Cytotechnology (2016) 68:1425–1433
DOI 10.1007/s10616-015-9902-9

ORIGINAL ARTICLE

Astrocyte/neuron ratio and its importance on glutamate toxicity: an in vitro voltammetric study

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Received: 6 April 2015 / Accepted: 9 July 2015 / Published online: 6 October 2015

Abstract The purpose of this study was to clarify the relationship between neuron cells and astrocyte cells in regulating glutamate toxicity on the 10th and 20th day in vitro. A mixed primary culture system from newborn rats that contain cerebral cortex neurons cells was employed to investigate the glutamate toxicity. All cultures were incubated with various glutamate concentrations, then viability tests and histological analyses were performed. The activities of glutamate transporters were determined by using in vitro voltammetry technique. Viable cell number was decreased significantly on the 10th day at 10^{-7} M and at 10^{-6} M glutamate applications, however, viable cell number was not decreased at 20th day. Astrocyte number was increased nearly six times on the 20th day as compared to the 10th day. The peak point of glutamate reuptake capacity was about 2×10^{-4} M on the 10th day and 10^{-3} M on the 20th day. According to our results, we suggested that astrocyte age was important to maintain neuronal survival against glutamate toxicity. Thus, we revealed activation or a trigger point of glutamate transporters on astrocytes due to time since more glutamate was taken up by astrocytes when glutamate transporters on the astrocyte were triggered with high exogenous glutamate concentrations. In conclusion, the present investigation is the first voltammetric study on the reuptake parameters of glutamate in vitro.

Keywords Glutamate-uptake · Neurotoxicity · Astrocyte · Neuron · In vitro voltammetry

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Introduction

Glutamate is known as the major excitatory neurotransmitter in the brain and present in millimolar concentrations in mammalian central nervous system (Coyle et al. 1981; Choi et al. 1987). Up to 40 % of all synapses are glutamatergic (Fairman and Amara 1999) and these synapses are distributed throughout the nervous system, prominently in the cerebral cortex and limbic regions of the brain (Kim et al. 2011). Increased extracellular glutamate causes nerve cell death in stroke or trauma (Rothman and Olney 1986). Lots of studies have been shown that astrocytes influence on glutamate toxicity in vitro (Aizenman et al. 1990; Brown 1999).

Voltammetry is the technique that measures the concentrations of compounds through their oxidation at an inert electrode, and it has been applied in vitro for measuring the release and reuptake amounts of neurotransmitters (Burmeister et al. 2000). Investigators have determined signaling dynamics of neurotransmitters, such as dopamine, norepinephrine, serotonin, acetylcholine, nitric oxide, glutamate by microelectrodes. Unlike the complimentary technique known as microdialysis, in vitro voltammetric methods allow for very rapid measurement of the dynamic properties of neurochemicals. However, the routine detection limits of such methods, which are in the 25–50 nM range for analytes such as glutamate, do not rival the picomolar detection limits of methods that are used to analyze microdialysis samples. Electrochemistry is really important for investigating high frequency stimulation of neurotransmitter systems and correlating behavioral phenomena with neurochemical changes, for which the utilization of microdialysis would be problematic. Electrochemistry especially in vivo voltammetry has been shown to overcome the limitations of microdialysis (Robinson et al. 2008; Kasasbeh et al. 2013). Thus, in vivo voltammetric methods have higher spatial and temporal resolution compared to microdialysis (Adams 1990; Burmeister et al. 2002; Bortz et al. 2013).

Na^+-dependent transport keeps extracellular glutamate at low levels (Nicholls and Attwell 1990), and this transport also terminates synaptic transmission (Kim et al. 2011). A rat brain has three different Na^+-dependent transporters: EAAC1, GLT-1 and GLAST. They are membrane-bound pumps. The first one is expressed in neurons and the other two in astrocytes (Storck et al. 1992; Pines et al. 1992; Rothstein et al. 1994; Schmitt et al. 1996; Swanson et al. 1997). Astrocytes play the major role in removal of glutamate from the extracellular compartment. This clearance limits the activation of glutamate receptors and affects the synaptic response (Rothstein et al. 1996; Genoud et al. 2006). The astrocyte-selective glutamate transporter EAAT2 (GLT-1 in rats) has been shown to be chief for keeping extracellular glutamate levels below excitotoxic levels. Astrocyte ratio might be important for the viability of neuron cells.

