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# Purinergic Receptor-Stimulated $IP_3$-Mediated $Ca^{2+}$ Release Enhances Neuroprotection by Increasing Astrocyte Mitochondrial Metabolism during Aging

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**Astrocytes play an essential role in the maintenance and protection of the brain, which we reported was diminished with age. Here, we demonstrate that activation of a purinergic receptor (P2Y-R) signaling pathway, in astrocytes, significantly increases the resistance of astrocytes and neurons to oxidative stress. Interestingly, P2Y-R activation in old astrocytes increased their resistance to oxidative stress to levels that were comparable with stimulated young astrocytes. P2Y-R enhanced neuroprotection was blocked by oligomycin and by Xestospongin C, inhibitors of the ATP synthase and of inositol (1,4,5) triphosphate ($IP_3$) binding to the $IP_3$ receptor, respectively. Treatment of astrocytes with a membrane permeant analog of $IP_3$ also protected astrocytes against oxidative stress. These data indicate that P2Y-R enhanced astrocyte neuroprotection is mediated by a $Ca^{2+}$-dependent increase in mitochondrial metabolism. These data also reveal a signaling pathway that can rapidly respond to central energy needs throughout the aging process.**

*Key words:* **intracellular $Ca^{2+}$; mitochondria; $IP_3$; P2Y-R; two-photon; metabolism**

## Introduction

The classical role of astrocytes is to protect and support neuronal function. They express receptors for a variety of neurotransmitters that are released from either neurons or glial cells (Araque et al., 2001). ATP appears to be the predominant extracellular signaling molecule in astrocytes (Verkhratsky and Kettenmann, 1996; Verkhratsky et al., 1998; James and Butt, 2002). Both metabotropic (P2Y) and ionotropic purinoreceptors (P2X) are expressed in astrocytes, and activation of either receptor subfamily results in an increase in intracellular $Ca^{2+}$ (Verkhratsky and Kettenmann, 1996; James and Butt, 2002). The metabotropic inositol triphosphate ($IP_3$) signaling pathway provides a mechanism whereby local extracellular signals can be rapidly transduced into increased intracellular ATP. G-protein-linked receptors increase the production of $IP_3$, triggering release of $Ca^{2+}$ from thapsigargin-sensitive stores in the endoplasmic reticulum. $IP_3$-mediated $Ca^{2+}$ release can, in turn, increase mitochondrial $Ca^{2+}$ and, consequently, increase respiration and ATP production (Denton and McCormack, 1985; McCormack et al., 1990; Hajnoczky et al., 1995, 2000). The production of intracellular ATP via $Ca^{2+}$-induced activation of matrix dehydrogenases is very rapid, occurring at levels 10-fold faster than stimulation by feedback from ATP/ADP pools (Territo et al., 2000). Although mitochondrial $Ca^{2+}$ uptake can increase ATP production, increased matrix $Ca^{2+}$ release also sensitizes cells to apoptotic stimuli and induces the opening of the mitochondrial permeability transition pore (MPT) (Bernardi et al., 1992; Petronilli et al., 1993; Zoratti and Szabo, 1995; Byrne et al., 1999; Szalai et al., 1999). Recent reports indicate that $Ca^{2+}$ sensitivity can be attributed to cyclophilin D, a prolyl isomerase located within the mitochondrial matrix, which plays a critical role in MPT opening (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005). Thus, a purinergic receptor-mediated increase in intracellular $Ca^{2+}$ release can signal either cell survival or cell death for an astrocyte.

The importance of $Ca^{2+}$ signaling in aged astrocytes remains mostly unexplored (Landfield and Pitler, 1984; Khachaturian, 1994; Disterhoft et al., 1996; Cotrina and Nedergaard, 2002). Deficiencies in cytosolic $Ca^{2+}$ buffering of neurons have been implicated in the pathogenesis of seizures (Sloviter, 1989), epilepsy (Miller and Baimbridge, 1983), and neurodegenerative diseases (Heizmann and Braun, 1992; Sutherland et al., 1993). We recently reported that purinergic receptor-mediated increases in intracellular $Ca^{2+}$ were significantly changed during the aging process (Lin et al., 2007). Several investigators have also reported that mitochondrial $Ca^{2+}$ levels decrease with age (Leslie et al., 1985; Peterson et al., 1985; Vitorica and Satrustegui, 1986a,b). In this study, we focused our investigation on the potential role of purinergic receptor $Ca^{2+}$ signaling on astrocyte survival. We show that a brief exposure of astrocytes to extracellular ATP significantly increases the ability of astrocytes, and neurons in co-

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culture, to resist oxidative stress. This protective effect is stimulated by $IP_3$-mediated $Ca^{2+}$ signaling, is dependent on intracellular mitochondrial ATP production, and is significantly stronger in old astrocytes when compared with nonstimulated young astrocytes. These studies identify astrocyte mitochondria as an important energy source that can be rapidly activated by metabotropic purinergic receptors.

## Materials and Methods

*Isolation, culture, and growth of astrocytes and neurons.* Primary cultures of astrocytes were prepared as described previously (Lin et al., 2007). C57Bl/6 male mice were purchased from the National Institute of Aging and maintained in standard accredited housing conditions at The University of Texas Health Science Center Animal Facility. Animal protocols were approved by our Institutional Animal Care Use Committee. Astrocytes were cultured in DMEM F-12 media (#11039–021; Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine, nonessential amino acids, 10% fetal calf serum, 100 U/ml penicillin/streptomycin (#17–602E; Cambrex, Walkersville, MD), and 1% fungizone (#15290–018; Invitrogen) at 37°C in an atmosphere containing 5% $CO_2$. The culture medium was changed every 3–5 d. Confluent cells were gently removed by a trypsin-EDTA solution and replated on 35 mm glass bottom chambers (#04200415C; Bioptechs, Butler, PA) before imaging experiments 3–4 d later. Cells were maintained in plastic culture flasks until the final reseeding of cells onto a glass bottom chamber and were passed up to three times for astrocyte-only experiments. For coculture experiments with neurons, primary astrocytes were plated directly onto corning transwell-clear permeable supports, which physically separates the two cell types by ~1 mm (#07-200-170; Fisher Scientific, Houston, TX). Astrocytes were cultured in transwells until they were ~70% confluent. Mouse cortical neurons (#M-CX-300; Cambrex) were then cultured onto six-well mat-tek glass bottom plates and maintained in Neurobasal Medium (#21103-049; Invitrogen), 2 mM L-glutamine (#17-605E; Cambrex), 100 U/ml penicillin/streptomycin (#17-602E; Cambrex), and B-27 with (#17504-044; Invitrogen) and without antioxidants (#10889-038; Invitrogen), which controls glia contamination to <0.5%. The transwell supports with cultured astrocytes were placed in the neuron-seeded glass bottom chambers for 4 d before imaging, and both cell types were then maintained in neuronal media. For neuron-only experiments, the astrocytes on the transwell supports were removed before treatments and imaging.

*$O_2$ consumption assay.* $O_2$ consumption was monitored with a Clark electrode (Mitocell S200 micro respirometry system; Strathkelvin Instruments, Motherwell, UK). Astrocytes (500 μl at $10^5$ cells/ml) in buffer (120 mM NaCl, 45 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4) were loaded into a 500 μl MT200A Respirometer Chamber, suspended by a fixed-speed solid-state magnetic stirrer inside the chamber, and maintained at 37°C by a circulating water bath. $O_2$ was monitored for 4 min before injecting 5 μl of ATP (200 μM) into the chamber. $O_2$ consumption was then monitored for another 4 min. Changes in $O_2$ levels were calculated by respirometry software. For control recordings, 5 μl of buffer was injected into chamber. Oligomycin astrocytes were treated with oligomycin (0.01 μM) for 30 min before trypsinization and loading into chamber.

*Intracellular ATP determination.* Intracellular ATP was measured using a luciferin-luciferase kit (#22066; Invitrogen). Plated astrocytes were trypsinized and washed three times with PBS buffer (#14190; Invitrogen). An aliquot of cells was set aside to measure the cell density with a hemocytometer. The cells were then suspended in a lysate buffer (4 mM EDTA and 100 mM Tris, pH 7.75) and immediately heated to 100°C for 5 min to minimize enzymatic changes in ATP levels. The supernatant was collected after a 10,000 × *g* centrifugation for 1 min and used with the luciferin–luciferase kit according to the manufacturer instructions. Luminescence was measured with a Synergy HT Multi-Detection Microplate Reader (BioTek, Burlingame, CA). ATP levels were normalized by cell density.

*Imaging acquisition and analysis.* Mitochondrial membrane potentials ($\Delta\Psi$s) were measured as described previously using the potential-sensitive dye tetra-methyl rhodamine ethyl ester (TMRE, #T-669; Invitrogen, Eugene, OR) (Lin and Lechleiter, 2002). Images were acquired with a 60× oil 1.4 numerical aperture objective on a Nikon (Tokyo, Japan) PCM2000 confocal microscope custom adapted for two-photon imaging. A Ti-sapphire Coherent Mira 900 Laser pumped with a 5W Verdi laser (Coherent, Santa Clara, CA) was used to excite TMRE at 800 nm. A neutral-density filter wheel was used to attenuate the laser intensity so that no detectable photobleaching of TMRE was observed.

