Decreased c-fos expression in experimental neonatal hydrocephalus: evidence for reduced neuronal activation

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Although neonatal hydrocephalus often results in residual neurological impairments, little is known about the cellular mechanisms responsible for these deficits. The immediate early gene, fos (c-fos), functions as a "third messenger" to regulate protein synthesis and is a good marker for neuronal activation. To identify functional changes in neurons at the cellular level, the authors quantified fos RNA expression and localized fos protein in the H-Tx rat model of congenital hydrocephalus. Tissue samples from sensorimotor and auditory regions were obtained from hydrocephalic rats and age-matched, normal litter mates at 1, 6, 12, and 21 days of age (four-six animals in each group) and processed for immunohistochemical analysis of fos and Northern blot analysis of RNA. At 12 days of age, hydrocephalic animals exhibited significant decreases in the ratio of fos immunoreactive cells to Nissl-stained neurons from both cortical regions, but no statistical differences were noted in fos expression. At 21 days of age, both the ratio of fos immunoreactive cells to Nissl-stained neurons and fos expression decreased significantly. The number of fos-positive neurons decreased in all cortical layers but was most prominent in layers V through VI. This decrease did not appear to be caused by neuronal death because examination of Nissl-stained sections revealed many viable neurons within the areas where fos immunoreactivity was absent. These results suggest that progressive neonatal hydrocephalus reduces the capacity for neuronal activation in the cerebral cortex, primarily in those neurons that provide corticofugal projections, and that this impairment may begin during relatively early stages of ventriculomegaly.

Key Words * hydrocephalus * neonatal * fos * cerebral cortex * immediate early gene * rat

Hydrocephalus is a common pediatric affliction and residual neurological deficits persist in many treated patients.[4,79] Analysis of the most recent studies suggests that the outcome in premature surviving infants with hydrocephalus is poor, with 78% exhibiting cerebral palsy; 72%, mental deficiencies; and
56%, epilepsy.[23,24] These impairments do not correlate with the thickness of the cortical mantle, except when it is less than 2 cm.[30,79] or to the degree of cerebral cortex reexpansion.[24,60,79] Thus, it is difficult to predict outcome based on routine observations of brain images, especially in patients with slowly progressing or moderate ventriculomegaly.

Several recent attempts have been made to assess neuronal function by evaluating cerebral metabolism. Investigators who in clinical studies used magnetic resonance (MR) spectroscopy and positron emission tomography have indicated that reduced metabolism, anaerobic metabolism, and possibly neuronal damage occur in infants with hydrocephalus and older patients with normal-pressure hydrocephalus.[61,68,74,75] In vivo MR spectroscopy has revealed that cerebral metabolism modestly decreases but not until ventriculomegaly has become severe.[6,12,49,54,73] The use of MR spectroscopy to analyze tissue extracts, which has the advantage of providing absolute values for metabolites, has demonstrated significant reductions of phosphocreatine and adenosine triphosphate during hydrocephalus.[34,35,43] Finally, quantitative analyses using $[^{14}\text{C}]$deoxyglucose have generally confirmed the trend toward reduced metabolism as hydrocephalus becomes more severe.[55,62,71] although Chumas, et al.,[10] have reported no changes in glucose utilization in cortical gray matter. One problem inherent in all these studies of regional cerebral metabolism is that the resolution is restricted to relatively large portions of brain. These techniques cannot detect functional alterations in different populations of cells. To study functional changes in small portions of the cerebral cortex and in individual neurons during the course of hydrocephalus, we chose to examine the immediate early gene, fos (or, c-fos).

Fos and fos-related antigens (FRAs) have been studied extensively because of their sensitivity to a wide range of stimuli. Expression of fos RNA and synthesis of fos protein are both elevated in adult brains within hours after trauma,[20,23,27,40,57,65,66,76] stress,[64,67] or normal stimulation.[19,26,38,39,45] Recently, Riley and Bernstein[63] have found that a protracted decrease in fos expression can occur in the sensorimotor cortex after lesions of the dorsal column nuclei are created. This finding may reflect functional inactivation of cortical neurons that have undergone deafferentation and may be pertinent to the alterations in connectivity that occur during hydrocephalus.[33,47,51,56,72] Thus, the presence of fos can be used as an acceptable marker for neuronal activation to help identify functional deficits that may occur at the cellular level.

The H-Tx rat is a well-established model of nontraumatic hydrocephalus in which the effects of progressive ventriculomegaly can be studied developmentally. In this strain, hydrocephalus caused by aqueductal stenosis develops naturally during the perinatal period.[41] Ventriculomegaly is mild at birth but becomes severe within 2 to 3 weeks, and untreated animals die by 4 to 6 weeks of age.

The present study was designed to assess the functional capacity of neurons in the hydrocephalic brain at various stages of ventriculomegaly. We hypothesized that as hydrocephalus progressed, neuronal function would decline, as reflected by reduced levels of fos. By relating these deficits to other pathophysiological findings, a critical period might be identified that demarcates the point of irreversible change in the hydrocephalic brain.

