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PKC γ Interneurons Mediate C-Fiber-Induced Orofacial Secondary Static Mechanical Allodynia, but not C-Fiber-Induced Nociceptive Behavior

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Abstract (250 words), **Introduction** (408 words), **Discussion** (1338 words)

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Brief Summary: Pain to innocuous punctate stimuli or static mechanical allodynia is a key symptom of secondary hyperalgesia. It requires the sensitization of lamina II; PKC γ interneurons. Such sensitization is driven by ROS and specifically GABA_Aergic disinhibition.

Abbreviated title: PKC γ neurons mediate static mechanical allodynia

Conflict of interest: The authors declare no competing interests.

ABSTRACT

Background: Tissue injury enhances pain sensitivity both at the site of tissue damage and in surrounding uninjured skin (secondary hyperalgesia). Secondary hyperalgesia encompasses several pain symptoms including pain to innocuous punctate stimuli or static mechanical allodynia. How injury-induced barrage from C-fiber nociceptors produces secondary static mechanical allodynia has not been elucidated.

Methods: Combining behavioral, immunohistochemical and western blot analysis, we investigated the cell and molecular mechanisms underlying the secondary static mechanical allodynia in the rat medullary dorsal horn (MDH) using the capsaicin model.

Results: Capsaicin injection into the vibrissa pad produces a spontaneous pain and a secondary static mechanical allodynia. This allodynia is associated with the activation of a neuronal network encompassing lamina I-outer lamina III, including interneurons expressing the gamma isoform of protein kinase C (PKC γ) within inner lamina II (II_i) of MDH. PKC γ is concomitantly phosphorylated. Mechanical allodynia and innocuous punctate stimulus-evoked laminae I-III neuronal activation can be replicated following intracisternally applied GABA_A receptor antagonist or reactive oxygen species (ROS) donor. Conversely, intracisternal PKC γ antagonist, GABA_A receptor agonist or ROS scavenger prevents capsaicin-induced mechanical allodynia and neuronal activation.

Conclusion: Sensitization of lamina II_i PKC γ interneurons is required for the manifestation of secondary static mechanical allodynia but not spontaneous pain. Such sensitization is driven by ROS and GABA_Aergic disinhibition. ROS released during intense C-fiber nociceptor activation might produce a GABA_Aergic disinhibition of PKC γ interneurons. Innocuous punctate inputs carried by A δ low-threshold mechanoreceptors onto PKC γ interneurons can then gain access to the pain transmission circuitry of superficial MDH, producing pain.

Introduction

Tissue injury is associated with increased pain sensitivity at the site of tissue damage (primary hyperalgesia) but also in surrounding normal skin (secondary hyperalgesia). Whereas sensitization of nociceptive afferents largely accounts for primary hyperalgesia, secondary hyperalgesia is attributed to sensitization of central nociceptive neurons (1). As a sign of central sensitization, secondary hyperalgesia might be a predictor of chronic neuropathic pain (2). Interestingly, the area of secondary hyperalgesia varies between subjects but is highly reproducible within individuals, suggesting that it can be regarded as a phenotypic characteristic (3). And magnetic resonance imaging reveals differences in brain morphology and noxious stimulation-induced neuronal activation between individuals developing large and small areas of secondary hyperalgesia (4). Altogether, this indicates that there are differences in injury-induced central sensitization according to phenotype and that a high-sensitization response is a risk factor for chronic pain. Identifying the mechanisms of secondary hyperalgesia is therefore becoming a public health priority.

Surprisingly, little is known about such mechanisms. It is established that distinct peripheral afferents mediate the induction and expression of secondary hyperalgesia: TRPV1-positive C-fiber input mainly contributes to induction whereas A-fibers are involved in transmitting inputs that produce pain (5,6,7,8,9). However, which A-fiber-mediated mechanical pathway is facilitated and how it is facilitated following a brief conditioning C-fiber input are still unclear.

Moreover, several pain symptoms are filed under the umbrella term of secondary hyperalgesia. Intradermal capsaicin generates a highly reproducible set of sensory changes: transient spontaneous pain followed by persisting mechanical hypersensitivity in the adjacent uninjured skin (5,8,10). Such secondary hyperalgesia includes dynamic mechanical allodynia

and hyperalgesia to punctate stimuli (5,11,12). Moreover, because hyperalgesia to punctate stimuli is characterized by a leftward shift of the whole stimulus-response function (5,7,13), it actually encompasses decreased pain thresholds and increased pain to suprathreshold stimuli, referred to as static mechanical allodynia and hyperalgesia, respectively. That static mechanical hypersensitivity covers a larger area (5,12) and requires smaller doses of capsaicin than dynamic mechanical allodynia (7) suggests that the mechanical pain symptoms characterizing secondary hyperalgesia are independent. They are therefore likely signaled by different mechanosensitive pathways.

Using behavioral, immunohistochemical and western blotting techniques, we specifically addressed the mechanisms of capsaicin-induced secondary trigeminal static mechanical allodynia within the rat medulla dorsal horn (MDH). Since hyperalgesia to punctate stimuli displays a much larger area than dynamic allodynia (5,12), these mechanisms could be assessed by stimulating the uninjured facial skin with innocuous von Frey filament (14) at a rather long distance from capsaicin injection site.

Materials and Methods

Animals

Adult male Sprague–Dawley rats (250 - 275 g) were obtained from Charles River (L'Arbresle, France). Rats were housed in plastic cages (3-4 rats per cage) with soft bedding and free access to food and water. They were maintained in climate- (23 ± 1°C) and light-controlled (12:12-h dark:light cycle), protected units (Iffa-Credo) for at least 1 week before experiments. All efforts were made to minimize the number of animals used. All behavioral experiments started at 10.00 h a.m. Experiments followed the ethical guidelines of the International Association for the Study of Pain (15), of the Directive 2010/63/UE of the European Parliament and of the Council on the protection of animals used for scientific purpose. Protocols applied in this study were approved by the local animal experimentation committee: CEMEAA “Comité d’Ethique en Matière d’Expérimentation Animale Auvergne” (n° CE 28-12). All experiments were conducted with the experimenters blinded to treatment conditions. Rats were randomized into treatment groups before any assessment was performed.

Chemicals

Capsaicin, bicuculline, muscimol, *tert*-butyl hydroperoxide (t-BOOH) and N-*tert*-butyl- α -phenylnitrone (PBN) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). KIG31-1 was obtained from Kai Pharmaceuticals (San Francisco, CA, USA). It is conjugated to Tat, a peptide carrier, via a cysteine-cysteine bond at its N terminus. KIG31-1 competes with activated PKC γ for binding to the isoenzyme-specific docking proteins, receptors for activated C kinase (RACK). This strategy prevents PKC γ translocation in an isoenzyme-specific manner (16,17). Linking of KIG31-1 to Tat enables efficient transfer of the peptide

into cells (18). Drug doses were selected based on literature (19,20) and our preliminary experiments. No motor impairment has been reported with intrathecal or intracisternal injections of muscimol up to 1 μg (21), PBN up to 100 μg (20) and KIG31-1 up to 500 μg (22).

Behavioral testing

For investigating the effects of capsaicin upon rubbing nociceptive behavior and static or dynamic mechanical allodynia, rats were first habituated to stand on their hindpaws on the experimenter sleeve and lean against the experimenter chest in a quiet room under red light, according to a method adapted from Ren (23). The habituation required 1/2 hour during which animals were tested with von Frey filaments or gentle air puffing using a calibrated pump onto a region between the right vibrissa pad and the upper right lip, with carefully avoiding touching any vibrissa. Ascending and descending series of von Frey filaments (1.0–12 g; Bioseb, France) were used. Each filament was tested five times at intervals of at least 5 s. The habituation session was repeated during 2 days. At the end of the second habituation session, all rats responded to 6-g von Frey filament with only a simple detection, showing a non-aversive response. The actual testing session took place on the 3rd day. Rats were then placed in an observation field (0.6 x 0.6 m square) under red light for a 30-min habituation period during which the experimenter reached into the cage to apply 6-g von Frey filament or gentle air puffing on the face of the animal. At the end of this habituation period, rats received an intradermal injection of capsaicin (25 μg in 25 μl endolipid) or vehicle alone into the right vibrissa pad using a 30G gauge needle as previously described (24). This is a relatively small dose to reduce as much as possible the area of dynamic mechanical allodynia. After 40 μg , a dose almost twice that used here, all subjects are hypersensitive to punctate stimuli whereas only a minority exhibit dynamic mechanical allodynia (7). After injection, animals were

placed back within the observation field for a 15-min observation period recorded by a digital camera (Sanyo, USA) followed by a 60-min mechanical-testing period. Mechanical stimuli were applied with 6-g von Frey filament and gentle air puffing (1 s long) every 3 min onto the right upper lip, 0.5 - 1 cm to the injection site, that is, well outside this site, in the anticipated region of secondary hyperalgesia. We selected the 6-g von Frey filament because it was, on one side, innocuous (14,25), but, on the other side, close to the aversive threshold (14). Each series of stimuli consisted of 5 stimuli, applied at an interval of at least 10 s. Stimulation was carried out when the rat was in a sniffing/no locomotion state: with four paws placed on the ground, neither moving nor freezing. The tip of the pump or von Frey filament was moved toward the target from behind the animal so that it could not see it. The 15-min recording time was divided into five 3-min blocks. For each block, a nociceptive score was determined by measuring the time (in s) animals spent rubbing the injected area (vibrissa pad) with the forepaw and not the hindpaw. The behavioral responses to mechanical stimulations were observed and quantified according to the method developed by Vos et al. (26). Rat responses to mechanical stimuli consisted of one or more of the following elements: 1) detection, rats turn head toward stimulus; 2) withdrawal reaction, rats turn head away or pulls it briskly backward when stimulation is applied (a withdrawal reaction is assumed to include a detection element preceding the head withdrawal and therefore consists of two responses elements); 3) escape/attack, rats avoid further contact with the stimulus, either passively by moving their body away from the stimulus, or actively by attacking the tip of the pump or the filament; 4) asymmetric grooming, rats display an uninterrupted series of at least three wash strokes directed to the stimulated area. The following rank-ordered descriptive responses categories were formulated: no response, non aversive response, mild aversive response, strong aversive response, prolonged aversive behavior. Each category was given a score (0-4) based on the number of observed response elements. This score is assumed to reflect the

magnitude of the aversiveness evoked by mechanical stimuli. Score was equal to zero in case of no response. A mean score value was then calculated for each stimulation series. Behavior was always analyzed by a second experimenter who was blind to animal treatment.

