IN VIVO COORDINATED INTERACTIONS BETWEEN INHIBITORY SYSTEMS TO CONTROL GLUTAMATE MEDIATED HIPPOCAMPAL EXCITABILITY

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
Ado	Adenosine
AMPA	α- amino-3-hydroxy-5-methylisoxazole-4-propionate
Asp	Aspartate
AUC	Area under the curve
ChAT	choline *O*-acetyltransferase
DBB	Diagonal band of Brocca
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
KW	Kruskal-Wallis
MK-801	Dizocilpine maleate
MS	Medial septum
NGS	Normal goat serum
NSE	Neuron specific enolase
OPA	*O*-phthaldialdehyde
PBS	Phosphate buffered saline
QNB	Quinuclidinil benzilate
Ser	Serine
Tau	Taurine

ABSTRACT

We present an overview of the long term adaptation of hippocampal neurotransmission to cholinergic and GABAergic deafferentation caused by excitotoxic lesion of the medial septum. Two months after septal microinjection of 2.7 nmol α- amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), a 220% increase of $GABA_A$ receptor labelling in the hippocampal CA3 and the hilus was evidenced, and also changes in hippocampal neurotransmission characterised by *in vivo* microdialysis and HPLC. Basal amino acid and purine extracellular levels were studied in control and lesioned rats. *In vivo* effects of 100 mM KCl perfusion and adenosine A_1 receptor blockade with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) on their release were also investigated. In lesioned animals GABA, glutamate and glutamine basal levels were decreased and taurine, adenosine and uric acid levels increased. A similar response to KCl infusion occurred in both groups except for GABA and glutamate, which release decreased in lesioned rats. Only in lesioned rats, DPCPX increased GABA basal level and KCl-induced glutamate release, and decreased glutamate turnover. Our results evidence that an excitotoxic septal lesion leads to increased hippocampal $GABA_A$ receptors and decreased glutamate neurotransmission. In this situation, a coordinated response of hippocampal retaliatory systems takes place to control neuron excitability.

Keywords: glutamate, GABA, taurine, adenosine, excitotoxicity, microdialysis.

Running title: hippocampal neuromodulator interactions

Neurochemical features of normal brain aging, in particular those associated with memory impairments and neurodegenerative diseases, have generated intense research activities over the last decades. As a classical model, lesions of the basal forebrain causing disruption of the septohippocampal pathway have consistently mimicked some of these impairments in cognitive processing and revealed reductions in cholinergic markers in the hippocampus (Waite et al. 1994; Zapata et al. 2000). However, it appears evident that age-related alterations in brain cholinergic activity cannot fully account for all those cognitive deficits and that other neurotransmitters are also involved. For instance, a reduction in the number of GABAergic neurons located in the medial septum (MS) and the diagonal band of Brocca (DBB) projecting to the hippocampus also contribute to some of these cognitive impairments (McAlonan et al. 1995; Venero and Hefti 1998). But among all neurotransmitters, glutamate (Glu) is considered of special relevance given its involvement in memory-related phenomena, such as long-term potentiation, and its central role in excitotoxicity.

The excitotoxic hypothesis of CNS injury is based on an excessive glutamate-mediated excitation that stands out as a critical factor common to a variety of neurological disorders (Obrenovitch and Urenjak 1997; Obrenovitch et al. 2000; Arundine and Tymianski 2004). It is generally accepted that excitotoxic injury to neurons results from excessive inward currents of Ca^{2+} and Na^{+} through glutamate-operated ion channels, supplemented by release of Ca^{2+} from intracellular stores subsequent to metabotropic Glu receptor activation, leading to intracellular Ca^{2+} overload (Arundine and Tymianski 2004). Excitotoxicity also involves an imbalance of transmembrane Na^{+}, Cl^{-} and K^{+} gradients, cell swelling (Katayama et al. 1995) and formation of calcium precipitates in most CNS areas (Nitsch and Scotti 1992; Mahy et al. 1995; Robledo et al. 1999). The complexity of

the mechanisms involved in glutamatergic neurotransmission makes it already apparent that a number of abnormalities, either pre-synaptic, post-synaptic or glial, alone or in combination, can be excitotoxic (Obrenovitch et al. 2000).

Given these toxic effects, adaptations that act to decrease synaptic accumulation of Glu can potentially be protective. A number of these protective mechanisms are conducted to inhibit Glu release during insults, and some of them involve retrograde signalling of inhibitory neurotransmitters and neuromodulators. Thus, GABA, taurine (Tau) and adenosine (Ado) present a retaliatory activity that has shown neuroprotective properties during Glu-mediated neuronal insults (see Sapolsky 2001 for a review). For example, GABAergic retrograde signalling in hippocampus is multisynaptic, i.e. collaterals from glutamatergic pyramidal terminate on GABAergic interneurons which, in turn, inhibit glutamatergic neurons (Saransaari and Oja 1997). Astroglial Tau release during insults derived from potassium and water uptake decreases presynaptic neuronal excitability by increasing chloride influx (Saransaari and Oja 2004). Ado neuroprotective activity is accomplished through binding to A_1 adenosine receptors linked by G proteins to both calcium and potassium channels (Pearson et al. 2004). In addition, the fine adaptation of the Glu-glutamine (Gln) cycle to neuronal activity and suffering is important to avoid excessive synaptic Glu and neuronal death (García and Massieu 2003; Massieu et al. 2003; Ramonet et al. 2004).

Despite the general agreement about the interdependency of all these processes to avoid excessive synaptic Glu, to our knowledge, the coordinated adaptation of the major retaliatory systems to a chronic brain lesion has not been investigated. To better understand these interactions in the hippocampus, we firstly characterised the α- amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-induced lesion in the MS in terms of

neuronal and receptor modifications. Then, *in vivo* alterations of hippocampal amino acid and purine release were investigated, with especial focus on A_1 receptor-mediated Ado modulation of excitatory and retaliatory neurotransmission.

MATERIALS AND METHODS

Materials

AMPA, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), *O*-phthaldialdehyde (OPA), amino acids, purines, mouse monoclonal anti-parvalbumin antibody, and rabbit policlonal anti-neuron specific enolase (NSE) were obtained from Sigma (St. Louis, MO, USA). Mouse monoclonal anti-choline *O*-acetyltransferase (ChAT, EC 2.3.1.6) antibody was purchased from Chemicon (Temecula, CA, USA). [^{3}H] quinuclidinil benzilate (QNB) was from Du Pont-NEN, [^{3}H] dizocilpine maleate (MK-801) was from ARC (Sant Louis, MO, USA) and [^{3}H]muscimol was from Amersham (Bucks, UK). CMA/12 microdialysis probes (membrane length 4 mm, outer diameter 0.64 mm, cut off 20 kDa) were from Carnegie Medicine (Sweden).

