



Regional changes in forebrain activation during the early and late phase of formalin nociception: analysis using cerebral blood flow in the rat

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Abstract

This is the first neural imaging study to use regional cerebral blood flow (rCBF) in an animal model to identify the patterns of forebrain nociceptive processing that occur during the early and late phase of the formalin test. We measured normalized rCBF increases by an autoradiographic method using the radiotracer [^{99m}Tc]exametazime. Noxious formalin consistently produced detectable, well-localized and typically bilateral increases in rCBF within multiple forebrain structures, as well as the interpeduncular nucleus (Activation Index, AI = 66) and the midbrain periaqueductal gray (AI = 20). Structures showing pain-induced changes in rCBF included several forebrain regions considered part of the limbic system. The hindlimb region of somatosensory cortex was significantly activated (AI = 31), and blood flow increases in VPL (AI = 8.7) and the medial thalamus (AI = 9.0) exhibited a tendency to be greater in the late phase as compared to the early phase of the formalin test. The spatial pattern and intensity of activation varied as a function of the time following the noxious formalin stimulus. The results highlight the important role of the limbic forebrain in the neural mechanisms of prolonged persistent pain and provide evidence for a forebrain network for pain. © 1998 International Association for the Study of Pain. Published by Elsevier Science B.V.

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1. Introduction

The formalin test is a widely used animal model for prolonged acute pain which is based upon injection of dilute formalin solution, usually into the subcutaneous tissue of one paw (Dubuisson and Dennis, 1977; Tjolsen et al., 1992). This algogenic stimulus elicits three prominent behavioral responses commonly assumed to indicate the presence of pain (Dubuisson and Dennis, 1977; Wheeler Aceto and Cowan, 1991; Abbott et al., 1995). Following a formalin injection, the paw is lifted or favored, licked and sometimes bitten and shaken, with the intensity of these behavioral

responses proportional to the concentration of formalin used in the test. This stimulus differs from conventional phasic pain stimuli in that it produces a mild tissue injury (Dubuisson and Dennis, 1977; Wheeler Aceto and Cowan, 1991; Tjolsen et al., 1992; Abbott et al., 1995). During the first (early) phase, which starts immediately after injection of formalin, pain behaviors are frequently elicited and are most intense. This phase continues for approximately 5 min, after which nociception is considerably reduced. The second (late) phase is marked by the return of moderate to high levels of pain-related behaviors beginning 10 to 15 min after formalin injection. These late phase pain-related behaviors can persist for up to 1 h or more depending on the concentration of formalin used as the noxious stimulus. The early phase is thought to be due to a direct activation of peripheral nociceptors by formalin; whereas the late phase is believed to be related to the development of inflammation and sensi-

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tization of central nociceptive neurons (Dubuisson and Dennis, 1977;Coderre et al., 1990;Coderre and Melzack, 1992). These features have resulted in the formalin test being accepted as a valid model of persistent clinical pain (Tjolsen et al., 1992).

Numerous studies have examined the effects of pharmacological agents on the pain-related behaviors in the formalin test (see review (Porro and Cavazzuti, 1993)). Electrophysiological investigations have also provided evidence for putative afferent (Puig and Sorkin, 1995), spinal cord (Dickenson and Sullivan, 1987b;Coderre et al., 1994) and brainstem (Pertovaara and Tukeva, 1989) mechanisms of formalin induced pain. Several imaging studies have attempted to identify the spatial pattern of glucose metabolism or the expression of immediate early genes (IEGs) in the spinal cord, or in brainstem nuclei during the formalin nociception (Presley et al., 1990;Porro et al., 1991a, 1991b;Abbadie et al., 1992). Because most investigations into the neural mechanisms of formalin-induced pain have focused on the spinal cord or brainstem, the functional role of forebrain structures is less clear.

Recent advances in functional brain imaging techniques now provide a powerful method for simultaneously assessing the activation of multiple brain regions during pain and other sensory experiences. Substantial evidence shows that increases in regional cerebral blood flow (rCBF) are tightly and positively coupled to increases in synaptic activity (Sokoloff, 1978, 1981a;Mraovitch et al., 1992;Malonek and Grinvald, 1996). Studies in humans using positron emission tomography (PET) have revealed unique patterns of changes in rCBF that are associated with the perception of pain (Talbot et al., 1991;Casey et al., 1994) (Coghill et al., 1994). Such studies provide insights into the function of the pain network as a whole; information which was previously unattainable from clinical data or individual pharmacological, stimulation, lesion or electrophysiological experiments.

We wished to investigate neural mechanisms involved in formalin induced pain using a method analogous to human PET. Accordingly, we developed an animal model for imaging localized changes in cerebral blood flow and employed it to investigate forebrain nociceptive mechanisms involved in the early and late phase of the formalin test. Our findings describe patterns of activation within forebrain structures during the prolonged and persistent pain induced by subcutaneous injection of formalin. In addition, they emphasize an increasingly important role in pain mechanisms for forebrain structures with limbic system connections.

2. Materials and methods

2.1. Animal subjects

Fifty-three male, Sprague–Dawley rats weighing between 225 to 350 g served as subjects for the experiments des-

cribed here. All animals were housed in groups of three and maintained on a 12:12 h light/dark cycle, with lights on at 0600 h. Ambient temperature in the animal facility was kept at $22 \pm 2^\circ\text{C}$. Food and water were given ad libitum. We divided the animal subjects into five experimental groups: an unstimulated control group, an early phase saline group, an early phase formalin group, a late phase saline group and a late phase formalin group.