Intracisternal injections

For experiments investigating the effects of KIG31-1 (50 pmoles, in 5 μ l Tat carrier), PBN (100 μ g, in 5 μ l aCSF) or muscimol (250 ng, in 5 μ l aCSF) upon capsaicin-induced rubbing nociceptive behavior or mechanical allodynia, animals were briefly (< 3 min) anesthetized using a mask with 2% halothane and received an intracisternal injection of either drug or vehicle alone (5 μ l) using a 10 μ l Hamilton syringe (14). Artificial CSF consisted of: 150 mM Na⁺, 3 mM K⁺, 0.8 mM Mg²⁺, 1.4 mM Ca²⁺, 155 mM Cl⁻, pH 7.4, 295 mosmol.kg⁻¹. Following recovery (< 2 min), rats were placed in the observation field for a 30-min habituation period. Immediately after habituation, animals received an intradermal injection of capsaicin and the rubbing nociceptive behavior and behavioral responses to mechanical stimuli were evaluated, as described above.

For experiments investigating the effects of bicuculline (0.05 μ g, in 5 μ l aCSF) or t-BOOH (50, 100 and 250 ng, in 5 μ l aCSF) upon mechanical cutaneous sensitivity, animals were briefly (< 3 min) anesthetized using a mask with 2% halothane and received an intracisternal injection of either drug or aCSF alone (5 μ l) using a 10 μ l Hamilton syringe. Immediately after recovery (< 2 min), rats were placed into the observation field for a 45-min (bicuculline) or 125-min (t-BOOH) test period. Behavioral responses to mechanical stimuli were evaluated as described above.

Immunohistochemistry

In a first series of experiments, we assessed extracellular-signal regulated kinase 1/2 phosphorylation (phospho-ERK1/2) under capsaicin-induced rubbing nociceptive behavior

and mechanical stimulus-evoked pain. Rats were anaesthetized deeply with urethane (1.5 g.kg⁻¹ intraperitoneal) as previously described (14). Twenty min after the injection, the depth of the anesthesia was assessed, and rats received an intradermal injection of capsaicin (25 µg in 25 µl endolipid) or vehicle alone into the right vibrissa pad using a 30 G gauge needle. Five or 30 min later, innocuous stimulation was applied using a 6-g von Frey filament during 3 min at a velocity of 0.5 Hz to the right upper lip, 0.5 - 1 cm to the injection site, that is, well outside this site, in the anticipated region of secondary hyperalgesia. Two min later, rats were perfused transcardially with warm heparinized saline followed by cold 0.1 M phosphate-buffered solution pH 7.6 (PB) containing 4% paraformaldehyde (PFA) and 0.03% picric acid. The brainstem was removed and post-fixed for 2 h in the same fixative solution at 4°C and then cryoprotected in 30% sucrose diluted in 0.05 M Tris-buffered saline pH 7.4 (TBS) at 4°C for 24 h. Thirty µm coronal sections were cut on a freezing microtome and collected in TBS before being processed. Free-floating sections were placed in 2% normal goat serum (NGS) diluted in TBS containing 0.25% bovine serum albumin (BSA) and 0.3 % Triton X-100 (TBS-BSA-T) for 2 h before incubation in a polyclonal rabbit primary antibody directed against phospho-ERK1/2 (1:3000, Cell Signaling Technology, USA) diluted in TBS-BSA-T overnight at room temperature. Sections were then incubated for 90 min with the secondary antibody goat anti-rabbit conjugated with peroxidase (1:400, Vector Laboratories, Les Ulis, France). Immunoreactivity was revealed using nickel-diaminobenzidine (Vector Laboratories). In all cases, sections were rinsed in TBS several times, between and after each incubation, and finally transferred onto gelatinized slides before being coverslipped using DPX mountant for histology. Specificity controls consisted of omitting the primary antibody and incubating sections in inappropriate secondary antibodies. In all these controls, no specific staining was evident. A few selected sections were mounted separately and slightly counterstained with cresyl violet to help delineate the limits of MDH.

In a second series of experiments, we assessed ERK1/2 phosphorylation in PKC γ -immunoreactive interneurons under capsaicin-induced mechanical allodynia. Free floating sections were first treated with 50 mM NH₄Cl diluted in phosphate buffer saline (PBS), pH 7.4 for 30 min at room temperature. After several washes in 0.1 M PBS with 0.2% Triton (PBS-Tx), sections were blocked by pre-incubation in 5% NGS in PBS-Tx for 1 h at room temperature. After washes in PBS-Tx, sections were incubated with a polyclonal rabbit primary antibody directed against phospho-ERK1/2 (1:1000, Cell Signaling) and a monoclonal mouse primary antibody directed against PKC γ (1:4000, Sigma-Aldrich), diluted in 5% NGS in PBS-Tx for 24 h at 4°C. Sections were then washed with 5% NGS followed by washes in PBS-Tx. Tissues were incubated with a Cy2 conjugated goat anti-mouse secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA, USA) and a Cy3 conjugated goat anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch) diluted in 5% NGS in PBS-Tx for 1 h at room temperature. Finally, sections were washed in 5% NGS, in PBS-Tx and then in PBS. Sections were transferred onto gelatinized slides before being coverslipped with DPX mountant for histology.

In a third series of experiments, rats were treated as in group 1 but received an intracisternal injection of KIG31-1 (500 pmoles, in 5 μ l Tat carrier), PBN (100 μ g, in 5 μ l aCSF) or vehicle alone 20 min after induction of anesthesia. Capsaicin was injected 30 min later, as previously described.

In a fourth series of experiments, we assessed ERK1/2 phosphorylation under bicuculline- or t-BOOH-induced mechanical allodynia. Rats were anaesthetized with urethane as previously described. Twenty min after induction of anesthesia, the depth of the anesthesia was assessed, and rats received an intracisternal injection of bicuculline (3 μ g, in 5 μ l aCSF) or t-BOOH (100 ng, in 5 μ l aCSF). Five min (bicuculline) or 60 min (t-BOOH) later, innocuous stimulation of the right upper lip was performed as described above. Tissues were

collected and ERK1/2 phosphorylation or ERK1/2 phosphorylation in PKC γ -immunoreactive interneurons were assessed as described above.

Western blot

Rats were deeply anesthetized with urethane as previously described. Twenty min after induction of anesthesia, the depth of the anesthesia was assessed, and rats received an intradermal injection of capsaicin (25 μ g, in 25 μ l endolipid) or vehicle alone into the right vibrissa pad using a 30 G gauge needle. Five min or 30 min later, innocuous stimulation was applied using a 6-g von Frey filament (at 0.5 Hz during 3 min) to the right upper lip, as described above. Immediately after, rats were decapitated for rapid tissue harvesting. Tissues containing the MDH were dissected out and cut from its rostral (0 μ m; obex aperture) to its caudal end (-2,400 μ m). These limits correspond to the 1) obex aperture and 2) end of the brainstem enlargement compared to the cervical spinal cord (27). The ventral part of the brainstem was not removed as MDH represents most of the tissue at this level. Ipsilateral and contralateral were divided by performing a cut in the midline of the tissue. Likely, ipsilateral and contralateral PKC γ interneurons were properly separated as these cells are located at at least 1 mm away from the central commissure (27). Tissue was then rapidly frozen on dry ice and stored at -80°C for later use. Proteins were extracted and homogenized in 500 μ l RIPA buffer (Sigma-Aldrich) containing 0.5% of protease inhibitors (Sigma-Aldrich) and 0.5% of phosphatase inhibitors (Sigma-Aldrich). The homogenate was centrifuged at 4°C for 30 min at 22,000 g to remove the debris. Protein concentration of the supernatants was measured with the Micro Lowry total protein kit (Sigma-Aldrich) with a spectrophotometer (Bio-Rad, Marnes-la-Coquette, France) at a wavelength of 750 nm. The supernatants (30 μ g) were heated for 5 min at 95°C and loaded onto 5% stacking/12% separating SDS-polyacrylamide gels for the protein separation. Electrophoresis was carried out in Tris 25 mM, glycine 192

mM, and 1% of SDS buffer for 2 h at 80 V, until the reference colorant reached the end of the gel. Proteins were then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Molsheim, France) in a buffer containing Tris 25 mM, glycine 192 mM, 10% of SDS and 20% of methanol for 45 min at 100 V. That sample loading was equivalent in each lane was confirmed by Ponceau Red membrane staining following the blotting. The membrane was blocked with 6% non-fat dry milk and subsequently incubated overnight at 4°C with the polyclonal rabbit primary antibody directed against the phosphorylated form of PKC γ (Thr514) (1:1000, Cell Signaling Technology) diluted in 0.05 M TBS, 5% BSA and 0.1% Tween[®] 20 with moderate shaking. The membrane was then incubated 1 h at room temperature with an HRP-conjugated donkey anti-rabbit secondary antibody (1:10,000, GE Healthcare, Velizy-Villacoublay, France) diluted in 0.05 M TBS, 6% non-fat dry milk and 0.1% Tween[®] 20. ECL Plus solution (GE Healthcare) was used to visualize blots. Blots were captured using an automated image acquisition system (Fusion FX7; Vilber Lourmat, Marne la Vallée, France) for 1–10 min. Blots were then incubated in a stripping buffer (Tris 62.5 mM, β -mercaptoethanol 100 mM and 2 % SDS, pH 6.7) for 30 min at 50°C and probed again 1 h at room temperature with a polyclonal rabbit primary antibody directed against β -actin (1:1000; Cell Signaling Technology) diluted in 0.05 M TBS, 3% BSA and 0.1% Tween[®] 20 as loading control.

