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Formalin-Induced c-fos Expression in the Brain of Infant Rats

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Abstract

In the fetal, infant and adult rat, injury induces a well-defined behavioral response and induces c-fos expression in the spinal cord dorsal horn. There is more limited information about the processing of noxious stimulation in the infant brain. We describe here the appearance of the Fos protein in the brain of fetal and infant rats following formalin-induced injury. Regions were chosen for analysis with a special focus on brain loci that express c-fos in the adult. No Fos positive cells were found in the brains of fetuses; newborns did not show increased Fos expression after formalin injection in any structure examined. At 3 and 14 days of age, there was a significant increase in staining induced by formalin in the ventral lateral medulla. In contrast, paraventricular and medial dorsal nuclei of the thalamus, the paraventricular nucleus of the hypothalamus and periaqueductal gray of the midbrain showed increased levels of Fos protein only at 14 days of age. We hypothesize that this developmental pattern is related not only to the maturation of pain perception but also to development of autonomic and defensive reactions to pain in the infant.

Perspective—Because the infant processes pain differently than the adult, knowledge of those differences informs pediatric clinical practice. Using Fos expression as a marker of neural activity in the rat, we show that the pattern of brain activation is immature at birth but is in place by 14 days of age.

Keywords

Pain; ontogeny; infant; spinal cord; c-fos; fetus

Pain can be a serious clinical problem for human neonates¹⁸, yet it is not known in detail how infants perceive and process painful stimuli. In the rat the substrates of nociception in the spinal cord mature during the late prenatal and early postnatal periods, roughly corresponding to the third trimester and early postnatal period in the human. Processing of noxious input differs, however, between infants and adults. Rat and human infants have lowered thresholds for some nociceptive stimuli (e.g. mechanical or pressure)^{2, 26} but are relatively non-responsive to others (e.g. mustard oil)^{11, 51}.

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The expression of immediate early genes, most notably *c-fos*, has been used to map activation of neural circuits under a variety of experimental conditions. In the fetus, infant, and adult rat, noxious stimulation of the paw induces a behavioral response and Fos expression in the dorsal horn of the spinal cord^{71, 26, 52, 53, 61}. The number of Fos positive cells increases from late gestation to the late preweaning period⁵². In the adult rat, *c-fos* is expressed in a variety of brain sites, although not in classic pain regions, following noxious stimulation of the hindpaw⁷¹. These sites include the periaqueductal gray of the midbrain (PAG), the ventral lateral reticular formation (VLR), dorsal midline thalamic nuclei (DMT), and the paraventricular nucleus of the hypothalamus (PVN). Although these are not the third order neurons that process noxious input, they are regions implicated in behavioral and autonomic responses to threat and thus Fos expression in these regions may be related to defensive and autonomic reactions of the animal to pain.

In the infant, autonomic responsiveness and defensive behaviors develop slowly and specific responses depend on the age of the animal, the response, and the nature of the threatening stimulus. For example, the cardiovascular response to threat is mediated differently by the autonomic nervous system moving from no response, to parasympathetic control and finally to sympathetic control around 30 days of age¹⁷. In response to the threat of a strange and potentially predatory adult male rat, infant rats defensively freeze for the first time at 14 days of age and that is correlated with the appearance of Fos expression in the PAG, PVN, and locus coeruleus⁴⁹. Little is known of brain sites that elicit those responses and whether or not similar processing of noxious stimulation occurs in the brain in the infant. Thus the goal of these experiments was to describe the early maturation of *c-fos* expression in the brain of the fetal and infant rat following formalin injected into the forepaw or hindpaw. Our hypothesis was that Fos as a marker of cellular activity in these structures would not appear in the fetus or neonate but would be seen in the older infant.

Materials and Methods

Subjects

Subjects were the offspring of Long-Evans Hooded rats (Blue Spruce) bred in our colony. Dams and sires were housed together in a room maintained at $23^{\circ} \pm 1^{\circ}$ C and the birth of litters checked twice daily at 9 AM and 6 PM. Pups were defined as 0 days of age on the day of birth; fetuses were from time-mated dams⁵³. We used a total of 42 fetal and infant rats [gestational day (GD) 20; postnatal days (PD) 0, 3 or 14]. There were between 4 and 9 pups per condition (age and injection type) in each experiment. However, because of experimental error, for saline injected 3-day-old pups, counts in the raphe nuclei were available for only two subjects. All experiments were approved by the Hunter College IACUC and followed the ethical guidelines of the Society for Neuroscience and the International Society for Developmental Psychobiology for treatment of animals.