Sensitivity of neurons to oxidative stress was assessed by treating cultures with 100 μM tert-butyl hydrogen peroxide (t-BuOOH; #B-2633; Sigma, St. Louis, MO) for 4 h. Neurons were then stained with Hoechst 33342 (10 μg/ml, #H-3570; Invitrogen) to label all cell nuclei and calcein AM (2 μM, #C3100; #C3100; Invitrogen). Neuronal death was quantified by counting the number of nuclei that did not colocalize with calcein-stained cells. Calcein fluorescence is only observed in live cells that have maintained their plasma membrane integrity. Images were acquired on an inverted Nikon TE300 microscope with a Hamamatsu (Bridgewater, NJ) ORCAER camera and Open lab software (Improvision, Lexington, MA).

$Ca^{2+}$ activity was imaged as described previously (Camacho and Lechleiter, 1995). In brief, cultured astrocytes were incubated with the fura-2 AM (#F-1221; Invitrogen) 30 min before the experiment. Images were acquired with two-photon excitation (800 nm) at the rate of 1.5 images/s. At this wavelength, only the $Ca^{2+}$-free form of fura-2 is significantly excited. Hence, a $Ca^{2+}$ increase is observed as a decrease in fluorescence. Images were analyzed with Image J (http://rsb.info.nih.gov/ij/) and ANALYZE software (The Mayo Foundation, Rochester, MN). The D-enantiomer of the $IP_3$-BM ester was synthesized by Stuart Conway in the Cambridge University Chemical Laboratory by using a modification of the protocol of Tsien and coworkers (Li et al., 1997).

One-way ANOVA or a paired *t* test was used for statistical analysis. Differences with a $p$ value $<0.05$ were considered statistically significant.

## Results

### Extracellular ATP protects astrocytes from oxidative stress

The presence of multiple purinergic receptor isoforms on astrocytes suggested to us that an increase in extracellular ATP could affect the sensitivity of astrocytes to oxidative stress during the aging process. To investigate this, primary cultures of astrocytes were prepared from the brains of young (4–6 months of age) and old (26–28 months of age) mice and replated on glass coverslips before each imaging experiment as described previously (Lin et al., 2007). Seeded glass coverslips were then sealed in an open chamber (Bioptechs Delta T) and perfused at 37°C with the potential sensitive dye tetra-methyl rhodamine ethyl ester (TMRE; 200 nM; Invitrogen) to label astrocyte mitochondria. Relative changes in mitochondrial membrane potentials ($\Delta\Psi$s) were measured every 5 min using two-photon microscopy (800 nm excitation, z-stack of six images in 1 μm steps). Oxidative stress was applied to the cells in the perfusate with t-BuOOH (100 μM). Cell viability was then monitored by the collapse of $\Delta\Psi$ (TMRE fluorescence) to 10% of its initial value. This value was chosen to ensure the potential indicator remained above the lower limit of fluorescence at de-energization, which has been attributed to TMRE partitioning into the membrane phase (Fink et al., 1998). Images of TMRE-labeled mitochondria from untreated young and old astrocytes are presented in Figures 1*a* and 2*a* at the indicated time points during oxidative stress. The time course of $\Delta\Psi$ collapse is plotted for three astrocytes in each culture (Figs. 1*b*, 2*b*). As reported previously (Lin et al., 2007), the time until $\Delta\Psi$ collapse was significantly faster for old astrocytes (3.62 ± 0.12 h; $n = 75$ cells; pooled from four experiments) compared with young astrocytes (4.87 ± 0.48 h; $n = 15$ cells; pooled from two experiments) (Figs. 1*e*, 2*e*). To test the impact of purinergic receptor activation, astrocyte cultures were initially exposed to extracellular ATP (10 μM) for 10 min before stressing the cells with

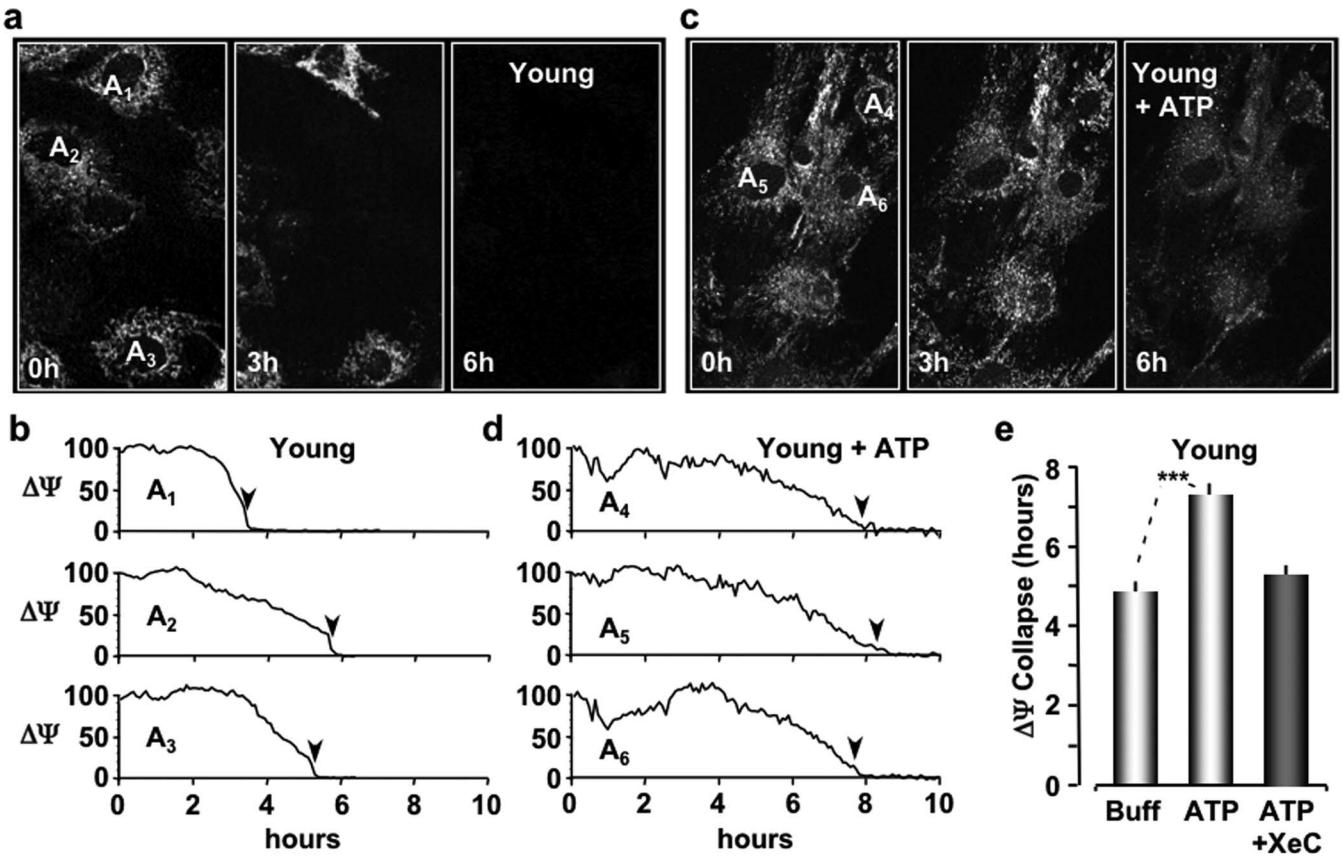


**Figure 1.** P2Y-R activation protects young astrocytes from oxidative stress. ***a***, Images of astrocytes cultured from young mice labeled with the mitochondrial potential-sensitive dye TMRE and exposed to oxidative stress for the indicated times. Each image is a maximum intensity projection of a z-stack of six optical sections (1 $\mu$m steps). Plated cells were continuously perfused at 37°C with culture medium containing TMRE (200 nM) and imaged with two-photon microscopy (800 nm). ***b***, Line plots of the mean TMRE fluorescence ($\Delta\Psi$) for the cells labeled in ***a***. Fluorescent units are scaled to 100% at 0 h. The times when the TMRE fluorescence collapses to 10% of the initial values are indicated by black arrowheads. ***c***, Images of young astrocytes pre-exposed to extracellular ATP (10 $\mu$M) for 10 min before adding t-BuOOH (100 $\mu$M) to the perfusate (0 h). ***d***, Line plots of the TMRE fluorescence for the cells indicated in ***c***. ***e***, Histograms of the mean times of $\Delta\Psi$ collapse for young astrocytes exposed to buffer only (Buff), ATP, or ATP plus Xestospongin C. ***$p < 0.001$.

t-BuOOH (100 $\mu$M). The perfusate again contained TMRE (200 nM) to monitor $\Delta\Psi$ every 5 min. Under these experimental conditions, we found that the average time for $\Delta\Psi$ to collapse was significantly increased to 7.35 ± 0.23 h ($n = 27$ cells pooled from four experiments; $p < 0.001$) in astrocytes from young mice (Fig. 1*c–e*). Similarly, a 10 min application of extracellular ATP to astrocytes cultured from old mice significantly increased the average time until $\Delta\Psi$ collapse to 6.51 ± 0.18 h ($n = 24$ cells pooled from five experiments; $p < 0.001$) (Fig. 2*c–e*). The collapse time for ATP-treated old astrocytes was comparable with treated young astrocytes during oxidative stress (Figs. 1*e*, 2*e*). We concluded from these data that a brief application of extracellular ATP to astrocytes established a prolonged period of protection against oxidative stress in astrocytes cultured from both young and old mice.

