Persistent depolarization and Glu uptake inhibition operate distinct osmoregulatory mechanisms in the mammalian brain

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Abstract

The ways of coupling neuronal with glial compartments in natural physiology was investigated in microdialysis experiments by monitoring extracellular concentration of amino acids in the brain of anaesthetized rats. We hypothesized that extracellular [Glu], [Gln] and [Tau] patterns would be state-dependent. This was tested by stimulation of N-methyl-D-aspartate (NMDA) receptors, by inhibition of Glu uptake or by local depolarization with a high-K+ dialysate, coupled with the addition of Co2+ to block Ca2+ influx. The results showed that (1) extracellular [Gln] was low whereas [Glu] and [Tau] were high during infusion of NMDA (0.5–1.0 mM) or high-K+ (80 mM) in the hippocampus and ventrobasal thalamus, (2) hippocampal extracellular [Glu], [Gln] and [Tau] were increased in response to the Glu uptake inhibitor, L-trans-pyrrolidine-2,4-dicarboxilic acid (tPDC, 0.5–3.0 mM), in a concentration-dependent manner, (3) high-K+ induced increase of extracellular [Glu] was partially blocked by the addition of 10 mM CoCl2 with the high-K+ dialysate in the hippocampus. Searching for main correlations between changes in [Glu], [Gln] and [Tau] by calculating partial correlations and with the use of factor analyses we found, the primary response of the mammalian brain to persistent depolarization is the neuronal uptake of [Gln] and release of [Tau] thereupon, acting independently of Glu changes. When glial and neuronal uptake of Glu is blocked, releases of Tau occur from neuronal as well as glial compartments accompanied by increases of [Gln] in the mammalian brain.

Keywords: L-trans-pyrrolidine-2,4-dicarboxilic acid; N-methyl-D-aspartate; High-K+; Co2+; Extracellular [Glu]; [Gln] and [Tau]; Microdialysis; Hippocampus; Vento basal thalamus; Rat brain

1. Introduction

Overstimulation by Glu and analogues can induce neuronal degeneration, both in vitro and in vivo (Rothman and Olney, 1987; Tapia et al., 1999 and references cited therein). By contrast, accumulation of extracellular Glu by inhibition of its uptake in the presence of L-trans-pyrrolidine-2,4-dicarboxilic acid (tPDC; Parpura et al., 1994; Massieu et al., 1995; Herrera-Marschitz et al., 1996; Nyitrai et al., 1999) does not induce neuronal damage in vivo (Massieu et al., 1995), suggesting that the responses of the brain to overexcitation and Glu uptake inhibition are different. Several lines of evidence suggest Tau functioning as a protectant against excitotoxicity (Pazdernik et al., 1990; Saransaari and Oja, 1997, 1999; Zielińska et al., 1999; and references cited therein). Furthermore, recent studies have suggested the glial Glu/Gln cycle as the major metabolic flux of total cellular Glu metabolism (Shen et al., 1999). Supposing altered interactions between glial, neuronal and extracellular...
compartments we addressed if extracellular concentration of Glu, Tau and Gln ([Glu], [Tau] and [Gln]) were changed under these different conditions. We monitored [Glu], [Tau] and [Gln] after tPDC, NMDA and high-K+ in combination with CoCl2 to block Ca2+ influx (Satoh et al., 1999), were dialysed into the hippocampus and ventrobasal thalamus of the rat. With the novel use of factor analyses we asked, whether there were significant changes in [Glu], [Gln] and [Tau] interrelations.

2. Materials and methods

2.1. Materials

tPDC, NMDA and mercaptoethanol were from Sigma. CaCl2, acetonitrile, orto-phtalalddehyde (OPA), phosphoric acid, tetraethylammonium hydroxide, and tetrahydrofuran were obtained from Merck. CoCl2, KCl, NaCl, MgCl2, KMnO4 and NaOH were purchased from Reanal (Budapest). The amino acid standards were from Serva.

