Mechanosensitive release of ATP through pannexin channels and mechanosensitive upregulation of pannexin channels in optic nerve head astrocytes: a mechanism for purinergic involvement in chronic strain

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Abstract

As ATP released from astrocytes can modulate many neural signaling systems, the triggers of and pathways for this ATP release are important. Here, the ability of mechanical strain to trigger ATP release through pannexin channels, and the effects of sustained strain on pannexin expression, were examined in rat optic nerve head astrocytes. Astrocytes released ATP when subjected to 5% equibiaxial strain or to hypotonic swelling. While astrocytes expressed mRNA for pannexins 1–3, connexin 43 and VNUT, pharmacological analysis suggested a predominant role for pannexins in mechanosensitive ATP release, with Rho kinases contributing. Astrocytes from *panx1*^{−/−} mice had reduced baseline and stimulated levels of extracellular ATP, confirming the role for pannexins. Swelling astrocytes triggered a regulatory volume decrease that was inhibited by apyrase or probenecid. The swelling–induced rise in calcium was inhibited by P2X7 receptor antagonists A438079 and AZ10606120, in addition to apyrase and carbenoxelone. Extended stretch of astrocytes *in vitro* upregulated expression of *panx1* and *panx2* mRNA. A similar upregulation was observed *in vivo* in optic nerve head tissue from the *Tg-MYOC*[Y437H] mouse model of chronic glaucoma; genes for *panx1, panx2* and *panx3* were increased while immunohistochemistry confirmed increased expression of pannexin 1 protein. In summary, astrocytes released ATP in

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response to mechanical strain, with pannexin 1 the predominant efflux pathway. Sustained strain upregulated pannexins *in vitro* and *in vivo*. Together these findings provide a mechanism by which extracellular ATP remains elevated under chronic mechanical strain, as found in the optic nerve head of patients with glaucoma.

Keywords

mechanosensitive signaling; ATP release; pannexin; P2X7 receptor; glaucoma; cell swelling

Introduction

Identifying the ways in which mechanical strain is translated into physiological signals is of considerable importance as over-simulation of these pathways can lead to pathophysiologic events in disease. Throughout the body, mechanical strain rapidly triggers the release of the transmitter ATP from both neural and non-neural cell types (Burnstock 1999; Grygorczyk et al. 2012). Astrocytes are emerging as central mediators of mechanical strain, and the ability of astrocytes to release ATP upon stretch or swelling has implications for neuronal signaling in various regions (Bennett et al. 2012; Darby et al. 2003; Halassa et al. 2009; Ostrow and Sachs 2005; Perez-Ortiz et al. 2008). However, the pathways involved in this release, the physiological effects of this release, and the consequences of chronic strain are only partially understood.

While astrocytes in many locations are subject to mechanical strain, optic nerve head astrocytes are particularly well-situated to examine the signals induced by mechanical strain. Elevated pressure in a closed system leads to a stretch of cells and their membranes (Landsman et al. 1995). The lamina cribrosa, where the astrocytes reside, is the focal center of mechanical strain produced in the posterior eye upon elevation of intraocular pressure (IOP) in glaucoma (Burgoyne 2011; Sigal and Ethier 2009). As glaucomatous eyes have both an increase in baseline IOP and a increased magnitude in the daily IOP fluctuations (Gao et al. 2012), optic nerve head astrocytes are subject to considerable stretch. These astrocytes have been implicated in the damage to retinal ganglion cell axons in chronic glaucoma (Hernandez et al. 2008; Morgan 2000), although the precise signaling mechanisms linking elevated strain to cellular injury remain unresolved. As moderate neuronal damage may occur over many years, the mechanisms that link the short term responses to mechanical strain with more chronic damage are key to understanding chronic neural degenerative diseases like glaucoma.

Several studies suggest a role for aberrant purinergic signaling in glaucoma. Human patients with either chronic or acute glaucoma have elevated levels of ATP in the anterior chamber relative to non-glaucomatous patients (Li et al. 2011a; Zhang et al. 2007). Mechanical perturbation of retinal Müller cells can evoke ATP release (Newman 2001), while increased ocular pressure triggers a release of ATP from the retina (Resta (Resta et al. 2007; Reigada et al. 2008). *In vivo* and *in vitro* studies demonstrate that excessive stimulation of the $P2X_7$ receptor for ATP on retinal ganglion cells can lead to rapid cell death (Hu et al. 2010; Resta et al. 2007; Zhang et al. 2005).

While rapid neuronal death is clearly important, the slow time course of ganglion cell death in the chronic disease suggests sudden neuronal death may not by the primary pathological mechanism. Instead, impaired axonal transport and compression in the unmyelinated axons passing through the lamina cribrosa implicates aberrant axonal signaling in the chronic neuronal degeneration (Howell et al., 2007; Calkins 2012). Within the body of the retina, the ATP release from Müller cells is likely to stimulate receptors on the retina ganglion cell soma, and purinergic signaling in Müller cells has been well described (Bringmann et al. 2002; Keirstead and Miller 1997; Metea and Newman 2006; Newman 2004; Pannicke et al. 2001; Reifel Saltzberg et al. 2003; Wurm et al. 2009). However, the ganglion cell axons would be closer to astrocytes in the optic nerve head, and mechanosensitive ATP release from these astrocytes would be more likely to modulate axonal signaling in glaucoma. As such, identification of the conduit for this ATP release may pinpoint potential targets for intervention.

Both vesicular and channel mediated ATP release from astrocytes have been documented in other tissues (Coco et al. 2003; Pascual et al. 2005). Of the non-vesicular release pathways, connexin hemi-channels, pannexin hemi-channels, maxi-anion channels and CALMH1 channels have all been implicated (Cotrina et al. 1998; Iglesias et al. 2009; Liu et al. 2008; Taruno et al. 2013). As the pannexin hemi-channel has been identified with mechanosensitive ATP release most consistently (Bao et al. 2004; Bruzzone et al. 2005; Dubyak 2009), we asked whether mechanical perturbations could trigger a pannexin-mediated release of ATP from optic nerve head astrocytes, whether this release had functional implications, and tested if sustained mechanical strain led to changes in pannexin expression.

Materials and Methods

Animal care and use

All procedures were performed in strict accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals" and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). All animals were housed in temperature-controlled rooms on a 12-h light, 12-h dark cycle with food and water *ad libitum*. Long-Evans rat pups of both sexes, aged postnatal day 2–5, were used as a source of optic nerve head astrocytes. $Panx1^{-/-}$ mice were obtained from Dr. Shestopalov (Dvoriantchikova et al. 2012), and the presence and absence of relevant constructs was confirmed with PCR. $Tg\text{-}MYOC^{Y437H}$ mice were obtained from Drs. Sheffield and Zode (Zode et al. 2011). IOP was measured from conscious litter mate C57BL/6N and $Tg\text{-}MYOC^{Y437H}$ mice monthly using a Tonolab rebound tonometer; each value was the mean of 9 measurements from the left and 9 from the right eye, performed between 2 and 5 pm.

Solutions and reagents

The composition of isotonic solution used in our experiments was as follows: (in mM) 105NaCl, 5KCl, 4NaHEPES, 6HEPES acid, 1.3CaCl$_2$, 5glucose, 5NaHCO$_3$, 60mannitol, pH7.4, 298mOsm. The composition of hypotonic solution was almost identical except that the mannitol was omitted. This yielded a solution that measured approximately 227mOsm,

using a VAPRO vapor pressure osmometer (Westcor, Inc.). In some experiments, hypotonic solution was created by diluting isotonic solution with dH_2O at a ratio of either 1:1 (50% hypotonic) or 1:2.3 (30% hypotonic). The osmolarity of isotonic solution diluted with dH_2O was approximately 206mOsm for 30% hypotonic and 148mOsm for 50% hypotonic. All reagents were purchased through Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted.

Astrocyte cell culture

Primary rat optic nerve head astrocyte (RONHA) cultures were grown according to a protocol modified from Mandal et. al. (2009). Rat pups were sacrificed by PD5, with the optic nerve proximal to the sclera defined as the optic nerve head. This optic nerve head tissue was digested for 1 hr using 0.25% trypsin, with periodic trituration to break up the clump and create a cell suspension. Cells were washed once with Dulbecco's Modified Eagle's Medium (DMEM)/F12 containing 10% fetal bovine serum (FBS) to inactivate the trypsin and re-suspended in medium comprised of DMEM/F12, 10% FBS, 1% penicillin/ streptomycin and 25ng/ml epidermal growth factor (EGF) before being plated on T25 cell culture flasks. Cultures were found to contain greater than 97% astrocytes, as defined by GFAP immunofluorescence staining (not shown). Of note, optic nerve astrocytes express more GFAP at baseline than cortical astrocytes, suggesting the cells were not necessarily reactive (Dowling 2011). Cells were trypsinized after 7 days and passaged; while no difference between cells of different passage number was observed, cells were generally used in passage 2–3. Mouse optic nerve head astrocytes were isolated from C57Bl/6J and $panx^{-/-}$ mice at postnatal day 15–20. The isolation procedure to obtain mouse astrocytes was the same protocol as for the rat isolation procedure, except that the optic nerve head tissue was incubated in 0.25% trypsin for only 20–30min.

