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Amyloid- β Induces a Caspase-Mediated Cleavage of P2X4 to Promote Purinotoxicity

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Abstract

Overproduction of the β -amyloid fragment 1-42 ($A\beta_{1-42}$) is thought to contribute to synaptic dysfunction and neuronal death in Alzheimer's disease. Mounting evidence suggests that purinergic receptors play critical roles in synaptic plasticity and neuronal survival, but the potential involvement of these receptors in $A\beta_{1-42}$ -induced synaptic dysfunction and neuronal death has not been addressed. Here we report that $A\beta_{1-42}$ promoted accumulation of the calcium permeable purinergic receptor P2X4 in neurons. We also report evidence that $A\beta_{1-42}$ induced a caspase-3 mediated cleavage of the receptor that slowed channel closure times and prevented agonist-induced internalization of the receptor. Molecular interference to reduce the expression of P2X4 in primary rodent neurons attenuated $A\beta_{1-42}$ -induced neuronal death while induced expression of P2X4 in a neuronal cell line that does not normally express P2-receptors enhanced the toxic effect of $A\beta_{1-42}$. Together these findings suggest that $A\beta_{1-42}$ -induced synaptic dysfunction and neuronal death may involve proteolytic processing of the purinergic receptor P2X4.

Keywords

Alzheimer's; Purinergic; P2X4; Receptor trafficking

Introduction

There is considerable evidence that suggests $A\beta$ promotes the synaptic dysfunction and death of mature neurons by mechanisms that involve a disruption in calcium handling (Mattson &

Furukawa 1997, Mattson *et al.* 1998, Harkany *et al.* 2000). A β -induced disruption of calcium homeostasis is thought to play a critical role in neuronal dysfunction and death by facilitating membrane lipid peroxidation, mitochondrial dysfunction and activation of apoptosis (Markesbery 1997, Mattson *et al.* 1997, Mark *et al.* 1996, Rottkamp *et al.* 2000). Although A β -induced neuronal cell death may not be directly mediated by glutamate (Busciglio *et al.* 1992), A β does increase the vulnerability of neurons to excitotoxic insult by increasing glutamate-evoked calcium flux and by prolonging membrane depolarization that results in an over activation of voltage-operated calcium channels (VOCC; Koh *et al.* 1990, Mattson *et al.* 1992). However, glutamate and VOCC antagonists/modulators tested thus far have shown limited efficacy in clinical trials to treat AD. One explanation for the limited success of glutamate and VOCC channel antagonists as therapeutics, is that other calcium permeable receptors are dysfunctional in AD and contribute to neuronal demise. Here we report findings that suggest the calcium permeable purinergic receptor P2X4 may be involved in A β -associated neuronal death.

Purinergic receptors (receptors for nucleotides, purines or pyrimidines) are abundant on neurons. There are two broad categories of purinergic receptors: P2X family members assemble in pentameric arrangements to form ligand-gated cation channels and P2Y family members are G-protein coupled receptors (Burnstock & Knight 2004). P2X4 is abundantly expressed in brain and is often expressed on neurons that are also glutamatergic (Rubio & Soto 2001). P2X4 is preferentially located at the peripheral portion of the postsynaptic specialization where it can form homomeric and heteromeric channels that are important for synaptic plasticity (Rubio & Soto 2001, Tanaka *et al.* 1996, Buell *et al.* 1996, Collo *et al.* 1996, Seguela *et al.* 1996, Soto *et al.* 1996, Sim *et al.* 2006). The calcium permeability of P2X4 receptors is comparable to that of NMDA-receptors; both $P_{Ca}/P_{Na} \sim 4$ suggesting that it may also play important roles in the regulation of neuronal survival (Evans 1996, Evans *et al.* 1996, Koshimizu *et al.* 2000). Indeed, accumulating evidence suggests purinergic receptor dysfunction may contribute to neuronal death (Merlo & Volonte 1996, Volonte & Merlo 1996, Volonte *et al.* 1999, Cavaliere *et al.* 2001a, Cavaliere *et al.* 2001b, Schwab *et al.* 2005, Casanovas *et al.* 2008).

Despite the abundant expression of P2 receptors on cortical and hippocampal neurons and the documented susceptibility of some neurons to purinergic insult, there has been surprisingly little research detailing the involvement of P2 receptors in neurological disorders. In particular, the potential role of purinergic receptors as mediators of neuronal dysfunction and death in Alzheimer's disease has not been addressed. Here we provide evidence that suggests a toxic form of A β can deregulate the expression and function of P2X4.

