ELSEVIER

Brain Research 673 (1995) 149–152

BRAIN RESEARCH

Research report

Glucose deprivation increases aspartic acid release from synaptosomes of aged mice

Marcos Martínez [a], Angel Hernanz [b], María Luisa Ferrándiz [c], Emilio De Juan [a], Inmaculada Sevila [a], Natividad Martínez [a], Jaime Miquel [a,*]

[a] *Instituto de Neurociencias and Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad de Alicante, Alicante, Spain*
[b] *Servicio de Bioquímica, Hospital La Paz del Insalud, Madrid Spain*
[c] *Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia, Valencia Spain*

Accepted 22 November 1994

Abstract

To investigate the possible existence of age-related changes in excitatory amino acid release in brain, and the influence of glucose deprivation on this process, we have determined the release of endogenous aspartate and glutamate from synaptosomes freshly isolated from the cerebrum of young (12 months old) and aged (24 months old) mice. We found that there are no age-related changes in the synaptosomal release of aspartic and glutamic acids. However, in the absence of glucose in the medium of incubation aspartate and glutamate release was higher in old than in young animals ($P < 0.05$, and $P = 0.09$ respectively). Our results suggest that the ability of cerebral synaptosomes to release glutamate and aspartate remains functionally intact in old cerebrum, but there is an age-dependent dysfunction in this process linked to energy metabolism disturbances.

Keywords: Brain ageing; Glutamate; Aspartate; Synaptosome; Glucose

1. Introduction

Amino acids participate in general metabolic processes, and in the equilibrium of ion distribution across cellular membranes [2]. Aspartate and glutamate are the major excitatory neurotransmitters in the mammalian brain [26]. The potent neuroexcitatory effects of dicarboxylic amino acids (glutamate and aspartate) were first described over 40 years ago [16] and they may be involved in cellular death in a wide range of neurological disorders [12,13,28]. Animal experiments suggest that excitatory amino acid neurotransmitter systems have a role in memory and learning [7]. Furthermore, there are reports showing changes in brain glutamate and aspartate levels [30] but unchanged *N*-methyl-D-aspartate receptor density in Alzheimer's disease [15,25].

To examine the possibility that there is an age-related dysfunction in excitatory amino acid release in nerve terminals and the influence of energy metabolism disturbances on this process, we have determined the release of endogenous aspartate and glutamate from synaptosomes freshly isolated from the cerebrum of young and aged mice and the effect of glucose deprivation on this release.

2. Materials and methods

Female mice of the albino swiss stock, randomly distributed in two groups of 12 ($n = 6$) and 24 month ($n = 12$) old animals, were maintained under controlled environmental conditions: temperature (24 ± 1°C), light (12 h light:12 h dark) and fed ad libitum standard maintenance pellets. This study was performed in accordance with the guidelines of the European Communities for the care and use of laboratory animals and was approved by the local animal care committee.

* Corresponding author. Departamento de Farmacología y Terapéutica, Facultad de Medicina, Apdo 374 San Juan, 03080 Alicante, Spain. Fax: (34) (6) 5940725.

SSDI 0006-8993(94)01423-X

Table 1
Viability of cerebral synaptosomes in various experimental conditions

Age	Control	$ClCl_2$ + KCl	EGTA + KCl	no-glucose + KCl
12 months	84.47 ± 0.83	82.31 ± 2.53	83.57 ± 4.32	79.71 ± 4.72
24 months	81.74 ± 4.88	80.19 ± 3.62	81.73 ± 3.92	79.16 ± 4.15

Viability is expressed as the percentage of total LDH activity preserved in synaptosomes under each experimental condition. Data are mean ± S.D.

The animals were sacrificed by cervical dislocation and their whole cerebrums were quickly dissected out in ice-cold isolation medium (in mM: sucrose, 320; K-EDTA, 1; Tris-HCl, 10, pH 7.4). The homogenates were obtained using a Teflon-glass homogeniser (Braun) by six up and down passes of the pestle (clearance 0.1 mm) at 800 rpm, and synaptosomes were isolated according to Lai and Clark [21]. Freshly isolated synaptosomes were incubated in Krebs-bicarbonate buffer (in mM: NaCl, 122; KCl, 3.1; $MgSO_4$, 1.2; KH_2PO_4, 0.4; $CaCl_2$, 1.3; $NaHCO_3$, 25; and glucose, 10) gassed previously (60 min) with 95% O_2/5% CO_2 in order to allow functional recovery. Synaptosomes were incubated in Krebs-bicarbonate-gassed buffer under the following conditions: control conditions; in glucose-free Krebs-bicarbonate gassed buffer to achieve conditions of 'hypoglycemia'; in Krebs-bicarbonate gassed buffer without $CaCl_2$ and 1 mM EGTA. Aliquots of synaptosomes at a final concentration of 1–1.5 mg protein/ml, were kept in eppendorf tubes for 10 min at 32°C in the above experimental conditions and depolarization was achieved by adding 10 μL of a KCl solution (50 mM final concentration). After 10 min the test tubes were kept in an ice bath, and aliquots of the supernatant (1800 g, 5 min) were removed and stored at −80°C for further analysis.

