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EFFECTS OF NEONATAL TREATMENT WITH 6-AMINONICOTINAMIDE ON BASAL AND ISOPROTERENOL-STIMULATED ORNITHINE DECARBOXYLASE ACTIVITY IN CEREBELLUM OF THE DEVELOPING RAT

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Abstract—6-Aminonicotinamide (6-AN) is a nicotinic acid (vitamin B_3) antagonist which, when administered to immature animals, has a profound influence on brain development. To explore the biochemical mechanisms which underlie these actions, we evaluated effects of 6-aminonicotinamide on ornithine decarboxylase, an enzyme involved in cellular replication and differentiation. The cerebellum of the neonatal rat was chosen for study because it represents a brain region which undergoes major maturational events postnatally. When given to neonatal rats, 6-aminonicotinamide (10 mg/kg, i.p., on days 1, 3, 5 and 7) caused a prompt and persistent inhibition of the enzyme well in advance of adverse effects on tissue weight or on general growth. In addition, the ability of the cerebellum to respond to trophic stimulation by a β-adrenergic agonist, isoproterenol, was attenuated markedly. Assessment of cerebellar morphology indicated an early adverse effect of 6-AN on granule cell division, resulting in eventual disruption of the characteristic laminar structure of this brain region. These data support the view that reduced ornithine decarboxylase activity and impairment of its reactivity to growth stimuli participate in the toxic effects of 6-aminonicotinamide on brain development.

6-Aminonicotinamide (6-AN) is a nicotinic acid (vitamin B_3) antagonist which has profound effects on the central nervous system [1, 2]. As an analogue of nicotinamide, 6-AN is rapidly incorporated into NAD and NADP to form the antimetabolites 6-amino-NAD and 6-amino-NADP, which then act as competitive inhibitors of enzymes which require NAD and NADP as cofactors [3–5]. In brain tissue, there is selectivity of action of 6-AN toward 6-phosphogluconate dehydrogenase [6, 7], which catalyzes the initial step in the pentose phosphate shunt; thus, 6-AN effectively interdicts glucose utilization through shutdown of this pathway [8, 9]. Because the shunt is more vital to neuroglial cells than to neurons [10], 6-AN has been utilized as a selective agent to damage developing glial elements, although neurons are also affected [1, 2, 11].

Prior studies from our laboratory have concentrated on the role of ornithine decarboxylase (ODC, EC 4.1.1.17), the first enzyme in the biosynthetic pathway for polyamines [12, 13], in growth and development of neural tissues [14–18]. ODC and the polyamines participate in replication, differentiation and migration of developing brain cells, and each brain region has its own archetypic developmental profile for ODC activity [19]. The importance of ODC is best exemplified in the cerebellum, a brain region which undergoes major maturational events postnatally in the rat and which therefore exhibits the highest ODC activity during that period [18, 19]; inhibition of cerebellar ODC by enzyme-targeted drugs such as α-difluoromethylornithine results in arrested cellular development as well as general disruption of the characteristic structural features of this region [17, 18]. This may reflect direct interference with ontogeny of neuronal elements. In addition, altered maturation of neuroglia could contribute significantly to the subsequent deficits in neuronal organization and synaptogenesis [11, 18, 20]: cerebellar granule cells migrate along the Bergmann glia and glia contribute to metabolic requirements of developing neurons [11, 20, 21]. Accordingly, the current study was undertaken in order to determine whether 6-AN influences ODC activity during cerebellar development. Trophic responses (including induction of ODC activity) of the immature brain to catecholaminergic input are thought to play an important role in cellular development [22–24], and cerebellar glia are particularly rich in β-receptors, which mediate the ODC increase [24–26]; thus, in addition to assessing the influence of the drug on basal ODC activity, we have also determined its effects on the ability of isoprotenenol to stimulate ODC.

