Peripheral Inflammation Increases the Functional Coherency of Spinal Responses to Tactile but not Nociceptive Stimulation

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Galhardo, Vasco, A. Vania Apkarian, and Deolinda Lima. Peripheral inflammation increases the functional coherency of spinal responses to tactile but not nociceptive stimulation. J Neurophysiol 88: 2096–2103, 2002; 10.1152/jn.00720.2001. Reorganization of central networks and plasticity of neuronal representations have been implicated in recent years in the dynamic expression of somatosensory responses. The functional properties of spinal cells were shown to change in the scale of minutes after peripheral high-intensity stimulations and to undergo profound alterations in their responses in experimental models of chronic pain. These observations, however, are restricted to recordings from individual cells, and no information exists on how these changes may be reflected on the activity of somatosensory neuronal networks involved in pain processing. To understand how spinal cord networks may be altered after the onset of hyperalgesia, we extracellularly recorded from groups of five to nine neighboring neurons in the hindlimb representation area of the dorsal horn. The multineuronal activity evoked by cutaneous innocuous and noxious stimulation was compared before and for 3 h after the subcutaneous injection of diluted formalin. Formalin caused immediate changes in response properties and mechanical threshold of activation for the majority of the neurons and induced the incorporation of previously unresponsive neighboring neurons to the functional network. Analysis of the temporal correlation within the neuronal population revealed that formalin-induced inflammation increased the functional coherence of the network to the nociceptive stimulation but not to the painful stimuli. This increase in the tactile acuity of populations of nociceptive neurons may be a basis for the emergence of touch-evoked pain.

INTRODUCTION

Peripheral somatosensory manipulations of a strong or repetitive nature are known to alter the response properties of central neurons and to induce long-lasting reorganizations in cortical and thalamic somatosensory areas (Buonomano and Merzenich 1998). Both physiological (Biella et al. 1997; Cook et al. 1987; Eblen-Zajjur and Sandkühler 1997) and morphological (Neumann et al. 1996; Ruda et al. 2000) studies show that similar plastic phenomena also occur in the spinal cord, typically including expansion of receptive fields and decreases in the pain-threshold of nociceptive neurons (Laird and Bennett 1993; Palecek et al. 1992). The time course of the emergence of these changes conforms well with the onset and development of behavioral manifestations of pain hypersensitivity, and hence they have been implicated in the genesis and maintenance of pathologic states (Dubner and Ruda 1992; Woolf and Doublé 1999; Woolf and Salter 2000). Some of these alterations may be explained by inflammation-induced abnormal afferent activity due to either peripheral sensitization of nociceptors (LaMotte et al. 1992) or ectopic discharges from damaged fibers at the lesion site (Kajander et al. 1992; Puig and Sorkin 1995). However, reversible but otherwise similar alterations occurring in the absence of significant peripheral damage or inflammation have also been described (Cook et al. 1987; Doubrovsky et al. 1976), suggesting that reorganization of central functional networks play an active role in painful somatosensory plasticity.

Touch-evoked pain (alldynia) is intimately linked to hyperalgesic states caused by peripheral inflammatory conditions (Cervero and Laird 1996), but can also occur as the result of central nervous lesions (Head and Holmes 1911; Leijon et al. 1989) or permanent deafferentations (Jensen and Nikolajsen 1999). Alldynia may be experimentally induced by the pharmacological disruption of the GABAergic/glycinergic spinal inhibitory system (Yaksh 1989). In addition to evoking allokinetic behaviors, these models have the double effect of immediately expanding the cutaneous receptive fields of spinal (Sorkin and Puig 1996) and thalamic (Sherman et al. 1997a,b) somatosensory neurons while decreasing their threshold for innocuous, sometimes previously ineffective, stimuli. The GABAergic/glycinergic spinal inhibition is assumed to act tonically, both pre- and postsynaptically, on the responses to activation of myelinated afferent fibers (Levy 1977; Todd et al. 1996). However, not only the responses of the low-threshold spinal units but also those of the nociceptive-specific neurons are modified during alldynic states (Lin et al. 1994; Sorkin and Puig 1996), suggesting that during the establishment of chronic pain, neighboring neurons reorganize functionally in a population-wise manner.

