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

Jaekwang Lee², M. Tommerdahl², O.V. Favorov², and B. L. Whitsel¹²

Department of Cell & Molecular Physiology¹
and
Department of Biomedical Engineering²
School of Medicine
University of North Carolina,
Chapel Hill, NC 27599-7545

Running title - ASTROCYTE STATUS & DORSAL HORN NEUROTRANSMISSION

Word count -
Abstract - 248
Introduction – 679
Materials & Methods – 1476
Results – 3362
Discussion – 1983

Statistics -
Pages - 38
Figures & legends - 9
Tables - 0

Corresponding author - B.L. Whitsel, Ph.D.
5109-A Neurosciences Research Building
University of North Carolina
Chapel Hill, NC 27599-7545
Email: bwhitsel@med.unc.edu
FAX: (919) 966-6927
Telephone: (919) 966-1291

Copyright © 2005 by the American Physiological Society.
ABSTRACT

In rat spinal cord slice repetitive electrical stimulation of the dorsal root at an intensity that activates C-fibers evokes a slow-to-develop and prolonged (30-50 sec) change in light transmittance (OIS_{DR}) in the superficial part of the ipsilateral dorsal horn (DHs). Inhibition of astrocyte metabolism (by bath-applied 400 μM fluoroacetate and 200 μM glutamine; FAc+Gln) or interference with glial and neuronal K\(^{+}\) transport (by 100 uM 4-aminopyridine; 4-AP) leads to dissociation of the OIS_{DR} and the postsynaptic DHs response to a single-pulse, constant-current dorsal root stimulus (P-PSP_{DR}). The OIS_{DR} decreases under FAc+Gln whereas the P-PSP_{DR} remains unaltered; under 4-AP the P-PSP_{DR} increases but the OIS_{DR} decreases. In contrast, both the OIS_{DR} and P-PSP_{DR} increase when K\(^{+}\)_{o} is elevated to 8 mM. These observations from slices from normal subjects are interpreted to indicate that the OIS_{DR} mainly reflects cell volume and light scattering changes associated with DHs astrocyte uptake of K\(^{+}\) and glutamate (GLU). In slices from subjects that received an intracutaneous injection (i.c.) of formalin 3-5 days earlier both the OIS_{DR} and the response of the DHs ipsilateral to the injection site to 100 msec local application (via puffer pipette) of 15 mM K\(^{+}\) or 100 μM GLU are profoundly reduced, and the normally exquisite sensitivity of the DHs to elevated K\(^{+}\)_{o} is decreased. Considered collectively, the observations raise the possibility that impaired regulation of DHs K\(^{+}\)_{o} and GLU_{o} may contribute to initiation and maintenance of the CNS pain circuit and sensorimotor abnormalities that develop following i.c. formalin injection.

Key words - spinal cord, glia-neuron interactions, pain mechanisms, hyperalgesia
INTRODUCTION

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\textsubscript{IR}-type membrane potassium channels (Huang et al., 2004; Kawahara et al., 2002; MacFarlane and Sontheimer, 1997; Olsen and Sontheimer, 2004), increased Cl\textsuperscript{−} conductance (Parkerson and Sontheimer, 2004; Walz, 2002), absence of the [Ca\textsuperscript{++}]\textsubscript{i} 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., 1999; 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 following exposure of skin or muscle to an algesic chemical, the relationship between the glial alterations and the neuronal / perceptual abnormalities that develop following such an exposure is incompletely understood. This gap in understanding motivated us to re-investigate the effects on the dorsal horn of intracutaneous (i.c.) injection of 5 % formalin. Our primary goal was to evaluate whether the glial activation that follows i.c. 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, i.c. formalin injection is accompanied by a large and prolonged elevation of $[K^+]_o$ in the superficial dorsal horn (Heinemann et al., 1990; Svoboda et al., 1988). Second, tetanic nerve stimulation, noxious skin stimulation, inflammation, or an experimentally-imposed elevation of dorsal horn $[K^+]_o$ is followed by long-term changes in the efficacy of C- fiber mediated synaptic activation of dorsal horn neurons (Ma and Zhao, 2002; Sandkuhler et al., 2000; Sandkuhler and Liu, 1998; for CNS effects of elevated $[K^+]_o$ see Somjen, 2002). Third, the hyperalgesia / persistent pain, glial activation and the abnormal elevation of dorsal horn $[K^+]_o$ that develop following i.c. formalin injection are either prevented entirely or substantially reduced by prior
local anesthetic block of action potential conduction in the afferents that innervate the injection site (Fu et al., 1999; Svoboda et al., 1988).

