Journal of Neuroscience Research 00:000–000 (2011)

A New Specialization in Astrocytes: Glutamate- and Ammonia-Induced Nuclear Size Changes

Chun Zhang Yang,[1] Hui Li Li,[1] Ye Zhou,[1] Rui Chao Chai,[1] Rui Zhao,[1] Yan Dong,[1] Zhen Yu Xu,[1] Lok Ting Lau,[1,2] Zhang Yingge,[3] Junlin Teng,[4] Jianguo Chen,[4,5] and Albert Cheung Hoi Yu[1,2,6,7]★

[1]Neuroscience Research Institute, Key Laboratory for Neuroscience (Ministry of Education), Key Laboratory for Neuroscience (Ministry of Public Health), Department of Neurobiology, School of Basic Medical Sciences, Health Science Center, Peking University, Beijing, China
[2]Hai Kang Life Corporation Limited, HKSAR, China
[3]Beijing Institute of Pharmacology and Toxicology, Beijing, China
[4]Key Laboratory of Cell Proliferation and Differentiation of Ministry of Education, State Key Laboratory of Bio-Membrane and Membrane Bio-Engineering, College of Life Sciences, Peking University, Beijing, China
[5]The Center for Theoretical Biology, Peking University, Beijing, China
[6]Infectious Disease Center, Peking University, Beijing, China
[7]Laboratory of Translational Medicine, Institute of Systems Biomedicine, Peking University, Beijing, China

We observed nuclear swelling in glutamate (Glu)-treated astrocytes that was concomitant with but independent of astrocytic cell swelling. We confirmed Glu-induced nuclear swelling with nuclei isolated from astrocytes. Ammonia is metabolically related to Glu and could induce a nuclear swelling in intact astrocytes but shrinkage in isolated nuclei. Other compounds such as glutamine, aspartate, taurine, glycine, and ATP did not cause any nuclear swelling in isolated nuclei of astrocytes. Surprisingly, Glu and ammonia did not induce nuclear swelling in microglia, C6, HEK 293, or Hep G2 cell lines in cultures and their isolated nuclei. The Glu- and ammonia-induced nuclear size changes appear to be a specific response of astrocytes to these two closely related metabolic compounds.

Key words: astrocyte; nuclear swelling; cytoplasmic swelling; glutamate; ammonia; atomic force microscopy

Glutamate (Glu) is the major excitatory neurotransmitter, transferring information among neural cells in the central nervous system (CNS). Glu released from neurons not only relates excitatory signals to neurons but also excites astrocytes (Zonta and Carmignoto, 2002; Haydon and Carmignoto, 2006; Yang et al., 2008; Bennett et al., 2009; Lehmann et al., 2009). More and more evidence has demonstrated that Glu-induced cell swelling is one of the most remarkable size changes observed in astrocytes (Hansson et al., 1994; Gunnarson et al., 2008, 2009). Glu was directly taken up by Glu transporters on the plasma membrane (Kimelberg et al., 1989), and each internalized Glu molecule would translocate over 400 water molecules into astrocytes (MacAulay et al., 2001, 2002; Yang et al., 2008).

Significant astrocytic swelling is also observed under high concentrations of extracellular ammonium ions (Gregorios et al., 1985; Norenberg et al., 2005; Jayakumar et al., 2006; Albrecht et al., 2010). Even under normal physiological conditions, astrocytes are generally exposed to higher levels of Glu and ammonia, and subsequent volume changes might be a normal response or function in vivo. Astrocytes can detoxify ammonia with Glu via glutamine synthetase to form glutamine (Gln), which not only is essential but also is a unique way to reduce the ammonia level in the CNS, which has no urea cycle (Schneider et al., 1992; Zwingmann, 2007; Brusilow et al., 2010; Butterworth, 2010). This could be considered as one of the specializations in

Contract grant sponsor: National Natural Science Foundation of China; Contract grant numbers: 30270426, 30470543, 30670644, 30870818, 31070974; Contract grant sponsor: Beijing Natural Science Foundation; Contract grant numbers: 7032026, 7051004, 7091004; Contract grant sponsor: National High Technology Research and Development Program of China (863 Program); Contract grant number: 2006AA02Z452; Contract grant sponsor: National Basic Research Program of China (973 Program); Contract grant number: 2011CB504400.

★Correspondence to: Prof. Albert Cheung-Hoi Yu, PhD, Neuroscience Research Institute, Peking University, 38 Xue Yuan Road, Beijing, 100191 China. E-mail: achy@bjmu.edu.cn

Received 13 December 2010; Revised 23 February 2011; Accepted 10 March 2011

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.22657

astrocytes, which has evolved to deal with toxic ammonia levels in the CNS through Glu, ammonia, and glutamine metabolism.

This study systematically analyzed the response of astrocytes to Glu and ammonia. We observed that Glu and ammonia induce a nuclear size change in the astrocytes that is concomitant with but independent of its cytoplasmic swelling. We also confirmed that Glu induced nuclear swelling but that ammonia induced nuclear shrinkage. These nuclear responses are specific and could not be simulated by Glu-related compounds or in some other cell types. Therefore, we might have found a new addition to the specializations of astrocytes.

