Regulation of the glial glutamate transporter GLT-1 by glutamate and δ-opioid receptor stimulation

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Abstract The excitatory effect of presynaptically released glutamate is tightly regulated and terminated by high affinity sodium-dependent glutamate transporters. The regulation of the glial glutamate transporter GLT-1 is potentially important in synaptic modulation. Using astroglial cultures prepared from the rat cerebral cortex, we found that the δ-opioid receptor agonist [D-pen^2,D-pen^5]-enkephalin decreases and glutamate increases the expression of the GLT-1 transporter mRNA. Corresponding changes in the uptake kinetics were found after incubation for 48 h with the respective agonists when glial glutamate uptake was measured in primary astroglial cultures. The data suggest that long-term receptor activation induces alterations in glial glutamate uptake properties.

Key words: Astroglia; Glutamate; [D-pen^2,D-pen^5]-Enkephalin; δ-Opioid receptor; Primary culture

1. Introduction

Glutamate transport is coupled to the co-transport of Na^+ and the counter transport of K^+, with no dependence on Cl^-. Glutamate has an intimate coupling to the extracellular potassium levels and the membrane potential. For each glutamate anion transported into the cell, three Na^+ (or two Na^+ and one H^+) ions are co-transported into the cell, and one K^+ ion is transported out of the cell [1].

Three brain glutamate transporter cDNAs have so far been isolated: excitatory amino acid carrier (EAAC1) [2], glutamate/aspartate transporter (GLAST) [3] and glutamate transporter GLT-1 [4]. EAAC1 has been reported to be localized to neurons [2], and GLT-1 and GLAST are expressed in glial cells [3,5]. Astrocytes are active in the maintenance of the physiological conditions in and around the synaptic cleft. The astroglial expression of uptake carriers for amino acid neurotransmitters is well documented [6–10]. Glial cells express receptors for a number of neuromodulators, including glutamate and opioid receptors [11–16].

One of the most abundant transmitters is the excitatory amino acid glutamate. This transmitter is released from nerve terminals. Glutamate diffuses and activates postsynaptic receptors. Thereafter, the transmitter is taken up into nerve terminals and astroglial cells [17]. The synaptic activation is thereby terminated. Furthermore, it has recently been shown that transmitter uptake in astrocytes is acutely regulated by surface receptors in both a stimulatory and an inhibitory way [18,19].

Glutamate is thought of as the key mediator of excitotoxic brain damage in, for example, cerebral ischemia or hypoglycemia [20]. Furthermore, disturbed glutamate transport has been proposed to be associated with amyotrophic lateral sclerosis [21,22].

It has been shown that chronic opioid administration induces specific plastic changes in the brain, which in some manner counteract the processes underlying the acute actions of these drugs [23]. Knowledge of the mechanisms underlying effects elicited upon chronic receptor agonist administration is only fragmentary. We have previously shown that long-term opioid receptor stimulation induces changes within the signal transduction components in glial cells [23].

The present study was initiated in order to investigate whether changes in GLT-1 expression could be induced within astroglial cells in vitro as a result of long-term stimulation with an excitatory (glutamate) and an inhibitory (δ-opioid) neurotransmitter. The results obtained may represent a glial mechanism for preventing synaptic overexcitation in situations with elevated levels of extracellular glutamate, such as epilepsy.

2. Materials and methods

2.1. Tissue culture

Primary glial cultures were prepared from newborn rat (Sprague-Dawley) cerebral cortex (B&K Universal AB, Stockholm, Sweden). The glial cells were grown in Dulbecco's modified Eagle's medium (DME/high modified; Sera-Lab Ltd, Crawley Down, Sussex, UK) supplemented with 250 000 IU/l penicillin; 0.5% streptomycin and 20% (v/v) fetal calf serum (Gibco, Bio-Cult. Labs., Ltd., London, UK). The glial cells were grown in plastic Petri dishes (NUNC A/S, Roskilde, Denmark) for 14 days, at which time the cultures became confluent and contained approximately 0.350 mg protein per culture well. The cultures were characterized concerning the cellular composition and no neuronal cells were found using the present protocol [12]. The medium (pH 7.3) was changed three times weekly and every 24 h during incubation of the astroglial cultures with the δ-opioid receptor agonist [D-Pen2, D-Pen5]-enkephalin (DPDPE, 10^{-9}–10^{-5} M), glutamate (10^{-9}–10^{-5} M) or potassium (7.5–56 mM). The neuroactive substances were added to the experimental cultures at every medium change, while control cultures received only fresh medium. The treatments lasted for 48 h and were initiated so that all experiments were terminated on day 14 in vitro.

