Optically Recorded Response of the Superficial Dorsal Horn: Dissociation From Neuronal Activity, Sensitivity to Formalin-Evoked Skin Nociceptor Activation

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Tissue injury, infection, or inflammation frequently is accompanied by transformation (activation) of the glia in the topographically corresponding region of the spinal cord dorsal horn (Watkins and Maier 2002). Glial activation involves changes in astrocyte shape and volume and altered communication between neighboring astrocytes via gap junctions (Meme et al. 2004; Olsen and Sontheimer 2004; Walz 2002). Astrocytes in the affected region of the dorsal horn exhibit increased expression of glial fibrillary acidic protein (GFAP) (Garrison et al. 1991), decreased expression of glutamate transporters and K<sub>cat</sub>-type membrane potassium channels (Huang et al. 2004; Kawahara et al. 2002; MacFarlane and Sontheimer 1997; Olsen and Sontheimer 2004), increased Cl<sup>−</sup> conductance (Parkerson and Sontheimer 2004; Walz 2002), absence of the [Ca<sup>2+</sup>], transients normally evoked by afferent activity (Aguado et al. 2002), and altered expression of genes regulating production and release of neuroactive cytokines, chemokines, growth factors, and NO (Meeuwsen et al. 2003; Milligan et al. 2001).

Application of algesic chemical to skin or muscle not only is followed by glial activation in the dorsal horn region that receives afferent projections from the tissue exposed to the chemical, but also by hyperalgesia/persistent pain and abnormal pain-related sensorimotor behaviors (Banna et al. 1986; Dickenson and Sullivan 1987; Fu et al. 2000; Porro and Cavazzuti 1993; Watkins and Maier 2002; Watkins et al. 1997). Moreover, the intensity and time-course of dorsal horn glial activation are strongly correlated with the pain-related sensorimotor behaviors triggered by the exposure to algesic chemical (Watkins and Maier 2002; Watkins et al. 1997), and neurons in the region of the horn that receives input from the exposed tissue display unusual receptive field and response properties consistent with subjects’ abnormal pain-related sensorimotor behaviors (Watkins and Maier 2002; Woolf and Salter 2000).

Although it is established that the morphological, biochemical, and functional properties of the glia of the dorsal horn modify after exposure of skin or muscle to an algesic chemical, the relationship between the glial alterations and the neuronal/perceptual abnormalities that develop after such an exposure is incompletely understood. This gap in understanding motivated us to investigate the effects on the dorsal horn of intracutaneous injection of 5% formalin. Our primary goal was to evaluate whether the glial activation that follows intracutaneous injection of formalin is accompanied by significant modification of the normally appreciable ability of astrocytes to homeostatically regulate local extracellular fluid composition. We regarded multiple lines of published evidence as consistent with the possibility that the vigorous C-nociceptor activity evoked by algesic chemical to skin or muscle (Klemm et al. 1989; McCall et al. 1996; Porro et al. 2003; Puig and Sorkin 1996) is accompanied by an impaired ability of astrocytes to regulate dorsal horn extracellular fluid composition. First, intracutaneous formalin injection is accompanied by a large and prolonged elevation of [K<sup>+</sup>]<sub>i</sub> in the superficial dorsal horn (Heinemann et al. 2005).
METHODOLOGY

Experiments were performed in accordance with National Institutes of Health guidelines for animal care and welfare. All protocols were approved in advance by the University of North Carolina Institutional Animal Care and Use Committee. Subjects were young adult rats (100–125 g; Sprague-Dawley, Charles River). Effort was made to minimize animal use and suffering.

Slice preparation

The subject was placed in a light-tight enclosure. An anesthetic gas mix (4% halothane in a 50/50 mix of nitrous oxide and oxygen) was delivered to the interior of the enclosure via a tube connected to an anesthesia machine (COMPAC-50, Forreger). After induction of general anesthesia, ketamine was administered intraperitoneally (0.5 ml of a 25 mg/ml solution) to prevent CNS excitotoxicity. Local anesthetic (lidocaine; 0.1–0.2 ml of a 1 mg/ml solution) was injected into the paravertebral musculature at three to four equally spaced sites on both sides of the vertebral column between levels T5 and S3 to reduce/eliminate the impact on the lumbar sacral cord dorsal horn of the vigorous afferent barrage that otherwise would accompany surgical isolation of the spinal cord and transection of the dorsal roots. After administration of the local anesthetic, the subject was transferred to a bed of ice (to lower body temperature). General anesthesia was maintained by administering the anesthetic gas mix via a face mask, and the vertebral column and spinal cord were excised, placed in ice-cold artificial cerebrospinal fluid (NaCl replaced by MgSO4, 1.25 NaH2PO4, and 10 glucose. A fine nylon mesh stabilized the slice in a submerged position. A total of 233 slices were studied—152 from 67 normal/untreated subjects, and 81 from 36 subjects in which the right hindpaw was injected with formalin 3–5 days before the experiment.

Optical intrinsic signaling imaging

The submerged slice was transilluminated using a controlled light source (Oriel), and images were obtained at ×2 or ×4 magnification using an inverted microscope (Diaphot 200, Nikon) and a cooled, slow-scan CCD camera (Photometrics). The optical response of each slice to a standard dorsal root stimulus consisting of a series of repetitive constant-current pulses (pulse duration, 0.2 ms; 2–4 times the intensity at which an optical response was first detected; train duration, 1.0 s; frequency, 20 Hz) was recorded. The standard stimulus was applied once every 30 s to the attached root at the dorsal root entry zone using a 50-μm-diam bipolar stimulating electrode, isolation unit, and programmable pulse generator (Master-8, AMPI).

