

Chronic, Selective Forebrain Responses to Excitotoxic Dorsal Horn Injury

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Intraspinal injection of the AMPA/metabotropic receptor agonist quisqualic acid (QUIS) results in excitotoxic injury which develops pathological characteristics similar to those associated with ischemic and traumatic spinal cord injury (SCI) (R. P. Yeziarski *et al.*, 1998, *Pain* 75: 141–155; R. P. Yeziarski *et al.*, 1993, *J. Neurotrauma* 10: 445–456). Since spinal injury can lead to partial or complete deafferentation of ascending supraspinal structures, it is likely that secondary to the disruption of spinal pathways these regions could undergo significant reorganization. Recently, T. J. Morrow *et al.* (*Pain* 75: 355–365) showed that autoradiographic estimates of regional cerebral blood flow (rCBF) can be used to simultaneously identify alterations in the activation of multiple forebrain structures responsive to noxious formalin stimulation. Accordingly, we examined whether excitotoxic SCI produced alterations in the activation of supraspinal structures using rCBF as a marker of neuronal activity. Twenty-four to 41 days after unilateral injection of QUIS into the T12 to L3 spinal segments, we found significant increases in the activation of 7 of 22 supraspinal structures examined. As compared to controls, unstimulated SCI rats exhibited a significant bilateral increase in rCBF within the arcuate nucleus (ARC), the hindlimb region of S1 cortex (HL), parietal cortex (PAR), and the thalamic posterior (PO), ventral lateral (VL), ventral posterior lateral (VPL), and ventral posterior medial (VPM) nuclei. All structures showing significantly altered rCBF are associated with the processing of somatosensory information. These changes constitute remote responses to injury and suggest that widespread functional changes occur within cortical and subcortical regions following injury to the spinal cord. © 2000 Academic Press

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INTRODUCTION

In recent years the excitotoxic model of spinal cord injury (SCI) has been used to study the morphological (47), physiological (46), and behavioral (45) changes

associated with excitotoxic injury to the cord parenchyma. In this model the intraspinal injection of the AMPA/metabotropic receptor agonist quisqualic acid (QUIS) is used to simulate injury induced elevations of excitatory amino acids, a well documented neurochemical sequela of SCI (19, 25, 35). The progressive pathological changes resulting from QUIS injections closely resemble those described following ischemic and traumatic SCI (3, 5), and the expanding cavities bear a striking similarity to the clinical condition of posttraumatic syringomyelia (23). The results of studies using the QUIS model of SCI have provided valuable insights into the structural and functional consequences of excitotoxic injury within the spinal cord.

An area of research that has been largely ignored with respect to the varied consequences of spinal injury is the pathophysiological and neurochemical changes at sites remote to the site of injury and specifically cortical and subcortical regions, i.e., supraspinal. Considering that the varied severity of spinal injury can lead to partial or complete deafferentation of supraspinal structures, it is likely that secondary to the disruption of spinal pathways these regions undergo significant reorganization following injury. Recently we described the molecular changes in the diencephalon following QUIS injury (4). Paralleling these molecular changes are likely to be the reorganization of neural substrates and functional changes that could contribute to the severe consequences of spinal injury, including chronic pain and spasticity. Consistent with this hypothesis are reports describing significant functional changes in the response properties of thalamic neurons and the reorganization of cortical somatotopic maps in human (18) and nonhuman primates (16) following spinal cord injury.

Recently, Morrow *et al.* (27) showed that autoradiographic estimates of regional cerebral blood flow (rCBF) can be used to simultaneously identify pain-specific alterations in the activation of multiple forebrain structures. In an attempt to understand the extent and nature of supraspinal changes triggered by SCI we have carried out the present study focusing on changes

in supraspinal blood flow following excitotoxic spinal cord injury. The results have shown that following spinal injury there are significant changes in blood flow at forebrain sites associated with the processing of somatosensory information. These changes constitute remote responses to injury and provide important information related to the widespread functional changes that occur within cortical and subcortical regions following injury to the spinal cord. Furthermore they provide insights into the potential mechanisms responsible for the onset of altered sensory states associated with SCI (43). A preliminary report of this study has been presented (26).

