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Activity-dependent potentiation of calcium signals in spinal sensory networks in inflammatory pain states

Ceng Luo [a,*], Peter H. Seeburg [b], Rolf Sprengel [b], Rohini Kuner [a,*]

[a] *Pharmacology Institute, University of Heidelberg, Im Neuenheimer Feld 366, Heidelberg 69120, Germany*
[b] *Max Planck Institute for Medical Research, Department of Neurobiology, Jahnstrasse 29, Heidelberg 69120, Germany*

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Abstract

The second messenger calcium is a key mediator of activity-dependent neural plasticity. How persistent nociceptive activity alters calcium influx and release in the spinal cord is not well-understood. We performed calcium-imaging on individual cell bodies and the whole area within laminae I and II in spinal cord slices from mice in the naïve state or 24 h following unilateral hindpaw plantar injection of complete Freund's adjuvant. Calcium signals evoked by dorsal root stimulation at varying strengths displayed a steep rise and slow decay over 15–20 s and increased progressively with both increasing intensity and frequency of stimulation in naïve mice. Experiments with pharmacological inhibitors revealed that both ionotropic glutamate receptors and intracellular calcium stores contributed to maximal calcium signals in laminae I and II evoked by stimulating dorsal roots at 100 Hz frequency. Importantly, as compared to naïve mice, we observed that in mice with unilateral hindpaw inflammation, calcium signals were potentiated to $159 \pm 10\%$ in the ipsilateral dorsal horn and $179 \pm 8\%$ in the contralateral dorsal horn. In addition to the contribution from NMDA receptors, GluR-A-containing AMPA receptors were found to be critically required for the above changes in spinal calcium signals, as revealed by analysis of genetically modified mouse mutants, whereas intracellular calcium release was not required. Thus, these results suggest that there is an important functional link between calcium signaling in superficial spinal laminae and the development of inflammatory pain. Furthermore, they highlight the importance of GluR-A-containing calcium-permeable AMPA receptors in activity-dependent plasticity in the spinal cord.

Keywords: Spinal cord; Hyperalgesia; AMPA receptors; NMDA receptors; Calcium-imaging; Primary afferents

1. Introduction

Activity-dependent neural plasticity is a prime mechanism underlying clinical transitions between physiological pain and chronic, pathological pain [19]. Various converging lines of evidence have revealed the importance of the second messenger calcium and calcium-dependent pathways in central sensitization. Mobilization of intracellular calcium upon neuronal activation is the main trigger for activation of a variety of signaling mediators, such as CamKII-alpha, Protein Kinase A and extracellular receptor activated kinases (ERK1/2); these, in turn, regulate the expression and functions of downstream proteins determining the excitability of neurons, which are involved in pain processing [19]. Mechanisms underlying activity-dependent changes in calcium signaling in the somatosensory pain pathway are therefore helpful towards understanding the development of chronic pain.

By virtue of housing the first, crucial synapse in the pain pathway, the spinal dorsal horn constitutes one of the most important sites in plasticity mechanisms leading to chronic pain. Most of the early work on

* Corresponding authors. Tel.: +49 6221 548289; fax: +49 6221 548549.
E-mail addresses: ceng.luo@pharma.uni-heidelberg.de (C. Luo) and rohini.kuner@pharma.uni-heidelberg.de (R. Kuner).

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understanding calcium regulation in the spinal cord was focussed upon imaging individual motor neurons, and only very few studies have addressed calcium signaling in spinal sensory neurons [13]. In particular, activity-induced changes in calcium dynamics in spinal sensory networks in states of chronic, pathological pain have not been addressed so far. Therefore, several critical issues remain to be understood: Are spinal calcium responses evoked by nociceptors and tactile afferents different in nature and duration? Are they potentiated upon persistent activation of nociceptors? Is calcium release in spinal laminae I and II altered in states of chronic pain? Which receptor systems mediate activity-induced changes in calcium mobilization in ensembles of spinal neurons and pathological transitions thereof?

Here, we have performed calcium-imaging in laminae I and II of spinal dorsal horn in acutely prepared mouse spinal cord slices to understand the pattern and magnitude of calcium alterations upon activation of tactile afferents and nociceptors. Using a model of unilateral hind paw inflammation, we show that calcium signals in spinal networks are potentiated in striking spatial patterns in states of inflammatory pain. Furthermore, using a combination of pharmacological approaches and genetically modified mouse mutants, we show that NMDA receptors and Ca^{2+}-permeable AMPA receptors are the primary mediators of activity-induced changes in spinal calcium mobilization in chronic inflammatory pain states.

2. Materials and methods

2.1. Preparation of spinal cord slices

Mice (11- to 15-day-old) were anesthetized with urethane (1.2 g/Kg, i.p.), and then lumbosacral laminectomy was performed. The lumbosacral spinal cord was removed and placed in pre-oxygenated Krebs solution at 1–3 °C. The spinal cord was mounted on a vibratome (HM 650 V, MICROM, Germany), and 400–500 μm-thick transverse slices each with an attached dorsal root were cut. The slices were stored in an incubation solution at 33 °C (in mM: NaCl, 95; KCl, 1.8; KH_2PO_4, 1.2; $CaCl_2$, 0.5; $MgSO_4$, 7; $NaHCO_3$, 26; glucose, 15; sucrose, 50; oxygenated with 95% O_2, 5% CO_2; pH 7.4).