**MATERIALS AND METHODS**

**Rat Model**

The H-Tx rat, the phenotype of which has been described previously,[41] is an excellent model of
fetal-onset hydrocephalus. Hydrocephalic animals can be identified by a domed head and expanded lateral ventricles at autopsy. The rat colony was maintained under 12-hour light-dark cycles at the University of Florida. Normal control and hydrocephalic animals of both sexes were killed, and the brains were removed and evaluated subjectively for the severity of hydrocephalus. Based on the size of the lateral ventricles and the thickness of the cerebral cortex, these brains were classified into categories of mild, moderate, and severe degrees of hydrocephalus. Normal brains exhibited no ventriculomegaly or cortical thinning. All procedures were performed in accordance with the animal care guidelines of the University of Florida and the Cleveland Clinic Foundation, both of which adhere to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Experimental Design**

Eight groups, each consisting of three to eight H-Tx rats, were examined: four experimental groups of untreated hydrocephalic rats, killed on postnatal Days 1, 6, 12, and 21, and four groups of age-matched, normal litter mates. Northern blot analysis was performed in all groups; routine histological and immunohistochemical analyses were performed only to evaluate 12- and 21-day-old rats because previous Northern blot data indicated no change in fos RNA. Two additional study groups were used for immunohistochemical experiments to establish positive controls for the fos antibody. The first group (three rats) consisted of normal 21-day-old H-Tx rats that received intraperitoneal injections of hypertonic saline to induce fos expression in the paraventricular hypothalamic nucleus.

These animals were killed at 60 to 90 minutes after injection. The second group (one rat) consisted of a normal 3-month-old Sprague-Dawley rat that received unilateral injections of quinolinic acid in the caudate putamen to create a lesion in the target area and produce a global cortical increase in fos-positive neurons in the overlying cerebral cortex.

Tissue samples obtained from normal control and hydrocephalic rats for immunohistochemical staining were collected unilaterally; Nissl staining was performed on tissue from both sides of the brain. Tissue from the occipital (posterior) cortex containing the auditory region was located 3 to 4 mm anterior to the interaural line. Sensorimotor tissue was analyzed in sections taken from the frontal (anterior) cortex at the level of the anterior commissure. For Northern blot analysis, fresh-frozen tissue samples were collected bilaterally from frontal and occipital cortices. The frontal cortex comprised the entire rostral half of both hemispheres and contained most of the sensorimotor region. Tissue designated as occipital cortex included auditory cortex as well as portions of visual cortex and hippocampus.

**Immunohistochemical and Routine Histological Analyses of Fos**

Methods for fos immunohistochemistry were adapted from previous studies. Rats were deeply anesthetized using sodium pentobarbital (Nembutal 40 mg/kg) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). The brain was removed, fixed overnight in 4% paraformaldehyde, and cryoprotected in a 30% sucrose/PBS solution for 24 to 48 hours. Serial coronal sections were cut at a thickness of 60 µm on a freezing microtome and collected from the brain regions described in the previous section.

Free-floating sections were treated with 1% hydrogen peroxide for 10 minutes. After rinsing with PBS, sections were incubated for 30 minutes in 3% normal goat serum followed by incubation overnight at 4°C in a 1:2000 dilution of c-fos antibody rabbit polyclonal antibody. After rinsing three times in PBS, sections were incubated for 30 minutes at room temperature in a 1:500 dilution of biotinylated goat anti-rabbit immunoglobulin G. Sections were again rinsed three times in PBS, incubated for 60 minutes
in avidin-biotin complex by using the ABC Elite Kit and then treated for 5 minutes with 3,3-diaminobenzidine tetrahydrochloride (DAB tablets). Sections were mounted on glass slides, coverslipped with glycerol, and examined using routine light microscopy to evaluate fos-positive staining. Because the c-fos antibody has a high specificity for the nucleus, intense nuclear staining was the criterion used for positive fos labeling. To assess the amount of nonspecific binding attributed to other reagents in the assay, negative control tissue sections were processed as part of each immunohistochemical experiment by omitting the primary antibody.

To analyze the cytoarchitecture of the cerebral cortex and the cytology of cortical neurons and glia, routine Nissl staining was performed on sections adjacent to those processed for fos immunohistochemical analysis.

To localize and quantify fos-positive cells, plots were made from three cortical sections at 180-µm intervals with the aid of a camera lucida attachment mounted on a Leica DMX microscope. On each section, an area of interest was selected that spanned the width of the cortical mantle from ventricular to pial surface and was located at 1-, 3-, and 5-o'clock positions of the neocortex dorsal to the rhinal sulcus. Each region contained intact tissue and was approximately 1 mm wide. Only fos-positive neurons found in a single focal plane near the surface of each section at X 400 magnification were plotted on these drawings. The area of each region was calculated using computer-assisted planimetry, and density was recorded as the number of fos-positive cells per unit area. The microscopist was blinded to the status of each brain.