2.2. Experimental procedures

Our Institutional Animal Care and Use Committee approved all experimental procedures. We conducted all experiments in accordance with the guidelines of the NIH for the ethical use of laboratory animals (NIH, 1996) and the IASP for use of conscious animals in pain research (Zimmermann and Brinkhus, 1983). Over a 1–2-week period the rats were handled extensively and acclimated to gentle restraint in a soft towel. This towel restraint allowed access to the tail and to the left hindlimb while restricting movement. During restraint, the left hindlimb and tail were frequently touched to accustom each subject to these manipulations by the investigator. We designed this procedure to reduce stress and anxiety due to handling that might affect rCBF and/or pain perception during actual experimental conditions.

On the day of rCBF measurement, we again placed each rat in the towel restraint and inserted a flexible 24-gauge intravenous catheter through the skin into the tail vein. A rubber capped injection port was attached to the catheter and flushed with approximately 0.25 ml of saline solution. Each animal was then permitted to rest quietly in the restraint for approximately 30–40 min to recover from the stress induced by tail vein catheterization.

2.3. Group procedures

2.3.1. Unstimulated controls ($n = 11$)

For baseline measurement of rCBF in the absence of intentional somatosensory stimulation, each rat was allowed to remain undisturbed prior to, during and for 2–5 min following tracer injection (see below).

2.3.2. Early phase formalin pain group ($n = 17$)

For examining changes in rCBF during the early acute pain phase of the formal test, we injected the left hindfoot with 0.05 ml of a 2.5% solution of formalin. Two minutes following formalin, during the early phase of the formalin test (Dubuisson and Dennis, 1977), we injected each animal with an intravenous bolus of radiotracer. It is important to note that while unrestrained rats will typically lick, bite or favor the formalin-injected paw, our restrained animals were restricted from making these behavioral responses. The restrained animals used here typically remained quiet and made few movements during the critical times for these procedures. Although subjects briefly withdrew the injected

hindlimb immediately after formalin administration, they were quiet at the time of radiotracer injection.

2.3.3. Early phase saline control group ($n = 10$)

A subcutaneous injection of saline into the hind foot served as a control for the pain due to formalin. We therefore measured rCBF by injecting with an intravenous bolus of radiotracer, 2 min after injecting one hind foot with 0.05 ml of 0.9% saline.

2.3.4. Late phase formalin pain group ($n = 9$)

For examining changes in rCBF during the late phase of the formal test, we injected the left hindfoot with 0.05 ml of a 2.5% solution of formalin. Twenty minutes following formalin, we injected the radiotracer via the tail catheter. This allowed us to examine blood flow changes occurring during the peak of the tonic pain phase of the formalin test (Dubuison and Dennis, 1977). Subjects were quiet and non-moving at the time of tracer injection.

2.3.5. Late phase saline group ($n = 8$)

As a control for evaluating changes in rCBF during the late (tonic pain) phase of the formal test, the left hindfoot was injected with 0.05 ml of 0.9% saline. As with the above late phase formalin group, we injected the radiotracer via the tail catheter 20 min after hind foot injection. Subjects were quiet and non-moving at the time of tracer injection.

2.4. Radiotracer preparation

The radiotracer was prepared according to the manufacturer's directions by addition of sterile pyrogen-free sodium pertechnetate (^{99m}Tc) in isotonic saline to a commercially available radiopharmaceutical kit (Ceretek[™]; Amersham, Arlington Heights, IL). This produces the highly lipophilic complex, exametazime [(RR,SS)-4,8-diaza-3,6,6,9-tetramethylundecane-2,10-dione bisoxime]. (Exametazime was formerly known as hexamethylpropylene amine oxime or HMPAO.) Paper chromatography was performed to measure the radiochemical purity of each prepared Ceretek kit and determine the fraction of technetium label (^{99m}Tc) as lipophilic exametazime (Neirinckx et al., 1987; Ballinger et al., 1988). All reconstituted radiotracer kits used in this study had a lipophilic exametazime content of 90% or higher. The lipophilic complex is relatively unstable and rapidly converts within about 30 min to a non-lipophilic form, which is unsuitable for imaging rCBF. Accordingly, we completed all radiotracer-related procedures within this critical half-hour period.

2.5. Radiotracer administration and tissue preparation

For imaging regional cerebral blood flow, 8–10 mCi of [^{99m}Tc]exametazime in a 0.5–1 ml total volume was injected through the tail vein catheter as a bolus over 10 to 15 s. The injection port was then flushed with 0.5 ml

sterile saline. Two 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. We then carefully removed the brain from the skull and prepared it for histological sectioning by rapid freezing with powdered dry ice. Standard 20- μm coronal frozen sections were cut at -18°C using a Hacker-Bright[™] cryostat. Three to four consecutive sections taken at fixed intervals were mounted on glass slides and rapidly desiccated on a hotplate at 60°C . This insured that there would be a sufficient number of sections through all sampled brain regions for later autoradiographic analysis. Slides were then arranged in a standard X-ray cassette. An autoradiogram was generated by direct apposition of the tissue to the emulsion side of Kodak BioMax[™] MR-1 Imaging film (Kodak, Rochester, NY) for a 1.5–3.0 h exposure. A commercially available [^{14}C]-standard (ARC-146B; American Radiolabeled Chemicals, St. Louis, MO) was also placed in the film cassette for film calibration. Immediately after the film was loaded in the cassette, the level of residual radioactivity in the initial injection volume of tracer was calculated. This value was used for the computation of film exposure time by proportional comparison with the optimal exposure time for a known level of radioactivity. After exposure, the film was removed from the cassette and processed using a Kodak XOMAT[™] automatic film processor. When the radioactivity level of the ^{99m}Tc -labeled slides had returned to background, they were stained with cresyl violet. We made precise structural identifications by careful comparison of cresyl violet-stained sections to coronal plates from the stereotaxic atlas of the rat brain (Paxinos and Watson, 1986).

