The essential amino acid lysine acts as precursor of glutamate in the mammalian central nervous system

Fabio Papes[a], Marcelo J. Surpili[a], Francesco Langone[b], Jose R. Trigo[c], Paulo Arruda[a,d,*]

[a] *Centro de Biologia Molecular e Engenharia Genetica, Universidade Estadual de Campinas, 13083-970, Campinas, SP, Brazil*
[b] *Departamento de Fisiologia e Biofisica, Instituto de Biologia, Universidade Estadual de Campinas, 13083-970, Campinas, SP, Brazil*
[c] *Departamento de Zoologia, Instituto de Biologia, Universidade Estadual de Campinas, 13083-970, Campinas, SP, Brazil*
[d] *Departamento de Genetica e Evolucao, Instituto de Biologia, Universidade Estadual de Campinas, 13083-970, Campinas, SP, Brazil*

Received 11 November 2000; revised 12 December 2000; accepted 12 December 2000

First published online 22 December 2000

Edited by Guido Tettamanti

Abstract **Lysine has long been recognized as an essential amino acid for humans and the lack or low supply of this compound in the diet may lead to mental and physical handicaps. Since lysine is severely restricted in cereals, the most important staple food in the world, the understanding of its biological roles must be a major concern. Here we show that lysine is an important precursor for de novo synthesis of glutamate, the most significant excitatory neurotransmitter in the mammalian central nervous system. We also show that the synthesis of glutamate from lysine, which is carried out by the saccharopine pathway, is likely to take place in neurons.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Lysine; Saccharopine; Amino acid catabolism; Neurotransmitter; Brain

1. Introduction

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system [1]. Although some reactions leading to the synthesis of glutamate have been studied during the last decade, the sources of nitrogen and carbon atoms for de novo synthesis have remained elusive, as has the involvement of astrocytes and neurons in this process [2,3]. Glucose has been described as the main precursor of the carbon skeleton of glutamate [4], whereas the branched-chain amino acids (leucine, isoleucine and valine) have been suggested as donors of amino groups for the synthesis of ~25% of the glutamate produced within the brain, by the action of the branched-chain aminotransferase (BCAT) in astrocytes [5–8]. However, the amino acids which act as nitrogen donors for the remaining 75% of the glutamate pool have not been identified as yet.

On the other hand, several amino acids have been pointed out as short-term precursors of glutamate, such as glutamine [9], alanine [6,8–10] and aspartate [11]. Glutamine is synthesized from glutamate in astrocytes and oligodendrocytes and then transported into neurons, where it is reconverted to glutamate [9], constituting the well-known glutamate–glutamine cycle.

The saccharopine pathway is the main route for lysine degradation in mammals [12–14] and plants [15,16], and its first two reactions (Fig. 1) are catalyzed by enzymatic activities known as lysine-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH), which reside on a single bifunctional polypeptide (LOR/SDH) [12,13]. LOR/SDH has been detected in a number of mammalian tissues, mainly in the liver and kidney, where it converts lysine into ketone bodies [12,13], and in the rat embryonic nervous system [17]. The net result of the sequential action of LOR and SDH is the transfer of the ε-amino group of lysine to glutamate (Fig. 1). This, together with the fact that lysine is present at a high concentration in the brain [18], suggested that the saccharopine pathway could be a putative glutamate-synthesizing route in the mammalian nervous system.

Here we show that the essential amino acid lysine, the availability of which is severely restricted in some of the world's major food staples, is an important precursor for de novo glutamate production in the mammalian central nervous system. We also show that the transfer of amino groups from lysine to glutamate, which occurs via the saccharopine pathway, is likely to take place in neurons.

2. Materials and methods

2.1. Tracing experiments with ^{15}N-labeled precursors

[ε-^{15}N]L-lysine (99% atom percent excess), [α-^{15}N]leucine (98% atom percent excess) and [α-^{15}N]glutamate (99% atom percent excess) were purchased from Isotech Inc. Cerebellum and cerebral cortex slices from 2 months old C57/Black6 mice were prepared and incubated with ^{15}N-labeled precursors according to previous studies [6]. Labeled leucine and glutamate were used at final concentrations of 300 μM, as described by Yudkoff et al. [8]. Labeled lysine was also used at a final concentration of 300 μM because its concentration in the central nervous system has been shown to be similar to that of leucine [18]. Sample preparation and analysis by gas chromatography-mass spectrometry (GC-MS) were performed as previously reported [8], using a Hewlett-Packard 5971 GC-MS system and a 12 m×0.32 mm DB-5 capillary also from Hewlett-Packard. Ion ratios for glutamate and leucine were as detailed elsewhere [8] and different ion ratios were used for lysine (*m/z* 174/175, 317/318 and 431/432) to analyze its α- and ε-amino groups separately. The percentage ^{15}N-enrichment ('atom percent excess' or APE), corrected for the natural abundances of the isotopes, was determined from those mass ratios [6]. The higher the APE of a given compound, the larger the percentage of its molecules which are labeled with ^{15}N.