To determine the density of Nissl-stained neurons, similar regions were selected from three sections adjacent to the fos-positive sections and the area of interest circumscribed by an eyepiece reticule. At X 400 magnification, every Nissl-stained neuron with a nucleolus and a diameter greater than 12 µm was counted throughout cortical layers I to VI. Density was recorded as the number of Nissl-stained neurons per unit area, and this number was used to calculate the ratio of fos cells to Nissl cells.

**Northern Blot Analysis**

Routine Northern blot analysis procedures were performed to quantify fos RNA expression. Nonanesthetized animals were decapitated, the brain excised on ice, and cortical regions were dissected, frozen immediately in liquid nitrogen, and stored at -70°C. Total RNA was extracted using the Trizol method[9] on samples of frozen tissue weighing approximately 50 mg. Prior to gel loading, total RNA was quantified photospectrometrically at 260 nm. For each sample, 20 mg of total RNA was denatured at 65°C, loaded onto a 1% agarose/2.2 M formaldehyde gel, and electrophoresis was performed for 3 hours at 100 V. The ethidium-stained gel was then photographed under ultraviolet light to determine RNA quality and lane loading consistency. After capillary transfer was performed, the nylon membrane was crosslinked and baked for 30 minutes at 80°C and prehybridized overnight at 42°C.

A 1-kb viral **fos** copy DNA, which is a homolog of the eukaryotic **c-fos** copy DNA, was used for hybridization according to standard methods.[11] Using a random prime kit, 50 ng of cDNA (**v-fos**) was labeled and approximately 10^6 cpm/ml of the v-fos probe was used for hybridization. The v-fos probe was hybridized on the membrane for 18 hours at 42°C. The membrane was washed sequentially and exposed to a radiation-sensitive screen at room temperature for 4 to 6 days, depending on the intensity of the signal. The membranes were then stripped of the v-fos probe by using standard procedures and probed again with beta-actin to determine the consistency of lane loading. Because of the possibility that beta-actin levels can change as hydrocephalus progresses, several blots containing experimental and
control tissue were probed a third time with cyclophilin, which is abundant in most eukaryotic cells and is used routinely as a standard to determine lane loading consistency.[14] Because the results obtained using a copy DNA donated by Dr. Wendy Macklin were the same as those obtained using beta-actin, the latter probe was used for all subsequent Northern blot analyses.

Densitometric readings of fos, beta-actin, and cyclophilin expression were determined using the Molecular Dynamics system. Background values were subtracted, and the readings were expressed as a fos:beta-actin ratio.

**Statistical Analyses**

The mean ratios of fos:beta-actin values from the Northern blot analysis were calculated for each group of control and hydrocephalic animals at each age. To compare group mean values in each tissue area at each sacrifice age t-tests were used. Because two areas were compared in each brain, Bonferroni's correction for an overall alpha level of 0.05 was used to minimize the possibility that changes in one area might influence the results obtained in the other area. Thus, individual probability values were considered significant at an alpha level of 0.025.

From the immunohistochemical analysis, the mean densities of fos-positive cells and the ratios of fos-positive to Nissl-stained cells were subjected to several statistical comparisons. The data were tested for normality using the Kolmogorov-Smirnoff test. Parametric data were compared within four groups (controls and untreated hydrocephalic tissue obtained from anterior and posterior cortices) at each age by using a one-way analysis of variance. Nonparametric data were compared using the Kruskal-Wallis nonparametric analysis of variance.

**Sources of Supplies and Equipment**

We obtained the Nembutal from Abbott Laboratories (Abbott Park, IL). Santa Cruz Biotechnology (Santa Cruz, CA) manufactured the rabbit polyclonal antibody. Both the DAB tablets and normal goat serum were obtained from Sigma Chemical Co. (St. Louis, MO), and the beta-actin and copy DNA were acquired from Oncor (Gaithersburg, MD). Vector Labs (Burlingame, CA) manufactures the ABC Elite Kit; Boehringer Mannheim (Indianapolis, IN), the random prime kit; and Molecular Dynamics (Sunnyvale, CA), the radiation-sensitive screen.

**RESULTS**

**Animal Groups**

For immunohistochemical analysis, four hydrocephalic animals were compared with four normal controls at 12 days of age. The body weights of these animals were not significantly different, with hydrocephalic and control rats exhibiting mean weights of 22 ± 1 g (standard error of the mean [SEM]) and 27.2 ± 0.8 g (SEM), respectively. At 21 days of age, eight hydrocephalic animals were compared with five normal controls, with mean body weights of 46.2 ± 2.4 g and 48 ± 1 g, respectively. Tissue samples for the positive controls were obtained from three normal H-Tx rats at 21 days of age (mean body weight 48.1 ± 1.6 g) and one adult Sprague-Dawley rat.

