK is involved in the maintenance of latent sensitization induced by both inflammation and nerve injury. It will be important to elucidate if BDNF is involved in these two forms of latent sensitization and whether they have the same maintenance mechanisms.

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Conflict of interest

The authors declare no competing financial interests or other conflicts of interest.

Authors contributions

W. Chen performed the surgeries, obtained behavioral measures and contributed to the design of the study. J.C. Marvizón designed the study, analyzed the data, made the figures and wrote the manuscript.

Data accessibility

Data will be archived in Figshare.

Abbreviations

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
CFA	complete Freund's adjuvant
ERK1	extracellular signal-regulated kinase 1
Fig.	Figure
i.th.	intrathecal
MEK1/2	mitogen-activated protein kinase kinases 1 and 2
NTX	naltrexone
NMDA	N-methyl-D-aspartate
PP2	3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine
PWT	paw withdrawal threshold
SEM	standard error of the mean

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Tables

Table 1 - Repeated measures two-way ANOVA of CFA-induced LS data in Fig. 2

Fig. 2 panel	*n*	Data analyzed	time	side	time x side
A	8	CFA in the paw	$p = 0.0006$ $F(5, 35) = 5.7$	$p = 0.0386$ $F(1, 16) = 45$	$p < 0.0001$ $F(5, 35) = 11$
B	8	day 28: vehicle i.th., then NTX i.th.	$p < 0.0001$ $F(4, 28) = 10.3$	$p = 0.203$ $F(1, 7) = 1.97$	$p = 0.305$ $F(4, 28) = 1.27$
C	8	day 35 NTX i.th.	$p < 0.0001$ $F(4, 28) = 18$	$p = 0.051$ $F(1, 7) = 5.5$	$p = 0.273$ $F(4, 28) = 1.36$
D	7	CFA in the paw	$p = 0.0001$ $F(5, 30) = 7.27$	$p = 0.073$ $F(1, 6) = 4.7$	$p < 0.0001$ $F(5, 30) = 9.0$
E	7	day 28: PP2 i.th., then NTX i.th.	$p = 0.312$ $F(4, 24) = 1.26$	$p = 0.0108$ $F(1, 6) = 13$	$p = 0.844$ $F(4, 24) = 0.34$
F	7	day 35 NTX i.th.	$p = 0.385$ $F(4, 24) = 1.08$	$p = 0.168$ $F(1, 6) = 2.4$	$p = 0.0269$ $F(4, 24) = 3.31$

Data in each of the panels of Fig. 2 were analyzed by repeated measures two-way ANOVA, matching by both factors: time of von Frey measures after CFA or NTX and side (ipsilateral vs. contralateral hindpaw). Panels A-C and D-F represent a group of rats each. Sample sizes (*n*) are number of rats in each experiment.

Table 2 - Repeated measures two-way ANOVA of SNI-induced LS data in Fig. 3

Fig. 3 panel	*n*	Data analyzed	time	side	time x side
A	8	SNI	$p = 0.0026$ $F(5, 35) = 4.58$	$p = 0.0017$ $F(1, 7) = 24$	$p < 0.0001$ $F(5, 29) = 8.3$
B	8	day 28: vehicle i.th., then NTX i.th.	$p < 0.0001$ $F(4, 28) = 16$	$p = 0.0085$ $F(1, 7) = 13$	$p = 0.0042$ $F(4, 28) = 4.8$
C	5	day 35 NTX i.th.	$p = 0.0039$ $F(4, 16) = 6.0$	$p = 0.063$ $F(1, 4) = 27$	$p = 0.403$ $F(4, 16) = 1.07$
D	7	SNI	$p < 0.0001$ $F(5, 30) = 12$	$p = 0.013$ $F(1, 6) = 12$	$p = 0.0028$ $F(5, 30) = 4.7$
E	7	day 28: PP2 i.th., then NTX i.th.	$p = 0.0027$ $F(4, 24) = 5.5$	$p = 0.901$ $F(1, 6) = 0.016$	$p = 0.029$ $F(4, 24) = 3.2$
F	7	day 35 NTX i.th.	$p = 0.048$ $F(4, 24) = 2.8$	$p = 0.0023$ $F(1, 6) = 26$	$p = 0.723$ $F(4, 24) =0.52$