Hypotonic ATP release experiments

Rat optic nerve head astrocytes were sub-cultured onto plastic 96-well plates and grown until 80% confluent. Before the challenge, cells were pre-incubated in either 50μl of isotonic solution (for control or hypotonic conditions) or 50μl isotonic solution containing an antagonist (hypotonic + drug) for 30 min. After the incubation, 50μl of either isotonic (control), dH_2O (to produce a 50% hypotonic solution), or dH_2O with antagonist (hypotonic + drug) was added to each well. To prevent ATP degradation, drug solutions contained 100μM of ATPase inhibitors β,γ-methylene ATP (β,γ-mATP) and ARL67156; 20μl of luciferin/luciferase working solution was then added to each well (Stock solution = 450 μl Isotonic + 50 μl water to 1 vial of ATP assay mix; Sigma, Catalog #FLAAM, working solution 100 μl of stock + 2.4 mL of isotonic solution). Luminescence was read 10x on a Luminoskan Ascent luminometer plate reader (ThermoFisher Scientific, MA) with 100 ms integration time per well. The values used here were obtained 1.5–2.5 min after addition of hypotonic solution, as trials indicate ATP levels are at their peak at 2 min and fluctuations from the solution change have subsided. Luminescence values were converted to ATP concentration by means of a standard curve performed separately for each solution used, and corrections were made for the effect of hypotonicity and the various antagonists on the curve as done previously (Reigada et al. 2006).

ATP release from mouse cells followed a similar protocol with the following modifications. C57Bl6J and panx$^{-/-}$ optic nerve head astrocytes (passage 2) were plated in adjacent wells in a 96-well plate and grown to confluence. After washing, 50μl isotonic + 10μl luciferin-luciferase working solution was added to each well. In some wells, 100μM β,γ-mATP was added to inhibit degradation of extracellular ATP by ecto-ATPases. After taking 50 measurements of baseline ATP levels, 50μl water + 10μl luciferase working solution was added using the internal dispensing system. One hundred measurements were recorded of the hypotonic induced ATP release with a 100 ms integration time. As in rat, measurements were made 1.5–2.5 min after addition of hypotonic solution to quantify ATP levels.

Mechanical stretch ATP release experiments

Rat optic nerve head astrocytes were sub-cultured onto sterilized silicone sheets and allowed to grow for 24 hours (Silastic, Speciality Manufacturing, Saginaw, MI). In some cases, media maintaining the cells was removed prior to the experiment and replaced with isotonic buffer solution containing 100μM β,γ-mATP and ARL67156 to prevent ATP degradation. Using a custom-designed apparatus (Winston et al. 1989), astrocytes were subjected to a 5% equibiaxial strain at approximately 0.3 Hz for 2 min. This mild degree of strain was below that calculated as the mean strain present in the lamina cribrosa with a rise in IOP to 50 mm Hg (Sigal and Ethier 2009; Sigal et al. 2005; Sigal et al. 2007); our imposition of cyclical strain is consistent with short term changes in pressure (ocular pulse) observed in primate eyes using remote telemetry (Downs et al. 2011). While the magnitude of this cyclic strain in the living ONH is currently unknown, the imposed values are not inconsistent with computations, accounting for the strain amplification that has been predicted by lamina microarchitecture. After stretch, the silicone sheet was examined to confirm cell attachment, and a 100μl aliquot of the extracellular solution was removed and stored at −80°C until ATP quantification. The ATP concentrations of the samples were determined via the luciferin/luciferase reaction in 96-well plates, as described above.

Lactose dehydrogenase (LDH) cytotoxicity assay

In order to rule out the possibility that increased extracellular ATP was due to cell death and lysis, we utilized a commercially available, colorimetric assay for quantifying cytolysis through LDH activity (Cytotoxicity Detection Kit PLUS LDH, Roche Applied Science, Germany). 100μl samples of the extracellular solution were taken before and after stimulation of cells by hypotonic solution or mechanical stretch as outlined above and measured for LDH activity following the manufacturer's instructions as described (Xia et al. 2012). LDH activity in each test sample was normalized to ATP levels in lysed cells as a maximum and isotonic solution as a minimal response to calculate percentile as 5=100x(max-test)/(max-min).

PCR/qPCR

For cultured astrocytes, media was aspirated from cultured cells and 1 ml of TRIzol reagent (Invitrogen/Life technologies, NY) was added for stretched cells on silicone supports. For cells in 96-well plates, 50μl Trizol was added per well. Total RNA was extracted using the protocol recommended by the manufacturer. For 96-well plates, 20 samples were combined. RNA concentration and purity was assessed using a Nanodrop spectrophotometer

(ThermoFisher Scientific). To create cDNA for PCR, 1 μg of total RNA was reverse transcribed using the First Strand Synthesis Kit (Applied Biosystems) using the manufacturer's instructions. PCR was performed using the Taq PCR Core Kit (Qiagen) and a thermocycler (Applied Biosystems). Primer sequences and expected product sizes are listed in Table 1. Primers were designed in house using Primer 3 software (http://primer3.wi.mit.edu/). PCR products were visualized on a 2.0% agarose gel in TAE buffer using ethidium bromide to stain DNA.

To quantify gene expression changes in cultured astrocytes following stretch or swelling, cells were grown to 80% confluence and subjected to either mechanical stretch (5% strain, 0.3 Hz for 4 hours then rested for 20 hours) or hypotonicity. Appropriate untreated controls were also grown simultaneously. RNA was isolated and cDNA created as described above. qPCR was performed using SYBR Green master mix and a real-time thermocycler (Applied Biosystems). Changes in mRNA expression were calculated using the CT method as described (Guha et al. 2013), using β-actin as a housekeeping gene. Analogous approaches were used for optic nerve head material from 8 month old C57Bl6N and $Tg\text{-}MYOC^{Y437H}$ mice, using mouse specific primers in Table 1.

Regulatory volume decrease (RVD)

Regulatory volume decrease was determined based on an approach used previously (Mitchell et al. 2002; Xia et al. 2012). Briefly, astrocytes were sub-cultured sparsely on glass coverslips and allowed to attach for 24 hrs. Cells were loaded with 4μM calcein-AM and 0.02% Pluronic at room temperature for 40 min, followed by a gentle wash. Cells were imaged with an inverted microscope equipped for fluorescence (Nikon Diaphot, Nikon USA every 20 sec, with the fluorescence excited at 488nM and emitted >520nm recorded with a camera and processed (all Photon Technologies International, Inc., Lawrenceville, NJ). Cells were first perfused at room temperature with isotonic control solution followed by hypotonic solution (addition of 30% H_2O). To determine cell area, images were thresholded to remove background fluorescence and cell area was defined as the number of non-black pixels after thresholding. The area values were converted to volume using standard equations, assuming the image represented the maximal cross sectional area. Regulatory volume decrease was defined as $100 \times (H\text{-}H_{30})/(H\text{–}I)$, where H = peak cell volume in hypotonic solution, H_{30} = volume after 30 min in hypotonic solution and I= volume in isotonic solution.

Intracellular calcium measurement

Pharmacological manipulation of intracellular Ca^{2+} levels was monitored with a plate reader as previously described (Reigada et al. 2005); this approach provides the most reproducible levels for comparisons. In brief, rat optic nerve head astrocytes from were grown to 80% confluence in black 96-well plates; the clear bottoms enabled inspection of cell density. Cells were loaded with 5μM Fura-2 AM for 30 min at room temperature in the dark. After washing, cells were pre-incubated with isotonic solution + drug for 10–30min. Pre-incubation solutions were removed and replaced with the drug in either isotonic or hypotonic solution. Experimental solutions and drugs included isotonic solution (Mg^{2+}-free), hypotonic solution (same composition as isotonic, but mannitol-free), and hypotonic plus 1U/mL apyrase, 10μM A438079, 10μM AZ10606120, or 10μM carbenoxelone.

Fluorescence was measured with a Fluoroskan Ascent Fluorometer (Thermo Scientific). Parameters were 340nm/380nm excitation and 527nm emission, 60ms integration time, 30s interval time, 11 measurements. The 340nm/380nm ratios in isotonic and hypotonic were normalized to the mean level in isotonic over the 5.5 minutes for each drug.

