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Kainic Acid and MPP+ Induce Upregulation of GLT-1 in Neuroblastoma and Glia Cells

Kainik Asit ve MPP+ GLT-1'in İfade Artışını Neuroblastoma ve Glia Hücrelerinde Düzenler

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ABSTRACT

Objective: Glutamate is the major excitatory transmitter in the brain. The excessive glutamate will lead to excitotoxicity. Glutamate transporter-1 (GLT-1) is the major transporter that performs 95% of the glutamate clearance contributing to normal neuronal function and preventing excitotoxicity. In this study, we investigated the effect of two toxins, kainic acid and MPP^+ (1-methyl-4-phenylpyridinium), on GLT-1 expression and excitotoxicity in neuroblastoma and glia cells (immortalized human astrocytes).

Methods: We treated neuroblastoma and glia cells with kainic acid and MPP^+, applied MTT assay to measure the cell viability. We identified the mRNA and protein levels of GLT-1 and also analyzed released glutamate levels using glutamate assay.

Results: The mRNA level of GLT-1 increased in neuroblastoma cells as a result of kainic acid or MPP^+ treatment while the protein expression of GLT-1 increased in glia cells after the treatment with MPP^+. Excess glutamate was found to be decreased after 12 h MPP^+ treatment. However, this decrease was no more prominent with further MPP^+ treatment.

Conclusion: Our results show that GLT-1 levels are elevated as a result of kainic acid or MPP^+ treatment as a survival mechanism to prevent excitotoxicity.

Keywords: Excitotoxicity, GLT-1, glutamate, MPP^+, kainic acid, mRNA

ÖZ

Amaç: Glutamat, beyindeki majör uyarıcı transmiterdir. Fazla glutamat eksitotoksisiteye yol açar. GLT-1 (Glutamat Transportır 1) beyindeki glutamatın %95'ini temizleyerek nöron fonksiyonunu sağlar ve eksitotoksisiteyi önler. Bu çalışmada kainik asit and MPP^+ (1-methyl-4-phenylpyridinium) isimli iki toksinin nöroblastoma ve glia (immortalize edilmiş insan astrositleri) hücrelerinde GLT-1 ve eksitotoksisite üzerindeki etkilerini incelemektir.

Yöntemler: Nöroblastoma ve glia hücreleri kainik asit ve MPP^+ ile muamele edilip hücre canlılığını ölçüldü. GLT-1'in mRNA ve protein ifadesi tespit edildikten sonra glutamat assay ile birikmiş Glutamate miktarı belirlendi.

Bulgular: GLT-1'in mRNA seviyesi kainik asit ve MPP^+ ile muamele sonucu nöroblastoma ve glia hücrelerinde artmıştır. GLT-1'in protein seviyesi de glia hücrelerinde MPP^+ muamelesi sonucu artmıştır. Salınan glutamatın 12 saatlik MPP^+ muamelesinden sonra azaldığı gözlenmiştir. Ancak bu azalış daha sonra geçerli olmamıştır.

Sonuç: Bulgularımız göstermektedir ki GLT-1 ifadesi kainik asit veya MPP^+ muamelesi sonucu yaşamsal bir mekanizma olarak eksitotoksisiteyi önlemek için artmaktadır.

Anahtar Sözcükler: Eksitotoksisite, GLT-1, glutamat, MPP^+, kainik asit, mRNA

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Introduction

Glutamate is one of the major neurotransmitters in the central nervous system. It helps to regulate the principal functions of the brain, among which are learning and memory (1). Glutamate transporter-1 (GLT-1), also known as excitatory amino acid transporter 2 (EAAT2), is the major GLT, performing 95% of glutamate clearance, contributing to normal neuronal function, and protecting against excitotoxicity (2). There is no other extracellular mechanism of glutamate catabolism. After glutamate is taken up by astrocytes, it is either catabolized by glutamate dehydrogenase and the tricarboxylic acid cycle, or it is converted to glutamine by glutamine synthetase. Glutamine is then transported out of the astrocyte, enters the glutamatergic neurons, and is converted to glutamate via the enzyme glutaminase (3). Glutamate interacts with ionotropic and metabotropic receptors on postsynaptic neurons following its release from glutamatergic neurons (4).

