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Prenatal Exposure to Ethanol Causes a Delay in the Developmental Expression of Neurofilament Epitopes in Cerebellum

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POLTORAK, M., W. J. FREED AND M. F. CASANOVA. *Prenatal exposure to ethanol causes a delay in the developmental expression of neurofilament epitopes in cerebellum.* PHARMACOL BIOCHEM BEHAV **35**(3) 693–698, 1990.—The expression of nonphosphorylated neurofilaments (nPNFs) and phosphorylated neurofilaments (PNFs) was examined in cerebella during development of C57/Bl/6J mice prenatally exposed to ethanol. The length of nPNF immunoreactive portions of primary and secondary dendrites of Purkinje neurons was reduced during early postnatal developmental stages. This difference disappeared by 2 months of age. These results indicate a delay in the maturation of nPNFs in Purkinje neurons of ethanol-exposed mice. There also appeared to be some underdevelopment of basket cell axons in terms of PNF expression, during early postnatal stages, as compared to normal control litters. These findings may reflect a general delay in neuronal maturation after ethanol exposure. Prenatal exposure to ethanol may, therefore, have profound effects on developmental events occurring during early postnatal life. We could not, however, exclude the possibility that the disturbances in neurofilament expression were due to malnutrition in the alcohol-treated animals.

Alcohol Neurofilament Purkinje cell Cerebellum Fetal alcohol syndrome Development

INTERMEDIATE filaments of cytoskeletal molecules include neurofilaments (NFs). Some epitopes on NFs are phosphorylated, whereas others are nonphosphorylated. The 200 kilodalton molecule is extensively phosphorylated, although all three NF proteins (200, 150 and 70 kD) are phosphorylated to some degree (10). Using monoclonal antibodies against NFs it has been demonstrated that nonphosphorylated neurofilament (nPNF) epitopes are present in neuronal cell bodies as well as some axons and dendrites (26,27). In paraffin-embedded material, normal rodent cerebellar tissue contains nPNFs localized in characteristic patterns. Particularly, nPNF epitopes are present in Purkinje cell bodies and dendrites (26,27). Electron microscopic studies using immunogold labeling with antibodies against nPNFs have demonstrated that NFs are abundant in perikarya of Purkinje cells with a "patchy" pattern of immunostaining, probably representing NFs prior to assembly (12).

NFs are produced in neuronal perikarya and slowly move through the axon, so they are predominantly axonal organelles. Initially, it was suggested that nPNFs present in neuronal cell bodies undergo posttranslational phosphorylation and modification, so that phosphorylated NFs (PNFs) would be present exclusively in axons. It has also been hypothesized that progressive NF phosphorylation mediates axonal transport (24, 26, 27). In paraffin-embedded rodent cerebellum, PNF immunoreactivity is characteristically confined to basket cell axons surrounding the cell bodies of Purkinje neurons, parallel fibers, and fibers of substantia medullaris cerebelli (27). As more antineurofilament antibodies and staining procedures have been tested, it has recently become apparent that some PNFs are also located in cell bodies, whereas others are mainly in axons (13,20). In several pathological conditions affecting the neuronal cytoskeleton, PNF immunoreactivity has been found to be increased in the soma of certain populations of cells (6, 8, 29). PNF immunoreactivity is also observed in normal cell bodies (13, 21, 22). Increased PNF expression in cell somata may, nevertheless, be an indication of neuronal abnormality, particularly in paraffin-embedded material.

Morphological disturbances in the central nervous system after prenatal exposure to ethanol are well known. In developing cerebellum, the loss of Purkinje cells (3,19), alteration of their dendritic arborization (17), loss of granule neurons (3) and delayed maturation of the internal granular layer (11) have been reported. It has been suggested that ethanol may cause delayed neuronal maturation by decreasing thyroid hormone levels (11). Disturbances in expression of nPNFs also occur in hypothyroid rats (14). These data suggest that cytoskeletal abnormalities may occur in neuronal cell populations after prenatal exposure to ethanol. We, therefore, investigated the evolution and distribution of PNFs and nPNFs during early postnatal development of cerebellum exposed in utero to ethanol.