To rule out the contribution of the nonspecific lysis of the synaptosomes on the observed release of endogenous amino acids, synaptosomal viability was estimated as the lack of a disturbance of plasma membrane integrity, by measuring lactate dehydrogenase (LDH) activity [20] in the synaptosome-free supernatant at 10 min of incubation under the different experimental conditions. A sample of synaptosome suspension was sonicated during 5 min for the measurement of total LDH activity.

Glutamic and aspartic acids were measured by high performance liquid chromatography (HPLC) following the Pico-Tag® method (Waters Assoc.) for physiological amino acids [5] with minor modifications [17].

All reagents were purchased from Sigma (St. Louis, MO, USA). Protein concentration was determined by the Lowry method [22] using cristaline BSA as standard. Differences between groups were analysed by the Mann–Whitney U test followed by Student's t-test for unpaired data. Comparisons between control values and those determined after the different conditions of incubation were made by the Wilcoxon's test followed by Student's t-test for paired data.

3. Results

There was no significant difference in synaptosomal viability between young and old animals under the various experimental conditions used (Table 1). Endogenous excitatory amino acid release induced by KCl was partially independent upon the presence of Ca^{2+} in the medium of incubation in synaptosomes from both young and old animals (Table 2). The Ca^{2+}-dependent release of glutamate and aspartate induced by depolarization with 50 mM of KCl in cerebral synaptosomes from 12- and 24-month-old mice is summarized in Table 2. These results show that there are no age-related changes in the synaptosomal release of aspartic and glutamic acids which suggest that the ability of cerebral synaptosomes to release glutamate and aspartate remains unchanged in aged mice. How-

Table 2
Excitatory amino acid release from cerebral synaptosomes in young and old mice

	L-Asp		L-Glu	
	12 months (n = 6)	24 months (n = 12)	12 months (n = 6)	24 months (n = 12)
Control	1.8 ± 0.73	1.36 ± 0.38	2.2 ± 0.90	1.92 ± 0.47
$CaCl_2$ + KCl	3.34 ± 0.98 $^{**\#}$	3.32 ± 0.62 $^{***\#\#}$	4.97 ± 1.13 $^{**\#}$	4.84 ± 0.74 $^{***\#\#}$
EGTA + KCl	2.33 ± 0.81 *	2.16 ± 0.44 **	3.13 ± 0.79 *	3.36 ± 0.58 **
no glucose + KCl	2.72 ± 0.62 *	4.06 ± 0.93 $^{**\#\#\$}$	3.54 ± 0.65 *	5.23 ± 1.29 $^{**\#}$

The K^+-evoked release (50 mM) of excitatory amino acids was performed in the presence ($CaCl_2$ + KCl) and absence (EGTA + KCl) of Ca^{2+}, and in the absence of glucose (no glucose + KCl). L-aspartate and L-glutamate are expressed as nmol/mg protein/10 min. Data are mean ± S.E.M.. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus control release; $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$ vs. release in absence of Ca^{2+}. $^{\$}$ $P < 0.05$ for statistically significant differences between old and young mice.

ever, in the absence of glucose in the medium of incubation, the release of aspartate induced by depolarization with 50 mM of KCl in cerebral synaptosomes was significantly higher in 24-month-old mice than in 12-month-old mice ($P < 0.05$; Table 2). The release of glutamate from cerebral synaptosomes in free-glucose buffer was also elevated in old animals in comparison to young mice but the difference did not reach statistical significance ($P = 0.09$) (Table 2).

4. Discussion

The absence of changes in the Ca^{2+}-dependent and the Ca^{2+}-independent release of glutamate and aspartate in cerebral synaptosomes in aged mice suggests that nerve terminals maintain intact the mechanisms involved in the release of excitatory amino acids. In agreement with our results other authors show no significant age-related changes in glutamic and/or aspartic acids [4,8,27,31] release from brain. Thus, despite the neuronal loss found in old brains, the neurons that remain seem to be functionally intact in terms of glutamate and aspartate release although we can not rule out that excitatory amino acid release is not affected by age-related changes in other neurotransmitters which regulate that mechanism [29].

