HIGH AFFINITY GLUTAMATE BINDING DURING POSTNATAL DEVELOPMENT OF RAT CEREBELLUM

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1. Introduction

There is strong evidence, although no definite proof, from electrophysiological, morphological or biochemical studies [1–4] that glutamate is a neurotransmitter in the mammalian central nervous system. Granule cells in cerebellum, where they represent by far the predominant neuronal cell population, might use glutamic acid as neurotransmitter [5–9]. Recently a high affinity binding site for [^{3}H]glutamate, associated with cerebellar membranes has been described [10]. Here we describe the property of such a binding site. In addition we report on its accumulation during cerebellar development. In rat cerebellum, granule cells originate from the postnatal multiplication of the cells of the external germinal layer. Parallel fibers, the axons of granule cells, make synapses mainly with the dendritic spines of Purkinje cells. Purkinje cells are present at birth, but their dendrites are formed postnatally simultaneously with granule cell formation and maturation.

2. Experimental

All the chemicals used were of analytical grade. The animals were inbred Wistar albino rats from our laboratory. To avoid discrepancies due to nutritional factors, mothers were fed ad libitum and each litter was reduced to 6 pups immediately after birth. Rats were killed by decapitation, the skull was opened and the cerebellum was removed by sectioning its pedunculi as close as possible to the cerebellum. Cerebella were:

(i) Rinsed with 0.32 M sucrose in 50 mM Tris–HCl buffer (pH 7.1);

(ii) Pooled (the number of cerebella in each pool depended on the age of the animals as is shown in fig.4B);

(iii) Homogenized (in the same sucrose-containing buffer) (1/10, w/v) using a Potter-Elvehjem Teflon–glass homogenizer.

The homogenate was centrifuged at 1000 × *g* for 10 min and the pellet (which contains cell nuclei, debris, some myelin and glomeruli) was washed once in the same sucrose containing buffer. The supernatants were pooled, centrifuged (60 min, 100 000 × *g*) and the resulting pellet, which corresponds to pooled 'crude mitochondrial' and 'microsomal' fractions, was stored at – 50°C. In order to eliminate mainly endogenous glutamate but also sucrose and ions, and before proceeding to the binding assay, the pellet was thawed and osmotically shocked by suspension in 10 mM Tris–HCl buffer (pH 7.1) for 1 h at room temperature, then centrifuged (60 min, 100 000 × *g*) and resuspended (1–2 mg protein/ml) in 50 mM Tris–HCl buffer (pH 7.1). This suspension is defined as 'crude membrane fraction'.

2.1. *Binding assay procedure*

Glutamate binding was determined by adding (in triplicate, and in 1.3 ml centrifuge cellulose acetate tubes (Du Pont de Nemours)) 200 μl of each crude membrane fraction (~0.2–0.3 mg protein in the same buffer as above) to the incubation medium (final vol. 1 ml) containing:

(i) L-[^{3}H]Glutamate (51 Ci/mmol, New England Nuclear) at the same concentration (~10^{-7} M or 5×10^5 dpm/ml);

(ii) Unlabelled L-glutamate and/or non-radioactive presumed inhibitors of glutamate binding, at different concentrations;

(iii) Bovine serum albumin (Sigma) (final conc. 0.1%) to reduce adsorption of the radioactive label to the surface of the tube.

A 30 min incubation at 25°C was chosen on the basis of the time and temperature curves of glutamate binding (fig.2). Incubation was terminated by centrifugation at 4°C for 1 h at 15 000 × *g*. Preliminary experiments in which centrifugation time and speed were assayed had shown that in the hypotonic medium used and after the elimination of most of the sucrose, all glutamate binding membranes sedimented in these conditions. The final pellet was superficially rinsed with ice-cold buffer, dissolved in 1 ml NaOH 1 M at 80°C and counted for ^{3}H. Points were rejected if counts differed by >10%. Protein was measured by the method in [11].

Thin layer chromatography (HPTLC precoated plates; solvent = methyl acetate 45/isopropanol 30/water 20) of extracts of the 'crude membrane fraction' after 30 min incubation shows that no radioactivity was detectable outside the glutamate spot and thus that L-[^{3}H]glutamate was not significantly metabolised during the incubation (results not shown).