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METHOD

Animals

Pregnant C57/Bl/6J mice were exposed to ethanol vapor for three hours daily in the morning, from seven days of gestation until birth of their litters. The inhalation model has been described in detail elsewhere except that no pyrazole was used in the present study (7). The concentration of blood ethanol inhalation after two hours was between 1.8 and 2.3 mg/ml as determined by a conventional enzymatic assay (Sigma Diagnostics) by removing 0.2 ml blood from four selected mice by cardiac puncture under ketamine (70 mg/kg) and xylazine (6 mg/kg) anesthesia. Pups did not survive well. Approximately one-third of animals prenatally exposed to ethanol died prior to the study. Animals from 14 litters were perfused with Bouin's fixative under chloral hydrate anesthesia, after 3 days (n=4), 7 days (n=8), 10 days (n=6), one month (n=6) and two months (n=5). Control animals were obtained from eight matched litters.

Immunocytochemistry

Animal brains were dehydrated, embedded in paraffin and whole brains were sectioned in the sagittal plane at a thickness of 8 μm. The immunocytochemical staining was performed using the peroxidase antiperoxidase method (27). Briefly, the sections were incubated for 30 min with 3% normal goat serum, then for 18 hours with primary antibody. Monoclonal antibodies against phosphorylated epitopes on neurofilaments (SMI 31, SMI 34; Sternberger-Meyer, Inc.) and against nonphosphorylated epitopes on neurofilaments (SMI 32; Sternberger-Meyer, Inc.) were diluted 1:1000 to 1:2000. The staining sequence was: rabbit anti-mouse IgG (Pel-Freez, Inc.) diluted 1:40 for 30 min, mouse PAP (Pel-Freez, Inc.) diluted 1:100, and finally 0.05% diaminobenzidine tetrachloride (Teknika Inc.) with 0.01% hydrogen peroxide for 8 min. All washes were in 0.05 M Tris buffer, 1.5% sodium chloride and all incubations were carried out at room temperature. Control staining was performed using normal mouse sera.

Morphometric Analysis

The individual who performed the quantitative analysis of nPNF-positive Purkinje cell dendritic arbors was blind to the treatment group. Stained sections were photographed twice in different areas and the resultant (13 × 18 cm) prints were studied with a LOATS (Loats & Assoc., New Munster, MD) computer image analysis system. The resultant magnification of the monitor's image was 1028× with a resolution of 1.53 μ. From each photograph, a field of view was randomly selected and five Purkinje cells having a well-defined nucleus were selected for our study. In cases where five neurons were not available in the initial field of view, the image was shifted to an adjacent field. A maximum of three fields were thus examined. Both the apical dendrites and their secondary divisions were taken from the same cells and traced with a cursor, following the contours of the dendrites. Length was measured manually with a line function in terms of numbers of pixels. Multiplication of the resolution by number of pixels gave the length. A total of 30 measurements were made for each animal. The statistical analysis was performed using a two-way analysis of variance for independent measures followed by Scheffé multiple comparisons. Morphometric analysis of the fine plexuses of basket cells surrounding Purkinje cells was not feasible with available methodology. No morphometric analyses of the intensity degree of nPNF and PNF immunoreactivity was performed, because of the variations in staining intensity using the peroxidase-antiperoxidase method.

RESULTS

Nonphosphorylated Epitopes on Neurofilaments (nPNFs)

In normal 3-day-old-cerebellum, the apical part of Purkinje cell bodies and proximal stumps of dendrites, as well as neuronal perikarya of some deep cerebellar nuclei were stained. In 3-day-old brains of animals prenatally exposed to ethanol, although the Purkinje cells showed some expression of nPNFs, the immunoreactivity was usually limited only to the apical part of the cell bodies. The deep cerebellar nuclei were highly stained (not shown).

On day 7, in normal brain, the apical part of Purkinje cell perikarya and large trunks of the primary and secondary dendrites were labeled. Terminal tertiary branches were not stained. The neurons of cerebellar deep nuclei also expressed nPNF immunoreactivity. The fibers in substantia medullaris cerebelli were slightly positive. In animals prenatally treated with ethanol at day 7, PNF immunoreactivity was largely confined to the apical part of Purkinje cell perikarya and the proximal stumps of apical dendrites. Often secondary branches were only slightly stained and the length of nPNF-positive dendrites was reduced. The soma of deep cerebellar nuclei showed positive nPNF immunoreactivity. The substantia medullaris cerebelli was relatively negative. Generally, the staining seemed less intense than in normal animals (Fig. 1A,B), Although the degree of immunoreactivity was not measured.