2.2. Microdialysis

Animal experiments were carried out as previously described (Nyitrai et al., 1996) on the basis of local ethical rules in accordance with the Guidelines on the Use of Living Animals in Scientific Investigations 1984. Microdialysis probes were prepared as described earlier (Juhász et al., 1989). The rats (350–400 g) were anaesthetised with 1% halothane in air and placed into a stereotaxic frame. The probes were implanted into the left hippocampus (A: −5.2, L: 5, V: −8 mm) or ventrobasal thalamus (A: −3.1, L: 3, V: −7 mm), according to the atlas of Paxinos and Watson (1997). We started to collect samples 40 min after the implantation of the microdialysis probe by perfusing artificial cerebrospinal fluid (ACSF) containing 144 mM NaCl, 3 mM KCl, 1 mM MgCl2 and 2 mM CaCl2 in water, bidistilled over KMnO4, at a rate of 1.3 μl/min. The pH of ACSF was adjusted to 7.4 with NaOH prior to the microdialysis experiment starting. The purity of ACSF used in the experiment was checked by HPLC. To establish the background [Glu], [Gln] and [Tau] each experiment started with the collection of three control samples (26 μl) in 1 h. To examine the effect of tPDC (with or without CoCl2), NMDA or high KCl (with or without CoCl2) on the background [Glu], [Gln] and [Tau] we applied these substances immediately after the control samples were collected with the following protocol: sampling in every 10 min (first hour) followed by sampling in every 20 min (second and third hour). After each experiment, the brain was removed and placed into 10% paraformaldehyde. Anatomical localisation of the dialysis probe was checked by histological analyses of Nissl-stained coronal sections.

2.3. Determination of the concentration of Glu, Gln and Tau in the microdialysis samples

Determination of the concentration of Glu, Gln and Tau in the microdialysis samples was performed as described (Nyitrai et al., 1996; 1999; Kardos et al., 1996). Precolumn derivatization with OPA was performed in the presence of mercaptoethanol at pH 10.4 followed by quantitative analysis of the OPA derivatized amino acids was assessed in an automated HPLC-combined fluorescence detection system (Pharmacia AminoSys) using 305–395 nm excitation and 430–470 nm emission filters and HP Hypersyl ODS reversed phase column with the following eluents: 0.1 M phosphate buffer containing 0.5% (vol/vol) tetrahydrofuran and 0.02% (vol/vol) tetraethylammonium hydroxide, pH 6.0 (A); 70% acetonitrile mixed with 0.1 M phosphate buffer adjusted to pH 6.0 with NaOH (B). The gradient profile was 3% B at 0 min, 11% B at 1.5 min, 22% B at 16 min, 40% B at 20 min, 60% B at 25 min, 100% B at 29 min, 2% B at 37 min. External standards of 10 μM amino acids were injected after every 10 samples. Chromatograms were evaluated by PE Nelson 2000 software. Detection limits for amino acids were 0.5–5 pmoles in 10 μl sample. Based on the in vitro calibration of the probes (Juhász et al., 1989) the estimated concentration of the drug proximal to the wall of the microdialysis probe is about 20% of its concentration inside the probe.

2.4. Evaluation of data

Since concentrations of amino acids in the dialysate are not identical with the [AA] (Benveniste and Huttemeier, 1990), concentrations of amino acids measured in dialysate samples collected during various drug applications were related to averaged concentration of amino acids of control samples collected for an hour previous to drug application and were given as percent changes of control. Values of the amino acid concentrations determined in sequential dialysis samples collected in different periods of time obtained from 3 to 4 animals were averaged (mean ± SD). For statistical analysis of data we used Student t-test. Percent change of control value was considered significant at p < 0.05 level.