METHODS

Experimental procedures were approved by the Animal Care and Use Committees of the University of Miami and the Ann Arbor Veterans Affairs Medical Center. We conducted all experiments in accordance with the NIH Guide for the care and use of laboratory animals (1).

Animal subjects. Twenty-five male, Long Evans rats weighing 225–250 g served as subjects for the experiments described. All animals were housed in groups of three and maintained on a 12 h on–12 h off light–dark cycle, with lights on at 06:00 h. Ambient temperature in the animal facility was kept at 22°C. Food and water were given *ad libitum*. Subjects were divided into two groups: an unoperated control group ($n = 12$) and a spinal cord injury (SCI) group ($n = 13$). It should be noted that two animals in the SCI group were excluded from blood flow analysis for technical reasons (see Results).

Surgical preparation for intraspinal injection. Rats were anesthetized with a mixture of ketamine, acepromazine, and xylazine (0.65/kg, sc). Supplemental doses of anesthetic were given, if necessary, when rats responded to a noxious pinch applied to the glabrous skin of the hindpaws. Animals were placed in a stereotaxic unit and spinal cords immobilized with a vertebral clamp. One injection window for intraspinal injection was made by laminectomy between spinal segments T12 and L3. Once the cord was exposed the dura was incised longitudinally and reflected bilaterally. A small hole for unilateral injections was made in the pia matter. After the QUIS injection, muscles were closed in layers and the skin closed with wound clips. Throughout surgery and during the postoperative recovery period animals were maintained at normal body temperature (36.5°C) with a feedback controlled heating blanket.

Intraspinal injection procedure. QUIS, 125 mM, was mixed fresh prior to injection, corrected to physiological pH, and injected unilaterally at one level of the

cord (T12–L2). This concentration was used because in previous studies it produced pathological changes comparable to those described following traumatic SCI, i.e., neuronal loss, cavitation, demyelination, glial response (45, 47). Injections were made at depths ranging from 600 to 1200 μm below the surface, between the dorsal root entry zone and the dorsal vein. These parameters placed injections in the center of the gray matter between spinal laminae IV and VI. The total volume of QUIS injected was 0.6 μl in three tracks (0.2 μl /track; distance between tracks 0.3 mm). A Hamilton microliter syringe with glass micropipet extension (tip diameter 5–10 μm) was used for intraspinal injections. The syringe was positioned in a microinjector unit (Kopf 5000) attached to a micromanipulator (Kopf, Tujunga, CA).

rCBF procedure. Regional cerebral blood flow was estimated using a technique described previously (27). Briefly, 24 to 41 days after SCI, we placed each rat in a soft towel restraint and an intravenous catheter was inserted into the tail vein. Subjects were then permitted to rest quietly in the restraint for approximately 40 min to recover from the stress of tail vein catheterization. For imaging rCBF, 10–12 mCi of $^{99\text{m}}\text{Tc}$ (technetium) exametazime was injected into the tail vein over 10 to 15 s. Approximately 2 to 5 min following tracer injection, the rat was deeply anesthetized with chloral hydrate (300 mg/kg, i.v.), removed from the restraint, and decapitated. The brain was removed from the skull and standard 20- μm coronal frozen sections cut at -18°C . Four consecutive coronal brain sections were taken at approximately 250- μm intervals and mounted on glass slides. Standard autoradiograms were generated by direct apposition of the mounted sections to the emulsion side of Kodak BioMax MR-1 imaging film. When the radioactivity level of the $^{99\text{m}}\text{Tc}$ labeled slides had returned to background, the slides were stained with cresyl violet. We made precise structural identifications by careful comparison of cresyl violet stained sections to coronal plates from the Paxinos and Watson stereotaxic atlas of the rat brain (30).

