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Age-Dependent Susceptibility of CNS Glial Populations *In Situ* to the Antimetabolite 6-Aminonicotinamide

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ABSTRACT

Intraperitoneal injections of the nicotinamide antagonist 6-aminonicotinamide (6-AN) were used to determine if there are regional differences in putative glial energy metabolism between the developing and adult rat CNS. 6-AN shuts down the hexose monophosphate pathway, which is used preferentially by astrocytes and oligodendrocytes. These cells subsequently undergo cytotoxic edema and cell death. Adult rats and pups ranging in age from 7 to 31 d received a single injection of 6-AN and were sacrificed after 24 h. As demonstrated with immunocytochemical staining for the astroglia-specific markers GFAP and S-100β, the 7–9-d-old animals exhibited a uniform appearance with edematous glial cells located throughout the CNS. However, with advancing age, a consistent pattern of progressively decreasing amounts of injured glia, which has not been previously described, occurred in cerebral and cerebellar structures. After 3 wk postnatal, the adult pattern was manifested in which glial degeneration occurred only in specific regions of the spinal cord, cerebellum, medulla, and thalamus, whereas the remainder of the CNS appeared normal. The results suggest the presence of heterogeneous populations of glia whose preferred use of the hexose monophosphate pathway is predicated on both the age of the animal and their location in the CNS.

Index Entries: Astroglia; astrocytes; hexose monophosphate pathway; pentose phosphate pathway; neocortex; hippocampus; cerebellum; spinal cord; postnatal development.

INTRODUCTION

It is now apparent that glial cells, particularly astroglia, are heterogeneous in terms of their ability to modulate the neuronal environment by controlling extracellular K^+, taking up and metabolizing neurotransmitters, and releasing neurotrophic factors (*see* Hansson, 1990; Wilken et al., 1990 for reviews). However, relatively little is known about differences in glial carbohydrate metabolism in various regions of the adult CNS or during development. In the adult rat, only 1–2% of the glucose in the brain is metabolized via the hexose monophosphate pathway (HMP) (Baquer et al., 1977), with the remainder entering the glycolytic pathway and tricarboxylic acid cycle. Although HMP enzymes are distributed in a wide variety of brain regions (Kauffman, 1972), they have been primarily localized in glial cells (Friede et al., 1963; Sotelo, 1967; Sims et al., 1974), blood vessel walls, and in catecholaminergic neuronal groups of adult rats (Sims et al., 1974). The HMP is more active in the early postnatal rat brain, and its contribution to glucose catabolism begins to decline after 3 wk postnatal (Baquer et al., 1977; Hakim et al., 1980; Bilger and Nehlig, 1992). Ontogenetic studies of CNS metabolism have focused primarily on brain tissue as a whole and have not differentiated glial from neuronal elements.

The functional significance of the HMP in the neonate appears to be related to its production of NADPH and ribose 5-phosphate for myelin formation and nucleic acid synthesis, respectively (Hakim et al., 1980; Bilger and Neglig, 1992). The role of the HMP in the adult is less clear; it functions during physiologically stressful events (Hakim and Moss, 1974; Harkonen and Kauffman, 1974; Hakim et al., 1976) and after electrical stimulation (Kimura et al., 1974). The HMP must be essential to the normal function of the adult brain as well, since severe damage occurs in specific CNS regions after its blockade by intraperitoneally injected 6-aminonicotinamide (6-AN) (Herken, 1992).

6-AN is an analog of nicotinamide that interdicts glucose utilization primarily within glial cells following its incorporation into NAD or NADP. The resulting abnormal pyridinenucleotides 6-aminoNAD or 6-aminoNADP cannot participate in hydrogen transfer reactions, and they are strong inhibitors of the HMP enzyme 6-phosphogluconate dehydrogenase, thus blocking this metabolic pathway and causing a large increase in 6-phosphogluconate. High levels of 6-phosphogluconate, in turn, cause a secondary blockade of the glycolytic pathway by inhibiting phosphoglucose isomerase (*see* Herken, 1992). Presumably, the ion pumps, e.g., $Na^+ + K^+$ ATPase, are deprived of sufficient metabolic energy and the cells begin to swell. Since a high concentration of HMP enzymes appear to be located in glial cells glia are thought to be more dependent on the HMP than are most neurons (Sotelo, 1967; Sims et al., 1974). However, the pattern of 6-AN damage in adult rats does not always coincide with regions exhibiting high HMP activity (Friede et al., 1963; Sims et al., 1974). Therefore, the