Data in each of the panels of Fig. 3 were analyzed by repeated measures two-way ANOVA, matching by both factors: time of von Frey measures after SNI or NTX and side (ipsilateral vs. contralateral). Panels A-C and D-F represent a group of rats each. Panel A was analyzed using mixed-effects analysis instead of ANOVA because a few baseline data were missing. Sample sizes (*n*) are number of rats in each experiment.

Figures and captions

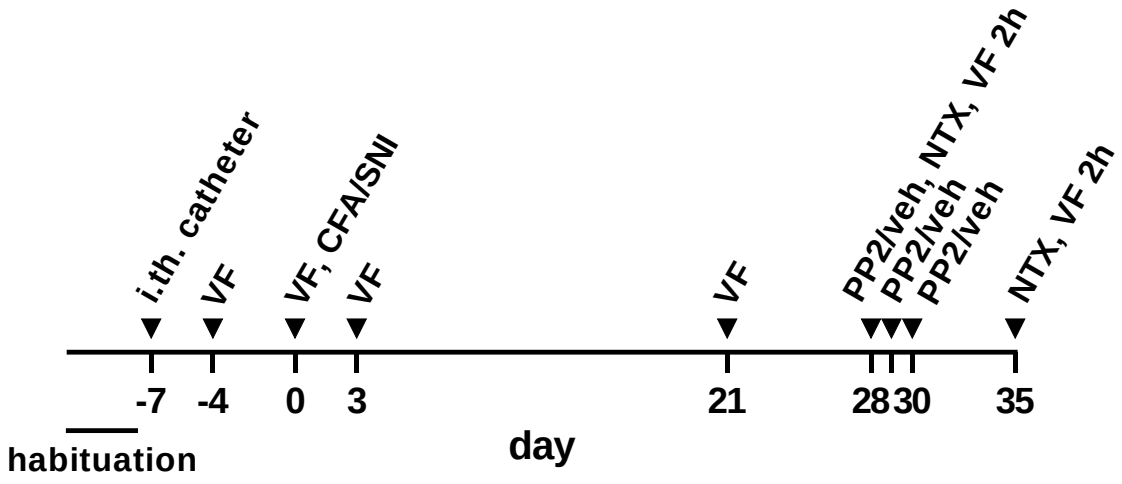

Figure 1. Timeline of the experiments: habituation - 30 min daily habituation to the von Frey cages for 3 days; i.th. catheter - intrathecal catheter implantation; VF - von Frey measures; VF 2h - von Frey measures for 2 h; CFA - 50 µl s.c. complete Freund's adjuvant injected in the paw; SNI - spared nerve injury; PP2 - 0.3 nmol PP2 i.th. in 10 µl; veh - 10 µl vehicle i.th.; NTX - 2.6 nmol naltrexone i.th. in 10 µl. CFA, SNI, vehicle or PP2 were given to four different groups of rats.