The imaged region of each slice was extensive: it always included all layers of the dorsal horn on one side and much of the neighboring ventral horn and white matter on the same side. The region of an image evaluated using quantitative analytical approaches was selected using a criterion independent of investigator interest/bias—i.e., the evaluated region always was the region that underwent an increase in light transmittance [the optical intrinsic signal (OIS)] (Asai et al. 2002; Murase et al. 1999, 1998; Sýkova et al. 2003) in response to the standard dorsal root stimulus. In every slice, the evaluated region was confined to the superficial dorsal horn (DHs; i.e., layers I, IIα, and IIβ).

The OIS not only develops in CNS regions in which a stimulus evokes increased spike discharge activity, but also in regions in which neurons undergo subthreshold decreases in membrane potential [i.e., excitatory postsynaptic potentials (EPSPs)] without action potentials (Kohn et al. 2002a,b; Shoham and Grinvald 2001). These attributes, together with its high spatial and temporal resolution, make the OIS imaging method well suited for detection, localization, and quantification of the population-level responses of the dorsal horn to both electrical stimulation of the dorsal root and direct application (using a puffe r pipette) of neuroactive chemicals.

A series of 30 images was acquired (image acquisition rate = 0.5/s) in a fixed temporal relationship to each application of the standard dorsal root stimulus (a trial). The first and second images in such a trial were obtained at 1000 ms and at 500 ms before stimulus onset (reference images); two poststimulus onset images were obtained during the standard stimulus and the remaining 26 images after termination of the stimulus. Trial duration was 15 s. An average difference image was generated from the images acquired during each trial. Each average difference image was formed by I) subtracting the reference image obtained at 500 ms before stimulus/puff onset from each image obtained in the same trial between 2.5 and 12.5 s after stimulus/puff onset (images 6–25; total of 20), and 2) at each pixel location by dividing the sum of the differences between the poststimulus/postpuff and reference images (same-trial) by the number of frames (20). An intensity value was calculated for each pixel in a difference image using the formula [sum]μTHij – μTref]/HTij, where THij is the intensity of the ith pixel in the jth image, and Tref is the intensity of the ith pixel in the reference image. The same approach was used to evaluate series of images acquired before, during, and after a brief local application of either 15 mM K+ or 100 μM glutamate (GLU; using a puffer pipette and controlling electronics; Picospritzer II, General Valve).

Mean intensity (ΔI/ΔT) of either the OISK+ or the optical response to puffer-applied K+ or GLU was determined by I) bounding the region in the DH that the transmittance values met or exceeded a criterion increase in transmittance (typically the upper 5–10% of pixel values in the image), and 2) computing the average intensity of all pixels within the bounded region. The effect of a treatment (e.g.,
bath-applied local anesthetic) on the mean intensity of the OISDR or the response to puffer-applied GLU or K+ was quantified as follows. The same region of the DHs used to determine $\Delta T/T$ for the control response also was used to measure $\Delta T/T$ for the response obtained under the treatment condition. Treatment effect was expressed in terms of the mean intensity of the control response: i.e., $\Delta T/T_{\text{control}} / \Delta T/T_{\text{treatment}} \times 100 = \%$. All average difference images shown in this paper were contrast enhanced using a standard histogram equalization procedure.

Field potential recording

Single-pulse (0.2 ms duration) constant-current stimuli were applied to the peripheral end of the attached dorsal root using a suction electrode, isolation unit, and programmable pulse generator (Master 8, AMP). The postsynaptic field potential evoked in the DH by the dorsal root stimulus (P-PSPDR) was recorded with a 1–2 mM NaCl-containing glass micropipette (shaft 1.2 mm OD; pulled with a vertical puller; Narishige PP-83). Each P-PSPDR was obtained by placing the tip of the recording micropipette in the substantia gelatinosa (SG; lamina II; all P-PSPDRs were obtained in the region of the SG where the intrinsic signal evoked by the standard repetitive dorsal root stimulus (OISDR) was maximal and, whenever possible, at a locus where the field potential consisted of clearly separated short- and longer-latency responses to single-pulse stimulation of the dorsal root (Hantman et al. 2004; Lu and Perl 2003; Ruscheweyh and Sandkuhler 2000). P-PSPDRs were evoked using stimulus currents two to four times the minimum (threshold; typically 50–80 μA) current. Currents of this intensity consistently activate both large- ($A_D$) and small-diameter ($A_S$ and C) dorsal root fibers (Asai et al. 2002; Ikeda et al. 1998; Murase et al. 1998, 1999; Ruscheweyh and Sandkuhler 2000, 2001). Recordings of P-PSPDRs were band-pass filtered (20–500 Hz) and sampled at 20 KHz using pClamp 6.0 (Axon Instruments).