At day 10, the antibody against nPNFs stained the cell bodies of Purkinje neurons. The entire dendritic arborization including the fine branches became positive. Neuronal cytoplasma of deep cerebellar nuclei, their proximal axons, and fibers in substantia medullaris cerebelli showed pronounced nPNF immunoreactivity. In brains of animals treated with ethanol, the expression of nPNF was present in the apical part of Purkinje cell perikarya, and the large trunks of primary and secondary dendrites. The visibility of nPNF immunoreactivity in Purkinje cells was lower than normal animals. Fibers of the substantia medullaris cerebelli were slightly positive (Fig. 1B,C).

In 4-week-old normal mouse cerebellum nPNF immunoreactivity was present in the primary, secondary and tertiary dendritic arbor and cell bodies of Purkinje cells. The fibers of substantia medullaris cerebelli were also highly reactive. There were no obvious differences between the immunostaining patterns in normal controls and age-matched alcohol-treated animals (not shown). In normal 8-week-old cerebellum the nPNF labeling was like that of the 4-week-old animals. There were no apparent differences between the alcohol-treated animals and controls (Fig. 1D,E).

The quantitative data are shown in Fig. 2. The data were analysed by two-way analyses of variance for independent measures. For apical dendrites, there were significant main effects of treatment group [alcohol vs. controls: $F(1,442)=25.28$, $p<1.0\times10^{-4}$] and time, $F(3,442)=62.58$, $p<1.0\times10^{-4}$, and a significant interaction, $F(3,442)=3.48$, $p=0.016$. For secondary branches, there were significant main effects of treatment, $F(1,442)=24.30$, $p<1.0\times10^{-4}$, and time, $F(3,442)=73.06$, $p<1.0\times10^{-4}$. The interaction was also significant, $F(3,442)=5.36$, $p<0.002$. Post hoc comparisons (Scheffé) revealed that at seven days postnatally, the lengths of nPNF-positive apical dendrites and secondary branches were significantly shorter ($p<0.05$) in ethanol-treated animals as compared to the controls. At day 10 postnatally, the differences between ethanol-treated and control mice in length of nPNF-positive apical dendrites and secondary branches were still statistically significant ($p<0.05$). In 4-week-old ethanol-treated mice, nPNF-positive apical dendrites were