Excitotoxicity is observed in age-dependent neurodegenerative diseases and also in stroke, epilepsy, and brain trauma (5). Preventing excitotoxicity will help to slow or halt the progression of these diseases. Excitotoxicity occurs as a result of excessive release of glutamate to the synapse after increased nerve cell stimulation. Excessive glutamate is taken up by GLT-1 present on astrocytes. Dysfunction or reduced expression of GLT-1 has been observed in many neurodegenerative disorders. Therefore, GLT-1 may be a therapeutical target for excitotoxicity (6).

Excitotoxicity may lead to neuronal death via apoptosis or necrosis (7). Overstimulation of glutamate receptors will trigger the entry of excessive Ca^{+2} and Na^{+} into cells via ion channels. Together with the Ca^{+2} released from mitochondria, this influx of Ca^{+2} ions will cause an overdose, resulting in necrosis. Excess Ca^{+2} influx will also lead to endoplasmic reticulum stress. Neuronal death may cause epileptogenic activities. Excitotoxicity is one of the major causes of epilepsy, which is a neurological disease associated with abnormal electrical activity in the brain, resulting in seizures and neuronal death (8). It may be diagnosed at all ages. Kainic acid is an AMPA/kainate receptor agonist and leads to excitotoxicity. Kainic acid is used as a model for status epilepticus in both cell (9) and animal models (10). It causes seizures, increased levels of glutamate in synapses, and neuronal death. It is also associated with neurodegeneration, behavioral phenotypes, oxidative stress, inflammation, mitochondrial dysfunction, and endoplasmic reticulum stress. Kainic acid is an agonist of kainate-class ionotropic glutamate receptors, which are generally excitatory (11). Excess stimulation by kainic acid induces excitotoxicity, which leads to apoptosis and epileptic seizures.

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, in which patients display motor deficit symptoms such as tremor, rigidity, bradykinesia, and akinesia (12). The pathology of PD includes progressive nigrostriatal dopaminergic degeneration and the presence of Lewy bodies. Excitotoxicity is one of the underlying molecular mechanisms of PD (12,13). Current studies indicate the decreased expression and function of EAATs in animal PD models (14). Additionally, increased expression and function of EAATs were shown to attenuate the death of dopamine neurons in the substantia nigra and striatum and ameliorate motor dysfunction in PD animal models. EAATs are potential effective drug targets for PD (14,15).

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a reliable and reproducible chemical model for PD (16,17). It produces damage of the nigrostriatal dopaminergic pathway after systemic administration. After passing through the blood–brain barrier, MPTP is converted to a toxic metabolite called 1-methyl 4-phenylpyridinium (MPP^{+}) by MaoB enzyme in glial cells. Afterwards, MPP^{+} is taken up by the dopamine transporter (17) into dopaminergic neurons, where it inhibits complex I in mitochondria leading to the generation of extensive ROS (17) and oxidative stress.

Neuroblastoma cells are used as a possible model in the study of glutamate receptors (18) and therefore used with kainic acid treatment in order to investigate the excitotoxicity and the molecular mechanism of epilepsy. Neuroblastoma cells are also used with MPP^{+} to investigate PD. Glial cells are also commonly used as a cellular model for elucidating the underlying mechanisms of neurodegenerative diseases.

Both neuroblastoma (N2A) and glial cell types are capable of glutamate release and uptake. Glial cells were shown previously to express kainate receptors (19-21). Kainic acid was demonstrated to destroy both oligodendrocytes and neurons and show its effects on both glia and neurons (22). The vast majority of cell lines, including glioma and oligodendritic cells, have been shown to contain ionotropic glutamate receptors co-existing with CySS/glutamate antiporters and metabotropic glutamate receptors (23).

In this study, we investigated the effect of two different toxins, kainic acid and MPP^{+}, on the expression of GLT-1 using two different cell lines.

Methods

Ethical Approval

Neither humans nor animals were used in the research; therefore, ethical approval was not required.

Cell Culture

Neuroblastoma (N2A) cells were obtained from ECACC. N2A cells were maintained in MEM with 10% FBS, 100 μg/mL penicillin/streptomycin, and 2 mM L-glutamine in 37 °C incubators with 5% CO2. Glial cells (IHA-immortalized human astrocytes) (24) were obtained from Dr. Yavuz Oktay at the IBG (Izmir Biomedicine and Genome Institute) and grown in DMEM with 10% FBS, 100 μg/ml penicillin/streptomycin, and 2 mM L-glutamine.