Algesic chemical administration/behavioral assessment

In a subset of animals, 10–25 μl of 5% formalin was injected into the digital and palmar skin of the right forepaw 3–5 days before the slice experiment. Sensorimotor behavior was monitored throughout the 2-h period after the injection, and once-daily thereafter, including the day of the experiment. During the 30 min to 1 h period immediately after the injection, every subject exhibited vigorous and frequent pain-related behaviors involving the injected limb (paw licking, shak- ing, guarding, limping, exaggerated avoidance responses to mechanical contact with the skin in the vicinity of the injection site). Over the 3- to 5 day period between the formalin injection and the slice experiment, most subjects that received the intracutaneous formalin injection (>80%) continued to react to mechanical contact with the injected paw in a manner consistent with hyperalgesia/persistent pain.

RESULTS

Studies of slices from untreated (control) subjects

OISDR DEPENDS ON STIMULUS-EVOKED NEUROTRANSMISSION. Figure 1 summarizes results obtained from two of the six slices in which the dependency of the OISDR on neurotransmission was evaluated. The top left image in Fig. 1, A and B, is a reference image that shows the superficial dorsal horn (DH) and several of the bordering anatomical structures (e.g., DC, dorsal column; LC, lateral column; VH, ventral horn). Comparison of each reference image with the corresponding prestimulus—poststimulus difference images (images at top right and bottom right in Fig. 1, A and B) shows that in both slices (1) the region in the slice that undergoes an increase in transmittance in response to dorsal root stimulation lies within the boundaries of the DHs, and 2) the stimulus-evoked increase in transmittance...
Temporal properties of the OISDR

While the effects of local anesthetic and Ca\(^{2+}\)-free ACSF show that the OISDR depends on stimulus-evoked neurotransmission, the slow temporal properties of the OISDR clearly differentiate it from stimulus-evoked dorsal horn neuroelectrical activity. The \(\Delta T/T\) versus time plots in Fig. 2 (bottom right) show the time-course of the OISDR at three different loci within the same dorsal horn. These plots show that 1) the magnitude of the stimulus-evoked transmittance increase was largest at DH\(_{\text{IV}}\) site 1, intermediate at site 2, and smallest at site 3, and 2) at each site, the stimulus-evoked increase in transmittance continued at near-maximal values for a prolonged period after stimulus termination. Although the \(\Delta T/T\) versus time plots are truncated at 6 s after stimulus onset, transmittance at site 3 (the site at which the OISDR was weakest) remained above background for \(\sim 20\) s after stimulus termination and above background for \(\sim 40\) s at sites 1 and 2 (sites at which the OISDR was near-maximal). The OISDR recorded in every slice in which the standard stimulus was delivered to the attached dorsal root (\(n = 48\)) exhibited similar slow temporal characteristics.

Dorsal horn optical response to local application of K\(^{+}\) or glutamate

Figure 3 shows that a 100-ms pressure-driven application (puff) of either K\(^{+}\) (15 mM; Fig. 3A), or glutamate (GLU, 100 \(\mu\)M; Fig. 3B) to a discrete locus in the DH\(_{\text{IV}}\) evokes an increase in transmittance that remains confined to the immediate vicinity of the tip of the puffer pipette (dark region in difference image identifies the region of increased transmittance). In the slice that provided the observations in Fig. 3, as in the other three slices studied in the same way, the focal increase in transmittance evoked in the DH\(_{\text{IV}}\) by a 15 mM K\(^{+}\) puff consistently was smaller in both magnitude and spatial extent than the response evoked at the same site by a 100 \(\mu\)M GLU puff (compare images in Fig. 3, A and B). Despite this considerable difference in magnitude, however, the temporal properties of the optical response of the DH\(_{\text{IV}}\) to locally applied K\(^{+}\) or GLU were similar—i.e., both responses developed relatively rapidly (peaked within \(\sim 1\) s of puff onset) and both declined over an extended time period (\(\geq 15\) s; \(\Delta T/T\) vs. time plots in Fig. 3C are truncated and thus do not show the total time that the increase in transmittance remained above background).

In the slice that provided the observations in Fig. 4A, application of a 100 \(\mu\)M GLU puff to layer IV of the dorsal horn evoked an increase in transmittance substantially weaker than that evoked when an identical GLU puff was applied to layer II. A similar result was obtained in all slices (\(n = 6\)) in which an identical GLU puff was applied to layer II and to layer IV in the same horn. The \(\Delta T/T\) versus time plots in Fig. 4B enable direct comparison of the response evoked by a GLU puff in layer II (plot with •; same slice as in Fig. 4A) and the OISDR evoked in the same horn by the standard dorsal root stimulus (plot with ○). Clearly, the OISDR not only develops more slowly than the response to the GLU puff, but its magnitude (peak \(\Delta T/T\) value) is substantially smaller. Similar discrepancies between the responses of the same dorsal horn to GLU puff versus dorsal root stimulation (OISDR) were observed in every slice (\(n = 7\)) that we studied in the same way.
While direct evidence is lacking, the authors presume that the above-described differences between the OIS_{DR} and the optical response of the DHs to a GLU puff are due to the temporally and spatially extended, but relatively modest, increase in [GLU]_o associated with the standard dorsal root electrical stimulus versus the abrupt, highly localized, and presumably much larger increase in local [GLU]_o achieved with the GLU puff.