Multineuronal recordings have shown that after disruption of peripheral afferents, the local dynamic equilibrium between excitatory and inhibitory afferents leads to a reorganization of the functional properties of thalamic and cortical somatosensory neurons (Faggin et al. 1997; Nicolesis et al. 1993); the

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rapid onset of this reorganization suggests that it may be due to the unmasking of previously latent synaptic contacts (Jacobs and Donoghue 1991; Schroeder et al. 1995). Deep dorsal horn neurons are also known to have subthreshold synaptic components (Woolf and King 1989), indicating the existence of wide connection schemes within the spinal populations. However, no multineuronal studies have been done in the spinal cord, and fundamental knowledge on spinal population dynamics is still lacking.

To address the populational effects of a peripheral partial deafferentation, we simultaneously recorded from groups of spinal deep dorsal horn neurons using a matrix of four tungsten electrodes. The responses of small populations of deep dorsal horn cells to cutaneous nociceptive and nonnociceptive stimulation were characterized before and after the subcutaneous injection of formalin in the hindpaw.

Parts of these results have been presented in abstract form (Galhardo and Lima 2000).

**METHODS**

Eleven adult male Sprague-Dawley rats (250–450 g) were used in this study. Housing, handling, and experimental protocols were approved by the National Ethical Committee for the Use of Experimental Animals. The animals were deeply anesthetized with urethane (1.25 g/kg ip), with supplements (a quarter of the initial dose) given every 2 h throughout the experiment. A laminectomy exposing spinal segments T1–L4 was done, and the animal firmly secured with two vertebral clamps and ear bars while the right hindpaw was fixed in a paraffin molding. Body temperature was maintained with a heating pad. Multi-unit extracellular recordings were done with matrices of four tungsten electrodes (4–7 MΩ impedance) aligned rostrocaudally in one row of between-tip intervals of 240 μm (FHC, Bowdoinham, ME). Spike waveforms with a twofold signal-to-noise ratio over the background activity were digitized at 20 kHz per channel and stored on-line (DataWave Technologies, Longmont, CO) for off-line clustering of single-unit neuronal activities (off-line sorter; Plexon, Dallas, TX).

Analysis of activity was done with NeuroExplorer (Nex Technologies, Winston-Salem, NC). Correlation strengths were calculated as the integral of the probability-based cross-correlation (Yamada et al. 1993)  

\[
I(X;Y) = \sum_{ij} p(y_i|x_j)p(x_i) \log \frac{p(y_i|x_j)}{p(y_i)p(x_j)} \tag{1}
\]

where \(X\) and \(Y\) stand for the time-ordered spike trains of pairs of neurons recorded simultaneously during the temporal interval defined by \(i\) and \(j\). \(\tau\) stands for the analysis time window used in the cross-correlation significance, and \(I\) stands for the quantitative measure of the information carried by the two spike trains. Cross-correlational methods based on information theory (Shannon 1948) provide a good estimate of synaptic connectivity, independently of the firing probability of the presynaptic unit (Borst and Theunissen 1999; Dan et al. 1998). All data are presented as means ± SE.