### The protective effect of ATP is blocked by pretreatment with Xestospongin C

The primary response of glial cells to extracellular ATP is an increase in intracellular $Ca^{2+}$ (Verkhratsky and Kettenmann, 1996; James and Butt, 2002). To investigate whether the protective effect of ATP could be initiated by a $Ca^{2+}$ response, we loaded cultures of astrocytes with the $Ca^{2+}$ indicator dye fura-2 AM (10 $\mu$M for 30 min). Fura-2 fluorescence was imaged with two-photon microscopy using 800 nm excitation (Fig. 3*a*). At this wavelength, fluorescence is primarily caused by the $Ca^{2+}$ free form of fura-2. Hence, we expressed increases in $Ca^{2+}$ as decreases in fura-2 fluorescence ($\Delta F$) normalized by the resting fura-2 fluorescence ($F_{rest}$). When young astrocyte cultures were exposed to extracellular ATP (10 $\mu$M for 10 min), the peak intracellular $Ca^{2+}$ ($-\Delta F/F$) was increased to 0.44 ± 0.02 ($n = 39$; pooled from four experiments). A similar increase in the peak $Ca^{2+}$ amplitude was observed for astrocytes cultured from old mice ($-\Delta F/F = 0.39 \pm 0.03$; $n = 21$; pooled from four experiments). A detailed analysis of the changes that occur in ATP-mediated intracellular $Ca^{2+}$ signaling in astrocytes during the aging process has been reported (Lin et al., 2007). Here, we tested whether the ATP-induced $Ca^{2+}$ response could be attributed to the metabotropic pathway, because $IP_3$-mediated $Ca^{2+}$ release can stimulate mitochondrial energy production as well as sensitize mitochondria to apoptotic stimuli (Hajnoczky et al., 1995, 2000; Szalai et al., 1999). Astrocytes cultured from old mice were pretreated with Xestospongin C (XeC) (25 $\mu$M for 30 min), which is a competitive inhibitor of $IP_3$ binding to the $IP_3$ receptor ($IP_3R$) (Gafni et al., 1997). When XeC-treated cultures were exposed to extracellular ATP (10 $\mu$M), the peak $Ca^{2+}$ response was significantly reduced to 0.21 ± 0.03 ($n = 30$; pooled from four experiments). These data suggested that at least part of the ATP-induced $Ca^{2+}$ response can be attributed to metabotropic P2Y receptor activation. This is consistent with other reports showing that activation of the ionotropic P2X receptor also increases $Ca^{2+}$ in cultured astrocytes (Verkhratsky and Kettenmann, 1996;

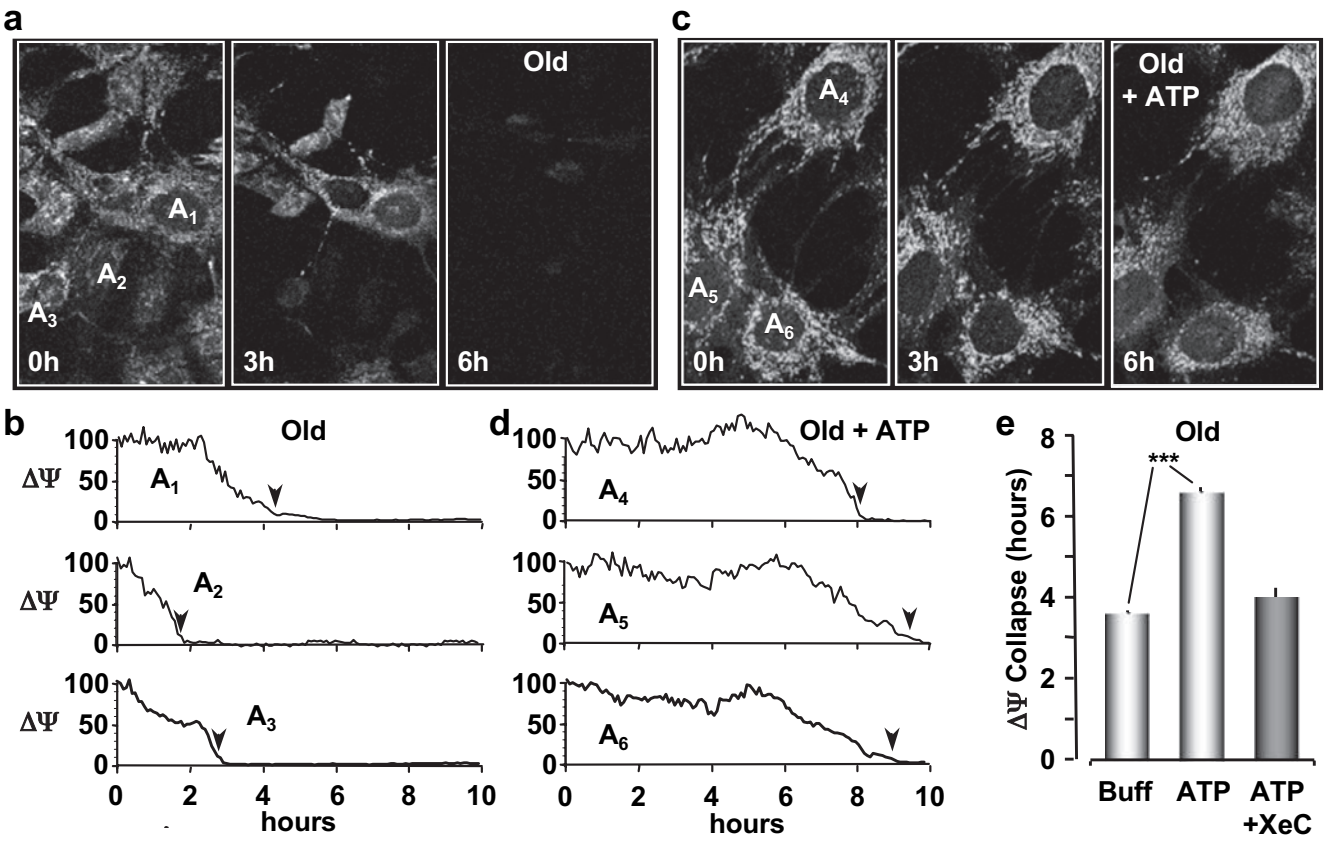


**Figure 2.** Old astrocytes are also protected from oxidative stress by pretreatment with extracellular ATP. ***a***, Images of astrocytes cultured from old mice with TMRE-labeled mitochondria under oxidative stress for the indicated times. Image acquisition parameters are the same as described in Figure 1. Intensity line plots of the mean TMRE fluorescence ($\Delta\Psi$) are shown in ***b***. ***c***, Images of old astrocytes treated with extracellular ATP (10 $\mu$M) for 10 min before oxidative stress (t-BuOOH; 100 $\mu$M). Intensity line plots of TMRE fluorescence are shown in ***d***. Histograms of the mean times of $\Delta\Psi$ collapse for old astrocytes exposed to buffer only (Buff), ATP, or ATP plus Xestospongin C are presented in ***e***. $^{***}p < 0.001$.

James and Butt, 2002). To determine whether the metabotropic pathway is involved with the protective effect of extracellular ATP, we subsequently exposed the XeC/ATP-treated astrocytes to oxidative stress. As described above, the perfusate contained t-BuOOH (100 $\mu$M) and TMRE (200 nM) to monitor $\Delta\Psi$. Under these experimental conditions, the average time until $\Delta\Psi$ collapse was reduced to 5.26 ± 0.25 h ($n = 26$; pooled from four experiments) in young astrocytes (Fig. 1*e*) and to 3.97 ± 0.17 h ($n = 30$; pooled from four experiments) in old astrocytes (Fig. 2*e*). These values were not significantly different from untreated astrocytes. We concluded that the protective effect of ATP on astrocytes was mediated by P2Y receptors (P2Y-Rs).