The optical imaging and neurophysiological observations reported in this paper strongly suggest that 3-5 days after i.c. injection of formalin astrocyte regulation of \([K^+]_o\) and \([GLU]_o\) is profoundly impaired in the region of the dorsal horn that receives direct afferent input from the injected skin site. The findings raise the possibility that impaired astrocyte regulation of the extracellular fluid composition contributes to the establishment of the dorsal horn neuronal circuit abnormalities that underlie hyperalgesia/persistent pain. Some of the results were described in a preliminary communication (Lee et al., 2003).
MATERIALS AND METHODS

Experiments were performed in accordance with NIH 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 (Forreger, COMPAC-50). Following induction of general anesthesia ketamine was administered intraperitoneally (i.p.; 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 3-4 equally-spaced sites on both sides of the vertebral column between levels T-5 and S-3 to reduce / eliminate the impact on the lumbosacral spinal cord dorsal horn of the vigorous afferent barrage that otherwise would accompany surgical isolation of the spinal cord and transection of the dorsal roots. Following 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 ACSF (NaCl replaced by sucrose), and the lumbosacral cord and attached dorsal roots freed from surrounding tissues by microdissection.

Transverse slices of the lumbosacral cord (400-800 µm thickness) were cut serially using an oscillating tissue slicer (OTS-4000, Electron Microscopy Sciences) and
placed in a reservoir containing ACSF warmed (30°C) and oxygenated (using a 95 % O₂ and 5 % CO₂ gas mix). Each slice remained in the reservoir until (never <1 hr) it was transferred (by pipette) to the recording chamber. The recording chamber was continuously perfused with warmed (28–30.8 °C) and oxygenated ACSF (perfusion rate 1.5–2 ml/min). Composition (in mM) of the ACSF was 124 NaCl, 3.0 KCl, 2.5 CaCl₂, 25 NaHCO₃, 1 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; 81 from 36 subjects in which the right hindpaw was injected with formalin 3-5 days prior to the experiment.

**OIS Imaging**

The submerged slice was transilluminated using a controlled light source (Oriel) and images 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 msec; 2-4× the intensity at which an optical response was first detected; train duration 1.0 sec; frequency 20 Hz) was recorded. The standard stimulus was applied once every 30 sec to the attached root at the dorsal root entry zone using a 50 μm diameter 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 – that is, 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; Sykova 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, IIo, and IIi).

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., 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 puffer pipette) of neuroactive chemicals.

A series of 30 images was acquired (image acquisition rate = 0.5/sec) in a fixed temporal relationship to each application of the standard dorsal root stimulus (a “trial”). The 1st and 2nd images in such a trial were obtained at 1000 msec and at 500 msec before stimulus onset (“reference” images); 2 “poststimulus onset” images were obtained during the standard stimulus, and the remaining 26 images after termination of the stimulus. Trial duration was 15 sec. An average difference image was generated from the images acquired during each trial. Each average difference image was formed by (1) subtracting the reference image obtained at 500 msec before stimulus / puff onset from each image obtained in the same trial between 2.5-12.5 sec 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 post-stimulus / post-puff 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 $\sum (T_{ij} - T_{i,ref})/T_{i,ref}$, where $T_{ij}$ is the intensity of the $i^{th}$ pixel in the $j^{th}$ image, and $T_{i,ref}$ is the intensity of the $i^{th}$ 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 15mM K+ or 100 µM GLU (using a puffer pipette and controlling electronics – Picospitzer II, General Valve).

Mean intensity ($\Delta T / T$) of either the OIS$_{DR}$ or the optical response to puffer-applied K+ or GLU was determined by (i) bounding the region in the DH$_s$ which the transmittance values met or exceeded a criterion increase in transmittance (typically the upper 5-10 % of pixel values in the image), and (ii) 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 OIS$_{DR}$ or the response to puffer-applied GLU or K+ was quantified as follows: The same region of the DH$_s$ 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., $\Delta T / T_{treatment} / \Delta T / T_{control} \times 100 = \%$. All average difference images shown in this paper were contrast enhanced using a standard histogram equalization procedure.