Alterations of the normal relationship between the OIS_{DR} and stimulus-evoked neuroelectrical activity

INCREASE OF \([K^+]/[H^+]\). Figure 5A shows the OIS_{DR} (dark regions in difference images) recorded before any treatment (control; top right), after bath application of ACSF containing 8 mM K^+ (high K^+; bottom left in A), and after return of the perfusate to normal ACSF containing 3.5 mM K^+ (washout; bottom right in A). On average (across 3 slices studied in the same way), bath-application of ACSF containing 8 mM K^+ led to a highly significant increase in the magnitude of the OIS_{DR} (to 172.63 ± 4.73% of control; \(P < 0.001\)). The ΔT/T versus time plots in Fig. 5B show (for the same slice that provided the images in A) the detailed time-course of the OIS_{DR} under each condition. After washout of the chamber with ACSF containing normal (3.5 mM) K^+, the magnitude of the OIS_{DR} at most times between 0.0 and 6.0 s after onset of the standard dorsal root stimulus was either the same or very similar to that measured in the same slice before exposure to elevated K^+.

Across the three slices studied in this way, OIS_{DR} magnitude after washout with ACSF containing 3.5 mM K^+ was 102.66 ± 2.77% of control (\(P = 0.391\); not significant).

Recordings of the field potential (P-PSP_{DR}) evoked in the DHs by dorsal root stimulation showed that bath application of
ACSF containing 8 mM K\textsuperscript{o} is accompanied by a progressive increase in the late (long-latency) but not the early component of the response (representative field potentials from an exemplary slice are shown in Fig. 5C). The results obtained from five slices studied in the same way are summarized in Fig. 5D. On average, the late component of the P-PSP\textsubscript{DR} increased progressively in the presence of 8 mM K\textsuperscript{o} (plot with ●), reaching 173.93 ± 12.41\% of control at 15 min after onset of the exposure to elevated K\textsuperscript{o} (\(p < 0.001\); \(n = 5\)), whereas the shorter-latency (early) component declined to 75.11 ± 3.78\% of control (plot with ○; \(p < 0.001\); \(n = 5\) slices). After the solution perfusing the recording chamber was returned to ACSF containing 3.5 mM K\textsuperscript{o} (washout), both the early and late components of the P-PSP\textsubscript{DR} recovered to near-control values.

That the late but not the early component of the P-PSP\textsubscript{DR} increased in the presence of ACSF containing 8 mM K\textsuperscript{o} was observed in each of the five slices. This outcome was not anticipated, but is of some interest because it raises the possibility that an elevation of K\textsuperscript{o} in the DH\textsubscript{s} selectively enhances the DH\textsubscript{s} response to input conveyed via small-diameter afferents in the dorsal root—i.e., afferents that in intact subjects terminate in peripheral nociceptors and terminate synaptically on neurons in the DH\textsubscript{s} (Hantman et al. 2004; Lu and Perl 2003; Ruscheweyh and Sandkuhler 2001, 2000).

INHIBITION OF ASTROCYTE ENERGY METABOLISM. The next experiments sought to evaluate the effects of a selective inhibitor of astrocyte metabolism FAc (Bacci et al. 2002; Hulsmann et al. 2003; Keysen and Pellmar 1994; also see Berg-Johnsen et al. 1993; Watkins et al. 1997) on both the optical and neuroelectrical responses of the DH\textsubscript{s} to dorsal root stimulation. To this end, P-PSP\textsubscript{DRs} and OIS\textsubscript{DRs} were recorded before and after exposure of the slice to ACSF containing 400 \(\mu\)M FAc. Figure 6 shows representative results obtained from one of the four slices studied in this way. The column of color-contoured images at the left of Fig. 6A shows the spatial extent and intensity of the OIS\textsubscript{DR} recorded before any treatment (control), after onset of bath application of 400 \(\mu\)M FAc, and subsequently, after replacement of the FAc-containing ACSF with ACSF containing FAc + Gln. The P-PSP\textsubscript{DRs} shown in the column on the right of Fig. 6A were acquired from a different slice than the slice that yielded the optical responses shown at the left, were obtained at times and under conditions corresponding to those under which the optical responses were obtained. Visual comparison of the optical and field potential observations in Fig. 6A shows that both the late component of the P-PSP\textsubscript{DR} (indicated by shading) and the OIS\textsubscript{DR} are depressed under FAc. Note that when the perfusate was switched from FAc to FAc + Gln, the magnitude of the late component of the P-PSP\textsubscript{DR} recovered to near-normal, but the magnitude of the OIS\textsubscript{DR} continued to decline to values well below those detected under FAc.

The plots in Fig. 6B summarize in a more quantitative way the temporal sequence of changes in the magnitude of both the OIS\textsubscript{DR} and P-PSP\textsubscript{DR} observed under the above-described FAc and FAc + Gln conditions. Two tendencies are apparent. First, the magnitude of both the OIS\textsubscript{DR} (average across 5 subjects; open symbols; error bars indicate ± 1SE) and the late component of the P-PSP\textsubscript{DR} (results from 2 subjects are plotted; solid lines) decline progressively in the presence of ACSF containing 400 \(\mu\)M FAc. Second, after the perfusate was switched to ACSF containing FAc + Gln (last 3 points in plots in Fig. 6B)
the OISDR continued to decline, whereas the magnitude of the late component of the P-PSPDR recovered and attained values approaching, if not exceeding, those recorded under control conditions (before exposure to FAc). In other words, the OISDR and the P-PSPDR became dissociated in the presence of FAc/H11001 Gln.

