Original Paper

Low Dose Administration of Glutamate Triggers a Non-Apoptotic, Autophagic Response in PC12 Cells

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Key Words

Glutamate • Chaperones • Stress • Excitotoxicity • autophagy

Abstract

Background/Aims: Increasing amounts of the neurotransmitter glutamate are associated with excitotoxicity, a phenomenon related both to homeostatic processes and neurodegenerative diseases such as multiple sclerosis. **Methods:** PC12 cells (rat pheochromocytoma) were treated with various concentrations of the non-essential amino acid glutamate for 0.5–24 hours. The effect of glutamate on cell morphology was monitored with electron microscopy and haematoxylin-eosin staining. Cell survival was calculated with the MTT assay. Expression analysis of chaperones associated with the observed phenotype was performed using either Western Blotting at the protein level or qRT-PCR at the mRNA level. **Results:** Administration of glutamate in PC12 cells in doses as low as 10 μ M causes an up-regulation of GRP78, GRP94 and HSC70 protein levels, while their mRNA levels show the opposite kinetics. At the same time, GAPDH and GRP75 show reduced protein levels, irrespective of their transcriptional rate. On a cellular level, low concentrations of glutamate induce an autophagy-mediated pro-survival phenotype, which is further supported by induction of the autophagic marker LC3. **Conclusion:** The findings in the present study underline a discrete effect of glutamate on neuronal cell fate depending on its concentration. It was also shown that a low dose of glutamate orchestrates a unique expression signature of various chaperones and induces cell autophagy, which acts in a neuroprotective fashion.

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Introduction

Glutamate is an essential excitatory neurotransmitter that is produced by the tricarboxylic acid cycle or as a product of glutamine deamination and plays a significant role in essential neural functions such as learning, memory, long-term potentiation and synaptic plasticity [1, 2]. Its extracellular concentration needs to be maintained at low levels since its excessive accumulation in the extracellular area leads to a disease-related state known as excitotoxicity, through over-activation of glutamate receptors and the concomitant toxic depolarisation of neurons [3]. Glutamate receptors, which are divided into metabotropic and ionotropic, drive excitotoxicity through the regulation of calcium influx, the production of NO and the orchestration of classical (caspase-mediated) and novel (GAPDH/Siah 1) apoptotic pathways [4]. Moreover, excessive glutamate concentration in the extracellular area promotes intracellular toxicity through reverse operation of the cysteine/glutamate antiporter (Xc⁻), a phenomenon called oxidative glutamate toxicity [5]. Glutamate-associated cell toxicity has been related to various neurodegenerative pathologies such as Huntington disease, Amyotrophic lateral sclerosis, stroke and brain trauma; hence, fine-tuning of its extracellular levels is important for the maintenance of neural homeostasis [6].

Glutamate-induced excitotoxicity is related to endoplasmic reticulum stress [7], which can trigger the unfolded protein response (UPR), a dynamic signalling network that restores proteostasis. UPR is often the leading cause of various neurodegenerative diseases and serves as an active field of interest for pharmacological intervention [8]. Recent studies have linked UPR to autophagy-related neuronal cell death, a fact that is however still being debated. Accumulative evidence supports a significant role of various chaperones either in UPR *per se* or in UPR-mediated autophagy. For example, GRP78 serves as a key player in autophagy activated as a protective mechanism that maintains ER homeostasis [9], while its close partner, GRP94, the endoplasmic member of the HSP90 family of stress-related proteins, orchestrates essential proteostatic events such as proper protein-protein interactions, protein translocation and secretion [10]. Moreover, GRP75, which is essential for proper functioning of the mitochondrial ATPase, acts as a member of the mitochondrial quality control system regulating mitophagy and apoptosis [11] and has been implicated in the inhibition of apoptosis by associating with and inactivating p53 [12]. Equally important, HSC70, a member of the HSP70 family of chaperones, participates in ER and mitochondrial stress responses while synchronising proper protein folding, clathrin-mediated translocation and autophagy [13].

