Research Paper

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Differential contributions of A- and C-nociceptors to primary and secondary inflammatory hypersensitivity in the rat

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Abstract

Primary hyperalgesia is characterized by increased responsiveness to both heat and mechanical stimulation in the area of injury. By contrast, secondary hyperalgesia is generally associated with increased responses to mechanical but not heat stimuli. We tested the hypothesis that sensitization in secondary hyperalgesia is dependent on the class of peripheral nociceptor (C- or A-nociceptor) rather than the modality of stimulation (mechanical vs heat). A- and C-nociceptors were selectively activated using contact heat ramps applied to the hind paw dorsum in animals with hind paw inflammation (primary hyperalgesia) and knee inflammatory arthritis (secondary hyperalgesia). Sensitization to A- and C-nociceptor activation in primary and secondary hyperalgesia was assessed by reflex withdrawal thresholds and by Fos immunocytochemistry in the dorsal horn of the spinal cord, as an index of neuronal activation. In primary hyperalgesia, only C-nociceptor-evoked withdrawal reflexes were sensitized. This was associated with increased spinal lamina I neuronal activation to both A- and C-nociceptor activation. Fos-like immunoreactivity (FLI) was unchanged in other dorsal horn laminae. In secondary hyperalgesia, only A-nociceptor-evoked withdrawal reflexes were sensitized, and FLI was increased in both superficial and deep dorsal laminae. Neurons in the superficial dorsal horn receive and process nociceptor inputs from the area of primary hyperalgesia, resulting in functional sensitization to C-nociceptive inputs. In inflammatory arthritis, secondary hyperalgesia is evoked by A-nociceptor thermal stimulation, suggesting that secondary hyperalgesia is A-nociceptor, rather than stimulus modality (mechanical vs thermal), dependent. Fos-like immunoreactivity evoked by A-nociceptor stimulation in secondary hyperalgesia suggests that the sensitization is underpinned by spinal neuronal sensitization in laminae I and IV/V.

Keywords: Hyperalgesia, A-nociceptor, c-fos, Spinal cord, Arthritis, Nociception

1. Introduction

Hyperalgesia, increased responsiveness to noxious stimulation, results from tissue damage and inflammation. There are 2 defined types of hyperalgesia, primary and secondary; primary hyperalgesia is found in areas of tissue damage, whereas secondary hyperalgesia is evident in undamaged skin adjacent,[37,48,68,72] or more distant, to the area of damage.[12,19,60,61] Heat and mechanical stimuli are the most commonly used stimulus modalities in behavioral and mechanistic studies of hyperalgesia. Increased responses to both modalities are often reported in primary hyperalgesia, whereas typically, only mechanical responses are enhanced in secondary hyperalgesia.[31,37,38,48,72] Thermal responses are, however, also sometimes reported to change in secondary hyperalgesia.[13,25,30,50,68]

High threshold mechanical stimuli used in experimental studies have been postulated to predominately activate A-nociceptors, whereas thermal stimuli are thought to principally activate C-nociceptors.[10,15,26,56,72] This distinction in nociceptor activation could explain the frequent absence of heat hyperalgesia (no C-nociceptor sensitization), in the presence of mechanical hyperalgesia (A-nociceptor sensitization) in studies of secondary hyperalgesia, for example after capsaicin application in humans (eg,[37]), or nerve injury in animals (eg,[22]).

It is generally agreed that the mechanisms that mediate primary and secondary hyperalgesia are distinct. Primary hyperalgesia is attributed to both peripheral nociceptor sensitization, at the site of injury, and central sensitization, whereas secondary hyperalgesia results largely from central sensitization, triggered and often maintained by enhanced afferent input. Secondary (mechanical) hyperalgesia, resulting from capsaicin injection[58] or tissue damage in man,[71] can be reduced by A-fiber blockade and is dependent on the activity in capsaicin-sensitive C-nociceptors[55,59,64] innervating the area of primary hyperalgesia.[4,30,58,60] These observations led Treede et al. to hypothesize that C-nociceptor drive leads to sensitization of central neurons to A-nociceptor activation, resulting in secondary punctate mechanical hyperalgesia.[37,71,72]

A- and C-nociceptors have different functions in pain sensation; activation of C-nociceptors evokes slow, burning poorly localized pain, whereas activation of A-nociceptors evokes sharp well-localized pain.[37,46] Thus, differences in nociceptor sensitization are likely to serve different functions; A-nociceptors are postulated to serve protective functions, signaling acute tissue damage,[11] whereas C-nociceptors may signal ongoing tissue damage.[14] In addition, as a result of the different distributions of C- and A-nociceptor input to superficial and deep dorsal horn[21] and their

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different properties and functions, the different dorsal horn laminae are functionally distinct.[28,47]

To determine whether there are different contributions of A- and C-nociceptor inputs in primary and secondary hyperalgesia, we used a method to preferentially activate either A- or C-nociceptors using thermal stimulation alone.[34,42,70] This method enables the interpretation of the consequences of A- and C-nociceptor activation, without the additional confound of different stimulus modalities. In these studies, we tested the following hypotheses (1) that inflammatory arthritis results in secondary hyperalgesia only to A- and not C-nociceptor stimulation; (2) that cutaneous inflammation results in primary hyperalgesia to both A- and C-nociceptor stimulation; and (3) that spinal neurons in specific laminae are sensitized to only A-nociceptor inputs in areas of secondary hyperalgesia but to both A- and C-nociceptor inputs in primary hyperalgesia.

2. Materials and methods

2.1. Animal preparation

A total of 92 male Wistar rats (250-300 g, Harlan, United Kingdom) were used in these experiments. All procedures involving experimental animals were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986 plus associated guidelines and with the approval of the University of Bristol Ethical Review Group.

Inflammation was induced by injection of 100 μL Freund's complete adjuvant (CFA, 29-gauge BD insulin syringes; Sigma, Gillingham, United Kingdom) under brief isoflurane anesthesia (2% in O_2). Primary hyperalgesia: inflammation was induced by subcutaneous injection into the dorsal hind paw (n = 32: electromyographic [EMG] studies, n = 14; Fos studies, n = 18) and the inflamed area of the hind paw stimulated for study of primary hyperalgesia. Secondary hyperalgesia: inflammatory arthritis was induced by intraarticular injection into the knee joint (n = 32: EMG studies, n = 14; Fos studies, n = 18) and the hind paw stimulated for study of secondary hyperalgesia. Naive animals were used as controls (n = 28: EMG studies, n = 14; Fos studies, n = 14).