2.2. Immunohistochemistry

Rabbit polyclonal antiserum against glial fibrillary acidic protein (GFAp; DAKO, Stockholm, Sweden) was used at a dilution of 1:100. Secondary fluorescein conjugated donkey anti-rabbit IgG (Amersham) diluted 1:300 was used as a fluorescence label. The primary antiserum was tested at different dilutions. The specificity of the staining obtained with the polyclonal rabbit GFAp antiserum was

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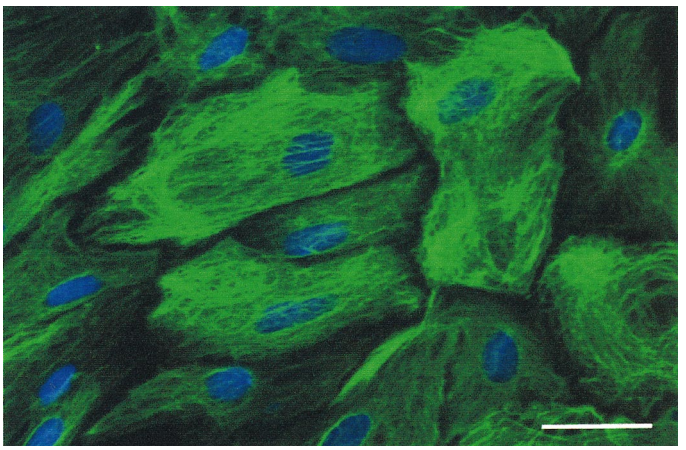

Fig. 1. Fluorescence micrograph of a representative astroglial culture consisting of type 1 astrocytes immunopositive for GFAp (green; FITC). Nuclei are stained blue with DAPI. The bar represents 5 μm.

compared with the staining obtained using normal rabbit immunoglobulins. Furthermore, the staining from incubations using only secondary antibodies was compared with the staining obtained after incubation with both primary and secondary antibodies.

2.3. Drugs

L-Glutamate (Sigma, Stockholm, Sweden) was used as a glutamate agonist. DPDPE (Sigma, Stockholm, Sweden) was used as a δ-receptor agonist. The doses used for DPDPE were based on previous experiments [14].

2.4. cDNA

A sequence corresponding to bases 438–1023 of GLT-1 [4] was subcloned from the base coordinates 398–985 of the GluT-1b cloned by Roginski and coworkers [24] (GenBank accession number U15098) into pBSK (Stratagene, La Jolla, CA, USA) and subsequently used to generate synthetic standard mRNA and antisense probe. ^{35}S-Labeled RNA antisense probes were generated from linearized plasmids (*Not*I) (Promega, Madison, WI, USA) using T7 (Promega, Madison, WI, USA) polymerase. Synthetic standard was generated using (*Sac*I) (Promega, Madison, WI, USA) linearized plasmid and SP6 (Promega, Madison, WI, USA) polymerase. The sense RNA was synthesized essentially as previously described [25] and quantified spectrophotometrically [26].