Results

P2X4 Receptor Levels are Decreased in Alzheimer's Brain

We first quantified P2X4 levels in brain tissues from AD patients with severe cognitive impairment and normal controls with no signs of cognitive impairment. Protein levels of P2X4 were decreased in the MFG and MTG but were similar in the cerebellum of subjects with AD compared to controls (Fig 1).

Modification of P2X4 Expression by Amyloid- β Peptide

We next exposed hippocampal neurons in culture to a toxic form of amyloid- β (A β ₁₋₄₂; Fig 2A) and quantified P2X4 levels by Western blot and immunocytochemistry. A β increased P2X4 receptor expression in cell bodies after 6 hours and in cell bodies and neurites after 12 hours (Fig 2B). By Western blot we observed that A β increased full length P2X4 within 6 hours and we noted the accumulation of a smaller ~ 13 kD fragment (Fig 2C,D). Based on evidence that

A β can increase caspase activity (Jhamandas & MacTavish 2004) and sequence information that suggests the C-terminal of P2X₄ (but not other P2X receptors) contains a putative caspase 3 cleavage site (amino acids 271-274, dtrd, that is internal to the antibody recognition site; Fig 3A), we thought it possible that the fragment was a cleaved product of P2X₄. In support of this hypothesis, we found that the caspase inhibitor ZVADfmk prevented A β -induced accumulation of the P2X₄ fragment (Fig 3B,C). To demonstrate that caspase 3 directly cleaved P2X₄, we next bound P2X₄ to protein-A beads and exposed the column to purified caspase 3 or 4. At pH 7.4, caspase was unable to cleave the P2X₄ C-terminal. However, when we lowered the pH to 6.8 and exposed the column to caspase 3, a fragment appeared that was consistent with the predicted size of the P2X₄ C-terminal (Fig 3D). Incubation with caspase 4 did not result in cleavage of P2X₄ (Fig 3D).

Induction of Caspase Activity Perturbs P2X₄ Trafficking

One means of signal termination following P2X₄ receptor activation involves internalization of the receptor by mechanisms dependent on the C-terminal. During agonist-induced receptor internalization, receptors traffic to an endosomal/lysosomal compartment where a fraction of these receptors are returned to the cell surface. Based on data that the C-terminal of P2X₄ plays an important role in the regulation of channel internalization (Bobanovic *et al.* 2002, Fountain & North 2006), we thought a caspase-mediated cleavage of the P2X₄ C-terminal would prevent agonist-induced channel internalization. To test this hypothesis we pre-treated primary neurons with a dose of A β that significantly increased caspase 3 activity over vehicle (Fig 4A) and triggered P2X₄ internalization by addition of ATP. In vehicle treated cultures, the amount of P2X₄ that co-localized with the lysosomal marker LAMP-1 increased following 6 minutes of stimulation with ATP and was slightly reduced but remained elevated after 30 minutes (Fig 4B,C). Pre-treatment of cultures with A β reduced agonist-induced internalization of the P2X₄ receptor (Fig 4B, C), suggesting that caspase prevented the internalization of P2X₄ receptors by cleaving the C-terminal. Indeed, A β -induced disruption of agonist-induced P2X₄ trafficking was prevented by pre-treatment of neurons with the broad-spectrum caspase inhibitor ZVADfmk (Fig 4D). ZVADfmk alone did not affect ATP-induced internalization of P2X₄ (data not shown). Similar caspase-dependent perturbations of agonist-induced P2X₄ internalization were obtained when we induced caspase activity with the pro-apoptotic agent staurosporine (data not shown).

Caspase 3 Modifies the Kinetics of P2X₄ Channel Function

Based on data that the C-terminal of P2X₄ is an important modifier of channel kinetics (Fountain & North 2006, Koshimizu *et al.* 1999), we next determined if A β -induced cleavage of P2X₄ modified channel function. Although the amplitude of ATP-evoked calcium responses were similar in vehicle and A β -treated cultures, the slope of decay in calcium transients was greater in A β -treated neurons (Fig 5A). A similar slowing of the decay rate in ATP-evoked calcium transients was observed in cultures treated with staurosporine (data not shown) and was prevented by pre-treatment of cultures with ZVADfmk (Fig 5A).

