Extracellular ATP Binding to P2Y1 Receptors Prevents Glutamate-Induced Excitotoxicity: Involvement of Erk1/2 Signaling Pathway to Suppress Autophagy

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Abstract

Glutamate-induced neuroexcitotoxicity causes neuronal death in neurodegenerative diseases including Alzheimer's disease and Parkinson's disease. Extracellular ATP exerts a wide variety of functions, such as attenuating A β -mediated toxicity, inhibiting NMDA receptor subunit combinations, and aggravating ischemic brain injury. However, the effect of extracellular ATP on glutamate-induced neuroexcitotoxicity remains largely unknown. Herein, we showed that extracellular ATP prevented the glutamate-induced excitotoxicity via binding to its P2Y1 receptors. We found that excessive glutamate triggered cellular ROS overproduction and mitochondrial membrane potential damage, which were significantly attenuated by extracellular ATP. Besides, glutamate activated autophagy, as illustrated by the increased protein level of autophagic marker LC3II and decreased level of p62, and glutamate-induced neuroexcitotoxicity could be completely abolished by autophagy inhibitor chloroquine. In addition, we revealed that extracellular ATP activated Erk1/2 signaling to suppress autophagy and to exert its neuroprotective effects, which was further reduced by autophagy agonist rapamycin and the selective Erk1/2 inhibitor PD0325901. Taken together, our findings suggest that extracellular ATP binding to P2Y1 receptors protected against glutamate-induced excitotoxicity via Erk1/2-mediated autophagy inhibition, implying the promising potential of ATP for the treatment of neurodegenerative disorders.

Introduction

Neuroexcitotoxicity caused by glutamate is a marked pathogenesis in a variety of neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD) [1–3]. Glutamate acts as an excitatory neurotransmitter and exert many functions in mammalian brain, such as learning and memory, via modulating Na⁺, Ca²⁺ and K⁺ influx through its several receptors [4]. Excessive glutamate release induces the overactivation of glutamate receptors and high levels of Ca²⁺ entry into neural cells, which in turn triggers excitotoxicity and ultimately neural death. Glutamate has been widely used as an excitotoxin to induce neural cell death *in vivo* and *in vitro*. For instance, glutamate treatment resulted in an increase of mitophagy and high levels of zinc ion in HT-22 cells [5], or induced apoptosis in the rat primary cortical neurons [6] and SH-SY5Y cells [7].

Adenosine triphosphate (ATP) has been reported to play important roles in the central nervous system (CNS). Massive extracellular release of ATP is involved in the etiopathology of some neurodegenerative disorders. For instance, extracellular ATP activated astrocytes to mediate motor neuron death [8]. Extracellular ATP also contributed to photoreceptor neurodegeneration in rodents and induced intracellular alpha-synuclein to damage lysosome functions [9, 10]. However, increasing evidence has showed that extracellular ATP could be neuroprotective. An analogue of ATP, ATP- γ -S-(α , β -CH₂), rescued PC12 cells from oxidative stress injury and amyloid beta-induced toxicity [11]. Besides, extracellular ATP administration protected astrocytes against H₂O₂-induced oxidative injury [12], or activated glutamate receptor to modulate synaptic plasticity in the hippocampus [13]. Importantly, ATP has recently been demonstrated as an endogenous inhibitor of N-Methyl-D-Aspartate (NMDA) receptors against glutamate-induced mitochondrial membrane potential disruption in cultured rat hippocampal neurons [14].

Therefore, these actual results suggest that extracellular ATP acts opposite effects in the CNS: neuroprotection and neurodegeneration. Although ATP has been reported to be neuroprotective against glutamate, the contribution of extracellular ATP on excitotoxicity induced by glutamate and its underlying mechanism in SH-SY5Y neuroblastoma cells still maintain largely unknown.

In the present study, we investigated the potential of extracellular ATP on glutamate-induced excitotoxicity and explored the underlying molecular mechanisms in SH-SY5Y cells. We clearly demonstrated that extracellular ATP suppress autophagy to attenuate glutamate-induced excitotoxicity via Erk1/2 signaling. Our works therefore highlight ATP as an effective agent in the treatment of neurodegenerative diseases.