### $IP_3$ butyryloxymethyl ester treatment protects astrocytes from oxidative stress

To directly investigate the role of metabotropic $IP_3/Ca^{2+}$ signaling pathway in the protective effect of P2Y-R activation, we examined the $Ca^{2+}$ response of astrocytes to butyryloxymethyl ester of $IP_3$ ($IP_3$-BM), a membrane permeant analog of $IP_3$ (Li et al., 1997). Cultured astrocytes were loaded with fura-2 and imaged with two-photon microscopy as described above. When cells were treated with $IP_3$-BM (25 $\mu$M), an increase in intracellular $Ca^{2+}$ was observed in astrocytes cultured from both young and old mice (Fig. 3*a–c*). The peak $Ca^{2+}$ response was 0.30 ± 0.02 ($n = 16$; pooled from five experiments) for young astrocytes and 0.24 ± 0.02 ($n = 24$; pooled from four experiments) for old astrocytes. Overall, the $Ca^{2+}$ responses of astrocytes treated with $IP_3$-BM were slower than the comparable $Ca^{2+}$ responses for ATP treatment. This was expected because of the amount of time required to accumulate and activate $IP_3$-BM within cells. After 15 min of $IP_3$-BM treatment, cultured cells were perfused with t-BuOOH, and $\Delta\Psi$ was monitored with TMRE fluorescence. We found that the time until $\Delta\Psi$ collapse was significantly increased to 6.48 ± 0.34 h ($n = 26$; pooled from four experiments) in young astrocytes and to 5.47 ± 0.13 h ($n = 33$; pooled from four experiments) in old astrocytes (Fig. 3*d*,*e*). Again, the time until $\Delta\Psi$ collapse in $IP_3$-BM-treated old astrocytes was comparable with the time until $\Delta\Psi$ collapse in treated cultures of young astrocytes. Pretreatment of young and old astrocytes with XeC (25 $\mu$M for 30 min) significantly reduced their $Ca^{2+}$ responses to 0.12 ± 0.01 ($n = 28$; pooled from four experiments) and 0.10 ± 0.01 ($n = 27$; pooled from three experiments) (Fig. 3*c*). Furthermore, XeC completely inhibited the protective effect of $IP_3$-BM in young and old astrocytes. The time until $\Delta\Psi$ collapse was reduced to 4.13 ± 0.22 h ($n = 20$; pooled from three experiments) in young astrocytes and to 3.69 ± 0.29 h ($n = 24$; pooled from three experiments) in old astrocytes (Fig. 3*e*). Neither of these values were significantly different from those observed in untreated cells. We concluded from these data that $IP_3$-mediated intracellular $Ca^{2+}$ release protects astrocytes from oxidative stress.

### Oligomycin blocks the protective effect of P2Y-R activation against oxidative stress

A potential mechanism by which $IP_3$-mediated intracellular $Ca^{2+}$ release could provide protection to a cell is by increasing intracellular production of ATP via a $Ca^{2+}$-mediated increase in

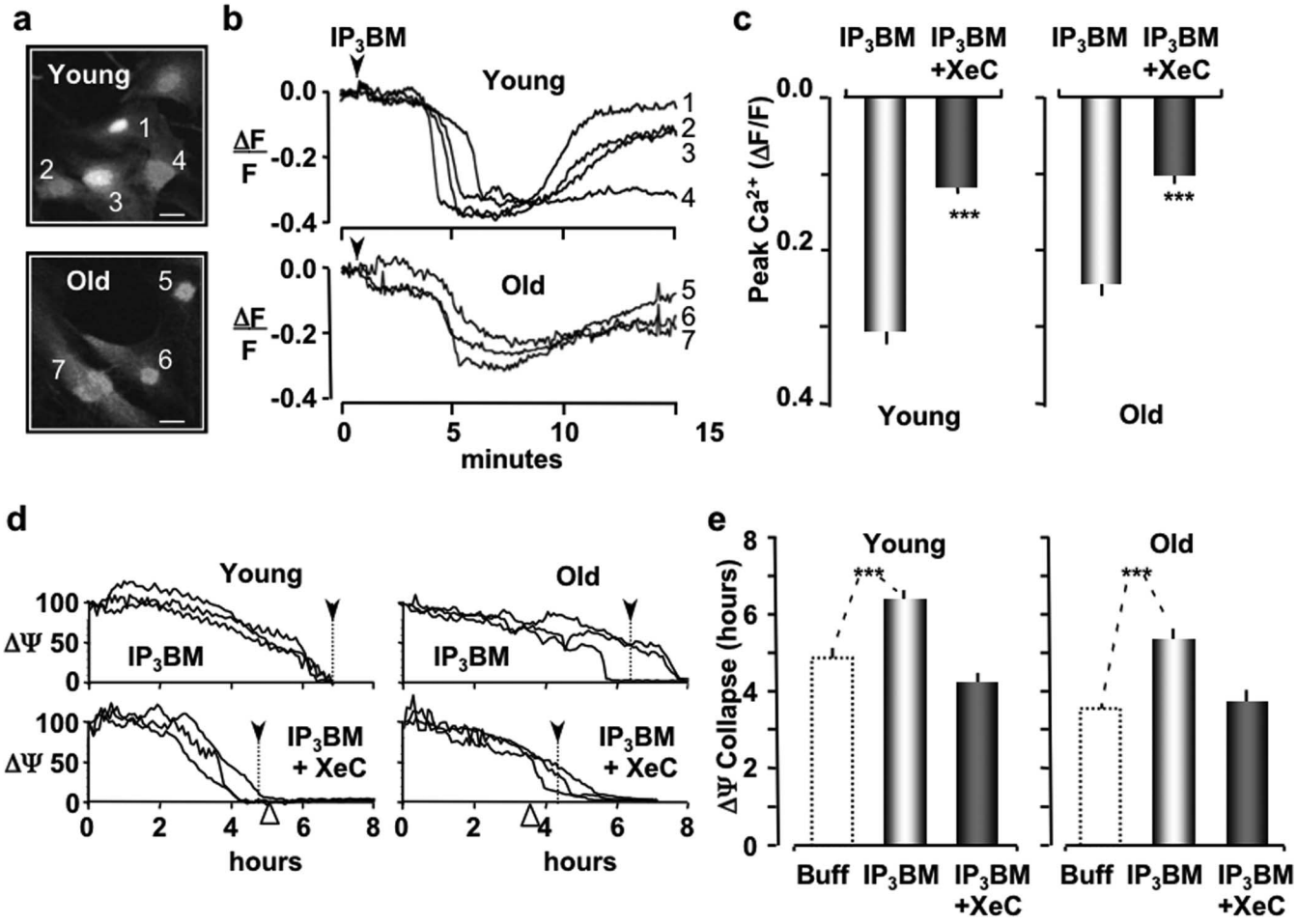


**Figure 3.** $IP_3$-mediated intracellular $Ca^{2+}$ release protects young and old astrocytes from oxidative stress. ***a***, Images of cultured young and old astrocytes loaded with fura-2 and imaged with two-photon microscopy at 800 nm excitation. ***b***, $IP_3$-BM (25 $\mu$M; black arrowhead) increases intracellular $Ca^{2+}$ in both young and old astrocytes. $Ca^{2+}$ increases for the indicated cell numbers are observed as decreases in signal intensity, because the $Ca^{2+}$ free form of fura-2 is preferentially excited at 800 nm. ***c***, Histogram plots of the peak $Ca^{2+}$ response for young and old astrocytes. $Ca^{2+}$ responses were significantly inhibited by pretreatment with XeC (gray bars). ***d***, Intensity line plots of the mean TMRE fluorescence ($\Delta\Psi$) for three representative astrocytes pretreated with $IP_3$-BM (25 $\mu$M; 20 min) and subsequently exposed to oxidative stress (t-BuOOH; 100 $\mu$M). $\Delta\Psi$ traces from cells that were initially exposed to XeC (25 $\mu$M; 1 h) before $IP_3$-BM treatment are presented in the bottom plots. The mean $\Delta\Psi$ collapse time for each plot is indicated by the vertical dotted lines and black arrowheads. The white arrowheads indicate the mean $\Delta\Psi$ collapse for buffer alone. ***e***, Histogram plots of the mean times until $\Delta\Psi$ collapse for astrocytes exposed to $IP_3$-BM or $IP_3$-BM plus XeC. Dotted line histogram bars are presented as control (Buff only) references for the data shown in Figures 1 and 2. ***$p < 0.001$.

mitochondrial respiration (Hansford, 1985; McCormack et al., 1990; Hajnoczky et al., 1995, 2000; Szalai et al., 1999). To initially test this mechanism of action, we pretreated cultured astrocytes with oligomycin (1 $\mu$g/ml for 30 min), a specific inhibitor of the mitochondrial ATP synthetase (Merck Index 13, 6902). We found that the time until $\Delta\Psi$ collapse was 4.64 ± 0.21 h ($n = 27$; pooled from three experiments) in young astrocytes and 4.05 ± 0.19 h ($n = 30$; pooled from three experiments) in old astrocytes exposed oxidative stress (t-BuOOH; 100 $\mu$M) (Fig. 4). These times until $\Delta\Psi$ collapse were comparable with control values of untreated astrocytes, suggesting the acute pretreatment of astrocytes with oligomycin did not affect their resistance to oxidative stress. However, when oligomycin-treated astrocytes were exposed to extracellular ATP for 10 min, we found that P2Y-R activation no longer enhanced their resistance to oxidative stress. ATP treatment in old astrocytes made them just as resistant to oxidative injury as young astrocytes. The mean time until $\Delta\Psi$ collapse was 4.69 ± 0.23 h ($n = 34$; pooled from three experiments) in young astrocytes and 2.97 ± 0.17 h ($n = 8$; pooled from three experiments) in old astrocytes (Fig. 4). Together, these data suggest that astrocyte mitochondrial ATP production is required for the protective effect of P2Y-R activation against oxidative stress.