2.6. Data analysis

Densitometric analysis of autoradiograms was performed using a microcomputer-assisted video imaging densitometer (MCID system; Imaging Research, 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 using an approach designed to ensure the accuracy and consistency of structural identification. For each coronal brain section, we overlaid matching transparent stereotaxic templates on the digitized brain images displayed on the video monitor and aligned the images using prominent landmarks (see example in Fig. 1). Section templates were adapted from the stereotaxic atlas of Paxinos and Watson (1986). The template served as a guide to sample the ROIs present at a given anterior–posterior level. This template matching method, combined with histological verification from stained sections provided sufficient resolution to identify and sample even small nuclear groups and provided for high inter-experimenter reliability.

For the purpose of this study, we limited the regions of interest (ROIs) to eighteen structures. Table 1 lists all struc-

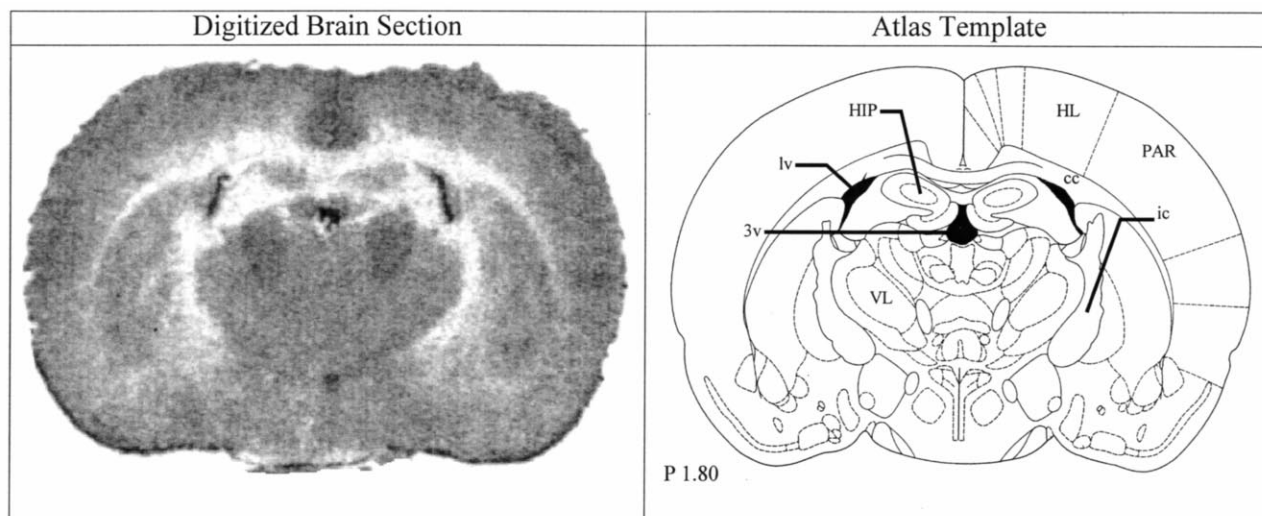


Fig. 1. Left panel shows a typical 256 level grayscale image digitized from the autoradiogram of a coronal brain section at AP level -1.80 . Right panel shows a scanned image of the transparent template for AP -1.80 modified from the stereotaxic atlas rat brain of Paxinos and Watson (1986). The outline drawing on the template is superimposed over the grayscale image of the brain and aligned with key structural landmarks. At this AP level, we align the hippocampus (HIP), 3rd ventricle (3v), lateral ventricles (lv), corpus callosum (cc), and internal capsule (ic) on the template to matching structures on the brain image. This overlay method permits precise localization of ROIs.

tures sampled in this study and provides a key for all anatomical abbreviations. We sampled structures believed to participate in pain mechanisms and others not considered part of traditional pain pathways. Some ROIs were previously identified in human PET as showing increased blood flow during acute pain due to noxious thermal stimuli (Talbot et al., 1991; Casey et al., 1994). Due to our specific interest in the forebrain, we restricted sampling primarily to cortical and thalamic structures and the brainstem PAG. The spinal cord was not examined.

We converted the film densities to apparent tissue radioactivity concentrations (nCi/mg) by comparison with the film optical densities of [^{14}C] standards to allow ROI comparisons across different films and animals. An index of activation (AI) was then calculated from individual ROI activities as a percent of average total activity of the entire brain.

$$\text{AI} = \frac{(\text{Activity}_{\text{ROI}} - \text{Activity}_{\text{Brain}})}{\text{Activity}_{\text{Brain}}} \times 100\% \quad (1)$$

where AI is the relative activation index computed as the percent difference from whole brain activity, $\text{Activity}_{\text{ROI}}$ is the activity of the ROI, and $\text{Activity}_{\text{Brain}}$ is the average activity of all brain sections.