preferential swelling of particular glial elements may be more indicative of quantitative differences in the need for metabolic energy necessary to maintain cellular function (Baethmann and van Harreveld, 1973). Moderate dosages of 6-AN cause cytotoxic edema in astrocytes and oligodendrocytes, but appear to have no direct affect on most neurons or on vascular endothelial cells (Schneider and Cervos-Navarro, 1974; Krum, 1992, 1994; Krum and Rosenstein, 1993). The glial lesions appear to be owing to direct effects of 6-AN after ip delivery; similar lesions appear locally with 6-AN is injected into the subarachnoid space (Deshpande et al., 1978) or directly into spinal cord white matter (Blakemore, 1975).

6-AN has been used in this laboratory to study the effect of perivascular astroglial injury on the development of the blood-brain barrier (Krum, 1992; Krum and Rosenstein, 1993). These studies led to the observation that glial cells exhibited a regional heterogeneity in their response to this antimetabolite that was dependent on the age of the animal. Thus far, there has been no description of the transition between neonatal glial vulnerability to 6-AN, which occurs throughout the CNS, and the region-specific pattern observed in adult animals. The purpose of this study is to characterize further the developmental pattern of glial susceptibility to 6-AN and to relate it to what is known about glial metabolism and gliogenesis in the maturing brain.

MATERIALS AND METHODS

A total of 27 Wistar rat pups ranging in age from postnatal day (P) 6 to P31 were taken from four separate litters. Each pup received a single ip injection of 6-AN (10 mg/kg body wt) dissolved in Earle's balanced salt solution (EBSS). Four 250-g adult rats were similarly injected. Age-matched experimental control rats (P6, P8, P10, P12, P14, P16, P22, P25, P31, and adult) were injected with EBSS alone. Twenty-four hours after injection, the animals were anesthetized and killed by intracardial perfusion with 4% paraformaldehyde in 0.1*M* cacodylate buffer with 3% sucrose added.

The brain and portions of cervical and lumbar spinal cord were removed from each animal and postfixed overnight in the same perfusion fixative. Portions of the paraformaldehyde-fixed brains and spinal cords were dehydrated, embedded in paraffin, sectioned at 6 μm, and stained with toluidine blue. Some slides were processed for immunocytochemical detection of the astroglial markers GFAP (1:200; Sigma, St. Louis, MO) and S-100 protein (1:1000; Sigma) using the PAP method. Sections were deparaffinized and washed in 0.05*M*, pH 7.6, Tris-buffered saline (TBS). Endogenous peroxidase activity was blocked in all sections incubation in 10% MeOH/0.3% H_2O_2 in TBS buffer. Immunocytochemical controls consisted of deleting either the primary or secondary (linking) antibody from

the reaction series. Selected sections were counterstained with toluidine blue prior to mounting with Permount.

The remainder of the tissue was osmicated (1% OsO_4) for 1–2 h and embedded in Epon resin. One-micron thick and ultrathin sections were cut and examined by light and electron microscopy, respectively.

RESULTS

Both adult and neonatal rats that were exposed to 6-AN became lethargic and exhibited spastic paresis of the hindlimbs by 24 h postinjection. Control animals that received EBSS alone showed no behavioral or histological changes; all immunocytochemical controls were negative.