Immunohistochemical staining

Rat optic nerve head astrocytes cells were grown to ∼80% confluence on 12mm glass coverslips and then fixed with cold methanol/acetone (80%/20%) solution for 20 minutes at 4°C. Cells were blocked with 10% goat serum in a phosphate buffered saline solution (PBS) for 90 min. A polyclonal rabbit anti-glial fibrillary acidic protein (GFAP, 1:250 dilution, EMD Millipore, MA) antibody was applied overnight at 4°C, followed by a goat anti-rabbit IgG Alexa-Fluor 488 secondary antibody (1:500 dilution, Abcam Inc, MA) for 60 min. For pannexin 1 staining of cells, the cells were fixed with 4% paraformaldehyde in PBS for 20 min. at room temperature, washed 3x with PBS and permeablized for 1hr. with PBS containing 5% donkey serum and 0.3% TritonX-100. Coverslips were incubated with goat polyclonal antibody against pannexin 1 (1:200, Abcam, Inc.) diluted in PBS containing 1% donkey serum and 0.3% TritonX-100 overnight at 4°C. A secondary antibody (donkey anti-goat FITC, 1:500 dilution in blocking solution, Abcam Inc.) was added for 2 hrs. Cells were incubated with DAPI and mounted using SlowFade Gold Anti-fading Media (Invitrogen). Images were acquired using a Nikon Diaphot microscope and ImagePro software (MediaCybernetics). Incubation with only the secondary antibody showed no non-specific binding (results not shown).

For immunostaining C57Bl6N and of *Tg-MYOC^{Y437H}* mouse optic nerve heads, mice were perfused with 4% paraformaldehyde and the eye enucleated. Eyes were fixed overnight and incubated in 30% sucrose for 24 h. Tissues were then embedded in O.C.T compound, and cryosectioned at 8μm. Sections were incubated with a blocking solution containing 0.1% Triton-X, and immunostained with a pannexin 1 primary antibody (goat polyclonal antibody #SC-49695; Santa Cruz Biotechnology). Secondary donkey anti-goat Alexa555 conjugated antibody was applied (1:500). Images were acquired using a Nikon A1 confocal microscope at the University of Pennsylvania Live Cell Imaging Center.

Data Analysis

Data are reported as mean ± SEM. Statistical analysis used a one-way ANOVA with appropriate post-hoc test, or tests on ranks where data were not normally distributed. Results with $p < 0.05$ were considered significant. In data from experiments with plate-readers, n=the number of wells with 3–7 plates typically used.

Results

Astrocytes release ATP in response to mechanical strain or osmotic stress

Initial experiments measured the unstimulated baseline levels of ATP in the bath surrounding optic nerve head astrocytes and examined the presence of ecto-ATPase enzymes on the cells. Baseline levels of ATP in the bath were low, although significantly distinguishable from background. However addition of the ecto-ATPase inhibitors β,γ-

methylene ATP (100μM) and ARL-67156 (100μM) to the extracellular media increased ATP levels over 9 fold to 1.9 nM (Fig. 1A). While these absolute levels are low, they represent the mean bath ATP levels sampled at a distance from the cell membrane and have thus been diluted many fold; concentrations at the membrane surface are expected to be several orders of magnitude greater (Beigi et al. 1999). These observations suggested that ATP is released basally at low levels from the astrocytes and that ectoATPase enzymes are present on the astrocyte membrane to rapidly control levels of released ATP.

To determine whether optic nerve head astrocytes released ATP in response to mechanical perturbation, cells grown on a silicone substrates were subjected to a mild 5% equibiaxial strain at 0.3 Hz for 2 min and concentrations of ATP in the bath were determined before and after the stretch. EctoATPase inhibitors were present in the bath to ensure focus on ATP release over degradation. Mild stretch doubled the extracellular ATP concentration (Fig. 1B). To determine whether this ATP release represented a general response to mechanical strain, astrocytes were swollen in a hypotonic solution. Swelling the astrocytes led to a 6-fold rise in the concentration of extracellular ATP when measured 2 min after initial exposure to hypotonic solution (Fig. 1C). In the absence of ectoATPase inhibitors, hypotonic swelling still increased extracellular ATP levels 8 fold, although levels were scaled down.

To confirm that the increased extracellular ATP observed was due to physiological release and not from a stress-induced rupture of the cellular membrane, we performed a lactate dehydrogenase (LDH) assay on aliquots of the extracellular solution of cells subjected to osmotic stress or mechanical stretch. Samples taken from either stretched or swelled cells exhibited low LDH activity, not significantly different from LDH activity in non-stimulated cells (Fig. 1D). In contrast, lysing cells led to a large increase in LDH activity. Together, these observations imply that mechanical strain leads to a physiological release of ATP from the astrocyte that is distinct from cell lysis.

Mechanisms of ATP release in astrocytes

Several approaches were used to try to determine which putative mechanisms were responsible for the mechanosensitive ATP release from optic nerve head astrocytes. The ability of various pharmacological agents to block release was tested first. Carbenoxolone reduced ATP release from cells exposed either to swelling (Fig 2A) or stretch (Fig. 2B). The ability of probenecid to prevent release was also examined; while probenecid has numerous targets, it has been reported to inhibit pannexins but not connexins (Silverman et al. 2009). Probenecid blocked the swelling-induced ATP release (Fig. 2C). In contrast to carbenoxelone and probenecid, neither 18α-glycyrrhetinic acid (Fig. 2D) nor $GdCl_3$ (Fig. 2E) significantly altered ATP release from swollen astrocytes.

The mechanosensitive opening of the pannexin channel can be mediated by the small GTPase RhoA pathway. Specifically, swelling-induced pannexin channel opening appears to be blocked by inhibitors of the Rho family of kinases in airway epithelial cells (Seminario-Vidal et al. 2011). In optic nerve head astrocytes, pretreatment with the non-specific Rho-kinase inhibitor GSK269962 partially decreased ATP release in response to hypotonic swelling. Release was blocked 35% by 0.1μM and 41% by 10μM GSK269962 (Fig. 3F).

While the observed pharmacological profile was consistent with pannexin channels being responsible for at least some of the mechanosensitive ATP release from optic nerve head astrocytes, none of the available drugs are specific for a particular class of channel. As such, we repeated the experiments in pannexin 1 knockout ($panx1^{-/-}$) mice. Optic nerve head astrocytes obtained from wild type mice showed a baseline ATP release that was increased by the inhibition of ectoATPases similar to that in rats (Fig. 3A). Baseline levels of ATP were decreased 10-fold in astrocytes from panx1$^{-/-}$ mice although ectoATPase inhibition elevated these levels too; ectoATPase inhibitors were thus included in all measures of ATP release. The amount of ATP released upon exposure to hypotonicity was significantly reduced in the $panx1^{-/-}$ mice (Fig. 3B). This supports the pharmacological characterization described above and implies that pannexin 1 makes a substantial contribution to the mechanosensitive ATP release from optic nerve head astrocytes, but that other conduits are also present.

Extracellular ATP released from optic nerve head astrocytes acts in an autocrine manner to regulate cell volume

ATP released via mechanical strain often autostimulates the cells from which it came, with the feedback regulating physiological processes influenced by mechanical strain (Wang et al. 1996). For example, the initial stretch from the influx of water that accompanies exposure to hypotonic solution is followed by ATP release and autostimulation of purinergic receptors leading to efflux of ions and a regulatory volume decrease (RVD, (Okada et al. 2001)). To determine if ATP also plays a role in RVD in optic nerve astrocytes, we examined the rate at which the astrocytes recovered their original size following stimulation with a hypotonic solution. Hypotonic solution initially increased cell area by approximately 30%, but area returned to normal within 15 min (Fig. 4A). However, in the presence of the ATP hydrolase apyrase, RVD was significantly slowed, with cells still approximately 12–15% larger than control 30 min after hypotonic stimulation (Fig. 4B–C). Similar results were observed in the presence of the pannexin channel inhibitor probenecid (Fig. 4B–C), consistent with a requirement for pannexin-mediated ATP release for RVD.

To provide further functional evidence for autocrine actions of released ATP, we examined the effect of swelling on intracellular calcium levels. When the astrocytes were subjected to hypotonic solution, intracellular Ca^{2+} levels increased significantly (Fig. 4D). Preincubation with apyrase inhibited the rise in Ca^{2+}, consistent with the autocrine stimulation above. Likewise, carbenoxelone prevented the rise in Ca^{2+} initiated by hypotonicity. As ATP released through pannexin channels is reported to autostimulate certain splice variants of the $P2X_7$ receptor (Xu et al. 2012), the ability of $P2X_7$ antagonists A438079 and AZ10606120 to prevent the rise in Ca^{2+} was examined. Both A438079 and AZ10606120 inhibited the Ca^{2+} response to hypotonicity. Taken together these findings suggest that mechanical strain leads both to ATP release and to autocrine activation of $P2X_7$ receptors in optic nerve head astrocytes.

Pannexin channel proteins are expressed in optic nerve head astrocytes

Given that pannexin 1 was implicated in the mechanosensitive ATP release from optic nerve astrocytes, release mechanisms were characterized on a molecular and protein level. ATP

release is a primitive response, and numerous pathways for release are present in mammalian cells (Corriden and Insel 2010). PCR analysis confirmed that the pannexin hemichannel was present in unstimulated astrocytes, with pannexin 1, and to a smaller extent pannexin 2, expressed (Fig. 5A). Levels of pannexin 3 were faint. PCR also indicated the presence of the vesicular nucleotide transporter (VNUT, also referred to as SLC17A9) and confirmed the presence of gap junction protein connexin 43 (Fig 5B). The ionotropic purinergic receptor $P2X_7$ and the ectonucleotidase nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) were also present. The cellular location of pannexin channels was determined using immunohistochemistry. Pannexin 1 was localized in discrete regions throughout the astrocytes (Fig. 5C). Some, but not all, staining for pannexin was associated with actin.