The nerve terminals from the cerebral cortex and the basal ganglia, retain the ability to release glutamate via exocytosis during development (Sanchez-Prieto et al. 1994). The age-related alterations in L-glutamate regulation may be the main contributor to the changed vulnerability of the aged brain to excitotoxic damages, such as stroke and trauma. In brief, putting the changes of the activities of glutamate transporters by day and astrocyte/neuron ratio together seems to be very important for revealing the age related toxicity of glutamate. In our opinion, this information will serve to understand the relationship between the duration of the culture and the toxic effects of the different glutamate doses on mixed cell cultures. Therefore, we evaluated astrocyte/neuron ratio and its importance on glutamate toxicity with an in vitro voltammetric study for the first time.

Materials and methods

Animal cell culture experiments were performed in accordance with the national guidelines for the use and care of laboratory animals and approved by the local animal care committee of the Ataturk University. 30 albino Wistar newborn rats, provided by the Ataturk University Medical Experimental Practice and Research Center, were used.

Neuronal/astrocytic cell cultures

Mixed cell cultures were prepared using rat astrocytes. In these co-cultures, rat astrocyte provides the required support for optimal synaptic function (Goudriaan et al. 2014; Wierda et al. 2007). Primary cultures of cerebral cortex cells were prepared from one-day-old albino Wistar newborn rat (Gepdiremen et al. 2000a, b). The newborn rats were decapitated and their cerebral cortexes were dissected. Cerebral cortex was divided into minor

pieces in a petri dish using a scalpel. They were suspended in 5 ml of calcium-free Hank's balanced salt solution (HBSS, Sigma Co., St. Louis, USA) with 2 ml of trypsin-ethylen diamine tetra acetic acid (0.25 % trypsin-EDTA; Sigma-Aldrich Co. Ltd., Irvine, UK) at 37 °C for 20 min. Trypsin digestion was ended by the addition of 10 ml of HBSS that contained DNAase type 1 (120 units per ml; Sigma Co., St. Louis, MO, USA). After 3 min centrifugation at 800 rpm, the pellet was re-suspended in 10 ml B27 neurobasal medium + 10 % fetal calf serum (Biol. Ind., Haemek, Israel) and was dissociated by repeated pipetting (Gepdiremen et al. 2002). Then the cells were plated at 3×10^5 cells per well in 24 micro titer plates (Corning Inc., Corning, NY, USA). The culture dishes were kept at 37 °C in humidified atmosphere containing 95 % air and 5 % CO_2. Culture media were changed twice a week and glutamate neurotoxicity tests were performed on the 10th and 20th days.

The cell cultures were treated with a range of L-glutamate concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) for 16 h except the control wells (Zhang and Bhavnani 2006). Sterile saline solution (0.90 % weight/volume of NaCl) was given to control groups. Cell viability was measured via using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after 4 h. The plates were incubated for 2.5 h with MTT. MTT reduction in live cells by mitochondrial reductase results in the formation of insoluble formazan. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. The plates were incubated overnight at 37 °C and absorbance read at 570 nm on the enzyme-linked immunosorbent assay (ELISA, MicroQuant, Reader, BioTek, Winooski, VT, USA). The values obtained for the solutions were compared with the control. Mixed cultures of rat cerebral cortex also contain non-neuronal cells like astrocytes, oligodentroytes, microglias and ependymal cells. These cells, especially astrocytes, are the largest component of the cultures. Cell viability values were shown as sample absorbance/control absorbance rate.

Platinum microelectrode arrays

Microelectrodes

The real-time monitoring of rapid changes in extracellular levels of glutamate and other neuro-active molecules in the central nervous system were provided by fast analytical sensing technology (FAST). We also used S2 type (for rats), glutamate oxidase and nafion-coated multisite ceramic microelectrodes in this study. FAST and microelectrodes were obtained commercially from Pronexus Analytical AB (Stockholm, Sweden). The microelectrodes have platinum (Pt) recording sites with Pt connecting lines.