**INHIBITION OF A-TYPE K+ CHANNELS.** Figure 7 shows representative results from a slice in which the OISDR and the postsynaptic response of the dorsal horn (P-PSP) were measured before and after perfusion of the recording chamber with ACSF containing 100 μM 4-aminopyridine (4-AP). The rationale for evaluating the effects of 4-AP was as follows. If, as the effects of inhibition of astrocyte metabolism (Fig. 6) suggest, the OISDR mainly reflects astrocyte uptake of K+ and/or GLU, interference with outward K+ transport in both astrocytes and neurons via A-type K+ channels also should alter the OISDR and P-PSPDR in opposite ways. More specifically, if the OISDR mainly reflects astrocyte clearance of K+ and GLU, 4-AP should decrease the OISDR (due to 4-AP block of astrocyte K+ uptake) but increase the P-PSPDR (because of the increase in neuronal excitability due to the block by 4-AP of outward K+ currents in both pre- and postsynaptic neurons).

As predicted, the optical and neuronal responses of the dorsal horn evoked by electrical stimulation of the dorsal root dissociated in the presence of 4-AP. More specifically, both the early and late components of the P-PSPDR increased under 4-AP (the late component exhibited the largest increase; plots in Fig. 7D; see also Fig. 7B), whereas the OISDR decreased (compare control and 4-AP images in Fig. 7A). Similar results were obtained in all four slices studied in the same way. On average, the early and late components of the P-PSP increased by 151.63 ± 5.41% (P < 0.001; n = 4) and 318.09 ± 50.66%...
respective, whereas the OISDR decreased to 26.19% ± 4.32% of control (P < 0.001; n = 4). Other experiments detected a dissociation of the dorsal horn optical and neuronal responses to dorsal root stimulation when the ACSF contained 5 mM cesium chloride (CsCl; data not shown)—CsCl was studied because it has been shown (Janigro et al. 1997) to block inwardly rectifying (KIR) potassium channels widely presumed to contribute to astrocyte-mediated K\(^{+}\) homeostasis (Kofuji and Newman 2004; Newman 2003; Olsen and Sontheimer 2004; Simard and Nedergaard 2004; Walz 2000).

Studies of slices from formalin-injected animals

**OISDR AFTER INTRACUTANEOUS FORMALIN INJECTION.** The pair of images on the right in Fig. 8 (Formalin) were obtained from the horn on the same side as the hindpaw that received an intracutaneous injection of 25 µl of 5% formalin 4 days before the experiment. In contrast, the image pair on the left (Fig. 8; Control) were obtained from the opposite horn in the same slice. The difference images at the bottom of Fig. 8 show the OISDR evoked in each horn by the standard dorsal root stimulus. Note that the OISDR recorded from the horn on the same side of the formalin injection (bottom right) is extremely small, whereas on the opposite side of the same slice (bottom left), the magnitude of the OISDR is typical of that recorded in the DH of an untreated subject. A similar result was obtained in each of the 10 slices from subjects that received an intracutaneous injection of formalin 3–5 days before the experiment. On average, the OISDR recorded from the DH of the ipsilateral horn to the formalin injection site was only 37.19 ± 5.92% (P < .001; n = 10) of the control OISDR recorded from the DH on the side contralateral to the injection.

In four subjects, the rostrocaudal extent of the attenuating effect of an intracutaneous injection of 5% formalin on the dorsal horn was determined by recording the OISDR not only from the ipsilateral (on the same side as the formalin injection site) and contralateral horns at the level of the cord at which most afferents from the injected skin site (volar hindpaw) enter the spinal cord (segmental level L_4) (Takahashi and Nakajima 1996; Takahashi et al. 2002), but also from both horns of slices cut from successively more rostral levels of the same spinal cord (i.e., at 0.8, 1.6, 2.4, 3.2, and 4.0 mm rostral to level 0; slice thickness was 400 µm). In three of the spinal cords studied in this way, the most rostral level studied (level 4.0) was L_1; in the remaining subject it was L_2.

At level L_4, the magnitude of the OISDR recorded from the DH on the same side as the formalin injection was only 51.5 ± 6.2% (P < .001; n = 4 slices) of the OISDR recorded in the contralateral horn, and this discrepancy remained essentially undiminished for ~1.6 mm rostral to L_4. At levels ~2.4 mm rostral to L_4, however, no effect of the intracutaneous formalin injection on the OISDR evoked in the ipsilateral DH was evident—i.e., at distances ≥2.4 mm above the level (L_4) at which most afferents from the hindpaw injection site enter the spinal cord, the average magnitude of the OISDR evoked in the ipsilateral DH by the standard stimulus did not differ significantly from the average magnitude of the OISDR recorded in the DH on the opposite side (i.e., at levels >2.4 mm rostral to L_4; the OISDR recorded in the ipsilateral DH was 89.43% of the OISDR recorded in the contralateral DH (P > 0.04; n = 6 slices; not significant).