Pannexin isoforms are up-regulated following stretch in isolated astrocytes

Exposure of optic nerve head astrocytes to mechanical strain can alter expression of numerous genes (Johnson et al. 2007). Given that chronic strain can make the cells more sensitive, we asked whether expression of pannexin genes was affected by mechanical strain using quantitative PCR (qPCR). Analysis of baseline levels indicated that pannexin 1 was the predominant isoform present in astrocytes (Fig. 6A). mRNA message for both pannexin 1 and 2 is up-regulated following an extended stretch (4 hours of 5% strain at 0.3 Hz and RNA extracted 20 hours later, Fig. 6B). mRNA for the ectonucleotidase NTPDase1 was also increased 1.6-fold; as NTPDase1 is a marker for chronic changes in extracellular ATP levels (Lu et al. 2007) this finding is consistent with a sustained elevation in ATP in response to extended stretch. Message for pannexin 3, the gap-junction protein connexin 43 and the $P2X_7$ receptor remained unchanged after stretch, however. It is worth noting that although expression of pannexin 2 rose twice as much as pannexin 1 upon stretch, initial expression of pannexin 1 was over 15 fold greater than pannexin 2 (Fig. 6B). As such, levels of pannexin 1 are expected to be greater after stretch too.

Pannexin isoforms are up-regulated in the Tg-MYOC[Y437H] mouse model of chronic glaucoma

While the use of isolated astrocytes enables the increase in gene expression to be examined in a cell-specific manner, we also examined pannexin mRNA expression in a genetic mouse model of chronic glaucoma in order to determine if pannexin expression is altered in this clinically-relevant model of sustained stretch. In the *Tg-MYOC[Y437H]* model of chronic glaucoma, transgenic mice expressing the Y437H mutation of the human myocilin gene exhibit increased IOP, increased retinal ganglion cell death and axonal degeneration as they age (Zode et al. 2011). For our experiments, the optic nerve heads from 8 month old wild-type mice and their transgenic *Tg-MYOC[Y437H]* litter mates were removed and the material tested for differences in pannexin gene expression. The optic nerve head of wild type mice expressed all three isoforms of pannexin, with pannexin 1 being the most abundant form (Fig. 7A). At 8 months of age, the IOP showed a modest but significant increase in *Tg-MYOC[Y437H]* mice as compared to age-matched controls (Fig. 7B). Relative gene expression for all three isoforms of pannexin was significantly increased in the optic nerve head of *Tg-MYOC[Y437H]* mice (Fig. 7C). This suggests that mechanosensitive ATP release via pannexin channels may be enhanced by stretch in chronic glaucoma.

The presence of pannexin 1 in optic nerve astrocytes was confirmed with immunohistochemistry (Fig. 7D, F). Globular staining in the optic nerve head surrounding nuclear staining for DAPI was consistent with astrocyte staining. This staining was greater in the optic nerve head of $Tg\text{-}Myoc^{Y437H}$ mice (Fig. 7E,G), suggesting the increased mRNA for pannexin in this model was also reflected at the protein level. Together, this increase in *panx* expression, combined with the identification of pannexin as a pathway for stretch-dependent ATP release, provides a mechanism for the upregulation of ATP release with sustained mechanical strain.

Discussion

This study indicates that pannexins contribute to the mechanosensitive release of ATP from optic nerve head astrocytes and that the expression of pannexins is likely increased by chronic stretch. In particular, the study has demonstrated that astrocytes release ATP upon stretch and swelling (Fig. 1). The preferential block by antagonists carbenoxelone and probenecid suggests that pannexins are the predominant pathway to the ATP release that accompanies mechanical strain, although other mechanisms may also contribute (Fig. 2). Both baseline and swelling-induced ATP release are reduced in optic nerve head astrocytes from $panx1^{-/-}$ mice, confirming a role for pannexin 1 in ATP release (Fig. 3). The mechanosensitive release of ATP makes a functional contribution to cell physiology, as the regulatory volume decrease was dependent on permeant pannexins and the presence of extracellular ATP while the rise in Ca^{2+} accompanying cell swelling was blocked by pannexin and P2X$_7$ receptor antagonists (Fig. 4). Pannexin 1 was present on both a molecular and protein level in the astrocytes (Fig. 5). Both pannexin 1 and pannexin 2 were increased after stretch in vitro. (Fig. 6). All three pannexin isoforms were increased in the optic nerve head of $Tg\text{-}Myoc^{Y437H}$ mice with a chronic elevation of IOP (Fig. 7). Together, these findings imply a role for pannexins in the response to acute stretch, and suggest a mechanism for enhanced involvement under conditions of prolonged mechanical strain.

Contribution of pannexins to the mechanosensitive release of ATP from optic nerve head astrocytes

The ability of probenecid and low levels of carbenoxelone to prevent the mechanosensitive release of ATP from the astrocytes implicates pannexins in this release (Ma et al. 2009). The 10μM level of carbenoxelone used in this study has been reported to preferentially inhibit pannexin channels (Bruzzone et al. 2005). However, carbenoxelone can also inhibit amino acid release from cortical astrocytes from P2X7$^{-/-}$ mice at this concentration (Ye et al. 2009), indicating the specificity may be complex even at this level. While the block by probenecid (Silverman et al. 2009), combined with the lack of block by Gd^{3+} or 18α-GA is consistent with pannexin channels as a conduit for the mechanosensitive ATP release, all these compounds have multiple sites of action and thus the pharmacological characterization is just suggestive. However, the reduction in both baseline and stimulated ATP levels in the $panx1^{-/-}$ mice provides a more definitive identification of pannexins in this release. This is consistent with the altered ATP release in cortical astrocytes from $panx1$ knock-out mice (Suadicani et al. 2012) and in astrocytes in which pannexin1 has been knocked down (Iglesias et al. 2009). Whether the residual ATP release in astrocytes from $panx1^{-/-}$ mice in

the present study reflects a contribution from pannexin 2 or from other conduits is unknown. The recent identification of leucine-rich repeat-containing 8 (LRRC8) channels as swelling-activated anion channels may be of relevance in this regard (Qiu et al. 2014; Voss et al. 2014). The permeability of LRRC8 to taurine (Voss et al. 2014) and the similarities with pannexin channels (Abascal and Zardoya 2012) suggest LRRC8 may also contribute to the mechanosensitive efflux of ATP, although this remains to be demonstrated directly.

Although pannexins are clearly involved in connecting mechanical strain to ATP release in astrocytes, the present data do not indicate whether they act as a mechanosensor or as a "middle man". Pannexins were shown to have intrinsic mechanosensitive properties in isolated patch experiments (Bao et al. 2004). Whether the association between pannexins and actin in Figure 5 indicates a more direct role in mechanosensitivity or is merely a reflection of the cultured condition, as found in cortical astrocytes (Oberheim et al. 2009), remains to be determined. An upstream role for TRPV4 has been implicated in the swelling-activated ATP release from pannexins in lens (Shahidullah et al. 2012) and airway epithelial cells (Seminario-Vidal et al. 2011). However, experiments to determine a role of TRPV4 in the present study were inconclusive due to the substantial effect of inhibitor HC067047 on the luciferase standard curve (not shown). Rho kinase was implicated in at least some of the mechanosensitive ATP release in the airway epithelial cells, consistent with the ability of Rho kinase inhibitor GSK269962 to prevent the mechanosensitive release in the present study. Interestingly, GSK269962 never blocked more than 42% of the ATP release even with a 100-fold rise in concentration; if the Rho kinase is involved in trafficking of pannexin it may not have impacted channels already in the membrane. However, it is also possible that several pathways for ATP release exist and not all of the release involves Rho kinase.

ATP release is a primitive response, and numerous pathways for release are present in mammalian cells (Corriden and Insel 2010). Astrocytes may use various mechanisms for ATP release depending upon the stimuli. Astrocytoma cells employ Ca^{2+} dependent vesicular mechanisms for ATP release triggered by receptors while the same cells use a non-vesicular pathway for ATP release when swollen (Joseph et al. 2003). The presence of a similar dual-response system in optic nerve head astrocytes would explain why VNUT was expressed but mechanosensitive release was largely blocked by pannexin-channel inhibitors. G-protein coupled receptors act synergistically with osmotic stress to modulate ATP release from 1321N1 astrocytes (Blum et al. 2010), and while such a synergistic mechanism is also possible from optic nerve head astrocytes, the above data indicate that it isn't necessary. Non-vesicular ATP release from cortical astrocytes can be regulated by circadian genes (Marpegan et al. 2011); as IOP also changes on a circadian basis, the temporal coordination between IOP levels and ATP release from optic nerve head astrocytes may have important implications.