Material And Methods

Reagents and Antibodies

Extracellular ATP, PD0325901, rapamycin, and chloroquine (CQ) were purchased from MedChemExpress (MCE, USA), and L-glutamate was obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin and phosphate-buffered saline (PBS) were purchased from Gibco (Thornton, Australia). The Cell Counting kit (CCK-8) was purchased from Dojindo Laboratory (Kumamoto, Japan). The JC-1 assay kit (5,50,6,60-tetrachloro-1,10,3,30-tetraethyl-imidacarbocyanine iodide) and RIPA lysis buffer were obtained from Beyotime Biotech (China). The ROS assay kit (DCFH-DA) was purchased from Meilun (China). The FITC-labeled Annexin V Apoptosis Detection kit was purchased from BD Biosciences (Canada). The antibodies of p-Erk1/2, Erk1/2, p-Akt, Akt, LC3, SQSTM1/p62, GAPDH and horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-P2Y1 receptor antibody was purchased from Alomone Labs (Jerusalem BioPark, Israel).

Cell Culture and Treatment

Human neuroblastoma cell SH-SY5Y cells were purchased from The Global Bioresource Center (ATCC, USA) and were cultured in DMEM with the addition of 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ incubator. SH-SY5Y cells were plated at a density of 2×10^4 /well in 96-well plates and 2×10^5 /well in 6-well plates for 24 h. Thereafter, SH-SY5Y cells were pretreated with either PBS or extracellular ATP followed by a stimulation of 10 mM glutamate for 24 h and experimental analyses, such as cell viability, mitochondrial membrane potential, ROS generation and protein expression, were performed.

Cell viability

Cell viability was quantified by CCK-8 kit following the manufacturer's instruction. After SH-SY5Y cells seeded in 96-well plates were treated with extracellular ATP and/or glutamate for 24 h, 10 μ L CCK-8

solution was added into each well and incubated at 37 °C for 1 h. Thereafter, the absorbance at 450 nm was determined using a microplate reader (BioTek).

Measurement of Intracellular Reactive Oxygen Species

The intracellular levels of reactive oxygen species (ROS) were measured with probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, ROS assay kit). Briefly, the treated SH-SY5Y cells were washed twice with PBS and incubated in serum-free medium with 10 µM DCFH-DA at 37 °C for 30 min. Fluorescent images were captured in six different areas of each well under a fluorescent microscope (AxioVert A1, Zeiss, Germany) and measured using Image-J v1.8.0 software. The percentage of ROS-positive cells was calculated.

Measurement of Mitochondrial Membrane Potential

The level of mitochondrial membrane potential was measured by a commercial cyanine JC-1 assay kit, either as red fluorescent J-aggregates or as green fluorescent J-monomers, as previous [15]. J-aggregates at higher mitochondrial concentrations reflect higher membrane potential whereas J-monomers at lower mitochondrial concentrations indicate lost membrane potential. Accordingly, fluctuation of mitochondrial membrane potential is represented as the J-aggregate/J-monomer (red/green) fluorescence intensity ratio. The treated SH-SY5Y cells were washed with PBS and then incubated with 1 mL JC-1 solution (5 µM) at 37 °C for 30 min. Subsequently, images of the cells were captured in six different areas of each well under the fluorescence microscope. The changes in mitochondrial membrane potential were presented as the red/green fluorescence intensity ratio using Image-J v1.8.0 software.

Western blot

After each treatment, cells were rinsed twice with cold PBS and lysed by RIPA lysis buffer containing proteases inhibitors (1:50, Roche, Germany), phosphatase inhibitors 1 and 2 (1:100, Roche, Germany), phenylmethanesulfonyl fluoride (PMSF, 1:100, Beyotime, China), and nuclease (1:1000, Biolog Biotech, China) on ice. The collected cell lysates were then vortexed and the insoluble cell debris were removed by centrifugation at $12000 \times g$ for 10 min at 4 °C. The total protein concentrations were measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The equal amount of protein extracts (10 µg) were separated on a 10%-15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T) at room temperature for 60 min and then incubated with the relevant primary antibodies at 4 °C overnight. The antibodies were diluted as follows: p-Erk1/2, Erk1/2, LC3II, p62, Akt and p-Akt and GAPDH in 1:1000 dilution. Afterwards, membranes were incubated with corresponding HRP-conjugated secondary antibodies at a 1:5000 dilution for 60 min at room temperature. Reactive bands were visualized by the Quantity One automatic imaging analysis system (Bio-Rad, Hercules, CA, USA) using enhanced chemiluminescence ECL (Millipore, Kankakee, IL, USA). Finally, the protein band intensities were calculated using Image-J v1.8.0 software.