**FIG. 7.** Effects of 4-aminopyridine (4-AP) on OISDR and P-PSPDR. A: reference image at top left. Top right: OISDR before treatment (Control). Bottom left: OISDR after 100 µM 4-AP. Bottom right: OISDR after return to drug-free ACSF (Washout). B: P-PSPDR under control and 100 µM 4-AP conditions. C: T/T vs. time plot showing that OISDR decreased after 4-AP (exposure to 4-AP indicated by rectangle). D: effect of 4-AP on early (●) and late (○) components of P-PSPDR.
INTRACUTANEOUS FORMALIN INJECTION REDUCES THE NORMAL IMPACT OF ELEVATED $[K^+]_o$ ON THE OIS$_{DR}$. When $[K^+]_o$ in the ACSF was 3 mM (control), the time-course of the OIS$_{DR}$ evoked in the DH$_i$ on the same side as the formalin injection site (ipsilateral; the formalin side) and in the DH$_o$ on the opposite side (contralateral; the normal side) are very similar (compare normalized $\Delta T/T$ vs. time plots with filled symbols in Fig. 9, A and B). More specifically, regardless of whether the horn receives afferent input from an untreated skin site or from a formalin-injected skin site (the $\Delta T/T$ values of all the plots in Fig. 9, A and B, are normalized; i.e., expressed in terms of percent of the maximal value recorded under each condition), the transmittance increase evoked in the DH$_i$ by stimulation of the dorsal root peaks at about 10 s after stimulus onset, and over the next 30–60 s declines toward baseline. In contrast, when the ACSF bathing the slice is elevated (contains 8 mM $K^+$), the temporal profile of the transmittance increase evoked in the DH$_o$ contralateral (the normal side) to a formalin-injected skin site is very different from the transmittance increase evoked in the horn ipsilateral (the formalin side) to the injection site (compare open symbols in Fig. 9, A and B). As described previously (e.g., Fig. 5), in the presence of ACSF containing 8 mM $K^+$, the increase in transmittance evoked by dorsal root stimulation in the DH$_i$ on the side contralateral to the formalin injection not only did not return to baseline during the 50 s period during which it was sampled (unlike the response of the same horn to the same dorsal root stimulus when $[K^+]_o$ is 3 mM), but the increase in transmittance remained near maximal over the entire period (60 s) during which images were acquired. Clearly, therefore the duration of the transmittance change evoked in the DH$_i$ contralateral to the formalin injection (the normal side) is substantially prolonged when $[K^+]_o$ is 8 mM, whereas in the DH$_i$ on the same side as the formalin injection (the formalin side), the duration of the transmittance change evoked by the standard dorsal root stimulus modifies only slightly in the presence of elevated $K^+$.

INTRACUTANEOUS FORMALIN INJECTION REDUCES THE DORSAL HORN OPTICAL RESPONSE TO LOCAL APPLICATION OF GLU. A final series of slices ($n = 3$) was studied to determine the effects of formalin on the optical response of the lumbosacral dorsal horn to puff application of 100 $\mu$M GLU. The difference images in Fig. 9C are representative and reveal that 4 days after intracutaneous formalin injection (volar hindpaw), a GLU puff evokes a prominent local increase in transmittance in the lumbosacral dorsal horn on the side opposite to the skin site that received the injection (dark region in control image), but an identical puff evokes a much smaller transmittance increase in the DH$_i$ on the same side as the skin site injected with formalin (formalin image). The $\Delta T/T$ versus time plots in Fig. 9D summarize, for the same slice shown in Fig. 9C, the time-course of the transmittance increase on the control versus formalin sides evoked by a 100-ms puff of 100 $\mu$M GLU. For the three slices studied in this way, the average (across-subject) DH$_i$ response to direct GLU application on the side of the formalin injection was only $32.6 \pm 5.2\%$ ($P < 0.001$) of the DH$_i$ response evoked by an identical puff on the control side.

DISCUSSION

Dorsal horn optical response—origins and underlying mechanisms

The findings obtained in the initial experiments (Figs. 1 and 2) show that, although the OIS$_{DR}$ depends on synaptic neuro-
transmission, its slow development and prolonged persistence
are uncharacteristic of stimulus-evoked neuronal activity. This
alerted us to the possibility—one already proposed by others
(Asai et al. 2002)—that the OISDR may be nonneuronal in
origin. Subsequent experiments showed (Figs. 3 and 4) that the
optical response of the dorsal horn to a 100-ms local applica-
tion of K⁺ or GLU is very prolonged, leading us to consider
that it, like the OISDR, primarily reflects nonneuronal mecha-
nisms/processes.

Although direct evidence is lacking, a wealth of published
observations is consistent with the possibility that astrocytes
are the major source of the transmittance increase that under-
lies both the OISDR and the dorsal horn optical response to
puffer-applied K⁺ or GLU. For example, astrocytes in the
dorsal horn and other CNS regions undergo morphological/
biophysical modifications (e.g., cell volume increases, light
scattering decreases) that are closely coupled to stimulus-
evoked increases in excitatory afferent drive. Furthermore,
astrocyte-mediated clearance of the excesses in extracellular
K⁺ and/or GLU that accompany increased postsynaptic neu-
ronal activity is accompanied by prominent and prolonged
alterations of cell volume and tissue light scattering. In addi-
tion, astrocyte uptake of GLU₀ and K⁺₀ is metabolism-depen-
dent and occurs via astrocyte-specific membrane transporters
(Huang et al. 2004; Kawahara et al. 2002; Rosenberg et al.
1992) and carrier- and channel-operated potassium chloride
uptake (Walz 2000), respectively.