Field potential recording

Single-pulse (0.2 msec 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, AMPI). The postsynaptic field potential evoked in the DH$_s$ by the dorsal root stimulus (P-PSP$_{DR}$) 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-PSP$_{DR}$ was obtained by placing the tip of the recording micropipette in the substantia gelatinosa (SG; lamina II; all P-PSP$_{DR}$s were obtained in the region of the SG where the intrinsic signal evoked by the standard repetitive dorsal root stimulus (OIS$_{DR}$) 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-PSP$_{DR}$s were evoked using stimulus currents 2–4× the minimum (“threshold”; typically 50-80 µA) current. Currents of this intensity consistently activate both large- ($A_{\beta}$) and small-diameter ($A_{\delta}$ 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-PSP$_{DR}$s 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 prior to the slice experiment. Sensorimotor behavior was monitored throughout the 2 hr period after the injection, and once-daily thereafter, including the day of the experiment. During the 30 min - 1 hr period immediately after the injection every subject exhibited vigorous and frequent pain-related behaviors involving the injected limb (paw licking, shaking, guarding, limping, exaggerated avoidance responses to mechanical contact with the skin in the vicinity of the injection site). Over the 3-5 day period between the formalin injection and the slice experiment most subjects that received the i.c. 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

The OIS\textsubscript{DR} depends on stimulus-evoked neurotransmission.

Figure 1 summarizes results obtained from 2 of the 6 slices in which the dependency of the OIS\textsubscript{DR} on neurotransmission was evaluated. The top left image in parts A and B of Figure 1 is a reference image that shows the superficial dorsal horn (DH\textsubscript{s}) 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 both A and B of Figure 1) demonstrates 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 DH\textsubscript{s}; (2) the stimulus-evoked increase in transmittance (OIS\textsubscript{DR}) is substantially reduced when the ACSF contains either 100 µM lidocaine (compare “control” vs. “lidocaine” images in A) - lidocaine at this concentration blocks action potential conduction in excitable tissue, or lacks Ca\textsuperscript{++} (“control” vs. “Ca\textsuperscript{++}-free ACSF” images in B) – Ca\textsuperscript{++}-free ACSF reduces transmitter release from the presynaptic terminals of dorsal root afferents. Figure 1 also shows that the OIS\textsubscript{DR} recovers to near-control values 30-60 min after the fluid perfusing the recording chamber is returned to drug-free ACSF. On average, bath-applied 100 µM lidocaine reduced the OIS\textsubscript{DR} to 37.74 ± 9.13 % of control (p<0.003; n = 3 slices); and zero Ca\textsuperscript{++} ACSF reduced the OIS\textsubscript{DR} to 53.42 ± 1.28 % of control (p<0.001; n = 3 slices). The ΔT/T vs. time plots at the bottom of Figure 1 show that at times between 200 msec - 6 sec after stimulus onset ACSF containing 100 µM lidocaine or lacking
Ca\(^{++}\) consistently suppressed the transmittance increase elicited by dorsal root stimulation.

**FIGURE 1 NEAR HERE**

**Temporal properties of the OIS\(_{DR}\).**

While the effects of local anesthetic and Ca\(^{++}\)-free ACSF demonstrate that the OIS\(_{DR}\) depends on stimulus-evoked neurotransmission, the slow temporal properties of the OIS\(_{DR}\) clearly differentiate it from stimulus-evoked dorsal horn neuroelectrical activity. The \(\Delta T/T\) vs. time plots in Figure 2 (bottom right) show the time course of the OIS\(_{DR}\) at 3 different loci within the same dorsal horn (see legend to Fig. 2 for methodological details). These plots demonstrate that (1) the magnitude of the stimulus-evoked transmittance increase was largest at DH\(_s\) 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 \(\Delta T/T\) vs. time plots are truncated at 6 sec after stimulus onset, transmittance at site #3 (the site at which the OIS\(_{DR}\) was weakest) remained above-background for ~20 sec after stimulus termination, and above-background for ~40 sec at sites #1 and #2 (sites at which the OIS\(_{DR}\) was near-maximal). The OIS\(_{DR}\) recorded in every slice in which the standard stimulus was delivered to the attached dorsal root (n=48) exhibited similar slow temporal characteristics.