Neonatal Animals

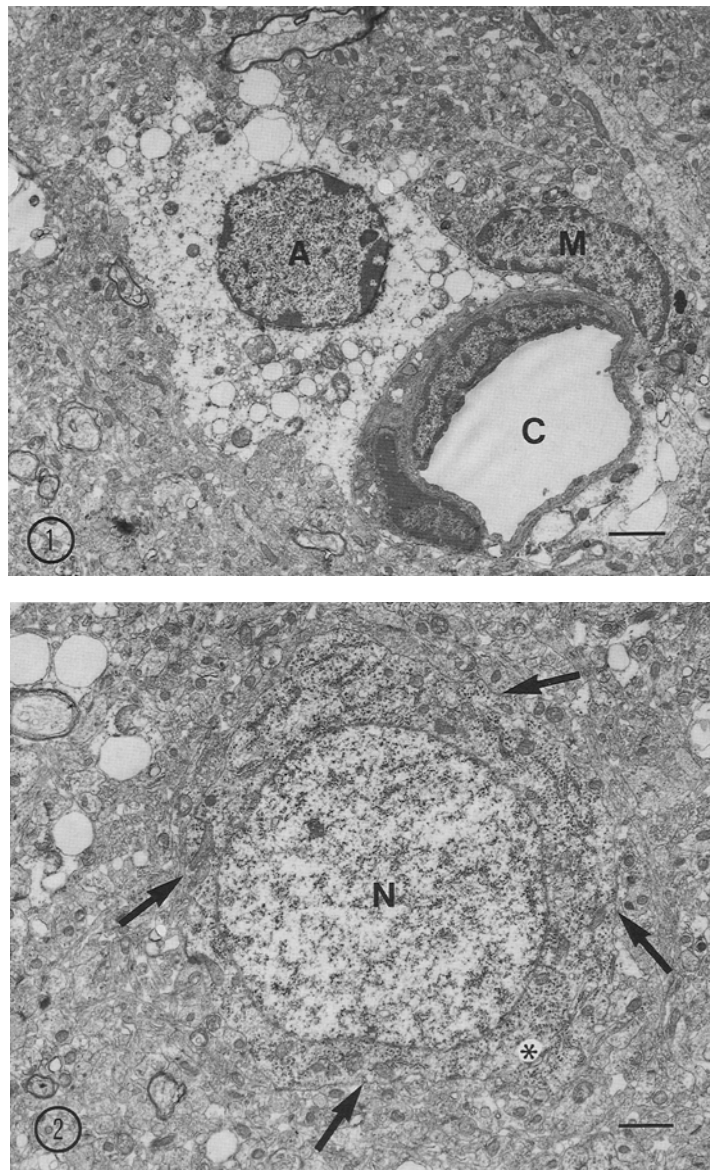
During postnatal maturation, 6-AN caused a reproducible, age-dependent pattern of glial degeneration in the CNS structures observed in this study. Neonates aged P7–P9 exhibited a uniform pattern of edematous glial cells throughout the CNS. However, with advancing age, a consistent pattern of progressively increasing numbers of intact, uninjured glia in cerebral, cerebellar, and hippocampal structures was observed. The adult pattern of regional glial degeneration appeared between P25 and P31.

The spongy appearance of the affected areas was owing primarily to the presence of swollen glial cells. Although both astroglia and oligodendroglia have been reported to be susceptible to 6-AN, the majority of the severely injured cells in this study were immunoreactive for S-100β or GFAP, indicative of their astrocytic lineage (Eng, 1985; Dahl et al., 1986). This may be the result, in part, of the fact that astrocytes are the most numerous cells in the CNS. At the ultrastructural level, it was possible to identify edematous oligodendrocytes as well. Moreover, in approximately 50% of the myelinated axons, the internal mesaxon was swollen.

The glial cell bodies were extremely swollen and appeared vacuolated; frequently, their nuclei were condensed. The cytoplasm was filled with flocculent material: membrane-bound vacuolated structures of various sizes that, in some cases, appeared to be dilated rough endoplasmic reticulum. In some of the affected glia, mitochondria were in various states of degeneration (Fig. 1). Notably, the majority of neurons did not appear to show any morphological alterations (Fig. 2).