Upregulation of pannexins upon chronic stretch

The ability of sustained stretch to upregulate expression of pannexin genes in isolated optic nerve head astrocytes, combined with the increase in pannexin expression in the optic nerve head of the *Tg-MYOC^Y437H* mice has important implications for the contribution of the channel to disease. In both cultured rat astrocytes and material from mouse optic nerve head,

baseline expression of mRNA for *panx1* was greatest (assuming analogous primer efficiency). However, the increase in *panx1*, and particularly of *panx2* in cultured astrocytes, signals the presence of an important positive feedback loop that may lead to increased ATP release with sustained stretch. This is strongly supported by the increased expression of *panx1*, *panx2* and *panx3* in material from the optic nerve head of the *Tg-MYOC^{Y437H}* mice, in which the intraocular pressure was moderately elevated for several months. Of relevance in this regard is the age-related increase in *panx1* expression in rats (Mawhinney et al. 2011), further supporting potential relevance of this channel activity to age-mediated progression of glaucoma.

It is interesting that *panx3* was elevated in the animal model but not *in vitro*. The relative contributions of the three isoforms to the optic nerve head response to stretch remains to be determined. While pannexin 1 is the most widely distributed isoform, all three have been associated with ATP release (Li et al. 2011b; Penuela et al. 2013). Both pannexin 2 and 3 have been associated with development (Vogt et al. 2005) and differentiation (Ishikawa et al. 2011); this role may be consistent with the stretch-dependent upregulation, although the signals connecting cell stretch to elevated transcription may differ from those involved in the developmentally-triggered changes.

Physiological implications

These data implicating pannexins in ATP release from optic nerve head astrocytes, combined with the observation that pannexin expression increases with sustained stretch, suggest a mechanism for maintaining an elevated extracellular ATP upon sustained mechanical strain. The increase in both pannexin and NTPDase1 message in swollen astrocytes above supports this connection as NTPDase1 can act as a marker for the maintained elevation of extracellular ATP in other ocular cells (Lu et al. 2007). The elevation of extracellular ATP in humans with chronic angle closure glaucoma also confirms this pattern has clinical relevance (Li et al. 2011a).

While the upregulation of pannexins provides a mechanism for sustained ATP release under chronic strain, the consequences of this release may be either beneficial or detrimental. ATP released from retinal glia can be converted to adenosine and protect retinal ganglion cells by stimulation A_1 and A_3 adenosine receptors (Hartwick et al. 2004; Newman 2001; Newman 2003; Zhang et al. 2006). In contrast, stimulation of the $P2X_7$ receptor for ATP can kill retinal ganglion cells both *in vitro* and *in vivo* (Hu et al. 2010; Zhang et al. 2005). As such, the net effect of increased extracellular ATP resulting from upregulation of pannexins may depend on the relative levels of adenosine and $P2X_7$ receptors. The ability of retinal ganglion cells to survive exposure to decades of mechanical strain suggests that the enhanced ATP release, mediated by pannexin upregulation, may in fact be beneficial.

In the brain, the increased expression of pannexin channels upon chronic stretch, combined with the identification of pannexins as conduits for ATP release from glial cells, may have important implications as the release of ATP from glial cells has recently been implicated in several key events. For example, an increased release of ATP from astrocytes led to an enhanced excitability of hippocampal neurons (Lee et al. 2013), while long-term depression of synaptic transmission in the hippocampus was also attributed to astrocytic ATP release

(Chen et al. 2013). The pannexin-mediated release of ATP from microglial cells influenced inflammatory responses (Orellana et al. 2013), while ATP released from astrocytes is implicated in sleep dynamics (Blutstein and Haydon 2013). An elevation in pannexin expression in response to mechanical strain, such as following traumatic brain injury or edema, could lead to chronic modification in any of these systems.