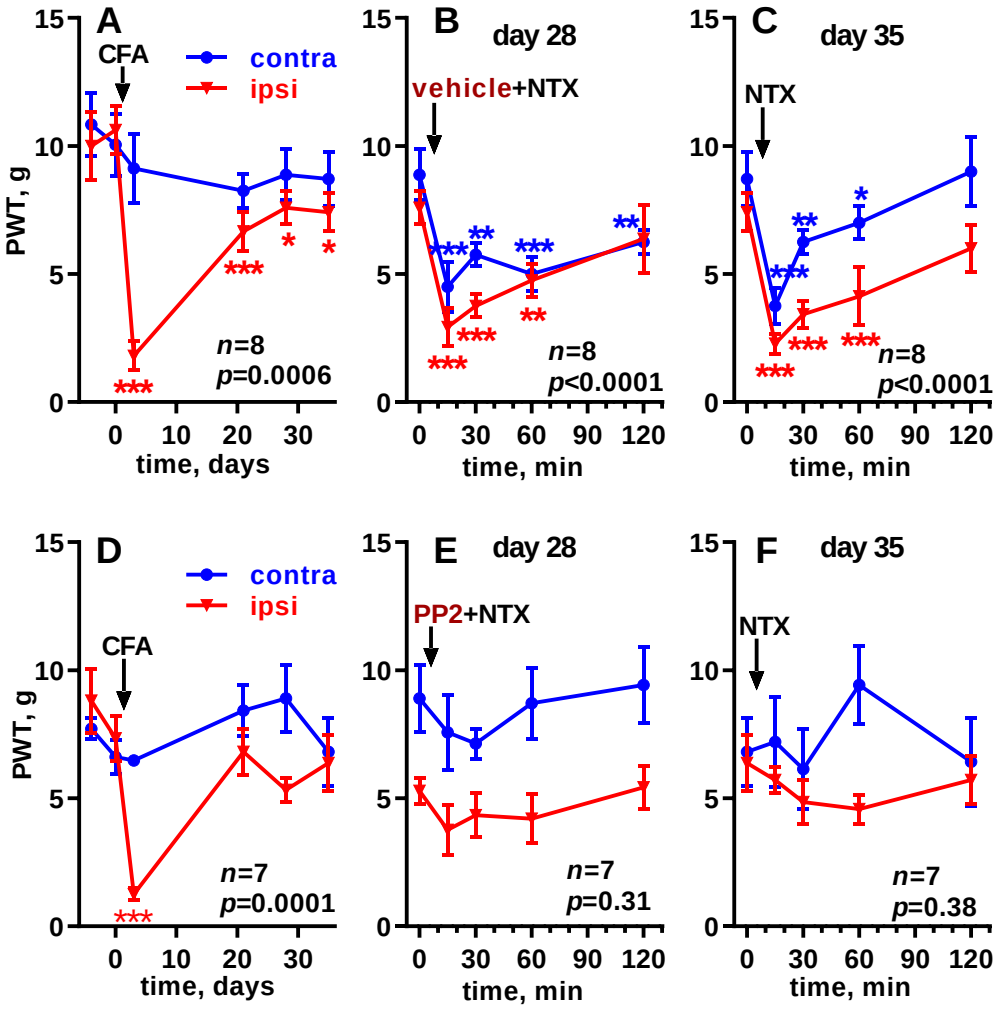

Figure 2. Effect of PP2 on NTX-induced allodynia in rats with CFA-induced LS. A, D: Rats (*n*=8 for A-C, *n*=7 for D-F) received 50 µl CFA in one hindpaw. Paw withdrawal thresholds (PWT, von Frey) were measured in the ipsilateral ('ipsi') and contralateral ('contra') hindpaws. **B, E:** On day 28, rats received i.th. injections of 10 µl vehicle (0.3% DMSO in saline, B) or PP2 (0.3 nmol in 0.3% DMSO, E), and 15 min later 10 µl NTX (2.6 nmol). Vehicle or PP2 were injected i.th. daily for two more days. **C, F:** On day 35, rats received i.th. injections of 10 µl NTX (2.6 nmol). Each panel was analyzed by RM (both variables) 2-way ANOVA; *p* values for 'time' (effect of CFA or NTX) are given in each panel. Holm-Sidak's post-hoc tests comparing to time 0: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