Animals

Adult male albino Sprague-Dawley rats (Charles River, Barcelona) weighing between 250-300 g were used in the experiments. They were kept on a 12 hour light/12 hour dark cycle, and were housed with free access to food and water. Animals were manipulated according to the European legislation (86/609/EEC) for animal handling and experimentation. Procedures were approved by the Ethic Committee of the Universitat de Barcelona, under supervision of the Generalitat de Catalunya. All efforts were made to minimise animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Surgery

Rats were anaesthetised with equithesin (a mixture of chloral hydrate and sodium pentobarbitone; 0.3 ml/100 g body wt, i.p.), and placed on a Kopf stereotaxic frame with the incisor bar set at -3.3 mm. Intracerebral injections aimed at the MS were performed through stainless steel cannulae connected by portex tubing to a Hamilton syringe activated by an infusion pump (CMA/100; Carnegie Medicine, Sweden). The cannula was inserted at an angle of 10º to a vertical line and positioned 0.7 mm rostral to bregma, 1.1 mm lateral, and 5 mm ventral from dura (Paxinos and Watson 1986).

Different groups of rats received either 0.5 µl of 5.4 mM AMPA dissolved in 50 mM phosphate buffer saline (PBS, pH 7.4) (n = 34), 50 mM PBS (sham, n = 11), or no treatment (n = 6). All injections were made over a period of 5 min, and the needle was left in place for an additional 5 min to allow for passive diffusion, and to prevent spread of the excitotoxin up the cannula track upon removal. The dose of AMPA was selected according to previous studies showing neurodegenerative processes (Ramonet et al. 2004; Rodríguez et al. 2004). As values from sham and non-operated rats did not significantly differ in any of the parameters studied, they were pooled in a group (n=17) called control.

Microdialysis procedure

Two months after the injection of AMPA, 40 animals (AMPA n= 28, control n= 12) were re-anaesthetised, placed on a stereotaxic frame, and microdialysis guide cannulae (Carnegie Medicine) were implanted unilaterally in the ventral hippocampus (5.2 mm caudal to bregma, ± 4.6 mm lateral to bregma, and 2.46 mm ventral from dura). In half of the animals, the probes were implanted in the right hippocampus, and in the other half they were placed in the left hippocampus.

Twenty-four hours after implantation of the guide cannulae, CMA/12 microdialysis probes were inserted into the hippocampus and 12 hours later they were connected to a microinjection pump (KDS220, Kd Scientific, Boston, MA, USA) as previously described (Boatell et al. 1995). The probes were continuously perfused with artificial CSF (126.5 mM NaCl, 2.4 mM KCl, 1.1 mM $CaCl_2$, 0.83 mM $MgCl_2$, 27.5 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 0.5 mM Na_2SO_4, and 5.8 mM glucose; pH 7.4.) at a rate of 0.2 μl/min for 12 hours. This procedure was carried out in order to ensure stability in basal amino acid release, and in order to minimise astroglial reaction. Then the perfusion rate was changed to 2.0 μl/min and, after a 30-min washout period, samples were collected in ice-cooled eppendorfs every 20 min during 460 min. Half of the total amount of each sample was used for amino acid detection and the other half for purine detection. Samples were then frozen immediately and stored at -80ºC until analysis. In these conditions basal amino acid and purine release was similar to that described elsewhere (Boatell et al. 1995; Britton et al. 1996).

All rats (AMPA n=28, control n=12) were initially perfused with CSF for 80 min (CSF 1), then with a high concentration of KCl (100 mM, KCl 1) for 40 min and again with CSF for 80 min (CSF 2). Subsequently, half of the rats (AMPA n=14, control n=6) were perfused with CSF for 40 min, then with 100 mM KCl for another 40 min and CSF for another 180 min. The other half (AMPA n=14, control n=6) were perfused with 0.1 μM DPCPX for 40 min (DPCPX), then with KCl for 40 min (KCl 2) and CSF for another 180 min (CSF 3).

The DPCPX concentration chosen was in the range of A_1 receptor specific saturation (Kaku et al. 1994; Lucchi et al. 1996) and its perfusion condition was selected to block the A_1 receptor in basal and KCl-stimulated initial states. Six microdialysis probes

were selected to test the probe recovery as previously described (Ballarín et al. 1991). The mean relative probe recovery for each studied compound were: 24 ± 3% for aspartate (Asp), 25 ± 2% for GABA, 22 ± 2% for Glu, 26 ± 2% for Gln, 20 ± 2% for glycine (Gly), 27 ± 3% for Tau, 20 ± 1% for Ado, 13 ± 1% for hypoxantine, 20 ± 2% for inosine and 50 ± 5% for uric acid. Extracellular concentrations of neurotransmitters were estimated according to these values.

Amino acid and purine analysis

Extracellular levels of Glu, Gln, GABA, Tau, Asp and Gly were analysed by HPLC with fluorescence detection (Waters, Barcelona, Spain), after derivatisation with OPA as previously reported (Boatell et al. 1995). Serine (Ser) was also included in our analysis as a control for non-specific changes. The content of amino acids in dialysate samples was quantified from peak areas using homoserine as an internal standard and a mixture of all the studied amino acids as external standard. The relation between peak areas and amount of amino acid injected was linear in the range of the dialysate sample concentrations.

Extracellular levels of Ado, inosine, hypoxantine and uric acid were analysed by HPLC with ultraviolet detection (Waters, Barcelona, Spain) at 260 nm, by modification of previous protocols (Ballarín et al. 1995). In order to maximise the detection of Ado, two 20-min samples were systematically pooled and directly injected on a C_{18} phase reverse column (μ-boundapack length 30 cm, 10 μm i.d.). Chromatograms were developed in isocratic 0.9 ml/min flow of 0.1 M Tris-HCl (pH 8.15) containing 10 % (v/v) methanol. The content of purines was quantified from peak areas using a mixture of all of them as external standard. The relation between peak areas and amount of purine injected was linear in the range of the dialysate sample concentrations.

Histochemistry and *in vitro* autoradiography procedures

Two months after the injection of AMPA, 11 animals (AMPA n= 6, control n= 5) were used for the *in vitro* autoradiography studies. Rats were anaesthetised, decapitated and the brain quickly removed and frozen with dry ice. Sections were then obtained at the level of the MS (+0.7 mm to bregma) and dorsal hippocampus (-3.3 mm to bregma). Adjacent sections were processed for *in vitro* autoradiography, for immunohistochemistry (see below), for acetylcholinesterase (AChE, EC 3.1.1.7) histochemistry (Koelle and Friedenwald 1949), or for Nissl standard staining with Cresyl Violet.

Muscarinic receptors were labelled with [^{3}H]QNB as described elsewhere (Nagasawa et al. 1994). In brief, sections were incubated for 90 min in 0.015 M phosphate buffer (pH 7.4) containing 1 nM [^{3}H]QNB (43 Ci/mmol). Non-specific binding was determined in presence of 1 μM atropine sulphate. $GABA_A$ receptor autoradiography was performed using [^{3}H]muscimol as previously described (Ban et al. 1994; Schliebs et al. 1997). In brief, after 15 min washing in an ice cold 0.05 M Tris-citrate buffer (pH 7.4) containing 2.5 mM $CaCl_2$, sections were incubated for 30 min in presence of 12 nM [^{3}H]muscimol (16.4 Ci/mmol) in the indicated buffer solution. The non-specific binding was determined by incubation with 100 μM GABA. Binding to the non-competitive site of the NMDA receptor complex was measured according to the procedure previously described (Beaton et al. 1992) and slightly modified by Petegnief et al. (1999). A 2-h incubation was performed in presence of 10 nM [^{3}H]MK-801 (20 Ci/mmol), at 0-4ºC in a 0.005 M Tris-acetate buffer (pH 7.9) containing 10 mM Gly and 10 mM Glu. The non-specific binding was determined in the presence of 10 mM (+)-MK-801. Rinses were performed in ice-cold 5 mM Tris-acetate containing 5 mM CGS-19755.