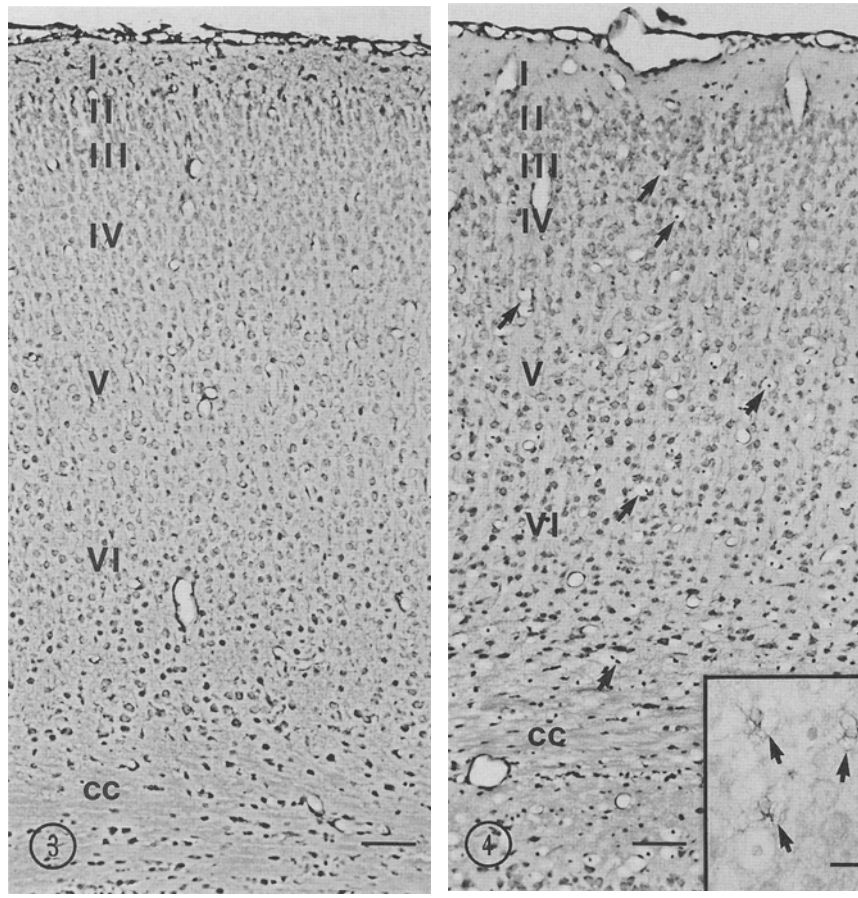
Parietal Neocortex

When compared to control neocortex (Fig. 3), P7–P9 rats exhibited glial swelling in all layers of the parietal cortex except for the molecular layer (I) (Fig. 4). Although GFAP immunoreactivity could not be demonstrated prior to P17 in either experimental or control animals in the paraformaldehyde-fixed paraffin sections used for this study, the majority of the swollen



Figs. 1 and 2. **(Fig. 1)** P20 neocortical perivascular astrocyte (A) is edematous, and contains vacuoles and swollen mitochondria. M, microglial cell; C, capillary lumen. Magnification bar = 1.6 μm. **(Fig. 2)** P20 neocortical interneuron (N) appears morphologically unaffected by 6-AN exposure. Arrows indicate neuronal plasma membrane. *, Artifactual defect in section. Magnification bar = 1.3 μm.

cells in the young neonates were immunoreactive for S-100β; the reaction product demarcated a fibrillar, stellate pattern within the swollen cells (Fig. 4, inset). Edematous cells that were not S-100β-positive were also present and likely represent oligodendrocytes. By P12, glial cells in layer II of the parietal neocortex were no longer affected by 6-AN, although those