Responding neurons were searched for in the deep dorsal horn (laiinae IV and V) of spinal segments L1 and L4, and the experimental protocol started if at least one of the sampled units responded both to hindpaw tapping and pinching in a graded manner (wide-dynamic range neuron; WDR). Mechanical stimuli were applied to the plantar hindpaw using two computer-driven servo-motors (Advanced Design, Tucson, AZ) that controlled a cotton brush (stimulus: 60 s, 4–5 Hz, nonnoxious tap) or a plastic grip (stimulus: 30 s, continuous noxious pinch, which was painful when applied to the human skin). In eight animals, after three trials of both stimuli (15-min interval between stimulations), a subcutaneous injection of formalin (50 μL, 5%) was done in the dorsal aspect of the hindpaw, and the stimulation trials were repeated every 20 min for up to a maximum of 3 h. In addition, three animals were used in control experiments performed without formalin injection. Neuronal activity was binned in 1-s intervals, and the responses to the cutaneous stimulation were statistically compared against the immediately preceding background activity using the Kolmogorov-Smirnov test (\(P < 0.01\)). Increased spontaneous activity immediately overlasting the cessation of tap- or pinch-evoked stimulation was classified as an afterdischarge event if, for a period longer than 30 s, the neuronal discharge rate overpassed by 2 SE the average spontaneous firing rate measured before the tapping stimulation of the same trial.

At the end of the experiment, the recording site was marked with a small electrolytic lesion (15 μA, 10 s), and the deeply anesthetized animal was perfused with 4% paraformaldehyde. The spinal lumbar segments were cut coronally in 50-μm sections and counterstained with cresyl violet to localize the recording site.

**RESULTS**

Recordings of spinal cells were obtained from eight small populations of neighboring neurons for a total number of 54 neurons. Only one population/site was recorded per animal. The number of well characterized neurons per recording site was 6.75 ± 0.45, with a maximum of nine cells in one recording. An additional 14 cells were obtained in three experiments in control animals. The majority of the recorded cells (44 of 54 neurons) were initially responsive to either the noxious or the nonnoxious cutaneous stimulation of the glabrous footpad; from the 10 neurons that were not responsive to the cutaneous stimulation, 8 presented ongoing spikes (e.g., neuron 1 in Fig. 1) while another 2 had no spontaneous activity before the formalin injection (e.g., neuron 3 in Fig. 1). Most of the recorded neurons (32/54) had spontaneous firing rates lower than 1 spike/s. No attempt was made to outline the cutaneous receptive fields of the units.

In all experiments, the response properties of the majority of the cells changed immediately or in the 90 min after the formalin injection; after that period fluctuations in the spontaneous and evoked firing rates between trials were common, but no shifts between response classes were observed. Therefore we will use the 90-min time point as the postformal period of reference of response properties, while the third of the trials of stimulation done before the injection will be taken as reference for preformal firing rates. Tap- and pinch-evoked firing rates were calculated as the average neuronal activity recorded over the entire period of stimulation. Spontaneous firing rates were calculated from the 60-s period immediately preceding each tap stimulation.

Ninety minutes after the formalin injection, 31 neurons (57.4% of the total) changed their type of response: 10 neurons that initially responded only to tapping started responding also to pinch, 9 neurons that responded only to pinch started responding also to tapping, 2 units that responded both to tap and pinch started responding only to tapping; the 10 units that were unresponsive before the formalin injection started responding to the footpad stimulation. Of these 10 neurons, 4 responded only to tapping, while the remaining 6 responded to both tap and pinch. The novel responses cannot be attributed to electrode movement because no changes were observed in the waveforms of the neurons that were recorded simultaneously.
stimulation (46 of the 54 neurons, or 75 ± 9% of neurons per recording—Fig. 2), and no neurons becoming silent or unresponsive.