We computed an average within subject AI for each of the eighteen ROIs sampled. The mean AIs for each ROI from all animals in each experimental group were then averaged together to compute within group means. The mean AIs of distinctly bilateral structures (AD, BLA, CC, CPU, HBC, HIP, HL, MT, PAR, PO, PVN, RS, VL, VM, VPL, VPM) were examined for side-to-side differences in rCBF using a paired t -test ($P < 0.05$). We tested for significant differences in activation for each ROI between

experimental groups, including the two midline structures (PAG, IPN) using ANOVA with post-hoc t -tests corrected for multiple comparisons using the Bonferroni correction. All statistical analyses were performed using the software package, SPSS for Windows (SPSS, Chicago, IL).

3. Results

3.1. Unstimulated control group

Table 2 shows the regional differences in cerebral blood flow for all ROIs sampled in unstimulated controls and in

Table 1

Anatomical abbreviations

AD	Anterior dorsal nucleus (thalamus)
BLA	Basal lateral amygdala
CC	Cingulate cortex
CPU	Caudate-putamen
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
VL	Ventral lateral nucleus (thalamus)
VM	Ventral medial nucleus (thalamus)
VPL	Ventral posterior lateral nucleus (thalamus)
VPM	Ventral posterior medial nucleus(thalamus)

Table 2

Regional differences in cerebral blood flow (rCBF) expressed as the mean percent difference from mean whole brain activity, the activation index (AI)

Region of interest	Control (n = 11)	Early phase formalin (n = 17)	Late phase formalin (n = 9)
AD	16.29 ± 2.50	18.82 ± 3.38	30.49 ± 3.11 ^{a,b}
BLA	-1.85 ± 1.19	1.27 ± 2.31	-2.41 ± 2.20
CC	8.98 ± 3.10	15.29 ± 2.57	14.81 ± 3.30
CPU	11.52 ± 3.23	1.68 ± 2.29 ^a	8.69 ± 2.54
HBC	41.51 ± 5.41	45.97 ± 3.62	58.03 ± 4.04 ^{a,b}
HIP	-3.68 ± 3.61	-4.41 ± 2.19	-0.49 ± 2.25
HL	12.36 ± 1.36	30.86 ± 2.41 ^{a,*}	31.35 ± 2.71 ^a
IPN	29.89 ± 7.20	35.93 ± 3.90	66.16 ± 2.31 ^{a,b}
MT	6.83 ± 3.80	5.23 ± 2.84 [*]	9.00 ± 2.92
PAG	-4.76 ± 3.39	7.88 ± 2.16 ^a	20.02 ± 3.20 ^{a,b}
PAR	15.81 ± 3.09	21.99 ± 3.22	29.90 ± 2.53 ^a
PO	4.23 ± 2.06	6.53 ± 3.74	4.92 ± 3.41
PVN	9.30 ± 6.48	7.40 ± 5.74	35.30 ± 9.89 ^{a,b}
RS	21.12 ± 1.97	31.12 ± 2.75 ^a	40.42 ± 3.82 ^a
VL	4.83 ± 1.84	5.01 ± 3.08	2.41 ± 2.38
VM	3.66 ± 2.17	6.41 ± 4.10	4.46 ± 2.60
VPL	4.28 ± 1.74	5.31 ± 3.76	8.67 ± 3.24
VPM	14.50 ± 2.56	14.38 ± 3.30	17.52 ± 4.94

Values calculated as group mean ± SEM (see Section 2) for all regions of interest (ROIs). ^aSignificant difference from control ($P \leq 0.05$, ANOVA); ^bsignificant difference from early phase formalin ($P \leq 0.05$, ANOVA). *Significant for side-to-side difference ($P \leq 0.05$, paired *t*-test).

the two formalin groups. The AI values (see Table 2) show that, under all experimental conditions, the pattern of cerebral blood flow displayed regional heterogeneity. There was considerable variation in blood flow among ROIs even in the unstimulated control subjects. However, control subjects showed no significant side-to-side differences in rCBF (see Fig. 2). Because lateralized differences were absent in the

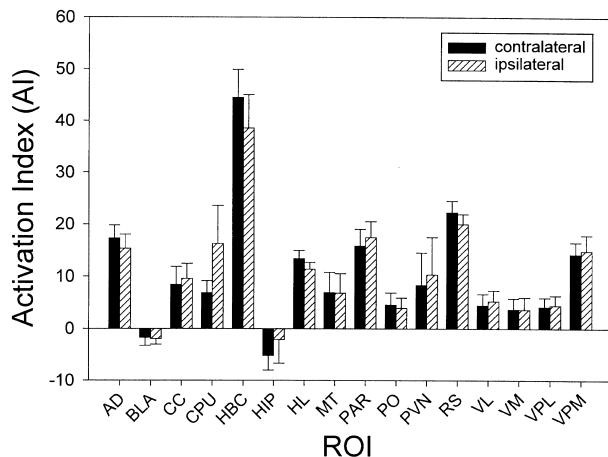


Fig. 2. This bar graph compares the side to side differences in regional cerebral blood flow (rCBF) in the unstimulated control group. The relative levels of activation are expressed as AI, the mean percent difference from total brain activity (see Section 2). Note the similarity in AI value between the contralateral and ipsilateral sides for each ROI. No significant lateralized differences in activation were found for any ROI in control subjects.