Seven days after, Freund's complete adjuvant injection rats were re-anaesthetized with halothane (2.5% in O_2), a branch of the external jugular vein was cannulated for anaesthetic maintenance (intravenous alphaxalone infusion, 25 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$), and the external carotid artery was cannulated to monitor blood pressure. Body temperature was maintained at approximately 37°C using a thermostatically controlled blanket.

2.2. Preferential activation of A- and C-heat nociceptors

A thermal stimulation apparatus was used to deliver slow or fast rates of skin heating, respectively, to the rat's hind paw dorsum in animals in experimental groups (n = 75: EMG studies, n = 42; Fos studies, n = 33) to preferentially activate either C- or A-nociceptors, as described previously.[42] In brief, heat from a sputter-coated projector bulb was focused onto a blackened copper disk positioned at the focal point. A T-type thermocouple (0.02-mm in diameter, made in-house) was fixed to the outer surface of the copper plate and therefore recorded the surface skin temperature when in contact with the hind paw dorsum. Using a constant bulb voltage, fast rates of heating (7.5 ± 1°C·s^{-1} measured over 2 seconds from the start of heating) were used to preferentially activate myelinated A-fiber heat nociceptors, whereas slow rates of heating (2.5 ± 1°C·s^{-1} measured over 4 seconds from the start of heating) were used to preferentially activate unmyelinated C-fiber heat nociceptors. Previous studies from our laboratory[34,42] have shown that these heating rates reproduce the subepidermal heating rates described by Yeomans et al.[69,70] that preferentially activate A- and C-nociceptors. The starting temperature of the heat lamp was 30°C, and the cutoff temperatures (controlled by a Spike2 script) of the heat lamp were 57°C and 55°C for fast and slow rates of heating, respectively, to prevent damage to the hind paw. Heating was controlled through a PC. For recording of withdrawal thresholds to noxious skin heating, alternate fast and slow ramps were applied at interstimulus intervals of 8 minutes in each animal as described previously.[28]

2.3. Determination of withdrawal thresholds to A- and C-nociceptor stimulation in areas of primary and secondary hyperalgesia

Electromyographic recordings were made in a total of 42 rats, naive (n = 14), dorsal hind paw cutaneous inflammation (primary hyperalgesia, n = 14), and knee joint arthritis (secondary hyperalgesia, n = 14). An intramuscular bipolar electrode was made from 2 short lengths of Teflon-coated, 0.075-mm-diameter, stainless steel wire (Advent Research Materials, Eynsham, United Kingdom). Teflon insulation was removed from the end of the wire to allow for electrical contact, and then the wire was inserted into the biceps femoris of the left hind leg to record EMG activity during the withdrawal reflex. The EMG signal was amplified (×10,000) and filtered (50 to 5 kHz; NeuroLog System; Digitimer, Welwyn Garden City, United Kingdom), before being captured for subsequent analysis through 1401plus (Cambridge Electronic Design, Cambridge, United Kingdom) onto a PC running Spike2 version 5 software (Cambridge Electronic Design).

2.4. Induction of Fos protein in spinal dorsal horn by A- or C-nociceptor stimulation in primary and secondary hyperalgesia

In a second series of experiments, a total of 50 animals were divided into 3 groups, naive (n = 14: unstimulated = 5, stimulated = 9), dorsal hind paw inflammation (primary hyperalgesia, n = 18: unstimulated = 6, stimulated = 12), and knee joint arthritis (secondary hyperalgesia, n = 18: unstimulated = 6, stimulated = 12). Unstimulated naive and hyperalgesic animals were also included to control for the effects of anesthesia and the inflammatory process. These 17 animals were anaesthetized and maintained for 4 hours but were not subjected to thermal stimulation.

After surgery, as described above, animals were allowed to stabilize for 2 hours, and then either A- or C-nociceptors were stimulated as described above.

After stimulation, animals were maintained under anesthesia for further 2 hours to allow for development of Fos expression in the spinal cord. At the end of experiments, animals were killed by overdose of alphaxalone and perfused transcardially with saline followed by paraformaldehyde (4% in 0.1 M phosphate buffer 300 mL). The spinal cords (L3-L5) were removed, postfixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose solution for at least 24 hours, and the L3-L5 region sectioned transversely at 40 μm on a freezing microtome.

2.5. Immunohistochemical processing for visualization of Fos protein

Free-floating spinal cord sections were incubated for 48 to 72 hours at 4°C with a polyclonal Fos antibody (1:5000; Santa Cruz Biotechnology, Heidelberg, Germany) in phosphate-buffered saline containing 1% bovine serum albumin, 0.1% Triton X-100, and 0.01% sodium azide. After rinsing in phosphate-buffered saline with 0.1% Triton X-100 (PBST), sections were incubated in

secondary biotinylated goat anti-rabbit antibody (1:500 in PBST; Sigma) for 1 to 2 hours at room temperature. The sections were rinsed in PBST, incubated in streptavidin-conjugated peroxidase (1:1000 in PBST; Sigma) for 1 to 2 hours, and visualized with 3,3-diaminobenzidine (0.015%; Sigma) and glucose oxidase (G-0543, 10,000 U/1.8 mL; Sigma). Finally, all sections were mounted onto gelatine/chrome alum–coated microscope slides and coverslipped with DPX mountant.

2.6. Cell counting

All spinal cord sections were scanned at low magnification for identification of Fos-like-immunoreactive (FLI) nuclei in the dorsal horn and of ~100 sections per animal, the 10 most heavily labeled sections were identified and total FLI was counted. We and others have previously used this method to determine maximum fos induction across all spinal segments under study.[7,28,66] Numbers of FLI dorsal horn neurons in laminae I, II, III, IV-V, X and the lateral spinal nucleus were then counted at higher magnification in these sections and their locations assigned to the appropriate laminae of the spinal cord as distinguished under dark field illumination, as described previously.[28] Data are presented as the total number of FLI nuclei counted in each animal to give a value to the overall FLI, not the mean number of FLI nuclei per section, which might not account for variation between different spinal segments.