Concerning the alteration induced in the firing rate in the overall neuronal population, there was no change in the spontaneous firing rate (1.06 ± 0.32 to 1.08 ± 0.28, $P = 0.96$, $t$-test, $n = 54$), a nonsignificant increase evoked by the pinch (7.79 ± 1.98 to 10.66 ± 2.28, $P = 0.18$, $t$-test, $n = 54$), and a significant increase in the tap-evoked firing rate (5.30 ± 0.76 to 8.71 ± 1.02, $P < 0.01$, $t$-test, $n = 54$). These net changes were not the result of a homogeneous trend: some neurons had important increases while others had big decreases in their evoked activity. As a broad rule, the formalin injection induced a decrease in the firing rates of the neurons with higher initial activities and an increase in the others. This was specially noteworthy for the tap-evoked activity where, from the 18 neurons that before the formalin were firing at more than 5 spikes/s (to a tap-stimulus frequency of 4 Hz), 17 dropped their activity from 11.78 ± 1.97 spikes/s to a final value of 8.08 ± 1.34 spikes/s; in contrast, of the remaining 36 neurons that were unable to follow the 4-Hz stimulation before the formalin, only one did not increase its evoked-activity, while the others had an average fourfold increase, from 2.06 ± 0.34 spikes/s to a final value of 9.01 ± 2.09 spikes/s. This corresponds to a 2.84 ± 1.47 increase in the tap-evoked activity signal-to-noise ratio per recording. Figure 3 shows an example of the increase in the populational signal-to-noise ratio for the innocuous stimulation. One hundred and fifty minutes after the formalin injection the onset of the tap stimulation was clearly distinguishable from the preceding background activity, although neurons 1, 4, 8, and 9 then had lower firing frequencies to tap stimulation than at the beginning of the recording (from, respectively, 10.3, 6.2, 6.1, and 0.8 spikes/s to final values of 8.7, 2.7, 0.9, and 0.7 spikes/s).

The incidence of afterdischarges was highly variable. In six of the eight populational recordings, 90 min after the formalin injection, 40–60% of the neurons had afterdischarges that outlasted the activity evoked by the pinch stimulus; in another recording all the neurons presented afterdischarges that, in four of the nine cells, lasted for a period longer than 5 min (neurons 1, 5, 6, and 9 in Fig. 3, 150 min after formalin injection). In the remaining recording, no cells presented afterdischarges. Of the 31 neurons that presented afterdischarges, 10 already presented them before the formalin injection, although in those cases the spontaneous activity subsided to basal levels in <1 min. Afterdischarges following the cessation of the tap-stimulation period were noted in only two neurons; those afterdischarges

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**FIG. 1.** Formalin-induced alterations of the response properties of simultaneously recorded neurons. Rate histograms of 1 recording of 6 deep dorsal horn neurons, before and 90 min after the formalin injection. [the periods of nonnociceptive (tapping: T) and nociceptive stimulation (pinch: P). The neurons are ordered according to their rostrocaudal orientation as recorded by the matrix electrode; neurons 3–5 were separated off-line from recordings from electrode number 3.]

Figure 1 shows an example of how the formalin injection alters the response patterns of six simultaneously recorded neurons: before the formalin injection neurons 2 and 4–6 responded to tap, while 2, 5, and 6 responded also to the noxious pinch; 90 min after the injection all the neurons responded to tap and only 1 and 3 remained unresponsive to pinch.

The overall number of neurons that had shifted response class (between being unresponsive, responding only to tap, only to pinch or to both) 90 min after the injection was of 3.75 ± 0.42 per neuronal ensemble recording. Accordingly, the number of different types of response classes present in each recording also dropped from an initial number of 3.25 ± 0.16 to only 2.00 ± 0.27, with the majority of the neurons responding 90 min after formalin injection both to noxious and innocuous stimulation.
were, however, different from the afterdischarges recorded following pinch because they presented no activity decay for over 10 min and disappeared after a pinch stimulus (data not shown).

The cross-correlational analysis of the multineuronal activity showed an almost complete lack of pairwise significant temporal correlations in the neuronal spike trains. The eight experiments resulted in a total of 124 pairwise cross-correlations from which only seven pairs (5.6%) had a significant correlation during the initial period of spontaneous activity; after the formalin injection, four of those seven pairs lost the correlated spontaneous activity. Temporal correlations during noxious pinch were even less numerous (only 5 pairs: 4.0%) but in contrast were not disrupted by the formalin injection. Cross-correlograms calculated for tap-evoked activity are, necessarily, biased by the neuronal synchronization induced by the discontinuous stimulation of primary afferents. However, the postformalin higher signal-to-noise ratio of the tap stimulation resulted in a significant increase in the strength of the overall temporal correlation between the neuronal pairs, in contrast to what happened during the noxious stimulations. Figure 4 shows the auto- and cross-correlograms of the activity evoked by 30 s of pinch and by 60 s of tapping in a nine neurons recording (data from the same experiment of Fig. 3). It is important to note that the formalin injection increased the population capacity to follow the tactile stimulation: before the formalin injection neurons 5 and 7–9 had cross-correlograms poorly linked with the stimulus timing, while 150 min after the injection their cross-correlograms are indistinguishable from the rest of the population.