MATERIALS AND METHODS

Timed pregnant Sprague–Dawley rats (Zivic–Miller, Allison Park, PA) were housed individually with a 12-hr light–dark cycle and allowed food and water *ad lib*. Pups from all litters were randomized and redistributed to the nursing mothers at birth (day 0). 6-AN (10 mg/kg, i.p.) was injected on days 1, 3, 5 and 7, whereas control pups received equivalent volumes of isotonic saline (1 μl/g body wt);

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this regimen of 6-AN has been shown to cause disruption of cerebellar development [11]. Experiments were conducted at four ages, 3, 5, 7 and 9 days; animals used on days 3, 5 and 7 were not injected on the day of sacrifice. To assess β-receptor-mediated stimulation of ODC activity, animals were given 5 μl intracisternal injections of either isoproterenol (200 nmoles/g brain wt), a dose shown previously to be fully effective for inducing ODC [24], or isotonic saline vehicle. Pups were killed by decapitation after 4 hr, a time period shown to be optimal for stimulation of cerebellar ODC [24]. Tissues were prepared and assayed for ODC activity as described previously [27], with final concentrations of 1.5 mM dithiothreitol, 50 μM pyridoxal-5′-phosphate and 4.4 μM L-[^{14}C]ornithine. Data are reported as means and standard errors, with significant differences assessed by Student's *t*-test (two-tailed).

For assessment of cerebellar morphology, pups were perfused transcardially with 10% formalin in normal saline. Brains were removed after 1 hr and stored in the fixative for 3–4 days at 4°. Cerebella were then dissected and cut into coronal frozen sections of 40 μm thickness. Sections were mounted on coated slides and stained with cresyl violet.

L-[^{14}C]Ornithine (sp. act. 50.3 mCi/mmole) was obtained from the New England Nuclear Corp. (Boston, MA); 6-aminonicotinamide, *l*-isoproterenol HCl and pyridoxal-5′-phosphate were from the Sigma Chemical Co. (St. Louis, MO); and dithiothreitol was from Bachem Feinchemikalien AG (Liestal, Switzerland).

RESULTS

Administration of 6-AN to neonatal rats produced a prompt inhibition of ODC in the cerebellum (Fig. 1). At 3 days of age, after only a single injection of 6-AN, enzyme activity was reduced by two-thirds; at subsequent stages, a progressive deficit was observed such that by day 9 there was little or no ODC detectable. In addition to causing a loss of basal ODC activity, 6-AN severely compromised the ability of isoproterenol to stimulate the enzyme (Fig. 2). Again, this was clearly evident even after only a single injection.

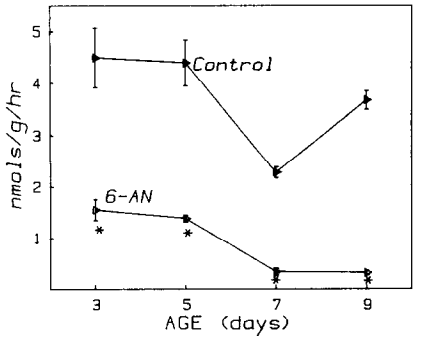

Fig. 1. Effects of 6-aminonicotinamide (6-AN) on basal ODC activity in cerebellum of the developing rat. Data represent mean ± S.E. of nine to twelve rats in each group at each age. Asterisks denote significant differences ($P < 0.05$ or less).

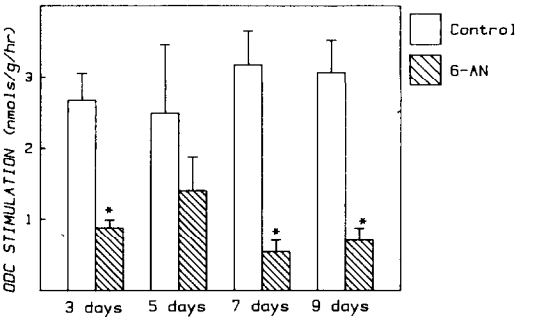

Fig. 2. Effects of 6-aminonicotinamide (6-AN) on isoproterenol-stimulated ODC activity in cerebellum of the developing rat. Animals received isoproterenol (200 nmoles/g brain) intracisternally and ODC was assayed 4 hr later. Data represent mean ± S.E. of nine to twelve rats in each group at each age. Asterisks denote significant differences ($P < 0.05$ or less).

6-AN had no effect on ODC *in vitro* (Table 1), even in concentrations orders of magnitude above those conceivably possible after *in vivo* treatment with drug.

Although 6-AN interfered with both tissue and body growth in the neonates (Table 2), these effects required a more prolonged period of treatment with drug than did the effects on ODC. At 3 days of age, body weights in the experimental group were within 9% of the normal values, and growth was not arrested until the end of the first postnatal week. Cerebellar growth was even less affected, with normal weight values maintained until 7 days of age; even then, "brain-sparing" was evident in that the cerebellum:body weight ratio was increased in the 6-AN group, indicating a greater effect on overall growth than on brain growth. Similarly, drug treatment did result in the signs of generalized toxicity typical of pellagra (skin crusting and exfoliation, listlessness and prolonged latency of the righting reflex); however, these were only barely discernible at day 5 and not fully in evidence until day 7.