*Corresponding author. Fax: (55)-19-788 1089.
E-mail: parruda@unicamp.br

Abbreviations: LOR, lysine-2-oxoglutarate reductase; SDH, saccharopine dehydrogenase

PII: S0014-5793(00)02401-7

2.2. RT-PCR and RNA gel blot analyses

Mouse brain and cerebellum RNAs were extracted from 2 months old C57/Black6 animals as previously described [12]. Reverse transcription (RT) was carried out with Superscript II (Gibco BRL), as recommended in the manufacturer's protocol. Oligonucleotides 5′-TAT GGG ACG GTG TTA AGT CGC-3′ and 5′-AAA ATC TAT GGA CCC TCC CGT-3′, which yield a 300 bp fragment of the mouse LOR/SDH cDNA (GenBank accession number NM013930), were used for the consecutive PCR (1 min at 94°C, 1.5 min at 62°C, 2 min at 72°C; 27 cycles). Oligonucleotides 5′-GGC TGT ATT CCC CTC CAT CGT-3′ and 5′-GAG CCA CCG ATC CAC ACA GAG-3′ were used to amplify β-actin as an internal control. Bands were quantified on a FLA-3000 Fluorescent Image Analyzer (Fujifilm). Human multiple tissue poly(A)$^+$ Northern blots (Fig. 3b,c) containing RNA samples from donors of varying ages and sex were purchased from Clontech (#7755-1; #7756-1) and hybridized with an SDH-specific probe, corresponding to nucleotides 1540–2235 of the human LOR/SDH cDNA (GenBank NM005763).

2.3. In situ hybridization

Digoxigenin-labeled antisense and sense run-off transcripts were synthesized using a DIG-labeling kit (Boehringer-Mannheim) and a PCR fragment containing 300 bp of the mouse LOR/SDH cDNA (bases 800–1112), which was flanked by T3 and T7 RNA polymerase promoters. In situ hybridization was performed on freshly cut cryosections (10–12 μm) of brain and cerebellum from C57/Black6 animals [19]. Probe concentrations were ~200 ng/ml of hybridization buffer. Hybridized labeled probes were visualized using alkaline phosphatase-coupled anti-digoxigenin Fab fragments (Boehringer-Mannheim) with BCIP/NBT as substrate [19]. Light microscope images were acquired using Ektachrome slide film (Kodak), which was then scanned at high resolution on a SprintScan 35 Plus (Polaroid) and processed electronically for photo-quality hard printing using a KDS 8650 digital printer (Kodak).

3. Results

3.1. Lysine is the precursor of a large proportion of glutamate in the mouse nervous system

To definitely establish the occurrence of the saccharopine pathway in the mammalian brain and to verify which fraction of the glutamate pool in the nervous system is actually derived from lysine via the LOR/SDH-catalyzed reactions, slices of cerebral cortex and cerebellum were incubated with ^{15}N-labeled lysine (Fig. 2). These incubations were done with lysine labeled in its ε-amino group so as to examine the fate of this group alone and, consequently, the participation of LOR/SDH (Fig. 1). In the cerebellum, as expected, labeled lysine was rapidly taken up by the slices: Its isotopic enrichment (APE) reached 40% after 1 h of incubation (Fig. 2a), decreasing afterwards due to the transfer of its ^{15}N-label to other cellular compounds. Glutamate ^{15}N-labeling rapidly reached a mean value of approximately 12% APE (Fig. 2a) and was already detectable after 10 min of incubation (Fig. 2a, inset). The results obtained for the cerebral cortex slices were similar (Fig. 2d), although the labeling of glutamate from lysine was lower than in slices from cerebellum (Fig. 2a). Since the incubations were done with lysine concentrations similar to those normally found in the brain extracellular fluid, these results indicate that approximately 33% (40/12) of all glutamate in the central nervous system is likely to be derived from lysine via LOR/SDH. This value could be an underestimate, since the transfer of the α-amino group from lysine to glutamate, which proceeds through the pipecolate pathway (Fig. 1) [20], was not assessed. Hence, lysine is probably a major nitrogen donor for glutamate synthesis in the mammalian central nervous system.