**FIGURES 2-4 NEAR HERE**

**The dorsal horn optical response to local application of K\(^+\) or glutamate.**

Figure 3 demonstrates that a 100 msec pressure-driven application (“puff”) of either K\(^+\) (15 mM; image sequence in A), or glutamate (GLU, 100 \(\mu\)M – image sequence in B) to a discrete locus in the DH\(_s\) 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 Figure 3, as in the other 3 slices studied in the same way, the focal increase in transmittance evoked in the DHs 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 µM GLU puff (compare images in A and B). Despite this considerable difference in magnitude, however, the temporal properties of the optical response of the DHs to locally applied K⁺ or GLU were similar – i.e., both responses developed relatively rapidly (peaked within ~1 sec of puff onset), and both declined over an extended time period (≥15 sec; ∆T/T vs. time plots in C of Figure 3 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 Figure 4A application of a 100 µM 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 ∆T/T vs. time plots in Figure 4B enable direct comparison of the response evoked by a GLU puff in layer II (plot with filled circles; same slice as in Fig. 4A) and the OISDR evoked in the same horn by the standard dorsal root stimulus (plot with open circles). Clearly, the OISDR not only develops more slowly than the response to the GLU puff, but its magnitude (peak ∆T/T value) is substantially smaller. Similar discrepancies between the responses of the same dorsal horn to GLU puff vs. 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 \textit{versus} the abrupt, highly localized, and presumably much larger increase in local [GLU]_{o} achieved with the GLU puff.

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

\textit{a. Increase of [K^{+}]_{o}}. Figure 5A shows the OIS_{DR} (dark regions in difference images) recorded (i) prior to any treatment (“control”; image at top right), (ii) after bath application of ACSF containing 8 mM K^{+} (“high K^{+}”; image at bottom left in A), and finally, (iii) after return of the perfusate to normal ACSF containing 3.5 mM K^{+} (“washout”; bottom right image 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 \(\Delta T/T\) vs. time plots in Figure 5B show (for the same slice that provided the images in part 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 - 6.0 sec after onset of the standard dorsal root stimulus is either the same or very similar to that measured in the same slice prior to the exposure to elevated K^{+}_{o}. Across the 3 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; ns).

\textbf{FIGURE 5 NEAR HERE}
Recordings of the field potential (P-PSP_{DR}) evoked in the DH_{s} by dorsal root stimulation showed that bath application of ACSF containing 8 mM K^{+}_{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 Figure 5C). The results obtained from 5 slices studied in the same way are summarized in Figure 5D. On average, the late component of the P-PSP_{DR} increased progressively in the presence of 8 mM K^{+} (plot with filled circles), reaching 173.93 ± 12.41 % of control at 15min after onset of the exposure to elevated K^{+}_{o} (p<0.001; n = 5), whereas the shorter-latency (“early”) component declined to 75.11 ± 3.78 % of control (plot with open circles; p<0.001; n = 5 slices). After the solution perfusing the recording chamber was returned to ACSF containing 3.5 mM K+ (“washout”) both the early and late components of the P-PSP_{DR} recovered to near-control values.

That the late, but not the early component of the P-PSP_{DR} increased in the presence of ACSF containing 8 mM K^{+} was observed in each of the 5 slices. This outcome was not anticipated, but is of some interest because it raises the possibility that an elevation of K^{+}_{o} in the DH_{s} selectively enhances the DH_{s}’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_{s}, (Hantman et al., 2004; Lu and Perl, 2003; Ruscheweyh and Sandkuhler, 2001, 2000).

b. Inhibition of astrocyte energy metabolism.

The next experiments sought to evaluate the effects of a selective inhibitor of astrocyte metabolism (fluoroacetate; FAc; Bacci, et al., 2002; Hulsmann, et al., 2003;
Keyser and Pellmar, 1994; also see Berg-Johnsen et al., 1993; and Watkins et al., 1997) on both the optical and neuroelectrical responses of the DHs 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 µM FAc. Figure 6 shows representative results obtained from one of the 4 slices studied in this way. The column of color-contoured images at the left of Figure 6A shows the spatial extent and intensity of the OIS\textsubscript{DR} recorded (1) before any treatment (control), (2) after onset of bath application of 400 µM FAc, and subsequently, (3) 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 Figure 6A were acquired from a different slice than the slice that yielded the optical responses shown at the left, but the P-PSP\textsubscript{DRs} recorded from this slice 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 Figure 6A demonstrates 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 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 Figure 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 ±1SEM) 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 µM
FAc. Second, after the perfusate was switched to ACSF containing FAc + Gln (last 3 points in plots in Figure 6B) the OIS_{DR} continued to decline, whereas the magnitude of the late component of the P-PSP_{DR} recovered and attained values approaching, if not exceeding, those recorded under control conditions (prior to the exposure to FAc). In other words, the OIS_{DR} and the P-PSP_{DR} became *dissociated* in the presence of FAc + Gln.