Cerebellar morphology was substantially normal in 3-day-old pups given 6-AN (Fig. 3); the Purkinje cells, deep cerebellar nuclei and the overall cytoarchitecture appeared not to have been damaged, but 6-AN clearly reduced the thickness and cell density of the external granule cell layer. At this stage, the internal granule cell layer has not yet developed in either controls or the 6-AN group. In contrast, by 9 days of age (after repeated injections of

Table 1. Lack of effect of 6-aminonicotinamide on ODC activity *in vitro**

Incubation	ODC activity (nmoles/hr/g)
Control	3.34 ± 0.13
6-AN, 10 mg/l	3.62 ± 0.09
6-AN, 100 mg/l	3.39 ± 0.16

* Data represent mean ± S.E. of at least eleven determinations in each group.

Table 2. Effects of 6-aminonicotinamide treatment on body and cerebellar weights in developing rats*

	Body wt (g)		Cerebellar wt (mg)		Cerebellum:Body (%)	
Age (days)	Control	6-AN	Control	6-AN	Control	6-AN
3	11.7 ± 0.3	10.7 ± 0.2†	25 ± 2	25 ± 1	0.22 ± 0.02	0.24 ± 0.01
5	16.0 ± 0.3	13.6 ± 0.2†	49 ± 1	48 ± 1	0.30 ± 0.01	0.37 ± 0.01†
7	19.6 ± 0.3	15.1 ± 0.3†	61 ± 1	56 ± 1†	0.30 ± 0.01	0.37 ± 0.01†
9	22.9 ± 0.4	15.1 ± 0.3†	84 ± 1	63 ± 1†	0.37 ± 0.01	0.43 ± 0.01†

* Data represent mean ± S.E. of values obtained from sixteen to sixty-one rats in each group at each age.

† Denotes a significant difference ($P < 0.05$ or less).

