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Extracellular ATP induces intracellular alpha-synuclein accumulation via P2X1 receptor-mediated lysosomal dysfunction

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Abstract

The pathological hallmark of Parkinson's disease (PD) is the accumulation of alpha-synuclein (α syn) in susceptible neurons in the form of Lewy bodies and Lewy neurites. The etiology of PD remains unclear. Because brain injury has been suggested to facilitate α syn aggregation, we investigated whether cellular breakdown products from damaged cells can act on neighboring healthy cells and cause intracellular α syn accumulation/aggregation. Using two neuronal cell models we found that extracellular ATP induced a significant increase in intracellular α syn levels between 24 to 48 hours after treatment. Further investigation revealed that the observed α syn accumulation is a result of lysosome dysfunction caused by extracellular ATP-induced elevation of lysosomal pH. Interestingly, P2X1 receptor appears to mediate the cells' response to extracellular ATP. Although Ca^{2+} influx via P2X1 receptor is necessary for α syn accumulation, Ca^{2+} influx per se is not sufficient for increased α syn accumulation. These findings provide new insight into our knowledge of the role of P2X receptors in PD pathogenesis and may be helpful in identifying new therapeutic targets for PD.

Keywords

alpha-synuclein; ATP; Luhmes cells; P2X1 receptor; Parkinson's disease; purinergic signaling

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, pathologically characterized by the formation of filamentous α -synuclein (α syn) neuronal inclusions and the loss of dopaminergic neurons in substantia nigra and dopaminergic

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Conflict of Interest

The authors declare that they have no conflict of interest.

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terminals in the striatum (Dauer and Przedborski, 2003). The etiology of PD remains unclear and no disease-modifying treatment currently exists. Misfolding and aggregation of α syn plays a central role in the pathogenesis of PD (Jellinger, 2012). Among the variety of aggregate species present, low-molecular weight oligomers, ranging from 2 to 30 mers are thought to be the primary neurotoxic species responsible for the impairment of neuronal function in PD (Planchard, et al., 2014). Traumatic brain injury (TBI) has been considered a causative factor for PD based on case-control studies (Lee, et al., 2012) and brain injury has been suggested to facilitate α syn aggregation and PD development (Bramlett and Dietrich, 2003, Jafari, et al., 2013). This notion is further supported in an experimental study showing that α syn expression and aggregation increased transiently following TBI in aged mouse brain (Uryu, et al., 2003). Focal cerebral ischemia was also found to induce both the aggregation of wild type and transgenic human mutant (A30P) α syn in mouse brain (Unal-Cevik, et al., 2011), and a recent study further linked ischemia to PD by showing early development of brain injury caused neuronal loss in substantia nigra in a mouse focal cerebral ischemia model (Rodriguez-Grande, et al., 2013). Despite these intriguing data, the exact mechanism(s) underlying brain injury-induced aggregation of α syn remain elusive. Considering that the aforementioned α syn aggregation only appears approximate to the injured area and α syn aggregates form in a relatively short time (from 3 days to one week) after brain injury (Uryu, et al., 2003), we hypothesized that cellular breakdown products from damaged cells may influence neighboring healthy neuronal cells leading to intracellular α syn accumulation/aggregation. Herein we tested whether extracellular ATP might be a key factor inducing α syn accumulation in the above scenario because ATP acts as a potent stimulus for inflammation and can be released from damaged cells. Extracellular ATP plays an important signaling role by activating broadly distributed cell surface purinergic receptors (P2 receptors) (Seminario-Vidal, et al., 2009). P2 receptors are divided into two subclasses, ATP-selective cation channels P2X and G-protein-coupled P2Y families. Functional P2X receptors are expressed on both neurons and glial cells (Volonte, et al., 2003). So far, seven members of P2X subfamily have been identified, P2X1-7 (Falzoni, et al., 2013). In present study, we confirm that extracellular ATP plays an important role in intracellular α syn aggregation in two different neuronal cell models and further demonstrate that lysosomal dysfunction is linked to extracellular ATP-induced α syn accumulation in neuronal cells. Importantly, we found that P2X1 receptor is key to mediating the observed effects.

2. Materials and methods

2.1. Chemicals

The following chemicals were used in the present study. ATP (Sigma, A7699), ADP (Sigma, A2754), AMP (Sigma, A1752) and Bafilomycin A1 (Sigma, 196000), Cycloheximide (Sigma, C7698), A23187 (Sigma, C7522), ATP γ S (R&D system, 4080), BzATP (R&D system, 3312), NF449 (R&D system, 1391), TNP-ATP (R&D system, 2464) and A438079 (R&D system, 2972), PSB-12062 (Glix Lab, 01919).

2.2 Generation of Tetracycline-off (Tet-off) inducible α syn-overexpressing H4 cells

A bidirectional and tetracycline-driven expression plasmid was constructed by combining pTRE3G-BI (Clontech, CA, USA) and the pc5DNA/FRT (Invitrogen, Carlsbad, CA, USA) plasmids. The pc5DNA/FRT fragment (1603-2963) including the FRT sequence, hygromycin ORF, and polyadenylation signal sequence were amplified by high-fidelity polymerase chain reaction (KOD Hot start, Novagen) with the following primers (forward: 5' GCGGCATGTGAAGTTCCTATTCCGAAG 3' and reverse: 5' GCGACATGCGGTTCGACGGTATACAG 3'). The amplified fragment was then digested and cloned into pTRE3G-BI at the NspI site (position 827). The resulting product, the empty pTRE3G-BI/FRT/Hygro vector, was verified by sequencing. The α syn fusion constructs; α syn-humanized *Gaussia princeps* luciferase (hGluc) C-terminal half and α syn-hGluc N-terminal half, were described previously (Putcha, et al., 2010). Syn-Luc1 was subcloned from rAAV-CBA-SYNUCLEIN-LUC1-WPRE (Danzer, et al., 2011) and inserted in the multi-cloning site 1 of pTRE3G-BI/FRT/Hygro vectors using the NotI and EcoRV restriction sites. Subsequently, syn-luc2 was inserted in the multi-cloning site 2 using the MslI and Acc65I restriction sites. The resulting plasmid, pTRE3G-BI/FRT/Hygro/synluc1(S1)/synluc2(S2) was verified by sequencing.

To generate stable cell lines, founder H4/Tetoff cells were transfected with plasmid pTRE3G-BI/FRT/Hygro/S1/S2. Clones were screened and amplified in medium containing Hygromycin B and G418. A single-cell clone with the highest expression of S1 and S2 was used in present study and referred to as "S1S2" cell line. S1S2 cells were maintained in OPTI-MEM (Invitrogen) medium supplemented with 10% fetal bovine serum (Invitrogen), 200 μ g/ml Hygromycin B, 200 μ g/ml G418 and 1 μ g/ml Tetracycline (Tet) (Sigma, T7760) and incubated at 37 °C.

For drug treatment, S1S2 cells were seeded into 24-well plates at the density of 1×10^5 cells/well or 96-well plates at the density of 2×10^4 cells/well. After 24 hrs, FBS-containing media was replaced with FBS-free media. 0, 1, 2, 3 mM ATP or 3 mM ADP or 3 mM AMP or 500 μ M ATP γ S or 0, 30, 100, 300, 1000 μ M BzATP were added for cells treatment. Compounds were prepared in DMSO or water which was used as control(s).

To optimize the concentration of P2X receptor antagonists, S1S2 cells were pretreated with 0, 0.5, 1, 2, 3, 4, 5 μ M NF449 (P2X1 receptor antagonist), or 0, 0.01, 0.1, 1, 5, 10 μ M TNP-ATP (P2X3 receptor antagonist) or 0, 0.1, 0.5, 1, 2.5, 5 μ M PSB-12062 (P2X4 receptor antagonist) or 0, 20, 40, 60, 80, 100 μ M A438079 (P2X7 receptor antagonist) for 30 min then subjected to 0 and 3 mM ATP treatment for 48 hrs, vehicles were used as control(s). Intracellular α syn oligomerization was evaluated by luciferase assay.