**FIGURES 6 & 7 NEAR HERE**

*a. Inhibition of A-type K+ channels.* Figure 7 shows representative results from a slice in which the OIS_{DR} 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 (Figure 6) suggest, the OIS_{DR} 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 OIS_{DR} and P-PSP_{DR} in opposite ways. More specifically, if the OIS_{DR} mainly reflects astrocyte clearance of K\(^+\) and GLU, 4-AP should decrease the OIS_{DR} (due to 4-AP block of astrocyte K\(^+\) uptake) but increase the P-PSP_{DR} (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-PSP_{DR} increased under 4-AP (the late component exhibited the largest increase; plots in Figure 7D; see also Figure 7B), whereas the OIS_{DR} decreased (compare control and 4-AP images in Figure 7A).
Similar results were obtained in all 4 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 % (p<0.001, n = 4), respectively, 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 5mM cesium chloride (CsCl; data not shown) – CsCl was studied because it has been shown (Janigro et al., 1998) 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**

The OISDR after i.c. formalin injection. The pair of images on the right in Figure 8 (“Formalin”) were obtained from the horn on the same side as the hindpaw that received an i.c. injection of 25 µl of 5% formalin 4 days prior to the experiment. In contrast, the image pair on the left (Figure 8; “Control”) were obtained from the opposite horn in the same slice. The difference images at the bottom of Figure 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 image on right) is extremely small whereas on the opposite side of the same slice (bottom image on left) the magnitude of the OISDR is typical of that recorded in the DHs of an untreated subject. A similar result was obtained in each of the 10 slices from subjects that received an i.c. injection of formalin 3-5 days prior to the experiment. On average, the OISDR evoked by the standard dorsal root stimulus in the horn ipsilateral to the
formalin injection site was only 37.19 ± 5.92 % (p<.001; n = 10) of the “control” OISDR recorded from the DHs on the side contralateral to the injection.

**FIGURE 8 NEAR HERE**

In 4 subjects the rostrocaudal extent of the attenuating effect of an i.c. 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 et al., 2002; Takahashi and Nakajima, 1996), 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 3 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 DHs 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 i.c. formalin injection on the OISDR evoked in the ipsilateral DHs was evident – that is, 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 DHs by the standard stimulus did not differ significantly from the average magnitude of the OISDR recorded in the DHs on the opposite side (i.e., at levels >2.4 mm rostral to L-4 the OISDR recorded in the ipsilateral DHs was 89.43 % of the OISDR recorded in the contralateral DHs (p>.04; n = 6 slices; ns).
Intracutaneous formalin injection reduces the normal impact of elevated $[\text{K}^+]_o$ on the OIS$_{\text{DR}}$

When $[\text{K}^+]$ in the ACSF is 3 mM (“control”), the time course of the OIS$_{\text{DR}}$ evoked in the DH$_s$ on the same side as the formalin injection site (ipsilateral; the “formalin side”) and in the DH$_s$ on the opposite side (contralateral; the “normal side”) are very similar (compare normalized $\Delta T/T$ vs. time plots with filled symbols in panels A and B of Figure 9). 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 A and B of Figure 9 are normalized – i.e., expressed in terms of percent of the maximal value recorded under each condition), the transmittance increase evoked in the DH$_s$ by stimulation of the dorsal root peaks at about 10 sec after stimulus onset, and over the next 30-60 sec declines towards baseline.

In contrast, when the ACSF bathing the slice is elevated (contains 8 mM $\text{K}^+$), the temporal profile of the transmittance increase evoked in the dorsal horn 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 normalized $\Delta T/T$ vs. time plots with open symbols in A and B of Figure 9). As described previously (e.g., Figure 5), in the presence of ACSF containing 8 mM $\text{K}^+$ the increase in transmittance evoked by dorsal root stimulation in the DH$_s$ on the side contralateral to the formalin injection not only did not return to baseline during the 50 sec period during which it was sampled (unlike the response of the same horn to the same dorsal root stimulus when $[\text{K}^+]$ in the ACSF was 3 mM), but the increase in transmittance remained near-maximal over the entire period (60 sec) during which images were acquired. Clearly, therefore, the duration of the transmittance change
evoked in the DH on the contralateral side to the formalin injection (the “normal side”) is substantially prolonged when $[K^+]_o$ is 8 mM, whereas in the DH 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 $K^+_o$.

