Biochemical Pharmacology, Vol. 25, pp. 1415–1419. Pergamon Press, 1976. Printed in Great Britain.

REGIONAL UPTAKE OF NEUROTOXIC AND NONTOXIC AMINO ACIDS *IN VIVO* BY THE INFANT MOUSE BRAIN*

VERNON J. PEREZ,† JOHN W. OLNEY,‡ CONSTANCE F. FROLICHSTEIN, JULIE F. MARTIN and WILLIAM O. CANNON

Neuropsychology Laboratory, Department of Psychiatry, Washington University School of Medicine, St. Louis, Mo. 63110, U.S.A.

(*Received* 8 *March* 1975; *accepted* 15 *August* 1975)

Abstract—The acidic amino acids, glutamate, cysteate and homocysteate, destroy neurons in the arcuate nucleus of the hypothalamus when administered subcutaneously to infant mice, but alanine, a neutral amino acid, does not. The neurotoxicity of glutamate, cysteate and homocysteate was confirmed in this study. It was further demonstrated that glutamate accumulates in the arcuate nucleus after the administration of glutamate but not after cysteate or homocysteate administration. This rules out the possibility that the neurotoxicity of the latter two compounds is mediated by the conversion *in vivo* of these compounds to glutamate. It supports the thesis that the similar neurotoxic manifestations of these compounds stem from the similar molecular structure they share. Alanine accumulated in the arcuate and ventromedial nuclei of the hypothalamus and, to a lesser extent, the medial nucleus of the thalamus but had no neuropathologic effects in any brain region. The failure of alanine to damage arcuate neurons is interpreted as evidence that, because of its dissimilar molecular structure, it either does not react or reacts very differently from the acidic neurotoxic amino acids at some receptor locus on neural membranes.

Several years ago Olney *et al.* demonstrated that glutamate (Glu) induces acute neuronal necrosis in the arcuate nucleus of the hypothalamus (ARH) when administered subcutaneously [1, 2] or orally [3] to infant mice. This observation has been confirmed repeatedly in mice [4–8] and extended to include rats [6, 9], chicks [10, 11], guinea pigs [12] and monkeys [13, 14]. Definitive agreement has not been reached, however, regarding the mechanism(s) of Glu neurotoxicity.

In 1957 Lucas and Newhouse [15] reported acute necrosis of neurons in the inner layers of the infant mouse retina after subcutaneous injections of either Glu or aspartate (Asp). They concluded, tentatively, that Glu was responsible for the neurotoxic activity and that Asp merely mimicked the effect by converting *in vivo* to Glu.

Perez and Olney [16] have shown that Glu, subcutaneously administered, accumulates in the ARH but not in other regions of the infant mouse brain and that the temporal course of Glu accumulation coincides with that of acute degeneration of arcuate neurons. This suggests that Glu influx into the arcuate region is a primary step in the pathogenesis of the ensuing neurotoxic reaction. It has been argued, however, that some metabolite of Glu might also accumulate in the arcuate region over the same time course and that such a metabolite, rather than Glu, could be responsible for the neurotoxic reaction [8].

Olney *et al.* [17] recently tested a large series of compounds and demonstrated that a select few—the exact group of amino acids which Curtis and Watkins [18, 19] have identified in microelectrophoretic experiments as neuroexcitatory amino acids—reproduce the Glu-type lesion in the ARH. These compounds (aspartic, cysteine sulfinic, cysteic, homocysteic acids and certain synthetic congeners of these amino acids), all of which are close structural analogues of Glu, were 1–100 times as potent as Glu in destroying arcuate neurons when given subcutaneously [17]. Members of the group previously shown [19] to be outstandingly potent neuroexcitants (DL-homocysteate and *n*-methyl-DL-aspartate) were found to be the most potent neurotoxins [17]. Olney [20] has suggested the term "excitotoxic amino acids" for this interesting group of neuroactive compounds and has postulated that their excitatory and toxic activities may be linked by a common mechanism, perhaps acting at a common receptor locus on neural membranes.

The present experiments were undertaken to explore further the postulate that excitotoxic amino acids destroy arcuate neurons, not by conversion to Glu but by virtue of molecular structural characteristics they share with Glu. If this postulate is correct, it should not require increased Glu concentrations in ARH for excitotoxic amino acids such as cysteic (Cys) or homocysteic (H-Cys) acids to induce a lesion there. The first portion of this study confirms this assumption. The second portion confirms another assumption, namely, that a nonexcitatory amino acid

*This research was supported by Grants NS-08909, DA-00259 and NS-01956.