Kainic Acid and MPP^{+} Treatment

Both kainic acid and MPP^{+} were dissolved in H_2O. Vehicle-treated cells were used as a control. N2A cells were treated with 50 μM, 200 μM, and 1000 μM kainic acid for 24 h. For the

MPP^+ experiment, N2A cells were treated with 10 μM, 50 μM, 200 μM, and 500 μM MPP^+, and IHA cells were treated with 50 μM, 500 μM, and 1000 μM MPP^+ for 24 h.

MTT Cell Viability Assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) Cell Viability Assay (Biotum) is a standard test to determine the number of living cells. MTT is a tetrazolium salt metabolized by living cells and converted to purple formazan crystals. It is dissolved in DMSO and measured at 570 nm. The assay was performed according to the manufacturer's guidelines.

Glutamate Assay

The Glutamate-Glo assay (Promega) is a bioluminescent assay for selective and sensitive detection of glutamate in a biological sample. The assay couples glutamate oxidation and NADH production with a bioluminescent NADH detection system and does not measure glutamic acid in the structure of peptides or proteins. The assay was performed according to the manufacturer's guidelines, and the measurement was conducted using a luminometer.

Western Blotting

Protein was isolated from whole cell extracts using RIPA Lysis buffer according to the manufacturer's guidelines (VWR). Protease and phosphatase inhibitors (MedChem Express-100X) were added. Cell extracts were loaded onto 4%-15% gradient SDS-PAGE gels (Bio-Rad) following incubation with loading buffer (126 mM Tris HCl pH 6.5, 25% glycerol, 5% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue at 95°C for 5 min. Proteins were immunoblotted with anti-GLT-1 (Elabscience) and anti-tubulin (Elabscience) antibodies at 1:1000 and 1:5000 dilutions, respectively. The membranes were then probed with horseradish peroxidase-conjugated secondary antibodies (Elabscience) and developed with enhanced chemiluminescence western blotting substrate (Thermo Scientific). Blots were exposed to HyBlot autoradiography film or visualized with a detection system. The protein bands were quantified by densitometry analysis using ImageJ software (NIH, Washington, DC, USA).

RNA Isolation, cDNA Synthesis, and Real-Time PCR

Total RNA was isolated from N2A cells using the RiboEx solution (GeneAll) following the manufacturer's standard protocols. RNA concentration and purity were determined and confirmed via spectrophotometry. RNA (1 μg) was reverse transcribed using the high-capacity RNA to cDNA kit (WizBio) according to the manufacturer's protocol and used in RT-PCR reactions.

The cDNA was subjected to quantitative PCR analysis with GLT-1- (GLT-1 fwd: AACAATATGCCCAAGCAGGT, GLT-1 rev: CTCCCAGGATGACACCAAAC) and β-actin-specific primers (β-actin fwd: AACTGGGACGACATGGAGAA, β-actin rev: GAAGGTCTCAAACATGATCTGG) using the SYBR Green Gene Expression Assay from GeneAll, and quantifications were performed according to the manufacturers' instructions. The relative abundance of mRNA was obtained by normalization to β-actin mRNA levels.

Statistics Analysis

All statistical analyses were performed using statistical software (GraphPad Software, Inc., San Diego, USA). Specifically, "unpaired, one-tail, equal variance and two-sample t-tests" were performed using Prism 5 software. Significant differences are shown by asterisks indicating *$p<0.05$. Error bars on figures represent the standard error of the mean.

Results

Kainic Acid and MPP^+ Treatment Leads to a Decrease In Cell Viability in N2A Cells

To determine whether kainic acid induces excitotoxicity and cell death, we analyzed cell viability using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. We treated N2A cells with 50 μM, 200 μM, and 1000 μM kainic acid for 24 h and observed a significant decrease in cell viability with the 1000 μM concentration (Figure 1A). These results showed that kainic acid-induced excitotoxicity leads to the lowest cell viability at the 1000 μM concentration after a 24-h treatment, when compared with the control.

We also treated N2A cells with 10 μM, 50 μM, 200 μM, and 500 μM MPP^+ for 24 h and observed a significant decrease in cell viability with both 200 μM and 500 μM concentrations (Figure 1B). These results showed that MPP^+-induced excitotoxicity leads to the lowest cell viability at the 500 μM concentration after a 24-h treatment, when compared with the control.