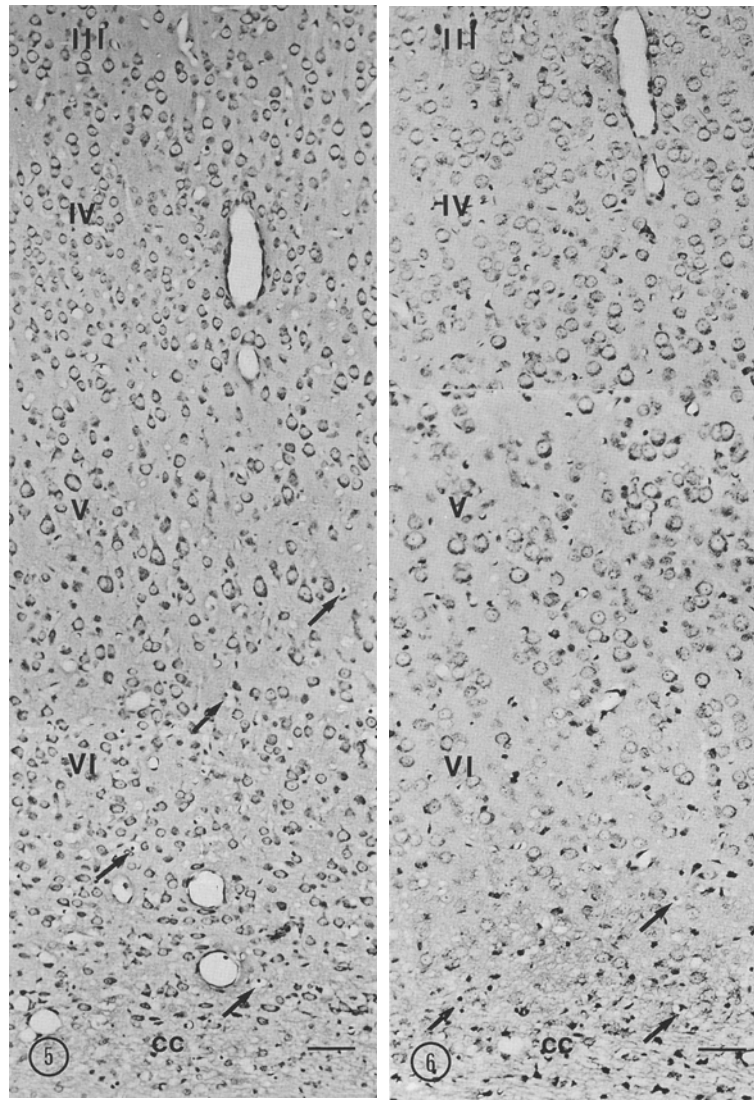


Figs. 3 and 4. **(Fig. 3)** P7 neocortex from control animal shows normal organization of neocortical layers; note the lack of vacuolation within the neuropil. cc, Corpus callosum. Magnification bar = 70 μm. **(Fig. 4)** P7 neocortex contains numerous vacuolated glia, many with condensed nuclei, in layers II–VI (examples depicted by arrows). cc, Corpus callosum. Magnification bar = 70 μm. Inset: Many (but not all) vacuolated neocortical astroglia are immunoreactive for S-100β (arrows). Magnification bar = 12.5 μm.

in the deeper layers were clearly injured. At P17, layers V and VI contained the majority of swollen glia (Fig. 5), and by P20, layer VI was primarily affected, particularly within the region adjacent to the corpus callosum (Fig. 6). At all postnatal ages examined prior to P31, glia within the corpus callosum were swollen. By P31, the glia within neocortex and corpus callosum were no longer affected, and these regions had a normal appearance.

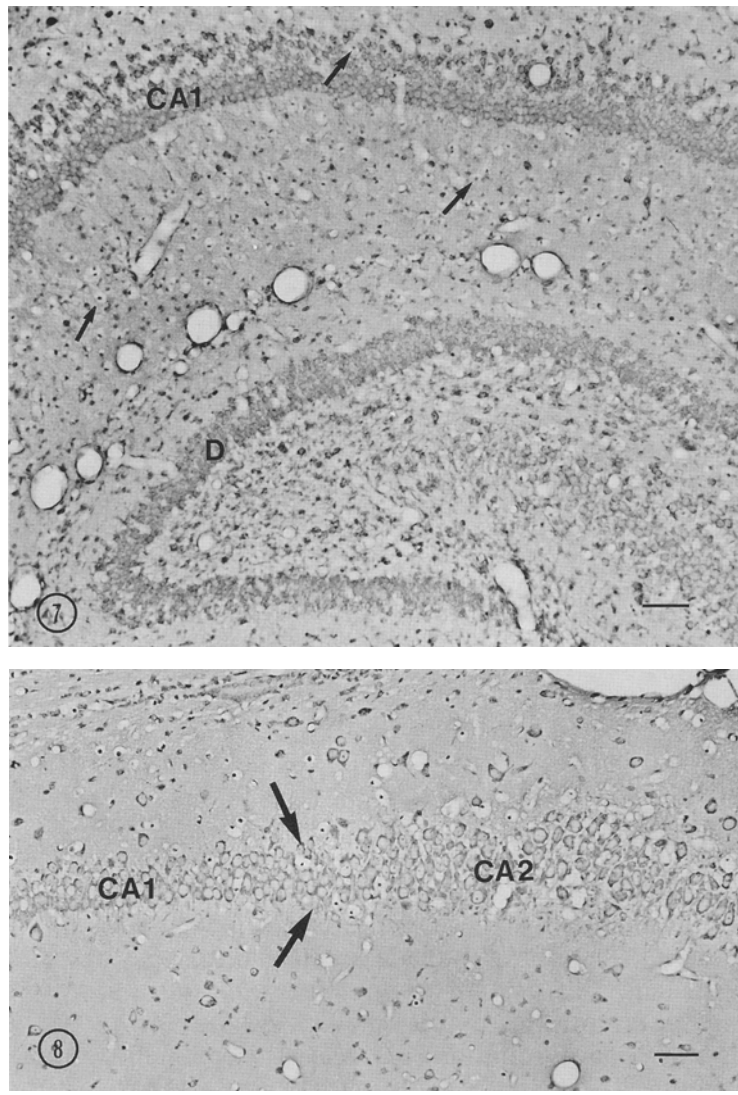
Hippocampus

From P7 to P14, swollen glia were apparent throughout all hippocampal regions (Fig. 7). By P14, the dentate gyrus was no longer affected.