2.5. Analysis of the relationship between the [AA] measured in the brain

In order to reveal and quantitatively characterise the relationship (intercorrelations) between changes of
[Glu], [Gln] and [Tau] ([Glu]–[Gln]–[Tau]) under different conditions two mathematical approaches were applied: (1) modeling of data by general linear least squares estimation which is a maximum likelihood estimator; (2) quantification of [Glu]–[Gln]–[Tau] intercorrelations by partial correlation coefficients followed by determination of the latent independent variables underlying the measured ones (changes of [Glu], [Gln] and [Tau]) by factor analysis.

The partial correlations represent the independent, unique contribution of V1 (variable 1) to the prediction of V2 (without the influence of V3 for both V1 and V2). It is computed by the calculation of the correlation between V1 and V2 after controlling for V3.

The measured [AA] ([Glu], [Gln] and [Tau]) can be considered as some measurable variables coming from the three-compartment system of neuron-glia-extracellular space. In order to identify the system the degrees of freedom (df) and the independent (canonical) variables of it are to be determined. Furthermore, to quantify behaviour, the dynamics of the system in the phase space spanned by the independent variables must be analyzed. Heuristically, the df may be defined as the number of independent (latent) variables describing the system which usually are not measurable. Here we interpret df as the intrinsic dimensionality of the phase space.

In case of the [Glu], [Gln] and [Tau] measurements in the brain, both the df and the independent variables as well as the intrinsic dimensionality of the phase space are unknown. Assuming that the measured extracellular concentrations contain all the information about the process Glu, Gln and Tau are involved in, a phase-space can be constructed by using changes of [Glu], [Gln] and [Tau] as coordinate axes. However, due to the possible interdependencies between the variables, this coordinate system might be oblique. To find independent variables (instead of the measured ones), one can transform it into a rectangular coordinate system. We used factor analysis as a tool of finding intrinsic dimensionality and independent variables.

3. Results

3.1. Determination of background [Glu], [Asp], [Gly] and [Tau]

Concentrations of Glu, Gln and Tau in the control dialysate samples remained constant for 1 h previous to drug application, and were found to be: 0.4 ± 0.1 μM Glu, 31 ± 5 μM Gln and 5.8 ± 2.0 μM Tau in the hippocampus and 0.6 ± 0.1 μM Glu, 25 ± 3 μM Gln and 1.4 ± 0.2 μM Tau in the ventrobasal thalamus of halothane-anaesthetised rats.

3.2. Effect of NMDA and high-[KCl] on [Glu], [Gln] and [Tau] in the hippocampus and ventrobasal thalamus

In pilot experiments, NMDA (0.5 mM) microdialysed into the hippocampus caused transient increases of [Glu] and [Tau] within 10 min ([Glu] = 166%) and 20 min ([Tau] = 244%), respectively. Thereafter, the [Glu] decreased below the control (37 ± 3%), where it remained for as long as it was recorded whereas [Tau] varied around the control value (116 ± 6%). The [Gln] showed a similar pattern like [Glu] but relative changes in [Gln] were smaller. Millimolar concentration of NMDA microdialysed into the ventrobasal thalamus caused significant and sustained changes: a decrease of the [Gln] (50 ± 5%, p < 0.05) and an increase of the [Tau] (160 ± 12%, p < 0.05) during the 180 min of drug application (Fig. 1A). The [Glu] did not differ significantly from the control (100%) but the standard error was relatively high (41%) indicating possible changes in [Glu] masked by the rate of sampling (Fig. 1A). Application of ACSF containing 80 mM KCl significantly (p < 0.05) increased the [Glu] (201 ± 32%) and [Tau] (346 ± 45) whereas the [Gln] (21 ± 2%) was significantly decreased in the ventrobasal thalamus (Fig. 1B) as well as in the hippocampus (data not shown).
3.3. Effect of tPDC on [Glu], [Gln] and [Tau] in the hippocampus