Statistical Analysis

Statistical comparisons were performed using one-way analysis of variance (ANOVA) following by a *post-hoc multiple-comparison Tukey* test. All quantified biological data were expressed as means $\pm$ standard error of the mean (SEM) of at least three independent experiments. Mean values were considered to be significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Extracellular ATP Reduces Glutamate-Mediated Excitotoxicity via Binding to P2Y1 Receptors in SH-SY5Y Cells

To study the vulnerability of SH-SY5Y cells to glutamate, SH-SY5Y cells were initially exposed to several concentrations of glutamate (2, 4, 6, 8, 10, 12, and 14 mM) for 24 h, and the cell viability was detected using CCK-8 kit. The result showed that glutamate caused a dose-dependent decrease in cell viability as compared to control (Fig. 1A and 1B). Glutamate at 4 mM led to a significant decrease in cell density ($P < 0.001$). Glutamate of 10 mM could induce almost half SH-SY5Y cell loss and thus was applied in all the following experiments. We then examined whether extracellular ATP exerted neuroprotective effect against glutamate-induced excitotoxicity in SH-SY5Y cells as that in rat hippocampal neurons. SH-SY5Y cells were incubated with 10 mM glutamate in the presence or absence of 5 μ M or 10 μ M ATP for 24 h, respectively. As shown in Fig. 1C and 1D, extracellular ATP at both concentrations significantly prevented glutamate-induced reduction of cell viability ($P < 0.001$, compared to the glutamate group), while 10 μ M extracellular ATP did not exhibit any cytotoxicity. In addition, extracellular ATP remarkably prevented reduction of EdU incorporation induced by glutamate (**Supplementary Figure 1**). Importantly, we demonstrated that purinergic P2Y1 receptors of extracellular ATP were expressed in SH-SY5Y cells, and MRS2500, the selective antagonist of P2Y1 receptors, completely abolished the neuroprotection of extracellular ATP (Fig. 1E and 1F). Finally, we tested whether glutamate triggered apoptosis in SH-SY5Y cells and no apoptotic cells were observed (**Supplementary Figure 2**), in accordance with the previous study [16]. Taken together, these results demonstrated that extracellular ATP protects SH-SY5Y cells from glutamate-mediated excitotoxicity via the P2Y1 receptors.

Extracellular ATP Reduces Intracellular ROS Overproduction and Mitochondrial Membrane Potential Damage

Previous studies have manifested that glutamate induced excessive ROS productions and mitochondrial dysfunction to cause cell death [17, 18]. Accordingly, we analyzed intracellular ROS production induced by glutamate in the presence or absence of extracellular ATP. As shown in Fig. 2A and 2B, ROS production was significantly induced by 10 mM glutamate, which was further prevented by either 5 μ M or 10 μ M extracellular ATP. We also monitored the mitochondrial membrane potential by JC-1 staining, and found that the red/green ratio was significantly reduced by 10 mM glutamate compared to the control group (Fig. 2C and 2D). However, administration of extracellular ATP substantially increased the red/green ratio

($P < 0.001$, compared to the glutamate group), suggesting that extracellular ATP could prevent mitochondrial membrane potential disruption. Therefore, extracellular ATP exerted neuroprotective effects by reducing ROS production and restoring mitochondrial functions.