Given the dubious role of glutamate in autophagy regulation, it could be speculated that stimulation of cells with this essential amino acid could lead to either apoptosis or cell survival depending on the activation status of the autophagic mechanism. To this end, the present study investigates a possible alternative role for glutamate in neural cell physiology and provides data in support of a non-apoptotic, autophagy-related mechanism induced by doses of glutamate as low as 10 μ M.

Materials and Methods

Cell Culture and treatment

All experiments were performed with rat pheochromocytoma PC12 cells, a generous gift from I. Papamatheakis (Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology, Heraklion Crete, Greece). The cells were cultured at 37°C in an incubator offering a humidified atmosphere of 5% CO₂ and atmospheric oxygen concentrations. The model of excitotoxicity employed utilised the exposure of PC12 cells to 10 μ M glutamate in low glucose complete Modified Eagle Medium supplemented with 15% horse serum, 2.5% fetal bovine serum and antibiotics. The same conditions were replicated for control cells, minus the addition of glutamate. Cells were collected at various time points after the addition of glutamate depending on the assay and washed three times in 1x PBS. Cells were then lysed and the lysate was subjected to various assays, as indicated in the figures.

Protein isolation, quantification and immunoblotting

Cell lysis and protein isolation was performed using the NucleoSpin TriPrep kit (Macherey-Nagel) and protein concentration was calculated using the protein quantification assay (Macherey-Nagel 740967). Electrophoresis was carried out on 10% SDS polyacrylamide gel in a Mini-PROTEAN® Tetra Cell (Biorad) and immunoblotting was performed in a Mini Trans-Blot® Cell (Biorad) for 90 minutes at 350 mA. Pre-stained Nippon Genetics (MWP02) was used as a molecular marker. Proteins were transferred on PVDF membrane (Perkin Elmer NEF1002001PK) followed by blocking with Blotto Non-Fat Dry Milk (Santa Cruz Biotechnology, sc-2324) and probing with primary antibodies. Primary antibodies α -GRP75 (H-155) sc-13967, α -GRP78 (H-129) sc-13968 and α -GRP-94 sc-11402 (H-212) were used at a 1:1000 dilution (Santa Cruz Biotechnology INC). Anti-HSC70 mouse IgG (AM03141PUN) and α -b-actin mouse IgG (AM0194PN) were purchased from Acris Antibodies and used at final dilutions of 1:2500 and 1:1000, respectively. Anti-GAPDH ABS16 was used at a 1:3000 dilution (Millipore). Anti-LC3 II D11 was purchased by Cell Signalling (3868) and used at a 1:1000 dilution. Goat anti-Rabbit IgG peroxidase AP132P was used at a 1:10000 dilution (Millipore) and goat anti-mouse IgG peroxidase A2554 at a 1:20000 dilution (Sigma Aldrich) as secondary antibodies. Signal detection took place using the Lumisensor reagent (Genescript, L00221V300) and Super RX films (Fujifilm, 4741008379). Image density analysis was performed with Image J software.

mRNA isolation and Real-Time PCR

Following the treatment of PC12 cells with 10 μ M glutamate for 30 minutes or one hour, mRNA was isolated with the NucleoSpin TriPrep kit and 1 μ g RNA was used as template for cDNA synthesis using the M-MLV reverse transcriptase and random primers (Promega, M1701, C1181). Real-Time PCR was carried out in duplicate using KAPA SYBR Fast® Mix (KK4603 Kapa Biosystems) in StepOne™ (Applied Biosystems). The PCR specifications were as follows: 95°C for 10 minutes, 40 cycles of 95°C, 60°C and 72°C for 30 seconds each, followed by a melting curve stage comprised of 95°C, 60°C and 95°C steps. The primers used were provided by Invitrogen, with the following sequences: F: 5'-AGA CCT TCA ACA CCC CAG CCA-3' and R: 5'-ATG GGC ACA GTG TGG GTG ACC-3' for b-actin, F: 5'-ACG GGA AGC TCA CTG GCA TGG-3' and R: 5'-CGC CTG CTT CAC CAC CTT CTT-3' for GAPDH, F: 5'-GCA CCC AGG CCA GTA TTG AGA-3' and R: 5'-CCA CGG AAC AGG TCA CAC TTC-3' for HSC70, F: 5'-TCC TGG AAA TTC AGA AAG GAG-3' and R: 5'-TCT GAA GTG CCA TGT TGT CTT-3' for GRP75, F: 5'-AAG AGT TCT TCA ATG GCA AGG-3' and R: 5'-GTC ATG ACA CCT CCC ACA GTT-3' for GRP78 and F: 5'-CAA GCT TGG TGT GAT TGA AGA-3' and R: 5'-GAC CCA GCC ATG AAG TAG ATT-3' for GRP94. All primers were designed to anneal to both human and rat sequences and their specificity was assessed using the NCBI BLAST software. B-actin was used as an internal control.