3. Results and discussion

3.1. *Crude membrane fraction*

As a first step for the quantitation of glutamate binding sites in the cerebellum, we preferred to use a membrane fraction which included all neuronal membranes rather than a more purified membrane fraction prepared from a 'nerve-ending subcellular fraction' since:

(i) The yield of a purified nerve ending membrane fraction is not quantitative;

(ii) Post-synaptic membranes do not always remain attached to the terminal buttons;

(iii) Nerve terminals in the developing central nervous system change in size and possibly in density during development. Moreover cerebellar neuronal plasma membranes, including those of nerve endings, can be prepared by simple osmotic shock-centrifugation of the pooled 'crude mitochondrial' and 'microsomal' fractions. Evidently this crude membrane fraction contains also glial cells plasma membrane, intracellular membranes, mitochondria and other organelles. Most of these membranes should not interfere with glutamate binding to neuronal plasma membrane whereas mitochondria could. However, in the conditions of incubation used glutamate appears not to be metabolised. Plasma membranes of the multisynaptic complexes called glomeruli, of some nerve endings, and of some myelinated axons are not present in this fraction since they sediment in the 'nuclear fraction'. The nuclear fraction was discarded to avoid problems due to the gelification of DNA during incubation. A study on glutamate binding to the membranes of the nuclear fractions, isolated by sucrose gradient centrifugation, is now in progress.

3.2. *Properties of glutamate binding*

As shown in fig.1, the binding of L-[^{3}H]glutamate to the 'crude membrane fraction' of adult rat cerebellum has two components: one is not saturable, and is considered as 'non-specific' binding; the other, determined by the method in [12], reaches a plateau at ~0.6 μM glutamate and is defined as 'specific binding'. Figure 3 shows the analysis (Scatchard plot and, in the insert, Hill plot) of 'specific' binding in a typical experiment. The dissociation constant (K_d) of specific 'binding' was 0.22 ± 0.13 μM. This is in good agreement with the data in [10,13,14]. However the density of sites (10.1 ± 2.2 pmol/mg protein) is much lower than the value found in [10]. The Hill number (n_H) was calculated and was statistically not different

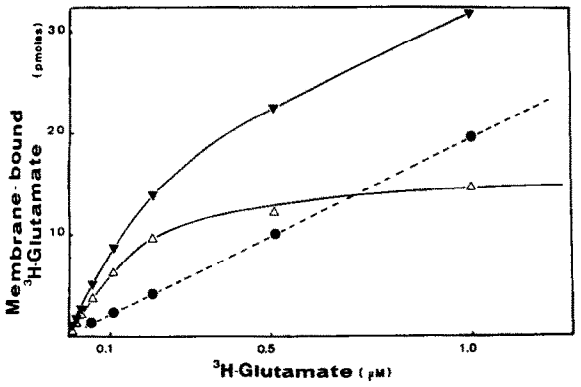

Fig.1. L-[^{3}H]Glutamate binding: Typical saturation curve obtained with a 'crude membrane fraction' prepared from adult rat cerebella. 'Specific binding' was calculated according to [4] as follows: non-specific binding (–•–) was extrapolated from the slope of the curve of total binding (—▼—) at high concentration. Specific binding (—△—) was obtained by subtracting, at each point, the calculated non-specific binding (–•–) from the total binding measured.