Figs. 5 and 6. **(Fig. 5)** P17 neocortex contains vacuolated glia (examples depicted by arrows) primarily in layers V–VI and in corpus callosum (cc). Magnification bar = 70 μm. **(Fig. 6)** P20. Swollen glia (arrows) predominate in deepest portions of layer VI and in corpus callosum (cc). Magnification bars = 70 μm.

Between the ages of P16 and P25, a sharp demarcation occurred between the intact CA1 subfield and the injured CA2 subfield (Fig. 8). Most, but not all, of the injured glia contained S-100β- and GFAP-positive material; they were concentrated in the pyramidal layer and stratum oriens of the CA2 and CA3 subfields. By P31, the hippocampal formation was completely devoid of spongy degeneration.



Figs. 7 and 8. **(Fig. 7)** 6-AN exposure at P7 results in glial swelling (examples depicted by arrows) throughout the hippocampal formation. D, dentate gyrus. Magnification bars = 70 μm. **(Fig. 8)** P17 hippocampus exhibits a rather abrupt change in numbers of vacuolated glial cells at the transition between CA1 and CA2 (arrows). Magnification bars = 70 μm.

Cerebellum

All layers of the cerebellar foliae were affected at P7 (*see* Krum and Rosenstein, 1993). However, between P12 and P16, only the molecular and Purkinje cell layers, as well as the white matter tracts, were lesioned. The GFAP-positive glia in the internal granule layer had a normal appearance.

By P20, spongiform lesions were present only in the white matter. The dentate nucleus contained injured glia at all postnatal ages, as in adult animals (Fig. 10).

Spinal Cord

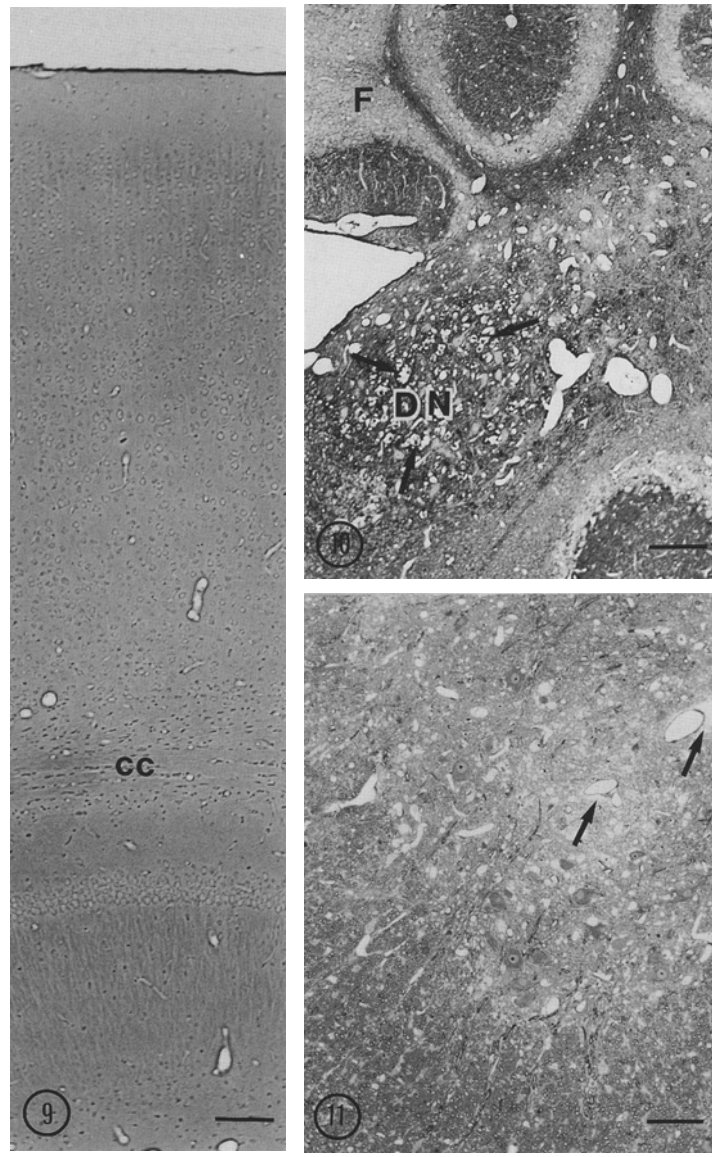
As in adult animals, the spinal cords of 6-AN-exposed neonates contained swollen glial cells throughout the gray matter at all ages examined. Neonates exhibited variable glial injury in the white matter.