Extracellular ATP Neuroprotects Against Glutamate-Induced Excitotoxicity via Suppressing Autophagy

There were numerous evidence showing the connections among mitochondrial damage, ROS overproduction and autophagy [19, 20]. To address whether autophagy was involved in glutamate-induced excitotoxicity in SH-SY5Y cells, the protein level of LC3II, a critical marker of autophagy, and the level of SQSTM1/p62 (p62), a marker for autolysosomal degradation, were analyzed. Apparently, exposure to glutamate significantly increased the level of LC3II compared to the control group ($P < 0.001$) whereas the expression of p62 was downregulated (Fig. 3A). Treatment with chloroquine (CQ), an autophagic inhibitor, further upregulated the amount of LC3II and p62, further indicating the activation of autophagy by glutamate (Fig. 3A-3C). In addition, CQ treatments significantly increased cell viability and prevented ROS overproduction induced by glutamate (Fig. 3D-3F). Administration of CQ also significantly ameliorated the mitochondrial membrane potential disruptions (Fig. 3G and 3H). Therefore, these above results clearly demonstrated that glutamate activates autophagy to cause excitotoxicity. Furthermore, we found that extracellular ATP restored the activated autophagy by glutamate, as evidenced by decreased expression of LC3II and upregulated expression of p62, implying that extracellular ATP suppresses autophagy to attenuate glutamate-mediated excitotoxicity (Fig. 3I-3L). Consistently, when cells were simultaneously pretreated with extracellular ATP as well as rapamycin, an agonist of autophagy, the protective effects of ATP against glutamate-induced excitotoxicity disappeared (Fig. 3L and **Supplementary Figure 3**). CQ did not significantly affect ATP neuroprotection against glutamate-induced excitotoxicity (**Supplementary Figure 4**). Taken together, these results suggested that extracellular ATP suppresses autophagy to inhibit glutamate-induced excitotoxicity.

Erk1/2 Signaling Pathway Is Involved in the Neuroprotective Effects of Extracellular ATP

Next, we examined the possible roles of several signaling pathways implicated in the ATP-mediated protective response to glutamate-induced neuroexcitotoxicity, such as PI3K/Akt and Erk1/2 signaling pathways [1, 21, 22]. We monitored the protein levels of phosphorylated Erk1/2 (p-Erk1/2) and phosphorylated Akt (p-Akt), and found that the levels of p-Erk1/2 were down-regulated by glutamate in SH-SY5Y cells; whereas the treatments of extracellular ATP significantly increased p-Erk1/2 expression (Fig. 4A and 4B), suggesting that ATP activates Erk1/2 to antagonize glutamate. However, the expression of p-Akt was not affected by extracellular ATP and glutamate (**Supplementary Figure 4E and 4F**). To further explore the potential of Erk1/2 signaling in ATP neuroprotection against glutamate-mediated excitotoxicity, PD0325901, a selective Erk1/2 inhibitor, was employed. As shown in Fig. 4C, the enhancement of cell viability treated by extracellular ATP against glutamate was totally abolished by PD0325901. In addition, PD0325901 prevented the decrease of ROS generation treated by ATP (Fig. 4C

and 4D), as well as inhibited the ATP-mediated recovery of mitochondrial membrane potential (Figure 4E and 4F). Furthermore, PD0325901 prevented the ATP-promoted increase in EdU incorporation (Supplementary Figure 5C and 5D). Together, these results suggested that the activated Erk1/2 pathway is involved in the neuroprotective effects of extracellular ATP against glutamate-induced excitotoxicity.

Extracellular ATP suppresses Autophagy via Erk1/2 Signaling Pathway

To further definite the neuroprotective mechanism of extracellular ATP against glutamate, we examined the connection between Erk1/2 and autophagy inhibition. SH-SY5Y cells were treated with glutamate and ATP in the presence or absence of PD0325901, and protein levels of LC3II levels and p62 were analyzed. Apparently, PD0325901 treatment entirely reversed the expression levels of LC3II and p62 mediated by extracellular ATP (Fig. 5A-5C). However, autophagy inducer rapamycin did not influence the expression of p-Erk1/2 affected by extracellular ATP (Fig. 5D and 5E). Therefore, we concluded that extracellular ATP activates Erk1/2 signaling pathway to inhibit glutamate-mediated activation of autophagy.

Discussion

Increasing studies have reported that extracellular ATP could be neuroprotective in the CNS. For instance, extracellular ATP could attenuate NMDA-mediated neurotoxicity in cultured hippocampal neurons by inhibiting the heterologous expression of NMDA receptors [23]. Extracellular ATP regulated amyloid precursor protein processing to inhibit A β production [24]. Enhancement of ATP production could ameliorate cognitive dysfunctions in a PD mouse model [25]. In current study, we demonstrated that extracellular ATP protected SH-SY5Y cells from glutamate-induced excitotoxicity via activating Erk1/2 to suppress lethally autophagy. These studies suggest that extracellular ATP could be able to develop as a therapeutic strategy for neurodegenerative disorders.