Data and statistical analysis

Neurons containing phospho-ERK1/2 immunoreactivity in the MDH were photographed using a Nikon Optiphot 2 coupled with a 3CCD Sony DXC-950P digital camera at x10 and x20 magnifications. Neurons containing phospho-ERK1/2 and/or PKC γ immunofluorescence in the MDH were photographed using a fluorescent Zeiss Axioplan 2 Imaging microscope coupled with a Hamamatsu C4742–95 digital camera, by switching between FITC and

Rhodamine filter sets at x20 and x40 and x100 magnifications. Each image was then analyzed with Fiji-ImageJ 1.47 program (<http://rsbweb.nih.gov/ij> ; 28). Phospho-ERK1/2 and/or PKC γ immunoreactive neurons were counted according to their location in the different laminae of the MDH from 7 different sections, each taken at a given rostrocaudal plane within MDH. Intervals of 400 μ m between planes ensured that cells were counted only once. The delineation of the MDH was based upon the Paxinos and Watson atlas (29) and our own myeloarchitectural atlas as determined by our previous work (27). The data are expressed as the sum of the total number of labeled cells counted from all 7 sections that were analyzed in each animal. Pictures were optimized for visual quality using Fiji-ImageJ 1.47 program at the end of analysis.

Data on capsaicin-induced rubbing behavior were analyzed using a Mann-Whitney test or an unpaired Student's *t* test after passed the Kolmogorov-Smirnov normality test. Data on capsaicin-, bicuculline- or t-BOOH-induced allodynia were analyzed using an ANOVA with repeated measures followed by a Bonferroni *post hoc* test. Data on dose efficiency of t-BOOH-induced allodynia were analyzed using a one-way ANOVA followed by a Dunnett's *post hoc* test. Data on phospho-ERK1/2 immunoreactive cells were analyzed using a Mann-Whitney test or a one-way ANOVA followed by a Newman-Keuls *post hoc* test. Bands obtained by Western blot were quantified by densitometry using Fusion capt[®] and Bio1D[®] software (Vilber Lourmat). Comparison of the means of densitometric analysis was made using a one-way ANOVA followed by a Newman-Keuls *post hoc* test. In all cases, $p < 0.05$ was considered to be statistically significant. All quantitative analysis, graphs and statistical tests were performed on GraphPad Prism[®] 5.0. and Statistica[®] 6.0. Figures were made using CorelDRAW[®] 12. All data is presented as Mean with SD.

Results

Capsaicin induces facial rubbing nociceptive behavior and secondary static but not dynamic mechanical allodynia

We first assessed the behavioral changes produced by an intradermal injection of capsaicin (25 μg in 25 μl) into the vibrissa pad. Capsaicin caused a nociceptive behavior (unpaired Student's *t* test, $P = 0.0008$; $n = 5$) (Fig. 1A). Spontaneous nociceptive behavior was maximum within the first 5 min after capsaicin injection and had completely disappeared after 15 min. We subsequently assessed the responses to mechanical stimuli applied 0.5 to 1 cm adjacent to the capsaicin injection site, i.e. within the zone of secondary mechanical hypersensitivity (see Methods), with innocuous static and dynamic mechanical stimuli: 6-g von Frey filament and air puff, respectively. Fifteen minutes after injection, mechanical stimulation with the 6-g von Frey filament evoked consistently the most aversive response categories (RM two-way ANOVA with post hoc Bonferroni test, $P < 0.0001$, $n = 5$) (Fig. 1B). Allodynia scores then steadily returned to pre-injection levels within 45 min. Importantly, capsaicin did not change response scores to air puff stimulation (Fig. 1B). Thus, rats exhibited secondary static, but not dynamic, mechanical allodynia at 0.5-1 cm away from the capsaicin injection site.

Laminae I-III_o MDH circuits are associated with capsaicin-induced secondary static mechanical allodynia

ERK1/2 has been reported to be quickly phosphorylated after noxious stimuli in neurons of spinal DH (30). But phospho-ERK1/2 signal can also be induced by tactile stimulation following inflammation or nerve injury (30). Therefore, we used phospho-ERK1/2 immunoreactivity as an anatomical marker to visualize MDH neurons that are activated by the

two types of stimuli, intradermal injection of capsaicin into the vibrissa pad alone or together with punctate mechanical stimulation at two delays after capsaicin injection: 5 min, when nociceptive behavior is maximum, and 30 min, when static mechanical allodynia is maximum. Capsaicin alone induced ERK1/2 phosphorylation mainly in ipsilateral superficial laminae I-II_o (within 5 min after injection; one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0115$, $n = 5$) (Fig.1C, E and F and Table 1). But ERK1/2 phosphorylation within ipsilateral MDH was further increased following stimulation applied on the area of secondary hypersensitivity with the 6-g von Frey filament (Fig. 1 D-F and Table 1). Interestingly, such mechanical stimulation-evoked ERK phosphorylation was observed only when the stimulation was applied 30 min (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0118$, $n = 5$) but not 5 min after capsaicin injection (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.8353$, $n = 5$) (Fig. 1E and Table 1). Moreover, it occurred within both superficial laminae I-II_o (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0357$, $n = 5$) and deeper laminae II_i-III_o (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0006$, $n = 5$) (Fig. 1 D, F). Importantly, there was no change in phospho-ERK1/2 immunoreactivity when stimulation was applied on the area of secondary hypersensitivity with a paintbrush, 30 min after capsaicin injection (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.4190$, $n = 5$) (Table 1). These results suggest that the expression of static mechanical allodynia is associated with the activation of a neuronal pathway within MDH encompassing laminae I to III_o interneurons.

Lamina II_i PKC γ interneurons are involved in capsaicin-induced secondary static mechanical allodynia

That genetic impairment of PKC γ prevents nerve injury-induced cutaneous hypersensitivity to punctate mechanical stimuli (31,32,33) suggests that activation of PKC γ is also a prerequisite

for the expression of secondary static mechanical allodynia. To test this hypothesis, we first examined the phospho-ERK1/2 signal in PKC γ -immunoreactive interneurons after mechanical stimulation applied adjacent to the capsaicin injection site with the 6-g von Frey filament. Using dual immunocytochemical labeling, we found that $30.5 \pm 5.5\%$ ($n = 4$) of the phospho-ERK1/2-immunoreactive cells within laminae II_i-III_o of the ipsilateral MDH were also PKC γ immunoreactive (Fig. 2A).

Expression of phosphorylated PKC γ (phospho-PKC γ) – the active form of PKC γ (34) – in MDH was directly measured using Western blot analysis (Fig. 2B) at the two delays after capsaicin injection: 5 and 30 min. Whereas capsaicin alone had no effect on phospho-PKC γ , stimulation applied 0.5 to 1 cm adjacent to the capsaicin injection site with the 6-g von Frey filament led to a strong increase in phospho-PKC γ . Interestingly, phospho-PKC γ protein level was only enhanced when cutaneous stimulation occurred 30 min ($451.4 \pm 79.2\%$ of baseline; one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0003$, $n = 4$), but not 5 min after capsaicin injection ($75.4 \pm 25.1\%$ of baseline; one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.5792$, $n = 3$).

Thus, our results indicate that the activation of PKC γ interneurons and the phosphorylation of PKC γ are associated with the manifestation of secondary static mechanic allodynia. To directly test whether activation of PKC γ is necessary for the expression of this allodynia, we examined the effect of intracisternal application of the selective PKC γ antagonist, KIG31-1 (50 pmoles in 5 μ l) on capsaicin-induced nociceptive behavior and static mechanical allodynia. KIG31-1 had no effect on the nociceptive behavior (Unpaired Student's t test, $P = 0.5379$, $n = 5$) (Fig. 3A) but completely prevented static mechanical allodynia (RM two-way ANOVA with post hoc Boneferroni test, $P < 0.0001$, $n = 5$) (Fig. 3B). In addition, it strongly attenuated mechanical stimulation-evoked phospho-ERK1/2 signal in laminae I-II_o as well as II_i-III_o (30 min after capsaicin injection: Mann-Whitney test, $P = 0.0286$, $n = 3-4$)

(Fig. 3C and Table 1). Altogether, these results suggest that PKC γ activation is required for the expression of secondary static mechanical allodynia.