2.7. Data analysis

Mean withdrawal thresholds in primary and secondary hyperalgesic animals were compared with those in naive animals receiving the same stimulus (A- or C-nociceptor stimulation) using analysis of variance (ANOVA) followed by planned Dunnett tests for comparison with naive controls. For some comparisons of number of FLI neurons, data sets were log transformed before analysis, as not all groups met standard conditions for parametric analyses. Comparisons of FLI-positive neurons in each spinal cord lamina between groups in (A) inflamed but unstimulated animals and (B) normal A- or C-nociceptor-stimulated animals (**Fig. 2**) were made using 2-way mixed-design ANOVA followed by Dunnett tests, with lamina as the within-subject variable and (A) inflammation state (hind paw or knee) or (B) stimulation modality as the between-subject variable. Two-way ANOVA was performed on raw untransformed data, and Gaussian distribution was therefore assumed. This was because some control animals exhibited no FLI in some laminae; log transformation therefore resulted in incomplete data sets, and within-subjects repeated-measures comparisons could not be made.

Altered FLI (neuronal activation) resulting from stimulation in primary or secondary hyperalgesia (**Figs. 4 and 5**) was determined by comparison of naive + stimulation, inflamed + no stimulation, and inflamed + stimulation groups in specific laminae, using between-groups one way ANOVA followed by Bonferroni or Dunnett planned comparisons as stated. Data are shown as mean ± SEM unless otherwise stated. Statistical analyses were performed using GraphPad Prism 5.0/6.0. Alpha was set at 0.05.

3. Results

3.1. Withdrawal thresholds to C- and A-nociceptor activation in areas of primary and secondary hyperalgesia

C-nociceptor-evoked withdrawal thresholds were significantly reduced, indicating sensitization, in the area of primary inflammatory hyperalgesia (ANOVA: $F(2,39) = 4.184$, $P = 0.023$, n = 14 per group) but were unaffected in arthritic secondary hyperalgesia. In cutaneous inflammation, C-nociceptor thresholds decreased significantly from 51.1 ± 0.2°C to 49.9 ± 0.5°C (mean ± SEM, $P < 0.05$ compared with naive animals), whereas thresholds in secondary hyperalgesia were 51.2°C ± 0.3°C (**Fig. 1A**). In contrast, A-nociceptor-evoked thresholds were unaltered in primary hyperalgesia (ANOVA: $F(2,39) = 5.842$, $P = 0.006$, threshold 52.7 ± 0.4 in naive compared with 52.6°C ± 0.4°C in primary hyperalgesia, **Fig. 1B**) but were significantly lower in arthritic secondary hyperalgesia, being reduced from 52.7 ± 0.4°C to 51.1 ± 0.2°C ($P < 0.05$, **Fig. 1B**) compared with naive animals.

3.2. Fos-like-immunoreactive–positive spinal dorsal horn neurons after 7 days of hind paw or knee joint inflammation

Cutaneous and arthritic inflammation had different effects on spinal FLI. Seven days of cutaneous inflammation of the hind paw resulted in an increase in FLI in ipsilateral L3-L5 lamina I, whereas 7-day knee joint arthritis had no effect in this lamina (naive: total FLI nuclei = 23 ± 3, cutaneous: 54 ± 11, arthritis: 26 ± 3, significant effect of lamina $F(5,70) = 32.9$, $P < 0.0001$; nonsignificant effect of inflammatory state $F(2,14) = 0.14$, $P = 0.14$, significant interaction $F(10,70) = 4.93$, $P < 0.0001$, **Fig. 2A**). Although neither dorsal hind paw inflammation nor knee joint arthritis had any significant effect

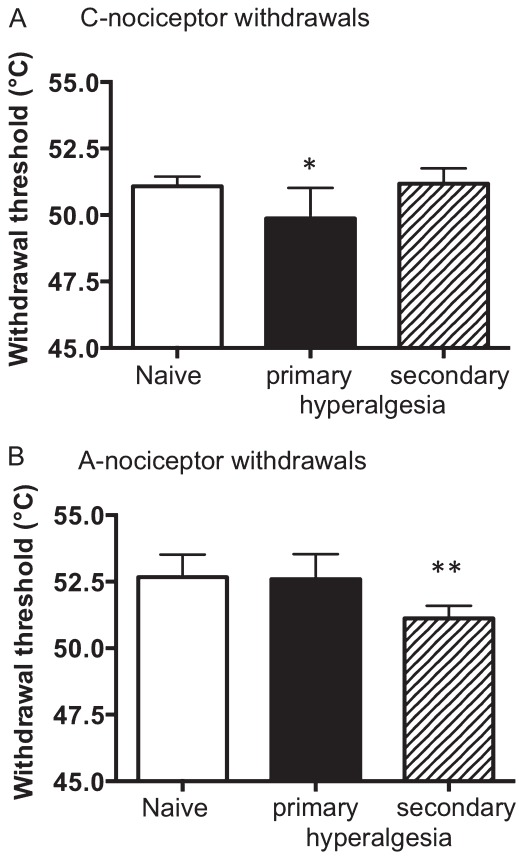

Figure 1. Withdrawal thresholds in response to A- and C-nociceptor stimulation in primary and secondary hyperalgesia. (A), Thresholds for C-nociceptor-stimulated noxious withdrawals were lowered only in the area of primary hyperalgesia in animals with hind paw inflammation and were unaltered in the area of secondary hyperalgesia in animals with knee joint arthritis. (B), Thresholds for A-nociceptor-stimulated noxious withdrawals were lowered only in the area of secondary hyperalgesia in animals with knee joint arthritis and not in the area of primary hyperalgesia in animals with hind paw inflammation (data are mean + 95% confidence interval, *$P < 0.05$, **$P < 0.01$, planned Dunnett tests, n = 14 all groups).