Neuroexcitotoxicity caused by high concentration of glutamate is one of pivotal pathological features in neurodegenerative disorders [26, 27]. Abnormally high concentration of glutamate stimulates the excessive activation of N-methyl-D-aspartate (NMDA) subtype of ionotropic L-glutamate receptors, subsequently results in an excessive Ca²⁺ influx, which is taken up by mitochondria and subsequently triggers excitatory biochemical processes and death pathways [28, 29]. Furthermore, glutamate-mediated excitotoxicity involves ROS overproduction that leads to neuroinflammation and synaptic dysfunctions in rat brain regions [30]. In the present study, we also demonstrated that glutamate remarkably caused ROS generation in SH-SY5Y cells (Fig. 2A and 2B), which was consistent with previous studies that glutamate caused oxidative damage in SH-SY5Y cells [31, 32]. Massive ROS production aggressed mitochondria and led to mitochondrial dysfunctions [33, 34]. Our previous study has shown that excessive ROS caused the loss of mitochondrial membrane potential and activated mitochondria-dependent cell deficits in HT-22 cells [15]. Besides, ROS overproduction stimulated by glutamate could result in lipid peroxidation and mitochondrial dysfunctions to the detriment of cellular growth [32]. Kushnareva *et al.* reported that excessive glutamate caused a reduction of mitochondrial ATP and an imbalance of homeostasis that

precede commitment to neuronal death [35]. Consistent with previous studies, we observed that exposure to 10 mM glutamate for 24 h indeed induced disruptions of mitochondrial membrane potential in SH-SY5Y cells (Fig. 2C and 2D). Furthermore, we showed that 10 mM glutamate prevented cell proliferation by EdU staining (**Supplementary Figure 1**). Finally, glutamate induced HT-22 cell ferroptosis mediated by massive ROS production [36]. However, our data showed that glutamate could neither affect glutathione peroxidase 4 levels nor glutathione expression, which suggested that glutamate-induced excitotoxicity in SH-SY5Y cells was not related to ferroptosis (data not shown). Flow cytometer analysis showed that glutamate could not induce SH-SY5Y cell apoptosis (**Supplementary Figure 2**). Characteristics of cell injury in response to glutamate shown in these studies suggest that glutamate-induced excitotoxicity is associated to multiple molecular mechanisms. Together, we confirmed that glutamate induced excessive ROS generation, triggered mitochondrial dysfunctions, and prevented cell proliferation.

ATP, a well-known intracellular energy currency, is a signaling molecule that influences cell proliferation, differentiation, survival, myelination as well as repairment in the CNS [37, 38]. Extracellular ATP not only counteracted H_2O_2 -promoted cell death in astrocytes [39], but also ameliorated cognitive impairments in a mouse model with PD [25]. Notably, extracellular ATP has been implicated to act two opposite roles when it combines and activates different purine receptors (P2X receptors and P2Y receptors) in ischemic brain injury: pathogenesis and neuroprotection. For instance, P2X1, P2X2, P2X4, P2X7 receptors participated in the pathogenesis of cerebral ischemia whereas P2Y1 receptor could induce neuroinflammatory responses to neuroprotect against ischemic neuronal injury [40–42]. These studies demonstrated that the activation of different purine receptors could be crucial for extracellular ATP neuroprotection. Our current study elucidated that extracellular ATP protected SH-SY5Y cells from glutamate-induced neuroexcitotoxicity. The observation showed that either 5 μ M or 10 μ M of extracellular ATP could significantly rescue SH-SY5Y cells from 10 mM glutamate-mediated excitotoxicity, including increasing cell viability, inhibiting ROS generation, and recovering mitochondrial functions (Fig. 1 and 2). Importantly, we found that P2Y1 receptors expressed in SH-SY5Y cells, and its selective antagonist, MRS2500, absolutely eliminated neuroprotective effects of extracellular ATP against glutamate (Fig. 1E and 1F), which was supported by a previous study that activated P2Y1 receptors were neuroprotective [42]. Evidence, on the contrary, demonstrated that P2Y1 receptor blockade could normalized activation of astrocytes and then attenuated cognitive dysfunctions in AD mouse models [43], indicating that activation of P2Y1 receptor could mediate neurodegeneration. The opposite roles of P2Y1 receptors in neurodegeneration and neuroprotection might be due to the activation of P2Y1 receptors in different neural cells: neuroprotection in neuronal cells and neurodegeneration in glial cells. In neurons, both ATP and its secondary metabolite ADP turn to AMP through CD39 or E-NTPDase and then is hydrolyzed into adenosine by CD73 or 5'nucleotidase. Adenosine is widely valued in the treatment of the CNS diseases [44]. In order to verify ATP neuroprotective effects, ADP was applied to pretreat SH-SY5Y cells incubated by glutamate, and CCK-8 assay showed that ADP did not rescue SH-SY5Y cells from glutamate-induced excitotoxicity (data not shown). Taken together, we concluded that extracellular ATP binding to its P2Y1 receptors protected from glutamate-induced excitotoxicity in SH-SY5Y cells.