Procedure

Formalin or saline was injected into either the forepaw or hindpaw at the ages described above (5 μ l for fetuses, 15 μ l for postnatal animals; 10%; see⁵³ for methods of treating the fetus). Two hours later they were overdosed with a barbiturate and when deeply anesthetized they were perfused transcardially with 4% paraformaldehyde. This time point was based on prior studies with brain and spinal cord showing that Fos levels were highest at 1.5 – 4 hours after the manipulation^{49, 53}. Brains were post-fixed in 4% paraformaldehyde for up to one week and placed in 30% sucrose for cryoprotection. Regions were chosen for analysis based on examination of sections from the anterior commissure to the area postrema with special focus on brain regions that express *c-fos* in the adult.

Immunohistochemistry

Thirty-micron sections were cut on a cryostat. Alternate sections were stained for nissl substance (cresylviolet). Free-floating sections were processed using a pan-Fos antibody (Oncogene Science) and the ABC method²¹. Sections were incubated for 48 hours at 40° C in the primary antibody diluted 1: 10,000–20,000 (w/v) in phosphate buffer with triton and 1% goat serum. The chromogen was diaminobenzidine enhanced with nickel. Tissue from several animals at different ages, including controls, was processed together. In prior experiments we found no significant assay-to-assay variability.

Cell Counting

The sections were scanned for a number of animals at each age in each condition to determine sites that were labeled. In these cases, when Fos positive cells were identified, the section was compared to an adjacent cresyl violet stained section to determine the region. Based on that preliminary analysis the following brain sites were targeted for quantification: ventral lateral medulla, the raphe nuclei (obscurus, pallidus, magnus), the rostral ventral gigantocellularis area (which included the lateral paragigantocellularis and gigantocellularis pars alpha), the periaqueductal gray of the midbrain, the paraventricular nucleus of the thalamus, the medial dorsal thalamus nucleus, and the paraventricular nucleus of the hypothalamus. The periaqueductal gray was further subdivided into rostral, medial and caudal regions for each of dorsal lateral, lateral and ventral lateral divisions⁴. Although these are not the third order neurons that process noxious input, they are regions implicated in behavioral and autonomic responses to threat in adults and infants. Hence Fos expression in these regions may be related to defensive and autonomic reactions of the animal to pain. Areas reported to express Fos in the adult, but on visual inspection showed no staining or no difference between formalin and saline included the dorsal medullary reticular formation, nucleus of the solitary tract, the locus coeruleus, parabrachial region and the lateral thalamic nuclei^{71, 19, 27, 61}. For each region all Fos positive cells were counted for all sections containing the region of interest under the microscope for both formalin and saline injected pups, without knowledge of the treatment condition. No effort was made to quantify the density of staining.

Statistical Analysis

Data from each site, except for the paraventricular nucleus of the hypothalamus (see below) were analyzed by a three way analysis of variance: between subject variables were age (0, 3 or 14 days), injection (formalin vs. saline) and paw of injection (forepaw or hindpaw). In addition the periaqueductal gray of the midbrain (PAG) was further divided into different subregions that were treated as within subject variables. These subregions were along the anterior-posterior plane (rostral, medial, caudal) and the dorsal-ventral level (dorsal, lateral, ventrolateral). Posthoc tests were Tukey's HSD. Alpha level was 0.05 in all cases.

Results

There was no significant difference between the forepaw and hindpaw injections for any brain site and all subsequent analyses combined those data. No staining was found in the brains of GD 20 fetuses. Newborns failed to show increased expression after formalin injection in any structure examined. All subsequent analyses were therefore of 3- and 14-day-old pups. Two raphe nuclei [obscurus (ROB), magnus (NRM)] failed to show Fos induction due to the formalin injection. The raphe pallidus (RPA) did show a significant increase in Fos staining following formalin injection, but the number of cells labeled was small in all cases. All three nuclei showed significant increases in the number of Fos labeled cells with increasing age, but failed to show any interaction between the age of the animal and the type of injection. These data for these three sites are presented in Table 1.