2.2. Fluorometric Ca^{2+} measurements in the spinal dorsal horn

Slices were incubated for 30 min at room temperature with Fura-2 AM (10 μM), pluronic acid (0.1%) and HEPES (20 mM), followed by $a \geqslant 30$ min de-esterification period as described in details previously [16]. Loaded slices were then transferred to a recording chamber mounted on a Zeiss Axiovert 135 microscope in a chamber with a glass coverslip bottom (13 μm in thickness) and visualized using an inverse air 10× objective with a numerical aperture of 0.5 NA. Slices were superfused (2.5 ml/min) by carbogen-aerated recording solution consisting of the following (in mM): NaCl, 127; KCl, 1.8; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 15 and illuminated with a monochromator and images were captured with a cooled CCD camera (TILL Photonics, Gräfelfing, Germany) at 2 Hz. Consecutive paired exposures to 340 and 380 nm were used to achieve digital fluorescence images, and Fura-2 emissions were recorded at 510 nm. Fura-2 being a dual excitation dye, the intensity of emitted light increases with increasing Ca^{2+} concentrations at an excitation wavelength of 340 nm and decreases at 380 nm. Measurement of the F340/F380 ratio overcomes artefacts evoked by the differences in background fluorescence, uneven dye loading, slice thickness and photobleaching. Only laminae I and II from the spinal segments L4 and L5 were imaged. Lamina II was identified via its translucence in the transmission mode (Fig. 1A), and the gray matter above lamina II constituted lamina I. During analysis of calcium transients, either the entire area of laminae I and II (whole area) or 10–20 individual somata within these two laminae were selected using the 'region of interest' tool and the corresponding calcium responses were then shown as either 'soma + neuropil' (corresponding to the whole area of laminae I and II) or 'soma alone' (corresponding to the average responses over the selected somata). Only those somata in laminae I + II which demonstrated good loading of Fura-2 and showed clear and reproducible responses upon phasic stimulation of primary afferents were selected in all slices. The selected regions were the same over all experiments. Measurements were made in at least 5–10 slices/group, as indicated.

2.3. Primary afferent stimulation

The dorsal root was stimulated via a suction electrode (WPI, Sarasota). Pulse (0.5 ms width) at different frequencies (2, 10, 100 Hz) with constant intensity (3 mA) stimulation or different intensities (25, 100, 500, 1000 μA) with constant frequency (20 Hz) stimulation over 1 s was applied, unless stated otherwise. Stimulation parameters used for recruitment of C-, A-δ and A-β fibers were employed based on the results described by Nakatsuka et al. (2000) [12] and Torsney and McDermott (2006) [18].

2.4. CFA model of chronic inflammatory pain

Calcium measurements were performed on the spinal cord slices obtained from mice injected with Complete Freund's adjuvant (CFA; 15 μl) or saline (control) into the plantar surface in the center of left hindpaw as described previously [6] 24 h before slice preparation.

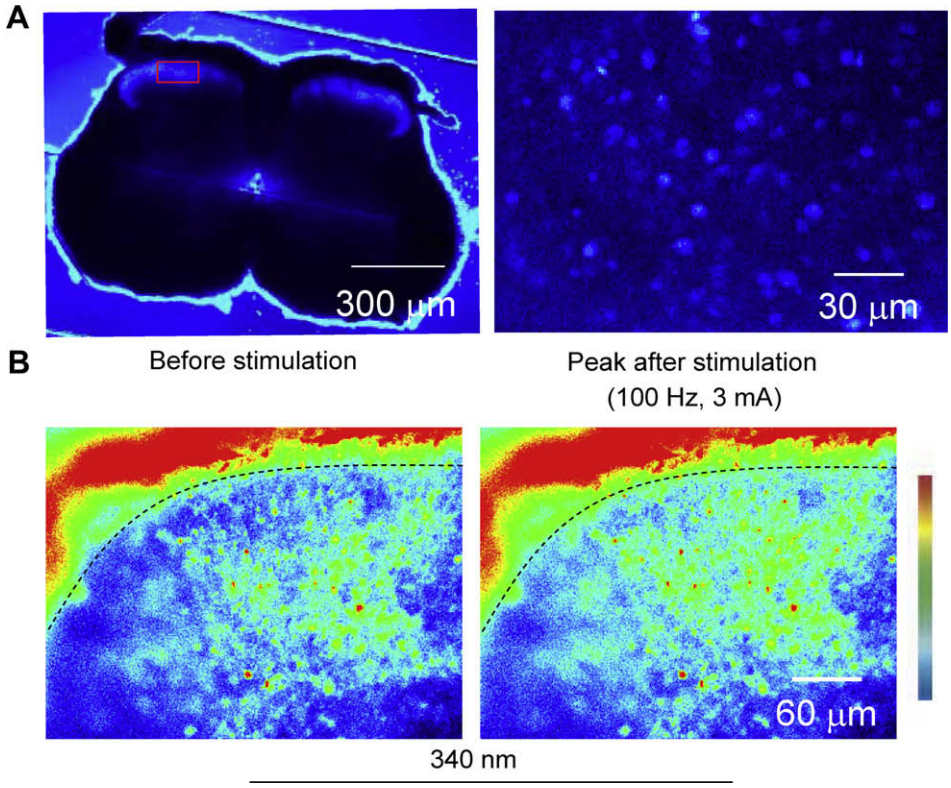

Fig. 1. Nature of calcium responses in laminae I and II of spinal cord slices and their dependence on the frequency and intensity of stimulation of the ipsilateral dorsal root (A) A spinal cord slice with attached dorsal root in the transmission mode; note the translucent nature of the substantia gelatinosa (lamina II), which aids selection of laminae I and II for Fura imaging. Fura-2-loaded neurons excited by light at 340 nm are magnified in the right panel. (B) Typical images captured in the superficial dorsal horn at 340 nm before stimulation (left) and after stimulation (right). Signals are shown in pseudocolour, and the intensity code is depicted on the scale at the right.