2.3. Luhmes cells maintenance and differentiation

Lund human mesencephalic (Luhmes) cells can be differentiated into morphologically and biochemically mature dopamine-like neurons (Lotharius, et al., 2005). The culturing and handling procedure of Luhmes cells was described previously (Prots, et al., 2013). Briefly, Luhmes cells were cultured in advanced DMEM/F-12/Glutamax medium supplemented with N2 supplement (all from Invitrogen) and 40 ng/ml FGF2 (R&D Systems, 4114-TC) at 37 °C

in a humidified 95% air, 5% CO₂ atmosphere. Plates or flasks were pre-coated with 50 µg/ml poly-L-ornithine (Sigma, P3655) and 1 µg/ml fibronectin (Sigma, F1141). For differentiation, cells were incubated with advanced DMEM/F-12/Glutamax/N2 medium (Invitrogen) containing 2 ng/ml human recombinant glial cell-derived neurotrophic factor (R&D Systems, 212-GD), 1 mM cAMP (Sigma, D0627) and 1 µg/ml tetracycline (Sigma, T7760) for 4 days.

2.4. Transfection of Plasmid DNA and siRNA

Plasmids carrying full length *Gaussia* luciferase or α syn fused with full length *Gaussia* luciferase have been described previously (Putcha, et al., 2010). The P2X1 receptor siRNA was purchased from Invitrogen (Carlsbad, CA, USA). The siRNA sequences of human P2X1 gene were as follows: sense- GGCCGAGAACUUCACUCUUt, antisense- AAGAGUGAAGUUCUCGGCCtc. S1S2 cells were plated 24 hours (hrs) prior to transfection and grown to 80–90% confluence prior to transfection. Transfection was performed using Superfect (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Next day, media was changed to serum-free Opti-MEM and subjected to different treatments for 48 hrs. After that, cells were washed and luciferase assay was performed or cells were harvested for western blot.

Transfection of differentiated Luhmes cells has been described previously (Schildknecht, et al., 2013). Briefly, cells were pre-differentiated for 2 days. 4 µL Lipofectamine 2000 (Invitrogen) and 50 nmoles of siRNA were used to prepare transfection complex for one well of a 6-well plate. Transfection complexes were transferred into 6-well plates and incubated for 20 min. Pre-differentiated, trypsinized Luhmes were then added in a volume of 1.7 ml differentiation medium to the siRNA containing solution at a density of 1.5×10^6 cells/well and thoroughly mixed. After an additional 48 hrs of differentiation, cells were harvested and subjected to western blot.

2.5. *Gaussia* luciferase protein-fragment complementation assay

Luciferase assays were performed as described previously (Danzer, et al., 2012). Briefly, cells were washed with PBS and replaced with serum-free and phenol red-free media. Luciferase activity from protein complementation was measured in live cells using a Wallac Victor 3 multilabel counter (PerkinElmer; Waltham, MA) at 480 nm following the injection of the cell permeable substrate, coelenterazine (20 µM, NanoLight).

2.6. Cell co-culture experiment

S1S2 cells expressing α syn in the absence of tetracycline for 1 week were seeded at the density of 2.5×10^5 per well in a 24 well plate with an individual insert.

For the co-culture of apoptotic cells, wild-type H4 cells (ATCC, HTB-148) were plated in the insert at the density of 5×10^4 per well. At the same time, G418 and Hygromycin B, the selective antibiotics for S1S2 cell maintenance, were added to media at a concentration of 200 µg/ml to induce apoptosis of wild-type H4 cells (Jin, et al., 2004). After 48 hrs, the majority of wild-type H4 cells had died and both the insert and old media were removed. S1S2 cells were washed with PBS and replaced with serum-free and phenol red-free media.

Luciferase activity was measured to evaluate intracellular α syn oligomerization. For the co-culture of necrotic cells, 5×10^4 naive H4 cells were subjected to freeze-thawing (Iyer, et al., 2009), then plated in the insert of the 24 well plate. After 48 hrs of co-culture, the insert and old media were removed. S1S2 cells were processed for Luciferase activity assay as described above.

2.7. ATP assay

ATP levels in the cell culture medium were analyzed using the ATP Colorimetric/Fluorometric Assay Kit (BioVision, K354-100) according to the manufacturer's instructions. Briefly, 50 μ L of each sample was mixed with 50 μ L of reaction mix, incubated at room temperature for 30 min, and then fluorescence was measured in a SpectraMax plate reader (Ex/Em = 535/587 nm) (Molecular device).

2.8. Dot blot

Cells were washed and scraped in cold PBS, then lysed in PBS buffer containing 1% Triton-X 100, 1mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche). Protein concentrations were determined using the Pierce BCA protein assay kit (Fisher). 10 μ g of each sample was applied to nitrocellulose membrane (pore size 0.22 μ m, Whatman Protran, Sanford, ME, USA) placed in a Dot blot apparatus (Bio-Rad) and incubated at room temperature for 1 hr allowing the samples to enter the membrane via gravity. The membrane was blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.01% Tween 20 (TBST) at room temperature for 1 hr then incubated with anti- α syn antibody (1:1000; BD, 610787) overnight at 4 °C with gentle agitation, washed three times with TBS-T, and incubated with horseradish peroxidase (HRP)-conjugated anti mouse IgG (1:1000, Southern Biotech, 1012-05) for 1 hr at room temperature. Immunoreactivity was visualized with Pierce ECL chemiluminescence kit from Thermo Scientific (Rockford, IL, USA).

2.9. Western blot

As previously described (Putcha, et al., 2010), cells were washed and scraped in cold PBS, then lysed with detergent-free lysis buffer (50 mM Tris/HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, pH 8.0) containing protease inhibitors (Roche) for native PAGE or with RIPA buffer (Millipore, 20-188) containing protease inhibitors for denatured PAGE. 30 μ g of protein from each sample was loaded on 4–12% Tris-Glycine gel (Invitrogen) for native electrophoresis or 10% Bis-Tris gel (Invitrogen) for denatured electrophoresis, then transferred to the polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with antibodies against: anti- α syn (1:1000, BD, 610787), anti-p62 (1:1000, BD, 610832), anti-LC3 (1:1000, Novus, NB100-2331), anti-P2X1 (1:100, Abgent, sc-42563-PR) and anti- β actin (1:5000, Sigma, A5316). Immunoreactivity was visualized with Pierce ECL chemiluminescence kit from Thermo Scientific (Rockford, IL, USA).

2.10. Measurement of intracellular calcium

Intracellular Ca^{2+} measurements were performed using a microscope-based imaging platform as previously described (Guha, et al., 2013). Confocal laser scanning microscopy with a Carl Zeiss LSM 510 with 40 $\times$ /1.3 oil objective was used to monitor the Fluo-4 AM

(Invitrogen) calcium indicator dye in real time. Carl Zeiss Zen software was used for image acquisition and quantification. Briefly, S1S2 cells were grown in LabTek 8-well chambers with glass bottom (Fisher) and loaded with 5 μ M Fluo-4 AM for 30 min at 37 °C. After washing three times with Hank's Balanced Salt Solution (HBSS) (Invitrogen), cells were pretreated with or without 3 μ M NF449 for 15 min. Time series images were taken every 2.5 sec to establish a baseline and changes in free intracellular calcium levels were recorded following 3 mM ATP stimulation with the excitation and emission wavelengths set at 488 and 526 nm respectively. The change of intracellular calcium concentration was quantified by the ratio of fluorescence signal at each time point to the basal signal (at 0 sec). Twenty control or NF449 pretreated cells from 3 independent cultures were analyzed.

2.11. Quantitative Real-time PCR

Total RNA isolated from cells using RNeasy Mini Kit (Qiagen) was reverse-transcribed with SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The reaction mix was subjected to Applied Biosystems 7900HT Fast Real-Time PCR System to detect expression levels of α syn using human quantitative PCR primers. The set of actin primers (Qiagen) was used as an internal control for amplification of genes of interest. The human α syn primers used were as follows: sense- ATCCGCACGGGCAAGTGTGT, antisense- GCCTGGCAAACCTGAAGTTG (Greco, et al., 2001).