For the RNA blot analysis, the 1- and 6-day-old animal groups were both composed of three hydrocephalic and three normal control animals. Body weights for the 1-day-old animals were not obtained, but at 6 days the mean weights for the hydrocephalic and control animals were 14.3 ± 2.1 g and
15.2 ± 0.2 g, respectively. At 12 days of age, six hydrocephalic animals were compared with four normal controls (mean body weights 21.8 ± 1.0 g and 25.0 ± 0.4 g, respectively). At 21 days of age, four hydrocephalic animals were compared with four normal controls (mean body weights 49.2 ± 2.2 g and 51.0 ± 2.9 g, respectively). No statistically significant differences were noted among the mean body weights of hydrocephalic and control animals at any age.

**Gross Morphological and Histological Examination**

Examination of gross anatomical features demonstrated that the brains obtained from hydrocephalic animals were nearly identical at 1 and 6 days of age. By 12 days, ventriculomegaly was moderate, and by 21 days most hydrocephalic brains were classified as moderate or severe. These observations were similar to those described in detail previously.[42] Histological assessments of cortical mantle thickness and lateral ventricle size confirmed the variability that was observed on gross inspection. For comparison, cortical tissue taken from both anterior and posterior regions of control brains at 21 days of age was approximately 2 to 3 mm thick and included a conspicuous layer of periventricular white matter. The lateral ventricles were consistently small, attaining a slitlike appearance in the posterior cortex.

Mildly hydrocephalic 21-day-old brains exhibited cerebral cortices that were somewhat thinner (approximately 2 mm thick) than normal controls with periventricular white matter slightly compressed. The cortices of moderately hydrocephalic brains obtained from rats at this age were much thinner (1-1.5 mm thick) than those of normal controls, and they were comparable to those described by Harris, et al.[31] as being 30 to 60% thinner. These tissue samples were demonstrated to have more pronounced white matter compression. The most prominent cortical pathological effects were observed in severely hydrocephalic 21-day-old animals. On gross inspection, the posterior cortex appeared translucent, and there were tears and holes in the caudal pole. Histological examination revealed that the posterior cortex was compressed to less than 30% of normal, and substantial white matter thinning was observed. In contrast, the anterior cortex of these same brains was less severely affected and considerably thicker. These features have been described previously in the brains of hydrocephalic H-Tx rats.[31,32,41,42]

In terms of age, more severe ventriculomegaly was generally demonstrated in 21-day-old rats than in 12-day-old hydrocephalic rats. Thus, when both age and cortical region were considered, the brains obtained from 12-day-old rats usually had mild thinning in the anterior cortex and moderate thinning in the posterior cortex. The brains obtained from 21-day-old rats usually had moderate thinning in the anterior cortex and severe thinning in the posterior cortex.

**Cytoarchitecture and Cytology**

Nissl staining of normal 21-day-old H-Tx auditory cortex revealed typical cytological and cytoarchitectural features (Fig. 1A). Neurons were organized into discernible laminae, with somata oriented perpendicular to the pial surface. White matter was intact, and there was no evidence of hyperchromatic cells in the cortical gray matter. Minimal changes were seen in the mildly hydrocephalic brains (not illustrated). In contrast, the auditory cortex obtained from severely hydrocephalic rats contained smaller neurons that were compressed and disoriented to the point that laminar boundaries were obscured. The white matter was extremely thin, and hyperchromatic neurons were present occasionally in layers V and VI. Nevertheless, nearly all of the neurons that populated the severely affected cortex were viable (Fig. 1B). Small darkly stained cells, presumably glia, were more conspicuous in deeper layers of the cortex. The most severe cytological damage was observed in the deepest portions of layer VI, where cells appeared flattened with the soma oriented parallel to the white
The sensorimotor cortex was thicker than auditory cortex in brains obtained from hydrocephalic rats. Thus, the cytoarchitecture of this region was better preserved, with clearly delineated cortical laminae. The primary histopathological features of the sensorimotor cortex were mild compression and increased density of neurons.