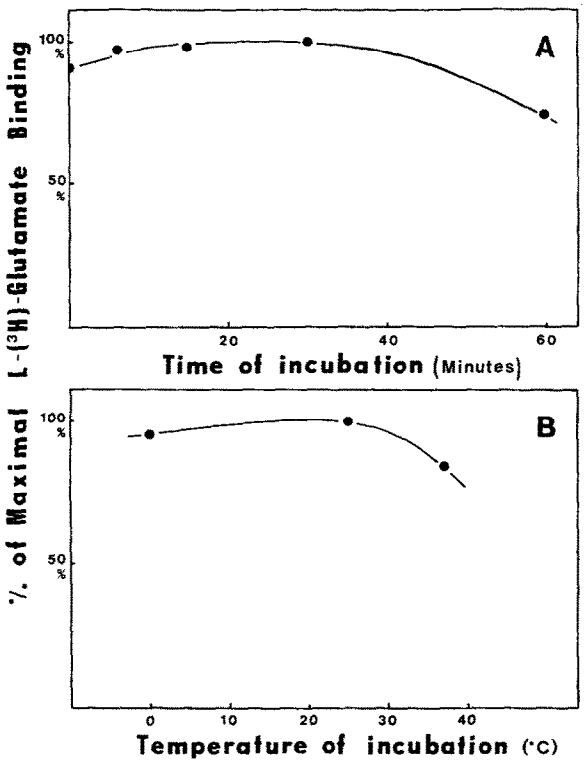

Fig.2. Physical properties of L-[^{3}H]glutamate binding. (A) shows the kinetics of binding of L-[^{3}H]glutamate for various incubation times. The experiment was performed at 25°C. (B) shows the temperature dependence. Incubation was for 30 min.

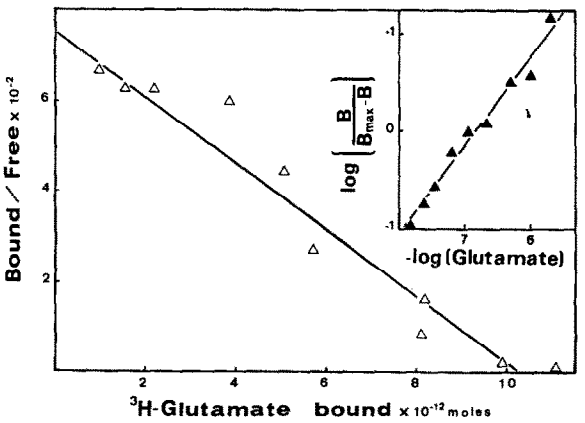

Fig.3. L-[^{3}H]Glutamate binding to a crude membrane fraction from 10 day old rats. Scatchard analysis of the data: the reciprocal value of the slope gives an estimation of the dissociation constant (K_d = 1.3 × 10^{-7} M). Hill plot, in the insert, shows that there is no cooperativity, n_H being statistically not different from unity (for technical procedure see section 2).

from unity and thus no positive or negative cooperativity is present in the conditions of our binding assay. Because of our experimental conditions (i.e., centrifugation time) the kinetics of association could not be determined.

'Specific' binding occurs in a sodium-free medium, it is slightly inhibited by increasing ionic strength of the assay medium (results not shown) and when the assay was performed at 37°C, the 'specific' binding slightly decreased (~10%) (fig.2B); the optimal pH is ~7.1 (results not shown). The fact that the K_d found in [10,13,14] is lower than any K_d measured for glutamate transport at high or even extra-high affinity and the fact that 'specific' binding occurs in sodium-free medium and at low ionic strength indicate that the sites involved in 'specific' binding are different from those involved in glutamate transport.

Several compounds added to the incubation medium affect to a different extent the 'specific binding' of L-[^{3}H]glutamate (table 1); D-glutamate is an inhibitor but it is much less effective than L-glutamate itself, hence 'specific binding' exhibits some stereospecificity for the ligand; D- or L-aspartate are also inhibitors but the concentration needed (10^{-5} M)

Table 1
Inhibition of L-[^{3}H]glutamate binding by various compounds

	Conc.	% Inhibit.
L-Glutamic acid	10^{-5} M	92
Kainic acid	10^{-6} M	23
Ibotenic acid	10^{-5} M	10
Glutamate diethyl ester	10^{-5} M	20
Glutamate γ-methyl ester	10^{-5} M	99
D-Glutamic acid	10^{-5} M	26
D-Aspartic acid	10^{-5} M	51
L-Aspartic acid	10^{-5} M	72
α-Amino adipic acid	10^{-6} M	45
D,L-homocysteic acid	10^{-6} M	50
GABA	10^{-6} M	n.s.
Muscimol	10^{-6} M	n.s.
Amino-oxyacetic acid	10^{-4} M	n.s.
Concanavalin A	10^{-6} M[a]	42