2.12. Evaluation of α syn turnover

S1S2 cells induced to express α syn for 1 week were used to evaluate α syn turnover. Briefly, growth media was replaced by serum-free Opti-MEM containing 1 μ g/ml Tet to turn off α syn expression. Cells were harvested at 0, 6, 12, 24, 36, 48 hrs after Tet addition in the presence or absence of 3 mM ATP. For differentiated Luhmes cells, 100 μ g/ml of cycloheximide (CHX) was added to the growth media to inhibit protein synthesis. Cells were harvested at 0, 6, 12, 24, 36, 48 hrs after cycloheximide addition in the presence or absence of 3 mM ATP.

Cell lysates from S1S2 and differentiated Luhmes cells harvested above were probed with antibodies to α syn and β -actin by western blotting for evaluation of α syn turnover.

2.13. Measurement of lysosomal pH

Lysosomal pH was measured as described previously (Guha, et al., 2013). In brief, differentiated Luhmes cells were grown in black 96-well plates, rinsed 3 times with isotonic solution [105 mM NaCl, 5 mM KCl, 6 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 mM Na HEPES, 5 mM NaHCO₃, 60 mM mannitol, 5 mM glucose, 0.5 mM MgCl₂, and 1.3 mM CaCl₂], then incubated with 5 μ M LysoSensor Yellow/Blue (Invitrogen, L7545) for 3 min. 15 min post-incubation, fluorescence was measured on a SpectraMax plate reader (Molecular device). Lysosomal pH was determined from the ratio of emission at 450 *versus* 510 nm (365 nm excitation) and calibrated by exposing cells to 20 μ M H⁺/K⁺ ionophore nigericin in 20 mM 2-(N-morpholino) ethanesulfonic acid (MES), 110 mM KCl, and 20 mM NaCl (pH 4.0–6.0) for 15 min. Comparisons were always made between measurements from the same plate and normalized for each plate.

2.14. Proteasome Activity Assay

Proteasomal activity was determined by Proteasome-Glo Chymotrypsin-Like Cell-Based Assay (Promega, G8660). Cells were incubated with Suc-Leu-Leu-Val-Tyr-AMC as a substrate to measure the chymotrypsin-like activity. Cells were seeded at the density of 1×10^4 per well of 96-well plate. After 48 hrs incubation with 3 mM ATP in serum-free medium, cells were washed three times and 100 μ L fresh serum-medium was loaded, next 100 μ L assay buffer containing 10 μ M of substrate was loaded. Medium and assay buffer were mixed at 700 rpm for 2 min, incubated with cells at room temperature for more than 10 min, and then luminescence was measured using a Wallac Victor 3 multilabel counter (PerkinElmer; Waltham, MA).

2.15. Statistical analysis

All quantified data excluding calcium quantification represent an average of triplicates. Data were analyzed using GraphPad Prism 5 (San Diego, CA) and are presented as mean $\pm$ SD or SEM. Statistical significance was determined using a Student's t-test or one-way ANOVA Tukey test. $p < 0.05$ was considered significant.

3. Results

3.1. Dying cells induce α syn oligomerization in surrounding healthy cells

Traumatic brain injury is a poorly understood risk factor for Parkinson's disease (Jellinger, 2012, Stern, 1991) and studies in animal models have shown that TBI induces the rapid accumulation of many key proteins that form pathologic aggregates in neurodegenerative diseases including α syn (Uryu, et al., 2007). Cells damaged by traumatic brain injury or focal cerebral ischemia can die by both apoptosis and necrosis (Bramlett and Dietrich, 2004, Clark, et al., 1997). To determine if either of these pathways can induce α syn aggregation, we measured α syn oligomer formation in response to apoptotic or necrotic stimuli using a tetracycline-regulated stable cell line (S1S2) developed to monitor α syn oligomerization *in situ* using a protein-fragment complementation assay (Danzer, et al., 2012). S1S2 cells were co-cultured together with either apoptotic wild-type H4 neuroglioma cells (induced by G418 and Hygromycin B) or necrotic wild-type H4 cells (induced by freeze-thaw) and then subjected to a luciferase activity assay after 12, 24 and 48 hrs of co-culture. Luciferase activity significantly increased in S1S2 cells co-cultured with apoptotic or necrotic cells for 24 hrs or 48 hrs, but not in cells co-cultured for only 12 hrs (Fig. 1A). These data suggest that our model of dying cells can induce α syn aggregation in neighboring healthy cells. Because cells undergoing apoptosis or necrosis release cellular breakdown products into the extracellular space, we reasoned that one of these breakdown products may be facilitating the accumulation of intracellular α syn. *In vivo* dying cells can release high concentrations of ATP to initiate a primary immune response (Bours, et al., 2011, Di Virgilio, et al., 2003). To determine if H4 cells undergoing apoptosis or necrosis release ATP, we measured ATP in the co-culture media. In the growth media of S1S2 cells alone the ATP concentration was below the level of detection however induction of necrosis by freeze-thaw resulted in an average concentration of 31.6 μ M ATP in the media 30 minutes after necrosis induction, which gradually declined to 12.9 μ M 48 hrs later. Induction of apoptosis in H4 cells using

antibiotics caused a gradual increase of ATP in the media to a maximum of 17.6 μ M after 48 hrs (Fig. 1B).

To determine if increased extracellular ATP is responsible for triggering α syn aggregation, we directly treated S1S2 cells with ATP and monitored intracellular α syn oligomerization using luciferase activity as a readout. Because ATP is rapidly degraded, we chose starting concentrations between 1 mM and 3 mM ATP based on reports in the literature (Kawamura, et al., 2012, Kurashima, et al., 2012, Liu and Wang, 2014, Trabanelli, et al., 2012) and treated cells for 24 hrs and 48 hrs to mimic our co-culture experiments. In this pilot experiment 1 mM, 2 mM, and 3 mM ATP all significantly induced α syn oligomerization without apparent cell toxicity (Fig. 1C). Because S1S2 cells co-cultured with necrotic or apoptotic cells only demonstrated increased α syn oligomerization after sustained exposure to dying cells for at least 24 hrs, we tested whether ATP was required to be continuously present in the media to induce this effect. 3 mM ATP was added to the media and incubated for various time-points from 1 hr to 48 hrs. At each time-point, media containing ATP was removed and replaced with normal growth media. After 48 hrs luciferase assays were performed on all treatment time-points. Interestingly, only cells treated for a minimum of 24 hrs had a significant increase in α syn oligomerization at 48 hrs indicating the need for continuous presence of ATP (Fig. 1D). In addition, we monitored the ATP concentration in the media over the course of the 48 hrs incubation. H4 cells were treated with 2 or 3 mM ATP for 48 hrs and extracellular ATP concentration was measured at 1, 24, and 48 hrs (Fig. 1E). As expected, ATP concentrations steadily decreased over 48 hours with a significant decrease in concentration apparent by 1 hr. After 48 hrs, 64.9 μ M ATP remained in the media following the addition of 3 mM ATP, while only 9.3 μ M ATP remained after 2 mM ATP treatment. Based on these data and the fact that 12.9 μ M ATP was necessary to induce a robust increase in α syn oligomerization after 48 hrs in the co-culture experiments, we chose 3 mM as the optimum starting ATP concentration for all subsequent experiments.

3.2. Extracellular ATP increases the level of intracellular α syn in S1S2 cells

Because ATP can be rapidly degraded to ADP and AMP, we treated S1S2 cells with 3mM ATP, 3 mM ADP, 3 mM AMP, and 500 nM ATP γ S, a non-degradable ATP analog. Intracellular luciferase activity of S1S2 cells treated with 3 mM ATP for 48 hrs was 200% higher than the control group ($p = 0.007$). ATP γ S induced a 55.4% increase in intracellular luciferase activity. ADP also increased intracellular luciferase but to a significantly lesser extent than ATP, however AMP had no effect on luciferase activity (Fig. 2A). Taken together, our data support ATP as the most potent inducer of intracellular luciferase activity.