Acknowledgments

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Main Points

- Mechanical strain triggers ATP release from astrocytes via pannexin 1

- Released ATP autostimulates $P2X_7$ receptors

- Sustained stretch increased pannexin expression

- Mechanism for sustained elevation of extracellular ATP under chronic stretch
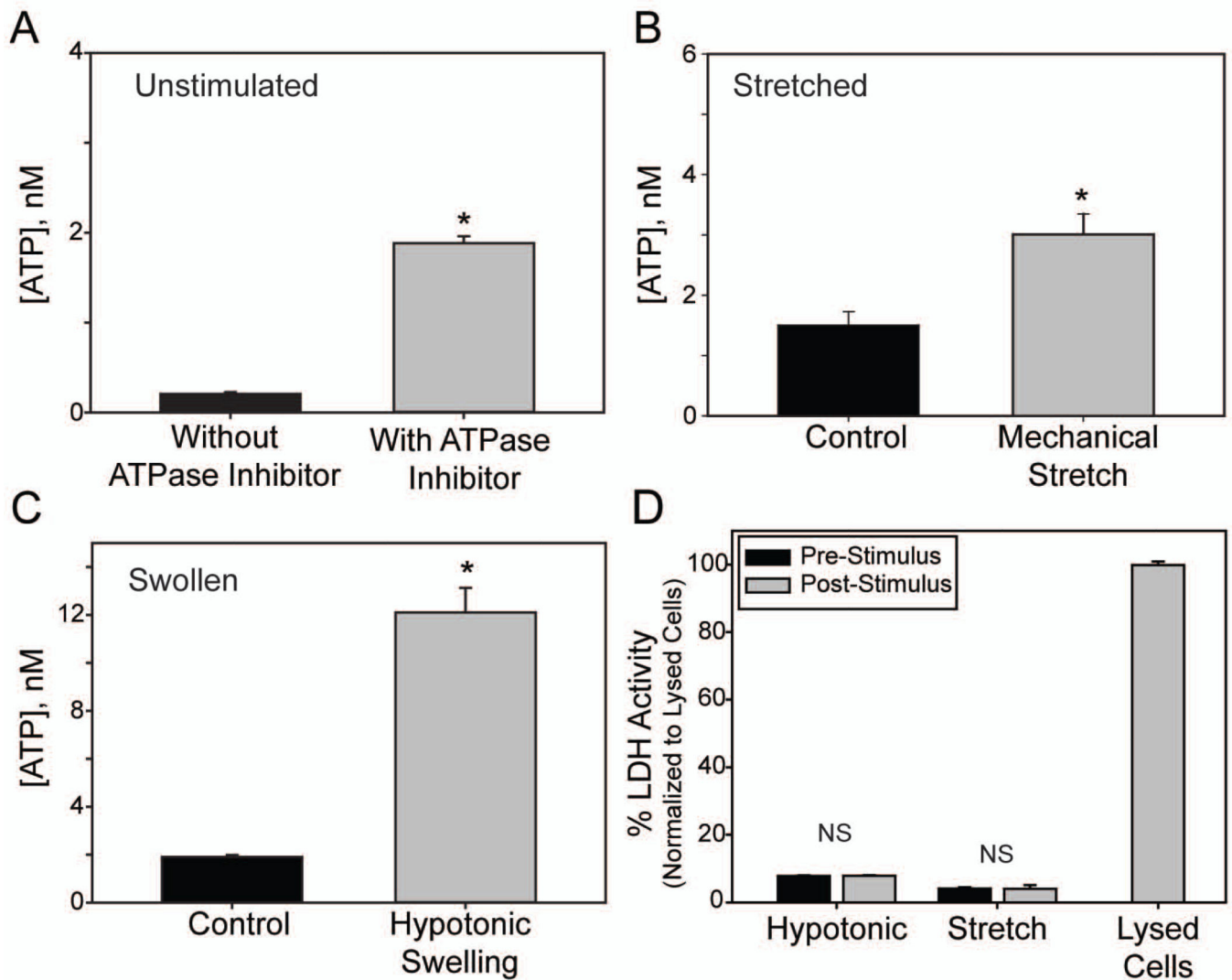
Figure 1.
Mechanical stretch and hypotonic swelling of astrocytes releases ATP. (A): Comparison of basal extracellular ATP concentrations from non-stimulated (control) cells in the absence or presence of the ecto-ATPase inhibitors β,γ-methylene ATP and ARL67156 (100μM each; n=385, 147 respectively) (B): Effects of mechanical stretch (5% equibiaxial strain, 0.3 Hz for 2 min) on extracellular ATP concentrations. ATP levels were determined from 100μl samples taken before and after cells were stretched, n=4 each. (C): Effects of hypotonic swelling (50% dH_2O) on extracellular ATP concentrations measured between 1.5–2.5min after addition of hypotonicity, n=140. All solutions in (B) and (C) had 100μM β,γ-methylene ATP and ARL67156. (D): Measurement of extracellular lactose dehydrogenase (LDH) as an index of cell lysis. LDH activity was measured before and after stimuli (hypotonic swelling or mechanical stretch). NS = no significant. Levels expressed as % of that measured from lysed cells; n=20 for hypotonic, 4 for stretch and 21 for lysed cells. *$p<0.05$, unpaired Students' t-test in A,C; paired in B,D.
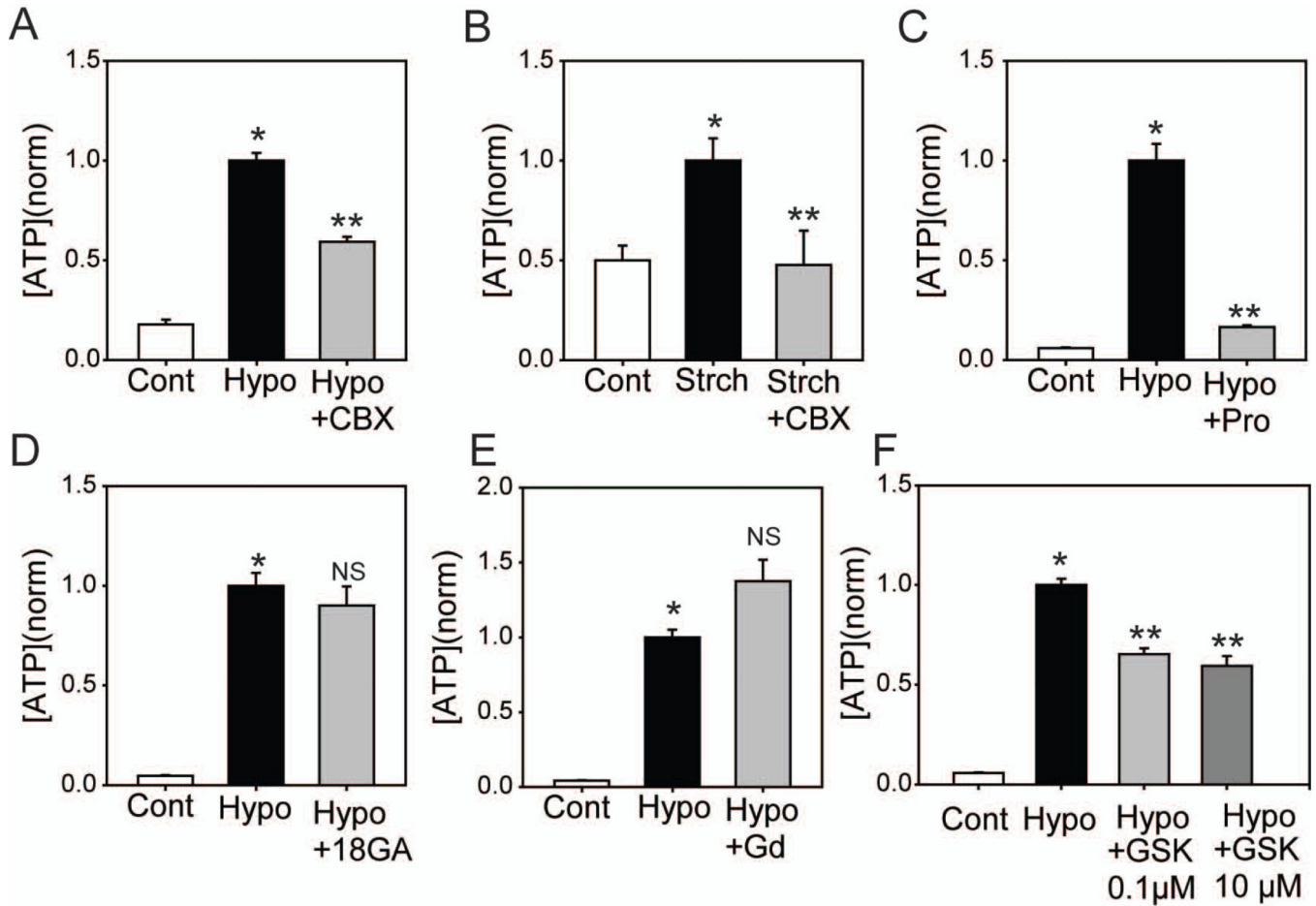
Figure 2.
Pharmacological characterization of mechanosensitive ATP release (A): Hypotonic swelling (Hypo) increased extracellular ATP levels over control (Cont) and this rise was reduced by 10μM carbenoxolone (CBX n=190). (B): Mechanical stretch (Strch, as in Fig. 1B) increased extracellular ATP levels over unstretched controls (Cont). Carbenoxolone (CBX, 10μM) inhibited this increase (n=4).(C) Probenecid also inhibited swelling-induced ATP release (2mM, n=60). (D): ATP release was not blocked by 18α-Glycyrrhetinic acid (18α-GA, 50μM, n=70), or (E) gadolinium chloride (GdCl$_3$, 50μM, n=66). (F): Increased extracellular ATP in response to hypotonic solution is partially reduced by the Rho kinase inhibitor GSK269962 at 0.1μM and 10μM (n=60). Throughout the Figure, ATP values were normalized to the mean levels attained upon swelling or stretching for each trial. * p<0.05 vs. Control, ** p<0.05 vs. Hypotonic alone.
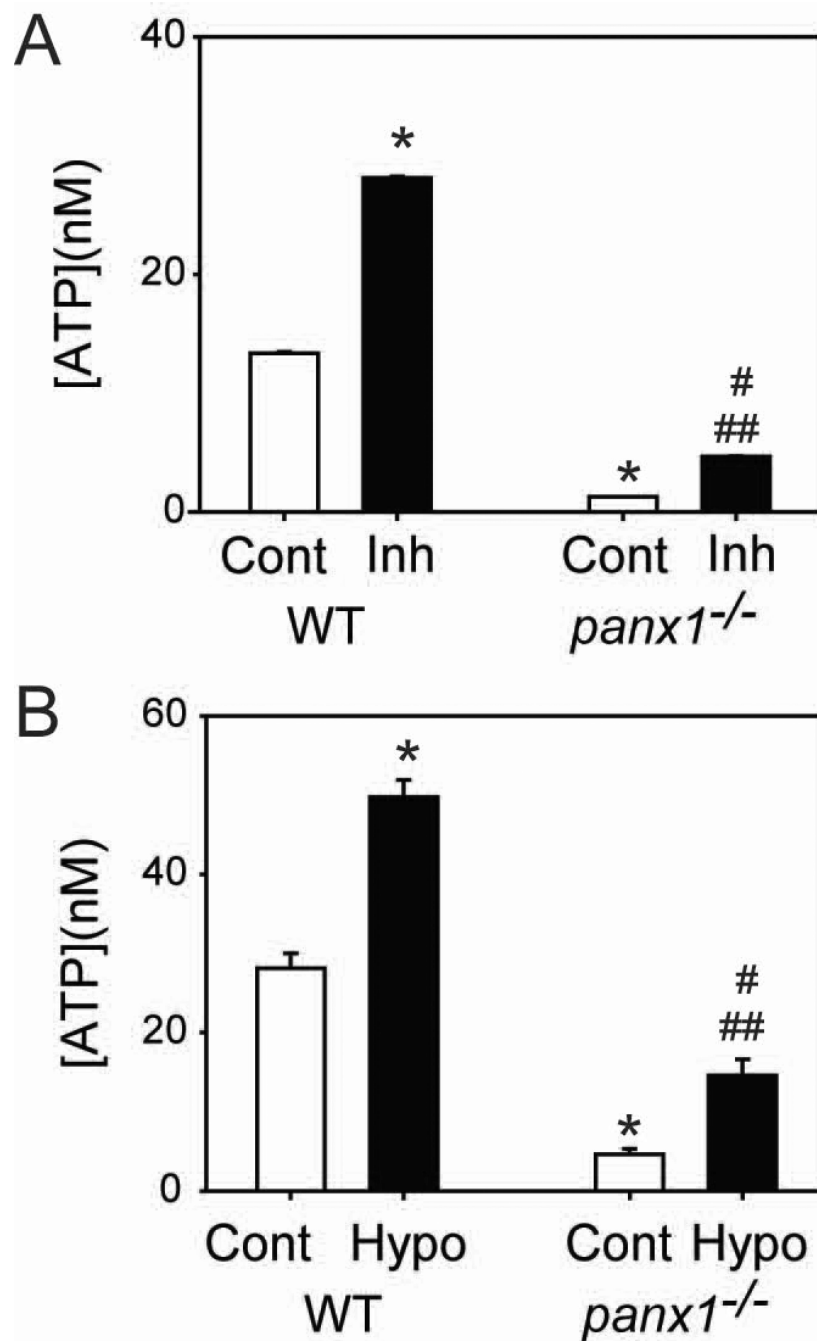
Figure 3.
ATP release from astrocytes of *panx1*−/− mice. (A) Baseline levels of ATP surrounding astrocytes were much lower with cells from *panx1*−/− mice than wild type although both were raised by 100μM β,γ-mATP (Inh). Baseline levels were much lower in astrocytes from *panx1*−/− mice (n=5). (B). While hypotonicity raised the ATP surrounding astrocytes from both wild type and panx1−/− mice, the magnitude of ATP release from the *panx1*−/− cells was reduced (n=5). Experiments in (B) were performed in the presence of β,γ-mATP. In