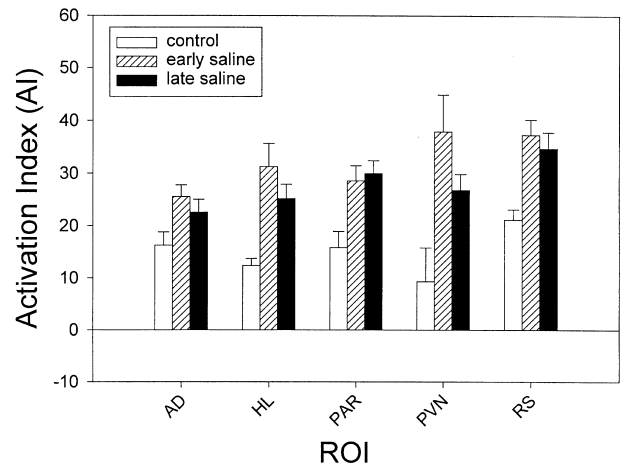


Fig. 3. This bar graph compares the average bilateral level of activation in several ROIs for control, early and late saline groups. Data are shown only for ROIs showing a significant difference in activation in either the early phase or late phase saline groups as compared to unstimulated controls. The relative levels of activation are expressed as AI, the mean percent difference from total brain activity (see Section 2). Note that all ROIs showed a tendency toward reduced activation in the late phase or a change in AI returning toward control values.

control group, we averaged the AI values from both sides for each ROI in control animals.

3.2. Early and late saline groups

Following injection of saline into one hindpaw, blood flow increases during the early phase (2 min post-injection) and late phase (20 min post-injection) showed significant differences in activation within several ROIs, including AD, HL, PAR, PVN and RS. Fig. 3 compares the activation indices (AI) of all sampled brain regions that exhibited significantly altered blood flow in either the early or late phase saline groups relative to unstimulated controls ($P < 0.05$, ANOVA). Although the AI of most ROIs showed a tendency to be slightly lower in the late as compared to the early phase saline group, this difference did not reach statistical significance. The midbrain PAG and several thalamic structures (MT, PO, VL, VM, VPL and VPM) showed a tendency toward reduced activation in both the early and late saline groups. We found no lateralized differences in rCBF among those regions of interest showing a significant change in activation with respect to controls. These data indicated that a subcutaneous injection of isotonic saline was not a neutral stimulus, and was therefore inappropriate as a control for noxious formalin stimulation.

3.3. Early and late formalin groups

We found lateralized differences in early phase formalin induced forebrain activation in only two of the 18 structures sampled (see Table 2), the hindlimb region of somatosensory cortex (HL) and medial thalamus (MT). As shown in Fig. 4, during the early phase, both HL and MT showed

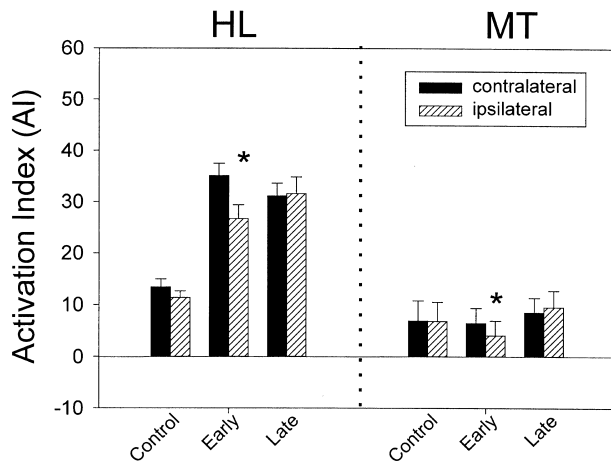


Fig. 4. This bar graph compares contralateral versus ipsilateral activation for HL and MT, the only two structures showing significant lateralized differences in rCBF during formalin nociception. The relative levels of activation are expressed as AI, the mean percent difference from total brain activity. Only during the early phase formalin was there a side to side difference in activation in HL and MT. In both cases the side contralateral to the site of stimulation had the greater AI. Lateralized differences in activation were absent during late phase formalin. Note that while MT showed a lateralized difference in blood flow, the AI was not significantly different from control. *Significant for side-to-side difference ($P \leq 0.05$, paired t -test).

significantly greater activation on the side contralateral to the site of formalin stimulation than on the ipsilateral side (paired t -test, $P < 0.05$). These side-to-side differences were exhibited only in the early phase and were no longer present 20 min later, during late phase formalin. In addition, superimposed on the lateralized difference, there was a significant bilateral increase in rCBF in hindlimb cortex as compared to unstimulated control animals. While we found a significant lateralized difference in activation within MT during the early phase, neither the contralateral nor the ipsilateral MT was significantly different from control values.

As shown in Table 2, three of the ROIs sampled in this study showed a bilateral increase in activation within 2 min after formalin injection, during the early phase of formalin nociception. These included two forebrain structures (HL and RS) and the midbrain PAG. Similarly, during the late phase of formalin nociception, the hindlimb cortex, RS and PAG continued to exhibit an increased rCBF as compared to controls. The late phase group, however, also showed increased activation in several ROIs not significantly activated in the early phase (see Table 2). The additional structures active only in the late phase animals included the parietal cortex adjacent to HL and four ROIs within the limbic system. These limbic structures included the AD, HBC, the IPN, and the PVN. Fig. 5 compares ROIs exhibiting statistically significant increases in rCBF during either the early or late phase following formalin injection as compared to controls. Also shown in Fig. 5 are three ROIs not significantly activated, including VPL and MT, representing

the spinothalamic system and one limbic structure, cingulate cortex. Note that with the exception of HL, all significant ROIs showed a greater activation during late phase formalin than during the early phase. As shown in Fig. 5, this late phase increase in relative blood flow was most prominent in IPN, PVN and PAG. A tendency toward greater activation during late phase formalin was also seen in two thalamic ROIs (MT and VPL; see Table 2 and Fig. 5). The trend toward greater forebrain activation in the late phase as compared to the early phase for formalin injected rats contrasts with our data from saline-injected animals where we found tendency toward reduced rCBF during the late phase in all but one ROI. Cingulate cortex also showed a subsignificant increase in rCBF during both phases of the formalin test. Only one structure, CPu, showed a significant reduction in blood flow during the early phase after formalin.