In some experiments, D-2-amino-5-phosphonovaleric acid (AP5; 5 mg/kg; Sigma) or Xestospongin C (XeC; 1 mg/kg; Sigma) were injected intraperitoneally once 10 min prior to intraplantar injection of CFA and again at 6 h after CFA treatment, and the slices derived from these mice were treated again with AP5 or XeC for 10 min prior to commencing calcium measurements as described below.

2.5. Bath application of drugs

Drugs were superfused into the recording chamber at defined final concentrations as indicated, and slices were incubated with drugs for at least 10 min. The following compounds were used (bought from Sigma unless otherwise stated): GYKI 52466 hydrochloride (100 μM), AP5 (50 μM), (+)-MCPG (1 mM), XeC (10 μM), Fura-2-AM (10 μM; AXXORA), pluronic acid (0.1%; Invitrogen). Stock solutions were prepared by dissolving D-AP5 in distilled water; XeC, Fura-2 AM in Dimethyl sulfoxide (DMSO); GYKI 52466 in 0.1 N HCl; (+)-MCPG in 0.1 N NaOH. The final concentrations upon dilution in the bath were as follows: HCl 0.0025 N, NaOH 0.002 N and DMSO 0.1% and vehicles used for dissolving drugs were employed as controls.

2.6. Statistical analysis

All data are presented as mean ± SEM. Analysis of variance (ANOVA) for random measures was performed followed by *post-hoc* Fisher test to determine statistically significant differences. $p < 0.05$ was considered significant.

3. Results

3.1. The features of calcium signals in spinal dorsal horn upon activation of primary afferents

Because our primary aim was to study calcium signals and their spread over populations of neurons in the superficial laminae of the spinal cord, it was imperative to bulk-label spinal cord slices. We observed that slices derived from mice which were older than 15 days did not take up Fura-2 sufficiently well to enable imaging, likely due to increased myelination. The data presented below are therefore derived from mice which were 11–15 days old. A large number of Fura-2-loaded cell bodies as well as processes were imaged over the entire laminae I/II (Fig. 1A and B), the main locations of synaptic connections between nociceptive Aδ- and C-fiber primary

afferents and spinal cord neurons. All cell bodies which demonstrated good loading with Fura-2 showed increased calcium signals after high-frequency stimulation. In general, calcium signals were larger in magnitude over the cell somata than in the surrounding neuropil (i.e. the space not occupied by somata, consisting of dendrites, axons, presynaptic terminals and glial processes). Using patch clamp analysis, a previous study has reported that a vast majority of Fura-2-stained dorsal horn cells in spinal slices represent neurons [13]. We observed spontaneous calcium waves in only 2 neurons of a total of 180 neurons analysed, although all 180 neurons showed evoked responses. The magnitude of spontaneous calcium waves was 25–30% of the maximal evoked calcium responses. It is conceivable that lack of segmental and supraspinal inputs in slice conditions alter spontaneous calcium signals.

In the somata as well as the neuropil, calcium signals displayed a steep rise and slower decay, with a return to near baseline values within 15–20 s (Fig. 2A; Fig. 1B). Peak amplitude of Ca^{2+} signals which were either averaged over the whole area or averaged over somata in laminae I and II increased with increasing frequency of dorsal root stimulation from 2 to 100 Hz at a constant (3 mA) intensity of stimulation (Fig. 2B; $n = 9$ slices). An increase in stimulation intensity, which leads to a complete recruitment of A-δ fibers (100 μA) over A-β fibers (25 μA) did not evoke significantly different calcium responses, suggesting that the contribution of A-δ fibers is less pronounced. Although it might be very revealing to examine the contribution of A-δ fibers independently of A-β fibers, as was done in electrophysiological studies by blocking A-β fiber conduction via anodal polarization [8], this could not be done here due to technical limitations. Importantly, upon additional recruitment of C-fibers via 500 μA stimulation [18] or via a supramaximal intensity of 3 mA at 20 Hz, peak Ca^{2+} responses increased significantly (Fig. 2C; $n = 6$).

Furthermore, we observed that the evoked spinal Ca^{2+} responses were not significantly different, when the dorsal root was stimulated at 20 Hz for 5 s or at 100 Hz for 1 s, (Fig. 2D; $p = 0.26$ for peak response and 0.86 for integrated Ca^{2+} response (data not shown); $n = 8$ slices). This suggests that activation of afferents at a lower frequency over a longer duration or by a brief high-frequency stimulation, which results in the same