Paraventricular nucleus of the hypothalamus

The paraventricular nucleus of the hypothalamus showed massive staining in the 14-day-old pups injected with formalin. There was no staining in younger animals in either treatment condition (not shown). In the 14-day-old pups given saline, Fos positive cells were diffusely distributed around the dorsal lateral aspects of the 3rd ventricle and not cleared delimited within the PVN; there was no substantial staining that was localized to the PVN. In contrast, formalin treated pups showed dense staining limited to the PVN. Because the staining localized to the PVN occurred only at 14 days of age and only in formalin treated pups, cells were not counted and the data for this region was not analyzed statistically.

Photomicrographs and camera lucida pictures are shown in Figures 1A and 1B.

Midline Thalamus

The midline thalamus, including the paraventricular nucleus (PVN) and medial dorsal nucleus, showed increased levels of the Fos protein that were age dependent [$F(2,30) = 8.60$; $p < .005$]. Posthoc tests showed that formalin increased Fos induction only at 14 days of age when compared to the saline treated pups and that the number of Fos positive cells in the 14-day-old animals was greater than that of the 0- and 3-day-old pups. These data are shown in Figures 2A–2C.

PAG

The PAG presented a complex picture, in part because of the differential development of labeling in the anterior-posterior plane and in the columns in the dorsal, ventral and lateral planes. First we divided the PAG into three caudal to rostral levels and the number of Fos positive cells was counted in the dorsolateral, lateral and ventrolateral subregions. The major effect was a significant three-way interaction among the age, injection type and rostral to caudal plane [$F(4,58) = 2.97$; $p < .05$]. There was a trend towards a significant 4-way interaction of age, injection type, rostral to caudal plane and dorsal to lateral plane [$F(8,116) = 1.97$; $p = .056$].

Posthoc analyses showed that the caudal PAG had higher levels of Fos protein than did either the rostral or medial PAG, except laterally. At 14 days of age, the dorsal and ventrolateral columns showed significantly more labeled cells following formalin injection. There was a trend ($p = .08$) for the saline treated 0-day-old pups to have more Fos positive cells in the dorsal PAG (Figures 3A, upper left panel and Figure 3B).

Ventral Lateral Medulla

Fos positive cells were increased by formalin injection, compared to controls, as early as 3 days of age [$F(2,32) = 3.37$, $p = 0.047$]. This was the earliest onset of a difference between saline and formalin injections. Here there was bilateral labeling with increased numbers on the side contralateral to the formalin injection. The anterior posterior extent was from the lateral reticular nucleus with some positively labeled cells as far rostral as the facial nerve, although the densest labeling was more caudal. The structures labeled included the lateral reticular nucleus, and rostral ventral reticular formation, and appeared to include A1, although without catecholamine labeling, this cannot be definitively stated. These are depicted in Figures 4A and 4B.

Discussion

The results of this study show that the ability of noxious stimulation to induce Fos in brain is postnatal and relatively late in development. Increased Fos labeling in the brain induced by the formalin injection was not seen in the fetus or the newborn. Fos expression first was induced in the infant at 3 days of age in the reticular formation and between 3 and 14 days of

age in the other regions examined. This is consistent with the late expression of Fos (PD10–PD14) in the amygdala, hypothalamus, hippocampus, superior colliculus and cortex in similar models^{1, 31}. In contrast Fos labeling is seen in the dorsal horn of the spinal cord as early as 21 days of gestation⁵³ and the fetus and neonate respond behaviorally to a formalin injection in a manner quite similar to that of the adult, although with obvious motor limitations^{26, 52, 53}.

Fos expression does not occur in all circuits that are active. For example, although animals respond with motor movement, ventral horn motor neurons do not express Fos. Thus, the lack of Fos expression in classic pain circuits both in young and adult animals does not imply that they are not activated. However the lack of Fos expression in the fetuses and neonates is not likely due to the immaturity of Fos pathways since Fos can be induced by other stimuli in brain of the rat fetus and newborn^{6, 33}.

Prior spinal cord Fos studies included saline injection as a control for the formalin treatment⁵² and that did not induce Fos expression or produce a prolonged behavioral response. Thus we followed that protocol. Consequently the change in Fos expression in brain is due to the more intense and prolonged signal induced by the formalin compared to the very mild and short-lived response induced by saline. It is possible, however, that the difference between the control and experimental conditions would have been greater had we omitted saline injection in the control group.