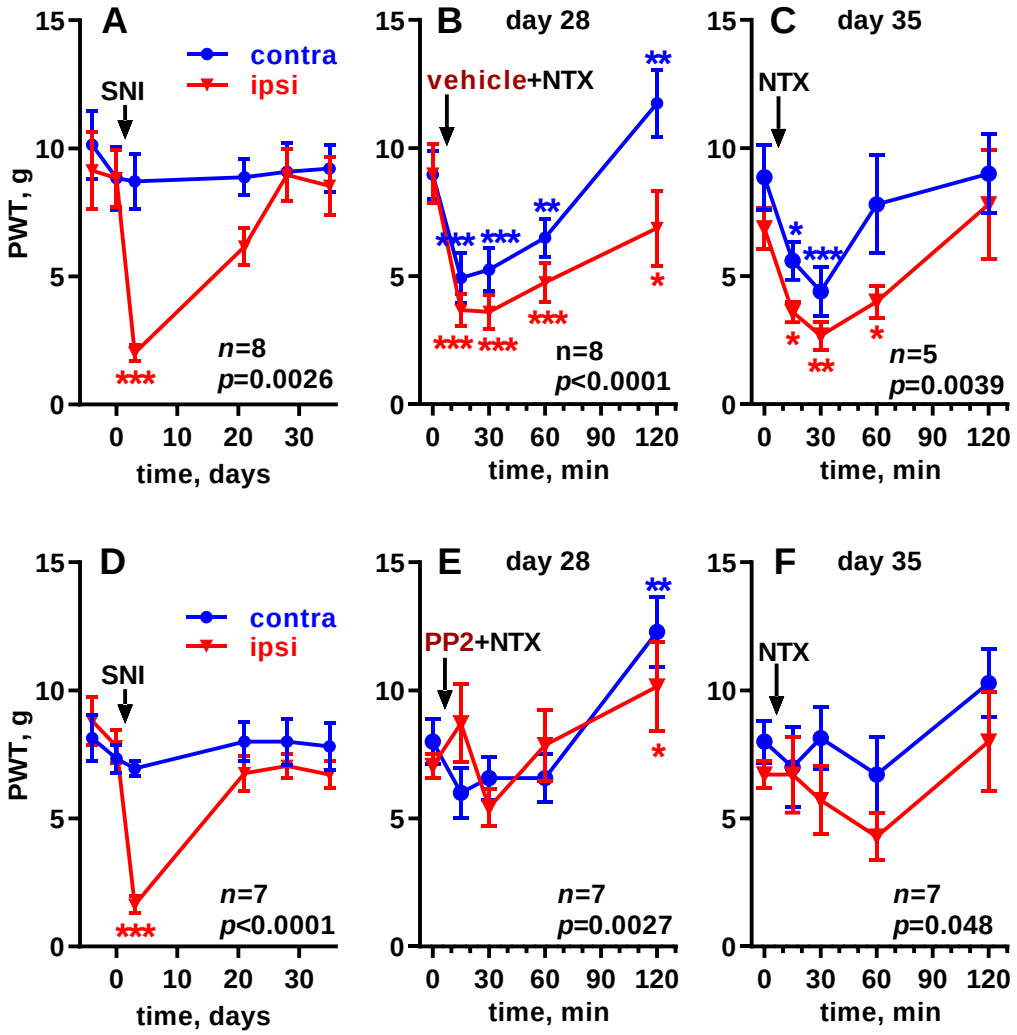

Figure 3. Effect of PP2 on NTX-induced allodynia in rats with SNI-induced LS. A, D: Rats (*n*=8 for A-C, *n*=7 for D-F) received SNI in one hindpaw. Paw withdrawal thresholds (PWT, von Frey) were measured in the ipsilateral ('ipsi') and contralateral ('contra') hindpaws. **B, E:** On day 28, rats received i.th. injections of 10 µl vehicle (0.3% DMSO in saline, B) or PP2 (0.3 nmol in 0.3% DMSO, E), and 15 min later 10 µl NTX (2.6 nmol). Vehicle or PP2 were injected i.th. daily for two more days. **C, F:** On day 35, rats received i.th. injections of 10 µl NTX (2.6 nmol) was injected again. Each panel was analyzed by RM (both variables) 2-way ANOVA; *p* values for 'time' (effect of CFA or NTX) are given in each panel. Holm-Sidak's post-hoc tests comparing to time 0: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.