Cerebral cortex and cerebellum slices were also incubated with ^{15}N-labeled glutamate to examine the reversibility of the LOR/SDH-catalyzed reactions (Fig. 2b,e). Although ^{15}N-labeling of lysine at its α-amino group was detected (Fig. 2b,e), indicating reversibility of the reaction which catalyzes the conversion of lysine into 2-keto-6-aminocaproate in the pipecolate pathway (Fig. 1), no labeling of lysine at its ε-amino group was observed. This finding suggests that the reactions catalyzed by LOR and SDH speed the conversion of lysine into glutamate up. As a control, the same set of incubations was performed with ^{15}N-labeled leucine (Fig. 2c,f) and reproduced already published data showing the production of glutamate from leucine via the enzyme BCAT [8].

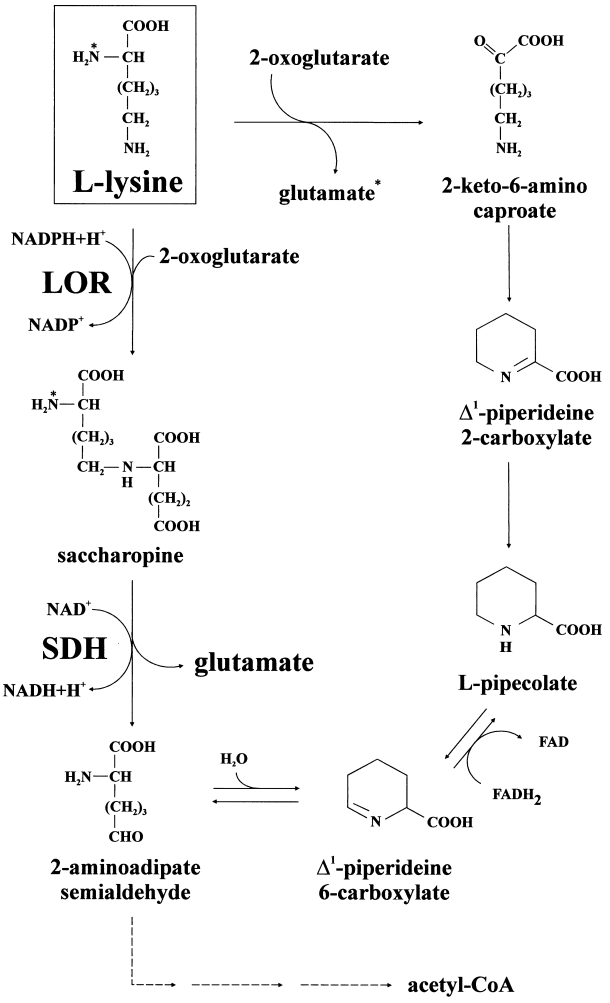

Fig. 1. Lysine catabolic routes in higher eukaryotes. The first two reactions of the saccharopine pathway (left) are catalyzed by the concerted activities of LOR and SDH, the net result of which is the transfer of the ε-amino group of lysine to 2-oxoglutarate, resulting in the synthesis of glutamate. The transfer of the α-amino group of lysine (marked by an asterisk) to glutamate occurs through the pipecolate pathway (right). The compound 2-aminoadipate semialdehyde, common to both routes, may be further degraded to acetyl-CoA through a series of reactions (broken lines), although the enzymatic activities involved have not been shown to occur in the brain yet.

3.2. The gene encoding the lysine-degrading enzyme LOR/SDH is conspicuously expressed in the mammalian central nervous system