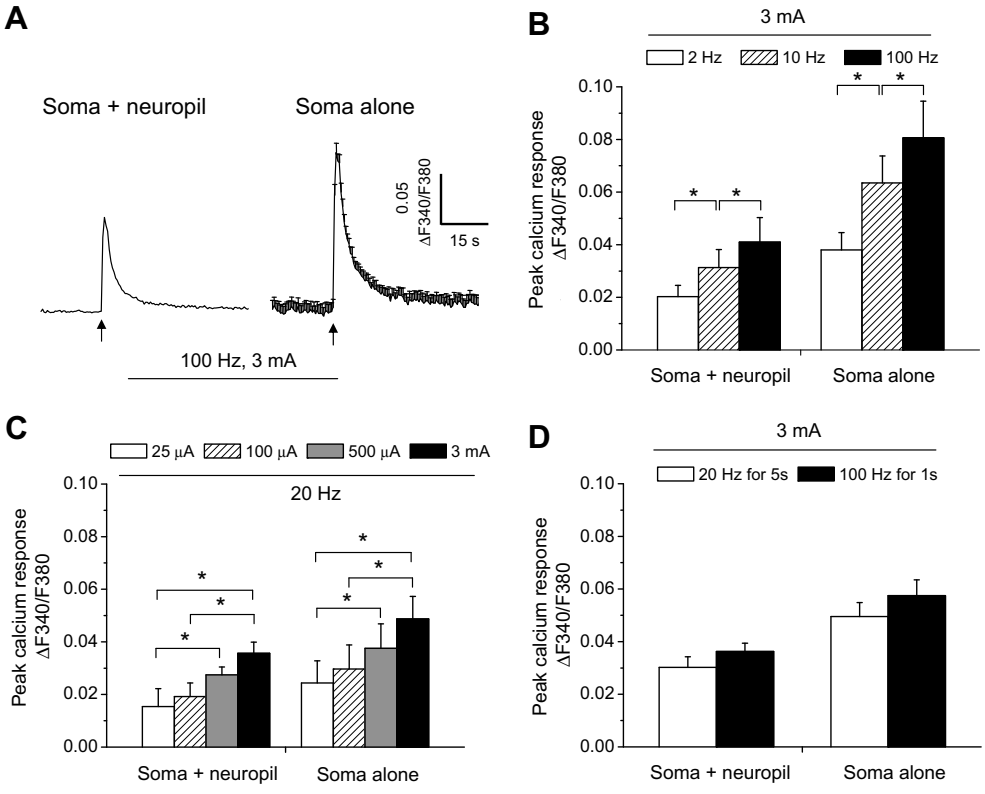

Fig. 2. Afferent-dependent properties of activity-induced calcium responses in the spinal dorsal horn. (A) Typical Ca^{2+} signals induced by stimulation of the dorsal roots in a region of interest in laminae I + II (soma + neuropil; left) or averaged over the soma of cells (soma alone; right) found in the same region of interest. Arrow indicates time of afferent stimulation. (B and C) Ca^{2+} responses induced by stimulation of dorsal roots increase progressively with an intensity- and frequency-dependent recruitment of Aβ, Aδ and C-fibers. Stimulation parameters are indicated. (D) Peak Ca^{2+} responses induced by stimulating dorsal roots at 20 Hz for 5 s or at 100 Hz for 1 s are comparable. $^{*}p \leqslant 0.05$, ANOVA followed by Fischer's test.

numbers of total stimulation pulses, leads to similar changes in post-synaptic calcium signals in the dorsal horn.

3.2. Contribution of diverse glutamate receptors to Ca^{2+} signals induced by activation of primary afferents

To investigate which receptor systems mediate spinal calcium responses evoked by repetitive firing of nociceptors, we pre-incubated spinal cord slices with receptor-selective antagonists, using concentrations which have been demonstrated to be specific in slice conditions. Repetition of the stimulation at 10-min intervals only led to a slight decline in response amplitude ($90 \pm 5\%$ of the first Ca^{2+} signal; $p = 0.07$ as compared to the first response; $n = 9$ slices). In the presence of AP5 (50 μM), an NMDA receptor antagonist, Ca^{2+} signals were reduced by $43 \pm 5\%$ in an entire area and by $35 \pm 6\%$ in the somata within laminae I/II in a reversible manner (Fig. 3A and B; $p < 0.05$; $n = 8$ slices). GYKI 52466 (100 μM), a drug which preferentially blocks AMPA receptors and binds kainate receptors minimally at this concentration, led to a reduction in the peak amplitude of the evoked calcium response over the whole laminae I/II by $46 \pm 8\%$ and the corresponding somata by $37 \pm 7\%$ in a reversible manner (Fig. 3A and B; $p < 0.05$; $n = 11$ slices).

In addition to evoking Ca^{2+} influx through ionotropic glutamate receptors, synaptic activity can mobilize Ca^{2+} release from intracellular stores through the activation of a variety of metabotropic receptors which signal via $G_{q/11}$, Phospholipase C (PLC) and Inositol-1,4,5-triphosphate receptors (IP_3R). Preincubation with XeC (10 μM), an inhibitor of IP_3R, reversibly suppressed the Ca^{2+} signal by $38 \pm 9\%$ in the entire laminae I and II and by $46 \pm 10\%$ in somata (Fig. 3A and B; $p < 0.05$; $n = 5$ slices). However, Ca^{2+} signals evoked by high-frequency afferent barrage were not altered by pre-incubation with MCPG (1 mM), an antagonist of the group I metabotropic glutamate receptors ($mGluR_{1/5}$) ($100 \pm 4\%$ in laminae I/II and $99 \pm 4\%$ in somata as compared to vehicle control; Fig. 3A and B; $p > 0.05$; $n = 5$ slices). Taken together, these results suggest that metabotropic receptors other than $mGluR_{1/5}$ receptors may be involved in intracellular calcium mobilization evoked by high-frequency activation of primary afferents.