Calibration

Calibration tests were performed with FAST-16 Data Acquisition Unit (Pronexus Analytical AB, Stockholm, Sweden). We used constant amperometric 0.7 voltage for in vivo voltammetry. The ceramic microelectrode amplifies head stage by being attached to a FAST 16 system. An Ag/AgCl commercial electrode also attaches to the head stage and functions as the reference electrode (Burmeister et al. 2000). Calibration tests involved placement of the electrodes in a stirred 40-ml of 0.1 M phosphate buffered saline (PBS; pH 7.4) solution. Different layers on microelectrode were shown in Fig. 1.

$$\text{L-glutamate} + H_2O + \text{GluOx/FAD} \rightarrow \alpha\text{-ketoglutarate} + NH_3 + \text{GluOx/FADH}_2$$

$$\text{GluOx/FADH}_2 + O_2 \rightarrow \text{GluOx/FAD} + H_2O_2 \text{ (Fig. 1)}$$

The generated peroxide can be efficiently oxidized by the Pt recordings sites at +0.7 V versus Ag/AgCl. The current generated by H_2O_2 oxidation on the surface of the electrode corresponded to the concentration of glutamate in solution. Glutamate selectivity versus ascorbic acid, sensitivity (slope) of glutamate detection, linearity of the dose–response curve (R2), and limit of detection (LOD) were evaluated with the microelectrode calibration results. For microelectrode calibration test; selectivity, R2, LOD values are considered acceptable >100:1, >0.990, <1 μM, respectively, in vitro experiments (Hascup et al. 2007). In our calibration test selectivity, R2, LOD results were >100:1, >0.990, <0.1 μM, respectively.

Reference electrode plating (Ag/AgCl) for in vitro recordings

To obtain records, a Teflon coated silver wire (bare inch AM Systems, Inc. 0008, coated in 0011, 8 inches

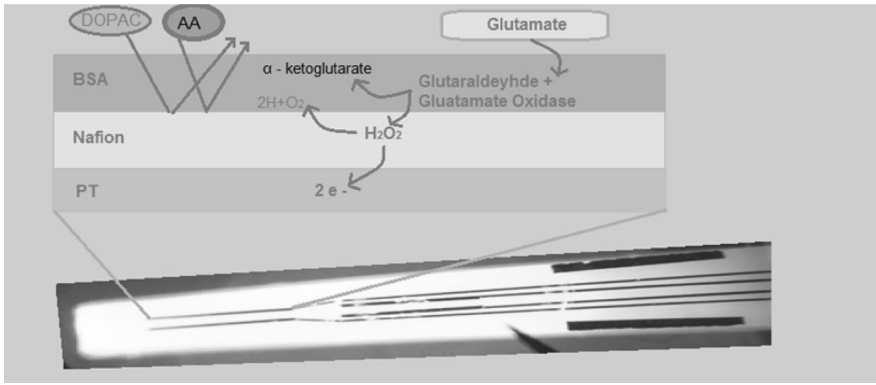

Fig. 1 Different layers on microelectrode. Platin record site, barriers and enzymes

in length) was used in vitro as a reference electrode. As is necessary in the preparation of a reference electrode, a silver wire was coated with chloride. The voltage source, wire, and Cl^- ions were activated, and the silver wire rod was kept in the bath for at least 10 min to ensure proper coating. After removal, a light gray wire tip color was obtained (Hascup et al. 2007).

In vitro voltammetry

Microelectrodes were used to record the reuptake parameters of glutamate from the cell culture wells. Before and after calibration, culture plates were placed in the middle of a circulating water bath that holds the temperature constant at 37 °C. An electrode manipulator that attached to electrode on a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA) was positioned above the culture dish. Our microelectrode had nafion and glutamate oxidase on the platinum surface. Glutamate oxidase converts glutamate to peroxide. On the other hand the nafion blocks interferents. The peroxide can pass through the nafion barrier and can be detected by the platinum side of microelectrode in voltammetry system. The peroxide level is correlated with the glutamate level. Finally, without breaking the tip on the bottom of the plate, the microelectrode is lowered into a culture well with the aid of a stereomicroscope to ensure that the Pt recording sites are immersed in the culture media. The coated tip of the Ag/AgCl reference electrode is placed into the same well (Hascup et al. 2007). Our laboratory used cell culture techniques with enzyme-based multisite microelectrodes to examine L-glutamate uptake in mix cell cultures. By ejecting a solution of different concentration of L-glutamate (10^{-8}–10^{-4} M) into the culture media, we determined the 80 % of reuptake time after peak point (t_{80}) time of uptake of the exogenously given L-glutamate into cultures. The mean ± SEM counts of reuptake time of glutamate (t_{80}) were determined.