After washes in ice-cold appropiate buffer, slides were dried under a stream of air and apposed to ^{3}H-sensitive films (Hyperfilm^{TM}, Amersham) for two to six weeks. Films were then developed and densitometrically analysed after calibration with plastic scales (^{3}H-microscales, Amersham) using a computer–assisted image analysis system (OPTIMAS®, BioScan Inc., Washington, USA). The average brain protein content was 8%. For each brain, eight sections were processed for total binding and two others for non-specific binding at both stereotaxic levels. AChE staining was densitometrically quantified using the same image analysis system. Morphometric evaluation was performed by measuring the hippocampal subfield areas in Nissl stained sections with the same image analysis system.

Immunohistochemistry procedure

Sixteen hours after microdialysis, each rat was anaesthetised and transcardially perfused, and then, the brains were isolated and frozen as previously described (Saura et al. 1995; Rodríguez et al. 2004). Cryostat sections (12 μm) were obtained at the level of the MS (+0.7 mm to bregma), dorsal (-3.3 mm to bregma) and ventral (-5.2 mm to bregma) hippocampus. Adjacent sections were either stained with Cresyl Violet in order to assess the septal lesion, and the microdialysis probe positioning, or processed for immunohistochemical methods. Only those animals properly lesioned and with correctly implanted probes were included in the study. Four rats were eliminated because of incomplete lesions. Also, adjacent sections of rat brain from the autoradiography study were processed for immunohistochemistry.

Immunohistochemistry was carried out by the avidin-biotin-peroxidase method. Sections were incubated at 4ºC separately with either, mouse monoclonal anti-parvalbumin diluted 1:1500 (v/v), mouse monoclonal anti-ChAT diluted 1:400 (v/v), or rabbit

policlonal anti-NSE diluted 1:400 (v/v) as previously described (Rodríguez et al. 2001; Ramonet et al. 2004). Cell counts were made at x100 magnification using an ocular grid. The total number of parvalbumin and ChAT-imunopositive neurons was counted in the MS-DBB region of all animals at the level of +0.7 mm to bregma. The border between MS and DBB was delimitated according to Mahy et al. (1995). NSE-imunopositive neurons were counted in the hippocampus at -3.3 mm to bregma. The cell numbers were corrected for section thickness using the method of Floderus (1944).

Data analysis

For each parameter, Kurtosis and Skewness moments were calculated to test the normal distribution of data. When normality was reached, Student's t-test and one-way ANOVA, followed by the LSD post-hoc test, were performed to compare the data. Otherwise, values of all groups were compared using the non-parametric Mann-Whitney U test and Kruskal-Wallis (KW) test. For histological and *in vitro* autoradiography studies, differences between control and AMPA lesioned rats were analysed using the Student's t or the Mann-Whitney U tests. For the microdialysis study, the effects of the lesion on basal and KCl-evoked neurotransmitter release were assessed with a two-way ANOVA: 1 between subject factor: *lesion* with 2 levels (AMPA and control), and 1 within subject factor: *treatment* with 2 levels (CSF, KCl). Following significant interactions, one-way ANOVA or KW test were used for individual comparisons. The effects of treatment with DPCPX on basal and KCl-evoked neurotransmitter levels in lesioned and control rats were evaluated with a three-way ANOVA: 1 between subject factor: *lesion* with 2 levels (AMPA and control), 1 between subject factor: *DPCPX* with 2 levels (CSF and DPCPX), and 1 within subject factor: *KCl* with 2 levels (CSF and KCl). Since each factor only had 2 levels, when significant two or three-way interactions were observed, individual

comparisons were performed using one-way ANOVAs. In addition to extracellular neurotransmitter content analysis, Glu and Ado turnover were also calculated by estimating the Gln/Glu and Uric acid/Adenosine ratios respectively for each time point. Then, the area under the curve (AUC) was calculated as the summatory of the semiareas for each treatment in order to compare the differences between groups, which were assessed by performing the Student's t test. In all cases, $p < 0.05$ was considered as significant. Results are expressed as mean ± SEM.

RESULTS

Assessment of the septal lesion

Two months after AMPA septal microinjection, neuronal damage was observed in the MS. Compared to control, cell counts showed a reduction of 70 ± 4% in ChAT-immunopositive neurons, whereas cholinergic neurons of the horizontal limb of the DBB were not modified (Figure 1). Reduction in GABAergic neurons of the MS was evidenced by the decrease of parvalbumin-immunopositive neurons (54 ± 5%) (Figure 1). Significant 7% AChE staining reduction was observed in the MS but not in the DBB nor lateral septum (data not shown).

FIGURE 1

This MS lesion did not induced changes in the size of the hippocampal area at –3.3 mm *to bregma*, but a 14% reduction was detected at this level in the area size of CA1-CA3 pyramidal layers with respect to control. NSE-immunopositive neuronal counts evidenced a discrete mean 10% neuronal loss in these layers and in granular dentate gyrus. Significant 6% AChE staining reduction was only observed in the pyramidal layer of the hippocampal CA3 (data not shown).

Receptor labelling by *in vitro* autoradiography

[^{3}H]muscimol specific binding showed medium levels (between 1,187 and 2,193 fmol/mg prot) in the septal area, caudate putamen and the internal layers of frontal cortex, with higher values in the external layers of this last area (Figure 2). In the hippocampus, [^{3}H]muscimol binding was specially low in all CA3 sublayers and the hilus, with values lower than 1,000 fmol/mg prot. The remaining hippocampal subfields presented medium [^{3}H]muscimol binding levels. Non-specific binding was homogeneous and very low, representing less than 10% of the total binding. The septal area of MS-lesioned rats did not show any modification in the amount of [^{3}H]muscimol binding sites with respect to controls. The same was true for the other brain areas studied except for the hippocampal formation that presented a 220% and a 198% increase in the CA3 and the hilus respectively (Figure 2).

FIGURE 2

[^{3}H]QNB binding was high (between 2,099 and 5,629 fmol/mg prot) in most brain areas, specially in the cerebral cortex and all hippocampal CA1 subfields. Non-specific binding was homogeneous and low, amounting to less than 12% of total binding in cortical areas. In MS-lesioned animals no significant change of [^{3}H]QNB binding was found in any of the studied areas.