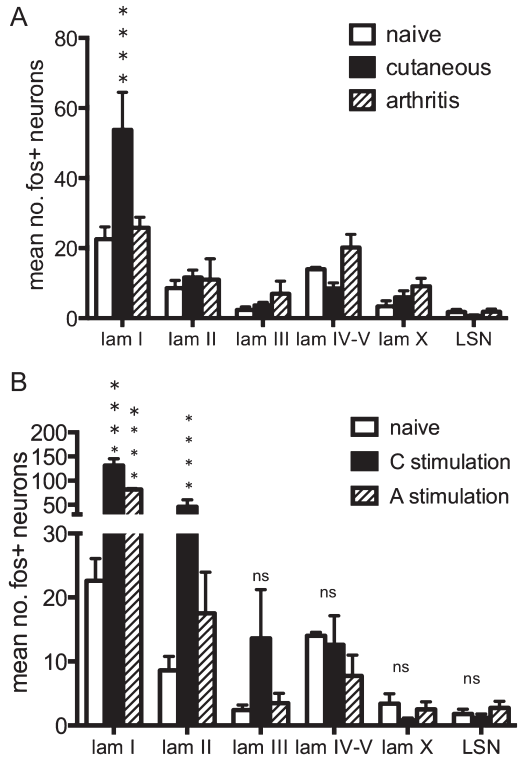

Figure 2. Different effects of inflammation or thermal stimulation on spinal c-fos-like immunoreactivity (FLI). (A), Cutaneous and arthritic inflammation has different effects on spinal FLI. Dorsal hind paw cutaneous inflammation resulted in an increase in FLI in lamina I, but in no other laminae (data are mean + 95% confidence interval, ****$P < 0.0001$, compared with naive, mixed-design 2-way analysis of variance, planned Dunnett tests; n = 5 naive, 6 cutaneous inflammation, 6 knee joint arthritis). (B), C- and A-nociceptor stimulation has different effects on FLI expression in nociceptive spinal laminae. Dorsal hind paw stimulation with heat ramps that preferentially activate either C- or A-nociceptors significantly increased FLI in lamina I. In the same animals, C-nociceptor activation also increased FLI in lamina II, whereas A-nociceptor activation had no effect in this or other laminae of the spinal cord (****$P < 0.001$ compared with naive, planned Dunnett tests; n = 5 naive, 5 C-stimulation, 4 A-stimulation). ns, not significant.

on FLI in any other laminae, in laminae IV-V, there was a trend for an increase in FLI in arthritis (**Fig. 2A**; naive: 14 ± 0.5, cutaneous: 9 ± 2, arthritis: 20 ± 4).

Generally, there were greater number of FLI-positive neurons in lamina I than in all other laminae, under all conditions (**Figs. 2 and 3**).

3.3. Fos-like-immunoreactive–positive spinal dorsal horn neurons after A- and C-nociceptor stimulation in naive animals

A- and C-nociceptor stimulation in naive animals evoked FLI in multiple laminae (**Figs. 2B and 3A-C, J, L, N**, 2-way ANOVA: effect of stimulation type $F(2,11) = 13.2$, $P = 0.0012$, effect of lamina $F(5,55) = 98.07$, $P < 0.0001$, interaction $F(10,55) = 19.67$, $P < 0.0001$). In the ipsilateral lamina I of L3-L5, both C- and A-nociceptor activation of the hind paw dorsum evoked significantly more FLI-positive neurons than in unstimulated naive rats (unstimulated [control], 23 ± 3; C-fiber-stimulated, 131 ± 14 [$P < 0.0001$]; A-fiber-stimulated, 82 ± 2 [$P < 0.0001$], **Figs. 2B and 3A–C, J, L**). C-nociceptor activation also increased the number of FLI-positive neurons in lamina II ($P < 0.0001$) with no effect in deeper laminae (**Figs. 2B and 3E, H, J, K**). There was a trend for an increase in lamina III attributable to a single outlier in this group (44 FLI neurons compared with the group mean 13.6 ± 7.6). When compared with unstimulated animals, A-nociceptor stimulation had no effect on FLI neurons in any area other than lamina I (**Figs. 2 and 3**).

3.4. Fos-like-immunoreactive–positive spinal dorsal horn neurons after A- and C-nociceptor stimulation in areas of primary and secondary hyperalgesia

3.4.1. Primary hyperalgesia

In lamina I, both A- and C-nociceptor activation in the area of primary hyperalgesia resulted in significantly more FLI-positive neurons compared with noninflamed animals, indicating a greater spinal activation to both C- (naive-stimulated total: 131 ± 14, inflamed-stimulated 243 ± 26, $F(3,18) = 43.77$, $P < 0.0001$, **Figs. 3B, E, J, K and 4A**) and A-nociceptive inputs (naive-stimulated: 82.2 ± 2, inflamed-stimulated: 154 ± 20, $F(3,17) = 26.5$, $P < 0.0001$, **Figs 3C, F and 4B**) in primary hyperalgesia. There were no changes in FLI in response to either A- or C-nociceptor activation in other laminae, (**Fig. 4C and D**; laminae IV-V shown for comparison). In primary hyperalgesia, there was, therefore, increased spinal activation only in lamina I neurons in response to both A- and C-nociceptor stimulation.

3.4.2. Secondary hyperalgesia

Both C- and A-nociceptor stimulation of the hind paw (in the area of secondary hyperalgesia) evoked significantly more spinal FLI-positive neurons in laminae I and II in animals with knee joint arthritis when compared with the unstimulated arthritic controls (eg, in lamina I, **Fig. 5A**. C-nociceptors: arthritis-unstimulated total: 26 ± 3; naive-stimulated: 131 ± 14; arthritic-stimulated: 184 ± 23, $F(3,18) = 62.99$, $P < 0.0001$. **Fig 5B**. A-nociceptors: arthritis-unstimulated: 26 ± 3; naive-stimulated: 82 ± 2; arthritic-stimulated: 179 ± 19; $F(3,17) = 69.13$, $P < 0.0001$). When C-nociceptors were stimulated in the area of secondary hyperalgesia, although there was a trend for an increase of ~30% in FLI, this did not reach significance compared with stimulation alone. The effect on spinal lamina I FLI can therefore be considered equivalent to that seen when C-nociceptors were stimulated in naive animals (**Fig. 5A**). In contrast, there were significantly more FLI neurons in lamina I after A-nociceptor stimulation in secondary hyperalgesia than after A-nociceptor stimulation alone (**Fig. 5B**), supporting the hypothesis that spinal neurons are sensitized to A-nociceptor inputs in secondary hyperalgesia.