4. Discussion

In the present study, we measured regional cerebral blood flow to identify the patterns of forebrain nociceptive processing that occur during the early and late phase of the formalin test. To our knowledge this is the first neural imaging study to use rCBF in an animal model to identify the neural correlates of prolonged noxious stimulation elicited by subcutaneous formalin injection. The results described here show that rCBF increases can be used to investigate pain mechanisms in animals.

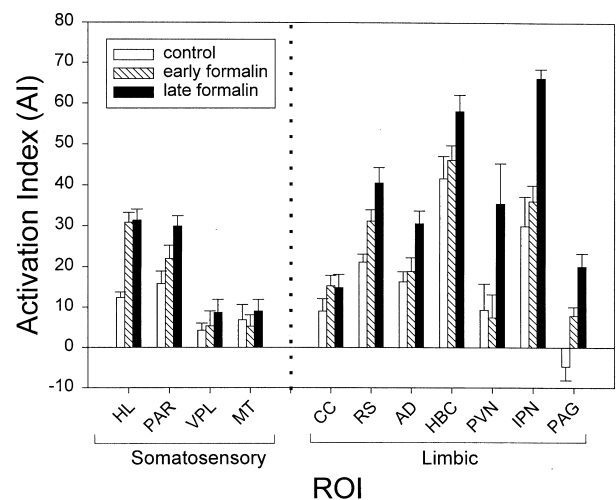


Fig. 5. This bar graph compares the average bilateral level of activation in several ROIs for control, early and late formalin groups. With the exception of VPL, MT and CC all bars presented here are for ROIs showing a significant difference in activation in either the early phase or late phase formalin groups as compared to unstimulated controls ($P \geq 0.05$, ANOVA). The relative levels of activation are expressed as AI, the mean percent difference from total brain activity (see Section 2). Unlike the saline group, there was a time-dependent increase in AI in all but two ROIs. Only HL and CC showed no difference in the degree of activation between the early and late phase.

Our data can be summarized as follows. Noxious formalin consistently produced detectable, well-localized increases in rCBF within multiple forebrain structures and the midbrain periaqueductal gray. Subcutaneous injection of physiologic saline is not a neutral stimulus because it is probably mildly noxious and activated several cerebral structures. Noxious formalin, especially during the late phase, primarily induced changes in brain regions within the limbic system. The somatosensory cortex was activated by noxious formalin and thalamic components of the spinothalamic pathway (VPL and MT) showed subsignificant increases during the late phase as compared to the early phase of the formalin test. Forebrain ROIs exhibited a bilateral pattern of activation and sometimes also showed superimposed lateralized differences. Finally, the spatial pattern and intensity of activation varied as a function of the time following the noxious formalin stimulus.

4.1. Methodological considerations

Although the measurement of regional glucose metabolism using ^{14}C -labeled 2-deoxyglucose (2DG) has been used previously to investigate spinal and brainstem mechanisms of formalin pain (Porro et al., 1991a,b; Aloisi et al., 1993) in rat, we chose to measure rCBF for two major reasons. First, due to tracer diffusion characteristics, conventional 2DG techniques have a spatial resolution of 100–200 μm (Smith, 1996) compared to the 25–50 μm resolution in the [$^{99\text{m}}\text{Tc}$]exametazime blood flow method we used here. Secondly, the blood flow method described in this paper provides a temporal resolution, relative to timing of the sensory stimulus, which is superior to 2DG. Cerebral blood flow can be used to identify changes in cerebral activation occurring in response to a relatively short duration sensory stimulus (<1 min duration). The identification of stimulus-evoked changes in brain activation with 2DG requires a prolonged period of stimulation (20–45 min) (Mao et al., 1992). Values obtained by the [^{14}C]2DG technique are also heavily weighted toward neural events occurring in the first 20 min of the experimental period (Sokoloff et al., 1977; Sokoloff, 1977, 1979, 1981b) While the use of the 2DG method provides important information regarding the relative degree of activation in different brain regions, in the final analysis this method has a lower temporal resolution than the blood flow imaging technique described in this paper.