Specific [^{3}H]MK-801 binding was low (less than 1,000 fmol/mg prot) in the MS and the internal layers of the frontal cortex. Medium [^{3}H]MK-801 binding levels were found in the lateral septum and caudate putamen, whereas high levels (more than 2,000 fmol/mg prot) were present in the cerebral cortex and the hippocampal formation. Non-specific binding was homogeneous and low, representing less than the 15% of the total binding. In MS-lesioned animals, no significant changes were found in any of the studied areas.

Extracellular hippocampal neurotransmitter levels

Two months after the MS lesion, hippocampal extracellular levels of GABA, Glu, Gln, Tau, Asp, Gly, Ser, Ado, uric acid, inosine and hypoxantine were estimated by *in vivo* microdialysis and HPLC. Negligible changes were observed in the hippocampal Ser extracellular concentration along the study. No lesion effect was found in the Ser basal level nor with the KCl and DPCPX treatments. Perfusion with CSF containing 100 mM KCl induced in control and lesioned rats a significative increase of all other amino acids and purine contents, with a 20-min delayed response for GABA, Tau, Ado and uric acid. Return to basal values was rapid for all compounds except for Tau, which level remained high for 100 min. In absence of DPCPX treatment, no differences were observed in the levels reached after the first and the second 100 mM KCl perfusion in any of the studied compounds (figure 3).

Extracellular hippocampal GABA basal level was 40% decreased by the MS deafferentation and its increase caused by 100 mM KCl infusion was 34% lower than in control animals ($F_{1,24} = 8.44$, $p < 0.01$ for AMPA effect; $F_{1,24} = 73.89$, $p < 0.001$ for KCl effect, and $F_{1,24} = 6.15$, $p < 0.05$ for AMPA-KCl interaction). Perfusion with 0.1 μM DPCPX did not modify GABA level in control rats, whereas in lesioned animals a marked tendency to increase that did not reach statistical significance ($p < 0.57$) was observed (Figure 3).

FIGURE 3

With regard to Glu, its basal level was 30% reduced and its increase after KCl reduced 34% by the MS lesion ($F_{1,24} = 7.53$, $p < 0.01$ for AMPA effect; $F_{1,24} = 105.53$, $p < 0.001$ for KCl effect, and $F_{1,24} = 4.99$, $p < 0.05$ for AMPA-KCl interaction). Perfusion with 0.1 μM DPCPX induced a 68% increase in the Glu basal level of control rats and this effect was not modified by the MS lesion (Figure 3). However, a significant interaction

between KCl and DPCPX treatments was observed in lesioned rats ($F_{1,20} = 4.62$, $p < 0.05$), showing a differential effect of KCl perfusion before and after 0.1 μM DPCPX. In MS lesioned rats, 100 mM KCl administered after DPCPX induced a higher outflow of Glu that took longer to recover ($F_{1,12} = 7.80$, $p < 0.01$).

Basal hippocampal Gln level was 34% decreased by the MS lesion. The KCl-induced increase of this level was not modified by the lesion ($F_{1,25} = 7.02$, $p < 0.014$ for AMPA effect; $F_{1,24} = 65.34$, $p < 0.01$ for KCl effect, and no AMPA-KCl interaction). Perfusion with 0.1 μM DPCPX significantly increased Gln level in control ($p < 0.021$) and in lesioned rats ($p < 0.012$) to similar values in both groups (Figure 3). The ratio Gln/Glu calculated as an estimation of hippocampal Glu turnover evidenced that 0.1 μM DPCPX treatment produced a decrease in the AUC of Glu turnover ($p<0.043$) that was still present during the subsequent 100 mM KCl perfusion ($p < 0.04$) (Figure 4).

FIGURE 4

MS-lesion did not induced any significant effect in the hippocampal Tau basal and 100 mM KCl-induced levels. However, when each individual time point for CSF treatment was compared (Figure 3) a significant increase was found in lesioned rats with respect to controls. Perfusion with 0.1 μM DPCPX did not modify basal nor 100 mM KCl-induced levels of Tau in either group.

Basal level of Asp in the hippocampus was not significantly different in MS-lesioned rats (Figure 5). Perfusion with 100 mM KCl produced a similar increase in the concentration of extracellular Asp in both groups and 0.1 μM DPCPX did not significantly affect basal nor KCl-evoked levels in any of the groups. Similarly, the MS-lesion did not modify the Gly extracellular basal nor KCl-evoked levels in the hippocampus (Figure 5). Perfusion with 0.1 μM DPCPX did not modify basal levels of Gly in either group, nor induced changes in the levels reached during the subsequent KCl perfusion.

FIGURE 5

No effect of the MS deafferentation was found in the extracellular hippocampal Ado levels when two-way ANOVAs were performed and the same was true for the 100 mM KCl treatment. However, one-way ANOVAs between groups receiving a similar treatment revealed a 60% higher Ado basal level in lesioned rats ($F_{1,6} = 6.07$, $p < 0.05$) and a 26% increase following 100 mM KCl perfusion ($F_{1,6} = 9.14$, $p < 0.05$) as compared to control animals (Figure 5). When 0.1 μM DPCPX was applied, a 63% increase in basal Ado level was evidenced in the lesioned group, which was not detected in control rats. In addition, 100 mM KCl perfusion following DPCPX increased Ado levels a 44% with respect to KCl treatment in control rats, but not in lesioned animals ($F_{1,14} = 42.58$, $p < 0.001$ for DPCPX effect, $F_{1,14} = 6.13$, $p < 0.05$ for the AMPA-DPCPX interaction and $F_{1,14} = 10.74$, $p < 0.01$ for AMPA-DPCPX-KCl interaction).

MS lesion resulted in 71% increase of basal hippocampal uric acid level but did not modify the 100 mM KCl response ($F_{1,17} = 13.32$, $p < 0.002$ for AMPA effect; $F_{1,17} = 17.63$, $p < 0.0006$ for KCl effect, and no AMPA-KCl interaction). 0.1 μM DPCPX presented no significant effects on uric acid levels and the subsequent 100 mM KCl perfusion in either group (Figure 5). We also calculated the ratio uric acid/Ado as an estimation of hippocampal Ado turnover (Figure 4). MS lesion induced an increase in the AUC of Ado turnover in lesioned rats in basal ($p<0.05$) and 100 mM KCl ($p < 0.02$) conditions. This increase disappeared during the 0.1 μM DPCPX and its subsequent 100 mM KCl perfusion.

In several cases, extracellular levels of hypoxantine and inosine were not detectable due to values of inosine and hypoxantine < 30 nM. However when they could be detected no significant changes were observed between groups (data not shown).

DISCUSSION

Two months after the AMPA-induced MS lesion, characterised by a significant decrease in cholinergic and GABAergic afferences to the hippocampus, a huge increase of $GABA_A$ receptor content and a discrete neuronal loss are observed in the hippocampus. The cholinergic deafferentation reduces the presynaptic function but not the acetylcholine hippocampal level, which remains unvaried (Waite et al. 1994; Zapata et al. 2000). NMDA receptor activation has been involved in maintaining choline availability for acetylcholine synthesis when choline is in short supply (Zapata et al. 2000). Thus, one of the putative agents for the maintenance of that level is NMDA receptor, since we here demonstrate that its hippocampal content is not modified by the lesion, as it happens with Gly level, a co-agonist of the receptor. This adaptation may also involve a reduction in the degradation of acetylcholine, since the AChE activity is decreased in the CA3. Nevertheless, the excitotoxic MS-lesion also leads to a lack of KCl-induced acetylcholine release in the hippocampus (Waite et al. 1994; Zapata et al. 2000). This presynaptic deficit, in combination with the stability of the muscarinic receptor content here found, evidences a MS-lesion-induced dysfunction of the hippocampal cholinergic system.