rCBF analysis. Densitometric analysis of autoradiograms was performed using a microcomputer-assisted video imaging densitometer (MCID system, Imaging Research Inc., St. Catharines, Ontario, Canada). Each brain section on film was digitized to produce a 256-level grayscale image. Anatomic location of selected regions of interest (ROIs) was determined by overlaying transparent stereotaxic templates (adapted from the Paxinos and Watson atlas (30)) on digitized brain images displayed on the video monitor. We limited ROIs to 22 supraspinal structures (see Table 1) including cortical and thalamic structures and the brain stem periaqueductal gray (PAG). As described previously (27), an index of activation (AI) was calculated for each ROI. Briefly, the MCID system converted sampled film

TABLE 1

Comparison of Bilateral Mean AI^a Values for Sampled ROIs in Control and SCI Animals

Region of interest	Control (<i>n</i> = 12)	Spinal cord injury (<i>n</i> = 11)
AD	30.88 ± 3.49	31.33 ± 2.51
ARC	38.53 ± 5.02	94.02 ± 6.92*
BLA	4.46 ± 2.59	9.63 ± 3.02
CC	17.37 ± 2.57	16.37 ± 2.90
CPU _{ad}	7.52 ± 2.43	9.70 ± 1.09
CPU _{pl}	20.51 ± 2.37	21.51 ± 3.36
DB	37.76 ± 2.83	36.45 ± 2.74
HBC	54.20 ± 2.51	57.39 ± 3.64
HIP	-6.17 ± 1.85	-4.94 ± 2.80
HL	23.67 ± 3.21	35.81 ± 3.84*
IPN	52.02 ± 4.84	53.57 ± 3.57
MT	10.70 ± 1.91	14.04 ± 2.12
PAG	-0.38 ± 3.23	4.09 ± 3.77
PAR	29.04 ± 1.49	52.33 ± 4.06*
PO	0.41 ± 2.91	9.25 ± 2.64*
PVN	53.53 ± 3.49	55.26 ± 5.89
RS	30.36 ± 2.48	34.72 ± 3.13
ST	23.05 ± 2.31	25.79 ± 3.93
VL	3.88 ± 2.06	11.38 ± 2.18*
VM	7.12 ± 2.30	13.78 ± 2.47
VPL	4.45 ± 1.77	15.79 ± 2.86*
VPM	12.56 ± 2.30	26.65 ± 3.43*

Note. AD, anterior dorsal nucleus (thalamus); ARC, arcuate nucleus (hypothalamus); BLA, basal lateral amygdala; CC, cingulate cortex; CPU_{ad}, caudate-putamen (anterior-dorsal); CPU_{pl}, caudate-putamen (posterior-lateral); DB, diagonal band of Broca; HBC, habenular complex; HIP, hippocampus; HL, hindlimb area of somatosensory cortex; IPN, interpeduncular nucleus; MT, medial thalamus; PAG, periaqueductal gray (midbrain); PAR, parietal cortex; PO, posterior group (thalamus); PVN, paraventricular nucleus (hypothalamus); RS, retrosplenial cortex; ST, subthalamic nucleus; VL, ventral lateral nucleus (thalamus); VM, ventral medial nucleus (thalamus); VPL, ventral posterior lateral nucleus (thalamus); VPM, ventral posterior medial nucleus (thalamus).

$$^a \text{AI} = \frac{(\text{sampled ROI activity} - \text{total brain activity})}{\text{total brain activity}} \times 100\%.$$

* Significant difference from control ($P \leq 0.05$, ANOVA).

optical densities to apparent tissue radioactivity concentrations (nCi/mg) by comparison with the optical densities of ¹⁴C standards also imaged on each film. The average total brain activity was then estimated by sampling all pixels in each brain section and averaging the activity across all sections for a given animal. AI values for each sampled ROI were then calculated as a percentage difference from the average total activity of the entire brain using the following formula:

$$\text{AI} = \frac{(\text{samples ROI activity} - \text{total brain activity})}{\text{total brain activity}} \times 100\%.$$