In contrast to our findings, Freeman and Gibson reported a higher efflux of endogenous L-glutamate both basal and K^+-evoked in mice aged 30 months in neostriatal and neocortical minislices [10]. The efflux of preaccumulated [^{14}C]aspartate and [^{14}C]glutamate from cerebrocortical synaptosomes was lower in rats aged 24–26 months than in those aged 4–6 months [1]. Moreover, the release of preaccumulated D-[^{3}H]aspartate evoked by electrical stimulation from hippocampal and temporal cortical slices was higher in rats aged 28–30 months than in those aged 8 months [24]. These conflicting results may be explained by differences in species, brain regions studied and the experimental models used. However, we think that the young animals (below 12 months in age) used in many gerontological studies may be unsuitable for the study of senescence since there is evidence that the maturation process may continue until 12 months in some brain regions of rodents [6]. In view of this, we have studied synaptosomes freshly isolated from whole cerebrum of 12-month-old and 24-month-old mice.

On the other hand it has been reported that cyanide markedly increased the K^+-stimulated efflux of aspartate and glutamate determined in presence and absence of Ca^{2+} in animals aged 3, 12, 24, and 37 months [27]. However, cyanide increases the cytosolic-free Ca^{2+} concentrations in synaptosomes, affecting Ca^{2+}-dependent and Ca^{2+}-independent processes [11]. Therefore, we have used glucose deprivation to study the influence of the impairment of energy metabolism on glutamate and aspartate release from synaptosomes in ageing. Our results show that in the absence of glucose there is a significant age-related increase in aspartate release induced by KCl, suggesting that under conditions of hypoglycemia the excitotoxic injury could be greater in aged nerve terminals.

The results reported here may be relevant to understand the relationship between excitatory amino acids and glucose metabolism impairment that has been implicated in the age-associated dementia of the Alzheimer type [14,18,19,23]. Our results suggest that neurons which are bioenergeticaly compromised [3,9] may be more vulnerable to amino acid excitatory toxicity.

Acknowledgements

The authors acknowledge the support of the Fondo de Investigaciones Sanitarias (FIS 94/5630 to M.M. and FIS 94/1348).

References

[1] Aprikyan, G.V. and Gekchyan, K.G., Release of neurotransmitter amino acids from rat brain synaptosomes and its regulation in aging, *Gerontology*, 34 (1988) 35–40.
[2] Basum, H., Forssell, L.G., Almkvist, O., Cowburn, R.F., Eklöf, R., Winblad, B. and Wetterberg, L., Amino acid concentrations in cerebrospinal fluid and plasma in Alzheimer's disease and healthy control subjects, *J. Neural. Transm.*, 2 (1990) 295–304.
[3] Bowling, A.C., Mutisya, E.M., Walker, L.C., Price, D.L., Cork, L.C. and Beal, M.F., Age-dependent impairment of mitochondrial function in primate brain, *J. Neurochem.*, 60 (1993) 1964–1967.
[4] Cobo, M., Exposito, I., Porras, A. and Mora, F., Release of amino acid neurotransmitters in different cortical areas of conscious adult and aged rats, *Neurobiol. Aging*, 13 (1992) 705–709.
[5] Cohen, S.A., Bidlingmeyer, B.A. and Tarvin, T.L., PITC derivatives in amino acid analysis, *Nature*, 320 (1986) 769–770.
[6] Coleman, P.D. and Flood, D.G., Neuron numbers and dendritic extent in normal aging and Alzheimer's disease, *Neurobiol. Aging*, 8 (1987) 521–545.
[7] Collingridge, G.L. and Lester, R.A., Excitatory amino acid receptors in the vertebrate central nervous system, *Pharmacol. Rev.*, 40 (1989) 143–210.
[8] Dawson Jr., R., Wallace, D.R. and Meldrum, M.J., Endogenous glutamate release from frontal cortex of adult and aged rats, *Neurobiol. Aging*, 10 (1989) 665–668.
[9] Ferrándiz, M.L., Martínez, M., De Juan, E., Díez, A., Bustos, G. and Miquel, J., Impairment of mitochondrial oxidative phosphorylation in the brain of aged mice. *Brain Res.*, 664 (1994) 335–338.
[10] Freeman, G.B. and Gibson, G.E., Selective alteration of mouse brain neurotransmitter release with age, *Neurobiol. Aging*, 8 (1987) 147–152.
[11] Gibson, G.E., Manger, T., Toral-Barza, L. and Freeman, G., Cytosolic-free calcium and neurotransmitter release with decreased availability of glucose or oxygen, *Neurochem. Res.*, 14 (1989) 437–443.