**Immunohistochemical Analysis for Fos Protein in Positive Controls**

Analysis of tissue obtained from control animals in which hypertonic saline injections had been made revealed an increase of fos immunoreactivity in neurons of the paraventricular nucleus of the hypothalamus (Fig. 2A) that was absent in noninjected controls. Fos immunoreactivity observed in these cells was predominantly nuclear. In adult quinolinic acid-injected animals, a global increase in the number of fos-positive neurons occurred in the ipsilateral cerebral cortex (Fig. 2B) compared with normal 2-day-old controls (Fig. 2C). This increase was observed throughout all cortical layers, along with enhanced staining intensity.
Fig. 2. Photomicrographs showing the number and intensity of fos-positive neurons in control and hydrocephalic tissue. A: Cells in the paraventricular hypothalamic nucleus obtained from a 21-day-old normal H-Tx strain rat that was injected with hypertonic saline, demonstrating induced nuclear fos immunoreactivity in this positive control. B: Similarly, cells obtained from a quinolinic acid-injected adult Sprague-Dawley rat used as a positive control, also demonstrated induced fos immunoreactivity throughout all cortical layers. C: In contrast to the positive controls, auditory cortex from a 21-day-old H-Tx normal control rat contains fos-positive cells located throughout all cortical layers. Neurons with both dark and light nuclear stain can be observed within the section. D: In mildly hydrocephalic auditory cortex, fos-positive cells can also be found throughout all cortical layers, but their number, especially those with dark nuclear staining, is decreased compared with the cortex obtained from normal control rats. E: In a 21-day-old severely hydrocephalic rat, many labeled neurons are found within a large patch that includes layers II through VI; only lightly stained cells were found in layer V. Areas adjacent to this patch contain relatively few fos-positive neurons. F: In the most severe case of hydrocephalus, fos immunoreactivity is limited to superficial layers II and III; deep layers were shown to exhibit only sporadic light staining. This photomicrograph was taken from the same section as that illustrated in Fig. 3H. Bar = 100 µm.

*Immunohistochemical Analysis for Fos Protein in Auditory and Sensorimotor Cortices*
Tissue obtained from normal 12-day-old rats exhibited light nuclear staining throughout all cortical layers and patchy areas of dark nuclear staining in cortical layers I through IV and VI (not illustrated). At this age, the auditory cortex of hydrocephalic animals contained qualitatively fewer fos-positive neurons (not illustrated). This decrease was most pronounced in layer V and appeared to be related to the severity of ventriculomegaly. Tissue samples obtained from mildly hydrocephalic rats at 12 days of age contained fewer fos-positive neurons primarily in layers V and VI. In moderate and severely hydrocephalic rats, fos-positive neurons were sometimes localized in patches or clusters and were observed primarily in cortical layers II, III, and VI.

At 21 days (Figs. 2D-F and 3), the number of fos-positive neurons decreased slightly in both mildly and moderately severe hydrocephalic cortices compared with age-matched controls. This decrease was observed throughout all cortical layers. Although the results were variable (Fig. 3E-H), more pronounced decreases in fos-positive neurons were demonstrated in the severely affected brains. Occasionally, this reduction occurred concomitantly with the appearance of patches or clusters of fos-positive cells. In one of the most severe cases, only a few fos-positive cells were observed, and these were confined to layers II and III (Fig. 3G and H).
Fig. 3. Nissl-stained photomicrographs (left column) obtained from sections adjacent to fos-immunolabeled sections drawn using a camera lucida (right column) in representative auditory cortex of 21-day-old H-Tx rats. Sections are oriented so that the pial surface is to the right. The Nissl-stained sections are illustrated at a higher magnification (bar = 100 µm) than the camera lucida drawings (bar = 400 µm) and are centered on cortical laminae III through VI. They illustrate the cytological and cytoarchitectural differences that result from variations in the severity of hydrocephalus. The camera lucida drawings depict all cells in a single focal plane that exhibited any degree of fos nuclear staining. In addition, these drawings illustrate the thickness of the cortical mantle. A and B: Cortex of a normal control exhibits typical cytological and cytoarchitectural features, as well as fos immunoreactivity that is evenly distributed throughout all cortical layers but is not present in the white matter (*). C and D: Cortex in mild hydrocephalus exhibits minor cytological changes in superficial and middle cortical layers, where some cells appear to be elongated. The density of fos-positive neurons appears to be slightly decreased throughout all layers of cortex, especially in layers II and III. E and F: Cortex in this case of severe hydrocephalus exhibits more compression of cortical laminae relative to mild hydrocephalus, as evident by an increased cell density. The neurons in layer VI also appear to be smaller. The fos-positive neurons are localized primarily to layers III and VI, with a distinct paucity in layer V. Note the dramatic reduction in cortical mantle thickness relative to control. G and H: The most severe case of hydrocephalus is characterized by smaller, disoriented neurons and more small, dark cells in an extremely thin cortex. In cortical layers IV through VI fos immunoreactivity is absent, whereas only intermittent staining is found in superficial layers. All sections are 60-µm thick.

The fos-positive neurons observed in the sensorimotor cortex of 12-day-old hydrocephalic animals did not differ notably from those demonstrated in the cortex of age-matched controls. The pattern was characterized by a patchy distribution of nuclear-stained neurons in cortical layers II through IV and VI, with light nuclear staining in layer V. At 21 days, hydrocephalic brains exhibited a qualitative decrease in the number of fos-positive neurons in the sensorimotor cortex compared with normal age-matched controls. The distribution of these cells was similar to that seen in the 12-day-old rats in that stained neurons were found in cortical layers II through VI.