Application of tPDC (0.5–3 mM) significantly ($p < 0.05$) increased [Glu] in the hippocampus and the extent of the increase was dependent on the concentration of tPDC: 0.5 mM: $341 \pm 12\%$; 1 mM: $915 \pm 39\%$; 3 mM: $783 \pm 145\%$ (Fig. 2). The [Gln] and [Tau] did not change when 0.5 mM tPDC was applied (Gln: $108 \pm 7\%$; Tau: $101 \pm 3\%$) and increased during or 3 mM tPDC application (1 mM: Gln: $182 \pm 15\%$; Tau: $159 \pm 9\%$; and 3 mM: Gln: $159 \pm 9\%$, Tau: $136 \pm 6\%$; Fig. 2). Phase-space representation of the measured [Glu], [Gln] and [Tau] triplets in the absence and presence of tPDC and the data modeling fitted by the maximal likelihood estimation represented as a surface is shown in Fig. 3.

3.4. Determination of variables controlling the dynamics of the three-compartment systems in the brain

First, the partial correlations were calculated. Fig. 4. shows the significant ($p < 0.05$) partial correlations coefficients highlighted. The fact that we found low as well as high partial correlation coefficients indicated the existence of factors. Supposing different strength of correlations between the measured variables (changes of [Glu], [Gln] and [Tau]) we searched for latent variables (factors).

Factor analysis was carried out on the three variables. Factor loadings were interpreted as correlations between the respective variables and the factors (Table 1). A conventional restriction is to choose the length of the factor to be equal to 1 (minimum eigenvalue = 1). In this case, factor analysis yielded a single factor which explained 68.78 and 66.82% variance for both conditions (inhibition of uptake by tPDC: Table 1 A and B panels; control: Table 1 C and D panels), respectively. To increase the variance explained and achieve a more detailed pattern of loadings, we selected a factor length of 0.5 (minimum eigenvalue = 0.5) and applied varimax factor rotation. In this case two factors presented themselves (Table 1). When tPDC was applied (Table 1 A), factor loadings showed factor 1 and factor 2 related mainly to changes of [Glu] and [Gln], respectively. Both factors 1 and 2 obeyed tight association with changes of [Tau]. Under control conditions (Table 1 C), factor loadings showed factor 1 related equally to changes of [Gln] and [Tau] while factor 2 is related mainly to changes of [Glu].

3.5. Effects of Co$^{2+}$ on background, high-K$^+$- and tPDC-induced changes of [Glu], [Gln] and [Tau] in the hippocampus

During Co$^{2+}$ infusion (10 mM CoCl$_2$ in ACSF) significant elevations of [Gln] occurred (Co$^{2+}$ experiment: $162 \pm 56\%$, $p = 0.0001$; Co$^{2+}$ + KCl experiment: $169 \pm 73\%$, $p = 0.0012$; Co$^{2+}$ + tPDC experiment: $146 \pm 43\%$, $p = 0.0001$; Fig. 5). Potassium evoked [Glu] responses were partially inhibited by inclusion of 10 mM CoCl$_2$ in the high-K$^+$ (80 mM KCl in ACSF) dialysate (Co$^{2+}$ + KCl experiment: $67.5 \pm 41\%$, $p = 0.0085$; Fig. 5) whereas [Tau] remained slightly elevated (Co$^{2+}$ + KCl experiment: $134 \pm 37\%$, $p = 0.0085$; Fig. 5). Co$^{2+}$ infusion significantly elevated tPDC (0.5 mM in ACSF) evoked [Glu] responses (Co$^{2+}$ + tPDC experiment: $179 \pm 52\%$, $p = 0.0003$) whereas [Tau] unchanged (Co$^{2+}$ + tPDC experiment: $93.1 \pm 18\%$, $p > 0.05$).