The structures within the brain that were labeled in the infant are congruent with those described in the adult and in areas other than those that are thought to be involved in the direction sensation of pain (e.g. VPL of the thalamus). The four structures that showed the most dramatic developmental change in Fos staining were the midline thalamic nuclei, the PVN of the hypothalamus, the PAG, and the ventral lateral reticular area including the lateral reticular nucleus. Because immediate early genes are generally not expressed in structures such as the ventral basal thalamus, are expressed largely bilaterally, [e.g.^{19, 54}], and because the maturation of Fos expression occurs long after the behavioral response directed towards the site of noxious stimulation appears^{32, 52}, it is likely that Fos labeling reflects more than the direct response to noxious stimulation. Given the nature of the sites that express immediate early genes in response to pain, it is possible that Fos labels sites that are part of the autonomic or “defensive or affective” component of pain [see for example, ²⁵]. This would be consistent with the slow maturation of adult-like tachycardiac responses to a formalin injection that first appear between 7 and 14 days of age⁵.

Ventral Lateral Medulla

Only the ventral lateral medulla showed a lateralization of staining; although there was bilateral staining, it was predominantly contralateral to the site of formalin injection. The VLM receives projections from the dorsal horn of the spinal cord, mostly from deeper lamina and especially the cervical and lumbar enlargements. This structure is involved in motor control³⁸ and although its role in nociception is less well documented, electrical stimulation of this region suppresses withdrawal reflexes from noxious stimulation^{10, 12, 35, 36, 41}. At its most rostral aspects, the ventral lateral reticular area is involved in cardiorespiratory regulation^{37, 39}. It is this region that may co-ordinate autonomic responses to pain²⁷, since it has a heavy projection to the intermediolateral cell column of the spinal cord¹⁵.

PAG

The PAG is a complex structure that has been hypothesized to be organized around longitudinal columns⁴. Although the exact location of these columns and the terminology

used to define them differs, there is agreement that there exists a dorsal column above the aqueduct, a dorsal lateral column, lateral column and ventrolateral column. The most reliable induction of Fos expression was in caudal PAG. Here formalin induced more Fos positive cells at both 3 and 14 days of age than at 0 days of age, although formalin and saline injections differed significantly from each other only at 14 days of age.

The PAG in part serves to integrate various physiological and behavioral responses to tissue damage and potential threat. The data presented here provide evidence for distinct developmental differences between the dorsal, lateral and ventral lateral columns in response to the formalin injection. This is consistent with the different pain-related functions served by these columns and their different developmental roles. The dorsal PAG preferentially mediates non-opioid dependent analgesia whereas the ventral PAG mediates an opioid dependent analgesia⁹. Similar differences occur in the infant rat where the effects of injections of glutamate and morphine into the dorsal or ventral PAG have unique effects at different ages, up to 14 days of age⁴⁴.

In the adult the PAG also regulates defensive responses such as freezing. Within the caudal PAG, ventral lateral injections of excitatory amino acids elicit quiescence, freezing and hypotension, whereas injections into the dorsal or lateral PAG are hypertensive and produce flight³. Similar differences are noted developmentally. In the infant, either a kappa opioid agonist or kainate acid injected into the PAG increases heart rate and defensive behaviors at 14 but not at 7 days of age^{13, 14}. Likewise Fos expression following exposure of infant rats to an unfamiliar and potentially infanticidal male rat first induces freezing and Fos expression in the PAG first at 14 days of age⁴⁹. Thus it appears that the PAG has a developmentally continuous role in the regulation of nociception and defensive behavior once developed but that its maturation is relatively late occurring. Moreover, this developmental course is identical whether the activating stimulus is actual tissue damage as in the formalin test or exposure to but not contact with a potential predator.

Thalamus and Hypothalamus

The PVN and medial dorsal thalamic nuclei are thought to integrate limbic and autonomic responses, and to mediate responses to innate threats⁴⁰. For the thalamus, formalin injection did not induce more Fos staining than saline until 14 days. These nuclei express Fos in response to stress²³. The role of the PVN of the hypothalamus is well documented (see²⁴ for a review). No Fos staining was noted in the PVN of the hypothalamus for either treatment until 14 days at which time formalin induced more staining. This is consistent with the late appearance, at 14 days but not 7 days of age, of Fos staining in the PVN of the hypothalamus following exposure to the threat presented by an unfamiliar male rat⁴⁹.