2.5. Northern blot

Total RNA was prepared essentially according to Chomczynski and Sacchi [27] with the addition of a proteinase K (Boehringer Mannheim, Bromma, Sweden) treatment. Briefly, cultured astroglial cells were scraped off and immediately homogenized in 4 M guanidinium thiocyanate. The homogenization solution was then diluted to 1 M guanidinium thiocyanate and proteinase K (1 mg/ml) was added. These cells were digested at 37°C for 45 min followed by phenol-chloroform extraction. 15 μg of total RNA was electrophoresed through an agarose (1%)/formaldehyde (2.2 M) gel containing ethidium bromide, transferred to Hybond-N (Amersham, Buckinghamshire, UK) with a vacuum transfer system (LKB, Stockholm, Sweden) and baked at 80°C for 3 h. The membranes were prehybridized at 60°C for 12 h in 50% formamide, 25 mM H_2NaPO_4, 25 mM HNa_2PO_4, 5×standard saline citrate solution (SSC) (20×SSC being 3 M NaCl, 0.3 M Na_3-citrate, pH 7.4), 0.1% $NaDodSO_4$ (SDS), 1 mM EDTA, 0.05% bovine serum albumin (BSA), 0.05% Ficoll, 0.05% polyvinylpyrrolidone (PVP), 200 μg/ml calf liver RNA, and 200 μg/ml salmon sperm DNA. Thereafter, hybridization was done in the prehybridization buffer for 18 h at 60°C with the addition of ^{32}P-labeled RNA probe. The membranes were then washed in 0.1×SSC and 0.1% SDS at 65°C. Autoradiography was done using Kodak XAR-5 film with intensifying screen (Eastman Kodak Co., Rochester, NY, USA).

2.6. Quantitative analysis of GLT-1 transporter mRNA

An RNase protection hybridization assay was used to quantify glutamate transporter mRNA. The solution hybridization RNase protection assay was performed essentially as described previously [23]. Briefly, four or five preparations consisting of 10–15 cultures each were used per agonist in each experiment, the cell material was pooled, and total RNA was extracted, as described above. The hybridization was done at 70°C for 24 h in 0.06 M NaCl, 20 mM Tris-HCl (pH 7.5), 4 mM EDTA, 0.1% $NaDodSO_4$, 10 mM dithiothreitol (DTT), 25% formamide, and ^{35}S-labeled probes. 100 μg herring sperm DNA (Sigma) was added and, thereafter, the samples were treated with 40 μg/ml RNase A and 2 μg/ml RNase T1 (Boehringer Mannheim, Bromma, Sweden). Protected radioactive probes were precipitated with trichloroacetic acid, washed with ethanol, and collected on glass-fiber filters (GF/C Whatman, Whatman International Ltd., Maidstone, UK) and measured for activity in a scintillation counter. In order to visualize the hybridization of labeled probe to mRNA a ribonuclease protection assay was performed using the RPA II kit (Ambion Inc., Austin, TX, USA). Briefly, samples of total cellular RNA were hybridized overnight with ^{35}S-labeled GLT-1 antisense probe and then digested with RNase according to the instructions provided by the manufacturer. The RNA:RNA hybrids were precipitated, resuspended and separated on a 6% polyacrylamide/8 M urea gel. The signal from the protected fragments was visualized using autoradiography using Kodak XAR-5 film with intensifying screen (Eastman Kodak Co., Rochester, NY, USA).

The amount of mRNA was calculated from a standard curve based on hybridization to known amounts of synthetic sense RNA. The total RNA content of the RNA preparations was analyzed spectrophotometrically [26]. The integrity of all RNA preparations was checked by electrophoresis in agarose (1%)/formaldehyde (2.2 M) gels with ethidium bromide. The results are expressed as pg mRNA/μg total RNA.

2.7. Astroglial glutamate uptake

One hour before the experiments, the culture medium was changed to serum-free Eagle's medium. The experiments were done according to Hansson and coworkers [9]. 1 μCi of ^{3}H-labeled glutamate (20.1 Ci/mmol) together with unlabeled glutamate was added per ml incubation medium to a final concentration range of 10^{-7}–10^{-3} M (12 different concentrations). The cells were incubated for 4 min with the ^{3}H-labeled solutions at 37°C on a heated plate. Thereafter, the cultures were immediately placed on ice and the medium was removed rapidly by suction. Samples were taken for liquid scintillation counting and excess radioactivity was removed by three rapid washes with non-radioactive ice-cold medium containing 35 mM Tris-HCl (pH 7.4), 140 mM NaCl, 3 mM KCl, 2.5 mM $MgCl_2$ and 20 mM glucose. The cells were scraped off and dissolved in 1 M NaOH, allowing samples to be taken for measurement of radioactivity. Protein content was measured according to Lowry et al. [28], with BSA as the standard.