Under normal physiological conditions, autophagy maintains neuronal homeostasis and neuronal survival [45]. Autophagy has been implicated to play a dominant role in neurodegenerative disorders, such as AD and PD. Autophagy could promote the metabolism of A β and the assembling of tau, and thus dysfunctions of autophagy might lead to the progress of AD [46, 47]. Moreover, autophagy processes could alter PD-associated genes, like SNCA, GBA, ATP13A2, and FBXO7 [48]. These studies suggested that selective agonists of autophagy might be developed as potential drugs for neurodegenerative disorders. However, excessive autophagy induces oxidative damage, neuroinflammatory injury, and then exacerbates neurodegeneration. Evidence has revealed that high concentrations of glutamate induced autophagy via the activation of the lysosomal Ca²⁺ channels TPC1 and TPC2 in neural cells [49]. In this study, we found that 10 mM glutamate significantly induced an increase of LC3 and a decrease of p62/SQATM1 (Fig. 3A), indicating that autophagy could be involved in glutamate-induced excitotoxicity. An inhibitor of autophagy, CQ, significantly suppressed glutamate-induced excitotoxicity, including increasing cell viability, decreasing ROS generation, and recovering mitochondrial functions (Fig. 3F-K). Our results demonstrated that glutamate-induced excitotoxicity was mediated by autophagy, which was supported by a previous study [49]. Importantly, the treatments of ATP were able to suppress autophagy induced by glutamate (Fig. 3 and **Supplementary Figure 3**). A study demonstrated that knockdown or pharmacological inhibition of purinergic P2Y12 receptors suppressed autophagy process in advanced atherosclerosis [50], which supported that extracellular ATP could suppress autophagy processes. Similarly, high concentrations of adenosine, one of ATP metabolites, inhibited autophagy in β cells [51]. Opposite to our study, a literature showed that extracellular ATP could be though P2X7 receptors to trigger autophagy in primary microglia [52]. In summary, these studies implied that extracellular ATP could exert different functions through activating different receptors in a certain condition. In the current study, we confirmed that the neuroprotective effects of ATP against glutamate-induced excitotoxicity was via preventing autophagy.

Multiple mechanisms were involved in the neuroprotective effects of extracellular ATP. For instance, extracellular ATP binding to P2Y1 receptors protected pancreatic duct epithelial cells via cAMP signaling pathway [53]. Extracellular ATP binding to P2Y13 purinergic receptors could neuroprotect astrocytes via PI3K/Akt/GSK3 pathway [54]. Also, extracellular ATP attenuated stroke via inhibiting glutamate release to neuroprotection of antioxidants against [55]. Our current study demonstrated extracellular ATP neuroprotected against glutamate-mediated excitotoxicity through Erk1/2 signaling pathway in SH-SY5Y cells (Fig. 4). PD0325901, a selective antagonist of Erk1/2 remarkably abolished the neuroprotective effects of extracellular ATP. Consistently, activation of Erk1/2 promoted PC12 cell survival by blocking the expression of the pro-apoptotic factor, BH3-only protein Bim [56]. A previous study showed that activated ATP-dependent potassium channels promoted glioma cell proliferation by upregulated Erk1/2 signaling pathway [57], which supported our findings that extracellular ATP pretreatment via Erk1/2 signaling pathway promoted SH-SY5Y cell proliferation (**Supplementary Figure 5**). Interestingly, extracellular ATP induced the S-phase cell cycle arrest of oral squamous cell carcinoma SAS cells via its binding to P2Y1 receptors-mediated Erk1/2 signaling [58]. This research showed the antitumor effects of extracellular ATP were through interaction with P2Y1 receptors to promote Erk1/2 signaling, which was opposite to its