We have previously isolated cDNAs encoding the mouse

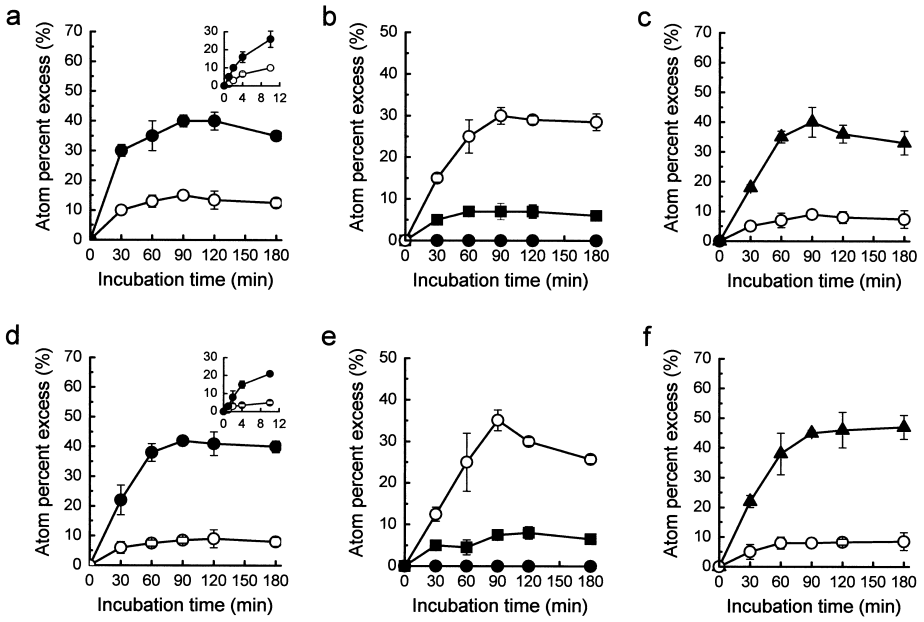

Fig. 2. Metabolism of ^{15}N-labeled amino acids in the brain and cerebellum. Slices of cerebellum (a–c) or cerebral cortex (d–f) were incubated with ^{15}N-labeled lysine (a,d), glutamate (b,e) or leucine (c,f) to assess the role of lysine as a precursor of glutamate in the mammalian brain. The percent ^{15}N-enrichment (atom percent excess) indicates the isotopic enrichment measured in a given amino group, namely the ε-amino group of lysine (filled circles), the α-amino group of lysine (filled squares), and the amino groups of glutamate (open circles) and leucine (filled triangles). Insets in a and d show earlier time points for incubations with lysine. Bars denote the S.E.M. of four independent experiments (slices obtained from four animals).

[12] and human LOR/SDH. These cDNAs were used in the analysis of LOR/SDH mRNA expression to demonstrate the occurrence of the saccharopine pathway in the mammalian central nervous system (Fig. 3). There was significant expression of LOR/SDH in the mouse cerebral cortex and cerebellum, with less prominent expression in the hypophyseal gland and in immature embryonic brain (Fig. 3a). In the cerebellum, the expression was even comparable to that in the liver (Fig. 3a). Expression in the cerebral cortex was lower than in the cerebellum, in agreement with the ^{15}N.data (Fig. 2). LOR/SDH expression also occurred in several regions of the human nervous system (Fig. 3b), paralleling the results obtained for mouse (Fig. 3a), with stronger expression in the occipital and frontal lobes and in the cerebellum. Expression was also evident in human embryonic tissues, including brain (Fig. 3c).

3.3. LOR/SDH is located in several regions of the mouse cerebellum, particularly in neurons

In situ hybridization was used to further investigate which regions and cell types of the brain and cerebellum express LOR/SDH (Fig. 4). LOR/SDH expression was found in almost every region of the adult mouse central nervous system, which was in keeping with results described in Fig. 3. The highest expression was seen in the cerebellum, with the granular and molecular cell layers showing lower staining and the Purkinje cell layer possessing the strongest staining (Fig. 4a–c). In the latter, the staining appeared to be concentrated in neuronal (Purkinje) cells (Fig. 4d, as compared with a Nissl-stained control section in Fig. 4e). There was no detectable expression of LOR/SDH in the cerebellar white matter (Fig. 4a). The hippocampus had conspicuous expression of LOR/SDH (Fig. 4f), mainly in the CA1 region, and less significantly in the CA3 region and dentate gyrus. The cerebral cortex also showed strong LOR/SDH expression (Fig. 4g,h), although somewhat less prominent than that in the cerebellum, in agreement with the RT-PCR results (Fig. 3a). There was no difference in the staining of the several gray matter cortical layers, and the white matter showed no staining at all (Fig. 4g), a finding similar to that seen in the cerebellum (Fig. 4a). In the brainstem, several nuclei possessed strong LOR/SDH expression (Fig. 4i) and, as in the cerebellum, the staining