As a control, we treated H4 cells expressing either *Gaussia* luciferase (Luc) alone or a full-length α syn-luciferase fusion protein (α syn-Luc) with ATP. Interestingly, we found that both ATP and ATP γ S significantly increased the luciferase activity in cells expressing α syn-Luc but not Luc (Figs. 2, B and C). AMP had no effect on either condition (Figs. 2, B and C). These results suggest that the increase in luciferase activity in S1S2 cells upon ATP treatment is specific to α syn.

The observed increase in luciferase activity in S1S2 cells could result from increased oligomerization with no change in total α syn levels or increased oligomerization as a result

of increased α syn expression or decreased α syn degradation. To determine if increased α syn oligomerization in S1S2 cells is the result of an increase in total α syn levels, cell lysates from ATP-treated S1S2 cells were resolved by native PAGE or SDS-PAGE followed by western blotting. Samples resolved by native PAGE revealed that α syn oligomers are significantly increased upon ATP treatment, consistent with the luciferase assay results (Fig. 2D). Samples resolved by SDS-PAGE showed that total α syn levels are significantly higher in ATP treated cells than control (Figs. 2, E and F).

3.3. P2X1 receptor mediates the increase of intracellular α syn induced by extracellular ATP

Extracellular ATP can activate P2 receptors on the cell surface. P2 receptors consist of two main subclasses, ATP-selective cation channels P2X and the G-protein-coupled P2Y families. In the nervous system, P2X receptors are broadly expressed in both neurons and glial cells (Volonte, et al., 2003). It is known that the P2X family is selectively activated by ATP, less activated by ADP, and not activated at all by AMP (North and Jarvis, 2013). There are seven P2X receptors, P2X1-7 and ATP is known to bind to and activate all seven P2X receptors (Hodges, et al., 2011). To determine if activation of P2X receptors are responsible for ATP-induced increase of α syn, S1S2 cells were pretreated with antagonists to the different subtypes of P2X receptors then exposed to 3 mM ATP. To determine if P2X antagonists could inhibit ATP-induced α syn aggregation we pre-treated S1S2 cells with a range of antagonist concentrations for 30 minutes before adding 3 mM ATP or vehicle for a total of 48 hrs (Figs. 3, A, B, C, D). Only NF449, a selective antagonist of P2X1, prevented ATP-induced α syn aggregation in a dose dependent manner (Fig. 3A) whereas the other antagonists tested had no effect. The role of P2X1 in mediating ATP-induced α syn aggregation was confirmed by the fact that 300 μ M BzATP, a P2X1 agonist, significantly increased intracellular α syn aggregation in S1S2 cells (Fig. 3E). Taken together, these data suggest the effect of ATP on α syn accumulation can be mediated via the P2X1 subfamily of receptors.

To further confirm a role for P2X1 receptor, we used genetic knockdown with a siRNA to P2X1. siRNA transfection significantly reduced P2X1 expression to 55.9% of the original level (Figs. 3, F and G) and importantly, prevented the ATP-induced increase in α syn oligomerization compared to control scrambled siRNA (Fig. 3H).

To determine whether the ATP-induced increase in α syn is an artifact of α syn overexpression in S1S2 cells, we repeated the above experiments in another cell model, differentiated Luhmes cells. Luhmes cells are a clone of the v-myc-overexpressing Tet-dependent human mesencephalic cell line MESC2.10 (Lotharius, et al., 2005). Tet down-regulates v-myc expression in this line and the cells can then be differentiated within 3–4 days into a dopaminergic phenotype when cultured in dibutyryl cAMP (cyclic adenosine monophosphate) and glial cell-derived neurotrophic factor (GDNF). Differentiated Luhmes cells are considered an ideal cell model of PD because the phenotype and morphology of the cells are closer to dopaminergic neurons *in vivo* (Schildknecht, et al., 2009) compared to other cell lines.

The P2X1 agonists, ATP and BzATP, both increased the level of endogenous α syn in Luhmes which was blocked by pretreatment with NF449. NF449 alone did not have a significant effect on α syn levels (Figs.4, A and B). Moreover, knockdown of P2X1 with siRNA in Luhmes cells also significantly inhibited ATP induced α syn accumulation (Figs.4, C–E). Together, the data from S1S2 and differentiated Luhmes cells indicate that P2X1 receptors play a role in mediating the effect of extracellular ATP on intracellular α syn accumulation.

3.4. Increased α syn accumulation is a result of decreased degradation

To determine if extracellular ATP-induced α syn accumulation is caused by increased transcription or decreased degradation, we used quantitative real-time PCR to measure the mRNA level of α syn in S1S2 and differentiated Luhmes cells with and without ATP treatment and detected no significant difference in α syn transcript levels in either cell type upon ATP treatment (Fig. 5A).

To monitor α syn protein degradation in S1S2 cells, we used Tet to “turn-off” α syn overexpression and monitored α syn levels in culture over time after the Tet addition. This approach has been used in previous studies for evaluation of protein turnover (Jiang, et al., 2013, Vogiatzi, et al., 2008). Briefly, S1S2 cells in the presence or absence of 3 mM ATP were harvested at 0, 6, 12, 24, 36, 48 hrs after the addition of Tet. Using dot blot to evaluate total levels of α syn, we found that the degradation of α syn significantly decreased in ATP-treated cells compared with control cells (Figs.5, B and C).

To monitor α syn degradation in differentiated Luhmes cells, we performed a quantitative cycloheximide chase assay. Luhmes cells with or without 3 mM ATP were harvested at 0, 6, 12, 24, 36, 48 hrs after the addition of cycloheximide. Cell lysates were resolved by SDS-PAGE followed by western blotting. Consistent with the results from S1S2 cells, α syn degradation in differentiated Luhmes cells was significantly slower in the presence of ATP (Figs.5, D and E). Together, these data support decreased degradation as a mechanism for ATP-induced α syn accumulation.

3.5. P2X1-induced lysosomal dysfunction results in α syn accumulation

We and others have shown that the ubiquitin-proteasome system (UPS) and autophagy-lysosomal pathway (ALP) are both responsible for α syn degradation in neurons (Ebrahimi-Fakhari, et al., 2011). To determine if either pathway is affected by extracellular ATP treatment we performed a proteasome function assay with and without ATP treatment, and found that ATP had no effect on proteasome activity in both S1S2 cells and Luhmes cells (Fig. 6A). To access ALP function, we compared the levels of autophagosome marker LC3II and macroautophagy substrate p62 with and without ATP treatment. We found that the expression of LC3II and p62 were significantly elevated in both cell models when treated with ATP. As a control, cells were treated with the lysosomal inhibitor, bafilomycin A1 (Figs.6, B–G). These data suggest that extracellular ATP treatment induces α syn accumulation by impairing the ALP pathway.

3.6. Extracellular ATP induced lysosomal dysfunction in neuronal cells was mediated by P2X1 receptor

Extracellular application of an ATP analog has been reported to induce lysosomal alkalization by activating P2X receptors (Guha, et al., 2013). To determine if this mechanism is applicable to our cellular systems, we monitored the fluorescence in live cells loaded with LysoSensor Yellow/Blue, which detects a change in the ratio of emission upon the change of lysosomal pH. Interestingly, we found that ATP treatment elevated lysosomal pH from 4.27 to 4.85 in S1S2 cells (Fig. 7A) and from 4.25 to 4.62 in differentiated Luhmes cells (Fig. 7B), respectively. More importantly, pretreatment with NF449, the P2X1 antagonist, significantly blocked the ATP induced increase in lysosomal pH in both cell models (Figs. 7, A and B), supporting a role of P2X1 in mediating ATP induced lysosomal dysfunction.