We also demonstrate that hippocampal GABAergic neurotransmission is substantially reduced 2 months after the MS lesion. This reduction is accompanied by a similar decrease in Glu and Gln levels, whereas Tau, Ado and uric acid are increased. The lack of variation of Asp, Gly and Ser levels argues for the specificity of these modifications and suggests the presence of compensatory neurotransmitter interactions to control Glu activity and avoid neuronal damage. To our knowledge, this is the first study where Asp and Glu have a different response to brain injury, which must be taken into account in further studies on Asp neurotransmission.

The decrease in hippocampal basal GABA level is in agreement with a tonic septal GABAergic input to this structure (Freund and Buzsáki 1996) whose neuronal loss is also supported by the decrease in KCl-stimulated GABA release observed in lesioned rats. Although one must consider that KCl does not mimick a purely neuronal stimulus and that the induced neurotransmitter release can be from a non-neuronal origin or from spreading depression effects. The enhanced $GABA_A$ receptor content in the CA3 layer and the hilus may reflect a compensatory mechanism to maintain, as possible, the functionality of this afferent connection. Additionally, the lack of excitatory cholinergic transmission (Zapata et al. 1998; Zapata et al. 2000) may also have contributed to the decrease in basal GABA outflow, since acetylcholine modulates GABAergic interneuronal activity through its action on muscarinic receptors (Freund and Buzsáki 1996), which labelling remains unvaried after the lesion.

In short-term fimbria-fornix lesions, Glu outflow from the hippocampus is increased (Herrera et al. 1993), indicating a prevalent action of acetylcholine over the GABA inhibitory effect. This Glu increase may also occur initially after the AMPA MS microinjection. However after the long-term deafferentation, the basal level and the KCl-induced release of Glu are decreased, whereas the content and distribution of NMDA receptors remain unvaried. This neuroprotective adaptation may result from a coordinated response that, with time, reduces glutamatergic activity through increased Ado and Tau activities. As this takes place when GABAergic activity is compromised, these two neuromodulators would not only control basal Glu activity, but also the KCl-induced release in a coupled synergistic inhibitory response. As shown previously, adaptation of the Glu/Gln cycle with a reduction of glutaminase (EC 3.5.1.2) activity also participates in the reduction of synaptic Glu (Ramonet et al. 2004). In conjunction with the cholinergic

and GABAergic lesion, the resultant glutamatergic reduction would thus account for part of the cognitive impairment associated to the MS lesion, limiting for instance long-term potentation. The maintenance of NMDA receptors here found would reflect the intent to preserve this function. According to all this, cognitive studies have shown that the memory deficits dependent on the cholinergic system appears between 15 days (McAlonan et al. 1995) and one month (Paban et al. 2005) after the MS lesion. As the post-lesion time increases new mnemonic impairments are detected, being a putative result of non-cholinergic system decompensations (Paban et al. 2005).

Enhancement of Tau released from glial cells decreases presynaptic excitability by binding to the $GABA_A$ (O'Byrne and Tipton 2000; Louzada et al. 2004) and Gly (Mori et al. 2002) receptors. By means of these interactions, Tau can help maintain the hippocampal inhibitory tone, as also suggested by the stability of Gly levels after the MS-lesion and the increased $GABA_A$ receptors. The enhanced hippocampal Ado level and turnover here described in lesioned rats may serve a neuroprotective role by inhibiting glutamatergic activity through binding to A_1 receptors (Simpson et al. 1992; Sapolsky 2001). In this situation, perfusion with DPCPX induced a major increase in basal Glu outflow, a reduction in Glu turnover, and a long-lasting increase in KCl-evoked Glu release. These results strongly suggest that the control exerted by Ado on basal and evoked Glu release through A_1 receptors is necessary to avoid further excitotoxic damage when cholinergic and GABAergic processes are compromised. CNS response to Ado is a balance between A_1 and A_2 receptor activation, and the stimulatory effects of A_2 receptors can be masked by activation of A_1 receptors (Okada et al. 1999; Dunwiddie and Masino 2001). Therefore, it is plausible that the increased KCl-evoked Glu release observed in our study with lesioned rats after DPCPX infusion could be due to the action of a new pathway

regulated by A_2 receptors. The A type of A_2 receptors are proposed not only to control Glu and acetylcholine release, but also to control the release of GABA and of noradrenaline, which are mostly insensitive to A_1 receptors (reviewed by Cunha 2005). However, in physiological conditions the effect of A_1 receptors must be prevalent in the hippocampus, since its density is considerably greater in this brain area (Fastbom et al. 1987; Svenningsson et al. 1999). With the MS lesion, this hippocampal A_1 receptor density may even increase, an effect that also would explain the DPCPX-induced Glu increase. On the other hand, the increase in Glu release may also be attributed to a decreased efficacy of the Glu transporter system, as described one month after fimbria-fornix lesions (Ginsberg et al. 1996), or to an increase in astroglial consumption of Glu to render energy (Haberg et al. 2000).

This Ado modulation of Glu activity also extends to the other studied systems. After MS lesion and following DPCPX perfusion, the extracellular concentration of GABA increases, reaching levels similar to those observed in control rats, suggesting an Ado modulation of GABA activity. This hypothesis is supported by previous data showing that following ischemia, Ado receptor agonists inhibit in the cortex the release of GABA (O'Regan et al. 1992). Furthermore, as shown in cardiovascular tissue (Andresen et al. 1999), Ado may modulate its own extracellular level through A_1 adenosine receptor stimulation, since DPCPX increases Ado level and normalises its enhanced turnover. In addition, an increased Ado turnover results in an enhancement of uric acid level. Uric acid, a potent antioxidant, preserves mitochondrial activity and acts as a neuroprotective agent against the rise in Glu-induced intracellular calcium concentration (Yu et al. 1998; Mahy et al. 1999).

We previously demonstrated that the long term MS lesion-induced neuronal loss in hippocampus is apoptotic with enhancement of neuronal glycolisis. This is linked to a cleavage of caspase 3, non necrotic processes, and Gln/Glu cycle displacement towards Gln production to reduce Glu synthesis (Ramonet et al. 2004). The reduction of extracellular Gln in MS-lesioned animals indicates that it could be expelled to vessels to reduce Glu released by injured neurons (Gorovits et al. 1997). In vessels, Gln exerts a vasodilatory effect through nitric oxide synthesis inhibition (Matés et al. 2002). Activation of adenosine A_1 receptors may also be involved in that Gln/Glu cycle modification through a modulation of Glu transport, since we observed a reduction in Glu turnover after perfusion with DPCPX. Activation of the apoptotic program evidenced by cleavage of caspase 3 requires energy consumption and underlies the neurodegenerative process (Nicotera et al. 1999). In this situation Glu signalling and neuronal energy metabolism are uncoupled and the retaliatory adaptations appear deficient.