In lamina II, C-, but not A-nociceptor stimulation resulted in significantly greater FLI than in arthritic animals alone (**Fig. 5C**, $F(3,18) = 7.73$, $P = 0.0016$), but the effect of C-nociceptor stimulation was equivalent in both naive and arthritic animals, indicating no additional sensitization to C-inputs in secondary hyperalgesia. A-nociceptor stimulation had no overall effect in lamina II compared with arthritis alone (**Fig. 5D, F**$(3,17) = 2.3$, $P > 0.05$). The effect of A-nociceptor stimulation in arthritic animals was slightly but not significantly greater than in arthritic animals alone (**Fig. 5D**) but was not greater than in stimulated naive animals, again, indicating no additional sensitization in lamina II neurons in secondary hyperalgesia. In deeper laminae IV-V, C-nociceptor stimulation had no effect on spinal FLI ($F = 2.47$, $P = 0.1$), whereas A-nociceptor stimulation in arthritis had equivalent effects to arthritis alone. A-nociceptor stimulation alone (**Fig. 5E**) evoked significantly less spinal FLI than either

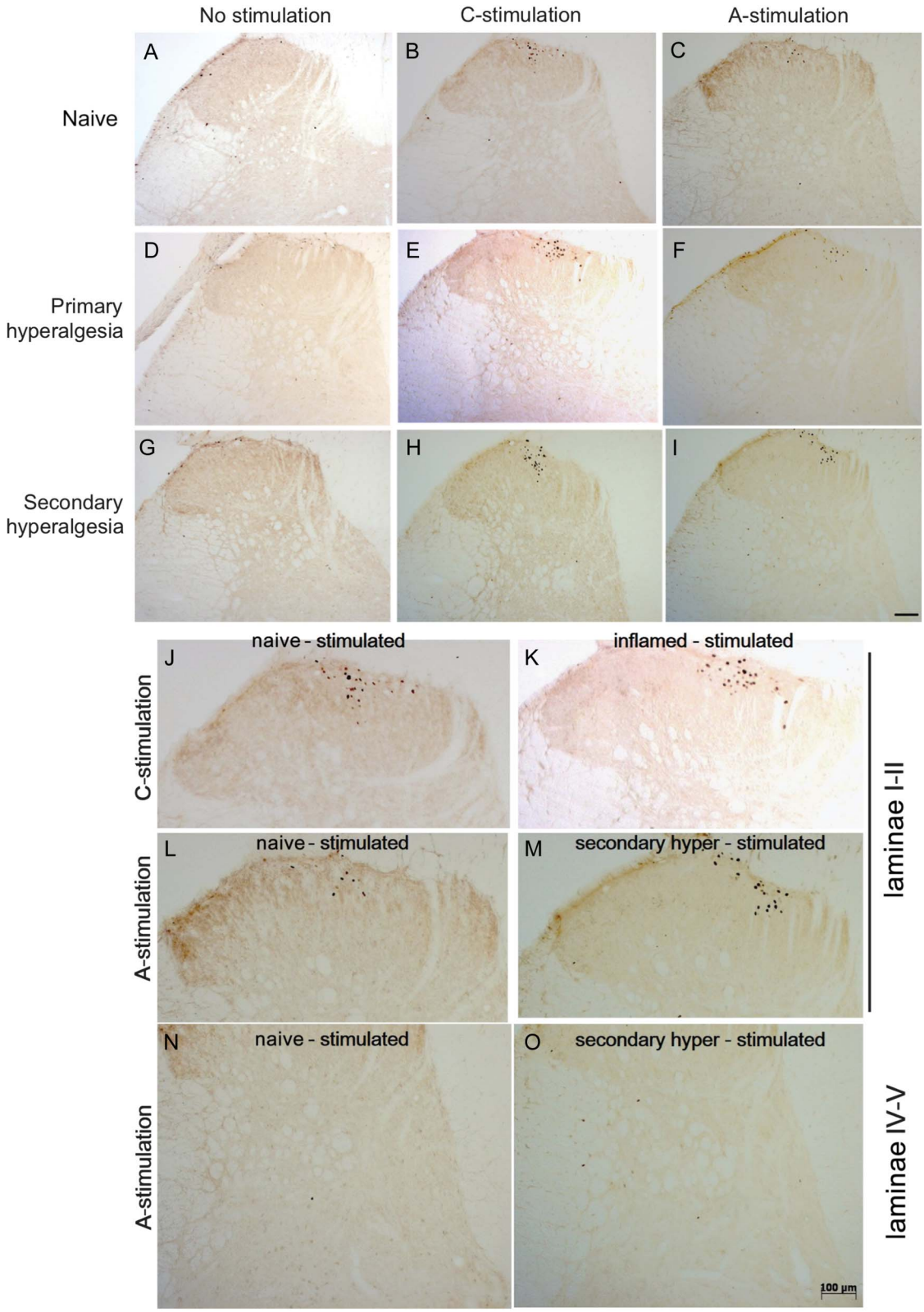

Figure 3. c-fos-like immunoreactivity (FLI) in spinal cord. (A), Photomicrographs of representative images showing FLI in naive rat and (B) the effect of C-nociceptor and (C) A-nociceptor stimulation on FLI. Note that data shown in Figures are the sum of FLI nuclei in 10 sections of spinal cord; hence, in each section, the number of FLI nuclei is approximately one-tenth of that in the associated graph. Both C- and A-nociceptor stimulation increased FLI in laminae I and II in naive rats. (D), In primary hyperalgesia, hind paw C-nociceptor stimulation resulted in increased FLI in lamina I, but not in other laminae. (E), C-nociceptor stimulation in primary hyperalgesia increased FLI, as did (F) A-nociceptor stimulation. (G), Knee joint arthritis had no effect on FLI compared with uninflamed animals, and (H) C-nociceptor stimulation in the area of secondary hyperalgesia did not increase the FLI compared with C-nociceptor stimulation alone (B). (I), A-nociceptor stimulation in the area of secondary hyperalgesia evoked a larger increase in FLI than the inflammation alone. (J and K), High-power image of the effect of C-nociceptor stimulation alone in laminae I and II in naive animals and in primary hyperalgesia. (L and M), A-nociceptor stimulation alone in laminae I and II in naive animals and in secondary hyperalgesia. (N and O), A-nociceptor stimulation alone in laminae IV-V in naive animals and in secondary hyperalgesia.