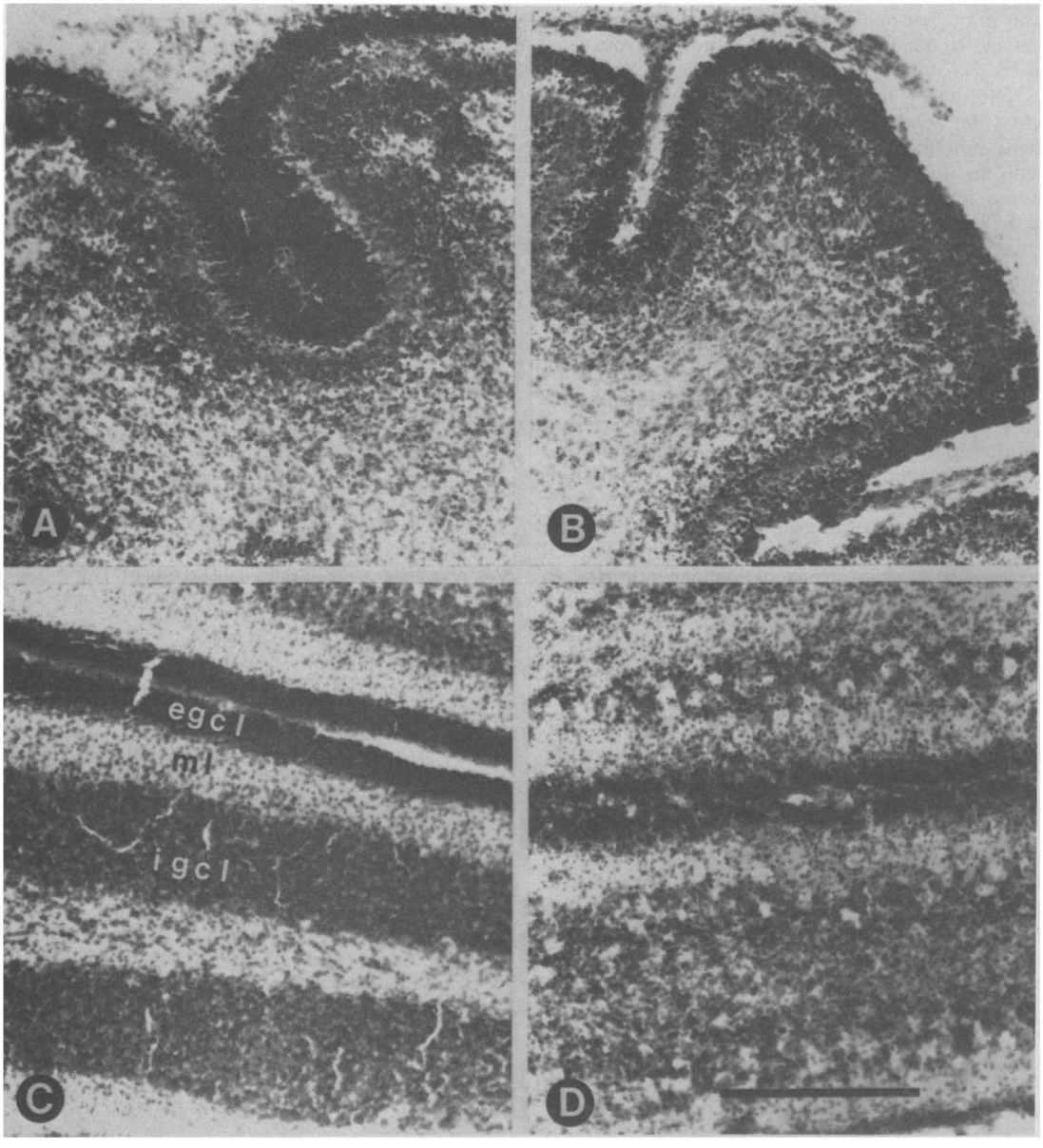

Fig. 3. Effects of 6-aminonicotinamide (6-AN) on cerebellar morphology. A and B are from control and treated 3-day-old rats, respectively; C and D are from control and treated 9-day-old rats. Thinning of the external granule cell layer (egcl) is apparent by 3 days in the 6-AN group. By day 9, few cells remain in the egcl, and the internal granule cell layer (igcl) has failed to develop; the Purkinje cells are still present and the molecular layer (ml) appears to be of normal width. Scale bar = 0.3 mm.

6-AN), there was profound disruption of cerebellar architecture; the external granule cell layer was much thinner than in controls and the internal granule cell layer was virtually nonexistent. Despite these alterations, the molecular layer still appeared to be of normal thickness and cell density.

DISCUSSION

ODC and its biosynthetic products, the polyamines, are obligatory for development of the central nervous system. A wide variety of studies of drugs, hormones and environmental factors indicate that perturbations in the ontogenetic patterns of ODC/polyamines contribute to subsequent alterations in nucleic acid and protein synthesis and thus to abnormal development and function of brain cells [14–18, 28–30]. In particular, prolonged inhibition of ODC during critical periods of nervous system maturation leads to cessation of replication, differentiation and migration of neurons with a consequent disruption of laminar organization and of synapse formation [15–18]. The finding that 6-AN caused marked inhibition of basal ODC in neonatal rat cerebellum supports the view that the developmental neurotoxicity of 6-AN may involve primary effects on polyamine biosynthesis. Indeed, ODC inhibition is detectable well before the general deterioration of growth processes (cessation of tissue and body weight gain, onset of pellagra-like symptoms) and in advance of major disruption of cerebellar cytoarchitecture. The thinning of the external granule cell layer and the failure to form an internal granule cell layer in the 6-AN group are consistent with immediate arrest of granule cell division and a consequent failure of migration of these cells to the internal layer. Similar disruptions of granule cell division and migration have also been reported to occur as a direct consequence of ODC inhibition by α-difluoromethylornithine [18], but the effects of 6-AN appear to be more immediate in onset and of greater intensity.

Of additional interest is the observation that 6-AN severely attentuated the ability of isoproterenol to stimulate ODC; recent studies [24, 31] from our laboratory have shown that ODC in developing brain is induced by adrenergic agonists acting at β-receptors, the type most prominent in glial cells [25]. Since catecholamines are thought to play a prominent role as trophic factors regulating macromolecule synthesis in developing cerebellum [17, 22, 24], this may provide a second route by which 6-AN, through effects on ODC, could influence maturation of the nervous system.

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