GABA_Aergic disinhibition induces a specifically static mechanical allodynia mediates capsaicin-induced mechanical allodynia

Activation of peptidergic C-fiber nociceptor central terminals reduces selectively GABAergic inhibitory signaling within spinal DH (35). Moreover, our group previously showed in anaesthetized animals that after a microinjection of the GABA_A receptor antagonist, bicuculline, into MDH, static, but not dynamic, mechanical stimulation of the face can elevate blood pressure, a reliable indicator of pain in anaesthetized animals (14). This suggests that local GABA_Aergic disinhibition also produces a static, but not a dynamic, mechanical allodynia. Therefore, we hypothesized that capsaicin produces a secondary static mechanical allodynia through GABA_Aergic disinhibition.

To test this hypothesis, we first confirmed in behaving rats our previous results obtained in anaesthetized animals. Intracisternal application of bicuculline (0.05 μ g in 5 μ l) induced a static, but not a dynamic, mechanical allodynia (Fig. 4; RM two-way ANOVA with post hoc Bonferroni test, $P < 0.0001$, $n = 5$) that lasted for about 30 min. Then, using phospho-ERK1/2 immunoreactivity, we examined which MDH neurons are activated by mechanical stimulation under GABA_Aergic disinhibition. As expected, no significant phospho-ERK1/2 signal was observed following intracisternal aCSF, with or without mechanical stimulation (Table 1). On the other hand, phospho-ERK1/2 immunoreactive neurons were observed 5 min after intracisternal bicuculline but only in superficial laminae I-II_o (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0423$, $n = 5$) (Fig. 5A, C). Finally, mechanical stimulation applied to the face with the 6-g von Frey filament under GABA_Aergic disinhibition, produced an even stronger phospho-ERK1/2 signal (Table 1). Phospho-ERK1/2-immunoreactive

neurons were now located within laminae I-II_o (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0006$, $n = 5$), II_i-III_o (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0019$, $n = 5$) and, to a smaller extent, III_i-V (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0139$, $n = 5$, Fig. 5B, C). Again, $20.7 \pm 3.1\%$ ($n = 4$) of phospho-ERK1/2-immunoreactive neurons in laminae II_i-III_o were also PKC γ -immunoreactive, indicating that PKC γ interneurons also participate in neuronal circuits activated by mechanical stimulation under GABA_Aergic disinhibition (Fig. 5D-F).

To confirm that capsaicin act through GABA_Aergic disinhibition, we examined the effects of intracisternal application of the GABA_A receptor agonist, muscimol, on capsaicin-induced nociceptive behavior and static mechanical allodynia. Intracisternal muscimol (250 ng in 5 μ l) completely prevented both spontaneous nociceptive behavior (Mann-Whitney test, $P = 0.0030$, $n = 4$ -5; Fig. 6A) and secondary static mechanical allodynia (RM two-way ANOVA with post hoc Bonferroni test, $P < 0.0001$, $n = 4$; Fig. 6B).

ROS release induces a specifically static mechanical allodynia

On one hand, the secondary static mechanical allodynia is produced by the capsaicin-evoked barrage from C-fiber nociceptors (36). On the other hand, present results indicate that local circuits associated with such allodynia involve lamina II_i PKC γ interneurons. But these interneurons are targeted by myelinated, but not unmyelinated, primary afferents (27,38). Consistently, no lamina II_i PKC γ interneurons showed phospho-ERK1/2 immunoreactivity following capsaicin injection alone. Therefore, how can the conditioning input, carried by C-fiber nociceptors, and the facilitated input, carried by A-LTMRs targeting lamina II_i PKC γ interneurons, interact with each other to induce a secondary static mechanical allodynia? One possibility is via a diffusible factor such as reactive oxygen species (ROS). They are released

during the capsaicin-induced barrage from peptidergic C- and some A δ -fiber nociceptors (38) and were shown to decrease selectively GABA release within DH (21).

We therefore tested whether intracisternal application of a ROS donor, t-BOOH, causes a static mechanical allodynia and unmasks the same neuronal circuit as GABA_Aergic disinhibition or intradermal capsaicin injection. Intracisternally-applied t-BOOH induced a static, but not a dynamic, mechanical allodynia (Fig. 7). Interestingly, increasing t-BOOH concentration did not enhance the maximum score of allodynia – the highest mean score was the same for 100 ng (2.92 ± 0.7 , $n = 5$) and 250 ng (3 ± 0.6 , $n = 5$) – but rather the duration of t-BOOH-induced mechanical allodynia (Fig. 7A). The area under the curve was thus correlated with the dose of t-BOOH (Fig. 7B). At the highest t-BOOH concentration (250 ng in 5 μ l), mechanical allodynia lasted for more than 2 h (Fig. 7A), and a transient dynamic mechanical allodynia was present during the first minutes (Fig. 7C).

We next examined, using phospho-ERK1/2 immunohistochemistry, which MDH neurons are involved in t-BOOH-induced static mechanical allodynia. The vibrissa pad was stimulated with the 6-g von Frey filaments, 60 min after intracisternal injection of t-BOOH (100 ng in 5 μ l), that is, at the peak of allodynic score at this concentration (Fig. 7A). t-BOOH alone had no effect on phospho-ERK1/2 levels (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.4528$, $n = 5$; Fig. 8A and Table 1), but mechanical stimulation led to a strong ERK1/2 phosphorylation (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0016$, $n = 5$; Fig. 8B and Table 1) within both laminae I-II_o (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0025$, $n = 5$) and II_i-III_o (one-way ANOVA with post hoc Student-Newman-Keuls test, $P = 0.0019$, $n = 5$) (Fig. 8B, C). Moreover, again, many phospho-ERK1/2-immunoreactive neurons in laminae II_i-III_o ($26.8 \pm 10.3\%$, $n = 4$) were also PKC γ -immunoreactive, indicating that PKC γ interneurons participate in the neuronal circuits associated with ROS-induced static mechanical allodynia

(Fig. 8D-F). It is interesting to note that such ratio of dually phospho-ERK1/2- and PKC γ -immunoreactive neurons within laminae II_i-III_o is rather similar to those in capsaicin- or bicuculine-treated rats: 30.5 ± 5.5 and $20.7 \pm 3.1\%$, respectively.

Intracisternal ROS scavenger prevents capsaicin-induced secondary static mechanical allodynia

That intradermal capsaicin and ROS release both produce a static mechanical allodynia associated with the activation of the same MDH neuronal circuits suggests that capsaicin induces a static mechanical allodynia through ROS release. To test this hypothesis, we examined the effect of intracisternal application of the ROS-scavenger, PBN, on capsaicin-induced nociceptive behavior and static mechanical allodynia as well as on mechanical stimulation-induced ERK1/2 phosphorylation within MDH. Intracisternal PBN (100 μ g in 5 μ l) had no effect on capsaicin-induced rubbing nociceptive behavior (unpaired Student's *t* test, $P = 0.6534$, $n = 5$) (Fig. 9A) but almost completely prevented static mechanical allodynia (RM two-way ANOVA with post hoc Bonferroni test, $P < 0.0001$, $n = 5$) (Fig. 9B). Consistently, the level of ERK1/2 phosphorylation within MDH evoked by a stimulation applied on the area of secondary hypersensitivity with the 6-g von Frey filament was significantly reduced (Mann-Whitney test, $P = 0.0286$, $n = 4$; Fig. 9C and Table 1), being equivalent with that following capsaicin alone (Fig. 1E).

Discussion

We investigated the mechanisms of trigeminal secondary static mechanical allodynia. Monitoring neural activity within MDH with phospho-ERK1/2 immunoreactivity shows that activation of laminae I-II_o and II_i-III_o neurons, including lamina II_i PKC γ -expressing interneurons, is associated with the expression of secondary static mechanical allodynia. Western blot analysis reveals that PKC γ is concomitantly phosphorylated. Either local GABA_Aergic disinhibition or ROS release is sufficient to initiate the same static mechanical allodynia and von Frey-induced laminae I-III_o neural activation as capsaicin. Conversely, intracisternal PKC γ antagonist, GABA_A receptor agonist or ROS scavenger prevents capsaicin-induced secondary static mechanical allodynia and associated von Frey-evoked neural activation.

Capsaicin injection into the rat vibrissa pad induced a static, but not dynamic, mechanical allodynia at 0.5-1-cm away from the injection site. Psychophysical studies in humans indicate that capsaicin-induced secondary hyperalgesia encompasses several pain symptoms such as dynamic mechanical allodynia and static mechanical hyperalgesia and allodynia, the two latter symptoms being filed under the umbrella term 'hyperalgesia to punctate stimuli' (39). These different pain symptoms appear to be independent from each other. And dynamic mechanical allodynia covers a much smaller area than static mechanical hypersensitivity (5,12). Therefore, our finding that in rats mechanical allodynia is specifically static at 0.5-1-cm from the capsaicin injection is consistent with the cutaneous sensitivity observed in humans inside the area of hyperalgesia to punctate stimuli but outside that of dynamic mechanical allodynia.