**FIGURE 9 NEAR HERE**

**i.c. formalin injection reduces the dorsal horn optical response to local application of glutamate.** 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 puffer application of 100 µM glutamate (GLU). The difference images in panel C of Figure 9 are representative and reveal that 4 days after i.c. 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 on the same side as the skin site injected with formalin (“formalin” image). The $\Delta T/T$ vs. time plots in panel D of Figure 9 summarize, for the same slice shown in panel C, the time course of the transmittance increase on the control vs. formalin sides evoked by a 100 msec puff of 100 µM GLU. For the 3 slices studied in this way the average (across-subject) DH 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 response evoked by an identical puff on the control side.
DISCUSSION

The dorsal horn optical response – origins and underlying mechanisms

The findings obtained in the initial experiments (Figures 1 and 2) demonstrate that although the OISDR depends on synaptic neurotransmission, 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 non-neuronal in origin. Subsequent experiments showed (Figures 3 and 4) that the optical response of the dorsal horn to a 100 msec local application of K⁺ or GLU is very prolonged leading us to consider that it, like the OISDR, primarily reflects non-neuronal mechanisms/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 underlies 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 neuronal activity is accompanied by prominent and prolonged alterations of cell volume and tissue light scattering. In addition, astrocyte uptake of GLUₒ and K⁺ₒ is metabolism-dependent 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 msec puff of 15 mM K+ (i.e., rapid onset, decay, followed by a secondary increase) reinforce the suggestion that the response is, in the main, attributable to astrocytes. For example, the rapid initial increase in transmittance appears explicable in terms of an initially rapid uptake of excess K+ 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 accompanied by a local release of GLU from presynaptic nerve terminals 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., Figure 4). This observation is in accord with the report (Svoboda, et al., 1988) that in normal subjects [K+]o 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 (along with water) may be greater than that of layer IV astrocytes. Interestingly, a differential
capacity of astrocytes in the superficial vs. deep dorsal horn to take up $K^+$ and GLU would be compatible with recent demonstrations that $K^+$ channels and aquaporins tend to co-localize 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 OIS$_{DR}$ 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 (OIS$_{DR}$) vs. neuronal (P-PSP$_{DR}$) responses to dorsal root stimulation of selective inhibition of astrocyte metabolism with bath-applied fluoroacetate – FAc (400 µM). The second experiments of this type evaluated the effects of bath-applied 4-aminopyridine – an agent that blocks A-type (rapidly inactivating) membrane potassium channels in both astrocytes and neurons.

a. Effects of fluoroacetate

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., 2000; 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 glutaminergic 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 (5) Provision of an adequate level of Gln_0 enables (a) replenishment of the GLU 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 (b) complete recovery of glutamnergic neurotransmission even though FAC continues to inhibit astrocyte energy metabolism (Bacci et al., 2002).

The observations obtained after inhibition of astrocyte metabolism with FAC (Figure 6) are interpreted as follows. First, the declines in the magnitude of both the OIS_{DR} and the P-PSP_{DR} (especially the late component of the P-PSP_{DR}) 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’s inhibition of astrocyte Gln production and release which, in turn, reduces the [Gln]_0 available for uptake by the presynaptic terminals of glutamnergic 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 Figure 6; the P-PSP_{DR} returns to control levels whereas the OIS_{DR} continues to decline) reflects restoration of stimulus-evoked dorsal horn glutamnergic neurotransmission due to restoration of [Glu]_i in presynaptic nerve terminals (i.e., by the Gln provided in the ACSF that bathed the slice). The OIS_{DR} continued to decline in the presence of FAC + Gln because of the continuing FAC-mediated inhibition of astrocyte metabolism.

b. 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-aminopyridine (4-AP) would antagonize K$^+$ uptake by astrocytes (and thus reduce or eliminate the OIS$_{DR}$), and at the same time enhance stimulus-evoked dorsal horn neurotransmission (and thus increase the magnitude of the P-PSP$_{DR}$ 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 expected, therefore, dissociation of the OIS$_{DR}$ and the postsynaptic response of dorsal horn neurons to dorsal root stimulation (P-PSP$_{DR}$) under 4-AP. The results illustrated in Figure 7 are viewed as fully consistent with this prediction.

**Dorsal horn astrocytes after i.c. injection of formalin**

Experiments on slices from subjects that had received an intracutaneous (i.c.) injection of 5% formalin to the volar hindpaw 3-5 days prior to 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 (Figure 8) and also by direct application of K$^+$ or GLU (Figure 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 OIS$_{DR}$ 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 OIS$_{DR}$ recorded in the lumbosacral horn on the same side as the hindpaw injected with formalin (panels A and B in Figure 9). This insensitivity of the OIS$_{DR}$ 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 GLU$_o$ that accompany increased dorsal horn neuroelectrical activity. As a consequence, $K^+_{o}$ and GLU$_o$ (and thus neuronal excitability) in the DH$_s$ 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 i.c. formalin injection**

Although it is recognized that perisynaptic astrocytes normally function as “active partners” in normal CNS synaptic neurotransmission (Newman, 2004), the results of the present 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 (Coull 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 – appears highly relevant to altered dorsal horn glia-neuron interactions and their potential contributions to hyperalgesia / persistent pain.