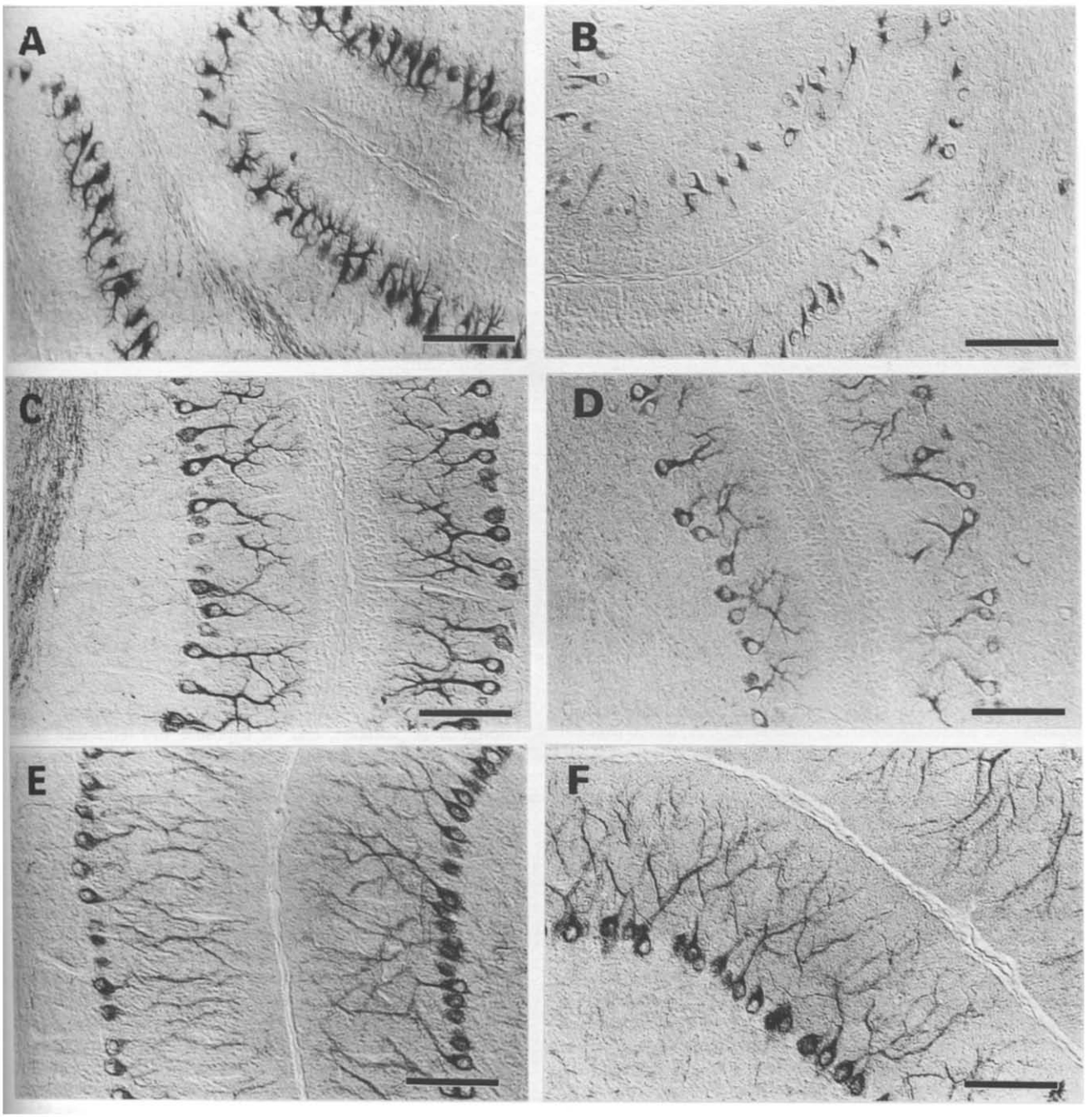

FIG. 1. Immunolabeling with antibody against nonphosphorylated epitopes on neurofilaments in mouse cerebellar cortex. (A, C, E) Control animals. (B, D, F) Animals after prenatal exposure to ethanol. (A, B) Mouse cerebella at postnatal day 7; (C, D) at day 10; (E, F) eight weeks postnatally. Note the reduction in the length with a large variation of immunoreactive neurofilaments, as well as a relative decrease in intensity of immunolabeling in cerebella of mice treated with ethanol at day 7 and 10 postnatally. In eight-week-old animals there is no obvious difference between ethanol-treated and age-matched control animals. Bars: A–F = 100 μm.

shorter than in control mice ($p<0.05$). The length of nPNF-positive secondary branches did not differ from control animals. In 8-week-old ethanol-treated mice the length of both nPNF positive apical dendrites and secondary branches were not significantly different from controls.

Phosphorylated Epitopes on Neurofilaments (PNFs)