The kinetic parameters V_{max} and K_m were then calculated using Lineweaver-Burk plots [29,30]. The transport velocity (V_{max}) of glutamate into the cells is expressed as nmol/mg protein/min. The affinity

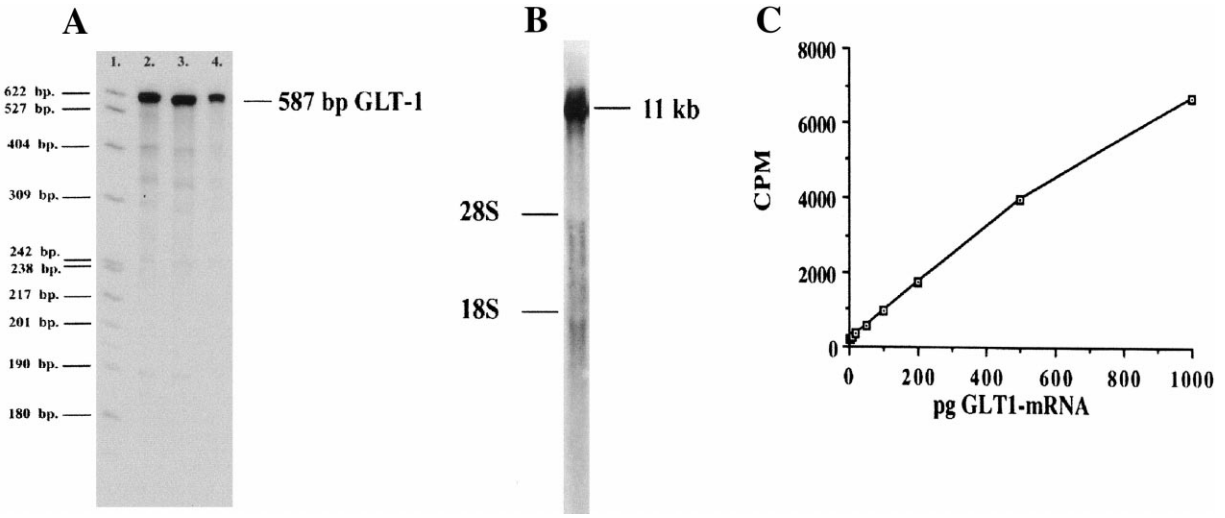

Fig. 2. A: The integrity and thus the specificity of the protected RNA:RNA hybrid was visualized using the ribonuclease protection assay. The result shows a major protected hybrid of 587 bp, corresponding to the size of the antisense GLT-1 probe used. Lane 1, size marker. Lane 2, 20 μg total RNA. Lane 3, 10 μg total RNA. Lane 4, 5 μg total RNA. B: Total RNA was electrophoresed, transferred and hybridized with a ^{32}P-labeled RNA probe under the conditions described in Section 2. One transcript with an estimated size of 11 kbp, corresponding to the GLT-1 transporter mRNA, is seen. C: The abundance of GLT-1 mRNA was estimated with the solution hybridization RNase protection assay and the amount of mRNA was calculated from a standard curve based on hybridization to known amounts of synthetic sense RNA.

of glutamate for the glial glutamate transporters (K_m) is expressed in mM.

2.8. Statistical analyses

Statistical analyses were performed using one way analyses of variance followed by Dunnett's *t*-test.

3. Results

3.1. Culture protein

No statistically significant differences in culture protein content were seen among the different treatment groups. The control mean protein content was 0.396 ± 0.018 mg. All cultures were inspected. No morphological differences were noted in any of the groups of cultures treated with opioids or glutamate compared with control cultures.

3.2. Immunohistochemistry

Immunohistochemical analyses of the cultures showed a homogeneous population of type 1 astroglial cells (GFAp$^+$ A2B5$^-$) (Fig. 1) with few type 2 astroglial cells (GFAp$^+$ A2B5$^+$) present in the cultures. Few non-astroglial cells were seen in the cultures.