To verify that A β -induced modification of channel closure time was the result of caspase activity and not due to diminished ability to remove calcium from the cytosol, we next performed patch-clamp experiments in GT1-7 cells transfected with P2X₄ (these cells do not normally express P2X₄) and administered purified caspase 3 through the recording pipette. ATP-evoked channel kinetics were similar in amplitude and closure time immediately following seal of the patch pipette ($173.8 \pm 49.1\%$ increase in current) and after 10 minutes ($169.6 \pm 37.0\%$ increase in current). When caspase 3 was introduced into the patch pipette, the initial ATP-evoked response was similar to control responses ($144.6 \pm 46.6\%$ increase in current), but the response to ATP after 10 minutes was increased in amplitude and showed slower channel closure times ($274.9 \pm 51.0\%$ increase in current). The inclusion of caspase 4

in the patch pipette did not modify P2X4 channel kinetics (data not shown). Thus, caspase 3 can rapidly modify P2X4 receptor function by increasing channel closure time.

P2X4 Expression Modifies Neuronal Function and Survival

To determine if P2X4 modified A β toxicity, we over expressed or knocked down P2X4 expression in primary neurons. Greater than 90% of the neurons were transfected by lentivirus as determined by quantification of V5-immunopositive cells (Fig 6A). P2X4 over expression was confirmed by immunofluorescent microscopy and by Western blot over a three day period (Fig 6A, B). Neurons that over expressed P2X4 exhibited exaggerated peak increases of cytosolic calcium in response to ATP application, but showed similar rates of decay in calcium transients (Fig 6C). The neurotoxic effect of A β was enhanced in cultures over expressing P2X4 compared to cultures expressing LacZ (Fig 6D). We next decreased P2X4 expression using RNA interference. Decreased P2X4 expression was confirmed by immunocemistry and Western blot over a three day period (Fig 6E,F) and functionally by measuring the calcium response to ATP (Fig 6G). The neurotoxic effect of A β was blunted in these cultures (Fig 6H), suggesting that P2X4 contributed to A β -induced neuronal cell death.

Discussion

Neurodegeneration in Alzheimer's disease (AD) is progressive and irreversible. The deposition of A β aggregates in brain that are closely associated with dystrophic neurons is thought to be a decisive event in the pathogenesis of AD (Tanzi & Bertram 2005, Selkoe & Schenk 2003, Yankner 2000, Hardy 1999). It has been repeatedly shown in tissue culture models of AD, that A β can induce neuronal dysfunction and death by promoting the exaggerated flux of calcium through NMDA and VOCCs (Brorson *et al.* 1995, Greenamyre *et al.* 1985, Canzoniero & Snider 2005). However, therapeutics designed to protect damaged and dying neurons by inhibiting the function of these calcium-permeable receptors have shown limited effectiveness in the clinic. Our findings suggest that the failure of NMDA or VOCC antagonists/modulators in AD may involve an unrestrained dysfunction of P2X4 receptors.

P2X4 levels were decreased in the medial frontal gyrus of end stage AD patients and increased prior to neuronal death in an *in vitro* model of A β -induced neuronal cell death. These findings suggest that P2X4 may accumulate concurrent with A β and contribute to neuronal demise. Similar to other oligomeric channels, the channel subunit composition can dramatically modify the ion permeability and kinetics of the channel. To date, 7 different P2X receptor subunits have been identified that can form functional homomeric and heteromeric channels. Thus, the complexity of the P2X system suggests that regulation of channel subunit availability may play a major role in regulating cellular function by altering in the calcium permeability of P2X channels. For example, P2X1, 2, 5, and 7 are down-regulated while P2X3, 4 and 6 are up-regulated during apoptosis in prostate (Slater *et al.* 2000). In addition, the permeability of P2X receptors is dynamically regulated as a function of the agonist concentration by agonist-induced alterations in the channel pore size (Hackos & Korenbrot 1999, Khakh *et al.* 1999). Brief (< 1sec) applications of ATP open a channel within milliseconds that is permeable to small cations and with longer applications (10-60s) the channel becomes permeable to larger cations such as N-methyl-D-glucamine and the propidium analog YO-PRO-1 (Virginio *et al.* 1999). Regulation of subunit composition and channel pore size may thus be important determinants that control calcium permeability of P2X receptors and cell survival. Indeed, it has been demonstrated that overabundance of extracellular ATP can induce the death of diverse cell types including fibroblasts, macrophages, lymphocytes, thymocytes, dendritic cells and glomerular mesangial cells by necrosis or apoptosis depending on the dose and length of stimulation due to alterations in calcium permeability (Nihei *et al.* 2000, Di Virgilio *et al.* 1990, Zanovello *et al.* 1990, Nagy *et al.* 2000, Freedman *et al.* 1999, Harada *et al.* 2000). Thus,