Histology

Cultured cells were fixed in ice-cold methanol for 10 min at room temperature. Then cells were washed twice with ice cold PBS. After that cells were incubated for 10 min with PBS containing 0.25 % Triton X-100. Then cells were washed cells in PBS three times for 5 min. Cells were incubated with 1 % BSA in PBST for 30 min to block unspecific binding of the antibodies. After that cells were incubated in the mixture of two primary antibodies, rabbit anti-microtubule-associated protein (MAP2, Abcam, Cambridge, MA, USA) mouse anti-glial fibrillary acidic protein (GFAP, Abcam, Cambridge, MA, USA) in 1 % BSA in PBST in a humidified chamber for overnight at 4 °C. The mixture solution was decanted and the cells were washed three times in PBS, 5 min for each wash. Cells were incubated with the mixture of two secondary antibodies (goat anti-mouse Ig G (Abcam) and goat anti-rabbit Ig G (Abcam) which were raised in different species) in 1 % BSA for 1 h at room temperature in dark. The mixture of the secondary antibody solution was decanted and washed three times with PBS for 5 min each in dark. Neuron and astrocyte cells for each group were counted in every well with an invert microscope at X20 magnification by a histologist. Astrocyte/neuron cell rates were compared before and after glutamate (10^{-8}–10^{-4} M doses) additions in 6 random areas in four different wells.

Statistical analysis

SPSS 18.0 (Statistical Package Program, Chicago, IL, USA) was used for the statistical analysis. The mean ± SEM counts of t_{80}, viability rates, astrocyte/neuron cell number ratio were determined. The data were analyzed statistically using ANOVA.

Results

Cell viability

The total cell numbers in all groups were compared with control groups in Fig. 2. Viability (sample absorbance/control absorbance) values decreased in the groups from 10^{-8} to 10^{-6} M glutamate on the 10th day cultures and in groups from 10^{-8} to 10^{-5} M glutamate on the 20th day cultures. The most toxic dose of glutamate was 10^{-6} and 10^{-5} M, for the 10th and 20th days, respectively. Surprisingly, in both cases, higher concentrations of glutamate resulted in increased viability values. Significant differences in viability values were not found at 10^{-8} and 10^{-7} M glutamate supplemented groups on the 20th day.

Voltammetric studies

In vitro voltammetry was used to understand the uptake time of glutamate in cultured cells. Glutamate was given in different concentrations to the different wells. The results of t_{80} are given in Table 1. The t_{80} time is related to 80 % reuptake time of supplemented glutamate (Fig. 3). The reuptake time was increased by concentrations ($<10^{-6}$ M) on the 10th day. But at 10^{-5} M glutamate concentration, t_{80} time was higher than at 10^{-6} M glutamate concentration for both 10th and 20th days. At 10^{-4} M, the t_{80} time was prolonged to 19.5 ± 4.9 s. At levels higher than 2×10^{-4} M of glutamate concentrations, glutamate amplitude did not return on the 10th day. The t_{80} time increment was found at 2×10^{-4} M glutamate on the 20th day (Table 1).

Histological analysis

The astrocyte and neuron cell numbers and ratios were higher at days in vitro (DIV) 20 compared to DIV 10.