### P2Y-R activation in astrocytes stimulates $O_2$ consumption and intracellular ATP production

To further investigate the importance of mitochondrial metabolism on the protective effect of P2Y-R activation, we first measured the rate of $O_2$ consumption for astrocytes with and without P2Y-R activation. Primary cultures of astrocytes were grown to ~70% confluency, gently removed by trypsin EDTA, and resuspended in PBS buffer. Astrocytes ($10^5$ cells/ml) were then loaded into a 500 $\mu$l Respirometer Chamber (MT200A) and maintained at 37°C by a circulating water bath. We found that the basal rate of $O_2$ consumption in astrocytes was 2.45 ± 0.24 nmol/min ($n = 3$) (Fig. 5*a*,*b*). This rate increased significantly ($p < 0.01$) to 3.67 ± 0.3 nmol/min ($n = 3$) when astrocytes were stimulated by a bolus of ATP (2 $\mu$M final concentration). The rate of $O_2$ consumption was significantly decreased ($p < 0.01$) to 0.97 ± 0.21 nmol/min ($n = 4$) after exposing the astrocytes to the ATP synthetase inhibitor oligomycin (0.01 $\mu$M; 30 min). Oligomycin treatment also

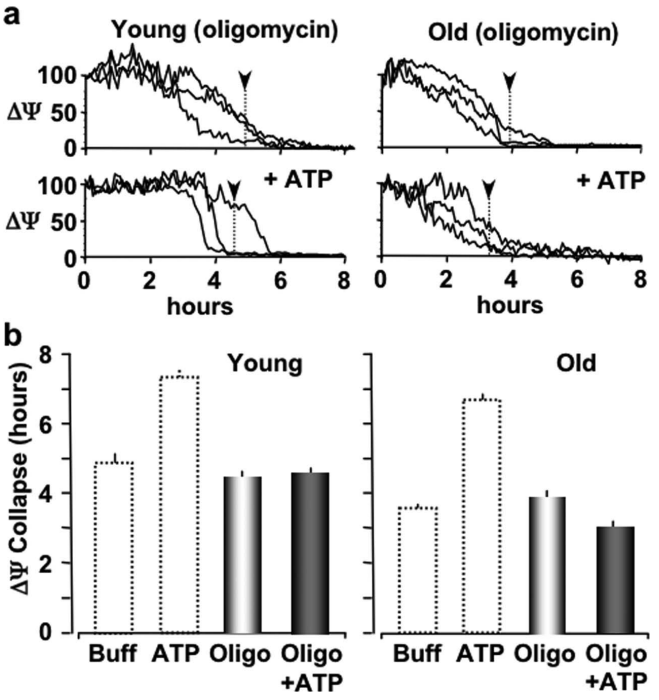


**Figure 4.** Oligomycin treatment of young or old astrocytes blocks their P2Y-R-enhanced resistance to oxidative stress. ***a***, Intensity line plots of the mean TMRE fluorescence ($\Delta\Psi$) for representative astrocytes pretreated with oligomycin (1 $\mu$g/ml; 30 min) and subsequently exposed to oxidative stress (t-BuOOH; 100 $\mu$M) with (top traces) and without P2Y-R stimulation (10 $\mu$M ATP; 10 min). Vertical dotted lines with black arrowheads indicate the mean $\Delta\Psi$ collapse times. ***b***, Histograms of the mean $\Delta\Psi$ collapse times during oxidative stress (t-BuOOH; 100 $\mu$M). Dotted line histogram bars are for reference showing control and ATP-enhanced responses from Figures 1 and 2.

completely inhibited the effects of extracellular ATP on the rate of $O_2$ consumption (1.19 ± 0.27 nmol/min; $n = 3$).

Our next approach to investigate the role of mitochondrial metabolism was to directly measure ATP levels in cultured astrocytes using a luciferin-luciferase assay (Invitrogen). Astrocytes were cultured as described above, trypsinized, and washed with buffer. The cells were then suspended in a lysate buffer and immediately heated to 100°C (5 min) to minimize enzymatic changes in ATP levels. After centrifugation, the ATP-dependent luminescence of the supernatant was measured with a microplate reader (BioTek). We estimated that the resting intracellular ATP concentration in cultured astrocytes was 0.31 ± 0.14 $\mu$M ATP/$10^4$ cells ($n = 3$). Treatment of cultured astrocytes with the $P2Y_1$-R specific ligand 2-MeSADP (2 $\mu$M) for 10 min increased ATP levels to 0.39 ± 0.16 $\mu$M ATP/$10^4$ cells. This increase was statistically significant when the data were expressed as a percentage of the control ATP levels for each experiment ($p < 0.04$; paired Student's $t$ test) (Fig. 5*c*). Oligomycin (0.01 $\mu$M; 30 min) significantly decreased the resting levels of ATP to 82.6 ± 3.2% of control ($p < 0.05$; $n = 3$). The same oligomycin pretreatment also completely inhibited $P2Y_1$-R-mediated increases in ATP (Fig. 5*c*). Finally, we tested whether ruthenium 360 ($Ru_{360}$; Calbiochem, La Jolla, CA), a polycation that inhibits the electrogenic mitochondrial $Ca^{2+}$ uniporter (Ying et al., 1991), affected mitochondrial ATP production. We found that $Ru_{360}$ treatment (1 $\mu$M; 30 min) significantly decreased the basal ATP levels to 60.5 ± 10.5% ($n = 3$) of control values and also completely blocked the ability of $P2Y_1$-R specific ligand, 2-MeSADP, to increase ATP levels (Fig. 5*c*). Together, these data strongly suggest that P2Y-R stimulation leads to an $IP_3$-mediated intracellular $Ca^{2+}$ release