3.3. Potentiated Ca^{2+} signals in inflammatory pain states

An important question which arises is whether spinal calcium responses evoked by the activation of nociceptive afferents are altered upon peripheral injury, as this might represent a direct correlate for nociceptive hypersensitivity. We employed a model of chronic inflammatory pain evoked by unilateral hindpaw inflammation upon intraplantar injection of complete Freund's adjuvant (CFA). Compared to saline-treated mice, spinal slices prepared from mice at 24 h after CFA injection showed significantly increased Ca^{2+} signals in the laminae I and II ipsilateral to CFA injection in response to stimulation of all primary afferents with 500 μA at 20 Hz (Fig. 3C–F). However, there were differences in the magnitude of inflammation-induced potentiation with respect to the types of recruited sensory neurons (Fig. 3C and D). In slices derived from CFA-treated mice, Ca^{2+} signals were potentiated to $209 \pm 8\%$ at 25 μA ($p < 0.05$; $n = 7$), $215 \pm 9\%$ at 100 μA ($p < 0.05$; $n = 7$) and $161 \pm 7\%$ at 500 μA ($p < 0.05$ as compared to saline group; $n = 7$; $p < 0.05$ as compared to stimulation at 25 or 100 μA in CFA group) of values in slices derived from saline-treated mice at 20 Hz. Thus, following inflammation, average potentiation of calcium signals evoked by Aß or Aß + Aδ neurons is higher than the net average potentiation evoked by Aß + Aδ + C-fibers, suggesting that spinal calcium responses to low intensity inputs potentiate to a larger extent than high-intensity inputs. Interestingly, although 100 Hz stimulation at 3 mA already represents a supramaximal stimulus frequently applied to evoke spinal long-term potentiation (LTP) [7], ipsilateral spinal Ca^{2+} signals evoked with these stimulation parameters were further potentiated following hindpaw inflammation ($159 \pm 10\%$ and $152 \pm 13\%$ of corresponding values from saline-treated slices in laminae I/ II and in somata, respectively; $p < 0.05$; $n = 19$).

Continuous blockade of NMDA receptors with AP5 during CFA-induced hindpaw inflammation reduced CFA-induced potentiation of ipsilateral spinal Ca^{2+} signals (Fig. 3E and F; $p < 0.05$ in the soma alone; $n = 5$). However, treatment with XeC during CFA-induced hindpaw inflammation did not clearly reduce CFA-induced potentiation of Ca^{2+} signals (Fig. 3E and F; $p > 0.05$; $n = 7$).

3.4. Role of Ca^{2+}-permeable AMPA receptors in potentiation of nociceptive activity-induced Ca^{2+} signals

Calcium permeability of glutamatergic AMPA receptors is determined by their subunit composition; AMPA receptors lacking the GluR-B subunit (GluR2) are permeable to calcium [1]. Although a majority of AMPA receptors in the CNS are calcium-impermeable, agonist-induced cobalt-uptake, a surrogate marker for agonist-induced calcium influx, has revealed a high density of Ca^{2+}-permeable AMPA receptors in the spinal dorsal horn, particularly in superficial laminae [3]. To address the role of Ca^{2+}-permeable AMPA receptors in nociceptive activity-induced Ca^{2+} signals, we used mice which constitutively lack either the GluR-A subunit (GluR-$A^{-/-}$; [20]) or the GluR-B subunit (GluR-$B^{-/-}$; [15]). Consistent with our previous observations regarding