The unusual temporal characteristics of the optical response
of the DHs to a 100-ms puff of 15 mM K⁺ (i.e., rapid onset,
decay, followed by a secondary increase) reinforce the sugges-
tion that the response is, in the main, attributable to astrocytes.
For example, the rapid initial increase in transmittance seems
explicable in terms of an initially rapid uptake of excess K⁺₀
by astrocytes in the vicinity of the puffer pipette (K⁺ uptake is
accompanied by obligatory uptake of water and astrocyte
swelling; Sykova et al. 2003), whereas such a secondary (delayed)
increase in transmittance is expected if local transient
neuronal excitation was evoked by the K⁺ puff. That is,
puff-evoked neuronal excitation not only would be accompa-
nied by a local release of GLU from presynaptic nerve termi-
nals but, after a delay, by astrocyte re-uptake (along with
water) of the released GLU, and a renewed (delayed) phase of
astrocyte swelling. Another possibility is that the secondary,
delayed increase in DHs transmittance that follows a 15 mM
K⁺ puff reflects astrocyte uptake of K⁺-evoked GLU release
from astrocytes (Volterra and Bezzi 2002).

A second example of an aspect of the dorsal horn optical
response to puffer-applied K⁺ or GLU that fits with the idea
that the response is astrocytic in origin is that the response of
lamina II to puffer-applied K⁺ or GLU consistently is larger in
spatial extent and magnitude than the response evoked by
application of an identical puff to lamina IV (e.g., Fig. 4). This
observation is in accord with the report (Svoboda et al. 1988)
that, in normal subjects, [K⁺]₀ is maintained at lower values in
the superficial dorsal horn (laminae I–II) than in the deeper
laminae (laminae III–V) and raises the possibility that the
capacity of laminae I–II astrocytes to take up K⁺ and GLU
(plus with water) may be greater than that of layer IV
astrocytes. Interestingly, a differential capacity of astrocytes in
the superficial versus deep dorsal horn to take up K⁺ and GLU
would be compatible with recent demonstrations that K⁺ channels and aquaporins tend to colocalize in astrocyte membranes, and aquaporin expression is higher in the superficial dorsal horn than in the deeper laminae (Asai et al. 2002; see also Binder et al. 2004).

If the OISDR is, in fact, attributable to light scattering changes that accompany astrocyte uptake of K⁺ and GLU, it should be possible to dissociate the dorsal horn optical and neuronal responses evoked by stimulation of the dorsal root. Two series of experiments were carried out to address this possibility. The first evaluated the effects on the optical (OISDR) versus neuronal (P-PSPDR) responses to dorsal root stimulation of selective inhibition of astrocyte metabolism with bath-applied FAc (400 μM). The second experiments of this type evaluated the effects of bath-applied 4-AP, an agent that blocks A-type (rapidly inactivating) membrane potassium channels in both astrocytes and neurons.

**EFFECTS OF FAC.** The following information about the cellular actions of FAc is essential for full appreciation of the results: 1) FAc inhibits the enzyme aconitase in the Krebs cycle of astrocytes, but does not affect neuronal metabolism-linked energy production (Clarke et al. 1970; Hassel et al. 1992, 1997; Hulsmann et al. 2003; Keyser and Pellmar 1994; Muir et al. 1986; Paulsen et al. 1987; Waniewski and Martin 1998); 2) FAc blocks both astrocyte production and release of glutamine (Gln) and thus reduces the availability of extracellular Gln for uptake by the presynaptic terminals of glutamatergic dorsal root afferents (Bacci et al. 2002); 3) glutaminergic neurotransmission fails in the presence of FAc due to decline of adequate GLU in the presynaptic terminals of dorsal root afferents (Bacci et al. 2002), but this decline can be avoided if an adequate supply of extracellular Gln is provided; and 4) provision of an adequate level of Gln in nerve terminals even in the continuing presence of FAc (because FAc does not affect the capacity of nerve terminals to take up and convert Gln to GLU), and enables complete recovery of glutaminergic neurotransmission even though FAc continues to inhibit astrocyte energy metabolism (Bacci et al. 2002).

The observations obtained after inhibition of astrocyte metabolism with FAc (Fig. 6) can be interpreted as follows. First, the declines in the magnitude of both the OISDR and the P-PSPDR (especially the late component of the P-PSPDR) that occurred during the exposure to FAc reflect, respectively, inhibition of astrocyte metabolism by FAc (because of the FAc-induced reduction of energy-dependent astrocyte uptake of K⁺ and GLU), and a disappearance of GLU in the presynaptic terminals of dorsal root afferents (due to FAc inhibition of astrocyte Gln production and release, which in turn, reduces the [Gln]o available for uptake by the presynaptic terminals of glutaminergic dorsal root afferents). Second, the dissociation of the dorsal horn optical and neurophysiological responses after switch of the bath to FAc + Gln (plots at bottom of Fig. 6; the P-PSPDR returns to control levels, whereas the OISDR continues to decline) reflects restoration of stimulus-evoked dorsal horn glutaminergic neurotransmission due to restoration of [Glu]o in presynaptic nerve terminals (i.e., by the Gln provided in the ACSF that bathed the slice). The OISDR continued to decline in the presence of FAc + Gln because of the continuing FAc-mediated inhibition of astrocyte metabolism.