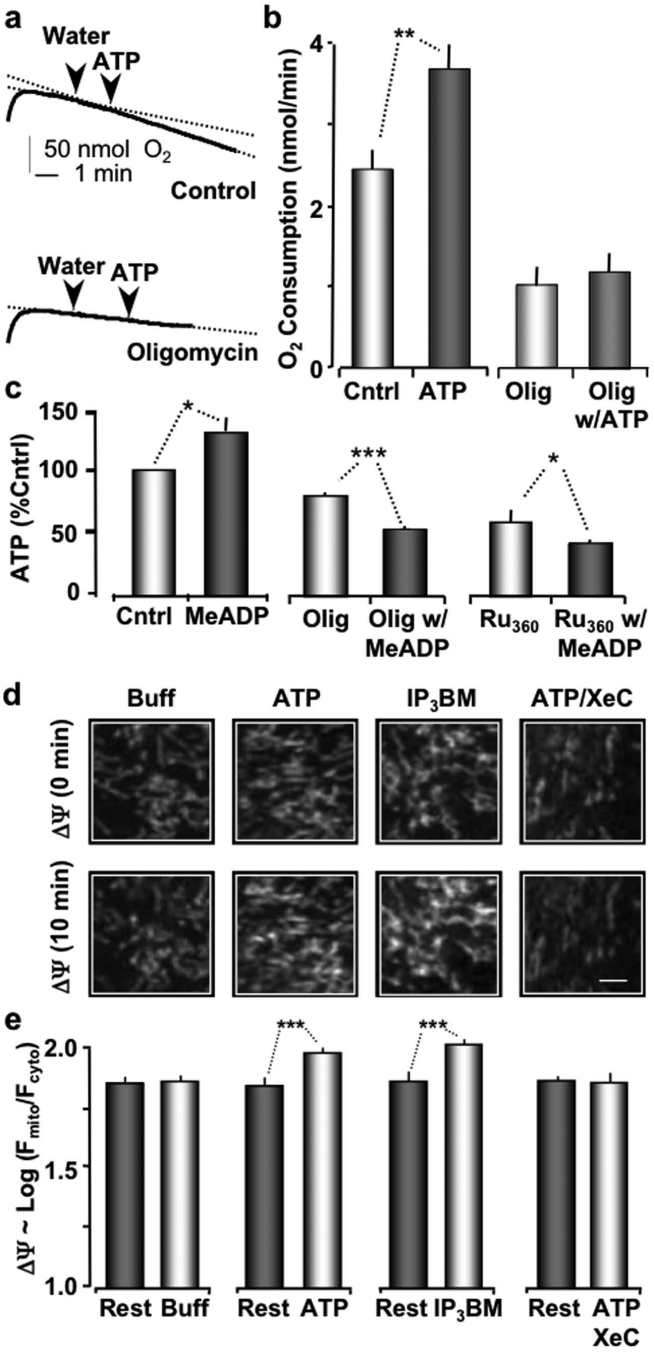


**Figure 5.** Stimulation of P2Y-Rs increases $O_2$ consumption, ATP production, and increases $\Delta\Psi$. ***a***, Plots of $O_2$ levels in suspended astrocytes as labeled. ***b***, Histogram shows the average change in $O_2$ consumption with 2 $\mu$M ATP compared with untreated cells (Cntrl). Bars on the right show $O_2$ consumption of astrocytes are preloaded with 0.01 $\mu$M oligomycin (30 min). ***c***, Direct measurement of intracellular ATP levels relative to untreated cells (control), P2Y-R stimulated (MeADP), then the same measurements in the presence of oligomycin (oligo) or $Ru_{360}$. ***d***, Astrocyte mitochondria (TMRE loaded) at 0 min (top panels) and 10 min after treatments as labeled (bottom panels). ***e***, Histogram plot showing average mitochondrial membrane potentials before and after treatment. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

that increases intracellular production of ATP via a $Ca^{2+}$-mediated increase in mitochondrial respiration.

### Extracellular ATP and $IP_3$-BM treatment increase $\Delta\Psi$

Our observation that P2Y-R enhanced protection of astrocytes was mediated by an increase in the intracellular production of

ATP suggested that mitochondrial membrane potential ($\Delta\Psi$) may also have been increased (Territo et al., 2001; Balaban et al., 2003). We confirmed this by directly measuring the effect of ATP, ATP plus XeC, and $IP_3$-BM treatments on $\Delta\Psi$. Cultured cells were labeled with TMRE (200 nM) and imaged with a two-photon microscope as described above. $\Delta\Psi$ was estimated as the log of ($F_{mito}/F_{cyto}$), where $F_{mito}$ is the peak fluorescent intensity observed in single mitochondria (Farkas et al., 1989; Lin et al., 2007). The mean values of individual mitochondria from a single cell were used for the mitochondrial potential estimate. $F_{cyto}$ represents the lowest value of TMRE fluorescence observed within the boundaries of the same cell. Before each treatment, the resting $\Delta\Psi$ was comparable between experiments. The mean resting $\Delta\Psi$s for untreated (Buffer), ATP-treated, ATP plus XeC-treated, and $IP_3$-BM-treated were 1.81 + 0.03 ($n = 264$ mitochondria; pooled from four experiments), 1.79 + 0.03 ($n = 269$ mitochondria; pooled from four experiments), 1.85 + 0.03 ($n = 303$ mitochondria; pooled from six experiments), and 1.82 + 0.02 ($n = 425$ mitochondria; pooled from six experiments), respectively (Fig. 5*d*,*e*). Astrocyte cultures were then exposed to their respective treatments, and the same fields of mitochondria were imaged 10 min later. As expected, the control untreated cells exhibited no change. $\Delta\Psi$ remained at 1.83 ± 0.02 ($n = 230$ mitochondria; pooled from four experiments). However, treating astrocytes for 10 min with ATP or $IP_3$-BM significantly increased $\Delta\Psi$ to 1.93 ± 0.02 ($n = 346$ mitochondria; pooled from four experiments) and 1.98 ± 0.02 ($n = 343$ mitochondria; pooled from six experiments), respectively. Furthermore, astrocytes pretreated with XeC and then exposed to ATP exhibited no significant change in $\Delta\Psi$, which was 1.80 ± 0.04 ($n = 460$ mitochondria; pooled from six experiments) (Fig. 5*d*,*e*). These data are consistent with the model that P2Y-R activation in astrocytes stimulates $IP_3$-mediated intracellular $Ca^{2+}$ release, which increases mitochondrial matrix $Ca^{2+}$, stimulating respiration and the subsequent production of intracellular ATP.

**P2Y-R activation in astrocytes increases neuroprotection**

To determine whether P2Y-R enhanced mitochondrial metabolism in astrocytes affected neuronal resistance to oxidative stress, we prepared cocultures of neurons and astrocytes using corning transwell-clear permeable supports, which physically separates the two cell types by ~1 mm (#07-200-170; Fisher Scientific). Primary cultures of astrocytes were directly prepared in transwell supports until they were ~70% confluent. Mouse cortical neurons were then cultured onto glass bottom mat-tek plates and maintained in B-27/Neurobasal medium to control glia contamination to <0.5% (Invitrogen). After 4 d, cocultures were treated with t-BuOOH (100 μM) for 4 h with and without prestimulation of ATP (2 μM; 10 min). Cells were then stained with the DNA intercalating dye Hoechst 33342 (10 μg/ml; #H-3570; Invitrogen) and calcein AM (2 μM; #C3100; Invitrogen). Cell viability was quantified by counting the number of nuclei that did not colocalize with calcein-stained cells, which is only observed in live cells that have maintained their plasma membrane integrity. We found that purinergic receptor activation significantly enhanced neuronal survival from 74 ± 2% ($n = 1332$ cells; pooled from four experiments) to 87 ± 2% ($n = 1914$ cells; pooled from five experiments) when cocultured with young astrocytes and from 77 ± 3% ($n = 1092$ cells; pooled from four experiments) to 87 ± 2% ($n = 2510$ cells; pooled from six experiments) when cocultured with old astrocytes (Fig. 6*a–c*). We then tested the neuroprotective effects of isoform-specific purinergic ligands 2-MeSADP ($P2Y_1$-R) and UTP ($P2Y_2$-R). A 10 min pretreatment of astrocytes with either 2-MeSADP (2 μM) or UTP (50 μM) was equally protective against oxidative stress-induced cell death. The percentage of neurons alive after 4.5 h of t-BuOOH treatment was 51 ± 4% ($n = 1664$ neurons; pooled from four experiments) for untreated controls, 70 ± 2% ($n = 2541$ neurons; pooled from four experiments) for 2-MeSADP, and 71 ± 3% ($n = 1189$ neurons; pooled from four experiments) for UTP-treated cocultures (Fig. 6*d*). P2Y-R-specific ligands also increased the resistance of primary cultures of astrocytes, in the absence of neurons. The percentage of astrocytes alive after 4.5 h of t-BuOOH treatment, in the absence of neurons, was 60 ± 4% ($n = 294$ cells; pooled from five experiments) for control, 77 ± 4% ($n = 294$; pooled from five experiments) for 2-MeSADP, and 79 ± 3% ($n = 333$; pooled from six experiments) for UTP-treated astrocytes.

Because it is known that B-27 supplement has antioxidant properties, we also repeated the above experiments using an antioxidant-free B-27 (also available from Invitrogen) to control glia contamination. Procedures were identical to those described above except that after 4 d of coculturing, dishes were treated with t-BuOOH (100 μM) for only 3 h with and without prestimulation of 2-MeSADP (2 μM; 10 min). Cells were then stained with Hoechst 33342 and calcein AM to ascertain cell viability. We found that the percentage of neurons alive after 3 h of treatment was reduced to 33 ± 2% ($n = 1322$ cells; pooled from four experiments) (Fig. 6, compare *c* and *f*). However, a 10 min treatment of cocultures with 2-MeSADP (2 μM) significantly enhanced neuronal survival to 43 ± 3% ($n = 1057$ cells; pooled from three experiments; $p < 0.03$) (Fig. 6*f*). In addition, oligomycin treatment significantly (0.01 μM; 30 min; $p < 0.01$) decreased neuronal survival to 25 ± 3% ($n = 1409$ cells; pooled from three experiments) and completely inhibited the protective effects of 2-MeSADP on neuronal survival (26 ± 3%; $n = 1048$ cells; pooled from three experiments).