3.3. Quantitative analyses of GLT-1 mRNA after long-term incubation

RNA extracted from astroglial primary cultures and analyzed by Northern blot with ^{32}P-labeled rat glutamate transporter cRNA probe revealed one major band with an estimated size of 11 kbp (Fig. 2B) corresponding to the reported size of the GLT-1 mRNA. The integrity and thus the specificity of the protected RNA:RNA hybrid was visual-

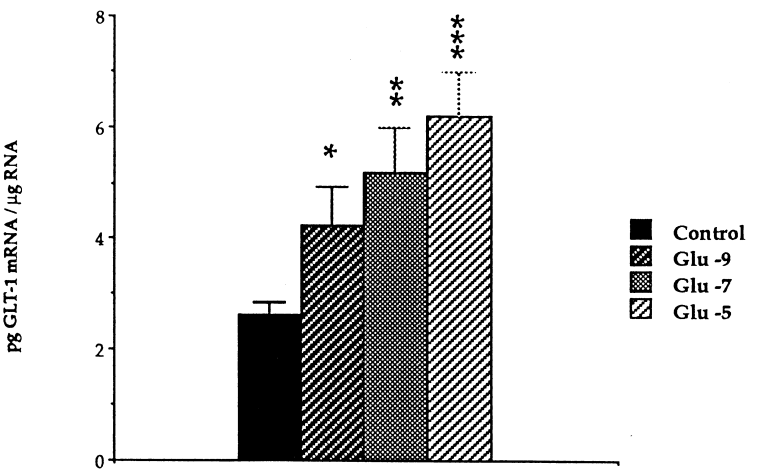

Fig. 3. Astroglial cultures were incubated with glutamate in concentrations of 10^{-9}, 10^{-7} or 10^{-5} M for 48 h. On day 14 in vitro, the cells were harvested and the total RNA was extracted. RNA from approximately 10 cultures was pooled for each concentration. Quantification of glutamate transporter GLT-1 mRNA was performed according to Section 2. The data represent the analysis of five different pooled preparations of RNA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

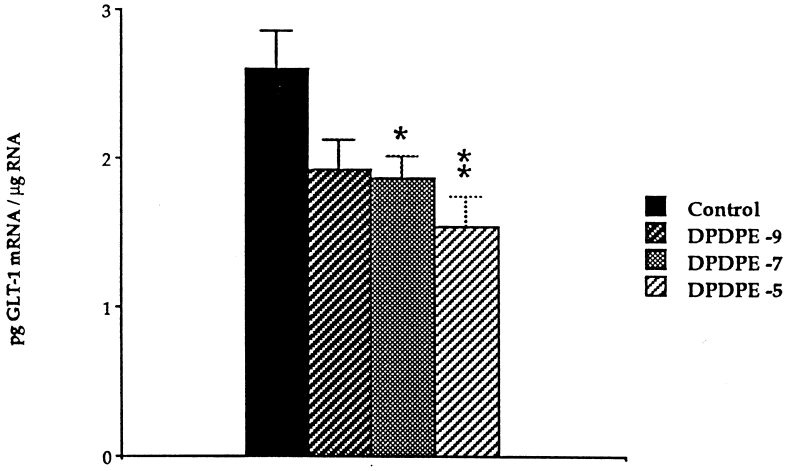

Fig. 4. Astroglial cultures were incubated with the selective δ-opioid receptor agonist DPDPE in concentrations of 10^{-9}, 10^{-7} or 10^{-5} M for 48 h. On day 14 in vitro, the cells were harvested and the total RNA was extracted. RNA from approximately 10 astroglial cultures was collected for each preparation. Quantification of glutamate transporter GLT-1 mRNA was performed according to Section 2. The data represent the analysis of five different pooled preparations of RNA. $^{*}P < 0.05$, $^{**}P < 0.01$.

ized using the RPA II kit (Ambion Inc., Austin, TX, USA). The result of RPA analysis showed a major protected hybrid of 587 bp, corresponding to the size of the antisense GLT-1 probe used (Fig. 2A).

The abundance of GLT-1 mRNA was estimated with a solution hybridization RNase protection assay and the amount of mRNA was calculated from a standard curve based on hybridization to known amounts of synthetic sense RNA (Fig. 2C). The detection limit for the assay was 5 pg GLT-1 mRNA. The intraassay coefficient of variation was 6.88%. All samples were analyzed in the same assay.