As in DH (40), capsaicin-induced ERK1/2 phosphorylation was restricted to superficial laminae I-II_o of MDH. But 6-g von Frey stimulation evoked ERK1/2 phosphorylation in both

laminae I-II_o and II_i-III_o, indicating that facilitation of a neural circuit encompassing laminae I-III is associated with secondary static mechanical allodynia. This circuit involves lamina II_i PKC γ interneurons: (i) 20-30% of lamina II_i phospho-ERK1/2-immunoreactive neurons after mechanical stimulation were also PKC γ -immunoreactive, (ii) mechanical stimulation-induced phosphorylations of PKC γ and ERK1/2 were detected at the same delay (+30-min) after capsaicin injection, and, (iii) intracisternal PKC γ antagonist prevented capsaicin-induced von Frey-evoked phospho-ERK1/2 immunoreactivity and associated static mechanical allodynia. Accordingly, genetic impairment of PKC γ was shown to reduce injury-induced mechanical hypersensitivity to innocuous punctate stimuli (31,32,33). Such pattern of neuronal activation is not consistent with A- or C-nociceptors transmitting inputs that produce pain: (i) in rats, TRPV1 receptors are expressed by most peptidergic and non-peptidergic C-nociceptors (41,42) and we show that neurons activated by capsaicin are restricted to laminae I-II_o; (ii) selectively stimulating A-nociceptors in the area of secondary hyperalgesia results in only lamina I neural activation (43). The laminae I-III pattern of neural activation rather resembles that associated with dynamic mechanical allodynia where A β -LTMR inputs to lamina II_i PKC γ interneurons can gain access to the lamina I pain transmission circuitry (14,23). Our results thus suggest that innocuous punctate inputs that produce secondary static mechanical allodynia are transmitted by LTMRs, which terminate within or below lamina II_i (44), and engage lamina I neurons by way of a dorsally-directed excitatory pathway including lamina II_i PKC γ interneurons. This circuit is polysynaptic as axonal terminals of lamina II_i PKC γ interneurons do not reach lamina I (45) but contact lamina II_o central neurons (46). Consistently, lamina I output neurons, which do not normally respond to innocuous stimuli, can be driven by static mechanical inputs in neuropathic rats (47), and by polysynaptic A β - and A δ -fiber inputs in DH slices, after GABA_A/glycinergic disinhibition (48). That capsaicin-induced secondary static and dynamic mechanical allodynias can manifest independently

from each other (see above) suggests that the two allodynias are signaled by different primary afferent pathways. Since dynamic mechanical allodynia is signaled by A β -LTMRs and PKC γ interneurons are exclusively targeted by myelinated primary afferents (27,37), secondary static mechanical allodynia is therefore likely signaled by A δ -LTMRs. Interestingly, mice genetically impaired for Piezo2, a major mechanical transducer in A β -LTMRs, exhibit no change in static mechanical allodynia in response to inflammatory mediators (49).

As previously reported (40,50), phospho-ERK1/2 was induced 5 min after capsaicin injection. But von Frey-evoked ERK1/2 activation – as PKC γ phosphorylation – was only detected 30 min after injection. This supports the idea that the signaling cascade initiating secondary static mechanical allodynia reaches a peak more than 5 min after the capsaicin-induced barrage from C-nociceptors. Consistently, the area of secondary hyperalgesia to punctate stimuli, immediately present after injection, grows to a maximum within 15-30 min after capsaicin injection (5).

The induction of secondary static mechanical allodynia using capsaicin requires the interaction of two primary afferent pathways: a conditioning one, carried by TRPV1-sensitive C-nociceptors terminating onto laminae I-II_o neurons, as indicated by the capsaicin-induced phospho-ERK1/2 immunoreactivity (40; present results), and a facilitated one, signaling innocuous punctate stimuli that produce pain, carried by A-LTMRs targeting lamina II_i PKC γ interneurons. How can such two mutually exclusive afferent pathways interact with each other? One possibility is through GABA_Aergic disinhibition. First, local GABA_Aergic disinhibition in our study produced a selectively static mechanical allodynia. In a previous study in anaesthetized rats, static, but not dynamic, mechanical stimulation could elevate blood pressure, a reliable indicator of pain, after bicuculline microinjection into MDH (14). Second, under GABA_Aergic disinhibition, innocuous static mechanical stimulation generated

a pattern of ERK1/2 activation across not only laminae I-II_o, as previously found in models of neuropathic pain (51), but also laminae II_i-III_o. Finally, such activation involved lamina II_i PKC γ interneurons. This supports the idea that the same local circuit is associated with capsaicin- and GABA_Aergic disinhibition-induced static mechanical allodynia. Accordingly, we found that intracisternal administration of a GABA_A receptor agonist prevents capsaicin-induced static mechanical allodynia. This suggests that A δ -LTMR inputs to PKC γ interneurons are under GABA_Aergic inhibition and, when this inhibition is lifted, these inputs can gain access to the lamina I pain transmission circuitry. Consistently, in DH slices, bath-applied capsaicin, which mimics C-nociceptor activation, reduces GABA_Aergic inhibition onto lamina II neurons (35,52). And local bicuculline application enhances DH neuronal responses to A δ -fiber-, but not A β -fiber-, mediated mechanical and electrical stimulation (53,54,55).

Another possibility is that the conditioning input, carried by C-nociceptors, sensitizes lamina II_i PKC γ interneurons via diffusible factors such as ROS. ROS are known to be released during the capsaicin-induced barrage from C-nociceptors (38) and to decrease selectively GABA release within DH (21). We found that the capsaicin-induced static mechanical allodynia and associated touch-evoked phospho-ERK1/2-immunoreactivity are replicated by intracisternal t-BOOH and, conversely, prevented by intracisternal PBN. Consistently, intrathecal ROS scavengers were shown to reverse the hypersensitivity to von Frey stimulation in neuropathic rats (21,56). Although a link between ROS release in DH and secondary hypersensitivity to punctate stimuli was previously described (Schwartz et al., 2008), the present results establish that ROS release leads to activation of the lamina I-III neuronal circuit associated with behavioral allodynia. Interestingly, alone, t-BOOH triggered no phospho-ERK1/2 immunoreactivity, indicating that ROS do not directly activate MDH neurons. On the basis of these results, we propose a model whereby ROS is released within

MDH during the capsaicin-induced barrage from C-nociceptors (38) and produces a selective GABA_Aergic disinhibition of lamina II_i PKC γ interneurons (Fig. 10).

Altogether, our results reveal a new mechanism for secondary hyperalgesia: sensitization of a polysynaptic, dorsally-directed excitatory circuit whereby static innocuous inputs likely carried by A δ -LTMRs terminating onto lamina II_i PKC γ interneurons can gain access to the pain transmission circuitry of superficial MDH/DH, producing static mechanical allodynia (Fig. 10). Thus both the primary afferent that transmits the input producing pain and the mechanisms of sensitization within MDH/DH of static mechanical allodynia are different from those of the two other mechanical pain symptoms characterizing secondary hyperalgesia: dynamic allodynia and static hyperalgesia. On one hand, secondary dynamic mechanical allodynia is signaled by A β -LTMR (39,57). A β -LTMR inputs onto PKC γ interneurons are controlled by selectively glycinergic feed-forward inhibition (14,22,46). Such glycinergic inhibition being lifted, A β -LTMR inputs can engage lamina I nociceptive neurons (22). On the other hand, secondary static mechanical hyperalgesia is signaled by A δ -nociceptors (6,9). And pain amplification likely relies on the unmasking and/or heterosynaptic strengthening of non-activated afferents converging onto MDH/DH neurons activated by C-nociceptors (58,59,60). Together with our result that static mechanical allodynia, but not capsaicin-induced pain behavior, is sensitive to both ROS scavenger and PKC antagonist, this indicates that individualized mechanism-based treatments are required for each of these symptoms.

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Competing financial interests

The authors declare no competing financial interests

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Figure legends

Figure 1. Intradermal injection of capsaicin into the vibrissa pad induces rubbing nociceptive behavior and static, but not dynamic, mechanical allodynia. (A) Bar histogram of the duration of the rubbing behavior in capsaicin (25 μ g)-treated and control rats (n = 5 per group). (B) Time course of changes in behavioral responses (allodynic score) evoked by static (6-g von Frey filament) and dynamic mechanical stimuli (air puff) applied 0.5-1 cm to the injection site, on the area of secondary hypersensitivity of the capsaicin-treated rats (n = 5). Capsaicin induces a static, but not dynamic, mechanical allodynia. (C, D) Images of phospho-ERK1/2 (phospho-ERK1/2) immunolabeling in the MDH of rats killed 30 min after intradermal injection of capsaicin with (D) or without (C) 3-min static mechanical stimulation (6-g von Frey filament) applied on the area of secondary hypersensitivity. Stippled lines indicate, from dorsal to ventral, the limits of lamina I and outer (II_o) and inner (II_i) lamina II. Phospho-ERK1/2 immunoreactive cells are exclusively located within the most superficial laminae I-II_o of MDH following rubbing nociceptive behavior whereas they encompass laminae I-II_o as well as laminae II_i-outer III (III_o) after static mechanical stimulation in capsaicin-treated rats. (E) Bar histogram of the total number of phospho-ERK1/2 immunoreactive cells within ipsilateral MDH, 5 and 30 min after either intradermal endolipid alone or capsaicin, with and without 3-min static mechanical stimulation (6-g von Frey filament) applied on the area of secondary hypersensitivity (n = 5 per group). Compared with capsaicin alone, mechanical stimulation leads to a larger number of phospho-ERK1/2 immunoreactive cells when it is applied 30 min, but not 5 min, after capsaicin injection. (F) Bar histogram of the number of phospho-ERK1/2 immunoreactive cells within laminae I-II_o, laminae II_i-outer III (III_o) and laminae inner-III (III_i)-V, 30 min after either intradermal endolipid alone or capsaicin, with and without 3-min static mechanical stimulation (6-g von

Frey filament) applied on the area of secondary hypersensitivity. Note that mechanical stimulation enhances the number of phospho-ERK1/2 immunoreactive cells in both laminae I-II_o and laminae II_i-III_o. Data are represented as the mean \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 2. PKC γ -immunoreactive interneurons are activated and PKC γ phosphorylated during expression of static mechanical allodynia. (A) Fluorescence images of PKC γ -immunoreactive interneurons (green), phospho-ERK1/2-immunoreactive cells (red) and double labeled neurons (white arrows) in lamina II_i of the MDH. Insets show the dually labeled neuron into the white stippled square. (B) Top, Western blots for phospho-PKC γ and β -actin on total MDH proteins for the four groups of rats as indicated below the bar histogram. Bottom, bar histogram of the quantification of Western blotting. Values in each blot are normalized to β -actin staining and then to that in endolipid-injected and mechanically-stimulated (6-g von Frey filament) rats. Treatments are indicated below the bars: (i) time after intradermal capsaicin at which mechanical stimulation is applied, just before animal death, (ii) 3-min static mechanical stimulation (6-g von Frey filament) applied 0.5-1 cm to the injection site, on the area of secondary hypersensitivity. Data are represented as the mean \pm SEM., n = 4 per group. *** P < 0.001.