In normal 3-day-old cerebellum irregular patches of PNF

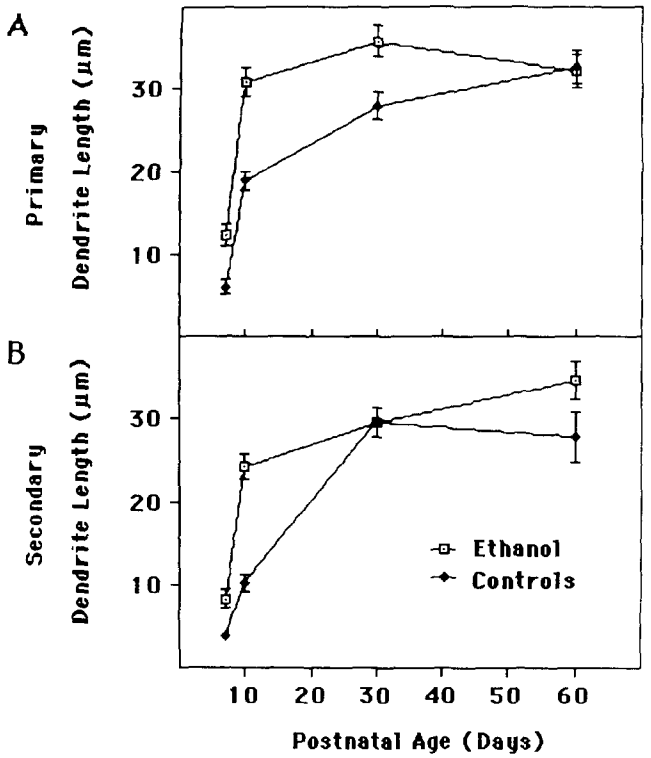

FIG. 2. The length of primary (A) and secondary (B) nonphosphorylated neurofilament (nPNF)-positive dendrites of Purkinje cells in cerebellar cortex of mice prenatally exposed to ethanol and the control group (μm: mean ± standard error) as a function of postnatal age. Data were analyzed by a two-way ANOVA for independent groups. Differences in primary dendrites were statistically significant (Scheffé) at 7, 10, and 30 days and at 7 and 10 days for secondary dendrite length.

immunoreactivity in substantia medullaris cerebelli could be seen. The cortex cerebelli was not stained. The distribution and intensity of PNFs in 3-day-old brains prenatally exposed to ethanol was similar to that of the controls. At 7 days in normal mouse cerebellum, the PNF reactivity was still confined to fibers in substantia medullaris cerebelli, but a greater number of fibers expressed PNFs. The distribution of PNF immunoreactivity was similar in ethanol-treated and control mice.

In normal 10-day-old mouse cerebella the PNF antibodies labeled more structures. The number of substantia medullaris cerebelli PNF immunoreactive fibers increased. PNF immunoreactivity was expressed on numerous regular basket cell neurites. At that time ethanol-treated animals were similar to the controls, although well-developed axons surrounding Purkinje cells were not visible in some areas.

In normal one-month-old cerebellum numerous regular basket cell axons surrounding Purkinje cells were present. PNFs were also present on substantia medullaris cerebelli fibers. In mice treated with ethanol, positive basket cell neurites were seen, although these fibers were shorter than those of the controls. This difference was not quantified (Fig. 3A,B).

In normal two-month-old cerebella PNF immunoreactivity was expressed on numerous regular basket cell neurites and the number of substantia medullaris cerebelli PNF immunoreactive fibers increased. At that time ethanol-treated animals were similar to the controls, although well-developed axons surrounding Purkinje cells were not visible in some areas.

PNFs in Neuronal Perikarya

We also looked for the expression of PNFs in neuronal perikarya in other brain areas. Cell bodies in the nucleus tractus mesencephalici nervi trigemini were positive in both groups of animals (Fig. 3C). Positive-labeled cell bodies of other neurons in brainstem and cortex cerebri were occasionally found in both groups. In the colliculus inferior, neuronal perikarya were often stained in brains of ethanol-treated animals (Fig. 3D).

DISCUSSION

The use of NF immunolabeling with monoclonal antibody SMI 32 to study Purkinje cell dendritic arborization has an advantage over classical neuropathological techniques. It labels extensively and consistently all Purkinje cells with dendritic arbors. Moreover, the staining is limited to Purkinje cells. In classical impregnation methods the labeling is attributed to only a small percentage of Purkinje neurons and the interpretation is complicated by staining of additional nonPurkinje cell fibers from different neuronal sources.

Our data suggest that early postnatal developmental expression of nPNFs in primary and secondary dendrites of Purkinje neurons was delayed in mice prenatally treated with ethanol as compared to age-matched controls. This inhibition was, however, transient and normal Purkinje cell features developed in the adult animals. These observations could reflect a more general delay in neuronal maturation after ethanol intoxication (3,28).