Forty-eight hours of glutamate incubation resulted in a dose-dependent increase in the abundance of GLT-1 mRNA from 2.6 (control) to 4.25 pg GLT-1 mRNA/µg RNA after stimulation with 10^{-9} M of glutamate ($P < 0.05$). Stimulation with 10^{-7} M of glutamate for 48 h resulted in an increase to 5.19 pg GLT-1 mRNA/µg RNA ($P < 0.01$). Stimulation with 10^{-5} M of glutamate for 48 h resulted in an increase to 6.20 pg GLT-1 mRNA/µg RNA ($P < 0.001$) (Fig. 3). Opposing effects were seen after 48 h of δ-opioid receptor stimulation. This treatment resulted in a dose-dependent decrease in abundance of glutamate transporter (Fig. 4). Forty-eight hours of DPDPE incubation resulted in a dose-dependent decrease in the abundance of GLT-1 mRNA from 2.6 (control) to 1.9 pg GLT-1 mRNA/µg RNA after stimulation with 10^{-9} M of DPDPE. Stimulation with 10^{-7} M of DPDPE for 48 h resulted in an decrease to 1.9 pg GLT-1 mRNA/mg RNA ($P < 0.05$). Stimulation with 10^{-5} M of DPDPE for 48 h resulted in an decrease to 1.5 pg GLT-1 mRNA/µg RNA ($P < 0.01$) (Fig. 4). We were unable to detect any statistically significant effects on the abundance of GLT-1 mRNA after 48 h of incubation with elevated levels of potassium in the medium in the concentrations 7.5 mM, 12 mM and 56 mM (Fig. 5).

3.4. Kinetics for astroglial glutamate uptake

The kinetic parameters K_m and V_{max} for the GLT-1 transporter were determined in acute glutamate uptake experiments

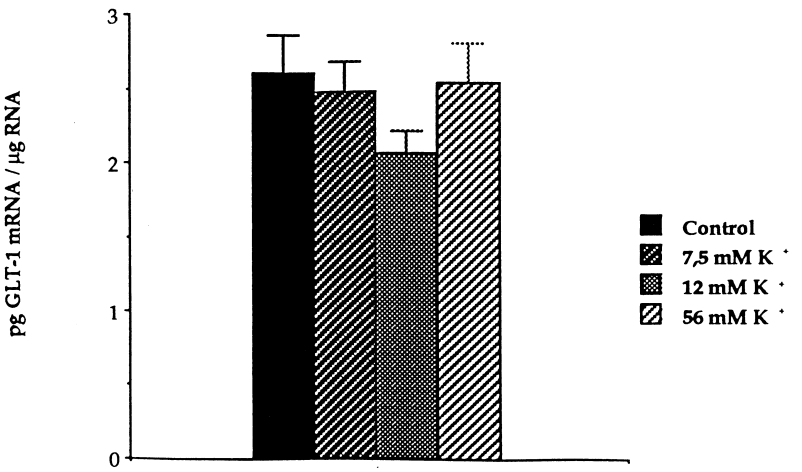

Fig. 5. Astroglial cultures were incubated with potassium 7.5, 12, or 56 mM for 48 h. On day 14 in vitro, the cells were harvested and the total RNA was extracted. RNA from approximately 10 astroglial cultures was collected for each preparation. Quantification of glutamate transporter GLT-1 mRNA was performed according to Section 2. The data represent the analysis of five different pooled preparations of RNA. No statistically significant differences in the abundance of GLT-1 mRNA were seen in cultures incubated with potassium compared with controls.