3.7. P2X1 mediated Ca^{2+} influx is partially responsible for extracellular ATP induced syn accumulation in S1S2 cells

According to the literature, P2X1 has high Ca^{2+} permeability and can be activated by extracellular ATP to trigger Ca^{2+} influx (Kew and Davies, 2010). To examine this in our model systems, we measured intracellular Ca^{2+} following ATP treatment and found that ATP increased Ca^{2+} influx in both S1S2 and differentiated Luhmes cells and that the increase can be significantly inhibited by pretreatment with the P2X1 antagonist, NF449 (Figs. 8, A and B) in S1S2 cells. This raised the possibility that P2X1-mediated Ca^{2+} influx is responsible for ATP induced lysosomal dysfunction and subsequent αsyn accumulation. Because long term Ca^{2+} depletion can result in cell death, we first determined the optimal time our cells could be maintained in Ca^{2+} free media without compromised viability and found that 15~30 min was the longest time. According to the literature, ATP triggered Ca^{2+} influx is a fast response and only lasts several seconds to 2 minutes (Inscho, et al., 1999, Korngreen and Priel, 1996, Tanaka, et al., 2010). Accordingly, S1S2 cells were treated with ATP in the absence of Ca^{2+} for 15 min, thereafter 1.5 mM CaCl_2 was added back to medium to support cell health. 48h later, cells were assayed for luciferase activity. Depletion of extracellular Ca^{2+} significantly inhibited the ATP-induced increase of αsyn in S1S2 (Fig. 8C), indicating that extracellular Ca^{2+} influx is involved in ATP-induced αsyn accumulation in S1S2 cells. By contrast the calcium ionophore 3 μM A23187, which induces a much higher Ca^{2+} influx in cells compared to ATP had no significant effect on luciferase activity (Fig. 8D). Therefore, Ca^{2+} influx alone does not appear to be sufficient for ATP-induced αsyn accumulation in cells. Instead, Ca^{2+} influx via P2X1 receptors appears to be a critical step.

4. Discussion

ATP is a potent signaling molecule abundantly present in the central nervous system (Apolloni, et al., 2009, Prasai, et al., 2011). Extracellular ATP induces various biological responses which are mediated by the activation of cell surface receptors including ligand-gated ion channels (P2X) and G-protein coupled (P2Y) receptor subtypes. High concentrations (~10 mM) of ATP are present in the cellular cytoplasm, whereas low concentrations (1~10 nM) can be found in the extracellular milieu under physiological

conditions (Di Virgilio, 2000) because ATP is released by regulated exocytosis to act as a neurotransmitter and/or neuromodulator (Sperlagh and Vizi, 2011, Trabanelli, et al., 2012). However, a large amount of ATP is released by dying cells following tissue injury, inflammatory reactions, or metabolic stress (Sperlagh and Vizi, 2011, Trabanelli, et al., 2012). Under these conditions, nearby healthy cells are exposed to high levels of extracellular ATP, especially if ATP release occurs at the site of cell-to-cell interaction. Increased extracellular ATP is also a pro-inflammatory danger signal for activation of the inflammatory response (Iyer, et al., 2009). It has been shown that extracellular ATP can accumulate during inflammatory reactions because 1) ubiquitous ecto-ATPases, known as ATP hydrolyzing enzymes regulating extracellular ATP concentration under normal conditions, can be inhibited during inflammatory reactions leading to the accumulation of extracellular ATP (Di Virgilio, et al., 2003), and 2) activation of inflammatory cells can also trigger release of potentially toxic mediators that may cause further tissue injury and release more ATP (Gourine, et al., 2007). In this regard, it has been suggested that neuroinflammatory processes in PD involve self-perpetuating deleterious events that lead to protracted neuronal degeneration (Hirsch, et al., 2012). Therefore, it is possible that sustained high levels of ATP under conditions of tissue injury or inflammation may contribute to α syn accumulation/aggregation in neurons in the PD brain.

Prior to our study, the role of P2X receptors in neurodegenerative diseases has been based mostly on studies of P2X7. It has been shown that blockade or absence of P2X7 receptor is neuroprotective in animal models of neurodegenerative disorders such as Alzheimer's disease (Ryu and McLarnon, 2008) and Huntington's disease (Diaz-Hernandez, et al., 2009), however, such protective effects have not been successfully reproduced in PD animal models (Hracsco, et al., 2011). Our present study demonstrates that the pharmacological blockade or genetic knockdown of P2X1 receptor can significantly inhibit extracellular ATP-induced α syn accumulation/ aggregation in PD cell models, whereas blocking other P2X receptors, including P2X7, does not have a comparable effect. These data indicate that at least in α -synucleinopathy cell models, P2X1 plays an essential role in mediating ATP-induced PD related pathology such as α syn aggregation although we cannot rule out the possibility that receptors other than the P2X family, such as P2Y receptors, may also play a role. Because the pharmacological blockade and genetic knockdown of P2X1 did not completely abrogate the effect of ATP on α syn accumulation/aggregation the possibility remains that other P2X subtypes may play a role in ATP-induced α syn aggregation, however the lack of specific P2X2, 5, or 6 antagonists make this difficult to assess. Regardless, the fact that P2X3, 4 and 7 antagonists had no effect supports a specific role for P2X1. Thus, our study is the first to reveal a role for P2X1 in mediating ATP-induced α syn accumulation/ aggregation in PD cell models.

The UPS and ALP are the major degradation pathways by which α syn is degraded in neurons. We have reported that the UPS is the main degradation pathway for α syn under normal conditions *in vivo* while increased α syn burden recruits the ALP (Ebrahimi-Fakhari, et al., 2011). Our study showed that upon ATP treatment, both S1S2 cells and Luhmes cells showed P2X1 mediated increase of α syn, autophagic markers LC3-II and P62, and lysosomal alkalization, indicating that P2X1-mediated lysosomal dysfunction upon ATP

treatment is responsible for the accumulation of the aforementioned proteins. Lysosomal alkalization can cause enzyme dysfunction, leading to accumulation of autophagosomes and delayed protein turnover (Stern, et al., 2012).

It has been shown that activation of P2X receptors is related to lysosomal alkalization and this process is dependent on calcium influx (Guha, et al., 2013). Among the seven P2X receptors, P2X1 receptor has relatively high calcium permeability (Kew and Davies, 2010, Rettinger and Schmalzing, 2003). Therefore, it is possible that ATP induced lysosome alkalization may occur via the P2X1/Ca²⁺ pathway. Further investigation demonstrated that depletion of extracellular calcium significantly prevented ATP-induced increase of intracellular α syn, indicating a possible involvement of Ca²⁺ influx. However, in our cell model treated with Ca²⁺ ionophore, which induced a much greater influx of Ca²⁺ than ATP, we did not see α syn accumulation/aggregation. Therefore, calcium influx may very well be necessary but is not sufficient for the accumulation of α syn induced by ATP.

In summary, our present study shows that intracellular α syn is significantly increased in neurons co-cultured with dying cells and that ATP released from dying cells plays an important role in this scenario. Mechanistically, intracellular α syn accumulation/aggregation results from impaired lysosome function that is mediated by ATP-induced P2X1 receptor activation. These findings provide new insight into our knowledge of the role of P2X receptors in PD pathogenesis and may be helpful in identifying new therapeutic targets for PD.