Adult Animals

Most regions of the adult rat brain, including the neocortex (Fig. 9), hippocampus (Fig. 9), and cerebellar foliae (Fig. 10), appeared completely unaffected and were indistinguishable from those of control animals. Spongy degeneration occurred only in the following areas: the gray matter of the spinal cord, particularly laminae VI–VII (Fig. 11); brainstem structures, including the medial reticular formation, the nuclei of cranial nerves V, VII, and VIII; the dentate nucleus of cerebellum (*see* Fig. 10); the red nucleus; the zona compacta and pars lateralis of the substantia nigra; the interstitial nucleus of Cajal; the medial habenular nucleus; and the nucleus ventralis of the thalamus. Occasional perivenous hemorrhages were observed in the affected regions. As in neonates, myelin sheaths in the affected areas exhibited swelling of the internal mesaxon.

DISCUSSION

Since 6-AN blocks a key enzyme of the HMP shunt and secondarily inhibits glycolysis, it would be expected that those cells having the greatest dependence on the HMP and/or those having the greatest energy requirement should exhibit cytoplasmic swelling. The results described above suggest that, as development proceeds, the majority of glial cells in the CNS depend less on the HMP shunt, which is in agreement with biochemical analyses of whole brain (Sims et al., 1974; Hakim et al., 1980; Bilger and Nehlig, 1992) and/or they require less energy to maintain normal function. This apparent change in glial metabolism occurs in an age-dependent, region-specific pattern. The reason for the initial widespread glial vulnerability to 6-AN during the early neonatal period could be the result of their dependence on the HMP, although it is conceivable that the CNS could take up more 6-AN during this time. However, studies have shown only a slight increase in nicotinamide uptake by isolated rabbit neonatal choroid plexus in comparison to adult choroid plexus (Spector, 1979). The choroid plexus, not the vasculature, is the primary site of uptake for both niacin and nicotinamide, which are then transferred to the cerebrospinal fluid (Spector and Kelley, 1979); it is presumed that 6-AN follows the same route.



Figs. 9–11. **(Fig. 9)** Glia within adult neocortex and hippocampal formation appear morphologically unaffected by 6-AN exposure. cc, corpus callosum. Magnification bars = 175 μm. **(Fig. 10)** Adult cerebellum, immunostained with S-100, exhibits glial swelling (arrows) within the dentate nucleus (DN), whereas the foliar structures (F) have a normal appearance. Magnification bar = 140 μm. **(Fig. 11)** Spongiform degeneration predominates in motor horn of adult lumbar spinal cord. Note perivascular swelling of astroglial processes (arrows). Large motor neurons, surrounded by swollen glia, appear intact. Magnification bars = 100 μm.

The significance of the adult pattern of glial HMP dependence, as determined by the susceptibility of these cells to 6-AN, is unknown, although Sotelo (1967) proposed that perineuronal neuroglia may present the neurons with NADPH for cellular biosynthesis. Perhaps more significantly, CNS regions that have a high glucose requirement are selectively damaged by a number of metabolic toxins (Cavanagh, 1988). Areas of high glucose consumption, as determined by 2-deoxy-D-[^{14}C]glucose, include some thalamic nuclei, deep cerebellar nuclei, and vestibular nuclei (Schwartz and Sharp, 1978), all of which are affected by 6-AN in the adult rat. It is likely that a higher overall energy requirement in the selectively vulnerable regions in adult rats underlies the pattern of 6-AN-induced gliopathy. The adult pattern of 6-AN damage is not owing to its inaccessibility to unaffected brain regions, since radiolabeled 6-AN is equally distributed throughout the CNS (Meyer-Esdorf et al., 1973).