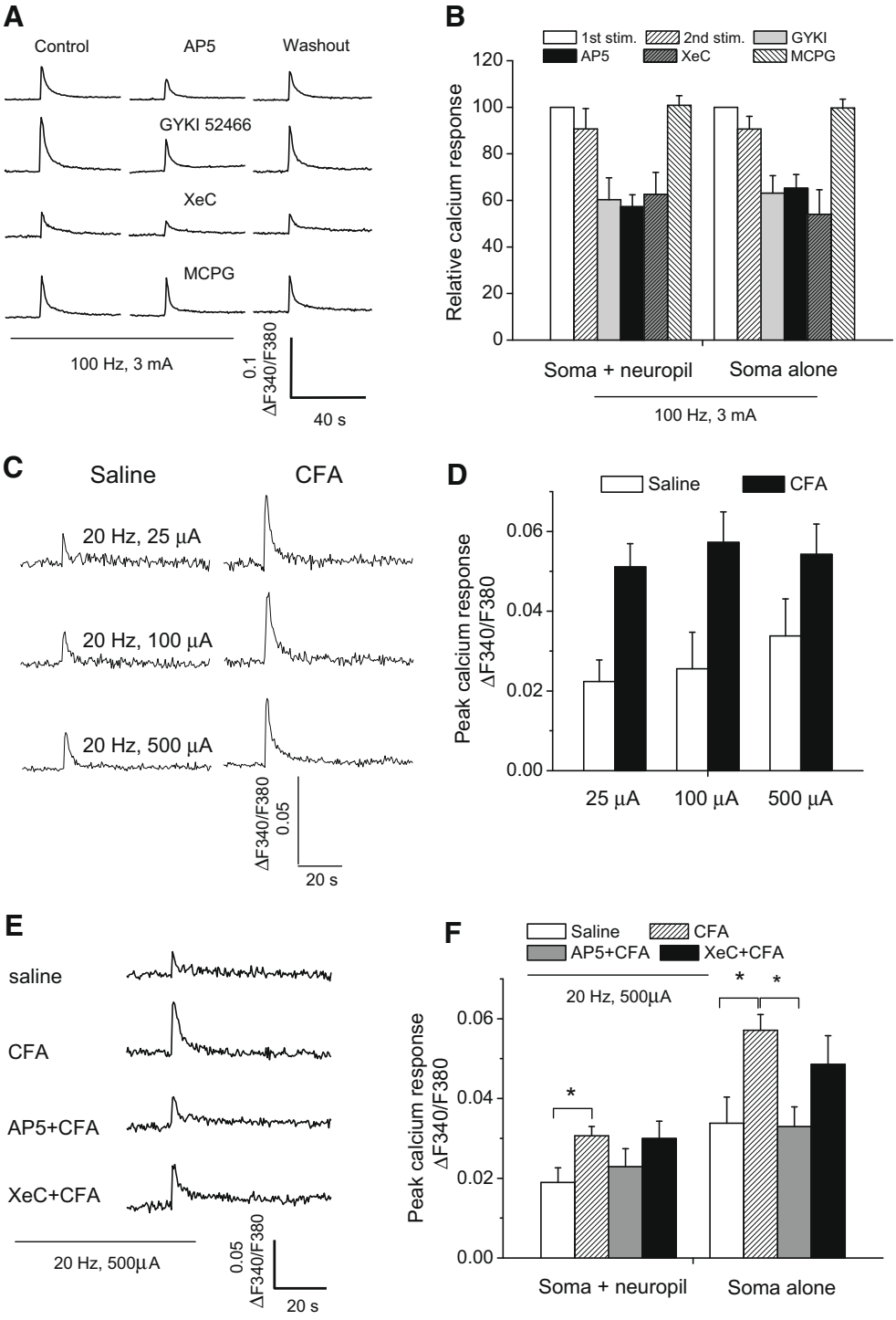

Fig. 3. Effects of pharmacological inhibitors of NMDA (AP5), AMPA (GYKI 52466) and IP_3 receptors (XeC) and of $mGluR_{1/5}$ (MCPG) on ipsilateral spinal Ca^{2+} signals in mice following unilateral hindpaw injection of saline or Complete Freund's Adjuvant (CFA). Shown are typical traces (A) or peak average amplitudes of Ca^{2+} signals (B) before and during application and at 20 min after washout of inhibitors. Typical examples (C) and peak average amplitudes of Ca^{2+} signals (D) in spinal slices of mice treated with intraplantar injection of saline or CFA or intraplantar CFA with simulatenous systemic administration of AP5 or XeC for 24 h. n, at least 10 neurons per slice and at least 5 slices per inhibitor. $^{*}p \leqslant 0.05$, ANOVA followed by Fischer's test.

magnitude of evoked EPSCs in spinal dorsal horn neurons or behavioral responses to heat [6], we observed differences in magnitude of evoked Ca^{2+} responses between mice of the C57Bl6 strain (background strain for GluR-$A^{-/-}$ mice) and the CD1 strain (background strain for GluR-$B^{-/-}$ mice). However, in all experi-

ments, knockout mice were only strictly compared to their wild-type littermates within the same strain. Ca^{2+} signals evoked by a high-frequency afferent barrage (100 Hz, 3 mA) did not differ significantly between GluR-$B^{-/-}$ mice and their wild-type littermates (Fig. 4A; $p > 0.05$; $n = 18$). In contrast, GluR-$A^{-/-}$ mice showed significantly reduced spinal Ca^{2+} signals during persistent activation of primary afferents compared to wild-type littermate (Fig. 4B; $p < 0.05$; $n = 8$). Importantly, inflammation-induced potentiation of Ca^{2+} signals in laminae I/II was also completely abrogated in GluR-$A^{-/-}$ mice (Fig. 4C; $p > 0.05$ in saline versus CFA groups; $n = 7$). In contrast, GluR-$B^{-/-}$ mice fully developed potentiation of Ca^{2+} responses following intraplantar CFA treatment as compared to vehicle treatment (Fig. 4D; $p < 0.05$; $n = 7$). These data show that GluR-A-containing AMPA receptors are required for activity-induced alterations in spinal calcium signaling in states of inflammatory pain, whereas GluR-B-containing (and therefore, calcium-impermeable) AMPA receptors do not play a major role.

3.5. Alterations in spatial spread of spinal calcium signals following peripheral inflammation

Supramaximal afferent stimulation at a high-frequency (100 Hz, 3 mA) produced small Ca^{2+} signals in the contralateral superficial dorsal horn in only 5 of 43 slices (Fig. 5A and B). In striking contrast, at 24 h after CFA injection in wild-type mice, contralateral Ca^{2+} signals were seen in 9 of 12 slices (Fig. 5A) and the peak amplitude of the contralateral Ca^{2+} response increased to 179 ± 8% of the responses seen in saline-treated mice (Fig. 5B and C; $p = 0.001$; $n = 13$; also see Supplementary Table 1 for comparison of peak amplitudes of the ipsilateral and contralateral calcium signals in control and CFA conditions). Similar results were seen in GluR-$B^{-/-}$ mice (Fig. 5A–C; $p = 0.002$; $n = 7$). Importantly, high-frequency afferent barrage entirely failed to elicit a potentiation of contralateral Ca^{2+} signals in GluR-$A^{-/-}$ mice (Fig. 5A–C; $p = 0.41$; $n = 6$). These results show that GluR-A-containing AMPA receptors are required for a spatial expansion of Ca^{2+} signals in states of inflammatory pain.

4. Discussion

The novel and most important finding of this study is that stimuli which trigger long-lasting changes and abnormalities in pain sensitivity produce strong changes in the nature and spatial patterns of calcium signals in spinal sensory laminae. Importantly, we observed that both the magnitude and the spatial spread of calcium signals in the spinal cord is potentiated at 24 h after peripheral injury. Furthermore, our results show that besides NMDA receptors, Ca^{2+}-permeable AMPA