In rodents, the Purkinje cells migrate from their transitory zone to their final position during gestation (2,23). Development of the dendritic arborization, however, takes place in early postnatal life (1,23). In our study, litters were exposed to ethanol during the last two-thirds of gestation, and the exposure was terminated at the birth. It is largely accepted that negative stimuli during brain development disturb primarily the cells and circuits undergoing rapid development at the time of stimulus operation. Nevertheless, the impact of ethanol exposure during gestation on neurofilament metabolism was found to extend to postnatal development. In rodents, NFs are synthesised in early gestation, but as an incomplete set without the 200 kD polypeptide (5). Production of the 200 kD subunit apparently increases slowly during normal neuronal development and postnatal maturation (5,25). Interestingly, the SMI 32 antibody recognizes both the 200 and 150 kD bands (27). It is conceivable that 150 kD subunit synthesis is suppressed by ethanol exposure during embryonic development, and this change results in a delayed appearance of both the 200 and 150 kD polypeptides. It is also possible that the altered appearance of nPNF-positive Purkinje cell dendritic arborization after prenatal ethanol exposure is influenced by disturbances in other cerebellar circuits, such as the development of granule cell axons, which provide major synaptic input to Purkinje cell dendrites.

Other studies have previously reported a delayed effect of ethanol on cerebellar histogenesis (9,11). Similarly, disturbances in migration of granule cells from the germinating layer to their final position in the internal granular layer as a result of prenatal ethanol exposure have been demonstrated (11). This event also occurs entirely postnatally. This change in granule cell migration was not primarily due to malnutrition, but instead was closely correlated with decreased tyroxine level in the pups (11). Recently, it has been suggested that experimental thyroid-deficiency deprives Purkinje cells and basket cell axons of nPNFs and PNFs (14), although these findings have been disputed by others (4). It