Haematoxylin/Eosin Staining

Following treatment with glutamate, cells were fixed in alcohol and then rehydrated by immersion in distilled water for 30 seconds with agitation; the slides were immediately dipped in a Coplin jar and incubated in Mayer's haematoxylin for 30 seconds with agitation. The samples were then rinsed 2-3 times in distilled water and subsequently stained with 1% eosin Y solution for approximately 30 seconds with agitation. Next, the sections were dehydrated with incubation twice in 95% ethanol and twice in 100% ethanol for 30 seconds each and the ethanol was extracted with two changes of xylene. Cells were finally mounted with glycerol, covered with coverslips and examined under an optical microscope.

Electron Microscopy

Following trypsinisation, cells were briefly centrifuged and the cell pellets were fixed with dropwise addition of 3% glutaraldehyde in 0.1M 1x PBS for 2 hours. The samples were subsequently washed in 1x PBS, post-fixed in 1% osmium tetroxide, rinsed with double-distilled water and finally stained in 1% uranyl acetate for 10-20 hours. The specimens were then ethanol-dehydrated and embedded in Epon 812. Semi-thin sections were stained with toluidine blue and observed under the light microscope in order to select areas for ultrathin sections which were stained with lead citrate and viewed under a Jeol Transmission Electron Microscope.

Statistical analysis

Graphs were created and statistical analysis of all data was performed with GraphPad Prism 5 software. Unpaired t-test was used for RT-PCR and MTT analysis between each time point and the relative untreated

control. All experiments were carried out at least three times. Data are presented as mean $\pm$ SEM. Asterisks indicate statistical significance at $p < 0.01$ (**) and at $p < 0.001$ (***).

MTT assay

Cell viability was evaluated by the 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyl-SH-tetrazolium bromide assay (MTT, Sigma-Aldrich) as originally described by Mosmann [14] and reproduced by Kritis et al. [15]; this was complemented with the Trypan Blue exclusion assay, which was clearly described by Strober [16]. Briefly, MTT was dissolved in MEM without phenol red at a final concentration of 5 mg/ml, and served as a stock solution. PC12 cells exposed to excitotoxicity were further incubated for 24h with 1 mg/ml MTT at 5% CO₂ at 37°C in a humidified incubator. At the end of the incubation period, MTT formazan crystals were solubilised with 0.1 N HCl isopropanol and absorption was detected at 570 nm, with background subtraction at 630 nm, using a micro-plate reader (Stat Fax – 2100, Awareness Technology Inc. USA).

Results

Low dose glutamate alters protein expression level of GRP78, GRP94, GRP75, HSC70 and GAPDH

Primary data from our laboratory that will be presented elsewhere supported a regulatory effect of glutamate on specific proteins detected with a 2D electrophoresis/mass spectrometry proteomic analysis. In order to verify these observations, PC12 cells were treated with 10 μ M glutamate for three hours and protein levels of GRP78, GRP94, GRP75, HSC70, and GAPDH were monitored with immunoblotting. Figure 1 shows that HSC70 and GRP78 were gradually up-regulated and peaked two hours after stimulation, while their levels subsequently subsided. GRP94 showed similar kinetics with a slower but steadier increase, while GAPDH levels showed a slight reduction and GRP75 levels rapidly decreased and remained low throughout the duration of the treatment.