necrotic death that follows shortly after acute injury such as stroke may involve the prolonged activation of P2X receptors that are over-stimulated by vast amounts of ATP made available from dead and dying cells. In AD, the death of neurons may involve alterations in the kinetics of P2X4 that provides a net increase of calcium flux. Because P2X4 is the only P2X receptor with a putative caspase 3 cleavage site located on the C-terminal, it is likely that P2X4 is the only P2X receptor subunit that is C-terminal truncated by caspase. Although, it is possible that the function of other purinergic receptors are altered in AD by caspase-independent mechanisms.

If indeed the putative caspase cleavage site on P2X4 resides on the ectodomain of the receptor, then it follows that caspase 3 cleaves P2X4 on the surface of cells, or during receptor trafficking. While it is possible that caspase 3 released from dead and dying cells could cleave extracellular epitopes of proteins on bystander cells, our biochemical and electrophysiological experiments suggest that cleavage of P2X4 occurs during trafficking. When immobilized P2X4 on an affinity column, caspases 3 but not caspase 4 directly cleaved the C-terminal of P2X4 receptors at a pH consistent with the endosomal/lysosomal compartment. Whole cell patch experiments where caspase 3 was directly infused into the cytosol confirmed the involvement of caspase 3 in modification of P2X4 receptor kinetics. Indeed, there are a number of endosomal proteins cleaved by caspases and caspase 3-like activity is found in exosomes (Santambrogio *et al.* 2005). Nevertheless, the compartment and exact conditions under which P2X4 is available to be cleaved by caspase 3 remain to be determined.

Our data suggest that a caspase-mediated cleavage of P2X4 slowed channel closure and prevented agonist-induced internalization of the channel. A similar proteolytic processing that alters the permeability of ion channels has been reported in the glutaminergic system and in IP₃-sensitive receptors found in the endoplasmic reticulum. Evidence for AMPA receptor proteolysis has been observed in AD brain and *in vitro* A β can promote the cleavage of the AMPA receptor subunits GluR1, GluR2/3, and GluR4 by a caspase-dependent mechanism (Chan *et al.* 1999). Calpains may also contribute to glutamate receptor destabilization by cleavage of the AMPA receptor subunit GluR1 and the NMDA receptor subunit NR2B (Bi *et al.* 1994, Simpkins *et al.* 2003). Additional evidence for the destabilization of calcium homeostasis by caspases was shown for the inositol 1,4,5-trisphosphate receptor type 1 receptor subunit (IP₃R1; (Hirota *et al.* 1999). The regulatory domain of the IP₃R1 receptor is necessary to keep the channel domain closed (Nakayama *et al.* 2004). Thus, cysteine proteases may promote cell death by destabilizing multiple calcium permeable receptors and assuring that cytosolic calcium levels remain sufficiently elevated to keep calcium-dependent apoptotic machinery active.

Our observation that cleavage of P2X4 enhances channel kinetics may be due to the position of the cleavage site. When we introduced a point mutation into the putative caspase 3 cleavage site of P2X4 and cloned this receptor into GT1-7 cells (that do not express endogenous purinergic receptors), there was a near complete loss of ATP-evoked calcium response and the lethal response to glutamate, staurosporine, potassium cyanide and iron were reduced (data not shown). These results are consistent with recent findings that showed disruption of the ATP binding pocket when the aspartic acid at position 280 was mutated to glutamine (Yan *et al.* 2005). The aspartic acid at position 280 of the P2X4 receptor is a negatively charged residue that is thought to participate in binding ATP complexed with magnesium. Magnesium may be an important structural component of the binding pocket that may stabilize the conformation of ATP and participate in catalysis. Magnesium likely modulates ion gating by providing an opposing charge that decreases the affinity of ATP for the binding site. Indeed, single-channel recording from cells expressing P2X4 receptors have shown that high Mg²⁺ reduces the amplitude of single-channel current (Li *et al.* 1997). Thus, when this portion of the receptor is cleaved by caspase, ATP may bind with higher affinity and activate the receptor for a prolonged

time. This slower disassociation of ATP from its binding site on P2X4 would slow the channel closure time and increase the amount of calcium flux. Thus, mutation to this site may create a conformational change which blocks the binding site for ATP, while truncation increases the affinity of the site for ATP by removing the binding site for Mg²⁺.