MATERIALS AND METHODS

Cell Cultures

Primary cultures of cerebral cortical astrocytes were prepared from newborn ICR mice as described previously (Yu and Hertz, 1982; Yu and Lau, 2000; Lau and Yu, 2001; Dong et al., 2009; Xu et al., 2009). Primary cultures of microglia were obtained as described by Liva and de Vellis (2001), with minor modification. Briefly, the cortices of newborn ICR mice were dissected and mechanically dissociated. In each T75 flask (Corning, Corning, NY), 2×10^7 cells were plated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; HyClone, Australia). The medium was changed at day 3, and the floating microglia were harvested at days 12–15. These microglia were plated at 1×10^6 per 35-mm dish in DMEM containing 10% FBS, incubated at 37°C with 5% CO_2 for 1 hr for cell attachment. Dishes were washed once with culture medium to remove nonadherent cells. Cells were grown for 2–3 days and then treated with Glu or NH_4Cl or processed for nuclei isolation. C6, HEK 293, and Hep G2 cell lines were grown in DMEM containing 10% FBS in 35-mm dishes. Cells were passaged by detaching with trypsin-EDTA, diluting 1:10 in fresh medium for reseeding. Cells were treated with Glu or NH_4Cl directly or processed for nuclei isolation.

Glu or NH_4Cl Treatment and Nuclear Area Measurements

Cultures were washed twice with prewarmed (37°C) DMEM and incubated with 1 mM monosodium glutamate (Glu; Sigma-Aldrich, St. Louis, MO) or 5 mM NH_4Cl (Sigma-Aldrich) in serum-free DMEM for 0, 4, 8, and 12 hr. For nuclear area measurement, cultures were washed twice with ice-cold phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and stained with Hoechst 33342 (2 μg/ml; Sigma-Aldrich). At least five images were taken randomly from each culture under a fluorescent microscope (Leica, Wetzlar, Germany), and nuclear areas in each field were measured by Image Pro Plus (V 6.0; Media Cybernetics, Silver Spring, MD).

Time-Lapse Imaging Analysis

Time-lapse imaging was performed on 2-week-old astrocyte cultures. Images of the same field were taken every 5 min for up to 6 hr. Images were analyzed by a MetaMorph Imaging System (V. 4.5 r 5; Universal Imaging Corp., Downington, PA), which converted light intensity to relative height and allowed comparison of the height changes of astrocytes.

Atomic Force Microscopy (AFM)

Astrocytes, after 12 hr of Glu or NH_4Cl treatment, were fixed for 30 min in 2.5% glutaraldehyde in 0.9% NaCl, washed three times with distilled water, cut into 1×1 cm^2 squares, and immobilized onto a steel disk with double-sided adhesive tape. AFM image was captured in contact mode with a SPM-9500J3 scanning probe microscope (Shimadzu Corporation, Tokyo, Japan) as described previously (Xu et al., 2009).

Nuclear Isolation and Treatments

Nuclei of cells in cultures were isolated by Optiprep iso-osmotic density medium (Nycomed, Norway). Cultures were washed twice with PBS and collected in 0.5 ml ice-cold homogenization solution (0.25 M sucrose, 25 mM KCl, 10 mM $MgCl_2$, 20 mM Tricine, pH 7.8). After incubation on ice for 10 min, the cell suspension was centrifuged at 600*g* for 10 min at 4°C, and the pellets were washed once with the homogenization solution. Then, the pellets were resuspended in 0.25 ml homogenization solution and 0.25 ml solution C [50% (w/v) Optiprep, 25 mM KCl, 5 mM $MgCl_2$, 20 mM Tricine, pH 7.8]. The suspension was underlaid with 0.3 ml 30% iodixanol and 0.2 ml of the 35% iodixanol before being centrifuged at 10,000*g* for 20 min at 4°C. The nuclei were collected at the 30–35% interface. Isolated nuclei were resuspended in cytosolic buffer (120 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1.1 mM EGTA, 0.1 mM $CaCl_2$, 10 mM HEPES, pH 7.4; Wang and Clapham, 1999).

Isolated nuclei were seeded onto 35-mm tissue culture dishes precoated with poly-D-lysine. After 30 min of incubation at 37°C, some of these attached nuclei were treated with hypotonic solution (150 Osm) to determine their integrity (Highland et al., 1991; Fig. 4B). Some Glu- and NH_4Cl-treated nuclei were fixed and stained with Hoechst 33342 (2 μg/ml). Twenty images were randomly taken from each sample, and the experiment was repeated for at least three times. Nuclear areas were estimated by Image Pro Plus as mentioned above.

Statistical Analysis

All experiments were performed at least three times with cultures from at least two different batches. Results were analyzed in Prism 4.0 software (GraphPad Software, San Diego, CA), and mean ± SEM were calculated using one-way ANOVA with Bonferroni posttest. The χ^2 test was used for grouped nuclear area comparisons. Results were considered statistically significant at $P < 0.05$.