†Present address: Department of Biochemistry, Texas Tech. University Medical School, Lubbock, TX 79404, U.S.A.

‡Recipient of Research Career Development Award MH-38894.

such as alanine (Ala), which lacks the structural specificities thought to confer excitotoxic activity, can accumulate in ARH without destroying ARH neurons. Elsewhere [3, 17] it was shown that Ala and various other nonexcitatory amino acids could be administered orally or subcutaneously to infant mice without an ARH lesion resulting [3, 17], but whether such compounds actually entered the ARH region of brain as Glu does was not ascertained. That subcutaneously administered Ala does accumulate in ARH without damaging ARH neurons is demonstrated here.

MATERIALS AND METHODS

Animals. Cox Swiss albino mice (Laboratory Supply Co., Indianapolis, Ind.) were bred in our laboratory and used in all experiments. The room in which they were housed was maintained at 25° and kept on a schedule of 10 hr dark and 14 hr light.

Chemicals. L-Cysteic acid (Cys) and L-α-alanine (Ala) were purchased from Sigma Chemicals (St. Louis, Mo.) and DL-homocysteic acid (H-Cys) from CalBiochem (La Jolla, Calif.). L-Glutamic acid (Glu) was obtained as the monosodium salt widely available under the trade name "Accent."

Treatment. Within 24 hr after birth, each litter was randomly culled to nine pups. At 4 days of age, mice were randomly selected from each litter, weighed on a triple-beam balance, and injected once subcutaneously over the back with an aqueous solution of the sodium salts of Glu, Cys, H-Cys or Ala at neutral pH, using 30 gauge hypodermic needles and 100-μl Hamilton syringes. The dose of Glu, Cys and Ala was 12 m-moles/g of body wt. H-Cys was given at a dose of only 0.3 m-mole/g of body wt because of its greater neurotoxic potency [17]. Concentrations of solutions were such that volumes of 80–100 μl were injected. Injections were spaced 5 min apart and after each the mice were put individually into cubicles of plastic ice-cube trays, the bottoms of which were covered with a thin layer of cedar shavings. The trays were kept in a Thelco model 2 incubator which was modified so that the inner glass door was replaced with one of Plexiglas, the bottom third of which was removed to guarantee free entry and circulation of air in the chamber. The temperature of the chamber was set at 37° at the start of a day's experiment. However, repeated opening and closing of the Plexiglas door to admit or remove mice from the chamber transiently caused as much as a 3° drop in temperature so that a range of 34°–37° was characteristic of a day's experiment.

Preparation of tissues and plasma. At 0 hr (nested controls, no injection), 15 and 30 min, and 1, 2, 3, 6 and 9 hr after injection, mice were killed by decapitation. Untreated controls were also killed by decapitation after being kept in the incubator for 1, 3, 6 or 9 hr. The heads were buried in powdered CO_2, and blood was collected from the neck in heparinized capillary tubes for the preparation of plasma. Heads and plasma were stored at −110°. Samples of the ARH, ventromedial nucleus of the hypothalamus (VMH) and medial nucleus of the thalamus (NMT) were dissected from cryostat sections cut 40 μm thick and lyophilized as described by Lowry [21] and Lowry and Passonneau [22, 23].

For histological purposes, randomly selected animals treated with each of the test agents were sacrificed at 3 hr by perfusion fixation and their brains processed as described elsewhere [2, 3] for microscopic examination of the hypothalamus.

Assay procedures. Glu and Ala were measured in the frozen-dried tissues and plasma according to the method of Young and Lowry [24] as adapted for regional studies on infant mouse brain by Perez and Olney [16]. Corresponding histochemical methods