Figure 3. Activation of PKC γ is required for the expression of capsaicin-induced static-mechanical allodynia but not rubbing nociceptive behavior. (A) Bar histogram of the duration of the rubbing behavior in capsaicin-treated rats which have preemptively received intracisternal Tat-carrier (50 pmole) alone or KIG31-1 (50 pmole) (n = 5 per group). The rubbing nociceptive behavior is not suppressed in KIG31-1-treated rats. (B) Time course of changes in behavioral responses (allodynic score) evoked by static mechanical stimuli (6-g

von Frey filament) applied 0.5-1 cm to the injection site, on the area of secondary hypersensitivity of the capsaicin-treated rats, preemptively treated with intracisternal KIG31-1 or Tat-carrier alone (n = 5 per group). Static mechanical allodynia is completely suppressed in KIG31-1-treated rats. (C) Bar histogram of the number of phospho-ERK1/2-immunoreactive cells in laminae I-II_o, II_i-III_o and III_i-V following 3-min static mechanical stimulation (6-g von Frey filament) applied on the area of secondary hypersensitivity, 30 min after capsaicin injection, in rats preemptively intracisternally injected with Tat-carrier (500 pmole) or KIG31-1 (500 pmole) (n = 4 per group). Intracisternal KIG31-1 prevents stimulation-induced elevation in the number of phospho-ERK1/2-immunoreactive cells in laminae I-II_o as well as II_i-III_o in capsaicin-treated rats. Data are represented as the mean ± SEM. * P < 0.05, *** P < 0.001.

Figure 4. GABA_Aergic disinhibition induces static, but not dynamic, mechanical allodynia. Time course of changes in behavioral responses (allodynic score) evoked by static (6-g von Frey filament) and dynamic mechanical stimuli (air puff) applied on the face of rats after intracisternal injection of bicuculline (0.05 µg in 5 µl) and aCSF. Intracisternal bicuculline induces a static, but not dynamic, mechanical allodynia lasting for about 30 min. Data are represented as the mean ± SEM., n = 5 per group. * P < 0.05, *** P < 0.001.

Figure 5. Static mechanical stimulation under GABA_Aergic disinhibition activates a neural circuit which encompasses both superficial and deeper laminae. (A, B) Images of phospho-ERK1/2 immunolabeling in the MDH of rats after intracisternal injection of bicuculline (3 µg in 5 µl) with (B) and without (A) 3-min static mechanical stimulation (6-g von Frey filament) of the face. Stippled lines indicate, from dorsal to ventral, the limits of laminae I, II_o and II_i. Whereas there is no or very few phospho-ERK1/2-immunoreactive cells

after bicuculline alone, there are many more after bicuculline and static mechanical stimulation, in laminae I, II_o, II_i and III_o. (C) Bar histogram of the number of phospho-ERK1/2-immunoreactive cells in laminae I-II_o, II_i-III_o and III_i-V, after intracisternal injection of aCSF or bicuculline, with and without 3-min static mechanical stimulation (6-g von Frey filament) of the face. Phospho-ERK1/2 signals are enhanced following bicuculline alone, but only in laminae I-II_o, whereas they are further increased in now laminae I-II_o, II_i-III_o and III_i-V after static mechanical stimulation under GABA_Aergic disinhibition. Data are represented as the mean ± SEM., n = 5 per group. * P < 0.05, ** P < 0.01, *** P < 0.001. (D-F) Fluorescence images of PKCγ-immunoreactive neurons (green; D), phospho-ERK1/2-immunoreactive cells (red; e) and dually labeled neurons (white arrows; F).

Figure 6. Intracisternal injection of the GABA_A receptor agonist, muscimol, prevents capsaicin-induced both rubbing nociceptive behavior and static mechanical allodynia.

(A) Bar histogram of the duration of the rubbing nociceptive behavior in capsaicin-treated rats which have preemptively received an intracisternal injection of aCSF (n = 5) or muscimol (250 ng; n = 4). Capsaicin-induced rubbing nociceptive behavior is suppressed by intracisternal muscimol. (B) Time course of the changes in behavioral responses (allodynic score) evoked by static mechanical stimuli (6-g von Frey filament) applied 0.5-1 cm to the injection site, on the area of secondary hypersensitivity of rats preemptively treated with intracisternal muscimol (n = 4) or aCSF (n = 5). Static mechanical allodynia is suppressed by intracisternal muscimol. Data are represented as the mean ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 7. Intracisternal application of the ROS donor, t-BOOH, induces a static, but not a dynamic mechanical allodynia. (A, C) Time courses of the changes in behavioral

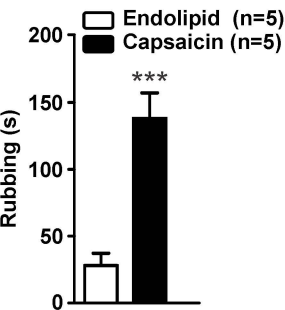
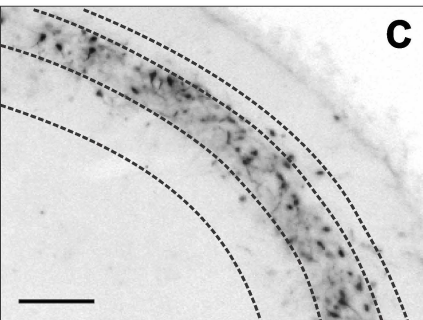
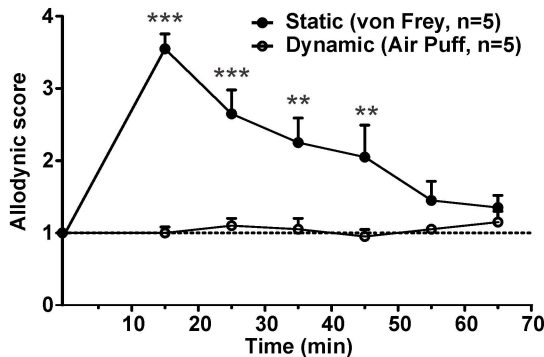
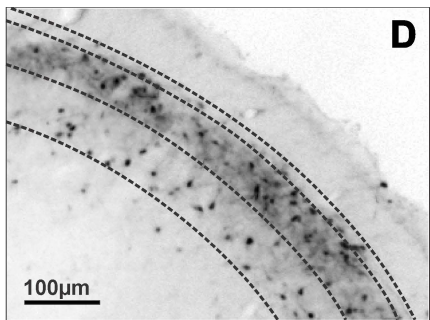
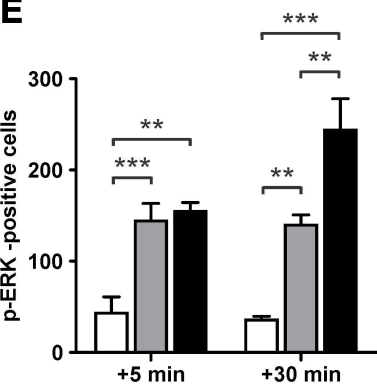
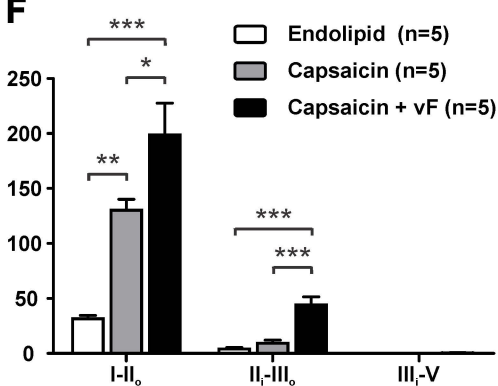
responses (allodynic score) to static (6-g von Frey filament; **A**) and dynamic mechanical stimuli (air puff; **c**) applied on the face of rats intracisternally injected with aCSF or increasing doses of t-BOOH: 50, 100 and 250 g. (**B, D**) Bar histograms of the corresponding areas under the curves for static (**B**) and dynamic mechanical stimuli (**D**). ROS mainly produce a static mechanical allodynia. Data are represented as the mean \pm SEM., n = 5 per group. * P < 0.05, ** P < 0.01, *** P < 0.001.