Elevation of GLT-1 mRNA Levels in N2A Cells After Kainic Acid or MPP^+ Treatment

To analyze GLT-1 expression in neuroblastoma cells, we treated N2A cells with kainic acid or MPP^+, isolated total protein, and checked for GLT-1 protein levels. However, we could not visualize GLT-1 protein via western blotting, although we tried several different antibodies. We believe that this was due to the lower level of GLT-1 expression in neuroblastoma cells, compared with that in glial cells. GLT-1 is mainly expressed on astrocyte membranes in order to take up excess glutamate. It is also expressed in neuroblastoma cells but at much lower levels, compared with that in glial cells. Therefore, we isolated total RNA from N2A cells after treatment with 1000 μM kainic acid or 500 μM MPP^+ for 24 h. We identified GLT-1 mRNA expression using real-time quantitative PCR (q-PCR) using β-actin as a reference gene. We observed that both MPP^+ and kainic acid treatment induced much higher expression of GLT1 mRNA in N2A cells than control untreated cells (Figure 1C and 1D).

MPP^+ Treatment Leads to a Decrease in Cell Viability in IHA Cells

To determine whether MPP^+ induces excitotoxicity and cell death, we analyzed cell viability using the MTT assay. We treated IHA cells with 50 μM, 500 μM, and 1000 μM MPP^+ for 24 h and observed a significant decrease in cell viability. The 1000 μM concentration of MPP^+ resulted in the lowest cell viability (Figure 2A).

Elevation of GLT-1 Protein Levels in IHA Cells After MPP^+ Treatment

In order to investigate the effect of MPP^+ treatment on GLT-1 expression, we isolated total protein from IHA cells and analyzed GLT-1 protein levels by western blotting. We treated IHA cells with 1000 μM MPP^+ for 24 h, isolated total protein, and checked for GLT-1 expression. In two different experiments, we observed that GLT-1 levels increase significantly after MPP^+ treatment in IHA cells, compared with those in control cells (Figure 2B).

Elevation of GLT-1 Levels Delays Excitotoxicity in Cells After MPP^+ Treatment

We then analyzed the excess glutamate released in both N2A and IHA cells after MPP^+ treatment as a result of excitotoxicity via a glutamate assay, which measures only secreted extracellular glutamate. Glutamate dehydrogenase uses glutamate and NAD^+ to produce α-ketoglutarate and NADH. The assay couples glutamate oxidation and NADH production with a bioluminescent NADH detection system. We treated N2A cells with 500 μM MPP^+, collected the cell culture media at certain time points such as 0 h, 12 h, 24 h, and 48 h and measured glutamate with the glutamate assay (Figure 3A). At the 12-h time point, the glutamate levels of MPP^+-treated cells were even lower than those of non-treated control cells (Figure 3A). At the 24-h time point, this difference in levels of secreted glutamate between non-treated and MPP^+-treated N2A cells was no longer present. At the 48-h time point, the levels of secreted glutamate of MPP^+-treated N2A cells started to increase and tended to be higher than those of control cells, although the difference was not significant (Figure 3A).

Similarly, we treated IHA cells with 1000 μM MPP^+, and then, at 0, 12, 24, and 48 h, cell culture media was collected, and the excess glutamate level was assayed (Figure 3B). Similar to N2A cells, at the 12-h time point, the glutamate levels of MPP^+-treated cells were even lower than those of non-treated control cells (Figure 3B). At the 24-h time point, this difference in levels