The Development of Defensive Response in Rats

The adult pattern of freezing and flight to threat and injury first appears around 12–14 days of age^{34, 42, 43, 47, 50}, paralleling the developmental course of Fos expression in the structures assessed here. Defensive responses exhibited by an animal depend on the certainty and proximity of the threat. Proximal threats (e.g. smell of the predator) alert the animal to visual, auditory, and olfactory cues in the environment and the animal lowers its body, moves carefully, and reacts vigorously to an unexpected stimulus. Injury results in behavioral guarding and recuperative behaviors. With the perception of actual but distant danger, the animal escapes if possible, or freezes to avoid detection if escape is blocked⁴⁵. These defensive responses are accompanied by opioid analgesia in the adult and infant^{18, 29, 47, 48}. The response of the infant is appropriate to its age and environment but different aspects of the defensive responses have different developmental courses⁴⁶.

Individual behavioral and autonomic defense responses emerge independently during the first three postnatal weeks in the rat. At about 14–16 days of age, the freezing response emerges^{22, 47} in response to a sudden novel or threatening stimulus and is mediated in part by the PAG^{13, 14}. Although infant rodents vocalize when separated from the dam and littermates from an early age, there are no detectable changes in cardiac response to isolation until after the first week. It is only at 14 days of age that tachycardiac responses to handling, isolation or to paw injury first appear²⁰. Infants respond differently to distal and proximal threats: localized immediate pain or isolation results in an increased heart rate that occurs at 14 days of age but not earlier⁵; the tachycardiac response to stimuli associated with shock (condition fear) does not appear until well after the freezing response first occurs⁸; and increased heart rate to white noise is mediated by parasympathetic withdrawal rather than sympathetic arousal until after 30 days of age^{16, 28}.

Pain and injury induce a multitude of responses that include immediate protective behavioral actions and activation of physiological systems adapted to the response to threats. The infant normally lives in a unique and protected environment and many of the adult-like stress responses are immature in the infant [e.g.³⁰]. To understand the mechanisms that mediate the short and long term effects of pain and stress on the neonate, these independent developmental trajectories must be considered.

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Figure 1.

The top panel (1A) shows photomicrographs of the staining in the paraventricular nucleus of the hypothalamus at low and higher magnification. The horizontal bars show the scale. There was no editing of any photomicrograph other than cropping. The bottom panel shows the camera lucida diagrams of representative sections. Each dot represents a single stained cell. There was no staining at 3 days of age and as can be seen, no staining localized to the hypothalamic PVN after saline injection.

Figure 2.

This figure shows the results for the midline thalamic structures (paraventricular nucleus and medial dorsal nuclei). Figure 2A shows quantification of FOS like positive cells from 0 to 14 days of age. The number of FOS like positive cells increased from 0 and 3 to 14 (§ $p < .001$) days of age and the difference between saline and formalin treated pups was significant only at 14 days (* $p < .001$). The error bars for all figures represent one SEM. Figures 2B shows staining at the level of the medial habenula as described in the legend to Figure 1. The camera lucida drawings (2C) are also at the level of the medial habenula.

Figure 3.

This figure shows the results for the PAG. The data were analyzed including the both the rostral to caudal planes and the dorsal, lateral and ventrolateral columns. Staining was greatest in the caudal aspects and in the dorsal and ventrolateral columns. * denotes differences between saline and formalin treated animals and § denotes differences of 14 days compared to younger pups. Figure 3B shows camera lucida drawings of representative sections at 0 and 14 days of age from the caudal PAG. Note the larger number of cells in the *saline* group at 0 days of age ($p=.08$) and in the formalin condition in the dorsal and ventrolateral columns at 14 days of age.

Figure 4.

There were significant increased Fos staining induced by formalin at both 3 and 14 days of age (Panel A). Although the omnibus ANOVA interaction of age by injection was significant ($p < .05$), none of the posthoc comparisons reached significance. 4B shows camera lucida drawing of representative animals at 0 and 14 days of age.

Table. 1

Fos expression in the raphe nuclei

		RPA	ROB	NRM
	<i>D0</i>	0.8±0.3	0.8±0.4	1.3±1.0
<i>Formalin</i>	<i>D03</i>	4.0±1.4	0.8±0.5	3.0±1.8
	<i>D14</i>	10.5±3.5	7.7±2.0	10.5±3.3
	<i>D0</i>	0.3±0.3	0.2±0.2	1.0±0.4
<i>Saline</i>	<i>D03</i>	0.0±0.0	0.0±0.0	0.0±0.0
	<i>D14</i>	4.3±1.1	3.3±0.9	3.3±1.0

Note. Entries are mean number of Fos positive cells per section ± SEM.