The average of the correlation strength of each neuron with its neighbors showed that immediately after the formalin injection there was a significant populational increase in the synchronization during the nonnociceptive stimulation but not during the noxious stimulus (Fig. 5). Prior to formalin injection, the overall correlation strengths were highest during the nociceptive stimulation (spontaneous activity: 0.0008 ± 0.0003; during tapping: 0.006 ± 0.003; during pinch: 0.015 ± 0.002). Immediately after the formalin injection, the correlation strength during tapping increased to 0.015 ± 0.009 while the correlation strength for pinch dropped to 0.011 ± 0.003. One-hundred and fifty minutes after the injection, the correlation strength during tapping continued to be high and peaked at a threefold increase (to a final value of 0.017 ± 0.003), while during pinch the correlation recovered slightly to a populational average of 0.012 ± 0.001.
In the control experiments, occasional changes in the firing rates of some cells, either spontaneously or in response to the cutaneous stimulation, were observed. At the end of the experiments almost all the control cells had an increase of more than 10% (maximum of 26%) from their initial noxious-evoked firing rate. However, in contrast with the neurons recorded in the formalin-injected animals, this activity increase developed slowly and steadily during the first hours of recording, suggesting that it was the result of the developing tissue sensitization caused by the repetitive stimulation.

**DISCUSSION**

The present study is the first report of somatosensory pain-related multineuronal activity in the dorsal horn of the spinal cord. Previous reports were restricted to the activity of single and two cells recorded either from the same electrode (Eblen-Zajjur and Sandkühler 1997) or from two independent electrodes (Biella et al. 1997). In several aspects, the information now gathered from the simultaneous activity of many neurons could not be identified by concatenating separate single-cell experiments.
It has long been known that the response properties of single spinal dorsal horn neurons are altered during hyperalgesic states: they have wider receptive fields, lower activation thresholds, and fire more prominently to the same noxious stimulus (Coderre et al. 1993). These alterations result, presumably, from the joint influence of alterations in both peripheral and central mechanisms (Woolf and Salter 2000). The present results expand our current understanding of the central plasticity occurring in the spinal cord because they suggest that the neuronal functional changes are not similar for all spinal cord neurons and that specific network rules govern the pain-induced reorganization.

The subcutaneous injection of a small volume of diluted formalin is a well-studied test of pain perception (Tjølseth et al. 1992) that causes two periods of pain-related behaviors: a short-onset acute phase of pain during the first 5 min after injection, and a later wave of tonic pain that develops 15–20 min afterward and lasts for about 20–40 min (Tjølseth et al. 1992). The first phase is assumed to be a direct effect of afferent fibers stimulation; the second phase results from the tissue inflammation developing at the injection site (Duboisson and Dennis 1977). It is known that the second phase is partially dependent on peripheral afferent drive (especially from sensitized C fibers) (Puig and Sorkin 1995), but it also depends on the hyperactivity of facilitated spinal circuits, because the blockade of the N-methyl-D-aspartate channel during the first wave of formalin-induced pain abolishes the occurrence of the second wave (Coderre and Melzack 1992). The single-cell studies performed to address the cellular mechanisms responsible for these behavioral responses have shown that the injection of formalin in the receptive field of somatosensory WDR neurons causes a double peak increase in their spontaneous neuronal discharges that is temporally correlated with the biphasic behavior response (Dickenson and Sullivan 1987a). The occurrence of this formalin-induced spontaneous biphasic discharge in WDR neurons was believed to be the cellular counterpart of the behavioral hyperalgesia because mechanical low-threshold cells respond only acutely during the first minutes following the subcutaneous injection (Dickenson and Sullivan 1987b). The present results, however, show a more complex picture in which WDR, low-threshold, and nociceptive-specific neurons alter their spatio-temporal responsiveness in a population-wise manner. The simultaneous occurrence of response changes in the three classes of somatosensory neurons is in direct accordance to what has been previously shown to occur to the lateral thalamus neuronal populations following a sciatic nerve injury (Brüggemann et al. 2001).