Conclusions

Our findings suggest that A β may promote the death of neurons by mechanisms that involve the deregulation of P2X4 receptor function. These findings contribute to the growing body of evidence that suggests purinergic receptors in the CNS play important roles in neuronal function and survival.

Methods

Brain Tissues

Brain tissues were obtained from the Alzheimer's Disease Research Centre at Johns Hopkins University School of Medicine. Fresh frozen tissue blocks of middle frontal gyrus (MFG), Middle temporal gyrus (MTG) and cerebellum were obtained from normal subjects (n = 12, MMSE score of 3.5 ± 2 and a mean age 79 ± 11) and cases of definite AD (n = 12, MMSE score 26 ± 4 ; mean age 83 ± 12) diagnosed according to CERAD criteria (Mirra *et al.* 1991).

Cell Culture

Hippocampal neuronal cultures were prepared from 18-day-old embryonic Sprague Dawley rats as previously described (Haughey *et al.* 2001). Hippocampal tissue was dissociated by gentle titration in a calcium-free Hank's balanced salt solution and centrifuged at $1000 \times g$. Cells were re-suspended in MEM media containing 10 % heat-inactivated fetal bovine serum and 1 % antibiotic solution (10^4 U of penicillin G/ml, 10 mg streptomycin/ml and 25 μ g amphotericin B/ml) in 0.9 % NaCl (Sigma). Neurons were plated at a density of 100,000 cells/ml on 25 mm diameter poly-D-lysine coated glass coverslips for calcium imaging, and at a density of 200,000 cells/ml on 12 mm diameter coverslips for apoptosis (survival) analysis. Three hours after plating, the media was replaced with serum-free Neurobasal media containing 1 % B-27 supplement (Gibco). Immunofluorescent staining for MAP-2 (neurons) and GFAP (astrocytes) showed that cultures were > 98 % neurons; the remainder of cells were predominantly astrocytes. Cultures were used between 14 and 21 days *in vitro*. GT17 cells were cultured in DMEM/F12 supplemented with 10%FBS and 30 mM NaHCO₃. Amyloid- β 1-42 (A β 1-42) was purchased from Bachem (Torrance, CA). A β was dissolved in Lockes buffer (10 mM stock) and incubated at 37°C for 24 hours prior to use.

Immunoblot Analyses

Brain tissue is homogenized in ice-cold RIPA buffer consisting of 62 mM Tris, 2 mM EDTA, 2 mM EGTA, 2 % SDS, 10 % glycerol and a protease inhibitor cocktail (Sigma, St Louis, MO), pH 6.0. Proteins (100 μ g/lane) were separated by SDS-polyacrylamide gel electrophoresis (10 - 15 % acrylamide) and transferred to a nitrocellulose membrane. Membranes were incubated for 30 m in the presence of 5% non-fat milk and incubated overnight at 4°C with the primary antibody P2X4 (1:500; Chemicon, Temecula, CA). Membranes are exposed to HRP-conjugated secondary antibody (1:3000; Jackson Immunological Research Laboratories Inc., West Grove PA) and immunoreactive proteins visualized using a chemiluminescence based detection kit according to the manufacture's protocol (ECL kit; Amersham Corp., Arlington Heights, IL).

Immunofluorescent Microscopy

For immunostaining, the cultures were fixed for 30 minute in a solution of 4% Para formaldehyde in PBS, and membranes were permeabilized by incubation for 10 minute in a solution of 0.2% Triton X-100 in PBS. Cultures were then incubated for 1 h in blocking solution (2% normal goat serum and 2% normal horse serum in PBS) and then primary antibodies were added and the cultures were incubated overnight at 4°C. The primary antibodies included: a mouse monoclonal antibody against the neuron-specific antigen Map-2 (1:500; Sigma Aldrech, St. Louis, MO); a mouse monoclonal antibody that identifies lysosomes LAMP1 (1:250; Calbiochem, San Diego, CA). Cultures were washed with PBS and then incubated for 1 h in the presence of appropriate fluorescently tagged anti-mouse and anti-rabbit secondary antibodies (AlexaFluor 633, 546 and 488; 1: 2000 dilution; Invitrogen/Molecular Probes, Carlsbad, CA). In some experiments cultures were further stained with propidium iodide or Hoescht 33342. Confocal images were acquired using a Zeiss 510 CSLM microscope.