We then computed an average AI for each ROI. Finally, the within-subject mean AIs for each sampled region were averaged across all subjects in an experimental group to compute within-group means for each ROI. Distinctly bilateral structures were examined for side to side differences in rCBF using a paired *t* test ($P \leq 0.05$). For each ROI, we tested for significant differences in AI between control and SCI groups using a one-way ANOVA. All statistical analyses were performed using the software package, SPSS for Windows (SPSS Inc., Chicago, IL).

Histological SCI assessment. Spinal cord segments containing QUIS injection sites were removed, post-fixed in buffered formalin, and cut on a freezing microtome (75 μm). Coronal sections were mounted on glass slides and stained with cresyl violet. Reconstructions of the QUIS induced neuronal loss were performed using a technique previously described (20, 44, 45). Briefly, sections were examined with light microscopy and drawings of damaged areas made using an overhead projector and camera lucida. For each animal a schematic representation of the injury site was made on a representative drawing of the spinal cord (see Fig. 1). To compare the areas of neuronal loss to those observed in a previous study (45, 46), the gray matter was divided into 11 regions.

RESULTS

Spinal Cord Histology

Histological analysis of the QUIS injected spinal cords showed characteristics similar to those described previously (45, 47). The photomicrographs and standardized grid drawings in Fig. 1 show examples of typical spinal injuries induced by the unilateral injection of QUIS in two animals. Note that although QUIS was injected into the dorsal horn on only one side of the cord, there was evidence of damage on the side ipsilateral and contralateral to the injection site. Although the behavioral consequences of QUIS injections were not evaluated in animals undergoing blood flow analysis, the pattern of neuronal loss was similar to that found in animals with spontaneous and evoked pain behaviors following excitotoxic spinal cord injury (45, 46). A pattern of bilateral injury similar to those in Fig. 1 was found in all but two rats in this study. Because the latter two subjects showed only damage to the side ipsilateral to the QUIS injection, we excluded these subjects from blood flow analysis.

Blood Flow: Control vs SCI Rats

Table 1 shows regional differences in cerebral blood flow for all ROIs sampled in the unoperated control and spinal cord injury groups. Animals in both groups displayed a regional heterogeneity in the pattern of