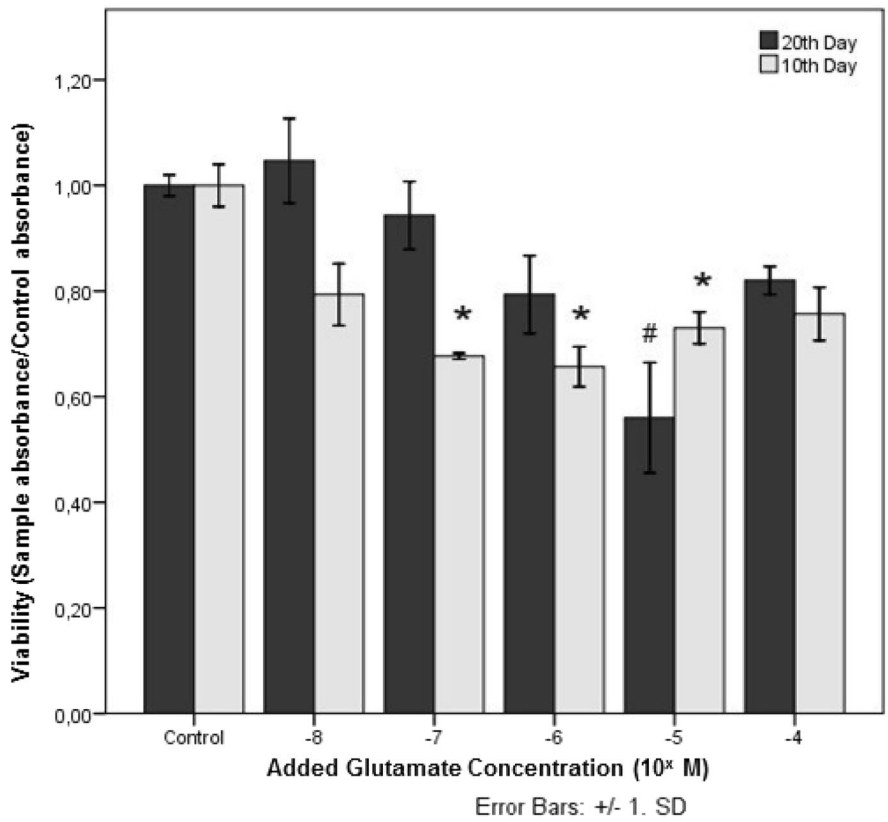

Fig. 2 Viability rates in newborn rat cerebral cortex cells after exposure to 10^{-8}–10^{-4} M glutamate doses for 16 h
* $p < 0.0001$ for 10th day,
# $p < 0.0001$ for 20th day are considered significant. Data were compared between the control group and the other concentration groups (n = 6)

Table 1 The voltammetry results in glutamate-treated mixed neuronal cultures after 10 and 20 days

Drug concentrations	10th day t_{80} (s)	20th day (s)
Control	2.67 ± 0.58	2.33 ± 0.58
10^{-8} M	3.75 ± 0.96	2.50 ± 0.58
10^{-7} M	3.50 ± 0.71	2.50 ± 0.71
10^{-6} M	5.75 ± 1.25*	4.25 ± 0.50
10^{-5} M	2.60 ± 0.55	2.60 ± 0.90
10^{-4} M	19.50 ± 4.90**	3.50 ± 0.70
2×10^{-4} M	∞**	60.50 ± 13.44**
5×10^{-4} M	∞**	92.00 ± 16.98**
10^{-3} M	∞**	∞**

t_{80}: 80 % of total reuptake time (t_{100}) of given glutamate; s: second

* $p < 0.05$; ** $p < 0.0001$

Astrocyte number was increased nearly 6 times in DIV 20 according to DIV 10 but there was no change in neuron cell number. We detected the highest difference between ratios DIV 20 and DIV 10 at 10^{-6} M glutamate concentration (Table 2).

Discussion

Glutamate levels are regulated in central nervous system synapses. There is equilibrium between the glutamate release and reuptake. Basal extracellular glutamate is maintained by its release from neurons and reuptake by astrocytes and neurons (Day et al. 2006; Robert 2011). Exogenous glutamate is also removed by both uptake and reuptake mechanisms (Rothstein et al. 1995). These mechanisms play an important role in the glutamate toxicity. In this study, a cerebral cortex mixed neuronal cultures of newborn rats were used to determine maximum toxicity levels of exogenous glutamate and relationship between the duration of cell culture and the exogenous glutamate uptake time.

In a toxicity study, it was shown that 5×10^{-4} M glutamate had no toxic effect on immature cortical neurons and glia (Choi et al. 1987). The cultured neurons expressed functional glutamate receptors and they became vulnerable to L-glutamate toxicity after 8 days (Mattson et al. 1993; Domoki et al. 2010). But they could not show glutamate transporter activity directly. In our study, we showed that glutamate was toxic at 10^{-5}, 10^{-6}, and 10^{-7} M doses on the 10th day and at 10^{-5} M on the 20th day. Interestingly, viability increased at concentrations above 10^{-5} M doses of glutamate in both cultures. In histological assessments this cell increment was related with astrocyte proliferation and they were more resistant to glutamate neurotoxicity (Table 2). This event could be explained by McKenna since he demonstrated that when the exogenous glutamate concentration increased above 10^{-4} M levels, the percent of glutamate which was oxidized in astrocytes via the tricarboxylic acid cycle increased (McKenna 2013).