Construction of pLentiviral-P2X4 and P2X4-SiRNA

The full length P2X4 receptor DNA was cloned into the Sall/EcoRI site of the entry vector pENTR (Invitrogen). The entry clone was conformed to pLenti6/V5-DEST expression vector and then transformed into Stab13 competent cells. The expression clone was selected following digestion with BamHI/ EcoRI and transfection into 293 FT cells at the concentration of 1.2×10^6 /ml using Lipofectamine2000 and ViraPower Lentiviral Packing Mix (Invitrogen). The medium was replaced with fresh complete media 24 h. after transfection. The virus-containing supernatant was harvested 48 h. post transfection. The pLenti6/V5-LacZ virus was prepared by using similar methods. For the siRNA lentiviral vector we purchased the pRNAT-U6.1/Lenti-siRNA construct for P2X4 from GenScript (NJ, USA). The sequence of the P2X4 siRNA used was: AAGTGGGGCTCATGAAC . The construct was transfected into 293FT cells (Invitrogen) at the concentration of 1.2×10^6 /ml by using Lipofectamine2000 (Invitrogen) and ViraPower Lentiviral Packing Mix (Invitrogen). The medium was replaced with fresh complete medium 24 h. after transfection and virus-containing supernatant was harvested 48 h. post transfection. Lentivirus were stocked at -80°C until use. Rat hippocampus neurons (11 DIV) were exposed overnight to lentivirus and experiments were conducted 24 – 72 h. later. The efficiency of infection was $> 80\%$ as determined by GFP or V5 expression.

Cell Survival

Quantification of cell survival was performed in cells stained with the fluorescent DNA binding dye Hoescht 33342 using methods described previously (Haughey et al., 2002). Nuclei were visualized and photographed under epifluorescence illumination (340 nm excitation and 510 nm barrier filter) using a 40 X oil immersion objective. Two-hundred cells from 3 fields in 3 separate cultures per experimental condition were counted without knowledge of experimental condition. Cells in which nuclear staining was diffuse were considered viable and cells where nuclear staining was condensed or fragmented were considered “apoptotic”.

Measurements of Intracellular Calcium

Cytosolic calcium concentrations ($[\text{Ca}^{2+}]_c$) are determined by imaging of the fluorescent probe Fura-2/AM using methods described previously (Haughey et al., 2001a). Cells are incubated for 25 minute at 37°C in Locke's buffer (154 mM NaCl, 3.6 mM NaHCO_3 , 5.6 mM KCl, 1 mM MgCl_2 , 5 mM HEPES, 2.3 mM CaCl_2 , 10 mM glucose; pH 7.4) with 10 % pluronic and 2 μM Fura-2/AM. Cells are washed with Locke's buffer to remove extracellular Fura-2 and incubated at 37°C for 20 minute to allow for complete de-esterification of the probe. Cultures are transferred to the stage of a Zeiss Axiovert microscope coupled to a CCD camera and a Zeiss AttoFluor calcium imaging system and cellular fluorescence is imaged using a 40X oil objective. The average $[\text{Ca}^{2+}]_c$ in individual cell bodies is determined from the ratio of the

fluorescence emissions obtained using two different excitation wavelengths (340 nm and 380nm). The system is calibrated by curve fitting using reference standards containing 0 – 10 mM calcium (Molecular Probes, Oregon).

Caspase Activity Assay

Hippocampal neurons were treated with staurosporine (50 nM) or potassium cyanide (50 μ M) for 0, 2, 6, 12 and 24 h. before harvest. Cell pellets were stored at -80°C until use. Caspase activity was measured using the EnzChek® Caspase-3 Assay kit (Molecular Probes, Oregon) according to the manufactures instructions. This procedure uses a bisamide derivative of rhodamine that is covalently linked to DEVD peptides. When this complex is cleaved by DEVD-specific protease activity, the bisamide substrate is converted to a fluorescent product that is detected using 496 nm excitation and 520 nm emission filters.