Effect of low dose glutamate administration on mRNA levels of GRP78, GRP94, GRP75, HSC70 and GAPDH

In order to check whether the observed changes in the protein levels follow a concomitant transcriptional deregulation of the molecules investigated, RNA was extracted from PC12 cells treated with 10 μ M glutamate for 30 minutes or 1 hour and the mRNA levels of GRP78, GRP94, GRP75, HSC70 and GAPDH were calculated with qRT-PCR. Interestingly, whereas GAPDH and GRP75 showed no significant changes, GRP78, HSC70 and GRP94 showed reduced mRNA levels upon treatment with glutamate (Fig. 2), thus excluding the possibility that the alterations seen in the protein levels of these three molecules reflected enhanced transcriptional activity.

Treatment of PC12 cells with low amounts of glutamate triggers an autophagic response

In order to check whether there is a phenotypic outcome of low dose glutamate, PC12 cells were treated with 10 μ M of the amino acid for three hours and subsequently stained with Haematoxylin/Eosin. Figure 3 shows increased acidification and cytoplasmic condensation as well as the presence of ghost cells (not actively metabolising cytoplasm), which is indicative of degeneration and autophagy-associated cell death. However, control cells treated with empty vehicle grew normally and did not show any significant morphological changes. In depth analysis of the glutamate effect on PC12 physiology with electron microscopy (Fig. 4) supported an autophagic phenotype with overt disorganisation and vacuolisation of the endoplasmic reticulum that promotes vesicular cytoplasmic degeneration and condensed irregularly-shaped nuclei. Induction of an autophagic response was further underlined through the up-regulation of LC3 II, used here as an autophagic marker.

Glutamate concentration sets the threshold of excitotoxicity induced cell death

Having shown that the treatment of PC12 cells with glutamate alters the expression of several chaperones and triggers an autophagic response, we then asked whether there was

Fig. 1. The effect of glutamate on expression of HSC70, GRP75, GRP78, GRP94 and GAPDH of PC12 protein extracts. (A) Representative western blots. (B) Quantitation of the protein levels relative to b-actin. Untreated samples were given the arbitrary value of '1'. The figure shows the median of three experiments $\pm$ SEM.

Fig. 2. mRNA expression levels of HSC70, GRP75, GRP78, GRP94 and GAPDH following glutamate treatment of PC12 cells. Relative expression analysis was achieved with the $2^{-\Delta\Delta Ct}$ method. All values were normalised to untreated samples and b-actin was used as internal control. The figure shows the median of three experiments $\pm$ SEM. Three asterisks (***) denote $p < 0.001$. $N = 3$.

a direct effect on cell survival. To answer this question we performed MTT assays on PC12 cells treated with various concentrations of glutamate for 24 hours (Fig. 5). Apparently, low doses of glutamate that fit our findings up to this point (Fig 1-4) showed a pro-survival rather a pro-apoptotic effect. However, increasing amounts of the amino acid reduce the survival

Fig. 3. Glutamate-induced morphological changes on PC12 cells after staining with H&E. (A) PC12 cells cultured in normoxic conditions. (B) PC12 cells cultured in the presence of 10 μ M glutamate for 3 hours.

Fig. 4. Low amounts of glutamate induce autophagy in PC12 cells. Representative cell images captured with electron microscopy (a) under normal culture conditions or (b) under treatment with 10 μ M glutamate for 3 hours. (c) Representative western blot of LC3 II protein levels following glutamate treatment of PC12 cells for the indicated time points.

Fig. 5. The dose-dependent effect of glutamate on PC12 survival. Increasing amounts of glutamate decrease the survival threshold of PC12 cells. Statistical analysis was performed with unpaired t-test (** $p < 0.01$, *** $p < 0.001$). The figure shows the median of three experiments $\pm$ SEM.

rate in a dose-dependent trend supporting thus the existence of a concentration threshold for glutamate above which the amino acid is highly toxic for the cells.

Discussion

In the Central Nervous System (CNS), glutamate is a major neurotransmitter playing a crucial role in the proper function of neuronal transmission and cell communication. On the other hand, glutamate is an important factor involved in many neurodegenerative diseases acting as a neurotoxin that leads to excitotoxicity and increased intracellular calcium influx [6].