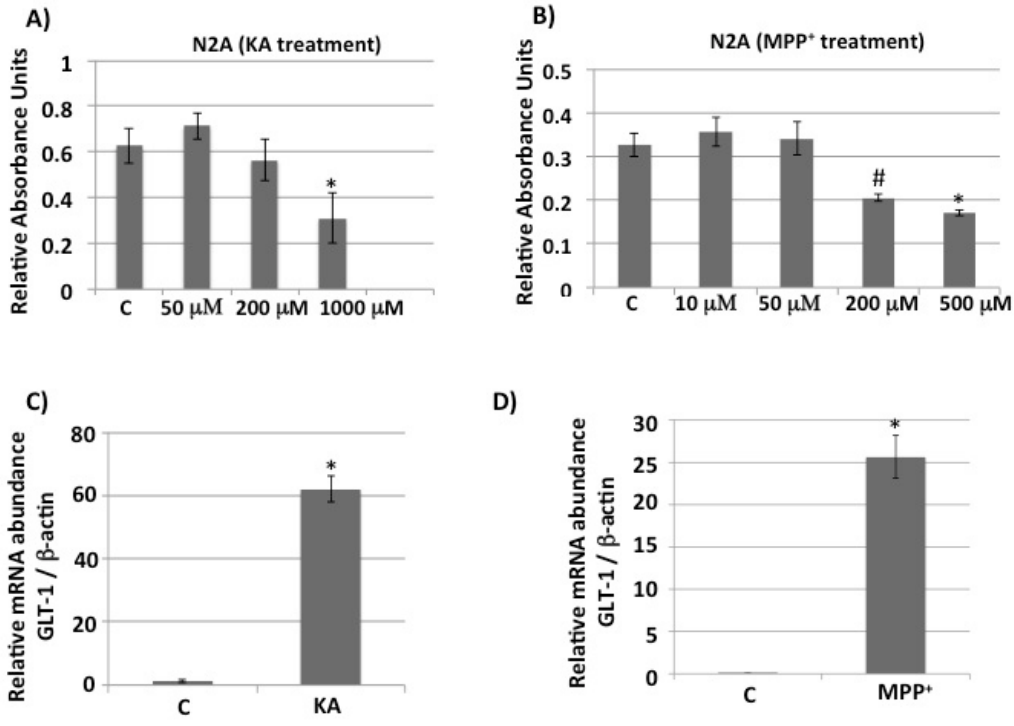

Figure 1. The effect of kainic acid and MPP^+ on cell viability and GLT-1 mRNA levels. Cell viability was measured using an MTT assay. The absorbance at 570 nm is expressed as arbitrary units. The values significantly different from the relative controls are indicated with symbols indicating $p<0.05$. **a)** N2A cells were treated with the indicated concentrations of kainic acid for 24 h. * denotes the comparison between control (C) and 1000 μM kainic acid treatment. **b)** The effect of MPP^+ on cell viability. Cell viability was measured using an MTT assay. The absorbance at 570 nm is expressed as arbitrary units. The values significantly different from the relative controls are indicated with symbols (*, #) indicating $p< 0.05$. N2A cells were treated with the indicated concentrations of MPP^+ for 24 h. * denotes the comparison between control (C) and 500 μM MPP^+ treatment. # denotes the comparison between control (C) and 200 μM MPP^+ treatment. **c)** GLT-1 mRNA expression in N2A cells. GLT-1 mRNA expression detected by q-PCR in non-treated or kainic acid treated N2A cells $p<0.05$. The numbers on y axis show the fold increase relative to β-actin levels. C: non-treated cells KA: Kainic acid treated cells. * denotes the comparison between C and KA. **d)** GLT-1 mRNA expression detected by q-PCR in non-treated or MPP^+ treated N2A cells $p<0.05$. The numbers on y axis show the fold increase relative to β-actin levels. C: non-treated cells MPP^+: MPP^+ treated cells. * denotes the comparison between C and MPP^+. The graph shows the average of three independent experiments

of secreted glutamate between non-treated and MPP+-treated IHA cells was no longer present. At the 48 h time point, the levels of secreted glutamate of MPP+-treated IHA cells started to increase as in the case of N2A cells (Figure 3A) and tended to be higher than those of control cells, although the difference was not significant (Figure 3B).

This result demonstrates that GLT-1 expression levels are elevated as a result of kainic acid or MPP+ treatment as a survival mechanism to take up excess glutamate and prevent excitotoxicity. This may be the reason why the levels of secreted glutamate of MPP+-treated IHA cells after the 12-h time point is even lower than that of control non-treated cells. However, if the insult persists, this transient elevation of GLT-1 might not be sufficient to take up excess glutamate as in the case of the 24 h and 48 h time points (Figure 3A and 3B). Another reason might be that, due to the death of cells after 12 h of treatment, the cell number decreases; therefore, the amount of glutamate secreted is decreased.