Electrophysiological Recordings

Patch-clamp recordings were performed on GT1-7 cells transfected with GFP-P2X4wt for ~ 3 days. Coverslips with transfected cells were mounted in a 0.5 ml recording chamber and placed on the stage of an Olympus IX70 fluorescence microscope. Cells were continuously perfused with bath solution (24°C) flowing at 1 ml/min. The bath solution contained (in mM): 150 NaCl, 5 KCl, 2 MgCl_2 , 2 CaCl_2 , 10 glucose, 10 HEPES, pH 7.4, osmolarity adjusted to 320 mOsm with sucrose. P2X4-expressing cells were identified by GFP under the fluorescence microscope. Cells were voltage-clamped at -70 mV in whole-cell configuration. The recording electrode internal solution contained (in mM): 110 Cs_2SO_4 , 2 MgCl_2 , 0.5 CaCl_2 , 5 TEA-Cl, 5 EGTA, 5 HEPES, pH 7.3. The junction potential was -9 mV and not adjusted. Recording electrode resistance was ~ 5 M Ω and the access resistance below 25 M Ω . ATP was rapidly applied to cells through a glass tube (500 μm in diameter) positioned 1.0 mm away from the recorded cells. The gravity-driven solution flow was electronically controlled by solenoid valves and triggered from a computer. The drug-on time was less than 100 ms. Effects of caspase on ATP-evoked currents were tested by inclusion of 10 nM caspase 3 or caspase 4 in the internal solution of electrode. ATP was applied at two time points, 30 second and 10 min after whole-cell configuration. Controls were experiments using electrode without caspase. Recording signals were amplified with Axopatch 200B (Axon Instruments), filtered at 2 kHz, and sampled at 5 kHz using pCLAMP 8 (Axon Instruments).

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Figure 1.

P2X4 receptor levels are decreased in Alzheimer's disease and increased by A β prior to neuronal death. P2X4 protein levels were determined in three brain regions by Western blot in Alzheimer's patients with severe cognitive impairment and extensive Alzheimer's pathology ($n = 12$, MMSE score 3.5 ± 2 ; mean age 79 ± 11) and compared to patients with no signs of cognitive impairment or brain pathology ($n = 12$, MMSE score 26 ± 4 ; mean age 83 ± 12). P2X4 levels were decreased in the medial frontal gyrus (MFG) and the medial temporal gyrus (MTG) but not different in cerebellum (CBLM) of Alzheimer's patients compared with non-demented controls. ** = $p < 0.01$. ANOVA with Tukey post-hoc comparisons.

Figure 2.

Aβ increased P2X4 expression prior to inducing the death of neuronal cells. Rat hippocampal neurons were exposed to Aβ₁₋₄₂ (10 μM) for the indicated time points and P2X4 was detected by Western Blot and by immunofluorescent microscopy. **A.** Aβ increased neuronal death within 24 h. **B.** Prominent increases of P2X4 were observed in the cell body and neurites at 12 h. Quantification of immunofluorescent intensity was conducted in a total of 60 cells per condition derived from three separate experiments. **C.** Aβ-induced an increase in P2X4 levels within 6 h and there was the accumulation of a 13 kDa fragment. **D.** Quantitative analysis of Western blot results from three separate experiments. *p<0.05; ** p<0.01, *** p<0.001; ANOVA with Tukey post-hoc comparisons.

Figure 3.

Caspase 3 directly cleaves P2X₄. **A.** The C-terminal sequence of P2X₄ is shown with the putative caspase cleavage site indicated in bold and the antibody recognition site underlined. **B.** The broad-spectrum caspase inhibitor ZVADfmk (20 μ M) prevented A β (10 μ M) from inducing accumulation of the 13 kDa P2X₄ C-terminal. **C.** Quantitative analysis of Western blot results for full length and c-terminal fragment of P2X₄. **D.** P2X₄ was immobilized from rat neurons on sepharose column and exposed to caspase 3 or caspase 4 at the indicated concentrations for 4 h. Western blots showing that only small amounts of the C-terminal fragment were detected when caspase 3 or caspase 4 was incubated with P2X₄ at pH 7.4. When the pH of the buffer was decreased to 6.8, the c-terminal fragment of P2X₄ was present at all

concentrations of caspase 3 tested, but was only present in small amounts in columns treated with caspase 4.

Figure 4.