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References

- Apolloni S, Montilli C, Finocchi P, Amadio S. Membrane compartments and purinergic signalling: P2X receptors in neurodegenerative and neuroinflammatory events. *Febs J.* 2009; 276(2):354–64.10.1111/j.1742-4658.2008.06796.x [PubMed: 19076216]
- Bours MJ, Dagnelie PC, Giuliani AL, Wesselius A, Di Virgilio F. P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. *Front Biosci (Schol Ed).* 2011; 3:1443–56. [PubMed: 21622280]
- Bramlett HM, Dietrich WD. Synuclein aggregation: possible role in traumatic brain injury. *Exp Neurol.* 2003; 184(1):27–30. [PubMed: 14637075]
- Bramlett HM, Dietrich WD. Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. *J Cereb Blood Flow Metab.* 2004; 24(2):133–50.10.1097/01.WCB.0000111614.19196.04 [PubMed: 14747740]
- Clark RS, Chen J, Watkins SC, Kochanek PM, Chen M, Stetler RA, Loeffert JE, Graham SH. Apoptosis-suppressor gene bcl-2 expression after traumatic brain injury in rats. *J Neurosci.* 1997; 17(23):9172–82. [PubMed: 9364064]
- Danzer KM, Kranich LR, Ruf WP, Cagsal-Getkin O, Winslow AR, Zhu L, Vanderburg CR, McLean PJ. Exosomal cell-to-cell transmission of alpha-synuclein oligomers. *Mol Neurodegener.* 2012; 7:42.10.1186/1750-1326-7-42 [PubMed: 22920859]
- Danzer KM, Ruf WP, Putcha P, Joyner D, Hashimoto T, Glabe C, Hyman BT, McLean PJ. Heat-shock protein 70 modulates toxic extracellular alpha-synuclein oligomers and rescues trans-synaptic toxicity. *Faseb J.* 2011; 25(1):326–36.10.1096/fj.10-164624 [PubMed: 20876215]

- Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron*. 2003; 39(6):889–909. [PubMed: 12971891]
- Di Virgilio F. Dr. Jekyll/Mr. Hyde: the dual role of extracellular ATP. *J Auton Nerv Syst*. 2000; 81(1–3):59–63. [PubMed: 10869701]
- Di Virgilio F, Ferrari D, Idzko M, Panther E, Norgauer J, la Sala A, Girolomoni G. Extracellular ATP, P2 receptors, and inflammation. *Drug Develop Res*. 2003; 59(1):171–4.10.1002/Ddr.10191
- Diaz-Hernandez M, Diez-Zaera M, Sanchez-Nogueiro J, Gomez-Villafuertes R, Canals JM, Alberch J, Miras-Portugal MT, Lucas JJ. Altered P2X7-receptor level and function in mouse models of Huntington's disease and therapeutic efficacy of antagonist administration. *Faseb J*. 2009; 23(6): 1893–906.10.1096/fj.08-122275 [PubMed: 19171786]
- Ebrahimi-Fakhari D, Cantuti-Castelvetri I, Fan Z, Rockenstein E, Masliah E, Hyman BT, McLean PJ, Unni VK. Distinct roles in vivo for the ubiquitin-proteasome system and the autophagy-lysosomal pathway in the degradation of alpha-synuclein. *J Neurosci*. 2011; 31(41):14508–20.10.1523/JNEUROSCI.1560-11.2011 [PubMed: 21994367]
- Falzoni S, Donvito G, Di Virgilio F. Detecting adenosine triphosphate in the pericellular space. *Interface Focus*. 2013; 3(3):20120101.10.1098/rsfs.2012.0101 [PubMed: 23853707]
- Gourine AV, Dale N, Llaudet E, Poputnikov DM, Spyer KM, Gourine VN. Release of ATP in the central nervous system during systemic inflammation: real-time measurement in the hypothalamus of conscious rabbits. *J Physiol*. 2007; 585(Pt 1):305–16.10.1113/jphysiol.2007.143933 [PubMed: 17901122]
- Greco NJ, Tonon G, Chen W, Luo X, Dalal R, Jamieson GA. Novel structurally altered P(2X1) receptor is preferentially activated by adenosine diphosphate in platelets and megakaryocytic cells. *Blood*. 2001; 98(1):100–7. [PubMed: 11418468]
- Guha S, Baltazar GC, Coffey EE, Tu LA, Lim JC, Beckel JM, Patel S, Eysteinson T, Lu W, O'Brien-Jenkins A, Laties AM, Mitchell CH. Lysosomal alkalization, lipid oxidation, and reduced phagosome clearance triggered by activation of the P2X7 receptor. *Faseb J*. 2013; 27(11):4500–9.10.1096/fj.13-236166 [PubMed: 23964074]
- Hirsch EC, Vyas S, Hunot S. Neuroinflammation in Parkinson's disease. *Parkinsonism Relat Disord*. 2012; 18(Suppl 1):S210–2.10.1016/S1353-8020(11)70065-7 [PubMed: 22166438]
- Hodges RR, Vrovlanis J, Scott R, Dartt DA. Identification of P2X(3) and P2X(7) purinergic receptors activated by ATP in rat lacrimal gland. *Invest Ophthalmol Vis Sci*. 2011; 52(6):3254–63.10.1167/iovs.10-7042 [PubMed: 21421865]
- Hracsco Z, Baranyi M, Csölle C, Goloncser F, Madarasz E, Kittel A, Sperlagh B. Lack of neuroprotection in the absence of P2X7 receptors in toxin-induced animal models of Parkinson's disease. *Mol Neurodegener*. 2011; 6:28.10.1186/1750-1326-6-28 [PubMed: 21542899]
- Incho EW, LeBlanc EA, Pham BT, White SM, Imig JD. Purinoceptor-mediated calcium signaling in preglomerular smooth muscle cells. *Hypertension*. 1999; 33(1 Pt 2):195–200. [PubMed: 9931104]
- Iyer SS, Pulsikens WP, Sadler JJ, Butter LM, Teske GJ, Ulland TK, Eisenbarth SC, Florquin S, Flavell RA, Leemans JC, Sutterwala FS. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A*. 2009; 106(48):20388–93.10.1073/pnas.0908698106 [PubMed: 19918053]
- Jafari S, Etminan M, Aminzadeh F, Samii A. Head injury and risk of Parkinson disease: a systematic review and meta-analysis. *Mov Disord*. 2013; 28(9):1222–9.10.1002/mds.25458 [PubMed: 23609436]
- Jellinger KA. Interaction between pathogenic proteins in neurodegenerative disorders. *J Cell Mol Med*. 2012; 16(6):1166–83.10.1111/j.1582-4934.2011.01507.x [PubMed: 22176890]
- Jiang P, Gan M, Ebrahim AS, Castanedes-Casey M, Dickson DW, Yen SH. Adenosine monophosphate-activated protein kinase overactivation leads to accumulation of alpha-synuclein oligomers and decrease of neurites. *Neurobiol Aging*. 2013; 34(5):1504–15.10.1016/j.neurobiolaging.2012.11.001 [PubMed: 23200460]
- Jin QH, Zhao B, Zhang XJ. Cytochrome c release and endoplasmic reticulum stress are involved in caspase-dependent apoptosis induced by G418. *Cell Mol Life Sci*. 2004; 61(14):1816–25.10.1007/s00018-004-4143-7 [PubMed: 15241557]