Discussion

These results show that, after an insult to the cell that increases excitotoxicity, such as MPP+ or kainic acid, the cell automatically increases GLT-1 expression as a survival mechanism in order to take up excess glutamate. This was the case in both cell types. When we analyzed the levels of secreted glutamate with the glutamate assay in N2A and IHA cells after MPP+ treatment, we observed that, in both cell types, glutamate levels showed a tendency to increase in MPP+-treated cells after 48 h of treatment.

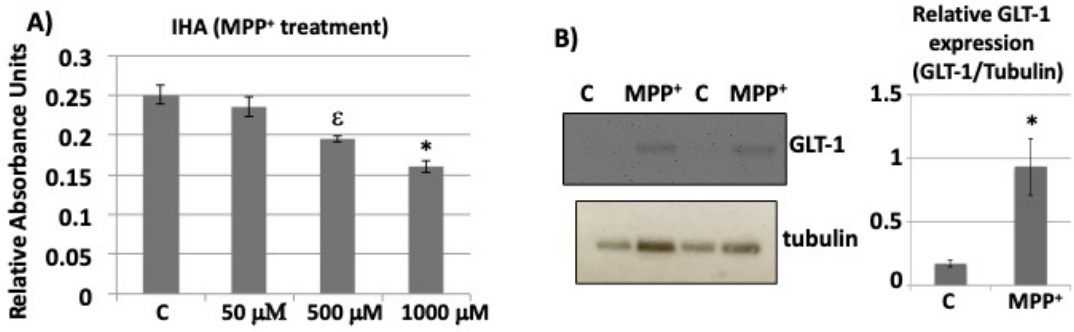

Figure 2. The effect of MPP+ treatment on IHA cells and GLT-1 protein expression level. **a)** IHA cells were treated with the indicated concentrations of MPP+ for 24 h. The values significantly different from the relative controls are indicated with symbols (*, ε) indicating p<0.05. * denotes the comparison between control (C) and 1000 µM MPP+ treatment. ε denotes the comparison between control (C) and 500 µM MPP+ treatment. **b)** A western blot of non-treated (C) or MPP+ treated IHA cell extracts show expression levels of GLT-1, with tubulin as a loading control. Relative protein levels from the blot (the average of two independent experiments) are quantified on the right. *p<0.05. C: non-treated cells MPP+: MPP+-treated cells. * denotes the comparison between C and MPP+

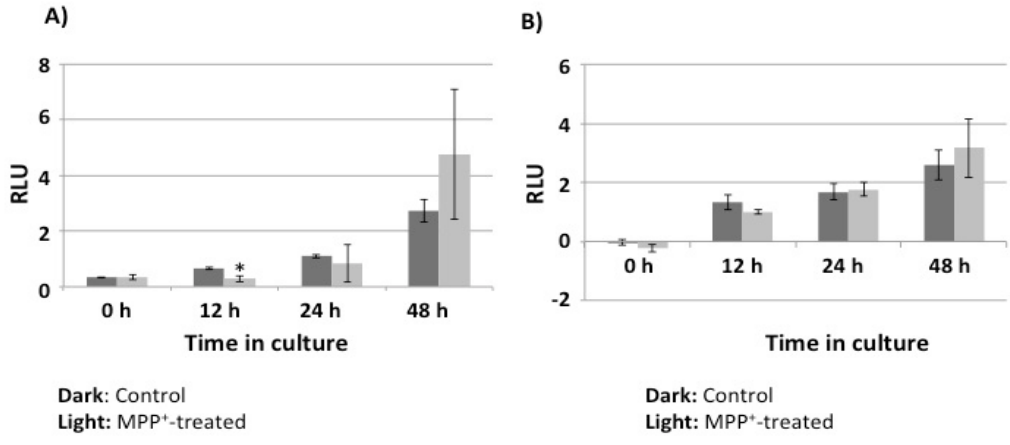

Figure 3. The measurement of secreted glutamate levels in MPP+-treated cells. **a)** The excess glutamate levels were measured from cell media using glutamate assay in non-treated and MPP+-treated N2A cells at indicated time points. p<0.05. C: non-treated cells. MPP+: MPP+-treated cells. * denotes the comparison between C and MPP+ at 12 h time point. RLU: Relative Luminescence Units. **b)** The excess glutamate levels were measured from cell media using glutamate assay in non-treated and MPP+-treated IHA cells at indicated time points. C: non-treated cells. MPP+: MPP+-treated cells. RLU: Relative Luminescence Units. Dark: Control (C), Light: MPP+-treated