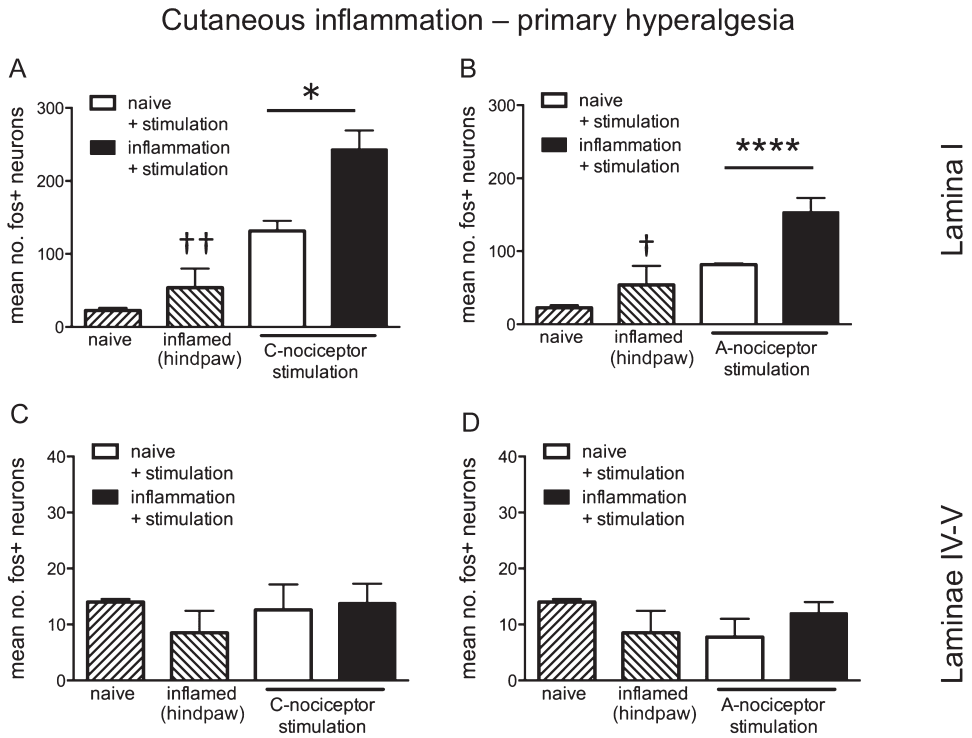

Figure 4. Spinal activation to both A- and C-nociceptor input is greater in primary inflammatory hyperalgesia than in naive rats. (A), C-nociceptor stimulation in hind paw inflammation resulted in an increased number of Fos-like-immunoreactive lamina I neurons than stimulation in naive animals, as did (B) A-nociceptor stimulation. Cross-hatched bars show naive and inflamed animals with no additional stimulation for comparison. (C and D), In laminae IV-V, neither C- nor A-nociceptor stimulation had any effect on Fos-like immunoreactivity (*$P < 0.05$, ****$P < 0.0001$ compared with stimulation in naives; †$P < 0.05$, ††$P < 0.001$ compared with both nociceptor-stimulated groups, Bonferroni planned comparisons, n = 6 hind paw inflammation, 4/5 naive + A/C-nociceptor stimulation, 6 inflamed + A/C-nociceptor stimulation).

arthritis, or arthritis plus A stimulation (**Figs. 3M, O and 5F**, F = 6.69, $P = 0.0035$). In secondary hyperalgesia, there was therefore an increased activation of lamina I spinal neurons as a consequence of A-nociceptor stimulation, but no effect after stimulation of C-nociceptors.

4. Discussion

Chronic pain and hyperalgesia are disabling and extremely difficult to treat, particularly secondary hypersensitivities such as allodynia and hyperalgesia, which occur in areas remote from the site of injury. Secondary hyperalgesia is usually reported as being evoked by mechanical but not thermal stimuli, in contrast to primary heat and mechanical hyperalgesia,[4,49] although some early studies did report secondary thermal hyperalgesia (eg,[19]). Our approach of preferential activation of A- and C-heat nociceptors[34,42] allowed us to compare hypersensitivity and spinal activation to A and C-nociceptor stimulation in areas of primary and secondary hyperalgesia, without the confound of a sensory modality–specific change (mechanical vs thermal stimulation).

In arthritic secondary hyperalgesia, we observed sensitization to thermal stimulation of A- but not C-nociceptors. Although A-nociceptor sensitivity in secondary hyperalgesia can also be regulated through peripheral mammalian target of rapamycin (mTOR)-dependent translational mechanisms,[45] there is no strong evidence to support peripheral nociceptor sensitization in areas of secondary hyperalgesia (Refs. 6,9,29,58; see also Ref. 53). As a result of a series of elegant experiments, secondary hyperalgesia has been hypothesized to result from central sensitization, driven by C-nociceptors,[67,71] which sensitize spinal neuronal responses to capsaicin-insensitive A-nociceptors.[29,37,60] For example, selective block of A-fiber afferents attenuates mechanical secondary hyperalgesia.[58,72] Here, we have shown for the first time that spinal nociception is facilitated in response to a thermal A-nociceptor stimulation in the area of secondary hyperalgesia, indicating that secondary hyperalgesia is actually A-nociceptor, rather than stimulus modality (mechanical vs thermal), dependent.

Given that sensitization of A-nociceptor-driven reflexes occurs through central mechanisms, sensitized spinal neurons might be expected to show enhanced responses to A-nociceptor but not to C-nociceptor stimulation in arthritic secondary hyperalgesia. We show increased FLI in both superficial and deep laminae in response to A-nociceptor stimulation in arthritis after 7 days, indicating relatively early onset of central sensitization compared with previous reports. In studies where stimuli did not discriminate between C- and A-nociceptor inputs, mechanical or thermal stimulation in secondary hyperalgesia increased FLI in superficial[2] and deep dorsal horn,[2,51] but significant changes were seen after approximately 3 weeks in monoarthritic rats. There were however no changes in spinal FLI after additional mechanical stimulation in secondary hyperalgesia resulting from deep muscle inflammation.[54] Sensitization of spinal neurons to A-nociceptor input may result from the unmasking of silent A-nociceptive inputs by C-nociceptor activation, which can occur within 7 days of inflammation onset, and/or by modulation of descending controls.[21,61] For example, descending inhibitory control from the periaqueductal gray (PAG) has differential effects on A- vs C-nociceptor-evoked spinal events,[28,41] preferentially suppressing C-fiber-mediated inputs, while preserving sensory-discriminative input conveyed by A-nociceptors.[21] The