effects in SH-SY5Y cells shown in the current study. This might be due to extracellular ATP exerted multiple functions in different microenvironments. Notably, our results showed that extracellular ATP through upregulation of p-Erk1/2 expression prevented autophagy against glutamate excitotoxicity (Fig. 5). Similarly, *Sun et al.* demonstrated that active MEK1 protected against H₂O₂-induced autophagy through Erk1/2 signaling pathway in neonatal rat cardiac ventricular cardiomyocytes [59]. Evidence, on the contrary, demonstrated that activation of Erk1/2 could contribute to glutamate-induced oxidative injury in primary cortical neurons [59, 60]. Another study also showed that PD98059, a blockade of Erk1/2 pathway, suppressed H₂O₂-induced cell death [61]. These studies illustrate that Erk1/2 signaling pathway can promote either neuronal survival or neuronal death under different paradigms. One possible explanation is that the magnitude and duration of Erk1/2 activation determines the different downstream effectors exerting the death-promoting capacity and cellular outcomes [59].

The current research is only based on non-clinical *in vitro* experiments, and a combination of *in vivo* and *in vitro* experiments is still needed to further explore the role of extracellular ATP as a potential candidate against excitotoxicity.

In conclusion, we clearly demonstrated that extracellular ATP binding to P2Y1 receptors prevented glutamate-mediated neuroexcitotoxicity through activating Erk1/2 to suppress lethally autophagy in SH-SY5Y cells (**Supplementary Figure 6**). Our present findings provide new insights into the neuroprotective molecular mechanism of extracellular ATP and extend the therapeutic strategy of neurodegenerative diseases.

Abbreviations

AD, Alzheimer's disease; PD, Parkinson's disease; CNS, central nervous system; ATP, Adenosine triphosphate; ROS, Reactive oxygen species; CQ, chloroquine; A β , Amyloid β ; NMDA, N-Methyl-D-Aspartate; LC3, light chain 3; SQSTM1/p62, Sequestosome 1; Erk1/2, extracellular signal-regulated kinases 1 and 2; p-Erk1/2, phosphorylated extracellular signal-regulated kinases 1 and 2; Akt, protein kinase B; p-Akt, phosphorylated protein kinase B; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Yiping Xiong performed most of the experiments. Yun Dong conceived the project and designed the experiments. Duanyang Zhou, Kai Zheng, and Wenchuan Bi participated in data analysis. All authors contributed to the article and approved the submitted version.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

The informed consent obtained from study participants.

Acknowledgements

Not applicable.

Availability of Data and Material

All data generated or analyzed during this study are included in this published article.

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Figures

Figure 1

Extracellular ATP prevents glutamate-induced excitotoxicity via binding P2Y1 receptors in SH-SY5Y cells

(A) Representative images of the cell morphology after cultured SH-SY5Y cells were treated with various concentrations of glutamate (Glu) for 24 h; the control group (Ctrl) consisted of the untreated cells. Scale bar = 100 μ m under 20 $\times$ magnification. (B) Cell viability was detected using CCK-8 kit. (C) Representative images of the cell morphology after pretreated by ATP (5 μ M and 10 μ M) or PBS following by the application of 10 mM glutamate for 24 h. Scale bar = 100 μ m under 20 $\times$ magnification. (D) Cell viability was calculated for ATP neuroprotection. Values are shown in percentage as compared to control cells. (E) The protein expressions of P2Y1 receptor, and expression of GAPDH was served as a loading control. (F) CCK-8 kit was performed to evaluated cell viability after the administration of MRS2500, and the changes were shown in histograms as percentage with control. All data represent mean $\pm$ SEM from at least three independent experiments. ** $P < 0.01$, *** $P < 0.001$ vs. control group or glutamate group. n.s., no significance.