[12] Goldberg, M.P., Monyer, H. and Choi, D.W., Hypoxic neuronal injury in vitro depends on extracellular glutamine, *Neurosci. Lett.*, 94 (1988) 52–57.
[13] Greenamyre, J.T., The role of glutamate in neurotransmission and in neurologic disease, *Arch. Neurol.*, 43 (1986) 1058–1063.
[14] Greenamyre, J.T., Neuronal bioenergetic defects, excitotoxicity and Alzheimer's disease: 'use It and lose It', *Neurobiol. Aging*, 12 (1991) 334–336.
[15] Greenamyre, J.T., Penney, J.B., D'Amato, C.J. and Young, A.B., Dementia of the Alzheimer's type: changes in hippocampal L-[^{3}H] glutamate binding, *J. Neurochem.*, 48 (1987) 543–551.
[16] Hayashi, T., Effects of sodium glutamate on the nervous system, *Keio J. Med.*, 3 (1954) 183–192.
[17] Hernanz, A. and Polanco, I., Plasma precursor amino acids of central nervous system monoamines in children with coeliac disease, *Gut*, 32 (1991) 1478–1481.
[18] Hoyer, S., Brain oxidative energy and related metabolism, neuronal stress, and Alzheimer's Disease: a speculative synthesis, *J. Geriat. Psych. Neurol.*, 6 (1993) 3–13.
[19] Hoyer, S. and Nitsch R., Cerebral excess of neurotransmitter amino acids subsequent to reduced cerebral glucose metabolism in early-onset dementia of Alzheimer type, *J. Neural. Transm.*, 75 (1989) 227–232.
[20] John, R.A., Photometric assays. In R. Eisenthal and M.J. Danson, (Eds), *Enzyme Assays: A Practical Approach*, IRL Press, Oxford, 1992, pp. 59–91.
[21] Lai, J.C.K. and Clark, J.B., Preparation and properties of mitochondria derived from synaptosomes, *Biochem. J.*, 154 (1976) 423–432.
[22] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 193 (1951) 265–275.
[23] Martínez, M., Frank, A., Diez-Tejedor, E. and Hernánz, A., Amino acid concentrations in cerebrospinal fluid and serum in Alzheimer's disease and vascular dementia, *J. Neural. Transm. (P-D. Sect)*, 6 (1993) 1–9.
[24] Meldrum, M.J., Glenton, P. and Dawson, R., [^{3}H] D-aspartic acid release in brain slices of adult and aged Fischer 344 rats, *Neurochem. Res.*, 17 (1992) 151–156.
[25] Palmer, A.M. and Burns, M.A., Preservation of redox, polyamine, and glycine modulatory domains of the *N*-methyl-D-aspartate receptor in Alzheimer's disease, *J. Neurochem.*, 62 (1994) 187–196.
[26] Palmer, A.M. and Gershon, S., Is the neuronal basis of Alzheimer's disease cholinergic or glutamatergic, *FASEB J.*, 4 (1990) 2745–2752.
[27] Palmer, A.M., Robichaud, P.J. and Reiter, C.T., The release and uptake of excitatory amino acids in rat brain: effect of aging and oxidative stress, *Neurobiol. Aging*, 15 (1994) 103–111.
[28] Plataikis, A., Glutamate dysfunction and selective motor neuron degeneration in amyotrophic lateral sclerosis: a hypothesis, *Ann. Neurol.*, 28 (1990) 3–8.
[29] Porras, A. and Mora, F., 'In vivo' interaction of glutamic acid and dopamine in the basal ganglia: studies of ageing, *Eur. J. Neurosci.*, 5 (1992) 100.
[30] Procter, A.W., Palmer, A.M., Francis, P.T., Lowe, S.L., Neary, D., Murphy, E., Doshi, R. and Bowen, D.M., Evidence of glutamatergic denervation and possible abnormal metabolism in Alzheimer's disease, *J. Neurochem.*, 50 (1988) 790–802.
[31] Sanchez, J., Herrero, I., Miras-Portugal, M.T. and Mora, F., Unchanged exocytotic release of glutamic acid in cortex and neoestriatum of the rat during, *Brain Res. Bull.*, 33 (1994) 357–359.