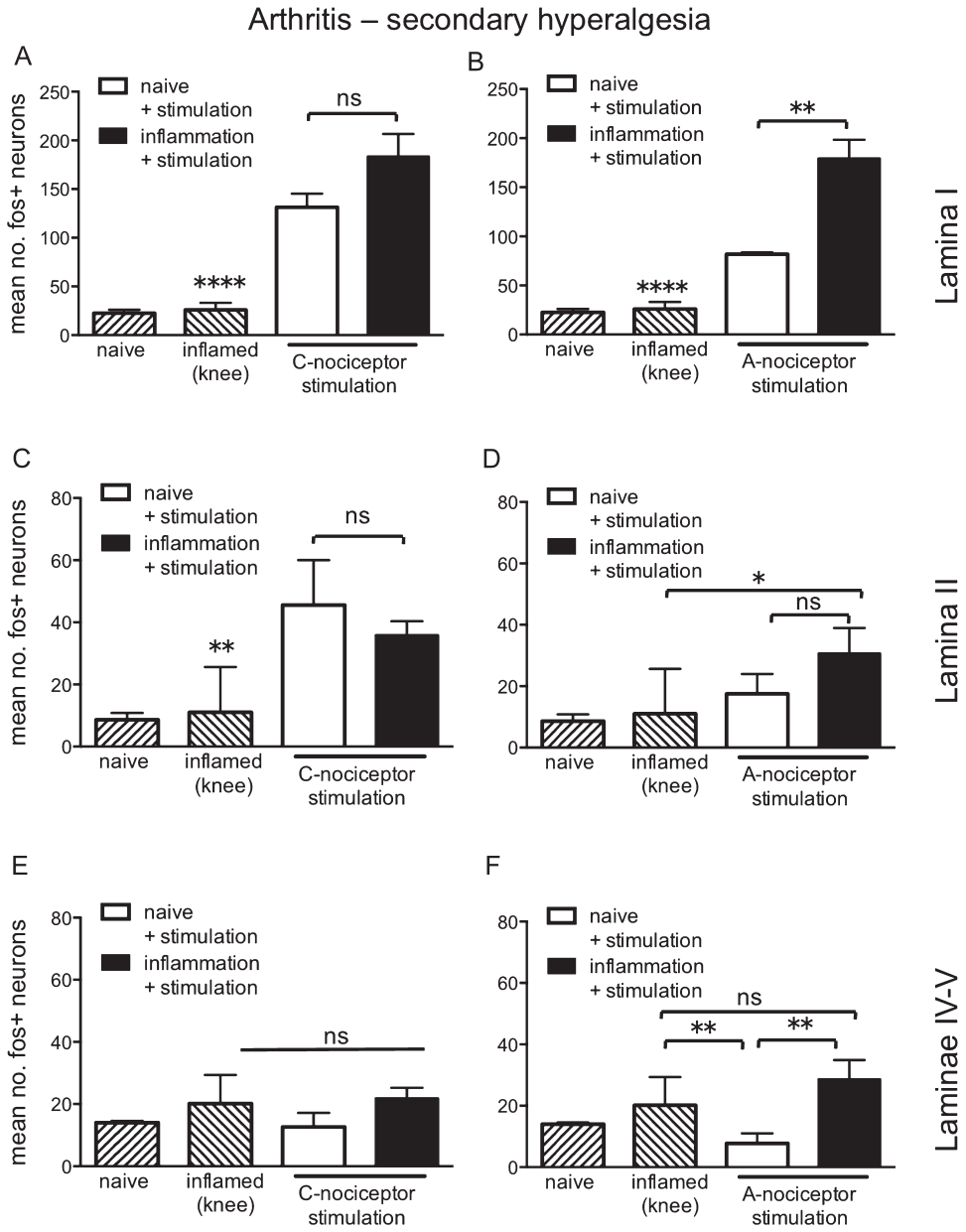

Figure 5. Spinal activation to A- but not C-nociceptor input is greater in secondary inflammatory hyperalgesia than in naive rats. (A), C-nociceptor stimulation resulted in greater Fos-like immunoreactivity (FLI) in lamina I than that caused by knee joint arthritis but had equivalent effects on both naive and arthritic animals, whereas (B) A-nociceptor stimulation in arthritic animals evoked significantly greater FLI than in naive animals. Cross-hatched bars show naive and inflamed animals with no additional stimulation for comparison. (C), In lamina II, C-nociceptor stimulation resulted in greater FLI than in arthritic animals, with no difference between these 2 groups, whereas (D) only arthritis plus A-nociceptor stimulation had any effect on FLI. (E), C-nociceptor stimulation had no effects in deep laminae IV-V. (F), A-nociceptor stimulation in arthritic animals evoked significantly greater FLI in deep laminae IV-V than in naive animals. This was due to lower FLI in stimulated naive animals than arthritic or stimulated arthritic animals. Unlike in more superficial laminae, arthritis alone evoked similar FLI to arthritis + stimulation (*$P < 0.05$, **$P < 0.01$ indicated groups; **$P < 0.01$, ****$P < 0.0001$ compared with both nociceptor-stimulated groups. Bonferroni planned comparisons, n = 6 knee joint arthritis, 4/5 naive + A/C-nociceptor stimulation, 6 arthritic + A/C-nociceptor stimulation). ns, not significant.

observations that the PAG differentially inhibits A- vs C-fiber-evoked responses in deep dorsal horn but inhibits both in superficial dorsal horn and that functional effects of PAG-evoked descending controls depend on the nature of the peripheral stimulus[39] may explain the differences in FLI/neuronal activation in our study compared with others. There was, interestingly, a trend for an increase in response to C-nociceptor-evoked spinal FLI in lamina I (**Fig. 5A**). Although this did not reach statistical significance, it is an intriguing observation that supports previous reports of descending inhibition of C-nociceptor inputs. This trend may indicate that spinal neurons are sensitized to C-nociceptor inputs from areas of secondary hyperalgesia but that they are modulated by descending inhibitory controls. Variable engagement of such descending inhibitory controls over C-nociceptor inputs may explain the variable nature of secondary behavioral thermal hyperalgesia.[13,25,30,31,37,38,48,50,68,72]

In contrast, in primary hyperalgesia in man where withdrawal thresholds to C-nociceptor stimulation are decreased, selective block of A-fiber afferents had little effect.[58,72] Both cutaneous and articular C- and A-nociceptors are sensitized in primary

inflammatory hyperalgesia,[5,44,52,65] but only C-nociceptor-evoked reflexes were sensitized herein. The increased FLI to both A- and C-nociceptor stimulation in spinal lamina I in primary hyperalgesia (**Fig. 4A and B**), together with the lack of sensitization of A-nociceptor reflexes, also suggests that there is differential spinal processing/descending control of A and C-nociceptor inputs in cutaneous inflammation/primary hyperalgesia. Dorsal hind paw inflammation is subject to potent descending inhibitory influences,[50,62] which could explain the lack of A-nociceptor-evoked reflexes. In acute inflammatory hyperalgesia (up to 3 hours) and after acute PAG stimulation, there is greater inhibition of C-nociceptor compared with A-nociceptor-evoked reflexes.[17,24] Our findings could therefore indicate a differential inhibition of A-nociceptor-evoked spinal nociception in more chronic cutaneous inflammation.[28,40,47]