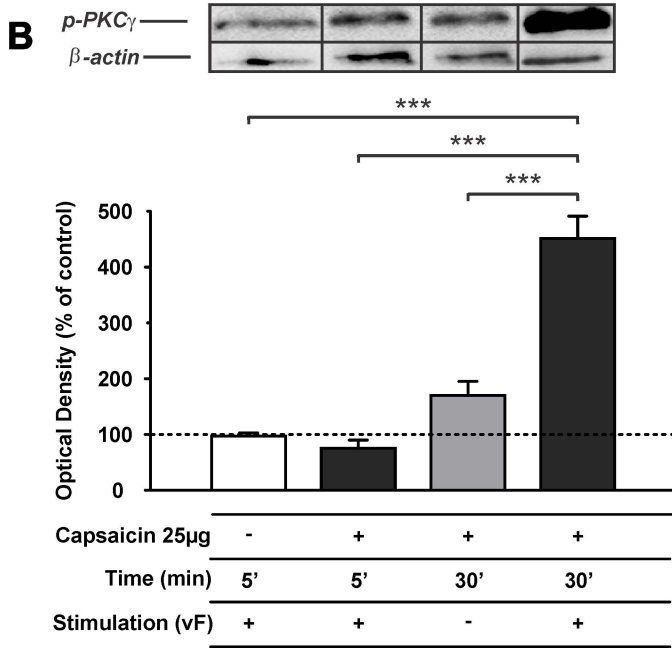
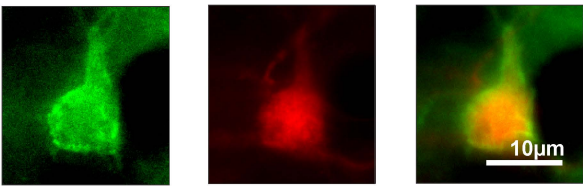
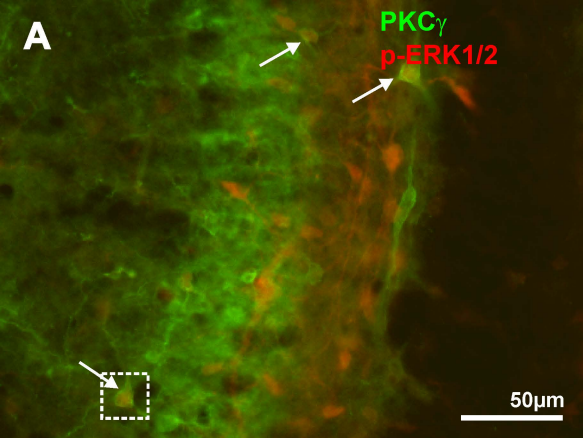
Figure 8. Static mechanical stimulation under ROS donor activates a neural circuit which encompasses both superficial and deeper laminae. (**A, B**) Images of phospho-ERK1/2 immunolabeling in the MDH of rats killed 60 min after intracisternal t-BOOH (100 ng) with (**B**) and without (**A**) 3-min static mechanical stimulation (6-g von Frey filament) of the face. Stippled lines indicate, from dorsal to ventral, the limits of laminae I, II_o and II_i. Whereas there are no or very few phospho-ERK1/2-immunoreactive cells after intracisternal t-BOOH alone, there are many more in laminae I, II_o, II_i and III_o after intracisternal t-BOOH with static mechanical stimulation. (**C**) Bar histogram of the number of phospho-ERK1/2 immunoreactive cells in laminae I-II_o, II_i-III_o and III_i-V, 60 min after either aCSF or t-BOOH (100 ng), with and without 3-min static mechanical stimulation (6-g von Frey filament) of the face (n = 5, 4 and 4 per group, respectively). Static mechanical stimulation enhances the number of phospho-ERK1/2 immunoreactive cells in laminae I-II_o as well as II_i-III_o when it is applied under ROS donor. Data are represented as the mean \pm SEM. ** P < 0.01. (**D-F**) Fluorescence images of PKC γ -immunoreactive neurons (green; **D**), phospho-ERK1/2-immunoreactive cells (red; **E**) and dually labeled neurons (white arrows; **F**) in lamina II_i of the MDH.

Figure 9. Intracisternal application of the ROS scavenger, PBN, prevents capsaicin-induced static mechanical allodynia but not rubbing nociceptive behavior. (A) Bar histogram of the duration of the rubbing nociceptive behavior in capsaicin-treated rats which have preemptively received intracisternal aCSF or PBN (100 μ g) (n = 5 per group). Rubbing nociceptive behavior is not suppressed in PBN-treated rats. (B) Time course of changes in behavioral responses (allodynic score) evoked by static mechanical stimuli (6-g von Frey filament) applied 0.5-1 cm to the injection site, on the area of secondary hypersensitivity of rats preemptively treated with intracisternal PBN or aCSF (n = 5 per group). Static mechanical allodynia is suppressed in PBN-treated rats. (C) Bar histogram of the number of phospho-ERK1/2-immunoreactive cells in laminae I-II_o, II_i-III_o and III_i-V in capsaicin-treated rats after 3-min static mechanical stimulation (6-g von Frey filament) applied on the area of secondary hypersensitivity of rats preemptively treated with intracisternal aCSF or PBN (n = 4 per group). Intracisternal PBN prevents stimulus-induced elevation in the number of phospho-ERK1/2-immunoreactive cells in laminae I-II_o as well as II_i-III_o in capsaicin-treated rats. Data are represented as the mean \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 10. Schematic diagram illustrating the putative DH neural circuit mediating static mechanical allodynia. Mechanical allodynia results from the activation of preexisting polysynaptic pathways that drive up tactile inputs toward lamina I output neurons. Such polysynaptic pathways are normally silent, under strong inhibitory control, but become unmasked after disinhibition (48) which likely occurs in pathological conditions. Lamina II_i PKC γ interneurons appear to be key elements for circuits activated after glycinergic (14,22) as well as GABA_Aergic disinhibition (present results). Recently, Lu et al. (46) provided evidence for convergence of glycinergic inhibitory and excitatory A β -LTMR inputs onto PKC γ interneurons. This feed-forward inhibitory circuit prevents A β -LTMR input from

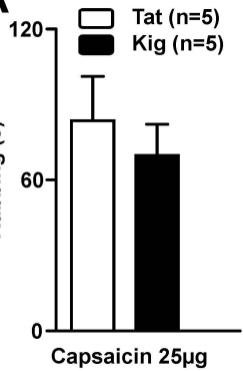
activating the nociceptive pathway. We assume a similar convergence of GABA_Aergic inhibitory and excitatory A δ -fiber inputs onto PKC γ interneurons. Such feed-forward inhibitory circuit would, in turn, prevent A δ -LTMR input from activating PKC γ interneurons. On the other hand, during the barrage from C-nociceptors, ROS is released within MDH and produces a selective GABA_Aergic disinhibition. A δ -LTMR inputs can then engage PKC γ interneurons and the dorsally-directed, polysynaptic excitatory pathways to lamina I. This diagram also illustrates a lamina II_o central neuron, one of the postsynaptic targets of PKC γ interneurons (46).

A**B****C****D****E****F**

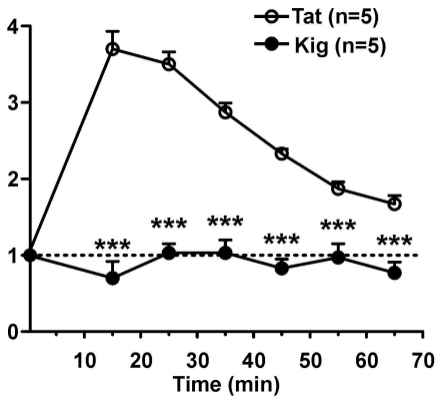


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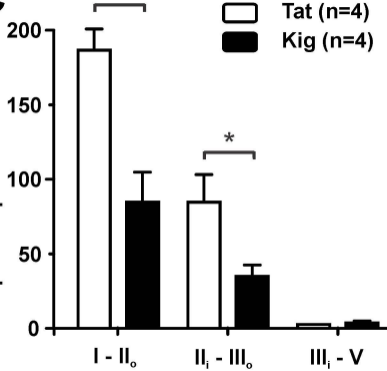
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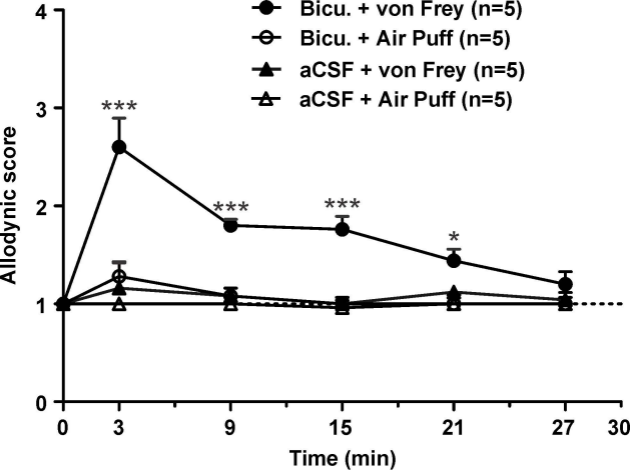
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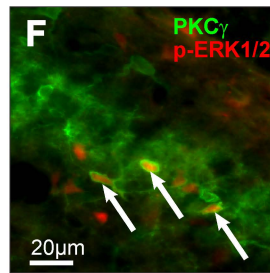
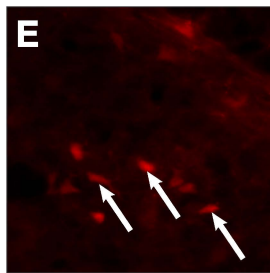
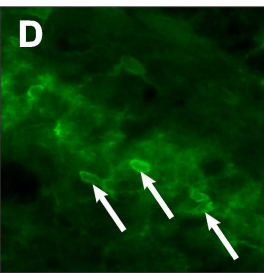
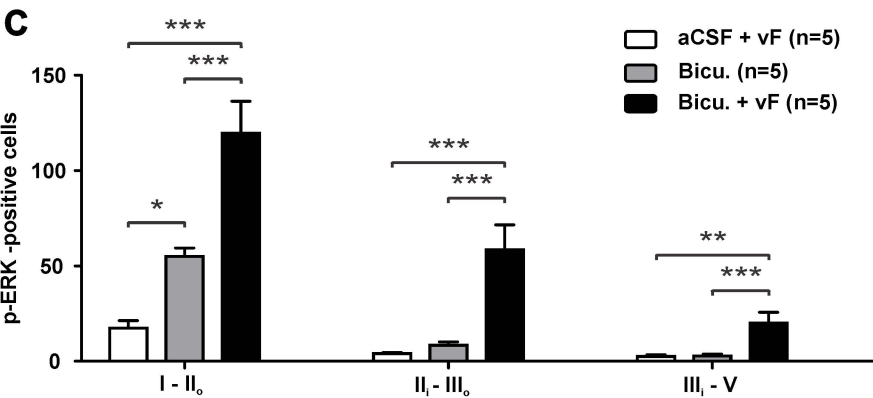
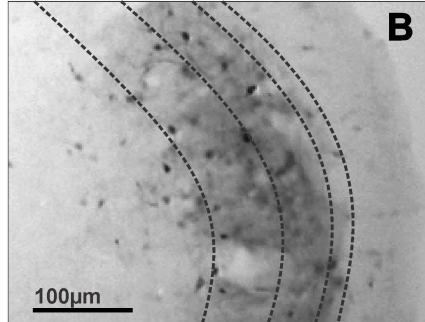
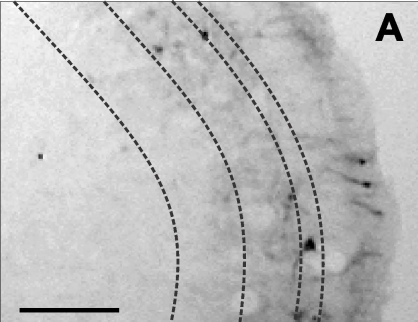
Allodynic score