**Quantification of Fos Protein by Immunohistochemical Analysis**

The density of fos-positive cells followed two general patterns (Fig. 4). Developmentally, reductions occurred between 12 and 21 days in both cortical regions; these changes were statistically significant in the sensorimotor region of both control and hydrocephalic animals (p = 0.016 and 0.008, respectively). Likewise, fos density was reduced in hydrocephalic animals compared with controls, with the exception of the sensorimotor region at 21 days. Values were decreased 62% in the sensorimotor cortex at 12 days, and 36% and 27% in the auditory cortex at 12 and 21 days, respectively. Nevertheless, none of these reductions was statistically significant.
To compare changes in the densities of fos-positive cells and Nissl-stained neurons within the same population of cells, ratios of these two parameters were calculated, and again, two patterns were observed (Fig. 5). Developmental decreases occurred consistently in all groups and were significant in the sensorimotor cortex at 21 days of age in both control (p = 0.016) and hydrocephalic (p = 0.006) rats. Hydrocephalus was also associated with decreased fos/Nissl ratios, especially in the sensorimotor cortex of rats at 12 days of age (69% reduction; p = 0.023) and in the auditory cortex of those at 21 days of age (73% reduction; p = 0.020).
Fig. 5. Graph depicting the ratios of the number of fos-positive cells to Nissl-stained neurons within the same area of anterior (sensorimotor) and posterior (auditory) cortices obtained from 12- and 21-day-old control (C) and untreated hydrocephalic (H) H-Tx rats. Data are expressed as the mean +/- SEM and are significantly different (p < 0.05 and 0.01, respectively) from the same phenotype at the previous age. The * indicates significance at p less than 0.05 relative to age-matched controls. These results indicate that decreases in fos occur without concomitant decreases in the number of neurons, as visualized in Fig. 3G and H.

**Expression of Fos RNA**

Ethidium bromide staining of total RNA in all gels revealed consistent 28S and 18S bands and, thus, verified that the extractions were of high quality and that lane loading was consistent. For Days 1 and 6, no fos RNA expression was detected in either region of the cerebral cortex in control or hydrocephalic animals (not illustrated). In control animals, expression in both the sensorimotor and auditory cortices was extremely low at 12 days, but expression had increased markedly at 21 days (Fig. 6, Table 1). In hydrocephalic animals, both cortical regions exhibited very slight, statistically insignificant decreases in fos RNA expression relative to controls at 12 days of age. By 21 days, however, a significant 46% decrease in fos RNA was observed in the sensorimotor cortex. Likewise, the auditory cortex exhibited a 55% decrease in fos expression at 21 days. Although this difference attained a probability value of 0.031, it was not statistically significant when the strict limits of the Bonferroni correction were applied.
DISCUSSION

Our results indicate that both the expression of fos RNA and the synthesis of fos protein in neurons were decreased in the cerebral cortex of infantile rats with inherited hydrocephalus. The reduction in the number of fos-immunoreactive cells began at 12 days of age, whereas the decrease in fos expression did not occur until relatively late stages of the disorder at 21 days of age. Grossly, in the more posterior auditory region ventriculomegaly caused more severe thinning than in the anterior sensorimotor cortex. However, fos alterations did not follow this gradient. For example, fos RNA expression exhibited 46% and 55% decreases in the sensorimotor and auditory cortices, respectively. Within the hydrocephalic cortex, a cytoarchitectural pattern appeared in which fos-positive neurons were sparse or absent in layers

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<tr>
<th>Cortical Region</th>
<th>12 Days of Age</th>
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<td></td>
<td>Control</td>
<td>Hydrocephalic</td>
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<td>sensorimotor</td>
<td>0.026 ± 0.031</td>
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<td>auditory</td>
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* Values are expressed as the mean ratios of v-fos to beta-actin density ± standard deviation.
† Significance was set at p < 0.025, as determined using the Bonferroni correction for multiple comparisons.
V and VI and were retained mainly in layers II and III. However, Nissl-stained normal neurons could still be found throughout all laminae of the thin cortical mantle. This observation, coupled with the significant reductions in the ratio of fos-positive cells to Nissl-stained neurons, indicates that cell death is not the main cause of diminished fos expression and cell number.

Fos as a Marker for Neuronal Activation

As an immediate early gene, fos (or c-fos) has been utilized repeatedly as a cellular marker for neuronal activation, as reviewed in several studies.[7,22,39,57,59,66] Nearly all studies have concentrated on short-term changes in fos message and protein production. For example, fos RNA and protein levels have been shown to increase dramatically within a few hours after various forms of traumatic brain injury,[23,65,66] hypoxia-ischemia,[20,27] and neurotoxicity,[40,76] and then to decline within 1 or 2 days. The possibility that this rapid response represents a form of neuronal activation has gained support from the fact that normal stimulation,[2] stress,[64] natural cell death,[25,70] growth factors,[65] and homeostatic manipulation[26,38,39,67] can elicit the same response as trauma.