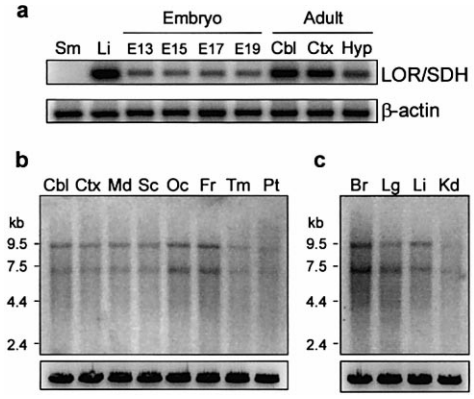

Fig. 3. Expression of LOR/SDH in the mammalian central nervous system. a: RT-PCR analysis showing the expression of LOR/SDH in mouse embryonic brain (stages E13–E19) and adult brain (Cbl, cerebellum; Ctx, cerebral cortex; Hyp, hypophyseal gland) compared to adult liver (Li) and skeletal muscle (Sm). b,c: Northern blot analysis showing the expression of LOR/SDH in human adult brain (b) and human embryo (c). Md, medulla; Sc, spinal cord; Oc, occipital pole; Fr, frontal lobe; Tm, temporal lobe; Pt, parietal lobe; Br, brain; Lg; lung; Kd, kidney. The 9.5 kb and 7.4 kb bands represent distinct LOR/SDH transcripts [12,29]. The bottom panels in b and c represent control hybridizations of the corresponding blots with a GAPDH probe.