In the present study, we did not inject the formalin directly in the receptive field of the cells. It is known that formalin induces hyperalgesic responses also from noninjected areas (Fu et al. 2001; Wiertalek et al. 1994). In addition it has been recently shown that the injected area becomes hypoalgesic, presumably due to fiber destruction by the formalin (Fu et al. 2001; Sweitzer et al. 1999). Hence we injected formalin in the dorsal hairy skin of the hindpaw (a region supplied by the superficial peroneal branch of the sciatic nerve) (Swett and Woolf 1985) while stimulating the glabrous footpad (supplied by the tibial branch) (Swett and Woolf 1985). This protocol is similar to the experimental conditions of the behavioral formalin test (Tjølseth et al. 1992). In contrast with previous studies that injected directly the receptive field (Dickenson and Sullivan 1987a; Raboisson et al. 1995), in only one of the experiments here reported, some of the recorded neurons did significantly increase their spontaneous discharge-rate in the 5 min following the injection. Even then—from the six neurons that were being recorded—only the two WDR cells responded. Despite the fact that most of the neurons did not respond directly to formalin, in all cases there were profound alterations in their response properties. Hence, an important observation is that the long-term central plasticity induced by peripheral nerve damage also develops in cells that do not directly respond to the insult. This phenomenon is similar to the central plasticity observed following intradermal injections of capsaicin in which touch-evoked sensitization occurs in neurons that did not respond to the affected fibers (Katz et al. 1999; Pettit and Schwartz 1996; Simone et al. 1989).

Most interesting of these differential alterations is the fact that the formalin injection induced the incorporation of neighboring unresponsive neurons into the functional networks. Single-cell mapping studies have demonstrated that side by side in the spinal cord co-exist neurons responding to nonadjacent receptive fields (Brown and Fuchs 1975; Woolf and Fitzgerald 1983). This was here reflected in the number of neurons (18%) that were initially unresponsive to the spatially restricted stimulation, although having, in most cases, spontaneous activity. Because the spike clustering was done off-line, it was not possible to delineate the receptive field boundaries during the experiment; therefore we cannot distinguish between neurons...
that were nonresponsive to peripheral stimulation from neurons that were responding to untested cutaneous areas. In any case, all the neurons that in the control state were unresponsive started responding along with the neighboring neurons. This can be due to an expansion of the initial receptive fields so that now all neurons acquired overlapping cutaneous representations. Alternatively, it may be a manifestation that spinal networks contain silent units that are switched on by specific painful conditions, as has been previously suggested (Cadden 1993; Morisset and Nagy 1998). The known existence of many subthreshold potentials in deep dorsal horn neurons (Woolf and King 1989) tends to favor the first hypothesis although further studies are needed before ruling out the existence of spinal units that act as functional switches (Cadden 1993).

The populational nonhomogeneity of changes, and also the recruitment of silent units, resembles very closely what has been observed in multi-electrode studies of the somatosensory thalamus (Apkarian et al. 2000; Brüggemann et al. 2001), suggesting that either common mechanisms operate in both areas or that spinal changes are instantly mirrored at higher levels of the somatosensory system.

Finally, the fact that neighboring cells that had different response properties before the formalin injection underwent different alterations in their stimulus-evoked activity suggests that spinal changes are instantly mirrored at higher levels of the somatosensory system.

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