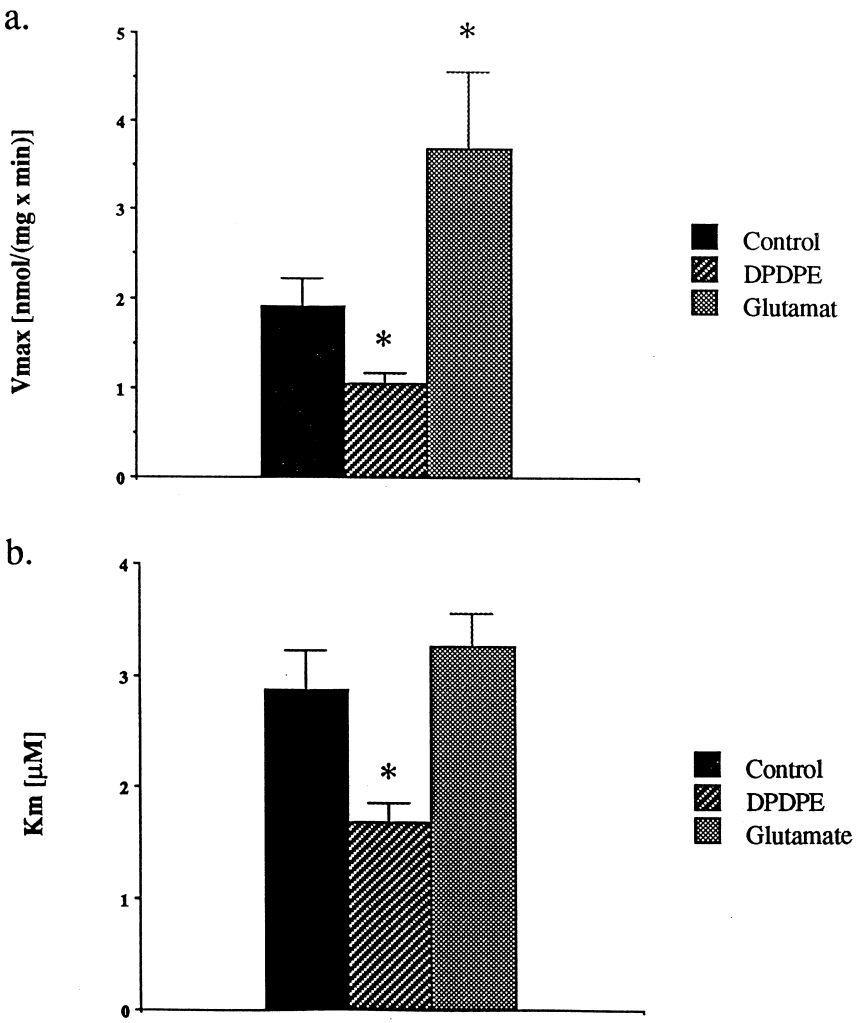

Fig. 6. Astroglial cultures were incubated with the selective δ-opioid receptor agonist DPDPE (10^{-5} M) or glutamate (10^{-5} M) for 48 h. On day 14 in vitro, the kinetic parameters K_m (mM) and V_{max} (nmol/mg/min) were determined. Four separate experiments consisting of 12 cultures were performed for each treatment and for the control group. S.E.M. values are given. *$P < 0.05$ vs. control.

in astroglial cultures after incubations with DPDPE, glutamate or potassium. Astroglial cultures prepared from the rat cerebral cortex were grown for 12 days and thereafter incubated with the δ-opioid receptor agonist DPDPE (10^{-5} M) for 48 h. This treatment resulted in a decrease in V_{max} and K_m of glutamate uptake compared with controls ($P < 0.05$) (Fig. 6). Glutamate (10^{-5} M) incubation for 48 h resulted in an increase in V_{max} of glutamate uptake compared with controls ($P < 0.05$) (Fig. 6).

4. Discussion

The primary astroglial cultures used in the present study have previously been characterized concerning cellular composition [12,31]. The presence of type 1 and type 2 astrocytes was investigated. This astrocyte subclassification is based on morphology and antigenic properties of cells in culture [32,33]. The type 1 astrocytes have a flat polygonal morphology and do not bind the A2B5 antibody, while the type 2 astrocytes bind the A2B5 antibody. Both type 1 and type 2 are GFAp positive. Only a small number of type 2 astrocytes are present in the cultures.

Due to the lack of access to antibodies against the glial specific glutamate (GLT-1) transporter and the subsequent inability to quantitate the transporter protein we decided to quantify the uptake of [^{3}H]glutamate into astroglial cells in culture and in parallel experiments also measured the amounts of GLT-1 mRNA. We consider the measurement of [^{3}H]glutamate uptake into astroglial cells a 'functional' assay.

The glutamate-mediated regulation of the GLT-1 mRNA and uptake kinetics observed in this study might be mediated via one or both of two major classes of glutamate receptors generally recognized on astroglial cells: the metabotropic (mGluR) or the ionotropic (iGluR) glutamate receptors [34–36].