Mixed cultures of rat cerebral cortex also contain non-neuronal cells like astrocytes, oligodentrocytes, microglia, and ependymal cells. These cells, especially astrocytes, are the largest component of the cultures, contributing up to 80–90 % of the total cellular mass at 3–5 weeks in vitro (Sinor et al. 2000).

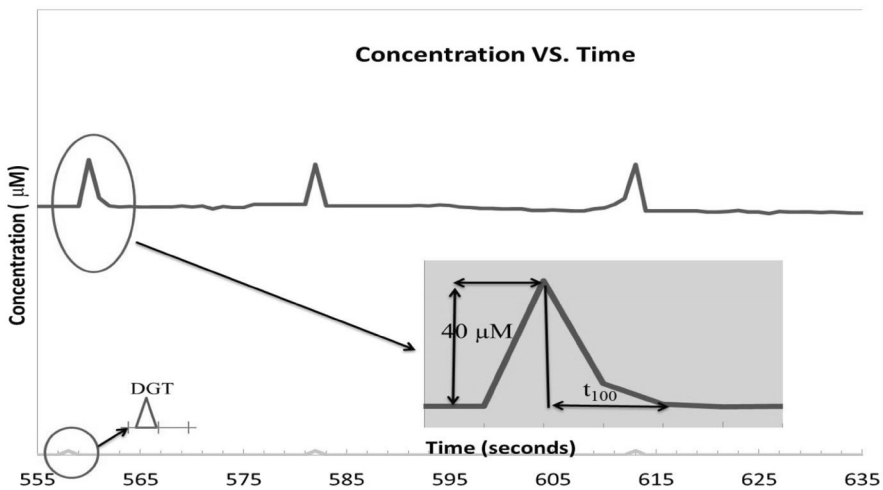

Fig. 3 Representative example of some signals produced acutely by exogenous glutamate application
t_{80}: 80 % of total reuptake time after peak (t_{100}) of given glutamate. Ejection times: 556, 581, 613 s on the 20th day. DGT: Drug given time

Table 2 Cell numbers and rates (astrocyte/neuron) in treatments with different glutamate concentrations on the 10th and 20th DIV

Glutamate concentrations	10th day			20th day		
	Neuron	Astrocyte	Ratio	Neuron	Astrocyte	Ratio
Control	5.3 ± 3.3	38.0 ± 16.0	7.1	6.0 ± 0.8	174.0 ± 23.7*	29.0
10^{-8} M	2.0 ± 0.8	40.0 ± 16.3	20.0	3.5 ± 0.6	175.0 ± 28.9*	50.0
10^{-7} M	2.0 ± 0.8	15.0 ± 6.1	7.5	6.0 ± 0.8*	171.0 ± 23.3*	28.6
10^{-6} M	2.3 ± 1.0	17.0 ± 7.2	7.6	1.5 ± 0.6	150.0 ± 57.7*	100.0
10^{-5} M	2.0 ± 0.8	20.0 ± 8.2	10.0	3.5 ± 1.3	175.0 ± 64.5*	50.0
10^{-4} M	5.6 ± 2.2	22.0 ± 8.3	3.8	3.4 ± 1.3	107.0 ± 39.5*	31.5

Data were compared between the 10th and the 20th days

n = number of wells; for each well six random areas were calculated

* $p < 0.05$

In another study glutamate administration caused hypertrophy and hyperplasia in microglia and astrocytic cells (Martinez-Contreras et al. 2002). In our study from 10^{-5} to 10^{-4} M concentration we saw cell viability increase in MTT analyses.