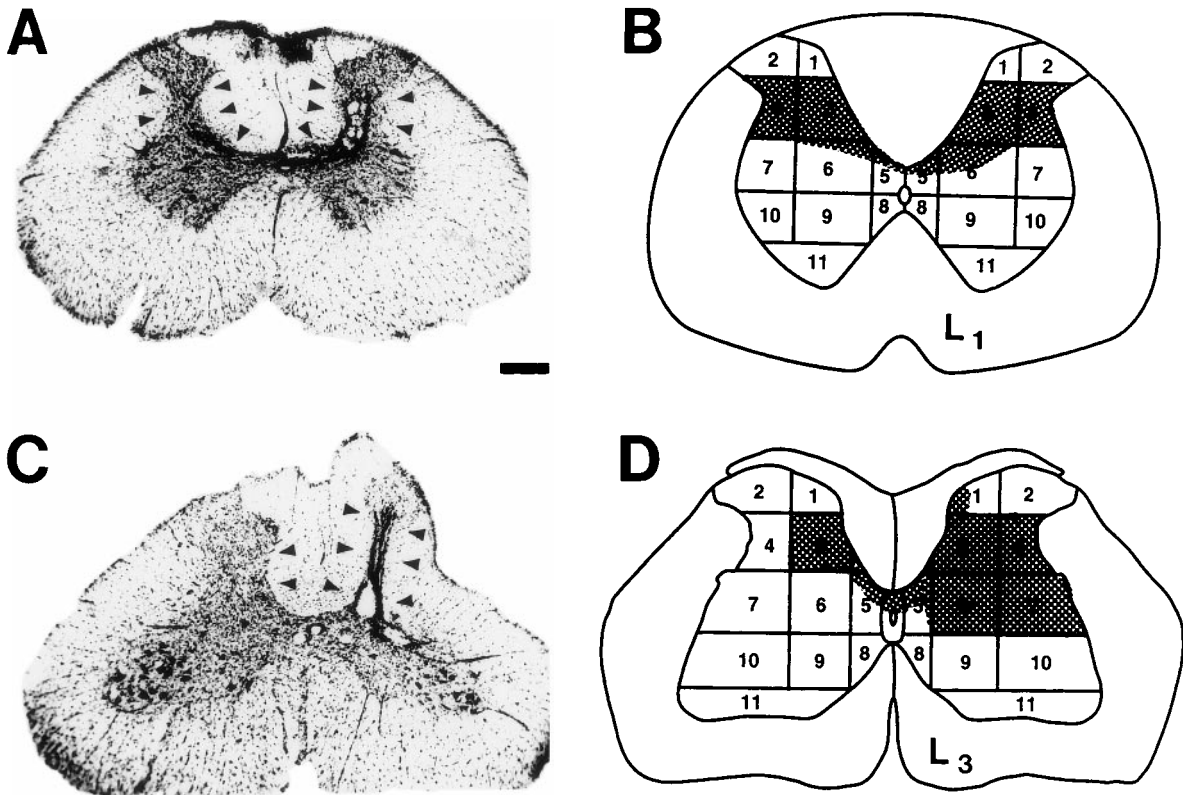


FIG. 1. Histological sections showing typical patterns of neuronal loss and cavitation at the epicenter of injection sites. Excitotoxic injury was achieved following unilateral injections (0.6 μ l) of 125 mM quisqualic acid (QUIS) on the right side of the cord at depths between 600 and 1200 μ m below the surface of the cord. Note the areas of neuronal loss (arrows) on both sides of the cord (A, C) and the presence of small cavities on the right side of the cord. The survival time for the animal in A was 23 days and 41 days for the animal in C. Schematic diagrams in B and D show the area of tissue damage for the sections shown in A and C, respectively. The 11 regions of spinal gray matter are the same as those used in a previous study to examine the morphological correlate of spontaneous and evoked pain behaviors following QUIS injections (45). Scale bar in A equals 297 μ m.

cerebral blood flow. Although there was considerable variation in AI values among ROIs even in the control subjects, we found no significant side-to-side differences in any of the 22 structures sampled in the two experimental groups. Accordingly for Table 1, we averaged the AI values from both sides for each ROI in each animal. This analysis was carried out for animals in both control and SCI groups.

As illustrated in Fig. 2, between 3 and 6 weeks following the injection of QUIS into the spinal cord on one side, SCI rats exhibited significant increases in relative blood flow within 7 of the 22 forebrain structures examined. As compared to controls, unstimulated SCI rats showed a significant bilateral increase in rCBF in ARC, HL, PAR, PO, VL, VPL, and VPM. No ROI exhibiting a significant change in rCBF showed a decrease in activation (a lower AI) when compared to control values. As noted above, we found no lateralized differences in SCI induced forebrain activation. Figure 2 compares the bilateral average activation indices (AIs) for all sampled brain regions that exhibited a significant blood flow change in the SCI group relative