**EFFECTS OF 4-AP.** Although the specific functional role(s) of rapidly inactivating A-type K⁺ currents in astrocytes remain(s) to be established, outward K⁺ currents are known to contribute to astrocyte-mediated spatial buffering and siphoning of K⁺ (Bekar and Walz 2002). With this in mind, the a priori prediction was that block of A-type K⁺ channels with 4-AP would antagonize K⁺ uptake by astrocytes (and thus reduce or eliminate the OISDR), and at the same time enhance stimulus-evoked dorsal horn neurotransmission (and thus increase the magnitude of the P-PSPDR evoked by dorsal root stimulation) due to the increased excitatory neurotransmitter release expected to accompany block of A-type K⁺ currents in the presynaptic terminals of dorsal root afferents. We therefore expected dissociation of the OISDR and the postsynaptic response of dorsal horn neurons to dorsal root stimulation (P-PSPDR) under 4-AP. The results shown in Fig. 7 are viewed as fully consistent with this prediction.

**Dorsal horn astrocytes after intracutaneous injection of formalin**

Experiments on slices from subjects that had received an intracutaneous injection of 5% formalin to the volar hindpaw 3–5 days before the experiment revealed that, in the horn on the same side (ipsilateral) as the injected skin site, the optical responses evoked by electrical stimulation of the dorsal root (Fig. 8) and also by direct application of K⁺ or GLU (Fig. 9) were substantially smaller than those recorded in the opposite horn (relative to the responses evoked in the contralateral dorsal horn). This outcome is interpreted to indicate that astrocytes in the dorsal horn on the same side of the formalin injection are relatively unresponsive to an elevation of [K⁺]o or [GLU]. Furthermore, in contrast to the marked prolongation of the time-course of the OISDR that occurs in an untreated subject during an exposure to elevated K⁺o (to 8 mM), the same elevation of K⁺o had little or no impact on the time course of the OISDR recorded in the lumbosacral horn on the same side as the hindpaw injected with formalin (Fig. 9, A and B). This insensitivity of the OISDR on the same side as the formalin-injected skin site to elevated K⁺o or GLU strongly suggests that astrocytes in the region of the dorsal horn that receives its afferent input from the formalin-injected skin region are unable to clear the extracellular compartment of excess K⁺ and GLU and do not take up water and swell (and thus tissue light scattering does not alter) in response to the increases in K⁺o and GLUo that accompany increased dorsal horn neuroelectrical activity. As a consequence, K⁺o and GLUo (and thus neuronal excitability) in the DH remain elevated for an abnormally prolonged period following stimulus-evoked dorsal horn neuronal activity.

**Contributions of impaired astrocyte homeostatic function to the abnormal dorsal horn neuron properties that develop after intracutaneous formalin injection**

Although it is recognized that perisynaptic astrocytes normally function as active partners in normal CNS synaptic neurotransmission (Newman 2004), the results of this study suggest that, under a variety of pathological conditions (i.e.,
inflammation, injury, infection), astrocytes are unable to function as fully active partners in CNS neurotransmission. The finding (Coul1 et al. 2003) that peripheral nerve injury is accompanied by trans-synaptic reduction in the expression of the potassium-chloride exporter KCC2 in dorsal horn lamina I neurons—an alteration that disrupts lamina I neuron anion homeostasis—seems highly relevant to altered dorsal horn glia–neuron interactions and their potential contributions to hyperalgesia/persistent pain.

Coul1 et al. (2003) showed that the nerve injury-induced decrease in the expression of KCC2 identified by results in the intracellular accumulation of Cl\(^-\) in lamina I neurons, resulting in a shift of the equilibrium potential for Cl\(^-\) that causes GABA\(_A\) receptor mediated synaptic currents (normally hyperpolarizing) to become depolarizing (excitatory). As a result, affected neurons in lamina I (and, presumably, also in the other layers of the DHs) exhibit increased excitability, acquire spontaneous activity, and develop abnormal responsiveness to non-noxious environmental stimulation that may account, at least in part, for the hyperalgesia/persistent pain detected by behavioral assays of nerve-injured subjects.

Because the decrease in lamina I neuron KCC2 expression occurs relatively late after nerve injury (15–17 days; Coul1 et al. 2003), it unlikely is a direct result of the injury. Instead, it may be the lamina I neuron response to a maintained increase in superficial dorsal horn [K\(^+\)]\(_i\) and [GLU]\(_o\), that accompanies the prominent dorsal horn glial activation associated with peripheral nerve injury (e.g., Milligan et al. 2001). In addition, if the demonstration (Kaila et al. 1997; using hippocampal pyramidal neurons; also Payne et al. 2003) that even a brief (seconds) exposure to a modest elevation of K\(^+\)\(_o\) (to \(-8\) mM) induces increases in neuronal [Cl\(^-\)] that account for a +16- to +18-mV shift in E\(_{GABA}\) applies to dorsal horn neurons, an impaired ability of astrocytes to buffer [K\(^+\)]\(_i\) and [GLU]\(_o\), following intracutaneous formalin injection may not only lead to abnormally prolonged elevations of neuronal excitability due to excess K\(^+\)\(_i\) and GLU\(_o\), but also to neuronal abnormalities characterized by a loss of GABA-mediated inhibition and hyperalgesia/persistent pain.

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