Neurons in the superficial dorsal horn receive and process both A- and C-nociceptor inputs from the area of primary hyperalgesia.[21,43] Ongoing firing in cutaneous nociceptors, such as that occurring in cutaneous inflammation,[18,27] is thought to drive spinal neuronal sensitization and activation and to result in increased number of FLI neurons in the superficial dorsal horn.[8] Additional peripheral stimulation would be expected to activate these sensitized nociceptors[35] and further increase activation of spinal neurons.[33,51] Indeed, mechanical stimulation of arthritic ankle joints and low-intensity touch stimuli in an area of cutaneous inflammation both increase the number of FLI neurons in both superficial and deep dorsal horn.[2,33] In contrast, our results indicate that additional A- and C-nociceptor stimulation increases FLI in the superficial dorsal horn alone. This suggests that spinal neuronal activation evoked from the area of primary hyperalgesia is restricted to the superficial but not the deep dorsal horn when selectively activating either A- or C-nociceptors, when any confound due to the concurrent activation of both A- and C-fibers activating local spinal networks and descending controls is removed.[21,28,43]

The expression of Fos protein has been widely used as a marker of neuronal activation in nociception,[20] in identifying populations of neurons activated by acute peripheral nociceptive input[23] or inflammation.[8,32] Cutaneous inflammation (7 days), without additional stimulation, activated neurons in only lamina I, and not deep laminae, consistent with previous findings after 2 weeks of cutaneous inflammation[32,36] and after acute thermal and chemical stimulation.[16,23] In contrast, CFA-induced arthritis alone had no effect on FLI in spinal dorsal horn, again consistent with previous findings, where changes in deep dorsal horn at 1 week were lower, or absent compared with earlier (1-2 days) or later (>3 weeks) time points.[1,3,32] There are therefore spatiotemporal differences in spinal cord neuronal activation between hind paw cutaneous and knee arthritic inflammation. The lack of increased FLI in spinal cord of arthritic rats may be attributable to engagement of multisynaptic networks, resulting in spinal inhibition.

In control animals, the distribution of FLI neurons activated by A- vs C-nociceptor stimulation is consistent with our previous study where the majority of FLI neurons are located in the superficial dorsal horn. Both A- and C-nociceptors synapse in this region, but there were more neurons activated by C-nociceptor as opposed to A-nociceptor stimulation.[28] Although a limited number of A-nociceptors also terminate in deep dorsal horn,[36,57] there was no significant difference in the number of activated neurons evoked by A- and C-fiber stimulation in control animals. Also, repeated noxious stimulation in naive animals can evoke potent descending inhibition (diffuse noxious inhibitory controls),[63] which could result in the lower FLI levels seen in these animals. Diffuse noxious inhibitory control has been hypothesized to "…constitute both a filter which allows the extraction of the signal for pain and an amplifier in the transmission system which increases the potential alarm function of the nociceptive signals."[63] This hypothesis is supported by our finding that activation of the protective "position-sense" A-nociceptors alone activates many fewer spinal neurons than the same stimulation in secondary hyperalgesia (**Fig. 5F**), as we hypothesize that the need for an "alarm function" would be greater in the face of existing damage, such as arthritis.

Taken together, our results identify distinct roles for A- and C-nociceptors in signaling inflammatory primary and secondary hyperalgesia. Importantly, we provide direct evidence that in secondary hyperalgesia, thermal responses to A-nociceptors are facilitated. We therefore conclude that secondary hyperalgesia is A-nociceptor, rather than stimulus modality (mechanical vs thermal), dependent and that it is underpinned by spinal neuronal sensitization to A-nociceptor inputs in laminae I and IV/V. In contrast, only C-nociceptor-evoked reflexes were sensitized in primary hyperalgesia. Our data suggest that neurons in the superficial dorsal horn receive and process A- and C-nociceptor inputs from the area of primary hyperalgesia (**Fig. 6**). C-nociceptor inputs drive the spinal hyperexcitability in superficial laminae, activating spinal and bulbospinal circuitry that results in a facilitation to A-nociceptor inputs in both the superficial and deep dorsal horn to mediate secondary hyperalgesia and to C-nociceptor inputs in primary hyperalgesia.

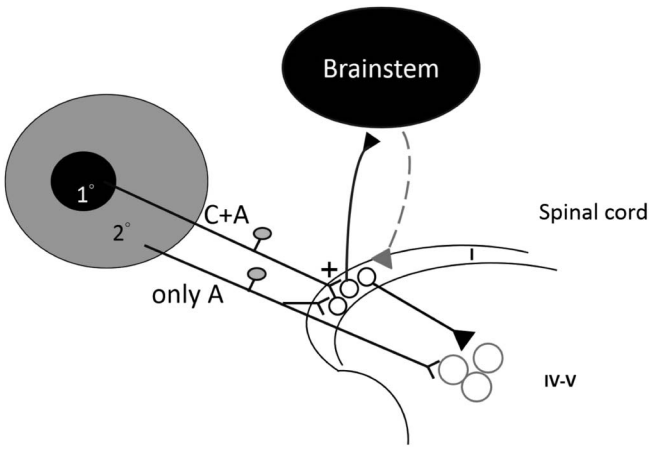

Figure 6. A model for inflammatory primary and secondary hyperalgesia. A model to explain how the neurons in superficial dorsal horn receive and process nociceptive input from the area of primary hyperalgesia; this leads to central sensitization and enhanced activation of superficial and deep dorsal horn neurons to A-nociceptor input from the area of secondary hyperalgesia. 1° and 2° represent primary and secondary hyperalgesia, respectively. Solid lines and black triangles represent excitatory synapses, gray dotted lines and triangles represent inhibitory connections.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Data included in this article have been presented in abstract form at the 14th World Congress on Pain by the IASP and are also included in M.-T. Hsieh's PhD thesis (University of Bristol, United Kingdom, 2014).

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