The DPDPE-mediated regulation of the glutamate transporter mRNA and uptake kinetics observed in this study is mediated via δ-opioid receptors, one of the three major opioid receptors besides μ and κ receptors. Subtypes of δ-opioid receptors have been proposed but not yet proven by molecular cloning [37]. DPDPE used in the present study selectively activates the δ1 site. We and others have recently shown that primary astrocytes from the cerebral cortex express δ-opioid receptors [14,16,38]. We have shown that antagoniz-

able δ receptors inhibit the accumulation of cAMP upon stimulation [38]. The second messenger responses elicited by stimulation of glutamate receptors and δ receptors share some features, i.e. increased PIP_2 hydrolysis and increased transmembrane Ca^{2+} flux, but also differ at significant points. The opposing effects on glutamate carrier mRNA and uptake kinetics seen after glutamate receptor stimulation compared with δ receptor stimulation could reflect differences in the complex signal transduction mechanisms involved in the respective receptor second messenger cascade. Our results show that glutamate and δ-opioid receptors couple to the regulation of glutamate transporter mRNA. One possibility could be that the regulation of GLT-1 is cAMP-dependent, with opioids decreasing the abundance via the known inhibition of adenylyl cyclase and glutamate increasing the abundance via cAMP induction through mGluRs. Prolonged δ-opioid receptor stimulation in astroglial cells increases the amount of G_{as} mRNA and decreases the amount of G_{ai2} mRNA [23], suggesting that these parts of the transduction system might be involved in mediating the effects observed after δ receptor stimulation and therefore might mediate the effects seen in the present study. This is in line with a recent study showing that the expression of GLT-1 was increased by elevation of cAMP while GLAST was constitutively expressed [39].

The results of Swanson and co-workers [39] might suggest that the decrease in affinity (K_m) seen after δ-opioid receptor stimulation might reflect the affinity for GLAST rather than GLT-1 and that the increase in V_{max} seen after glutamate reflects an increase in GLT-1 uptake capacity in the present study. The decrease in abundance of GLT-1 mRNA after DPDPE incubation might reflect the general inhibitory nature of opioid signaling in the central nervous system. Long-standing opioid-mediated synaptic depression might induce a decrease in e.g. glutamate neurotransmission. This possibility could make an opioid-mediated down-regulation of glutamate uptake a physiological adaptation of synaptic glutamate transmission. Such a mechanism might also contribute to the increased excitability associated with opioid abstinence after long-term opioid administration.

Glutamatergic denervation has previously been shown to down-regulate GLT-1 expression, suggesting a glutamate receptor-mediated regulation [40]. It is tempting to speculate that the increased abundance of GLT-1 mRNA and V_{max} after glutamate receptor stimulation reflect compensatory mechanisms aiming at the protection of the central nervous system against e.g. excitotoxicity. Considering this, it seems functional to up-regulate the astroglial uptake capacity for glutamate in order to ameliorate or prevent further excitotoxic damage in synaptic areas with increased glutamate transmission.

No alterations were seen in the abundance of GLT-1 mRNA after prolonged depolarization of the glial cells. One possible explanation for this might be that more long-standing differences in glial membrane potential might be physiological, as compared to long-standing alterations in synaptic neurotransmitters such as glutamate and opioids. One must also consider the lack of specificity of a long-standing membrane depolarization, which might be one explanation for why the central nervous system seems unable to adapt or quench seizure frequency in epileptic disorders.

Glutamate receptor-induced changes in glial glutamate uptake systems may be an endogenous mechanism for preventing synaptic overexcitation in situations with elevated levels of extracellular glutamate, such as ischemia, and might also be associated with the paradoxical neuroprotective effects of transient ischemia prior to a prolonged ischemic insult [41].

Apparently, a coupling also seems to exist between astroglial receptors and the uptake of glutamate, although this is only indirectly shown in this study. This coupling is interesting, since it suggests that the transport of glutamate can be regulated by the presence of a transmitter and, therefore, might be of pharmacological relevance.

Manipulation of astroglial glutamate uptake during massive extracellular glutamate load might provide novel neuroprotective strategies.

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