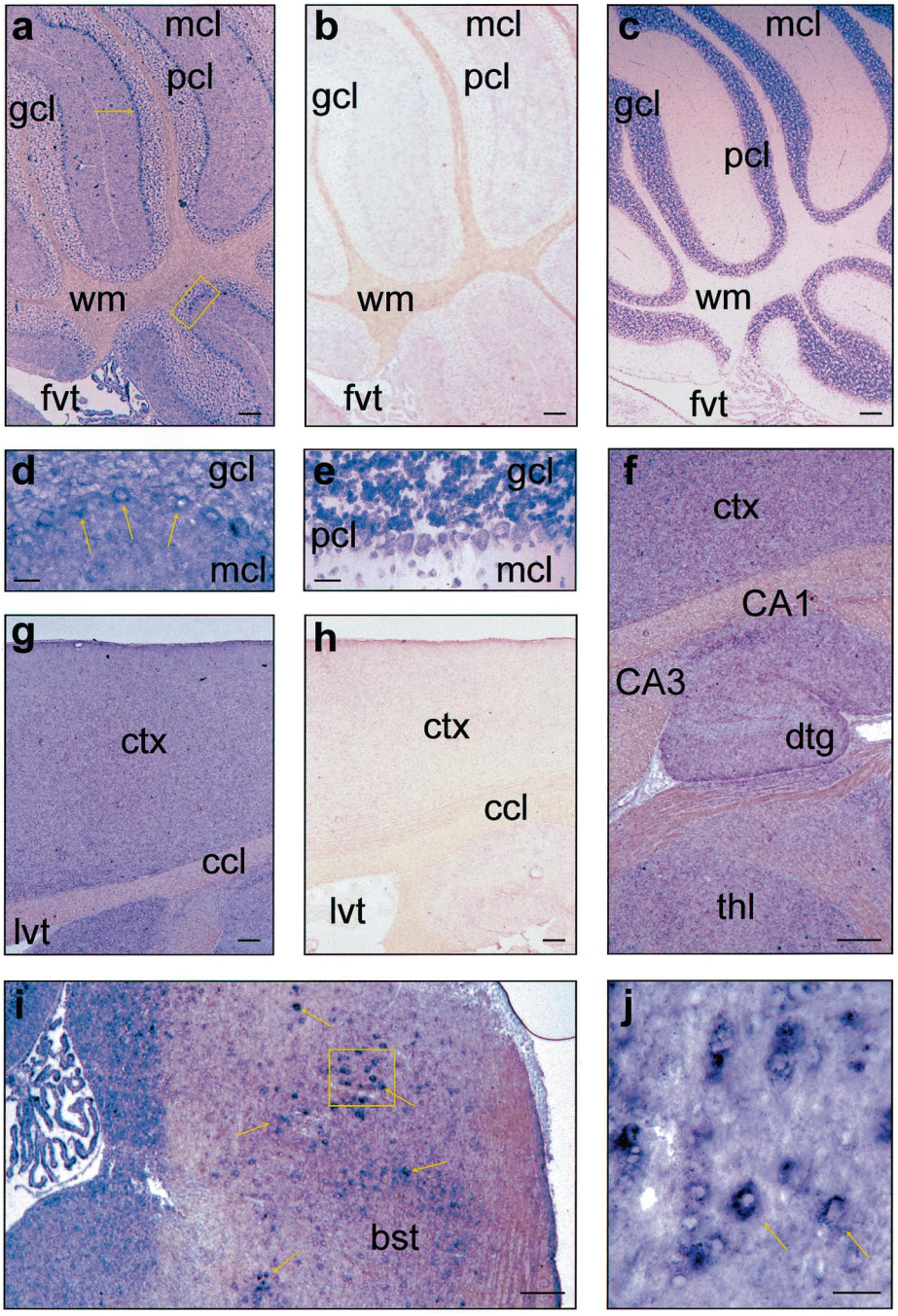

Fig. 4. In situ hybridization showing the expression of LOR/SDH in the mouse central nervous system. a,b: Cerebellum sections hybridized with LOR/SDH antisense (a) and sense (b) probes, showing conspicuous staining in the Purkinje (arrow, pcl), molecular (mcl) and granule (gcl) cell layers. The region delimited by the yellow box in a is shown at a higher magnification in d, and illustrates the expression of LOR/SDH in Purkinje neurons (arrows). c,e: Nissl-stained control sections of a and d, respectively. f: Expression in the hippocampus and in the thalamus (thl), showing strong staining in the CA1 region and less staining in the CA3 region and dentate gyrus (dtg). g,h: Cerebral cortex sections hybridized with LOR/SDH antisense (g) and sense (h) probes, showing uniform expression throughout cortical layers. i: Several brainstem (bst) nuclei with strong expression of LOR/SDH (arrows), mostly in neurons, as detailed in j (a higher magnification of the yellow-boxed region in i). Scale bars in a–c,f–i: 100 μm; scale bars in d,e,h: 21.5 μm. Other abbreviations: fvt, fourth ventricule; ccl, corpus callosum; lvt, lateral ventricle.

seemed to be located mainly in neurons (Fig. 4j), although concomitant expression in glia could not be ruled out. The detection of LOR/SDH in neuronal cells suggests that the saccharopine pathway may be a de novo glutamate-synthesizing route for neurons, in contrast to the BCAT-catalyzed glutamate synthesis, which seems to be restricted to astrocytes [2,3,5].

4. Discussion

Our data show the widespread distribution of the lysine-degrading enzyme LOR/SDH in the mammalian central nervous system and indicate that lysine is an important precursor of the neurotransmitter glutamate (via LOR/SDH) in several brain regions, in adult as well as in embryo. This biosynthesis

was shown to occur in neurons and it is the first time a de novo glutamate-synthesizing route has been ascribed to these cells. Moreover, this represents a glutamate synthesis which is independent of the astrocytic glutamate–glutamine cycle. GABAergic neurons may also use this route to produce GABA since this neurotransmitter is derived directly from glutamate [9]. Glutamate-mediated neurotransmission is essential for the proper functioning of the central nervous system and is especially required during brain maturation and immediately after birth, when most cellular communications are being established and refined [1,21–23]. Any substances associated with the production of glutamate in these phases will thus be essential, and their absence may probably result in brain malfunctioning. Indeed, lysine has long been recognized as an essential amino acid, the lack or low supply of which may lead to mental and physical retardations [24–27]. This seems to be a very important issue since the lysine content of cereals, the most consumed staple food in the world, is severely restricted. Thus, the results described here provide a link between glutamate synthesis, neurotransmission, nervous system development and nutritional requirement for lysine.

Moreover, since glutamate is involved in a number of excitatory brain injuries, such as those associated with stroke, hypoglycemia, epilepsy and ischemia [1,28,29], LOR/SDH may turn into an important molecular target for the prevention and therapy of such damages. Lastly, LOR/SDH has been implicated in several kinds of human familial hyperlysinemias [30], some of them frequently accompanied by severe physiological and morphological defects in the nervous system, which will be better understood and treated upon further studies on the human and mouse LOR/SDH and on their participation in glutamate synthesis in the brain.