- Kawamura H, Kawamura T, Kanda Y, Kobayashi T, Abo T. Extracellular ATP-stimulated macrophages produce macrophage inflammatory protein-2 which is important for neutrophil migration. *Immunology*. 2012; 136(4):448–58.10.1111/j.1365-2567.2012.03601.x [PubMed: 22564028]
- Kew, JNC.; Davies, CH. Ion channels: from structure to function. 2. Oxford University Press; Oxford: 2010.
- Kornegreen A, Priel Z. Purinergic stimulation of rabbit ciliated airway epithelia: control by multiple calcium sources. *J Physiol*. 1996; 497 (Pt 1):53–66. [PubMed: 8951711]
- Kurashima Y, Amiya T, Nochi T, Fujisawa K, Haraguchi T, Iba H, Tsutsui H, Sato S, Nakajima S, Iijima H, Kubo M, Kunisawa J, Kiyono H. Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors. *Nat Commun*. 2012; 3:1034.10.1038/ncomms2023 [PubMed: 22948816]
- Lee PC, Bordelon Y, Bronstein J, Ritz B. Traumatic brain injury, paraquat exposure, and their relationship to Parkinson disease. *Neurology*. 2012; 79(20):2061–6.10.1212/WNL.0b013e3182749f28 [PubMed: 23150532]
- Liu J, Wang YT. Allosteric modulation of GABAA receptors by extracellular ATP. *Mol Brain*. 2014; 7:6.10.1186/1756-6606-7-6 [PubMed: 24456563]
- Lotharius J, Falsig J, van Beek J, Payne S, Dringen R, Brundin P, Leist M. Progressive degeneration of human mesencephalic neuron-derived cells triggered by dopamine-dependent oxidative stress is dependent on the mixed-lineage kinase pathway. *J Neurosci*. 2005; 25(27):6329–42.10.1523/JNEUROSCI.1746-05.2005 [PubMed: 16000623]
- North RA, Jarvis MF. P2X receptors as drug targets. *Mol Pharmacol*. 2013; 83(4):759–69.10.1124/mol.112.083758 [PubMed: 23253448]
- Planchard MS, Exley SE, Morgan SE, Rangachari V. Dopamine-induced alpha-synuclein oligomers show self- and cross-propagation properties. *Protein Sci*. 2014; 23(10):1369–79.10.1002/pro.2521 [PubMed: 25044276]
- Prasai P, Stefos GC, Becker W. Extracellular ATP activates NFAT-dependent gene expression in neuronal PC12 cells via P2X receptors. *BMC Neurosci*. 2011; 12:90.10.1186/1471-2202-12-90 [PubMed: 21943104]
- Prots I, Veber V, Brey S, Campioni S, Buder K, Riek R, Bohm KJ, Winner B. alpha-Synuclein oligomers impair neuronal microtubule-kinesin interplay. *J Biol Chem*. 2013; 288(30):21742–54.10.1074/jbc.M113.451815 [PubMed: 23744071]
- Putcha P, Danzer KM, Kranich LR, Scott A, Silinski M, Mabbett S, Hicks CD, Veal JM, Steed PM, Hyman BT, McLean PJ. Brain-permeable small-molecule inhibitors of Hsp90 prevent alpha-synuclein oligomer formation and rescue alpha-synuclein-induced toxicity. *J Pharmacol Exp Ther*. 2010; 332(3):849–57.10.1124/jpet.109.158436 [PubMed: 19934398]
- Rettinger J, Schmalzing G. Activation and desensitization of the recombinant P2X1 receptor at nanomolar ATP concentrations. *J Gen Physiol*. 2003; 121(5):451–61.10.1085/jgp.200208730 [PubMed: 12719485]
- Rodriguez-Grande B, Blackabey V, Gittens B, Pinteaux E, Denes A. Loss of substance P and inflammation precede delayed neurodegeneration in the substantia nigra after cerebral ischemia. *Brain Behav Immun*. 2013; 29:51–61.10.1016/j.bbi.2012.11.017 [PubMed: 23232501]
- Ryu JK, McLarnon JG. Block of purinergic P2X(7) receptor is neuroprotective in an animal model of Alzheimer's disease. *Neuroreport*. 2008; 19(17):1715–9.10.1097/WNR.0b013e3283179333 [PubMed: 18852683]
- Schildknecht S, Karreman C, Poltl D, Efremova L, Kullmann C, Gutbier S, Krug A, Scholz D, Gerding HR, Leist M. Generation of genetically-modified human differentiated cells for toxicological tests and the study of neurodegenerative diseases. *Altex*. 2013; 30(4):427–44. [PubMed: 24173167]
- Schildknecht S, Nagel D, Leist M. The human dopaminergic neuronal cell line LUHMES as in vitro model for Parkinson's disease. *J Neurochem*. 2009; 110:13.
- Seminario-Vidal L, Lazarowski ER, Okada SF. Assessment of extracellular ATP concentrations. *Methods Mol Biol*. 2009; 574:25–36.10.1007/978-1-60327-321-3_3 [PubMed: 19685297]

- Sperlagh B, Vizi ES. The role of extracellular adenosine in chemical neurotransmission in the hippocampus and Basal Ganglia: pharmacological and clinical aspects. *Curr Top Med Chem.* 2011; 11(8):1034–46. [PubMed: 21401497]
- Stern MB. Head trauma as a risk factor for Parkinson's disease. *Mov Disord.* 1991; 6(2):95–7.10.1002/mds.870060202 [PubMed: 2057017]
- Stern ST, Adiseshaiah PP, Crist RM. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. *Part Fibre Toxicol.* 2012; 9:20.10.1186/1743-8977-9-20 [PubMed: 22697169]
- Tanaka K, Swanson SJ, Gilroy S, Stacey G. Extracellular nucleotides elicit cytosolic free calcium oscillations in Arabidopsis. *Plant Physiol.* 2010; 154(2):705–19.10.1104/pp.110.162503 [PubMed: 20671112]
- Trabanelli S, Ocadlikova D, Gulinelli S, Curti A, Salvestrini V, Vieira RP, Idzko M, Di Virgilio F, Ferrari D, Lemoli RM. Extracellular ATP exerts opposite effects on activated and regulatory CD4+ T cells via purinergic P2 receptor activation. *J Immunol.* 2012; 189(3):1303–10.10.4049/jimmunol.1103800 [PubMed: 22753942]
- Unal-Cevik I, Gursay-Ozdemir Y, Yemisci M, Lule S, Gurer G, Can A, Muller V, Kahle PJ, Dalkara T. Alpha-synuclein aggregation induced by brief ischemia negatively impacts neuronal survival in vivo: a study in [A30P]alpha-synuclein transgenic mouse. *J Cereb Blood Flow Metab.* 2011; 31(3):913–23.10.1038/jcbfm.2010.170 [PubMed: 20877387]
- Uryu K, Chen XH, Martinez D, Browne KD, Johnson VE, Graham DI, Lee VM, Trojanowski JQ, Smith DH. Multiple proteins implicated in neurodegenerative diseases accumulate in axons after brain trauma in humans. *Exp Neurol.* 2007; 208(2):185–92.10.1016/j.expneurol.2007.06.018 [PubMed: 17826768]
- Uryu K, Giasson BI, Longhi L, Martinez D, Murray I, Conte V, Nakamura M, Saatman K, Talbot K, Horiguchi T, McIntosh T, Lee VM, Trojanowski JQ. Age-dependent synuclein pathology following traumatic brain injury in mice. *Exp Neurol.* 2003; 184(1):214–24. [PubMed: 14637093]
- Vogiatzi T, Xilouri M, Vekrellis K, Stefanis L. Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. *J Biol Chem.* 2008; 283(35):23542–56.10.1074/jbc.M801992200 [PubMed: 18566453]
- Volonte C, Amadio S, Cavaliere F, D'Ambrosi N, Vacca F, Bernardi G. Extracellular ATP and neurodegeneration. *Curr Drug Targets CNS Neurol Disord.* 2003; 2(6):403–12. [PubMed: 14683468]

Highlights

- Extracellular ATP increases intracellular α syn levels between 24 to 48 hours after treatment
- α syn accumulation is a result of lysosome dysfunction caused by extracellular ATP-induced elevation of lysosomal pH
- P2X1 receptor appears to mediate the cells' response to extracellular ATP
- Although Ca^{2+} influx via P2X1 receptor is necessary for α syn accumulation, Ca^{2+} influx per se is not sufficient for increased α syn accumulation

Figure 1. Dying cells release ATP and induce α syn oligomerization in surrounding healthy S1S2 cells

(A) Stable S1S2 cells were co-cultured with necrotic wild-type H4 (Ne H4) or apoptotic wild-type H4 (Ap H4) for 12, 24 and 48 h and the extent of intracellular α syn oligomers was evaluated by luciferase assay. (B) S1S2 cells were co-cultured with necrotic H4 (Ne H4) or apoptotic H4 (Ap H4) for 0.5 h, 24 h and 48 h. An ATP assay was performed to measure the ATP concentration in the growth media at different time points. (C) S1S2 cells were treated with 0, 1, 2, 3 mM ATP for 24 h and 48 h and the extent of intracellular α syn oligomers was evaluated by luciferase assay. (D) S1S2 cells were incubated with 3 mM ATP for 1 h~48 h before being removed and replaced with normal growth media for a total of 48hrs. S1S2 cells without ATP were used as control. Luciferase assay was performed to monitor the intracellular oligomerization. (E) 2 mM and 3 mM ATP was added to S1S2 cells and ATP concentration in the media was measured at different time points by ATP assay. The data are plotted as mean $\pm$ SD (n=3). N.S., Not significant. *, $p < 0.05$. **, $p < 0.01$ compared to control.