Coull et al. (2003) demonstrated 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 converts GABA_A receptor mediated synaptic currents (normally hyperpolarizing) to depolarizing (excitatory) currents. 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 responsivity to nonnoxious environmental stimulation that may account, at least in part, for the hyperalgesia / persistent pain detected by behavioral assays of nerve-injured subjects.

Since the decrease in lamina I neuron KCC2 expression occurs relatively late after nerve injury (15-17 days; Coull 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⁺]_o and [GLU]_o which 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⁻]_i that account for a +16 to +18 mV shift in E_GABA_A applies to dorsal horn neurons, then an impaired ability of astrocytes to buffer [K⁺]_o and [GLU]_o following i.c. formalin injection might not only lead to abnormally prolonged elevations of neuronal excitability due to excess K⁺_o and GLU_o, but to neuronal abnormalities characterized by a loss of GABA-mediated inhibition and hyperalgesia / persistent pain.
ACKNOWLEDGEMENTS

Supported, in part, by NIH grant NS037501 (B. Whitsel, P.I.) and NIH grant NS050587 (M. Tommerdahl, P.I.). The authors thank Carol Metz for expert technical assistance, Dr. Adam Hantman for advice on slice preparation and maintenance, Dr. Adam Kohn for advice on imaging and neural recording issues, P.M. Quibrera for assistance with statistical data analysis, and Dr. Alan Light for helpful discussion of dorsal horn pain mechanisms and dorsal horn glia-neuron interactions.
REFERENCES


Kawahara, K, Hosoya, R, Sato, H, Tanaka, M, Nakajima, T, and Iwabuchi, S.  Selective blockade of astrocytic glutamate transporter GLT-1 with dihydrokainate


FIGURE LEGENDS

Figure 1. Dependency of OIS\textsubscript{DR} on neurotransmission. A - effect of lidocaine; B – effect of zero Ca\textsuperscript{++}. Top left image in A and B - reference image in which superficial dorsal horn (DH\textsubscript{s} = layers I, II\textsubscript{o} and II\textsubscript{i}) and ventral horn (VH) appear white; heavily myelinated regions (e.g., the dorsal and lateral columns – DC and LC) appear black. Oval containing label “E” indicates position of stimulating electrode. Grayscale images in A and B: prestimulus-poststimulus difference images showing OIS\textsubscript{DR} under control condition (“Control”), during exposure to 100 µM lidocaine (“Lidocaine”) or zero Ca\textsuperscript{++} ACSF (“Ca\textsuperscript{++} free ACSF”), and after return of perfusate to normal ACSF (“Recovery”). Region of slice in which light transmittance increased in response to dorsal root stimulation (dark pixels) corresponds to the DH\textsubscript{s}. Pixel size (spatial resolution) = 7 µm. ∆T/T vs. time plots at bottom show time course of stimulus-evoked increase in transmittance (0 point on abscissa indicates time of stimulus onset) under each control, treatment, and recovery condition.

Figure 2. Spatial and temporal characteristics of the OIS\textsubscript{DR}. Sequence of difference images obtained between 0.0-5.0 sec after stimulus onset. Bar at top of images between 0.2-0.8 sec indicates period during which repetitive dorsal root stimulus was applied. Plots at bottom right show time dependency of OIS\textsubscript{DR} at each of 3 sites within the DH\textsubscript{s} (sites indicated by arrows in reference image at bottom right). Plot of ∆T/T vs. distance below each difference image shows spatial distribution of transmittance increase along mediolateral path within the DH\textsubscript{s} (path indicated by bar drawn through DH\textsubscript{s} in reference image). ∆T/T vs. time plots at bottom right show time course of OIS\textsubscript{DR} at each site.
Figure 3. Effect of local elevation of $[K^+]_o$ or $[\text{GLU}_o]$. Sequence of difference images in A - time course of DH$_o$ optical response to 8 mM K+ puff (onset at time 0); sequence in B – time course of response to 100 µM GLU puff. $\Delta T/T$ vs. distance plots in C show rapid development and persistence of response to K+ or GLU puff. D - puff amplitude 10 psi; duration 100 msec.

Figure 4. Dorsal horn optical responses to electrical stimulation of the dorsal root vs. local (puffer) application of GLU. A - reference image at top left. Slice orientation as in previous figures. Arrow heads indicate layer II and layer IV sites where tip of puffer pipette made contact with slice. Top right image - OIS$_{DR}$ evoked by standard dorsal root stimulus (“Electrical Stim.”). Bottom images labeled “Layer II GLU Puff” and “Layer IV GLU Puff” – layer II and layer IV response to GLU puff, respectively. B - time course of transmittance increase evoked by standard dorsal root stimulus (open symbols) vs. layer II GLU puff (filled symbols).