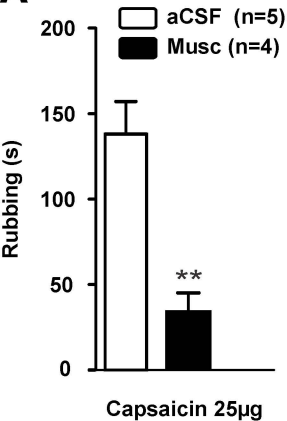
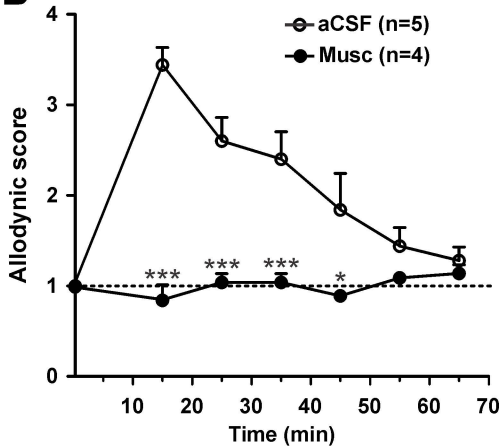
**C**

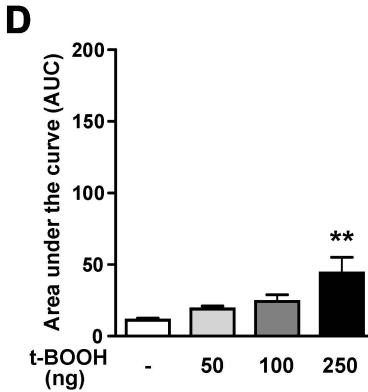
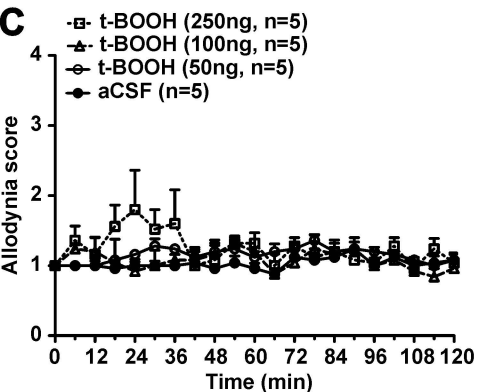
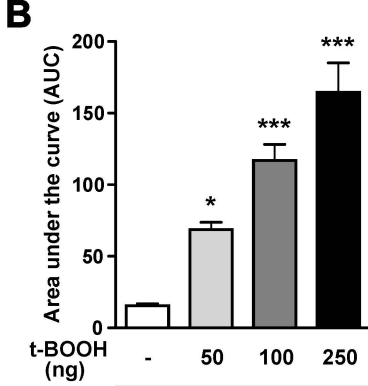
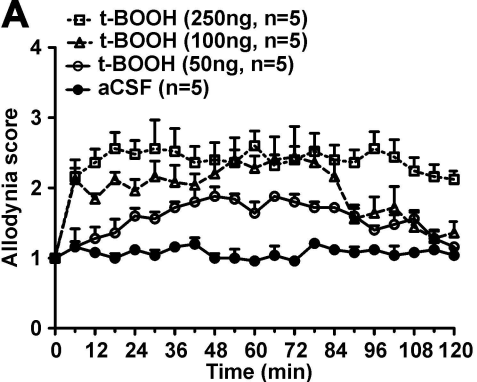
p-ERK - positive cells

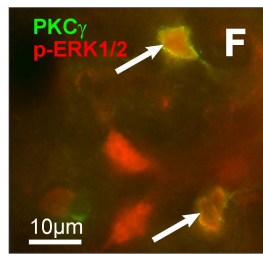
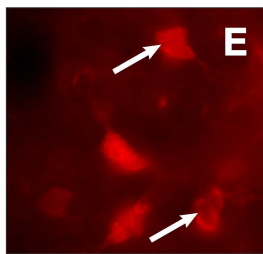
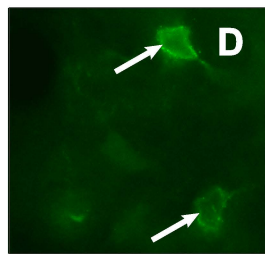
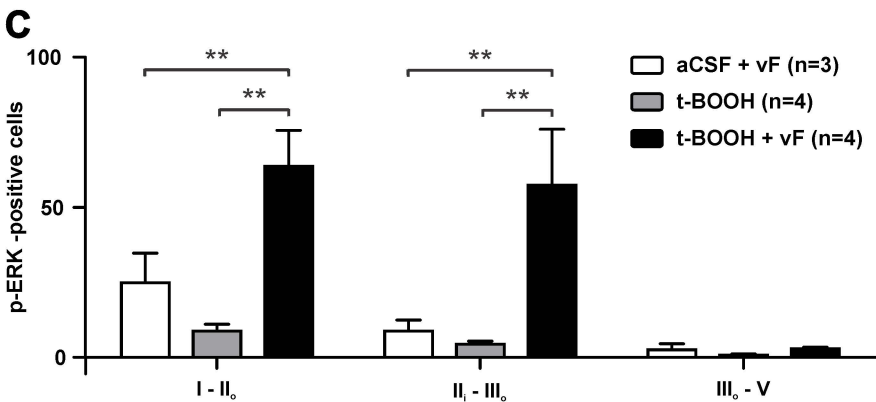
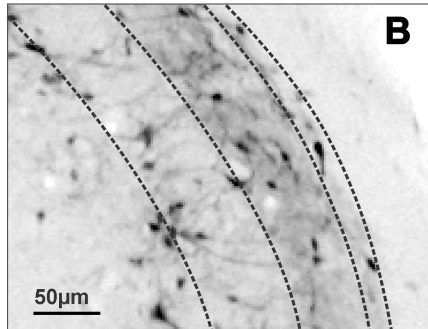
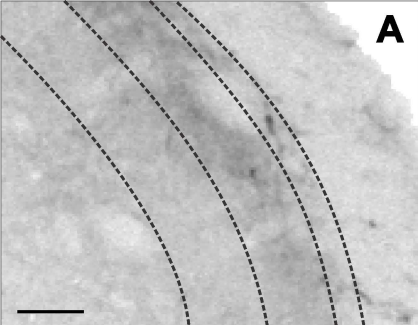


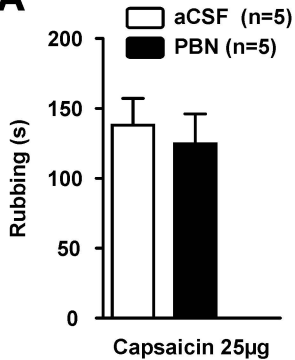
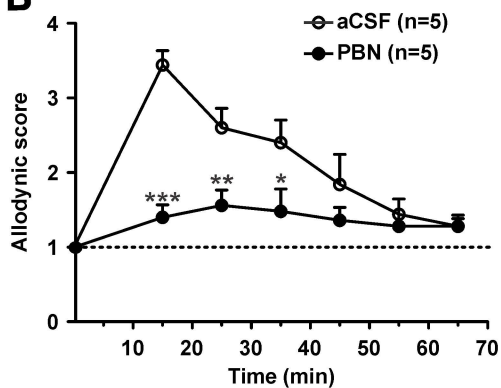




A**B**





A**B****C**