both panels, * = p<0.05 vs. WT control, # =p<0.05 vs. *panx1*$^{-/-}$ control, and ## = p<0.05 vs. WT hypo.
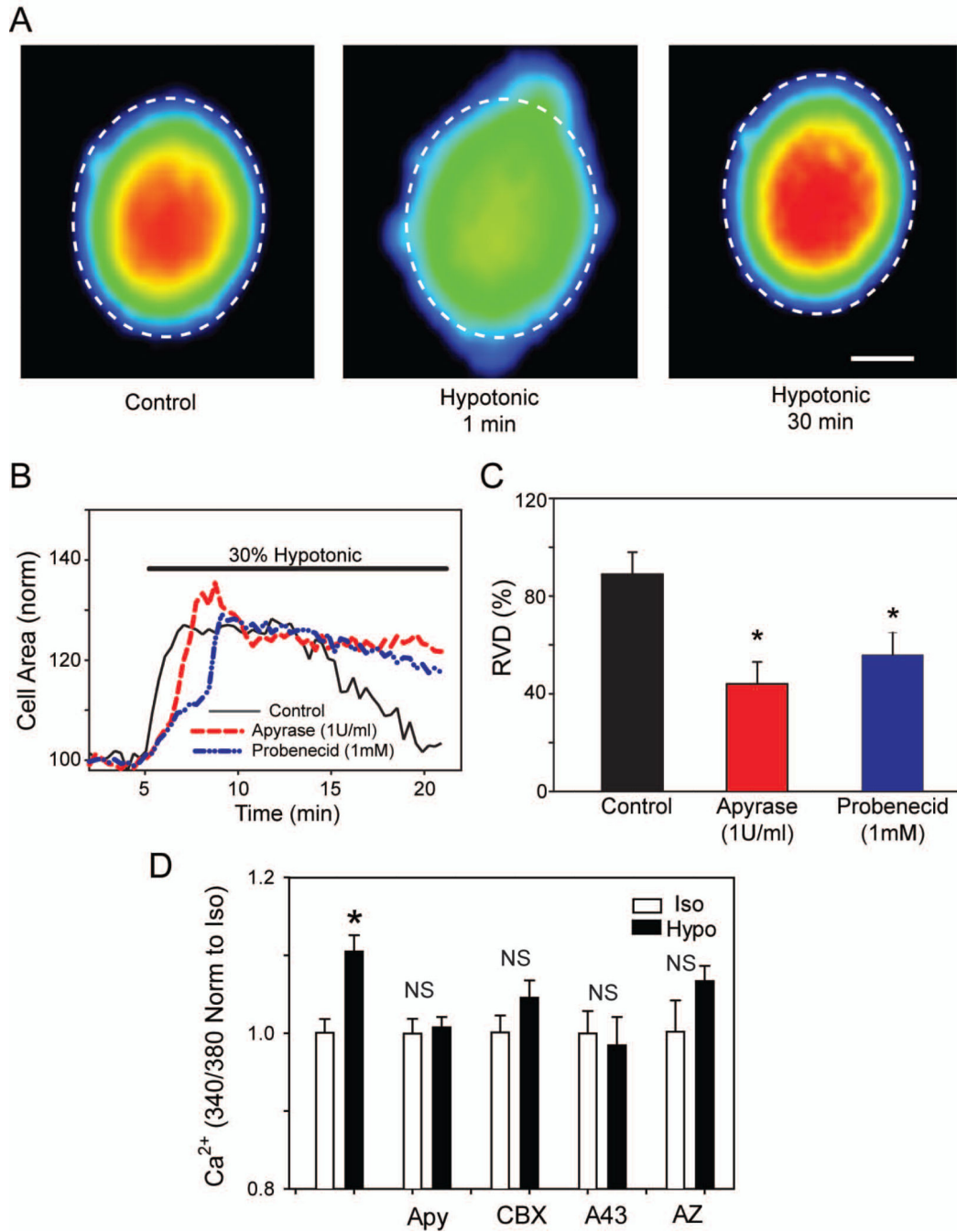
Figure 4.
Extracellular ATP plays a role in regulatory volume decrease and calcium signaling. (A): Example of increase in cell area with hypotonic solution and subsequent RVD. All three images are of the same control cell at different time points in the experiment. Dashed line indicates the circumference of the cell in isotonic conditions. Color indicates strength of calcein staining, with blue representing low intensity and red indicating high intensity, bar = 5μm. (B) Representative time courses depicting the changes in cell area of rat optic nerve head astrocytes following perfusion with a 30% hypotonic solution with or without

treatment with compounds that decrease extracellular ATP concentrations. Control cells are represented as a solid black line; cells treated with apyrase (1U/ml) are represented as a dashed red line and cells treated with the pannexin antagonist probenecid (1mM) are represented as a dashed blue line. (C): Summary of the extent of the regulatory volume decrease 30 minutes after start of perfusion of hypotonic solution (defined in Methods); n=16, 11 & 14, respectively. *p<0.05. (D). The calcium rise induced by exposure of astrocytes to hypotonic solution is reduced by apyrase (Apy, 1U/ml), carbenoxelone (CBX, 10μM), A438079 (A43, 10 μM) and AZ10606120 (AZ, 10μM). The ratio of light excited at 340 and 380 nm in cells loaded with fura-2 is used as an index of Ca^{2+}, with ratios normalized to the mean value in isotonic solution for each drug. *=p<0.05, NS = no significant difference, n=7–23.
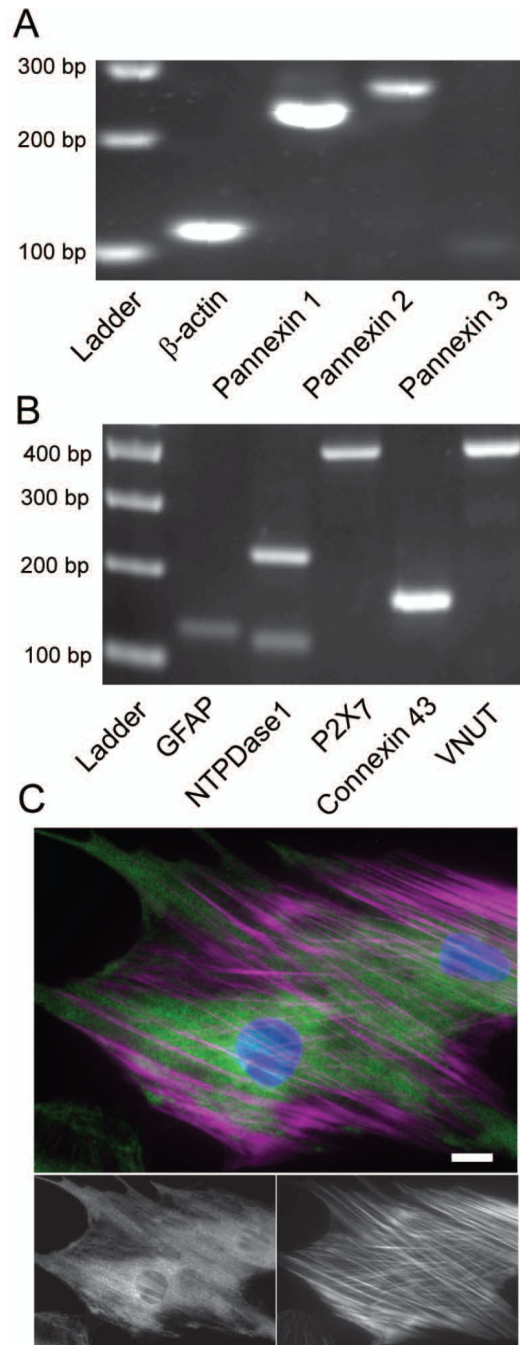
Figure 5.
Rat optic nerve head astrocytes express a number of possible mechanisms for releasing ATP. (A): Micrograph of PCR products for pannexin hemichannel isoforms as well as the positive control β-actin. (B): PCR products for other targets that have been shown to play a role in ATP release and signaling, plus the positive control glial fibrillary acidic protein (GFAP). NTPDase 1: Ectonucleoside triphosphate diphosphohydrolase 1. VNUT: Vesicular Nucleotide Transporter. Expected product sizes are listed in Table 1. (C). Immunofluorescence of cultured rat optic nerve head astrocytes stained for pannexin 1