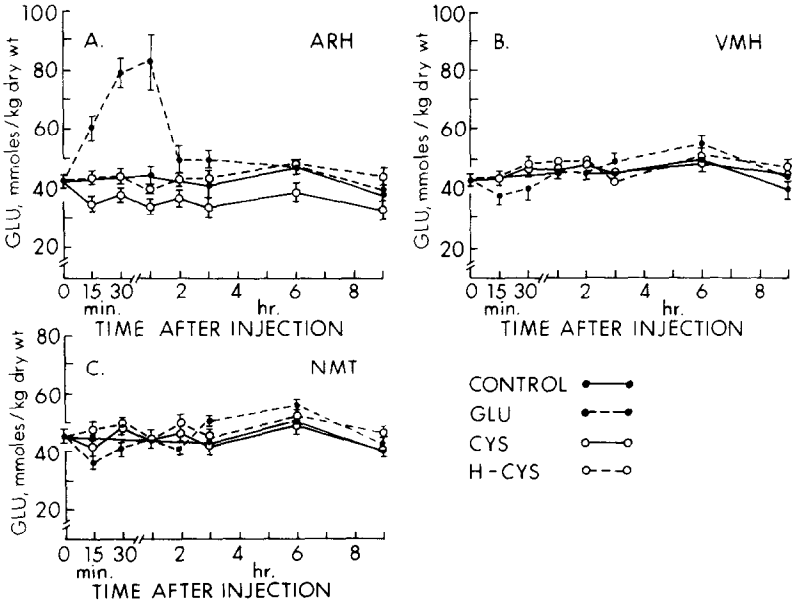

Fig. 1. Glutamate (Glu) content (m-moles/kg dry tissue wt ± S. E. M. in (A) the arcuate (ARH) and (B) ventromedial (VMH) nuclei of the hypothalamus, and (C) the medial nucleus of the thalamus (NMT) in 4-day-old mice injected subcutaneously with Glu, cysteic (Cys) or homocysteic (H-Cys) acids, and in untreated controls (Con). Doses of the amino acids are given in the text.

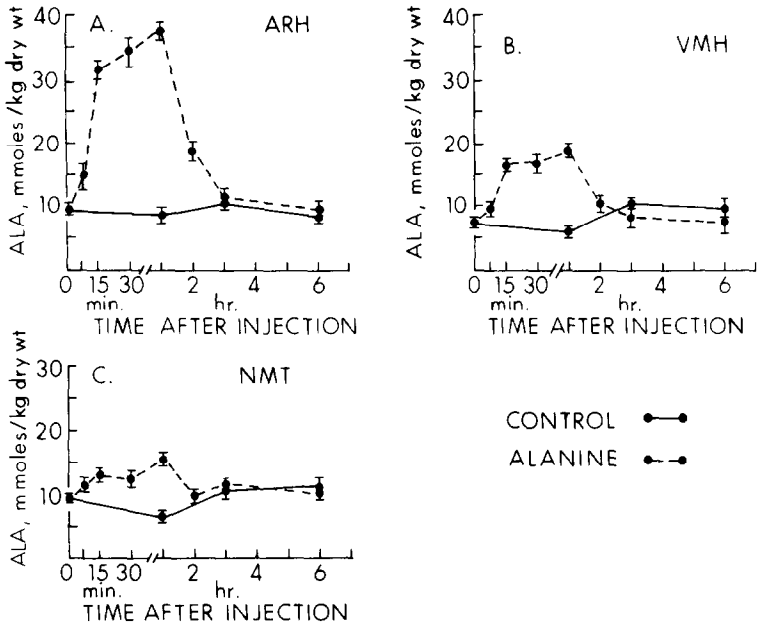

Fig. 2. Alanine (Ala) content (m-moles/kg dry tissue wt ± S. E. M.) in (A) the arcuate (RH) and (B) ventromedial (VMH) nuclei of the hypothalamus, and (C) the medial nucleus of the thalamus (NMT) in 4-day-old mice injected subcutaneously with Ala, 12 m-moles/kg of body wt.

for measuring Cys and H-Cys are not yet available. Standard solutions of Glu, Glu + Cys, and Glu + H-Cys were assayed for Glu as just described to determine if the presence of Cys or H-Cys interfered with the accurate measurement of Glu. Standard curves for Glu were included in every assay for the amino acid in tissue and plasma.

RESULTS

Standard curves for solutions containing Glu, Glu + Cys, and Glu + H-Cys did not differ from each other (arbitrary fluorescence units), indicating that the presence of Cys or H-Cys in the tissues of animals injected with these compounds did not influence the Glu assay.