The neocortex could be used as a model to discern possible reasons for the developmental pattern of glial 6-AN vulnerability, wherein glia in the deeper neocortical layers remain susceptible to 6-AN into the late postnatal period. Both neocortical neurons and astroglia populate the neocortex in a roughly "inside-out" gradient, where the most immature cells are present in the upper neocortical layers (Schmeckel and Rakic, 1979; Ichikawa et al., 1983; Misson et al., 1991; Miller and Robertson, 1993; Levison and Goldman, 1993). The relatively immature glia in the upper neocortical layers are not affected at later postnatal times, although this might be expected since the HMP is more active in younger brain tissue, which has high levels of nucleic acid synthesis and myelin formation (Baquer et al., 1977). Postnatal neocortical synaptogenesis also occurs in an "inside-out" manner (Voigt et al., 1993), so it is also unlikely that any potential astroglial role in synaptogenesis (Meshul et al., 1987) is responsible for the increased energy requirement. The production of permissive substrates by radial glia or astrocytes may be important for facilitation of fiber ingrowth into the neocortex (Barbe and Levitt, 1993), and the manufacture of these substances may necessitate the need for HMP-generated NADPH. However, thalamocortical and callosal axons enter the deep neocortex between P2 and P4 and P2 and P8, respectively (Norris and Kalil, 1991; Götz et al., 1992). Both the spatial pattern and time-course of fiber ingrowth from these areas are inconsistent with the pattern of 6-AN-induced glial injury observed. These particular events, which involve trophic interactions between neurons and glia, either do not coincide with the pattern of glial 6-AN susceptibility or occur too early in postnatal development to be linked with the putative changes in glial metabolism described herein.

An alternative role for the NADPH produced by the glial HMP is the maintenance of glutathione in the reduced form, which is needed to detoxify free radicals (Baquer et al., 1977). Several studies have determined

that reduced glutathione is primarily localized in glial cells in adult brain (Slivka et al., 1987; Philbert et al., 1991), and that cultured astrocytes contain and release much larger amounts of glutathione than do neurons (Raps et al., 1989; Yudkoff et al., 1990). The glutathione efflux from glial cells may protect neurons from locally produced oxidants, and it has been hypothesized that it could serve as a precursor to neurotransmitter glutamate (Yudkoff et al., 1990; Makar et al., 1994). In this regard, it is curious that there is minimal neuronal damage in neonates, even after chronic 6-AN administration (Krum and Rosenstein, 1993). Conceivably, upregulation of glutathione production may be necessary in specific regions during postnatal development, although there is a dearth of information available about regional glutathione localization in neonates. Inhibition of glutathione production in newborn rats is often fatal and produces mitochondrial swelling in CNS structures, lethargy, and intermittent tremors or minor fits (Jain et al., 1991).

Another potential use for HMP-generated NADPH is as a cofactor for nitric oxide synthase (NOS), which has been implicated in cell-cell signalling. Rat cerebral cortical astrocytes grown in vitro express both constitutive and inducible NOS (Simmons and Murphy, 1992; Ma et al., 1994). To date, however, only the inducible form of NOS has been associated with astroglia in vivo, and then only following ischemia (Endoh et al., 1994) or trauma (Wallace and Bisland, 1994). During postnatal development, constitutive NOS is predominantly neuronal, and it is expressed in an "inside-out" gradient in the neocortex, with NOS-positive neurons located in the deeper layers during the first postnatal week; the adult pattern of NOS expression, in layers VIb and II/III, is in place by P14 (Yan et al., 1994). Although it is possible that glial cells possess an isoform of constitutive NOS that is unknown or undetectable at present, it is difficult to connect the putative HMP-dependence of glial populations with NOS expression.

Glial cells, particularly astrocytes, now appear to play an important role in the metabolic support of neurons. There is recent evidence that astrocytes are primarily responsible for the increased glucose uptake observed during neuronal activation (Pellerin and Magistretti, 1994; Peng et al., 1994), and it has been postulated that astroglia provide lactate as an energy substrate for neurons via glutamate-activated glycolysis (Pellerin and Magistretti, 1994). To what extent this pathway is operational in the developing CNS is presently unknown. To clarify the significance of findings of the present study, future research could be directed toward defining patterns of change in regional astrocytic glutamate uptake, glutathione production, and NOS isoform expression during CNS maturation. An examination of the relationship between glial metabolic enzyme activity and specific types or classes of neurons could provide further insight about the supportive function of glia in the developing and mature CNS.

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