In summary, we present evidence that, following long-term lesion of the MS, in the hippocampus, extracellular GABA levels are reduced and $GABA_A$ receptor content increased. Moreover, extracellular levels of Glu are decreased and blockade of A_1 adenosine receptors reverses this reduction. These results demonstrate a direct control by Ado on Glu neurotransmission through A_1 adenosine receptor stimulation, and unveil a coordinated interaction between GABA, Tau and Ado systems to control neuronal excitability. Further experiments need to be performed in order to determine the neuronal and glial contribution to this interactions, and to characterise the adenosine A_{2A} receptor involvement in the control of extracellular hippocampal levels of Glu and GABA.

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FIGURE LEGENDS

Figure 1: Number of neurons in the medial septum-diagonal band complex. Illustrative microphotographs of ChAT (a,b) and parvalbumin (Parv) (c,d) immunostained sections of control and AMPA rats. Histograms show the quantification of ChAT (e) and Parv (f) immunopositive cells in adjacent sections. Values are mean ± SEM. * $p < 0.05$; ** $p < 0.01$ different from control (Student's t-test), (n =12 control + 24 AMPA). Bar = 75 μm.

Figure 2: Specific *in vitro* binding of [^{3}H]muscimol to rat brain sections after long-term MS lesion. Distribution of the $GABA_A$ receptor labelling to rat brain sections at the level of MS and hippocampus of control and AMPA rats. Units are fmol/mg prot. (I-III) means I to III cortical layers; (IV-VI) means IV to VI cortical layers. *, $p<0.05$ related to control (Mann-Whitney U test), (n =5 control + 6 AMPA).

Figure 3: Longitudinal study of extracellular concentrations for GABA, glutamate (Glu), glutamine (Gln), and taurine (Tau) in the hippocampus after the long-term MS lesion. Extracellular hippocampal GABA (a), Glu (b), Gln (c) and Tau (d) levels were assessed by *in vivo* microdialysis with (right column) and without (left column) DPCPX administration. CSF 1, KCl 1 (100 mM), CSF 2, DPCPX (0.1 μM), KCl 2 and CSF 3 correspond to the microdialysis procedure phases (see for details Materials and Methods section). (n = 6 control + 14 AMPA for each experimental condition) * $p < 0.05$ different from control values at the same time point (Student's t-test).

Figure 4: Hippocampal effects of MS lesion on glutamate and adenosine turnover. Longitudinal study of extracellular Gln/Glu (a) and uric acid /adenosine (b)

ratios of control and lesioned rats. CSF 1, KCl 1 (100 mM), CSF 2, DPCPX (0.1 μM), KCl 2 and CSF 3 correspond to the microdialysis procedure phases (see Materials and Methods section). The inset histograms correspond to the percent change of the area under the curve with respect to the control group (100%; discontinuous line) during the treatments. * $p< 0.05$ different from control (Mann-Whitney U test).

Figure 5: Extracellular hippocampal concentrations of amino acids and purines after the long term MS lesion. Aspartate (a), Gly (b) Ado (c) and Uric acid (d) levels were assessed by *in vivo* microdialysis. CSF 1, KCl 1 (100 mM), CSF 2, DPCPX (0.1 μM), KCl 2 and CSF 3 correspond to the microdialysis procedure phases (see Materials and Methods section). The values for CSF 1, KCl 1 and CSF 2 represent pooled data of the rats dialysed in the two experimental conditions * $p < 0.05$, ** $p < 0.01$ different from CSF 1; # $p < 0.05$, ## $p < 0.01$ different from control values (LSD, post-hoc test).

FIGURE 1:

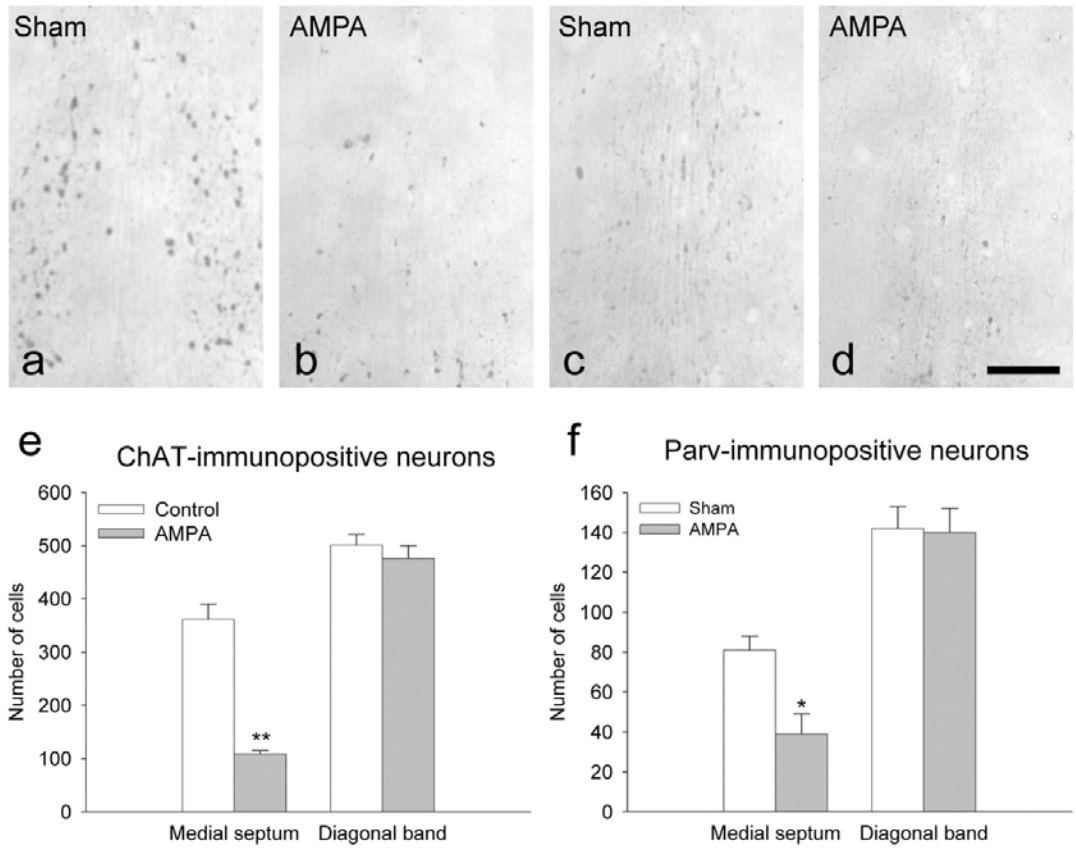

Sham
AMPA
Sham
AMPA
a
b
c
d
e
ChAT-immunopositive neurons
Control
AMPA
Number of cells
600
500
400
300
200
100
0
**
Medial septum
Diagonal band
f
Parv-immunopositive neurons
Sham
AMPA
Number of cells
160
140
120
100
80
60
40
20
0
*
Medial septum
Diagonal band