In contrast to fos increases reflecting heightened neuronal activity, the results of a recent study have supported the possibility that functional deficits in neuronal systems can be detected by decreases in fos expression and that these decreases occur several days after the initial insult.[63] The authors of this study reported decreases in fos RNA expression in the contralateral sensorimotor cortex 3 days after lesions were placed in cervical portions of the dorsal columns. This protracted decrease in fos expression followed the expected increase in fos RNA that occurred within hours of creating the primary lesion in the dorsal column nuclei. These results demonstrate that molecular changes in fos can occur at distant sites in multisynaptic pathways and that these decreases occur later than the "immediate" response typically observed with fos. With regard to our current study, this observation also lends credibility to the concept that decreases in fos can be indicative of functional impairments in neurons.

In addition, it is important to recognize that hydrocephalus represents a chronic, slowly progressing process. Most likely, the dynamic effects of steadily enlarging cerebral ventricles may take days or weeks to cause detectable changes in the function of neurons. Individual insults probably occur in such small increments that their additive effect is not realized for quite some time. Thus, in hydrocephalus, neurons may continue to exhibit baseline activity in response to homeostatic stimulation. However, it appears that at some point either intrinsic cellular response mechanisms become impaired or the afferent conduction pathways, which provide input to these cortical neurons, are disrupted. In either situation a reduction in fos RNA and/or fos protein production could result.

Technical Concerns

Because the positive staining of neurons observed in the present study can be a reflection of baseline neuronal activity, it was important to maintain consistent levels of normal stimulation at the time when the animals were killed. Other than the stimulation involved in handling the animals during sacrifice, neither control nor hydrocephalic rats were subjected to additional stimuli. All rats were killed using similar handling procedures at approximately the same time of day so that diurnal rhythms would not influence the results. Thus, it is unlikely that large variations existed in the amount of baseline stimulation to each animal.

It is now recognized that many FRAs are expressed in the normal brain,[1,3,8,19,58,67] and thus the antibody used may detect these important isoforms as well as authentic fos protein. Our polyclonal
antibody did recognize several isoforms of FRAs in the 33- to 37-kD range on protein blot analysis (data not shown). Thus, it is likely that the immunohistochemical findings also reflect reductions in FRA protein. Chronic increases in the 35- and 37-kD FRAs, derived from the more acute 33-kD deltaFosB form, have been reported following drug treatments,[8] seizure activity,[3] and injury.[58] Because these deltaFosB proteins exhibit DNA-binding activity and exert potent transactivating effects on reporter genes, it is believed that these long-term changes mediate neuronal plasticity. Thus, our findings of reduced fos and FRAs in viable neurons may have important consequences for differentiation and plasticity during a critical developmental period.

It is important to realize that glial cells can express fos RNA and produce fos protein when stimulated.[21,23,37] Because a considerable amount of gliosis has been observed in the periventricular white matter of hydrocephalic brains,[15,16] it is worth noting that fos-positive cells were rarely found in the white matter of our hydrocephalic rats. Thus, it is unlikely that any of the other fos-positive cells found throughout the gray matter were glia.

Finally, because the process of quantifying immunohistochemical preparations is fraught with potential errors and variation, we attempted to standardize the results by expressing the fos data as a ratio of fos-positive cells to Nissl-stained neurons that were counted within the same area of tissue. This approach had the added advantage of demonstrating that cell death appears to play a minor role in the pathogenesis of this disorder.

**Developmental Aspects of Fos Alterations**

In previous studies the authors have demonstrated that the levels of fos expression can vary during postnatal development.[1,25,53] During normal development of the rat brain, fos levels decrease initially but then rise to normal steady-state values by 3 weeks of age.[28] In the present study our results confirm this temporal pattern in normal rats of the H-Tx strain; fos expression was so low in the rats at 1 and 6 days of age that it was undetectable, but it increased four- to fivefold between 12 and 21 days of age. This developmental pattern was similar in both sensorimotor and auditory cortices. In contrast, a reverse pattern of fos immunoreactivity was observed in control animals, such that the density of fos-positive cells was dramatically lower at 21 days. This finding suggests that fos transcription and translation are not coupled during early postnatal development.

In the developing brain, alterations in fos transcription and protein synthesis may have profound and long-lasting effects on neuronal function. As a "third messenger," activated by intracellular signal transduction pathways, fos protein can reenter the nucleus to influence subsequent transcription of many other structural or regulatory proteins, as reviewed in several studies.[22,38,59] Thus, decreased fos in the developing brain may have an adverse effect on cellular responses that must occur normally at a precise time in neuronal maturation.