Figure 5. Effects of elevated $K^+$. A - Reference image (top left), and OIS$_{DR}$ obtained under “Control” (top right), “High K+ (8 mM)” (bottom left), and “Washout” (bottom right) conditions. B - time course of OIS$_{DR}$ under each condition. C - P-PSP$_{DR}$s recorded under same conditions. Each P-PSP$_{DR}$ average of responses to 10 stimuli – brackets with arrows define “early” and “late” components of the P-PSP$_{DR}$. D - time course of effect (average across 5 slices studied in the same way) of 8 mM K+ on late vs. early components of P-PSP$_{DR}$. Error bars = ±1SEM.
Figure 6. Effects of fluoroacetate (FAc; 400 µM) on optical and neuronal responses of the dorsal horn. A - reference image at top left; top right image – OIS\textsubscript{DR}. Top-bottom sequence of color-contoured images in left column - spatial distribution of OIS\textsubscript{DR} under control condition (“Control”), during exposure to bath-applied 400 µM FAc (“FAc”), and after return to ACSF containing 200 µM glutamine (Gln) + 400 µM FAc (“FAc + Gln”). Top-bottom sequence of field potentials in right column - P-PSP\textsubscript{DR} before (“Control”), during 400 µM FAc (“FAc”), and after switch of perfusate to ACSF containing 200 µM Glutamine + 400 µM FAc. Shaded area in P-PSP\textsubscript{DR} indicates “late” component (response between 10 msec after stimulus onset and baseline). Each P-PSP\textsubscript{DR} average of responses to 10 stimuli. B - open symbols indicate average across-slice magnitude of the late component of P-PSP\textsubscript{DR} (5 slices; error bars indicate ±1 SEM); solid lines show OIS\textsubscript{DR} magnitude for 2 slices studied under same conditions. Horizontal bar indicates time of exposure to FAc; rectangle indicates exposure to FAc + Gln.

Figure 7. Effects of 4-aminopyridine (4-AP) on OIS\textsubscript{DR} and P-PSP\textsubscript{DR}. A - reference image at top left. Top right - OIS\textsubscript{DR} prior to treatment (“Control”); bottom left – OIS\textsubscript{DR} after 100 µM 4-AP; bottom right – OIS\textsubscript{DR} after return to drug-free ACSF (“Washout”). B - P-PSP\textsubscript{DR} under control and 100 µM 4-AP conditions. C - ∆T/T vs. time plot showing that OIS\textsubscript{DR} decreased after 4-AP (exposure to 4-AP indicated by rectangle). D - effect of 4-AP on early (filled circles) and late (open circles) components of P-PSP\textsubscript{DR}. 
Figure 8. **Intracutaneous formalin injection attenuates the OIS$_{DR}$ in the ipsilateral dorsal horn.** A: Top row – reference images from same slice showing horns contralateral (“Control”; image on left) and ipsilateral (“Formalin”; image on right) to formalin injection site. Bottom row – OIS$_{DR}$ recorded contralateral (image on left) and ipsilateral (image on right) to injection site.

Figure 9. **Formalin injection attenuates effect of elevated K$^+$ on the OIS$_{DR}$ and suppresses the response to puffer-applied glutamate.** A and B - $\Delta T/T$ vs. time plots for horn contralateral (“Normal / Control”) and ipsilateral (“Formalin”) to formalin-injected skin site. Filled symbols indicate average (across 3 horns studied in the same way) transmittance increase evoked by standard dorsal root stimulus under control condition. Open symbols indicate average transmittance increase during perfusion with ACSF containing 8 mM K$^+$. Error bars indicate ± 1 SEM. C - reference (top row) and difference images (bottom row) showing response to puffer-applied GLU on both control and formalin sides of same slice. D - $\Delta T/T$ vs. time plots showing magnitude and time course of optical response (average across 3 slices) of control vs. formalin horns to puffer-applied 100 µM Glu. Error bars indicate ±1 SEM.
A. Normal

\[ \Delta T/T \]

\[ \text{Time (s)} \]

- Control
- High K^+

B. Formalin

\[ \Delta T/T \]

\[ \text{Time (s)} \]

- Control
- High K^+

C. Control vs. Formalin

D. Control vs. Formalin

\[ \Delta T/T \]

\[ \text{Time (s)} \]