Acknowledgements: We thank Claudia Morelli for her technical help with ^{15}N.experiments, and Dr. Leslie Vosshall (Columbia University, New York) for suggestions on in situ hybridizations. This work was supported by grants to F.P., M.J.S., J.R.T. and P.A. from FAPESP.

References

[1] Michaelis, E.K. (1998) Progr. Neurobiol. 54, 369–415.
[2] Daikhin, Y. and Yudkoff, M. (2000) J. Nutr. 130, 1026S–1031S.
[3] Hertz, L., Dringen, R., Schousboe, A. and Robinson, S.R. (1999) J. Neurosci. Res. 57, 417–428.
[4] Sibson, N.R., Dhankhar, A., Mason, G.F., Rothman, D.L., Behar, K.L. and Shulman, R.G. (1998) Proc. Natl. Acad. Sci. USA 95, 316–321.
[5] Yudkoff, M. (1997) Glia 21, 92–98.
[6] Jones, P. and Bachelard, H.S. (1999) Neurochem. Res. 24, 1327–1331.
[7] Kanamori, K., Ross, B.D. and Kondrat, R.W. (1998) J. Neurochem. 70, 1304–1315.
[8] Yudkoff, M., Daikhin, Y., Lin, Z.P., Nissim, I., Stern, J., Pleasure, D. and Nissim, I. (1994) J. Neurochem. 62, 1192–1202.
[9] Westergaard, N., Sonnewald, U. and Schousboe, A. (1995) Dev. Neurosci. 17, 203–211.
[10] Erecinska, M., Nelson, D., Nissim, I., Daikhin, Y. and Yudkoff, M. (1994) J. Neurochem. 62, 1953–1964.
[11] Erecinska, M., Pleasure, D., Nelson, D., Nissim, I. and Yudkoff, M. (1993) J. Neurochem. 60, 1696–1706.
[12] Papes, F., Kemper, E.L., Cord-Neto, G., Langone, F. and Arruda, P. (1999) Biochem. J. 344, 555–563.
[13] Markovitz, P.J., Chuang, D.T. and Cox, R.P. (1984) J. Biol. Chem. 259, 11643–11646.
[14] Markovitz, P.J. and Chuang, D.T. (1987) J. Biol. Chem. 262, 9353–9358.
[15] Arruda, P., Kemper, E.L., Papes, F. and Leite, A. (2000) Trends Plant Sci. 5, 324–330.
[16] Kemper, E.L., Cord-Neto, G., Papes, F., Moraes, K.C.M., Leite, A. and Arruda, P. (1999) Plant Cell 11, 1981–1993.
[17] Rao, V.V., Pan, X. and Chang, Y.-F. (1992) Comp. Biochem. Physiol. 103B, 221–224.
[18] Smith, Q.R. (2000) J. Nutr. 130, 1016S–1022S.
[19] Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L. and Hediger, M.A. (1997) Nature 388, 482–488.
[20] Chang, Y.-F. (1976) Biochem. Biophys. Res. Commun. 69, 174–180.
[21] Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vockler, J., Dikranian, K., Tenkova, T.I., Stefovska, V., Turski, L. and Olney, J.W. (1999) Science 283, 70–74.
[22] Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M., Geuze, H.J. and Sudhof, T.C. (2000) Science 287, 864–869.
[23] Ichise, T., Kano, M., Hashimoto, K., Yanagihara, D., Nakao, K., Shigemoto, R., Katsuki, M. and Aiba, A. (2000) Science 288, 1832–1835.
[24] Levitsky, D.A. and Strupp, B.J. (1995) J. Nutr. 125, 2212S–2220S.
[25] Levitsky, D.A. and Barnes, R.H. (1970) Nature 225, 468–469.
[26] Chase, H.P., Lindsley, W.F.B. and O'Brien, D. (1969) Nature 221, 554–555.
[27] Abelson, P.H. (1969) Science 164, 17.
[28] During, M.J., Symes, C.W., Lawlor, P.A., Lin, J., Dunning, J., Fitzsimons, H.L., Poulsen, D., Leone, P., Xu, R., Dicker, B.L., Lipski, J. and Young, D. (2000) Science 287, 1453–1460.
[29] Rossi, D.J., Oshima, T. and Attwell, D. (2000) Nature 403, 316–321.
[30] Sacksteder, K.A., Biery, B.J., Morrell, J.C., Goodman, B.K., Geisbrecht, B.V., Cox, R.P., Gould, S.J. and Geraghty, M.T. (2000) Am. J. Hum. Genet. 66, 1736–1743.