After 12 h of treatment, the levels of secreted glutamate in MPP^+-treated cells were lower than those in control cells. At the 24-h time point, this difference was no longer prominent. This shows that, in the first 24 h of MPP^+ or kainic acid treatment, GLT-1 levels increased immediately in treated cells. Therefore, glutamate levels were even lower in MPP^+-treated cells, compared with non-treated cells due to the increase in GLT-1 following treatment. However, after 24 h, first, the difference was no longer present (24-h time point), and then the glutamate levels started to increase in MPP^+-treated cells (48-h time point). The reason for this might be that the transient increase in GLT-1 expression might have returned to normal levels. Another reason for the reduction in glutamate levels after 12 h might be cell death. Since the number of living cells decreases, the amount of secreted glutamate is reduced.

We could not detect GLT-1 expression in the total proteins we isolated from N2A cells, although we tried different antibodies. The explanation for this might lie the fact that neurons express much less GLT-1 protein than glial cells.

Our experimental system does not distinguish between the secreted and absorbed glutamate, nor does it measure dynamics. The glutamate assay only measures the secreted and accumulated glutamate in the medium. Obviously, using a neuron-glia mixed culture could be ideal for studying this system; however, since we are interested in the change in expression of GLT-1, which is expressed by both cell types, using one cell type enabled us to analyze the response of that particular cell line to kainic acid or MPP^+.

Any stress to the cell will lead to an activation of the stress-response mechanisms (25). Administration of a toxin to a cell line is also a stress and triggers certain defense mechanisms. Both kainic acid and MPTP are known to induce oxidative stress (26,27). After the induction of stress in the cells due to treatment with kainic acid or MPP^+, glutamate release increases. As a response to this event, GLT-1, whose task is to clear up the excess glutamate, automatically increases. Since the upregulation of GLT-1 increases glutamate uptake (28), the cell attempts to get rid of the excess glutamate by increasing the expression of GLT-1 after the administration of kainic acid or MPP^+. However, the elevation of GLT-1 levels might not be adequate to cope with the increase in excitotoxicity due to the continuation of the stress.

A previous study showed that when kainic acid was administered (intraperitoneally 25 mg/kg) to SIRT4 KO mice, they displayed increased seizure activity (29). This result shows that the lack of SIRT4 in the brain increases the excitotoxicity induced by kainic acid. The molecular mechanism was found to be the decrease in glutamate uptake and also the decrease in the cell surface expression of GLT-1. GLT-1 is known to be internalized from the cell surface when there is no excess glutamate and degraded in the cell via ubiquitination (30-32). Its surface expression increases when there is an increase in extracellular glutamate levels. When the excitotoxicity decreases, GLT-1 might be internalized via vesicles and degraded (30-32).

Study Limitations

In the future studies, glutamate could be measured before the 12-h time point to capture the level of glutamate secretion before cell death. In this way, we can rule out a reduction in glutamate secretion due to the decrease in cell number.

Conclusion

Our study showed that both cell types, neuronal and glial, showed an autonomous survival mechanism as a result of an excitotoxic insult (MPP^+ or kainic acid) by significantly increasing GLT-1 expression levels in order to take up the excess glutamate to protect the cell. GLT-1 is the major transporter that performs 95% of the glutamate clearance contributing to normal neuronal function and protecting against excitotoxicity. Therefore, increasing GLT-1 expression may protect from excitotoxicity and cell death. Memantine, an FDA-approved drug for Alzheimer's Disease and other dementias, targets glutamate excitotoxicity (33). Rilutek (Riluzole) was the first drug approved by the U.S. Food and Drug Administration (FDA) in 1995 to treat amyotrophic lateral sclerosis. The exact mechanism of Rilutek is not known. However, it is believed that the drug blocks the release of glutamate from nerve cells (34). Therefore, targeting molecular pathways regarding the increase of GLT-1 expression or activity may lead to therapeutics of neurodegeneration and pave the way to enlarging our understanding of brain diseases on the cellular level.

Ethics

Ethics Committee Approval: Neither humans nor animals were used in the research; therefore, ethical approval was not required.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: G.D.Y., Design: G.D.Y., Data Collection or Processing: R.U., C.A., Analysis or Interpretation: R.U., C.A., G.D.Y., Literature Search: R.U., C.A., G.D.Y., Writing: G.D.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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