Araque et al. (1998) showed that astrocyte stimulation reduced excitatory and inhibitory synaptic transmissions through the activation of selective presynaptic metabotropic glutamate receptors in mixed cultures that include astrocytes and neurons (Araque et al. 1998). Additionally, another report showed that extracellular glutamate levels in the neonatal cortex were significantly elevated in GLT1 knock-out mice (Takasaki et al. 2008). GLT-1 transporters are especially placed on astrocytes. It was postulated that when the concentration of glutamate exceeds a threshold level, astrocytic glutamate transporters were activated, and there was a decrease in the extracellular glutamate concentration. This threshold was probably changing with the days in vitro. It was found that this threshold was 10^{-6} and 10^{-5} M, on the 10th and 20th day, respectively (Fig. 2). To detect the threshold levels we used in vitro voltammetry technique and we confirmed the results at the 10th day (Table 1).

In another study, a significant increase was found in the V max parameter of glutamate uptake in older astrocyte cultures (Pertusa et al. 2007). Uptake rates by with [^{3}H] a radioactive labeling method were found to be higher in 10^{-3} M glutamate than in 10^{-4} M glutamate on the 10th day (Pertusa et al. 2007). These findings support the important increase in viability rates at a glutamate concentration of 10^{-5} and 10^{-4} M at DIV 20 and decreased viability rates at DIV 10 (Fig. 2). Reuptake parameters of glutamate could not be understood clearly in astrocyte cultures. There can be two possible explanations for this; glutamate transporter expression may be higher in older cultures and/or their activity may be higher at higher concentrations. Thus, when glutamate transporters were triggered, more glutamate can be taken up by 20 day cultures. Moreover, transporter development on astrocyte continued between the 10th and the 20th days. Immunofluorescence studies demonstrated that GLT and GLAST proteins are present in the CNS from early developmental stages at very low concentrations initially. However, immunoblotting studies have shown that the expression of both transporters increases, and show adult levels nearly 20 days after birth (Danbolt 2001; Kugler and Schleyer 2004). In a long time period, in vitro oxidant parameters can harm neurons (Pertusa et al. 2007), so our experiments were performed on the 10th day (after functional glutamate receptors were expressed) and on the 20th day (before oxidant harm occurs).

In our study, it was thought that the threshold point for glutamate, which triggers the transporters, was elevated and that transporter activities were also accelerated in older cells. In other words, transporter activity starts at high glutamate levels but may be more accelerated in older cells. For clarifying this point, we used in vitro voltammetry technique on different days to detect glutamate reuptake time (t_{80}).

There were no significant difference in glutamate reuptake time (t_{80}) at lower glutamate concentrations

($<10^{-6}$ M) when compared to control groups on the 10th day. At a concentration of 10^{-6} M, t_{80} time (5.75 ± 1.25 s) was increased when compared to the control groups on the 10th day. When we increased the glutamate dose to 10^{-5} M, a reuptake trigger mechanism may have played a role, perhaps due to the transporters. The acceleration on reuptake time at 10^{-5} M of glutamate were seen (2.60 ± 0.55 s). When we increased the concentration of glutamate to 10^{-4} M, we saw the t_{80} times extend to 19.5 ± 4.9. Moreover, when the concentration was passed to 2×10^{-4} M, the t_{80} time could not return to basal glutamate levels. Voltammetric results on the 20th day showed that a delay in t_{80} time started at the doses two times higher (2×10^{-4} M) compared to the recordings on the 10th day. At 5×10^{-4} M concentrations, glutamate amplitude returned to basal levels lately (92 ± 16.98 s). At the concentrations of 10^{-3} M of glutamate, t_{80} became infinite on the 20th day (Table 1).

The astrocyte/neuron rate in vitro was nearly four times higher on the 20th day compared to the 10th day. Astrocyte numbers were shown to increase compared to neurons between the 10th and the 20th days (Table 2). It was hypothesized that astrocytes were activated at 10^{-6} M concentration of glutamate on the 10th day culture and 10^{-5} M on the 20th day culture, and they took up the toxic glutamate from the synaptic cleft (Table 1). In conclusion, astrocyte age was important to maintain neuronal survival; thus, it was proposed that the activation point or trigger point of glutamate transporters on astrocytes changes time dependently. These changes were shown via in vitro voltammetry in cultured cells for the first time.

Acknowledgments This study was supported by grants from The Scientific and Technological Research Council of Turkey (TUBİTAK-Project No: 107S067) and Atatürk University BAP (Project No: 2005/160 and 2011/270-271)

Conflict of interest The authors declared that there are no conflicts of interest.

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