Activation of neuronal P2X receptors has been reported to be neurotoxic (Di Virgilio et al., 1998; Norenberg and Illes, 2000; Koles et al., 2005). Consequently, we further examined the effects of P2Y-R activation on neuronal survival in the absence of astrocytes. Neurons were cocultured with astrocytes in transwell dishes that were removed from the cultures just before treating the neurons with purinergic ligands. Neurons in the absence of astrocytes were then exposed to t-BuOOH (100 μM; 4.5 h with normal B-27, 3 h with antioxidant-free B-27), and cell viability was assessed by the ability of neurons to retain calcein. Under these conditions, we discovered that purinergic receptor activation by ATP treatment decreased cell viability, consistent with published reports (Fig. 6*e*,*g*). With normal B-27 supplement, the percentage of neurons alive was significantly decreased to 56 ± 7% ($n = 1192$ cells; pooled from four experiments; $p < 0.005$) by ATP treatment, whereas control, untreated neurons were at 80 ± 2% ($n = 1049$ cells; pooled from four experiments). When the neurons were pretreated with 2-MeSADP, the percentage of neurons alive (74 ± 3%; $n = 892$ cells; pooled from four experiments) was not significantly different from control (Fig. 6*g*). The same results were found for neurons initially cocultured with old astrocytes. The percentage of neurons alive was 77 ± 2% ($n = 1057$ cells; pooled from four experiments) for untreated controls, 49 ± 6% ($n = 1197$ cells; pooled from four experiments; $p < 0.001$) for ATP-treated neurons, and 72 ± 3% ($n = 1182$ cells; pooled from four experiments) for 2-MeSADP-treated neurons (Fig. 6*f*). We obtained similar results when using the antioxidant-free B-27 as a supplement. The percentage of neurons alive was significantly decreased to 18 ± 2% ($n = 763$ cells; pooled from three experiments; $p < 0.01$) by ATP treatment,

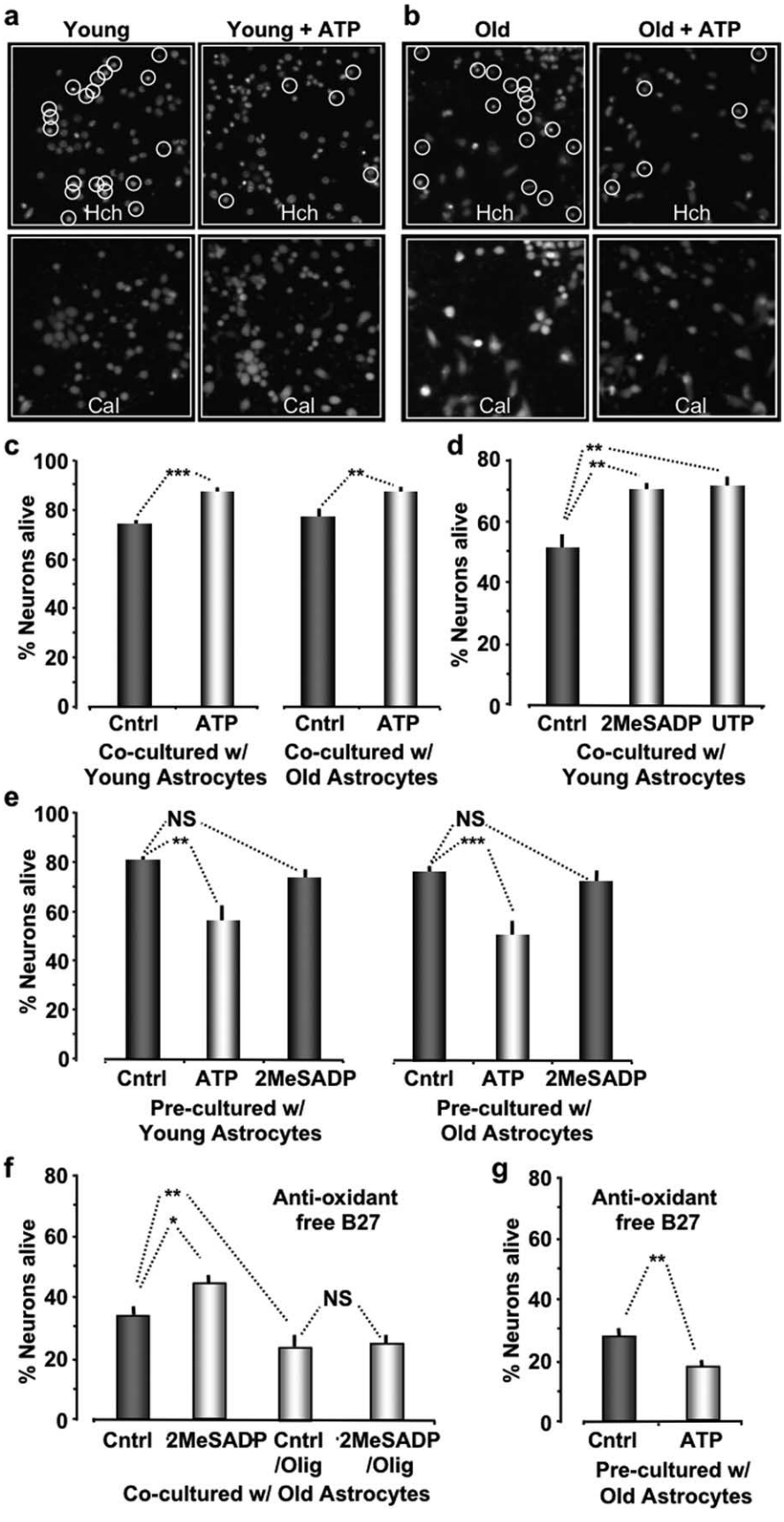


**Figure 6.** Stimulation of P2Y-Rs in astrocytes enhances their ability to protect neurons from oxidative stress. ***a***, ***b***, Images of cortical neurons cocultured with young (***a***) and old (***b***) astrocytes acquired 4 h after incubation with t-BuOOH (100 $\mu$M). Cocultures pretreated with ATP (2 $\mu$M; 10 min) are presented in the right panels. The top images show Hoechst (Hch) stained nuclei. The bottom images show the same field of neurons stained with calcein (Cal), which only stains the cytoplasm of cells that are alive. Hoechst-labeled nuclei that are calcein negative are circled and counted as cell deaths. ***c***, Histogram plots of the percentage of neurons alive when cocultured with young or old astrocytes. ***d***, Histogram plots of the percentage of neurons alive pretreated with either 2-MeSADP (2 $\mu$M) or UTP (50 $\mu$M) for 10 min. ***e***, Histogram plots of the percentage of neurons alive that were precultured with astrocytes but were separated before pretreating (10 min) the neurons with either ATP (2 $\mu$M ATP; 10 min) or 2-MeSADP (2 $\mu$M) and subsequently exposed to oxidative stress (100 $\mu$M; t-BuOOH; 4.5 h). ***f***, Histogram plots of percentage of neurons alive cocultured with old astrocytes using anti-oxidant free B-27 supplement for 3 h. ***g***, Same experiment as in ***e*** except using anti-oxidant free B-27 supplement. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

whereas control, untreated neurons were at 27 ± 3% ($n$ = 840 cells; pooled from three experiments) (Fig. 6*g*). We conclude from these data that the neurotoxic affect of purinergic stimulation is likely mediated by P2X receptors, as reported by others, whereas P2Y-R stimulation in neurons has no significant toxicity.

## Discussion

In this study, our studies focused on the capacity of astrocytes to resist oxidative stress and to protect neurons during the aging process. Unexpectedly, we discovered that a brief, transient application of extracellular ATP significantly increased the resistance of astrocytes to oxidative stress. The protective effect initiated by extracellular ATP was active throughout the aging process and made old astrocytes nearly as resistant to oxidative injury as stimulated young astrocytes. Assuming that a significant source of extracellular ATP under physiological conditions is presynaptic release, our data suggest that increased neuronal activity during the aging process can significantly increase the resistance of cells to oxidative stress and, presumably, to the aging process itself.

The protective effect of ATP was inhibited by the pretreatment of astrocytes with XeC, a competitive inhibitor of $IP_3$ binding to the $IP_3R$. These data indicated that the protective effect was mediated by P2Y purinergic receptors and the subsequent production of $IP_3$. This was confirmed by the protective effect of isoform-specific P2Y-R ligands. The ATP stimulated $Ca^{2+}$ responses were not completely blocked by XeC treatment. This was expected, because P2X purinergic receptors can also increase intracellular $Ca^{2+}$ by opening plasma membrane $Ca^{2+}$ permeable channels (Verkhratsky and Kettenmann, 1996; James and Butt, 2002). We directly tested the ability of $IP_3$ to protect astrocytes by pretreatment with a membrane permeant

analog of $IP_3$. $IP_3$-BM increased intracellular $Ca^{2+}$ and significantly increased the time until $\Delta\Psi$ collapse under conditions of oxidative stress. This protective effect could also be blocked by pretreatment with XeC, strongly supporting the identification of $IP_3$-mediated intracellular $Ca^{2+}$ release as the specific pathway involved in protection. Recent work by Shinozaki et al. (2005) has also uncovered a protective role for P2Y-R Gq/11 signaling in astrocytes against oxidative stress. However, the underlying mechanism of protection reported by Shinozaki et al. (2005) is very distinct from ours. Protection required prolonged and continuous exposure of astrocytes to extracellular ATP (12–24 h) and could be attributed to the upregulation of oxidoreductase genes, including thioredoxin, and schlafen-1 (Shinozaki et al., 2005). In our case, protection was activated by a transient application of extracellular ATP (10 min) and was dependent on mitochondrial ATP production. ATP treatment in both old and young astrocytes produced similar resistance to oxidative injury. The ability of P2Y-R activation to increase mitochondrial metabolism was further supported by data showing that increases in $\Delta\Psi$ were only observed in experimental paradigms that resulted in protection against oxidative stress. Ultimately, it will be important to measure mitochondrial pH in both young and old astrocytes, because the proton gradient is responsible for ATP synthase activity. However, our data appear to rule out the possibility that the observed $\Delta\Psi$ differences between old and young astrocytes can be solely attributed to differences in TMRE lipid partitioning (e.g., because of changing phospholipids content). Rather, the TMRE fluorescent measurements reported here indicate real differences in mitochondrial $\Delta\Psi$ in old and young astrocytes, although P2Y-R activation enhances neuroprotection to comparable levels.