[a] On the basic of the monomer molecular weight n.s., non significant

Final L-[^{3}H]glutamate concentration was 1.15 × 10^{-7} M in the assay medium. Inhibitors were present at the indicated concentrations

for this inhibition is much higher than that of L-[^{3}H]-glutamate (10^{-7} M); this is consistent with a specific process. Structurally restricted glutamate analogues such as kainic acid or ibotenic acid are weak inhibitors in contrast to glutamate-γ-methyl ester which almost completely inhibited the binding of L-[^{3}H]-glutamate. This effect of γ-methyl-glutamate agrees with the physiological evidence of a strong antagonist effect of γ-methyl-glutamate on the receptor of crustacean neuromuscular junction [19]. Glutamate dietyl ester is a weak inhibitor of L-[^{3}H]glutamate binding; this agrees with the results in [10] but is in contrast with [2–4]. Specificity of glutamate binding sites is also indicated by the lack of effect on this binding of γ-amino-butyric acid, muscimol and amino-oxyacetic acid (an inhibitor of glutamic acid decarboxylase). The pharmacological properties reported here are in agreement with the results obtained with the mammalian central nervous system [10,13,15–18]. Also in agreement with the results in [16], Concanavalin A inhibits glutamate binding; this suggests that the binding site of glutamate could involve a sugar moiety of, possibly, a glycoprotein.

3.3. *Glutamate 'specific' binding during cerebellar development*

The K_d and n_H calculated in all the experiments at each age remain constant during development. This indicates that only 1 type of receptor might be present throughout cerebellar development. The only changes of glutamate binding sites detected during cerebellar development are quantitative changes (fig.4).

At the fourth postnatal day, the amount of 'specific' binding sites is very low as shown in fig.4A,B. It increases dramatically during the second and third weeks of postnatal life, reaching a plateau around day 20 after birth (fig.4A). In the cerebellum, the amount of protein increases more slowly during ontogenesis than that of 'specific' glutamate binding sites and continues to increase after day 20. This explains the peak of 'site density' (amount of binding sites/mg protein) around postnatal day 20 (fig.4B).

The developmental curve of accumulation of the glutamate binding sites on a per cerebellum basis (fig.4A), has the same shape and a slightly retarded chronology as compared to that of postnatal accumulation of DNA in the cerebellum [20]. DNA accumulation is mainly due to the formation of granule cells which are all formed in the second and third postnatal week. The formation of each granule cell is followed within a few days by its migration, axonal growth, and the establishment of synapses between the parallel fibers and dendritic spines of Purkinje cells and of cerebellar folium interneurones other than granule cells [21–23]. Thus our results indicate that the formation of the glutamate binding sites described here occurs approximately at the same time as the formation of synapses between parallel fibers and the dendritic spines of their postsynaptic partner cells. Further studies are needed however to verify if the glutamate binding sites described here correspond to the receptor for glutamate and to localize it.

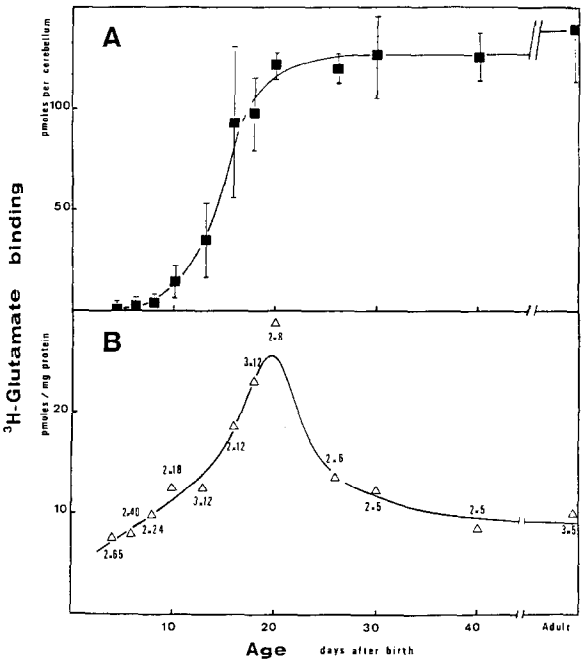

Fig.4. Glutamate binding sites during postnatal development of rat cerebellum. Changes of the amount of [^{3}H]glutamate 'specifically' bound to 'crude membrane' fractions prepared from cerebella of rats of different ages. For each point the pmoles of specifically bound [^{3}H]glutamate were determined by Scatchard analysis. The values, for the same experiments, are expressed in: (A) on a per cerebellum basis (■, average values ± SD); (B) on a per mg protein basis (△, average values). The no. expt (before the X) and the no. pooled cerebella (after the X) for each experiment are reported near each experimental point. 'Day 0' is the day of birth.

Acknowledgements

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