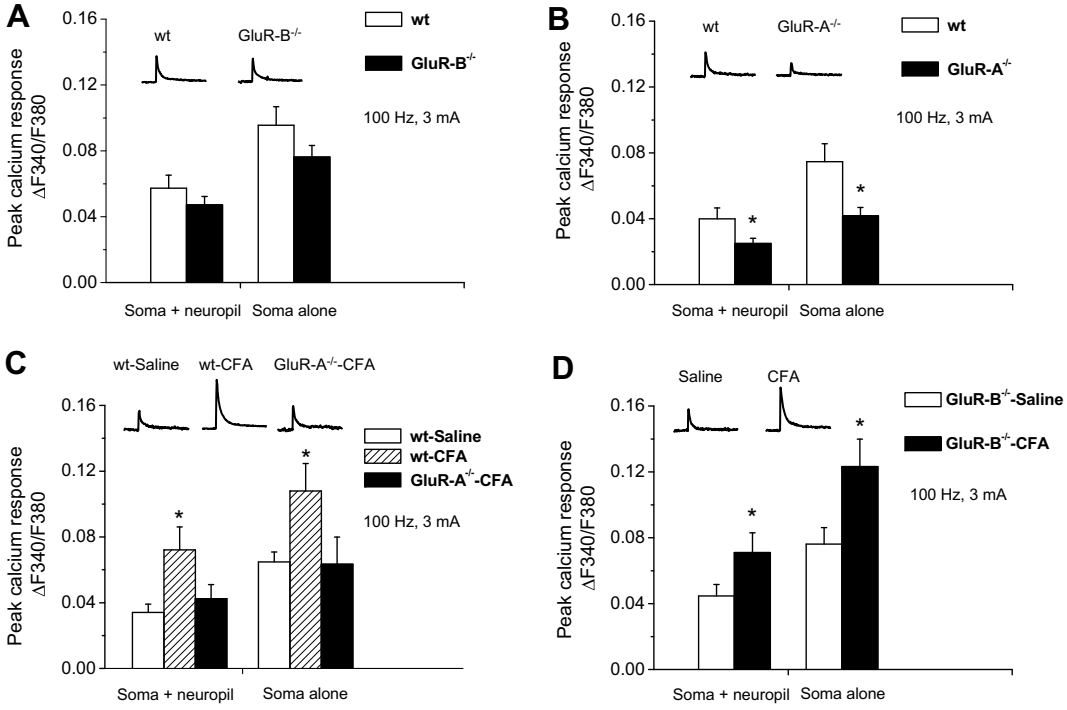

Fig. 4. Role of AMPA receptor subunits in spinal Ca^{2+} responses induced by repetitive, high-intensity activation of primary afferents in the naïve state (A and B) or following intraplantar injection of saline or CFA (C and D). Shown are typical somatic traces and average peak responses in mutant mice lacking the AMPA receptor subunits GluR-A (GluR-$A^{-/-}$) or GluR-B (GluR-$B^{-/-}$) as compared to littermate-controlled wild-type mice. n, at least 10 neurons per slice and at least 8 slices derived from at least 5 mice per genotype. $^{*}p \leqslant 0.05$, ANOVA followed by Fischer's test.

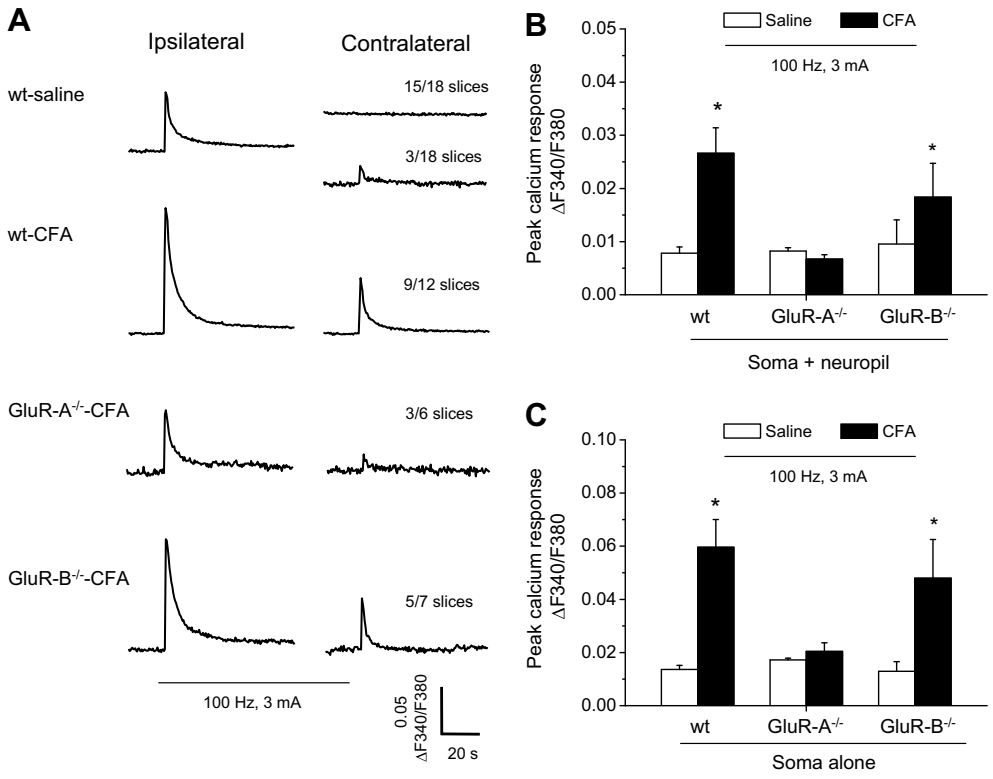

Fig. 5. CFA-induced unilateral paw inflammation leads to potentiation of calcium responses in the ipsilateral as well as the contralateral dorsal horn of wild-type mice and GluR-$B^{-/-}$ mice, but not in GluR-$A^{-/-}$ mice. Shown are typical somatic traces (A) and average peak responses (B and C). n, at least 7 slices derived from at least 5 mice per genotype. $^{*}p \leqslant 0.05$, ANOVA followed by Fischer's test.

receptors are key mediators of nociceptive activity-induced amplification of spinal calcium responses.