Figure 2. Extracellular ATP treatment induces intracellular α syn oligomerization in S1S2 cells (A) S1S2 cells were treated with or without 3 mM ATP, 500 μ M ATP γ S, 3 mM ADP or 3 mM AMP for 48 h and the level of intracellular α syn oligomers was evaluated by luciferase assay. Wild-type H4 cells transfected with (B) human α syn-luciferase or (C) luciferase were treated with 3 mM ATP or 500 μ M ATP γ S or 3 mM AMP for 48 h. Luciferase assay was performed to monitor the intracellular oligomerization. (D) Native-PAGE was performed to assess α syn oligomers in S1S2 cells treated with or without 3 mM ATP for 48 h. S1S2 cells without α syn expression (Tet+) were used as a negative control. 30 μ g proteins was loaded per lane. (E) S1S2 cells were treated with or without 3 mM ATP for 48 h and cell lysates were analyzed by denaturing SDS-PAGE. 30 μ g protein was loaded per lane. (F) Quantification of intracellular α syn levels in S1S2 cells in SDS-PAGE. The data are plotted as mean $\pm$ SD (n=3). N.S., Not significant. *, $p < 0.05$. **, $p < 0.01$ compared to control.

Figure 3. P2X1 mediates extracellular ATP induced accumulation of intracellular αsyn
 S1S2 cells were pretreated with (A) 0, 0.5, 1, 2, 3, 4, 5 μM NF449 (P2X1 receptor antagonist), (B) 0, 0.01, 0.1, 1, 5, 10 μM TNP-ATP (P2X3 receptor antagonist), (C) 0, 0.1, 0.5, 1, 2.5, 5 μM PSB-12062 (P2X4 receptor antagonist) or (D) 0, 20, 40, 60, 80, 100 μM A438079 (P2X7 receptor antagonist) for 30 min then subjected to 0 and 3 mM ATP treatment for 48 h. Intracellular αsyn oligomerization was evaluated by luciferase assay. (E) S1S2 cells were treated with 0, 10, 30, 100, 300, 1000 μM BzATP (P2X1 receptor agonist) for 48h. Intracellular αsyn oligomerization was evaluated by luciferase assay. (F) S1S2 cells were transfected with scrambled siRNA or P2X1 siRNA for 72 h, Cell lysates were analyzed by western blot, 30 μg protein/lane, and (G) statistical quantitation of P2X1 levels were performed to validate the effect of knockdown. (H) S1S2 cells transfected with scrambled siRNA or P2X1 siRNA were treated with or without 3 mM ATP for 48 h. The intracellular αsyn oligomerization was evaluated by luciferase assay. The data are plotted as mean ± SD (n=3). Asterisk(s) indicates significant differences between the treated group and control (N.S., Not significant. *, p < 0.05, **, p < 0.01). Number sign(s) indicates significant differences between bracketed groups (N.S., Not significant. #, p < 0.05. ##, p < 0.01).

Figure 4. Extracellular ATP increases endogenous αsyn in differentiated Luhmes cells through P2X1 receptor

(A) Differentiated Luhmes cells were treated with either 3 mM ATP, 300 μM BzATP, 3 μM NF449, or 3 μM NF449 plus 3 mM ATP for 48 h. Cell lysates were analyzed by SDS-PAGE. 30 μg protein was loaded per lane. (B) Quantitation of intracellular αsyn levels. (C) Luhmes cells were transfected with scrambled siRNA or P2X1 siRNA for 24 h, and then treated with or without 3 mM ATP for 48 h. Cell lysates were analyzed by western blot. 30 μg protein was loaded per lane. (D) Quantification of P2X1 and (E) intracellular αsyn levels. The data are plotted as mean ± SD (n=3). N.S., Not significant. *, p < 0.05. **, p < 0.01 compared to control. Number sign(s) indicate significant differences between two bracketed groups (N.S., Not significant. #, p < 0.05).

Figure 5. Extracellular ATP treatment inhibited αsyn degradation in both S1S2 cells and differentiated Luhmes cells

(A) The mRNA levels of αsyn in S1S2 cells and differentiated Luhmes cells with and without 48 h of 3 mM ATP treatment were compared by quantitative real-time PCR. (B) S1S2 cells were first cultured in media without Tetracycline (Tet) for 7 d to reach maximum αsyn expression. Then 1 μg/ml Tet was added back to shut down αsyn induction. Cells were harvested at 0, 6, 12, 24, 36, and 48 h after Tet addition in the presence or absence of 3 mM ATP. Cell lysates were analyzed by dot blot. 10 μg protein was loaded per well. (C) Quantification of total αsyn levels. (D) Differentiated Luhmes cells were treated with 400 μg/ml Cycloheximide (CHX) to inhibit proteins synthesis. Cells were harvested at 0, 6, 12, 24, 36, and 48 h after CHX addition in the presence or absence of 3 mM ATP. Cell lysates were analyzed by western blot. 30 μg protein was loaded per lane. (E) The change in total αsyn at different time points was quantified. The data are plotted as mean ± SD (n=3). N.S., Not significant. *, $p < 0.05$. **, $p < 0.01$ compared to control.

Figure 6. Extracellular ATP treatment inhibits autophagy in both S1S2 cells and differentiated Luhmes cells

(A) The proteasomal function of S1S2 cells and differentiated Luhmes cells treated with or without 3 mM ATP for 48 h or 500 nM Lactacystin for 24 h was evaluated using a Proteasome activity assay. (B) S1S2 cells and (E) Differentiated Luhmes cells were treated with or without 3 mM ATP or 200 nM Bafilomycin A1 (BA1) for 48 h. Cell lysates were analyzed by western blot. 30 μ g protein was loaded per lane. (C) Quantitation of the levels of LC3II and (D) p62 in S1S2 cells and (F, G) Luhmes cells. The data are plotted as mean $\pm$ SD (n=3). Asterisk(s) indicate significant differences between the treated group and Control (N.S., Not significant. *, $p < 0.05$. **, $p < 0.01$).

Figure 7. Extracellular ATP increased lysosomal pH in both S1S2 cells and differentiated Luhmes cells

(A) S1S2 cells and (B) differentiated Luhmes cells were pretreated with or without 3 μ M NF449 for 30 min in serum-free medium, 3mM ATP was loaded to the medium and incubated with cells for 48 h. Cells treated with 200 nM Bafilomycin A1 (BA1) for 24 h as a control. The lysosomal pH value was evaluated by measuring and calibrating LysoSensor Yellow/Blue fluorescence. The data are plotted as mean $\pm$ SEM (n=3). Asterisk(s) indicated significant differences between the treated group and Control (N.S., Not significant. *, $p < 0.05$. **, $p < 0.01$). Number sign(s) indicate significant differences between two bracketed groups (#, $p < 0.05$).

Figure 8. Depletion of Ca²⁺ prevents ATP-induced oligomerization of intracellular αsyn
(A) Using the Fluo-4 AM dye as a marker for intracellular Ca²⁺, the time course effect of ATP-evoked calcium influx was measured and quantified in S1S2 cells. 3 μM A23187, the calcium ionophore was used as a control. F/F₀ represents the ratio of fluorescence signal at each time point to the basal signal (at 0 s). The data are plotted as mean ± SEM (n=20). **(B)** The peak value of calcium fluorescence was analyzed and plotted as mean ± SD (n=20). **, p < 0.01 compared to ATP group. **(C)** S1S2 cells were treated with 3 mM ATP in Ca²⁺-free medium for 15 min, then 1.5 mM CaCl₂ was added back to the medium. 48 h later, luciferase activity assay was performed to evaluate the level of intracellular αsyn oligomerization. The data are plotted as mean ± SD (n=3). Asterisk(s) indicated significant differences between the treated group and Control (**, p < 0.01). Number sign(s) indicate significant differences between two bracketed groups (##, p < 0.01). **(D)** S1S2 cells were treated with ionophore, 3 μM A23187 in serum-free medium for 48 h. Luciferase activity was measured to monitor the level of intracellular αsyn oligomerization. The data are plotted as mean ± SD (n=3). N.S., Not significant compared to control.