Levels of Glu measured in the brain of 4-day-old mice injected with Glu, Cys or H-Cys, and in untreated controls are illustrated in Fig. 1, panels A, B and C, for the ARH, VMH and NMT respectively. Consistent with previous findings [16, 25], Glu concentrations rose in the ARH, but not VMH or NMT, of mice injected with Glu, reaching a maximal concentration of 82.9 m-moles/kg dry tissue wt approximately 1 hr after injection (Fig. 1A). Glu concentrations did not rise in ARH (Fig. 1A) or in the other regions studied (Fig. 1, panels B and C) after injections of Cys or H-Cys. It is noteworthy that, at each time after injection of Cys, the amount of Glu measured in the ARH (Fig. 1A) was appreciably less than that measured in untreated controls or in H-Cys mice. This was not the case in the VMH (Fig. 1B) or NMT (Fig. 1C).

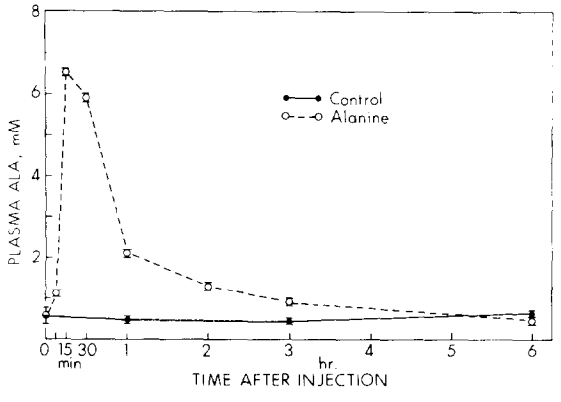

Fig. 3. Plasma alanine (Ala), mM ± S. E. M., in 4-day-old mice injected subcutaneously with Ala, 12 m-moles/kg of body wt.

Concentrations of Ala in the ARH, VMH and NMT at various times after an Ala injection are shown in Fig. 2, panels A, B and C respectively. Like Glu (Fig. 1A), Ala readily entered the ARH, reaching 37.7 m-moles/kg by 1 hr, a 4-fold increase over the 0-hr value of 9.7 m-moles/kg. Unlike the influx of Glu, which was restricted to the ARH (Fig. 1A), substantial increases in Ala—two to three times higher than corresponding control values—were detectable at 1 hr in the VMH (Fig. 2B) and NMT (Fig. 2C). The time course of the Glu (Fig. 1A) and Ala (Fig. 2A) accumulations in ARH after Glu and Ala injections, respectively, were similar with each reaching maximal levels about 1 hr after injection and returning to near control levels 1–2 hr later. The pattern of increase of Ala in plasma (Fig. 3) corresponded well with that for an equimolar dose of Glu reported earlier [16] with the maximal Ala concentration of 6.5 mM being reached 15–30 min after the injection and return to control levels of about 0.5 mM occurring within 6 hr.

Histological examination of the hypothalami of treated infants revealed typical Glu-type lesions in those treated with Glu, Cys (Fig. 4A) or H-Cys but

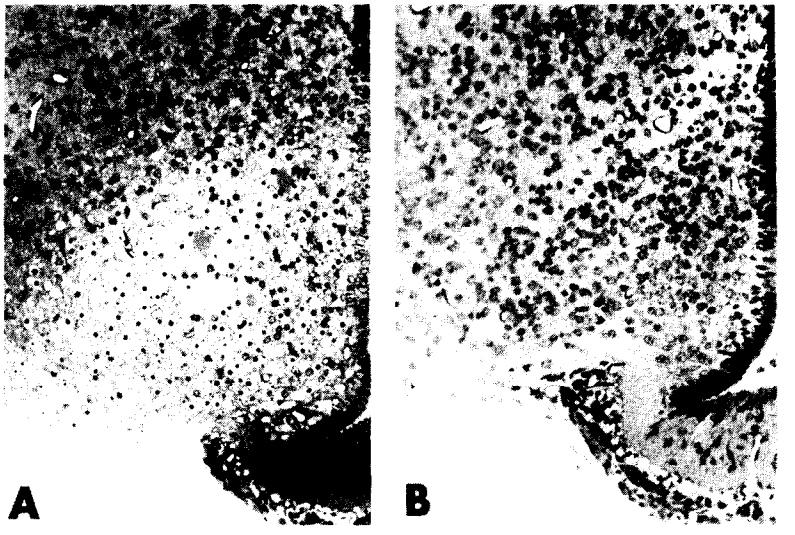

Fig. 4. Light micrographs of the arcuate nucleus of the hypothalamus (ARH) in the 4-day-old mouse injected subcutaneously with 12 m-moles/kg of body wt of (A) cysteic acid (Cys) or (B) alanine (Ala). Magnification (× 180).

not in those treated with Ala (Fig. 4B). The lesion induced by H-Cys was smaller than that induced by Glu and Cys, which suggests that a slightly higher dose of H-Cys, perhaps 0.4 to 0.5 mg/g, might have been suitable for these experiments. However, we have noted in other experiments [17] that the more potent excitotoxic amino acids, including H-Cys, induce lethal seizures in infant mice at very nearly the same dose as is required to induce extensive hypothalamic damage. It was to avoid this problem that we chose the lower dose.