We chose [$^{99\text{m}}\text{Tc}$]exametazime over other radioligands more frequently used for autoradiographic blood flow measurement, [^{14}C]iodoantipyrine (IAP) and [^{123}I]iodoamphetamine (IAMP), for several reasons. Although the uptake and distribution of all these tracers exhibits a relatively good correspondence with blood flow (Bok et al., 1987; Neirinckx et al., 1987; Hoffman et al., 1988; Lear, 1988a, b), IAP and IAMP freely cross the blood–brain barrier in both directions. This is a disadvantage because it makes experimental timing critical. Brain removal and freezing after

decapitation must be performed very rapidly to prevent diffusion of the tracer from regions of high blood flow to regions of lower blood flow, which would result in an underestimate of regional blood flow. In contrast, [$^{99\text{m}}\text{Tc}$]exametazime accumulates in the brain and rapidly compartmentalizes without significant redistribution for more than 1 h (Lear, 1988a,b). The high retention and low back diffusion of [$^{99\text{m}}\text{Tc}$]exametazime makes timing less critical and allows for testing multiple subjects during a single experiment. This radioligand is also used to study rCBF in humans with single positron emission tomography (SPECT). Technetium ($^{99\text{m}}\text{Tc}$) is primarily a gamma-2 emitter (mean energy 140.5 keV) which decays by isomeric transition with a physical half-life of 6.03 h. The high energy and short half-life of this radiotracer makes it ideal for use in these animal experiments by minimizing both film exposure time and the storage time required for complete decay of radioactive wastes.

Although the approach we used does not directly measure rCBF, it was sufficiently sensitive to allow detection of regional differences in blood flow even in the unstimulated subjects. The use of similar methods has been validated and used in other laboratories for autoradiographic studies that measured regional glucose metabolism (Porro et al., 1991a,b). Furthermore, we avoided the stress of surgery for the placement of an arterial catheter, which is required to sample arterial blood for direct blood flow measurement. An additional factor that affected our ability to detect changes in cerebral activation involved our method of sampling optical densities in each ROI. To avoid investigator bias, we chose to include the entire extent of each ROI present in a given brain section. For structures having spatially segregated or somatotopic organization of afferent inputs, such as the VPL thalamus, this sampling method would lead us to sample a greater area of that structure than could possibly be activated by formalin. We found that the MT and VPL showed a rCBF increase during the late phase that was significantly larger than during the early phase, but these differences were not statistically significant. Similarly, we grouped several medial and intralaminar thalamic nuclei together into one sample, including the central medial, central lateral, medial dorsal, and parafascicular nuclei. If only a small fraction of one or all of these nuclei was activated by formalin, the small relative increase in film optical density would be lost in the combined average for the entire MT nuclear group. Likewise, we may not have identified significant formalin induced changes in activation in VPL because the hindlimb nociceptors innervate only a small fraction of this nucleus and we sampled the total area of VPL on each section. Our conservative sampling technique biased our experiments toward identifying only robust changes in blood flow. By refining our sampling procedures in future studies, we may be able to demonstrate the predicted statistically significant increase in the activation of specific thalamic nuclei.

One disadvantage of blood flow (including human PET)

and other metabolic imaging techniques (2DG), is the inability to determine the functional valence of brain activation. Although there is substantial evidence that the activity of local neurons is a major factor regulating the specific distribution of blood flow throughout the brain (Sokoloff, 1981a; Lou et al., 1987; Iadecola et al., 1996; Malonek and Grinvald, 1996), current methods cannot distinguish between excitatory and inhibitory synaptic activity.

4.2. Changes in rcbf: early and late phase saline groups

In both the early and late saline injected groups, we found activation within several forebrain structures, including AD, HL, PAR, PVN and RS. Porro et al. (1991b), using 2DG, showed that saline injected into one hindpaw failed to produce metabolic activation in rat spinal cord, and therefore pooled the data from their control and saline groups. Our data, however, indicated that the saline injection was in fact not neutral and suggested that these groups were not appropriate choices for control comparison with the formalin groups. This conclusion is supported by the evidence for prolonged C-fiber activation following the cutaneous injection of saline (Puig and Sorkin, 1995). Therefore, we statistically compared all blood flow data obtained during stimulated conditions (saline and formalin) against that obtained from the unstimulated control group.

4.3. Changes in rCBF: early and late phase formalin groups

Cohen et al. (1991) using 2DG did not find a significant change in the activation of limbic or forebrain structures due to noxious stimulation with either formalin or tail immersion. In contrast, we found significant changes in forebrain activation during both the early (2 min) and late (20 min) phases following formalin injection. This apparent discrepancy suggests a lack of adequate sensitivity in the 2DG method to delineate areas of the brain mediating acute nociception in rats, a conclusion also suggested by the authors. There is also evidence that 2DG may in itself induce analgesia in the rat (Bodnar et al., 1979). These results highlight the greater sensitivity of blood flow imaging techniques for the study of pain mechanisms in certain circumstances.

Because pain-related behaviors in the formalin test are normally displayed only in the hindlimb ipsilateral to the injection of formalin (Dubuisson and Dennis, 1977; Abbott et al., 1995), we expected to see differences in activation primarily on the contralateral side in those structures known to be part of the ascending spinothalamic system. However, we consistently found bilateral changes in forebrain activation, with concurrent side-to-side rCBF differences occurring only during the early phase of formalin nociception in HL and MT. By the late phase, these lateralized differences were no longer evident. Our finding of primarily bilateral activation is supported by the work of Aloisi et al. (1993) who describes the appearance of 'mirror pain', the licking of

the contralateral untreated hind paw, 10–60 min after a unilateral formalin injection. The appearance of 'mirror pain', however, depended on the concentration of the formalin solution and required a 5–10% solution for consistent expression. Although we used only a 2.5% solution of formalin, this concentration was sufficient to induce bilateral changes in the forebrain rCBF. Using 2DG imaging, other studies also showed that levels of functional metabolic activity after unilateral hind limb injection with formalin are increased bilaterally in the lumbar spinal cord and brainstem of rats (Porro et al., 1991a,b; Aloisi et al., 1993). Human PET studies (Casey et al., 1996) have also described bilateral changes in the rCBF of forebrain structures during painful tonic deep cold stimulation of one arm. Furthermore, using 2GD, other laboratories have found bilateral activation in the brain (Mao et al., 1993) and spinal cord (Coghill et al., 1991; Mao et al., 1992) of rats due to chronic pain induced by a chronic constriction injury of the sciatic nerve in one hindlimb.