(green), F-actin (magenta) and nuclear DNA (blue), bar: 20µm. Pannexin 1 staining alone is shown on the bottom left while F-actin alone is shown on the bottom right.
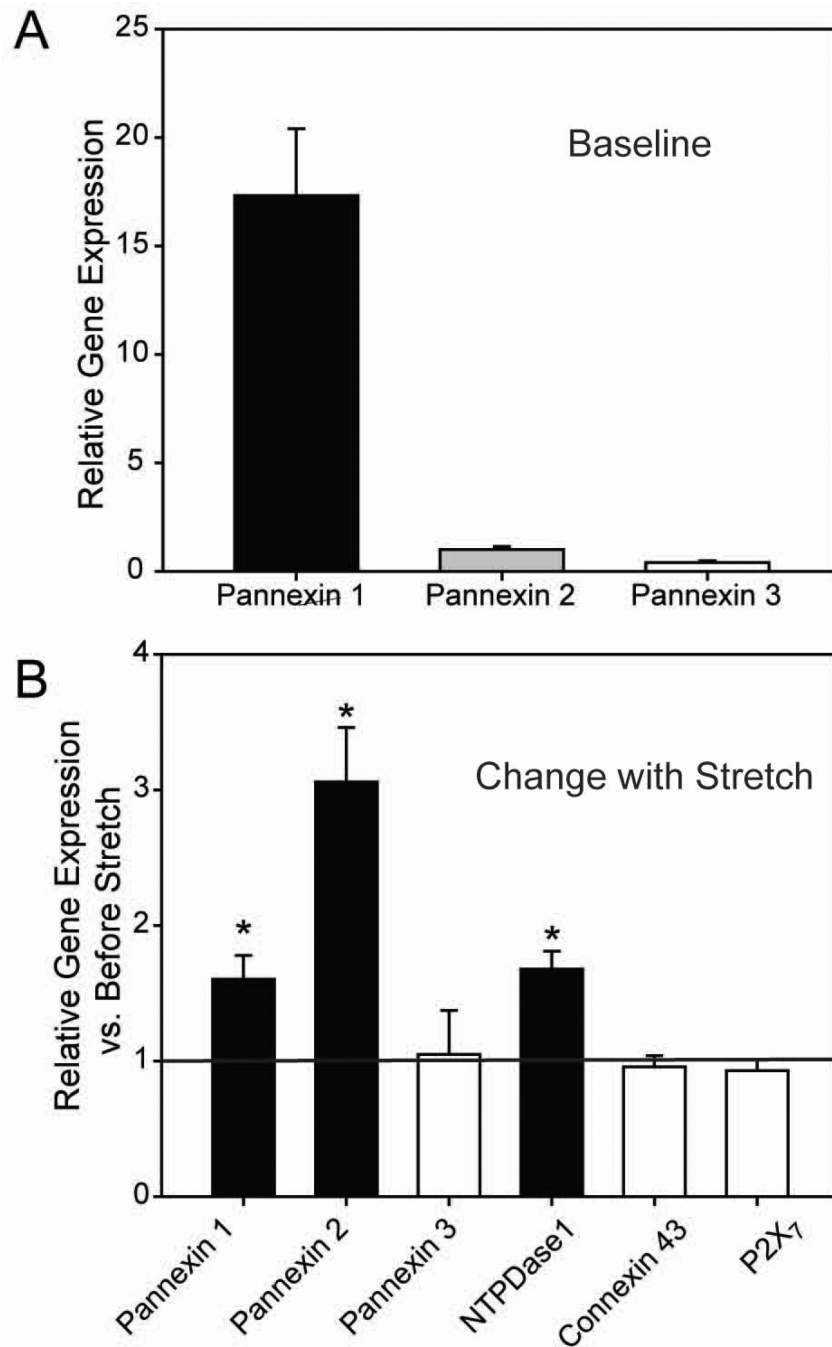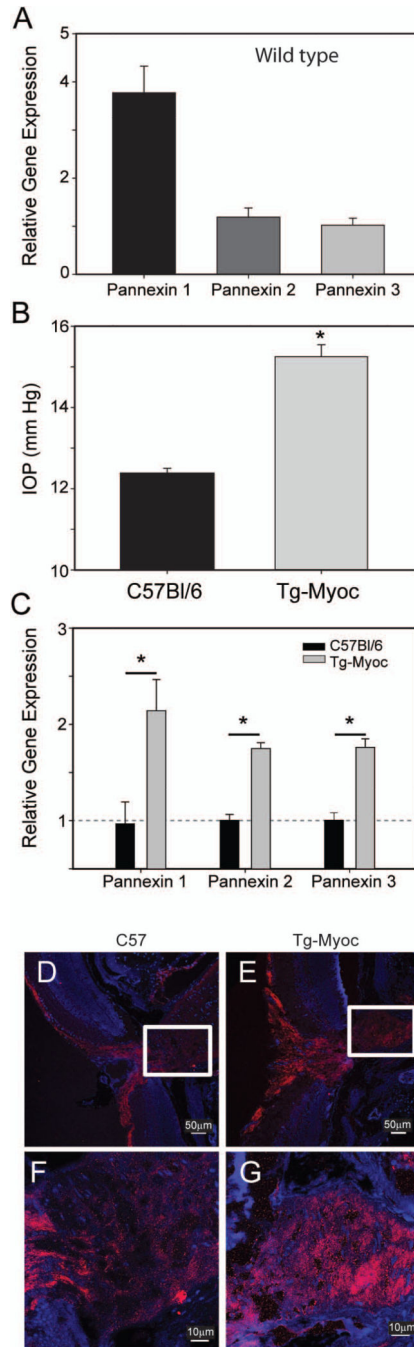
Figure 6.
Mechanical stretch alters gene expression in cultured rat optic nerve head astrocytes. (A): Relative expression of pannexin isoforms in unstretched astrocytes. Gene expression is normalized to pannexin 2 levels. Base pair (bp) sizes are indicated at left (B): Mechanical stretch of cultured rat optic nerve head astrocytes (5% equibiaxial strain, 0.3Hz for 4 hours followed by rest for 20 hours) significantly increased expression of pannexin 1, pannexin 2 and NTPDase1 mRNA, while pannexin 3, connexin 43 and P2X$_7$ mRNA remained

unchanged. *p<0.05 as compared by paired Students' t-tests to non-stretched controls (indicated by the dashed line). n=3 for each column.

Figure 7.
Increased pannexin expression in the optic nerve head on *Tg-MYOC^Y437H* mice with elevated IOP. (A): Relative gene expression for pannexin isoforms in the optic nerve head of wild type mice. Results are an average of optic nerve head tissue taken from 3 separate mice and are normalized to the lowest expressed isoform, pannexin 3. (B): IOP in wild-type C57Bl/6N mice and *Tg-MYOC^Y437H* (Tg-Myoc) mice at 8 months of age. n=7,8 mice, respectively. (C): Increases in expression of pannexin isoforms1-3 in *Tg-MYOC^Y437H* (grey), as compared to wild-type mice (black). n=3. * = p<0.05 vs. wild type. (D–G).

Immunohistochemistry confirming localization of pannexin 1 in optic nerve head astrocytes. Staining for pannexin 1 (red) and nuclei (blue) in a cross section of the optic nerve head demonstrating staining in the nerve fiber layer and along the optic nerve head of wild type (D) and *Tg-MYOC^{Y437H}* mice (E). The optic nerve head region inside the box is magnified below, showing that both wild type (F) and *Tg-MYOC^{Y437H}* (G) mice have staining surrounding nuclei, consistent with astrocytes. The staining is greater in the *Tg-MYOC^{Y437H}* mice.

Table 1

List of PCR primers used in the study.

Gene Name (NCBI Accession #)	Species	Sequence (5' → 3')	Expected Product Size (bp)
NTPdase1 (NM_022587.1)	Rat	F: AGGAGCCTGAAGAGCTACCC R: GTCTGATTTAGGGGCACGAA	224
P2X7 (NM_019256.1)	Rat	F: GGAAGATCCGGAAGGAGTTC R: GACATGGACGGGAGAGAAAA	392
Pannexin1 (NM_199397.2)	Rat	F: GCTGTGGGCCATTATGTCTT R: GCAGCCAGAGAATGGACTTC	234
Pannexin2 (NM_199409.2)	Rat	F: CAAGGGGAGTGGAGGTGATA R: GTGGGGTATGGGATTTCCTT	264
Pannexin3 (NM_199398.1)	Rat	F: TTTCCCTTGCTAGAGCGGTA R: GGGGCTCTAGAAGGCTCTGT	104
Connexin43 (NM_012567.2)	Rat	F: TCCTTGGTGTCTCTCGCTTT R: GAGCAGCCATTGAAGTAGGC	167
β-actin (NM_031144.3)	Rat	F: AGCCATGTACGTAGCCATCC R: ACCCTCATAGATGGGCACAG	115
GFAP (NM_017009.2)	Rat	F: GCAGGAGTACCAGGATCTAC R: ATCTGGAGGTTGGAGAAAGT	129
VNUT (NM_001108613.1)	Rat	F: GGAACTCCACCTCGGATGTA R: GTTTTGAACTGTGGGCCTGT	396
β-actin (NM_007393.3)	Mouse	F: TTGCTGACAGGATGCAGAAG R: ACATCTGCTGGAAGGTGGAC	141
Pannexin 1 (NM_019482.2)	Mouse	F: GGCCACGGAGTATGTGTTCT R: TACAGCAGCCCAGCAGTATG	247
Pannexin 2 (NM_001002005.2)	Mouse	F: TGCCCATCTCCTACCTATGC R: CAAGACGATGTCCACACCTG	176
Pannexin 3 (NM_172454.2)	Mouse	F: TTTCCCTTGCTAGAGCGGTA R: TTGCTGCACTGGAGACACTC	271