DISCUSSION

Glu, Cys and H-Cys, three excitotoxic amino acids, were administered subcutaneously to infant mice to determine how this would influence Glu concentrations in the ARH over the same time period during which ARH neurons are known to undergo degeneration from the administration of these compounds. The findings rule out the possibility that Cys and H-Cys produce neuronal necrosis in the ARH by increasing the concentrations of Glu to toxic levels in that hypothalamic nucleus. Administration of Cys and H-Cys was not associated with an increase of Glu in the ARH. In fact, Cys administration led to a reduction in ARH concentrations of Glu. Unfortunately, measurement of Cys and H-Cys in the ARH after administration of these compounds was not possible because suitable procedures for microassay of these compounds in minute brain samples remain unavailable. In the absence of such procedures, one can only speculate that Cys and H-Cys did enter the arcuate nucleus like Glu and that each of these compounds is independently capable of interacting with arcuate neurons in such a manner as to trigger degeneration of these neurons. Our findings do not directly rule out the possibility that some unidentified compound, which is a common metabolite of Glu, Cys and H-Cys, is the toxic agent. However, in the absence of evidence that Glu, Cys and H-Cys (and all other excitotoxic amino acids which have thus far been identified [17, 26] including certain heterocyclic analogues of Glu [27]) have a common metabolite which has neurotoxic activity, this hypothesis seems barely tenable. Furthermore, an important observation not taken into account by the common metabolite hypothesis is the potency differences among excitotoxic amino acids, some being at least 100 times more potent than Glu [17, 26, 27]. The more potent compounds need only be given in minute doses to destroy arcuate neurons, and in such doses they would not be expected to generate anywhere near as much of a toxic by-product as a compound such as Glu given in much higher doses.

Ala readily entered the ARH in the 4-day-old mouse after subcutaneous administration with a percentage increase over baseline levels greater than that for Glu, yet Ala produced no toxic reaction in ARH neurons. Thus, the previous observation by Olney *et al.* [3, 17] that Ala, even after such a heavy dose as 3 mg/g, induces no ARH damage cannot be attributed to failure of the amino acid to penetrate ARH. Glu, Cys and H-Cys all have excitatory properties, presumably because their acidic molecules are sufficiently similar to allow them to interact similarly at a common receptor site on neural membranes. We would postulate that the nonacidic Ala molecule is sufficiently dissimilar so that it either interacts differently or not at all at this receptor site and that this is why Ala does not destroy arcuate neurons. We suspect that Glu and related excitotoxic analogues which depolarize neural membranes exert their toxic action by effecting a sustained increase in membrane permeability. Ala has been reported to inhibit neuronal firing when introduced by microelectrophoresis [18], presumably by hyperpolarization mechanisms. Thus, an interesting interpretation suggested by our findings would be that sustained hyperpolarization of neural membranes has less dire toxicological implications for the neuron than sustained depolarization.

An apparent difference between blood brain barriers for Ala and Glu was demonstrated here in that Ala readily entered all brain regions tested but Glu

only entered ARH or, at least, did not accumulate anywhere but ARH. Our data suggests that brain regions other than ARH tend to discriminate rigorously even in early infancy against an accumulation of Glu (and probably its excitotoxic analogues) but not as rigorously against Ala. Oldendorf [28] has presented evidence that, in general, essential amino acids are admitted freely to brain and nonessential amino acids are denied entry. However, both Ala and Glu are nonessential amino acids, but we found that only Glu is rigidly prevented from accumulating in the infant rodent brain, making necessary some other explanation for our data. For example, a protective barrier may be operative in specific relation to such neuroactive compounds as Glu and its excitotoxic analogues because they could seriously interfere with CNS function if allowed free access to brain from blood. Why the arcuate nucleus of the hypothalamus is less well protected than other brain regions from hematogenous exposure to such amino acids remains an enigma.

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