4. Discussion

Searching for main correlations between changes in [Glu], [Gln] and [Tau] with the use of partial correlation and factor analyses we found, that in control conditions, the primary independent variable is related to increases of [Gln] which is coupled to increases of [Tau] whereas the secondary is related to changes of [Glu]. Decreases of [Gln] with increases of [Tau], were quantitatively more important under persistent depolarization by either NMDA or high-K$^+$. Similar responses to amphetamine in the prefrontal cortex of awake rats were also reported recently (Del Arco et al., 1998). The underlying neuronal uptake of [Gln] and release of [Tau] thereupon, acting independently of Glu changes, can be considered typical of hypoxic-motic conditions (Lehman, 1990; Schousboe and Pasantes-Morales, 1992). One may conclude that the mammalian brain responds to excessive excitation primarily by neuronal swelling, however mechanisms by which neuronal swelling and changes of extracellular Glu occur are different. In accordance, inhibition of
Fig. 3. Phase-space representation of the extracellular concentrations of amino acid vector values ([Glu], [Gln] and [Tau] triplets) as measured in the absence and presence of tPDC and the data modeling fitted by the maximal likelihood estimation represented as a surface. A: control. B: tPDC (0.5–3 mM).
Ca\(^{2+}\) influx significantly potentiated the potassium responses for [Tau] and [Gln] whereas potassium evoked [Glu] responses were significantly attenuated. Others (Lo et al., 1998) have classified potassium evoked responses for [Gln], [Tau] and [Glu] as calcium-independent and calcium dependent, respectively, by inclusion of \(\alpha\)-conopeptide MVIIC or high-Mg\(^{2+}\) and omission of Ca\(^{2+}\).

In vitro results demonstrated that high-K\(^+\) induces delayed \([\text{H}]\)taurine release from cerebral cortex neurons in culture as well as from resealed cerebrocortical nerve endings (Schousboe et al., 1990). Also, we observed a delayed increase of [Tau] as compared to [Glu] during NMDA application. Reportedly, the release of Tau evoked by NMDA receptor activation is store-operated in hippocampal slices (Menendez et al., 1993), suggesting that the observed neuronal increase of [Tau] could possibly be due to mobilization of intracellular Ca\(^{2+}\) stores in vivo.

When Glu uptake is blocked, separate independent variables can be related to increases of [Glu] and [Gln], whereas increases of [Tau] are dependent on both. The possibility of neuronal Glu release, secondary to neuronal uptake of glial Gln (Shen et al., 1998 and references cited therein) can be excluded on the basis of factor analysis. Thus, separate [Glu]- and [Gln]-coupled [Tau] increases in the extracellular compartment may indicate neuronal as well as glial swelling (Schousboe and Pasantes-Morales, 1992). Inhibition of Ca\(^{2+}\) influx by Co\(^{2+}\) significantly potentiated the tPDC responses for [Glu]. Others (Fallgren and Paulsen, 1996) have found dihydrokainate evoked responses for [Glu] as calcium-independent by inclusion of high-Mg\(^{2+}\) and omission of Ca\(^{2+}\). Mechanism by which the [Gln] was increased in the presence of Ca\(^{2+}\) is not clear.

![Fig. 4. Partial correlations between the measured extracellular concentrations of Glu, Gln and Tau. The highlighted figures are significant \(p < 0.05\) values. The value, 0.295, represents a borderline significance, \(p = 0.052\).](image)

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<td>[Gln]</td>
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\(a\) Upper panels A and B: uptake inhibition by tPDC. Lower panels C and D: control.
of the Glu uptake inhibitor are unknown at present, however, one may suspect that of glial origin (Huang et al., 1994). It is to note in this respect that inhibition of ischemia-induced Glu and Tau release by an astrocytic Glu uptake inhibitor in vivo was reported recently (Seki et al., 1999).

Astrocytically derived Gln is not only a precursor for transmitter amino acids but is also an energy substrate for neurons in vivo (Hassel et al., 1995). The increased [Tau] could also act neuroprotectively (Pazdernik et al., 1990; Saransaari and Oja, 1997, 1999; Zielińska et al., 1999; and references cited therein). Taking these results together one may suggest that by inhibiting the glial Glu transporter, the increase of both amino acids, Tau and Gln, in the extracellular compartment can possibly be beneficial, counteracting long-lasting increase of excitability and possible excitotoxic effects of elevated [Glu] in the mammalian brain.

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