FIGURE 2:

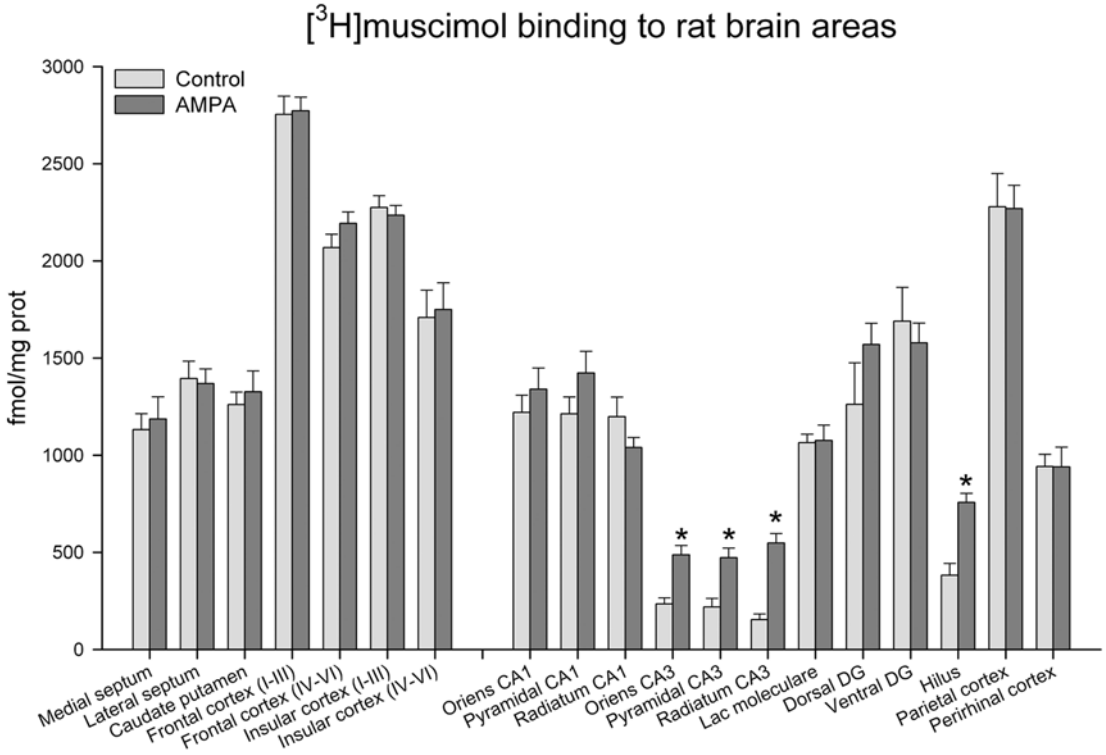

[3H]muscimol binding to rat brain areas
Control
AMPA
fmol/mg prot
3000
2500
2000
1500
1000
500
0
*
*
*
*
Medial septum
Lateral septum
Caudate putamen
Frontal cortex (I-III)
Frontal cortex (IV-VI)
Insular cortex (I-III)
Insular cortex (IV-VI)
Oriens CA1
Pyramidal CA1
Radiatum CA1
Oriens CA3
Pyramidal CA3
Radiatum CA3
Lac moleculare
Dorsal DG
Ventral DG
Hilus
Parietal cortex
Perirhinal cortex

FIGURE 3:

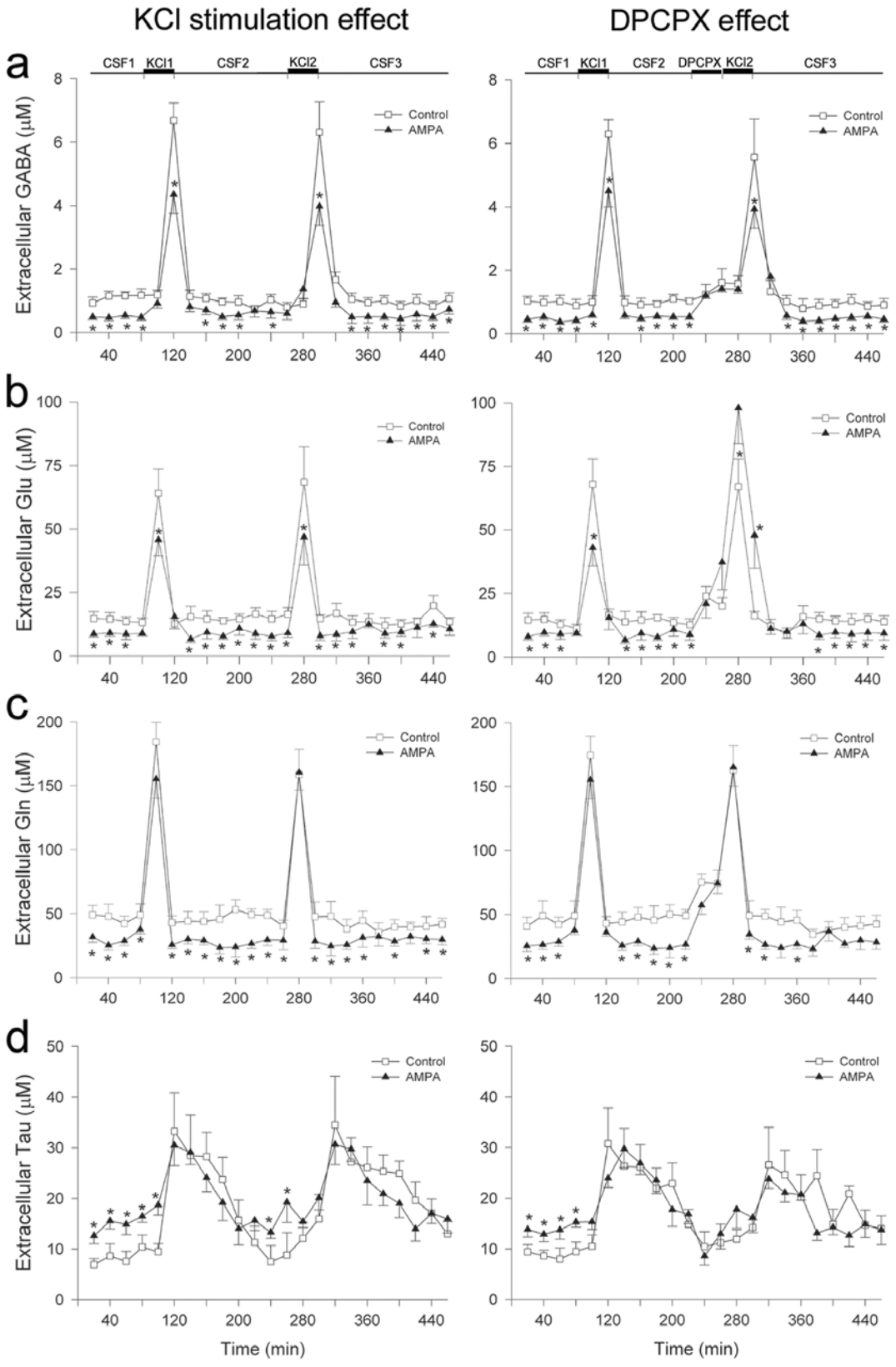

KCl stimulation effect
DPCPX effect
a
b
c
d
CSF1
KCl1
CSF2
KCl2
CSF3
DPCPX
Control
AMPA
Extracellular GABA (μM)
Extracellular Glu (μM)
Extracellular Gln (μM)
Extracellular Tau (μM)
Time (min)