Figure 2

Extracellular ATP reduces ROS overgeneration and restored mitochondrial membrane potential functions induced by glutamate

Cultured SH-SY5Y cells were incubated in different concentrations (5 μ M and 10 μ M) of extracellular ATP, following by the application of 10-mM glutamate for 24 h; the control group consisted of the untreated cells. **(A)** SH-SY5Y cells were stained with DCFH-DA. Covered glasses with cells were observed under a fluorescent microscope. Scale bar = 100 μ m under 20 $\times$ magnification. **(B)** Fluorescent density mean of ROS level was calibrated. **(C)** JC-1 staining in SH-SY5Y cells. Scale bar = 100 μ m under 20 $\times$ magnification. **(D)** The ratio of the red/green fluorescence optical density were analyzed by image J. All

data in bar charts present mean $\pm$ SEM from at least three independent experiments. *** $P < 0.001$ vs. glutamate group or control group.

Figure 3

Extracellular ATP prevents glutamate-induced excitotoxicity via suppressing autophagy in SH-SY5Y cells

(A) The protein expressions of LC3 and p62 were determined by western blot in SH-SY5Y cells treated with 10-mM Glu in the presence or absence of ATP (5 μ M and 10 μ M), and expression of GAPDH was served as loading control. (B and C) The quantitation of LC3 and p62 expressions was calibrated. (D) The protein expression of LC3 and p62 were determined by western blot in SH-SY5Y cells treated with 10-mM Glu in the presence or absence of CQ (20 μ M), and expression of GAPDH was served as loading control. (E and F) The quantitation of LC3 and p62 expressions were calibrated. (G) cell viability was evaluated. (H and I) Treated SH-SY5Y cells were stained with DCFH-DA, and the changes were shown in histograms as percentage with control. Scale bar, 100 μ m under 20 $\times$ magnification. (J and K) Treated SH-SY5Y cells were stained using JC-1, and the changes were shown in histograms as percentage with control. Scale bar, 100 μ m under 20 $\times$ magnification. (L) cell viability was detected. All data in bar charts present mean $\pm$ SEM from at least three independent experiments. *** $P < 0.001$ vs. ATP treated group or control group. n.s., no significance.

Figure 4

Extracellular ATP inhibits glutamate-induced excitotoxicity through Erk1/2 signaling pathway

Cultured SH-SY5Y cells were treated with 10 mM Glu in the presence or absence of ATP (5 μ M and 10 μ M). The control group consists of the untreated cells. **(A)** The protein expression of p-Erk1/2 and Erk1/2 were determined by western blotting. Expression of GAPDH was served as loading control. **(B)** The

quantitation of p-Erk1/2 and Erk1/2 expressions were calibrated. **(C)** CCK-8 kit was performed to evaluated cell viability. **(D)** All treated SH-SY5Y cells were stained with DCFH-DA. Covered glasses with cells were observed under a fluorescent microscope. Scale bar = 100 μ m under 20 $\times$ magnification. **(E)** Fluorescent density mean of ROS level was calibrated. **(F)** JC-1 staining in SH-SY5Y cells. Scale bar = 100 μ m under 20 $\times$ magnification. **(G)** The ratio of the red/green fluorescence optical density were analyzed. All data in bar charts present mean $\pm$ SEM from at least three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ vs. ATP treated group. n.s., no significance.

Figure 5

Upregulated p-Erk1/2 by extracellular ATP prevents autophagy induced by glutamate

Cultured SH-SY5Y cells were treated with or without ATP (5 μ M and 10 μ M), followed by the application of glutamate (10 mM) in the presence or absence of PD0325901 (50 nM) or rapamycin (50 nM) for 24 h; the untreated cells was the control group. **(A)** The expressions of LC3 and p62 were determined by western blot. Expression of GAPDH served as loading control. The quantitation of **(B)** p62 expression and **(C)** LC3 expression were calibrated. **(D)** The expressions of p-Erk and Erk were determined by western blot. Expression of GAPDH served as loading control. **(E)** The quantitation of p-Erk expression was calibrated. All data in bar charts present mean $\pm$ SEM from at least three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ vs. glutamate group or ATP treated group. n.s., no significance.

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