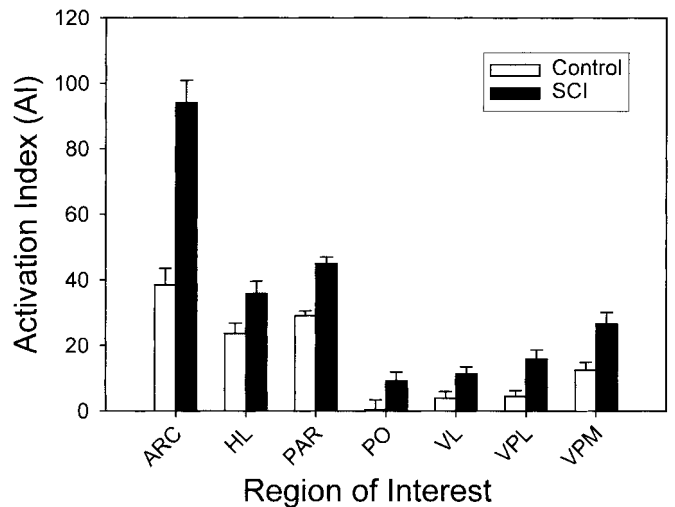


FIG. 2. The bar graph compares the average bilateral level of activation in seven ROIs between control and SCI groups. The data shown are only for ROIs exhibiting a significant difference in activation in the SCI group as compared to unoperated controls ($P \leq 0.05$, ANOVA). The relative levels of activation are expressed as AI, the mean percentage difference from total brain activity.

to unoperated controls. Note that all structures showing an increase in blood flow are sites previously associated with some aspect of somatosensory processing or sensory-motor integration.

DISCUSSION

In the present study, we identified changes in the pattern of forebrain activation following excitotoxic spinal cord injury using an autoradiographic estimate of regional cerebral blood flow. A unilateral injection of QUIS consistently produced bilateral damage in the spinal cord and bilateral increases in rCBF within multiple forebrain structures associated with some aspect of somatosensory processing or sensory-motor integration. To our knowledge, this is the first neuroimaging study to use rCBF in an animal model to identify changes in supraspinal activation following spinal cord injury.

Methodological Considerations

While there is substantial evidence that the activity of local neurons is a major factor regulating the specific distribution of blood flow throughout the brain (15, 22, 24, 36), current neuroimaging techniques cannot distinguish between excitatory and inhibitory synaptic activity. This inability to determine the functional valence of brain activation is common to other neuroimaging techniques as well, including procedures which measure metabolic activity (e.g., 2-deoxy-glucose and functional MRI). However, this inherent weakness is overshadowed by the greatest strength of this method, the ability to identify spatial and/or temporal patterns of neuronal activation within multiple brain regions simultaneously.

Although the autoradiographic approach we used did not directly measure rCBF, it was sufficiently sensitive to allow detection of regional differences in blood flow even in unstimulated subjects. Similar indirect methods have been used in autoradiographic studies that measured regional glucose metabolism using 2-deoxy-glucose (31, 32). Our blood flow estimates when sampling somatotopically organized thalamic structures (i.e., VPL and VPM) were probably biased toward identification of primarily robust changes in regional blood flow. Because we sampled the entire extent of each ROI present in any given brain section, small very localized changes in rCBF could have been underestimated or missed in the combined average for an entire nucleus.

Possible Mechanisms of SCI Induced Alterations in Supraspinal rCBF

Because we had made QUIS microinjections into the spinal cord on only one side, we had originally expected

rCBF changes to occur primarily in thalamic and cortical regions contralateral to the site of QUIS injection. However, histological analysis showed that all but two of the SCI rats had damage to the dorsal horn on both sides. Our finding of only bilateral alterations in supraspinal rCBF in SCI rats, with no detectable side-to-side differences was therefore consistent with the histological identification of bilateral cord damage.

We found increases in activation in unstimulated SCI rats that suggest pathophysiological changes may have occurred not only at spinal levels but also in supraspinal structures. Several ROIs showing SCI-induced increases in blood flow are well known to participate in somatosensory processing including HL, PAR, PO, VPL, and VPM. Our experimental protocol restricted SCI to the T12-L3 spinal segments, which receives input from the hindlimbs and lower trunk. However, we found a significant increase in rCBF in the thalamic VPM, which receives input from the head and neck. This finding may be the result of an injury-induced reorganization of thalamic receptive fields. Significant alterations in the somatotopic organization of the thalamus have been reported for patients with spinal cord injuries (18), as well as for nonhuman primates with spinal cord or peripheral nerve damage (16). Jain and colleagues (16) also reported that deafferentation resulting from spinal cord or peripheral nerve injury leads to the immediate expression of previously subthreshold inputs due to a release of inhibition resulting from a lack of excitatory inputs to inhibitory cells. Furthermore, deafferentation-induced plastic changes in the somatosensory pathway can occur immediately and simultaneously at cortical and subcortical levels (12).