Aβ perturbed P2X4 trafficking. **A.** Aβ (10 μM) increased caspase 3 activity in rat hippocampal neurons within 6 h. Caspase activity assays was conducted in a triplicate of 8 wells. **B-C.** Neurons were pre-treated with vehicle or Aβ for 6 h then pulsed with ATP (100 μM) for the indicated time points and the amount of P2X4 that co-localized with the lysosomal marker Lamp-1 was quantified. In vehicle treated cultures the amount of P2X4 that co-localized with Lamp-1 increased after a 350 sec exposure to ATP and declined to levels that remained above baseline values after 1800 sec. In cultures pre-treated with Aβ, ATP did not increase the amount of P2X4 that co-localized with Lamp-1 at any time tested. Immunofluorescent pictures are examples of Lamp-1 (red) and P2X4 (green) taken 350 sec after ATP pulse. **D.** The caspase inhibitor ZVADfmk (20 μM) rescued ATP-evoked trafficking of P2X4 in cultures treated with Aβ. The time point 350 sec following ATP pulse is shown. Co-localization experiments were conducted in 30 cells per condition derived from three separate experiments. ## = $p < 0.01$ decrease compared with time 0. *** = $p < 0.001$ increase compared with time 0. ANOVA with Tukey post hoc comparisons.

Figure 5. Caspase 3 directly modifies the kinetics of P2X4 receptor function

A. In primary neurons treated with $A\beta$ (10 μ M for 6 h), peak ATP-evoked responses were similar to vehicle but the slope of decay in ATP-evoked (100 μ M) calcium transients was slower. **B.** Quantification of the slope of decay in ATP-evoked calcium transients showing a greater slope of decay in cultures treated with $A\beta$ compared with vehicle treated cultures. Pre-incubation of cultures with the caspase inhibitor ZVADfmk (20 μ M) prevented staurosporine-induced slowing of the decay rates in ATP-evoked calcium transients. Calcium imaging was quantified in a total of 44 - 59 cells per condition derived from 3 — 5 separate experiments. **C.** In GT1-7 cells transfected with P2X4, ATP (100 μ M) evoked a large inward current with similar amplitude and channel closure times immediately after patch and following a 10 min delay. When caspase 3 (50 nM) was included in the patch pipette, the initial ATP evoked current was similar to those produced under control conditions. After a 10 min delay, ATP-evoked current amplitude and channel closure time were increase two-fold. Electrophysiological experiments are representative of three experiments with results summarized in the bar figure. * = $p < 0.05$. ** = $p < 0.01$. ANOVA with Tukey post hoc comparisons.

Figure 6.

Adjustment of P2X4 expression modifies neuronal susceptibility to A β -induced death. P2X4 was expressed in rat hippocampal neurons using a lentiviral delivery system **A**. 100X magnification of hippocampal neurites stained with the neuronal marker Map-2 (red) showing P2X4 (green) in untreated cells (NT), in cells expressing LacZ and in cells expressing P2X4. **B**. Western blot showing P2X4 levels at baseline and after a 1, 2 and 3 day transfection with Lac Z or P2X4. **C**. Neurons that over express P2X4 for 24 h show enhanced peak increase of cytosolic calcium in response to ATP (100 μ M). **D**. Neurons over expressing P2X4 are more sensitive to A β -induced (5 μ M) death. **E**. 100 X magnification of a rat hippocampal neurite (red) showing P2X4 immunoreactivity in untreated neurons (NT) and neurons transfected with a lentiviral vector expressing an SiRNA to P2X4. (P2X4 SiRNA). **F**. Western blot showing P2X4 expression levels at baseline and a reduction in P2X4 levels after a 24 and 48 h transfection with P2X4 SiRNA. **G**. Neurons transfected with P2X4 SiRNA for 24 h show decreases in the peak amplitude of ATP-evoked calcium transients (100 μ M). **H**. Decreasing P2X4 expression protected neurons from the A β -induced (5 μ M) death. Western blots are representative of 3 experiments. Calcium imaging was quantified in 36-44 cells per condition. Neuronal death was quantified by nuclear morphology in a minimum of 600 cells per condition derived from three separate experiments. *** $p < 0.001$ compared with NT or LacZ. # = $p < 0.05$, ## = $p < 0.01$ compared with A β or LacZ + A β ; ANOVA with Tukey post-hoc comparison.