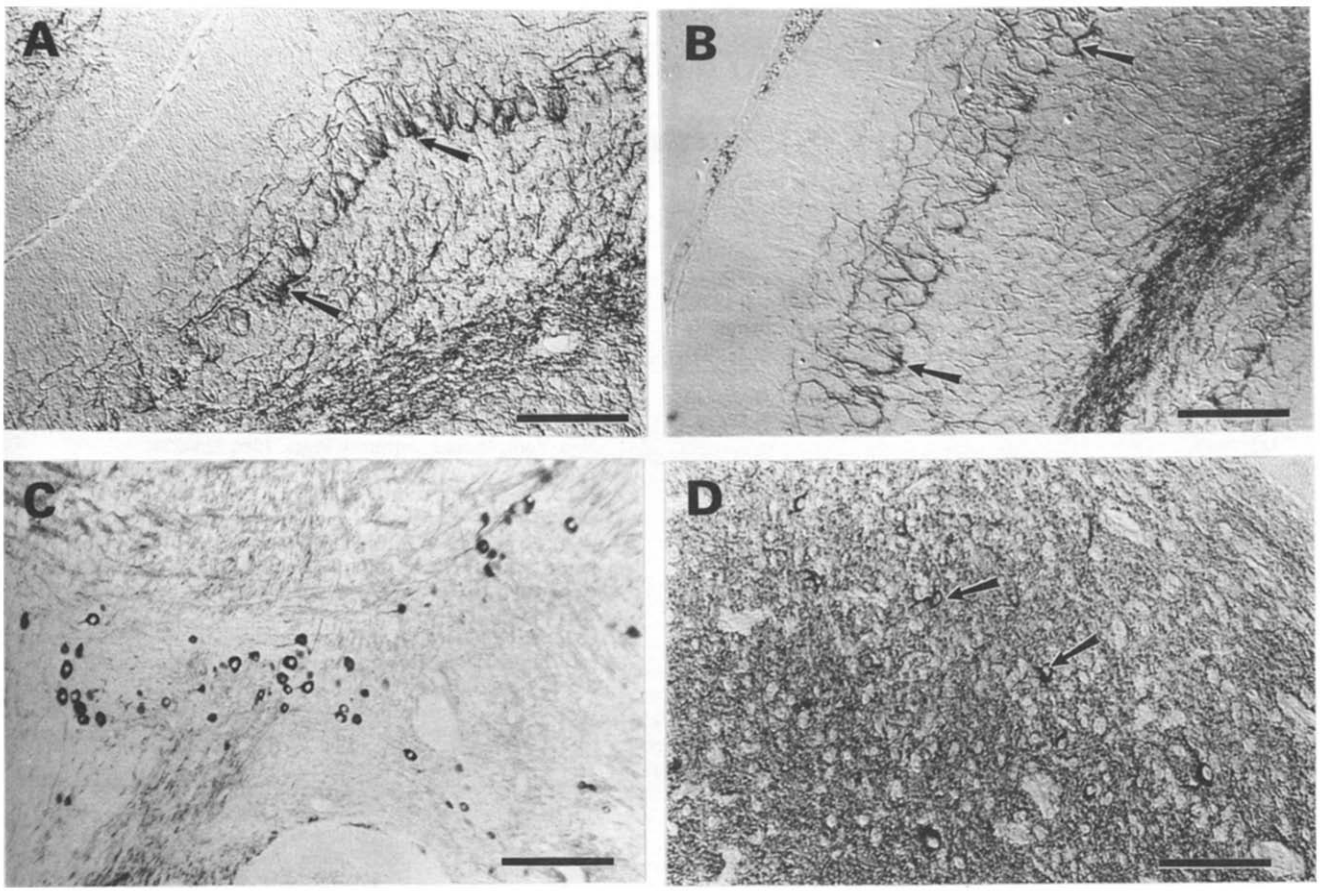

FIG. 3. Immunostaining with antibody against phosphorylated epitopes on neurofilaments (PNF). (A) Cerebellar cortex of two-week-old mice prenatally exposed to ethanol. (B) Control age-matched animals. (C) PNF-immunoreactive somata of neurons in nucleus tractus mesencephalici nervi trigemini. (D) Positive-stained neuronal perikarya of posterior colliculus in brains of ethanol-treated animals. Bars: A, B = 100 μm, C, D = 240 μm.

is possible that the present observations have a similar basis.

Many studies on undernutrition during fetal and early postnatal life have demonstrated extensive changes in the central nervous system [for review (15)]. Cerebellar cortex neurons in particular demonstrate a variety of alterations. In Golgi-impregnated preparations a decrease in overall vertical extent of the dendritic tree has been demonstrated, along with greatly increased diversity in the shape of the dendrites (30). In granule and basket cells a decrease in dendritic length, thinning of dendrites, and a decrease in dendritic spine density have been found in malnourished animals (30). In our study we cannot exclude the possibility that the disturbances in NF expression are secondary to the poor health and probable malnutrition of the ethanol-treated animals. Approximately one-third of litters prenatally exposed to ethanol died prior to study. Therefore, the issue of whether the findings are primary or secondary to malnutrition cannot be answered in the present study.

Although during early postnatal cerebellar development there appeared to be a slight alteration in PNF expression in basket cells, we were not able to quantify this observation by morphometric techniques. It should also be noted that, besides PNFs, SMI 31 and SMI 34 antibodies have recently been shown to recognize phosphorylated epitopes in microtubule associated proteins (16). This cross-reactivity may complicate the interpretation of the results of PNF immunoreactivity.

We have also found numerous PNF-positive perikaryonal constituents in colliculi inferiori in ethanol-treated animals. This could reflect a disturbance in auditory circuits of these animals (18). Occasionally, however, similar features were found in normal control animals, so that PNF-positive perikarya in colliculus inferiori were not an exclusive feature of ethanol-exposed animals.

In conclusion, our results point out the possibility that some stimuli which are damaging to central nervous system during a restricted gestational period, instead of inducing changes closely connected to the time during which the stimulus is active, can be manifest as later more subtle abnormalities. In the present study ethanol was administered during Purkinje cell migration. The delayed perturbations are probably a consequence of putative early alterations, which produce a series of subsequent changes finally resulting in detectable abnormalities. The changes that were detected were only transient, and development of Purkinje cell NFs subsequently advanced normally. Overall brain development proceeds according to a precisely timed sequence with innumerable interdependent component; therefore, it might be conjectured that the delay in Purkinje cell maturation found here results in additional more subtle alterations in related brain circuits that persist for longer periods.

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