It is possible that the blood flow changes we found are due to abnormal hyperactive discharges of spinal cord neurons within segments adjacent to the site of injury. Transient ischemic injury to (14) or partial transection of (10) the spinal cord in rat is reported to produce an increase in the excitability of wide dynamic range (WDR) neurons. Similarly, Yezierski and Park (46) also reported an increased level of background discharges, abnormal responses, and prolonged afterdischarges in rat dorsal horn neurons adjacent to the site of excitotoxic spinal injury.

Supraspinal mechanisms may also play an important role in producing the increases in forebrain blood flow described here. Deafferentation supersensitivity resulting from the loss of spinal inputs to supraspinal sites could have caused the development of abnormal generators, which in turn may be responsible for increased rCBF seen in forebrain structures. Hyperactive thalamic and cortical activity has been described in rats following dorsal rhizotomy (21). Studies have described spontaneous and evoked hyperactivity in the VPL nucleus of the cat following an ipsilateral transection of the spinothalamic tract (17). Similar findings

were reported by Lenz *et al.* (18) in a study examining thalamic neuronal activity in patients with spinal cord injury. Faggin *et al.* (12) also showed that deafferentation produces an immediate and simultaneous change in neuronal activity in rat thalamus and cortex. Recent work by Brewer *et al.* (4) shows that excitotoxic SCI lesions also cause changes in gene expression within the diencephalon.

Relationship to Possible Supraspinal Mechanisms of Chronic Pain

Although we did not behaviorally test our animals, it is likely that they had developed abnormalities in somatosensory processing consistent with results described previously (45, 46). Ischemic or traumatic spinal cord injury (SCI) in rats is well known to result in behavioral symptoms indicative of a chronic pain state, including mechanical allodynia, thermal hyperalgesia, and autotomy (9, 10, 34, 42). A role in nociceptive processing for many of the thalamic, cortical, and adjacent brain regions activated in the present study is well documented (see review (40)). Consistent with our data, Ness *et al.* (28) recently described increased activation in thalamus and cortex using PET in spinal cord injured patients during the perception of chronic pain. Nociceptive activation within cortical regions has also been reported in previous animal and human studies (8, 11, 27, 33, 38, 41).

In the present study, the arcuate nucleus of the hypothalamus (ARC) showed the greatest SCI-induced increase in activation. This finding is also consistent with the presence of an SCI-induced state of chronic pain. Recent studies have described direct spinothalamic projections from superficial laminae (I–II) and the base of the dorsal horn (6, 7). Other studies also support a role for ARC in both pain modulation (2, 13, 37, 39) and the physiological stress response induced by prolonged pain (29).

Based on the data presented here and the evidence from other studies presented above, it is reasonable to speculate that following SCI, the onset and especially the maintenance of chronic pain-related behaviors may in part be the result of alterations in the pattern of neuronal activation at supraspinal levels. Contributing to this elevated state of activation would be the relay of discharges from spinal generators of abnormal activity as well as the effects produced secondary to deafferentation of supraspinal structures. To address this hypothesis it will be important to record neuronal activity from areas identified in the present study as having elevated levels of rCBF. Since the results of the present study were carried out using an “unstimulated paradigm” it will also be interesting in the future to carry out the same study using noxious and nonnoxious stimulus conditions in injured versus uninjured animals. Results of such an investigation will be helpful in

gaining a better understanding of the extent of “remote,” e.g., supraspinal, changes and specifically the functional reorganization in the somatotopic map secondary to spinal injury.

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