The involvement of limbic structures in nociceptive processing and of structures associated with pain modulation is expected. The limbic system has long been believed to play an important role in neural mechanisms related to the affective-motivational dimension of pain (Casey and Melzack, 1967; Melzack and Casey, 1968; Hylden et al., 1986; Geisler et al., 1994; Chapman, 1996; Willis and Westlund, 1997). Especially during the late post-injection phase, formalin nociception primarily induced an increase in rCBF within limbic forebrain structures, including the AD, HBC, PVN and RS, as well as in the midbrain PAG and IPN. Recent studies using positron emission tomography also report significant increases in regional blood flow in limbic forebrain structures and PAG during acute or chronic pain perception in humans (Talbot et al., 1991; Casey et al., 1994, 1996; Coghill et al., 1994; Hsieh et al., 1995, 1996; Vogt et al., 1996). Anatomical studies have shown that many limbic structures receive spino-reticulo-thalamic input via connections from the thalamic medial-intralaminar nuclei and thalamic reticular nucleus (Kaitz and Robertson, 1981; Robertson and Kaitz, 1981). In addition, there is evidence for extensive reciprocal interconnections between many of these cortical and thalamic regions (Kaitz and Robertson, 1981; Robertson and Kaitz, 1981; Thompson and Robertson, 1987; Cavada and Goldman-Rakic, 1989; Lozsádi, 1994), thus providing a neural substrate for complex interactions.

Activation of limbic forebrain structures may be related to either the perception or the modulation of nociceptive inputs. An increase in the activation of the interpeduncular nucleus, which connects to the HBC via the fasciculus retroflexus, may be partly responsible for the hyperalgesia seen in the formalin test. Hentall and Budhrani (1990) showed that electrical stimulation in IPN of rat excites 'on cells' and inhibits 'off cells' in the nucleus raphe magnus. The effect of such stimulation should be hyperalgesic based on descriptions of the response properties of neurons in the

raphe spinal system (Barbaro et al., 1989; Heinricher et al., 1989; Heinricher and Tortorici, 1994). In contrast, activation of other limbic structures may lead to a reduction in pain perception. The role of the PAG in endogenous antinociception is well accepted and has been extensively investigated by physiological, pharmacological and behavioral approaches (Sherman and Gebhart, 1975; Giesler and Liebeskind, 1976; Basbaum and Fields, 1984; Manning et al., 1994; Traub et al., 1996; Sandkuhler, 1996; Willis and Westlund, 1997). Electrical stimulation of (Cohen and Melzack, 1986, 1993) or microinjection of morphine into (Cohen and Melzack, 1985) the habenular complex also markedly attenuates nociception in the formalin test. In addition, lesion studies involving the PVN suggest a complex role for this limbic structure in the production of stress induced analgesia (Cohen and Melzack, 1986; Truedell and Bodnar, 1987; Pacak et al., 1995; Fuchs and Melzack, 1996).

In the present study, noxious formalin elicited a marked activation of both cortical (RS) and subcortical (AD) limbic structures and a subsignificant activation in cingulate cortex (CC). These structures indirectly receive input via the pulvinar from one or more of the thalamic nuclei implicated in pain mechanisms, including the reticular, the intralaminar (centromedian, centrolateral and parafascicular) and the posterior nuclei (Tekian and Afifi, 1981). The extensive projections of the pulvinar to several cortical regions including the cingulate gyrus may in part represent a means by which cortical and limbic structures are activated during pain sensation. The subcortical connections of the pulvinar may provide an anatomical substrate for the activation of CC and RS during nociception. Recent work by Traub et al. (1996) also shows that restraint as well as the activation of visceral nociceptors by colorectal distension can induce the expression of the immediate-early gene, c-Fos in many limbic areas. Some of these same brain regions were activated by formalin stimulation in the present study including AD, CC, RS and PAG. In addition, the extensive reciprocal connections between RS and AD could account for the increased rCBF in AD.

Our results suggest that prolonged nociception and not acute phasic pain may provide the primary stimulus for pain-induced physiologic activation of structures involved in endogenous pain modulation. Consistent with this idea, we found that both the PAG and HBC, structures involved in antinociception, showed a greater increase in rCBF during the late phase of formalin pain as compared to the early phase.

The time course of evoked activity in both peripheral A δ - and C-fiber nociceptors (Puig and Sorkin, 1995) and spinal WDR neurons (Dickenson and Sullivan, 1987a) correlates with the biphasic pattern of behavior in which pain scores during the late phase are typically lower than those during the early phase. Based on these data, we had expected to find a similar temporal pattern in the intensity of activation in the forebrain of formalin-injected rats. In contrast, our blood flow data showed that most forebrain ROIs exhibited max-

imum activation during the late phase. In addition, we saw a spatial spread of activation over time to other forebrain regions. Several forebrain structures not activated in our early phase formalin group became activated in the late phase group. These findings suggest that the prolonged tonic phase (late phase) of the formalin nociception may in part be due to pain-induced changes in the activity of forebrain structures.

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