Various cellular correlates have been described to account for hyperalgesia and allodynia resulting after peripheral injury and inflammation, including hyperexcitability of peripheral and central neurons, LTP at nociceptive synapses and morphological rearrangements in the connectivity of sensory fibers [19]. Here, we observed that calcium transients in superficial spinal neurons are strongly potentiated in a nociceptive activity-dependent manner. Interestingly, experiments based upon diverse stimulation intensities and frequencies of dorsal root suggested that calcium signals evoked by low-threshold inputs potentiate to a larger extent than those evoked by high-threshold C-fibers in inflammatory states, which may become apparent due to a saturation effect. Functional reorganisation and recruitment of synaptic connections of low-threshold Aß fibers in the substantia gelatinosa has been suggested to occur in activity-dependent manner in certain pain states (e.g. [9,18]). Because Aß innervation of laminae I and II is believed to persist at the postnatal stages studied here [4], it is not clear whether the above phenomenon contributes to our observations. Importantly, however, our results provide a functional basis for observations from electrophysiological studies on spinal neurons showing a significantly greater potentiation of responses to brush (low-intensity inputs) than pinch (high-intensity inputs) following peripheral inflammation (e.g. [2]). Thus, a high level of potentiation of calcium responses to Aß inputs in superficial spinal neurons may well represent a cellular correlate of allodynia.

Ionotropic glutamate receptors of the NMDA and AMPA-types as well as $mGluR_{1/5}$ are densely expressed in the spinal dorsal horn. Here, we systematically delineated their relative contributions to nociceptive activity-induced calcium dynamics in the spinal dorsal horn. We observed that NMDA receptors mediate a significant proportion of the dynamic changes in calcium responses upon rapid, repetitive stimulation of nociceptors as well as in inflammatory pain states, consistent with their role as key regulators of the gain of spinal nociceptive transmission [19]. Surprisingly, we did not find clear evidence supporting a strong contribution of metabotropic receptors in activity-induced spinal calcium dynamics. Preventing mobilization of calcium from the internal stores via IP_3R blockade inhibited the rapid summation of calcium signals induced by high-frequency activation of nociceptors, but did not clearly inhibit inflammation-induced potentiation and a blockade of $mGluR_{1/5}$ inhibited neither form of facilitation.

In contrast to NMDA receptors and $mGluR_{1/5}$, AMPA-type glutamate ion-channels have been usually only regarded as mediators of fast nociceptive neurotransmission in the spinal cord. However, a subset of AMPA receptors on spinal superficial neurons constitutively lack GluR-B and express GluR-A, and are therefore predicted to be Ca^{2+}-permeable, based upon analysis of kainate-induced cobalt-uptake [3,6]. Furthermore, recent electrophysiological studies suggest that synaptically positioned Ca^{2+}-permeable AMPA receptors directly contribute to low-threshold as well as high-threshold sensory afferent drive into the dorsal horn [17]. However, whether AMPA receptors can directly regulate spinal calcium levels, particularly in states of chronic pain, had not been addressed so far. Here, our combined analysis of GluR-$B^{-/-}$ mice and GluR-$A^{-/-}$ mice revealed that AMPA receptors containing GluR-A and lacking GluR-B not only contribute to the rapid summation of post-synaptic calcium levels upon persistent sensory stimulation, but also mediate chronic changes in spinal calcium homeostasis following peripheral inflammation. These results provide a mechanistic basis for the requirement for calcium-permeable AMPA receptors in activity-dependent plasticity in the spinal dorsal horn [5,6]. Furthermore, the strong agreement between these calcium-imaging data and the significant reduction of pain-associated behaviors in mice lacking the GluR-A subunit in several preclinical models of chronic inflammatory pain [6]; (Hartmann, Sluka, Gebhart & Kuner, unpublished), unequivocally brings out the functional significance of calcium signaling pathways in injury-induced hyperalgesia and allodynia. In addition to a post-synaptic function, NMDA and AMPA receptors localised to presynaptic terminals of primary afferent fibers have been implicated in the modulation of neurotransmitter release in the spinal dorsal horn [10,11]. Neither the pharmacological agents used here nor the global (classical) GluR-mutant mice provide specificity of anatomical locus of action. Therefore, studies on conditional, region-specific mouse mutants will be required to dissect out a potential contribution presynaptic mechanisms to the activity-induced alterations in spinal calcium signaling reported here.

Another very intriguing observation of this study entailed the striking spread of calcium signals into the contralateral spinal cord in mice with peripheral paw inflammation. Our findings, taken together with the observations that calcium waves spread across the spinal midline upon pharmacological blockade of inhibitory GABAergic and glycinergic pathways [13] suggest that connections between neurons in the two halves of the spinal cord may be potentially inhibited by spinal interneurons under normal conditions and get activated in pathological states. Importantly, our analysis of specific GluR-mutant mice demonstrates that spinal Ca^{2+}-permeable AMPA receptors are required for inflammation-induced disinhibition to occur. This presents a very attractive mechanism for explaining a wealth of literature reporting enhanced functional and nociceptive behavioral responses and as well as biochemical changes, e.g. IEG induction and cytokine release, on the contralateral side following unilateral inflammation or injury (reviewed in [14]). Furthermore, an inflammation-induced contralateral spread of calcium responses may also underlie the remarkable symmetry and mirror-pain observed clinically in several chronic inflammatory diseases, such as rheumatoid arthritis [14].

In summary, this study shows that peripheral inflammation induces activity-dependent potentiation and exaggerated spatial spread of calcium responses via the activation of NMDA- and Ca^{2+}-permeable AMPA receptors and suggests that this may represent a mechanistic correlate for inflammation-induced hyperalgesia, allodynia and mirror-pain at the cellular and network levels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2008.09.008.

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