The observation that mitochondrial $Ca^{2+}$ uptake via $Ca^{2+}$ release from the $IP_3R$ protected astrocytes was, to some extent, unexpected. It is well established that increased matrix $Ca^{2+}$ can stimulate $Ca^{2+}$-sensitive dehydrogenases in the citric acid cycle, which in turn increase the supply of reducing equivalents to the respiratory chain and ultimately increase ATP production (Denton and McCormack, 1985; Hansford, 1985; McCormack et al., 1990; Hajnoczky et al., 1995, 2000; Robb-Gaspers et al., 1998). However, $IP_3$-mediated $Ca^{2+}$ release has been demonstrated to depolarize mitochondria and to sensitize many cell types to apoptotic stimuli. Specifically, the pro-apoptotic stimuli ceramide and staurosporine sensitize mitochondria to depolarize in response to $IP_3$-mediated $Ca^{2+}$ release in HEPC2 cells (Szalai et al., 1999). These sensitized cells exhibited significantly increased levels of apoptosis compared with control cells that were not challenged with $IP_3$ stimuli (Szalai et al., 1999). Mitochondrial $Ca^{2+}$ uptake has also been shown to stimulate reactive oxygen species (ROS) production (Chacon and Acosta, 1991; Paraidathathu et al., 1992; Richter, 1993), and mitochondrial $Ca^{2+}$ cycling can result in a self-propagating cascade that leads to loss of ATP and $\Delta\Psi$ (Richter, 1997). Furthermore, oxidative stress induced by t-BuOOH has itself been reported to increase mitochondrial free $Ca^{2+}$, ROS formation, and stimulate opening of the MPT (Byrne et al., 1999). Consistent with these published reports, we found that $IP_3$-induced intracellular $Ca^{2+}$ release sensitized human embryonic kidney (HEK293) cells to pro-apoptotic stimuli (our unpublished observations). We induced apoptosis in an HEK293 cell line overexpressing type 1 muscarinic acetylcholine receptors (Lechleiter et al., 1989) by exposing the cells to either t-BuOOH (100 $\mu$M; 3 h) or ceramide (40 $\mu$M; 12 h). For both stimuli, apoptotic cell death was significantly higher in the presence of ACh (1 $\mu$M). It is not clear why astrocytes are not sensitized to apoptotic stimuli by $IP_3$-mediated $Ca^{2+}$ release. The appropriate $Ca^{2+}$ binding targets in the mitochondria of astrocytes may be absent or they may be actively inhibited in astrocytes. ADP is known to be a more effective inhibitor of the MPT than ATP (Halestrap and Davidson, 1990; Halestrap et al., 1997; Kantrow et al., 2000). Consequently, it is possible that the adenine nucleotide translocator of astrocytes is less sensitive to $Ca^{2+}$ and ADP levels. Higher mitochondrial potentials are also known to inhibit the mitochondrial permeability transition pore opening (Bernardi et al., 1992; Petronilli et al., 1993; Zoratti and Szabo, 1995). However, our measurements show that $\Delta\Psi$ is significantly lower in old astrocytes compared with young astrocytes (Lin et al., 2007), whereas $IP_3$-BM-enhanced neuroprotection is comparable. Thus, it appears unlikely that inherently higher $\Delta\Psi$s in astrocytes could account for their lack of sensitivity to apoptotic stimuli during $IP_3$-mediated $Ca^{2+}$ release. Another possible explanation is that the ability of astrocytes to rapidly stimulate oxidative metabolism is itself protective. This could minimize the initial depolarization, which generally leaves the mitochondrial permeability transition pore more susceptible to apoptotic stress (Bernardi, 1996). Enhanced respiration is known to increase the ability of mitochondria to accommodate large $Ca^{2+}$ influxes without diminution of $\Delta\Psi$ (Carafoli, 1987; Gunter and Pfeiffer, 1990). In addition, work from Bruce-Keller et al. (1999) indicated that superoxide scavenging by the mitochondrial-specific superoxide dismutase (MnSOD) enhances the ability of mitochondria to sequester $Ca^{2+}$ by preventing the loss of $\Delta\Psi$. The preservation of $\Delta\Psi$ by MnSOD is apparently sufficient to modulate programmed cell death. Regardless of the underlying reason, astrocyte mitochondria do not appear to depolarize in response to $IP_3$-stimulated $Ca^{2+}$ release. In fact, our data quite clearly demonstrate that $\Delta\Psi$ is increased by mitochondrial $Ca^{2+}$ uptake.

It is generally presumed that metabolic activity generates oxidative stress via an increase in the production of ROS (Paraidathathu et al., 1992; Dawson et al., 1993; Richter, 1997). This only occurs when there is an imbalance between the generation and scavenging of ROS. In addition, ROS increases with pH and often falls under conditions of increased ATP production, presumably because the proton gradient is being partially consumed. The mitochondrial theory of aging proposes that degeneration of physiological processes over the course of a lifetime is fundamentally a result of the accumulation of oxidative damage (Sohal and Brunk, 1992; Shigenaga et al., 1994; Sohal and Dubey, 1994; Warner, 1994; Cortopassi and Wang, 1995; Gadaleta et al., 1998; Cortopassi and Wong, 1999). Little is known about the cumulative effects of damage on astrocytes, the primary function of which is to protect and support neuronal activity. However, it seems likely that a degradation of their supportive and neuroprotective functions would by itself contribute to the aging process (Amin and Pearce, 1997; Robb and Connor, 1998). Mitochondrial dysfunction also plays a key role in a variety of forms of cell death, including ischemia (Pastorino et al., 1993; Zahrebelski et al., 1995), excitotoxic neurodegeneration (Beal et al., 1993), oxidant-induced stress (Dawson et al., 1993), and apoptosis (Newmeyer et al., 1994; Petit et al., 1995; Zamzami et al., 1996; Kroemer, 1997). Our observation that mitochondrial $Ca^{2+}$ uptake in astrocytes stimulates cell survival places the $IP_3$-stimulated $Ca^{2+}$ signaling system of these cells in a unique regulatory position. It is particularly well adapted for the role of transducing information about local synaptic activity and metabolic status into appropriate trophic and/or protective responses. It is also well positioned to permit a rapid response to neuronal injury. Astrocytes are known to increase their metabolic activity

in response to neuronal injury in a process referred to as reactive gliosis (Cotrina and Nedergaard, 2002).

The specific underlying process (s) that become energy-limited during oxidative stress is unknown. One clue comes from the use of transwell dishes for our astrocyte-neuronal protection assays. This configuration unequivocally shows that oxidant protection is mediated by a diffusible messenger. Given the sensitivity of astrocyte neuroprotection to the inclusion of anti-oxidants in the glia-suppressant, B-27, it is reasonable to speculate that the diffusible substance is also an anti-oxidant. Consequently, a prominent candidate has to be the antioxidant, glutathione (GSH). Neuronal *de novo* synthesis of GSH is critically dependent on GSH efflux from astrocytes as the source of cysteine (Sagara et al., 1993, 1996; Wang and Cynader, 2000). In addition, *de novo* synthesis of GSH in astrocytes is controlled by two ATP-dependent enzymes, glutamate cysteine ligase and glutathione synthetase (Suzuki and Kurata, 1992; Papadopoulos et al., 1997). Oxidative stress would rapidly deplete GSH levels and stimulate energy-dependent *de novo* synthesis in astrocytes. We suggest that the increased energy load overwhelms glycolysis. Astrocyte mitochondrial ATP production is then well positioned to rapidly and abundantly respond to the increased energy demand during stress.

The identification of G-protein-coupled, $IP_3$-$Ca^{2+}$ signaling as a key pathway in the metabolic regulation of astrocytes suggests that it is an attractive therapeutic target to address many neuropathological processes. Regardless of their potential restorative roles, our data show that astrocytes increase their resistance to oxidative stress in response to a transient exposure to extracellular ATP. Although the source of extracellular ATP could be either glial or neuronal, this metabotropic-mediated protective mechanism can also be viewed as the molecular basis for why increased glia-neuronal activity is physiologically beneficial. Quite literally, brief episodes of neuronal activity are likely to increase the ability of the neurons to resist degeneration and premature cell death and, as a consequence, significantly slow degeneration of the brain during the aging process.

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