**Consequences for Cortical Connectivity**

The effects of progressive hydrocephalus on the laminar pattern of fos-positive neurons may correlate with specific functional deficits in the cortical systems.[39] In general, immunohistochemical analysis revealed that layer V neurons were the first cells to be affected by hydrocephalus in both sensorimotor and auditory cortices. In the most severe cases of hydrocephalus, the only remaining fos-positive neurons were located in laminae II to III. This finding suggests that the important projections from cortex to thalamus, neostriatum, superior colliculus, red nucleus, pons, medulla, and spinal cord may be incapable
of functional activation by normal stimuli.

Possible Mechanisms for the Functional Deficits

Although the cause for these alterations in fos expression and cellular protein remains a matter of speculation, several mechanisms are more likely to play important roles. First, neuronal death could contribute to the overall loss of fos-positive cells and the reduced expression of fos RNA, especially because pyknosis and decreased density of neurons have been observed previously in the 30-day-old H-Tx rat,[31,42] as well as in young cats with kaolin-induced hydrocephalus.[29,78] The findings in these reports suggest that neuronal death is most pronounced in the deeper layers of the cerebral cortex, and they may explain, in part, the absence of fos-positive neurons in layers V and VI of the most severely affected brains in our study. Nevertheless, examination of Nissl-stained sections in our study revealed that a considerable number of apparently normal neurons remain in the hydrocephalic cortex and that cortical lamination is relatively preserved until late stages of hydrocephalus. Further support for the preservation of neurons has been reported recently by Del Bigio and Zhang,[18] who found very few apoptotic neurons in the cerebral hemisphere of juvenile rats with kaolin-induced hydrocephalus. The remaining neurons may be compressed and smaller, with appreciable dendritic deterioration,[3,51] but they still appear quite viable. Although it is not known if neurons in early phases of degeneration exhibit decreases in fos, examination of the Nissl-stained tissue suggests that changes observed in fos are most likely not caused by cell death.

Second, it is possible that the functional deficits observed in the present study could be caused by ischemia. The authors of several experimental[13,16,36,44,46,77] and clinical [69] studies have demonstrated that cerebral blood flow is diminished in the hydrocephalic cortex. Although the findings obtained in a few studies have indicated that cerebral blood flow deficits can be reversed by placement of a shunt,[13,68,69] Del Bigio and associates[16] have provided evidence that the pathological effects of ischemia on the cortical white matter persist long after surgical decompression has been performed. Such chronic effects could influence the functional capacity of neurons directly or indirectly.

Third, because many authors have observed axonal degeneration and demyelination in the periventricular white matter of hydrocephalic brains (as reviewed in Del Bigio[15] and McAllister and Chovan [50]), it is conceivable that deafferentation of the cerebral cortex could be responsible for decreased activation of cortical neurons. This possibility gains support from the observations that synaptic density is significantly reduced in severe cases of hydrocephalus.[5,47,52] Thus, it may be that our results, which were obtained from a chronic model of trauma to the periventricular white matter, are similar to the protracted fos decreases that occur after interruption to the dorsal column-medial lemniscus-internal capsule pathway.[63]

Reversibility of Functional Deficits

Finally, it remains to be determined if the functional changes observed in the present study can be reversed. Clinically, ventricular drainage can cause reexpansion of the cortical mantle and improvements in neurological function. The results of similar treatments in experimental animals have indicated that some, but not all, morphological, biochemical, and behavioral features can be restored.[17,50] In terms of function, metabolic studies in which investigators use MR spectroscopy to assess cortical tissue obtained from H-Tx rats have indicated that levels of \( N \)-acetyl aspartate, energy metabolites, and membrane phospholipids do not fully recover after placement of shunts in these hydrocephalic animals at 10 to 11 days of age.[29,30] These indicators of neuronal damage (such as \( N \)-acetyl aspartate) and energy
depletion suggest that functional perturbations have occurred at 12 days of age, which could be reflected in the reductions we have observed in fos immunoreactivity. However, because these MR spectroscopy studies did not include 12-day-old untreated hydrocephalic animals, it is possible that metabolic and other functional deficits occurred immediately after 12 days of age and that they continue into the postshunt period. Continuation of the present studies in hydrocephalic ventricular shunt-treated animals will help evaluate the potential for functional recovery at the cellular level.

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References

7. Chaudhuri A: Neural activity mapping with inducible transcription factors. **Neuroreport** 8:5-9, 1997


hypoxia-ischemia. Mol Brain Res 18:228-238, 1993


32. Harris NG, Jones HC, Williams SC: MR imaging for measurements of ventricles and cerebral cortex in postnatal rats (H-Tx strain) with progressive inherited hydrocephalus. Exp Neurol 118:1-6, 1992


58. Pennypacker KR, Hong JS, McMillian MK: Implications of prolonged expression of Fos-related


75. Waldemar G, Schmidt JF, Delecluse F, et al: High resolution SPECT with [99mTc]-d,l-HMPAO in
normal pressure hydrocephalus before and after shunt operation. *J Neurol Neurosurg Psychiatry* **56**:655-664, 1993


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