FIGURE 4:

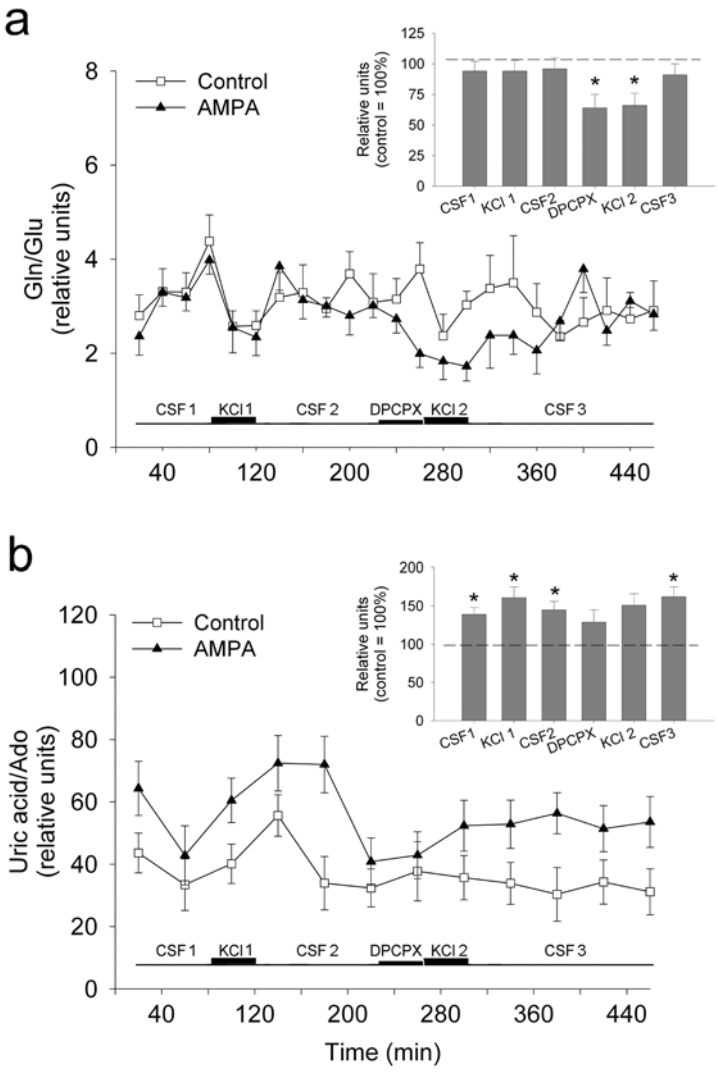
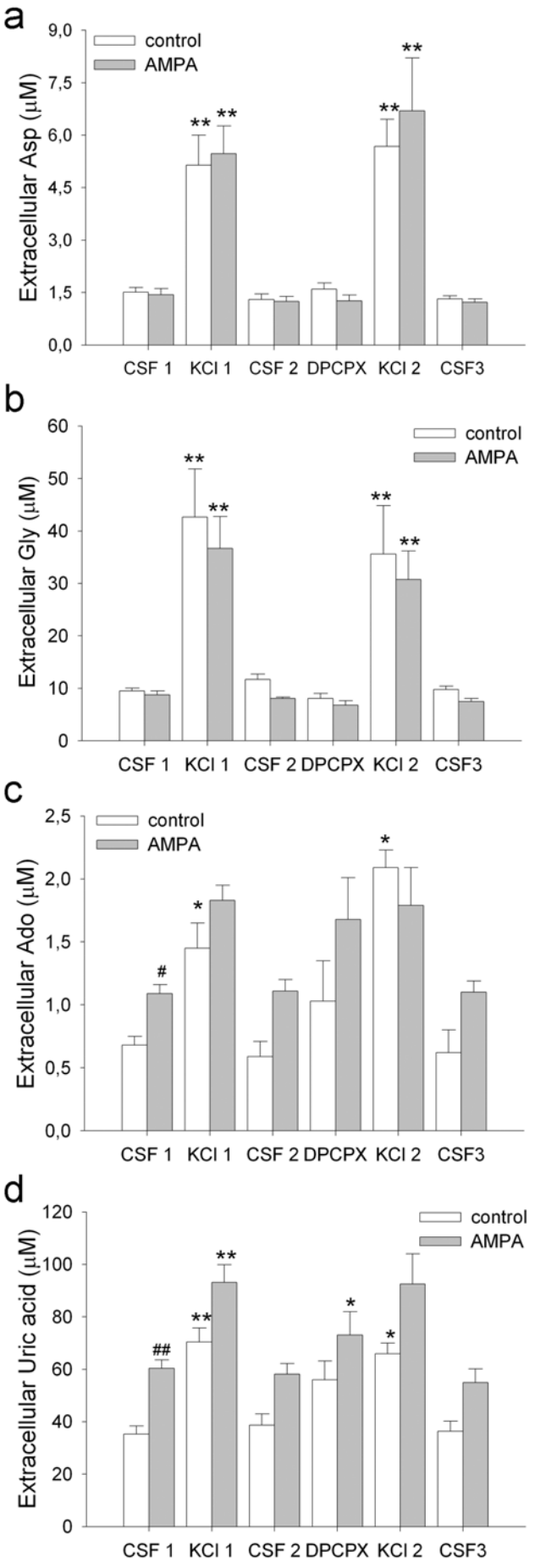

a
Control
AMPA
Gln/Glu
(relative units)
Relative units
(control = 100%)
CSF1
KCl 1
CSF2
DPCPX
KCl 2
CSF3
b
Uric acid/Ado
(relative units)
Time (min)

FIGURE 5:


a
control
AMPA
Extracellular Asp (μM)
9,0
7,5
6,0
4,5
3,0
1,5
0,0
**
**
**
**
CSF 1
KCl 1
CSF 2
DPCPX
KCl 2
CSF3
b
control
AMPA
Extracellular Gly (μM)
60
50
40
30
20
10
0
**
**
**
**
CSF 1
KCl 1
CSF 2
DPCPX
KCl 2
CSF3
c
control
AMPA
Extracellular Ado (μM)
2,5
2,0
1,5
1,0
0,5
0,0
#
*
*
CSF 1
KCl 1
CSF 2
DPCPX
KCl 2
CSF3
d
control
AMPA
Extracellular Uric acid (μM)
120
100
80
60
40
20
0
##
**
**
*
*
CSF 1
KCl 1
CSF 2
DPCPX
KCl 2
CSF3