In this work we investigated the impact of low dose glutamate administration on PC12 pheochromocytoma cells. Our results (Fig. 5) show that treatment with glutamate at final concentration as high as 10 mM reduced cell viability in total agreement with previously published data [17]. To our surprise however, lower amounts of the amino acid did not support a pro-apoptotic role for glutamate and this led us to speculate that there might be a concentration threshold that triggers glutamate-induced cytotoxicity. Morphological analysis of PC12 cells treated with low amounts of glutamate (10 μ M) revealed ghost cells indicative of autophagic process (Fig. 3). This observation was further verified by electron microscopy that showed disorganized endoplasmic reticulum and vesicular cytoplasmic degeneration. Moreover, immunoblotting of LC3 II, used here as an autophagosome marker, shows elevated levels (Fig. 4) which is a common feature of autophagic responses [18]. Overall, the results support the notion that in the presence of low amounts of glutamate, cells undergo an autophagic pro-survival procedure. This may reflect the fact that under mild stress conditions cells may develop an autophagic reaction, which in turn selectively removes damaged organelles as a cytoprotective mechanism [19]. Alternatively, prolonged or and/or enhanced stimulation of the cells with glutamate could lead progressively to cell death either through apoptosis or another death mechanism [20]. Conclusively, the final outcome of glutamate activity is highly depended on its concentration as well as the duration of its action and this observation follows a global pattern of glutamate-related signalling outcomes as recently reviewed in [21].

In support of previous unpublished data from our laboratory, treatment of PC12 cells with low amounts of glutamate alters the protein levels of various GRPs and HSC70 underlying thus a specific signature of chaperone expression (Fig. 1). Interestingly enough, a similar expression pattern was also evidenced under hypoglycaemia-induced stress [22] which leads to the hypothesis that these chaperones may act in a global fashion under different kinds of stress to dictate the response of a cell depending on the potential of the external stimulus. A more detailed analysis of the data shows an up-regulation of GRP78, HSC70 and GRP94. This supports the induction of a chaperone-mediated neuroprotective mechanism since all these proteins enhance cell viability [23, 24]. The observed expression profile of these chaperones however, may be context-dependent since it has been found that upregulation of GRP78 for example is associated with enhanced ER stress and cell death in isoproterenol-induced cardiomyocyte hypertrophy [25] or under cold stress [26]. On the other hand, chaperones such as GRP75 act constantly in favour of stress-induced cell death and their downregulation such as in our case is directly connected to cell survival [11]. Finally, GAPDH that can also trigger a death signal cascade in cells undergoing stress shows a relatively low reduction in expression [27]. Conclusively, our data support a chaperone-mediated autophagic mechanism induced by low amounts of glutamate that acts in favour of cell survival.

An interesting finding in the present study is also the fact that GRP78, HSC70 and GRP94 protein up-regulation does not coincide with their mRNA levels, which follow opposite kinetics (Fig. 2). This comes as no surprise, as glutamate can induce an endoplasmic reticulum stress-associated death signalling cascade in neuronal cells [28] resembling the unfolded protein response known to promote a global transcriptional reprogramming [29]. The particular finding also supports the speculation that the observed up-regulation in protein levels of these chaperones is caused probably by post-translational modifications known to block protein turnover and promote protein stability, such as ubiquitination [30], which needs further investigation.

In conclusion, our findings support a novel role for glutamate, an amino acid known mainly for its participation in excitotoxicity-associated mechanisms. We demonstrate in the present study that minor augmentation in the concentration of glutamate drives a cytoprotective mechanism in neuronal cells. Based on our data, this is achieved through the deferential regulation of various chaperones that orchestrate an autophagic response supporting cell survival.

Abbreviations

UPR (Unfolded Protein Response); ER(Endoplasmic Reticulum); GRP78 (Glucose Regulated Protein 78); GRP94 (Glucose Regulated Protein 94); HSC70 (Heat Shock Cognate protein 70); HSP90 (Heat shock Protein 90); LC3 (Microtubule-associated protein 1A/1B-light chain 3); GAPDH (Glyceraldehyde 3-phosphate dehydrogenase); NO (Nitric Oxide); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); qRT-PCR (quantitative Real Time Polymerase Chain Reaction).

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Disclosure Statement

The authors declare there are no conflicts of interest.

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