RESULTS

Time-Lapse Imaging Analysis of Astrocytes in Primary Cultures under Glu and NH_4Cl Treatment

Swelling of astrocyte in primary cultures treated with Glu (1 mM) or NH_4Cl (5 mM) was recorded from

the same field for 6 hr (Fig. 1A,B). Glu induced significant swelling within 5 min of treatment; the entire cell body became visible and surrounded by a dark gray ring (Fig. 1Aa, arrow). The cell body area appeared to decrease in the first 3 hr, probably as a result of cell lifting caused by the swelling. Cell processes began to come into focus (Fig. 1Aa, white arrowheads). After 4 hr, the spreading cell boundary indicated a continuous cytoplasmic swelling (Fig. 1Aa, arrows). The nuclei became phase bright at 2 hr (Fig. 1Aa, star), which was probably caused by a decrease in refractive index resulting from water entry. By 3–6 hr, the nuclei had significantly increased in size.

Astrocytes treated with NH_4Cl did not swell or lift compared with those under Glu treatment. Instead, the swelling caused cell bodies to spread and result in a reduction of the extracellular spaces (Fig. 1Ba). The cells did not bear cell processes as obvious as those under Glu treatment. Nuclei showed swelling, rounding and moving into focus by 1 hr (Fig. 1Ba, star). The increase in nuclear size was not as fast and dramatic as under Glu treatment. The swelling was apparent only at 5 and 6 hr. The swollen nuclei under NH_4Cl treatment appeared to be less phase bright at 6 hr. This is possibly due to the concealment of nuclear swelling by increased cytoplasmic coverage or confined movement of the spreading cell, pulling them out of the focal plane.

Swelling was more obvious after reconstruction of the phase-contrast images into three-dimensional color images (Fig. 1Ab, Bb). The cell processes were the first to become red within 1 hr of Glu treatment (Fig. 1Ab), and the edges of the nuclear area followed. This indicated that the cytoplasm became progressively swollen, followed by the nuclear swelling. Relative height changes of the cell body were not significant under NH_4Cl treatment, but changes around the nucleus were obvious between 1 and 5 hr (Fig. 1Bb). Figure 1C is a schematic diagram illustrating the differences in morphological changes in astrocytes under Glu or NH_4Cl treatments.

AFM Analysis on Nuclear Swelling and Nanochange of Plasma Membrane

AFM could be used to estimate the nuclear volume changes by measuring changes in the height of the highest membrane area (Oberleithner et al., 2003). We used this parameter of height changes to illustrate further the nuclear swelling in astrocytes under Glu and NH_4Cl treatment. Control astrocytes exhibited a relatively smooth membrane as indicated by its maximum height deviation (~392 nm), with subcellular structures under the membrane not clearly distinguishable and cell-to-cell boundaries not clearly defined (Fig. 2Ab, thin arrow).

The maximum height deviation increased to ~609 nm at 12 hr of Glu treatment as a result of swelling (Fig. 2B). Cell boundaries were clearly distinguishable (Fig. 2Bb, thin arrow), and dense finger-like structures were present on the membrane (Fig. 2Bc, d, arrowheads). The nucleus (Fig. 2Bb, d, thick arrows) could be distinguished based on the height difference from the cell body. Cytoplasmic swelling under 12 hr NH_4Cl treatment was not as apparent as under Glu treatment, and the cell boundary appeared to be less distinct (Fig. 2C, thin arrow). Large, tuber-like structures (Fig. 2Cc, d, arrowheads) were located on the membrane, and the protruding swollen nucleus was easily detected (maximum height deviation ~726 nm), with the nucleus (light yellow) being the highest area.

Nuclear Area Changes in Astrocytes under Glu and NH_4Cl Treatment

The control nuclei at 0 hr had a mean area of 155.9 $\pm$ 35.3 μm^2 (n = 833), which was only slightly and insignificantly increased to 164.8 $\pm$ 42.9 μm^2 (n = 625) after 12 hr. Treatment with 1 mM Glu increased the relative nuclear area by 6.7% $\pm$ 1.5% (n = 426) at 4 hr and 11.2% $\pm$ 0.9% (n = 500) at 8 hr (Fig. 3C). Although reduced to 4.9% $\pm$ 1.3% (n = 516) at 12 hr, this mean area was still significantly higher than that of the control (Fig. 3C). The numbers of cells measured were increased to ~1,500 for each time point after finding a greater variation in nuclear sizes under 5 mM NH_4Cl treatment. Nuclear area was not changed significantly at 4 hr (1.1% $\pm$ 0.8%, n = 1,538) or 8 hr (0.8% $\pm$ 0.9%, n = 1,469; Fig. 3C). However, the relative nuclear area at 12 hr of treatment was found to be equivalent to that of 12 hr Glu treatment (4.3% $\pm$ 0.8%, n = 1,316; Fig. 3C).

Statistical analysis at the 95% confidence level for all the measured nuclear areas in the control cultures revealed that the nuclear area could be split into three categories: small, <124 μm^2; medium, 124–184 μm^2; and large, >184 μm^2 (Fig. 3D). The nuclear area distribution in control cultures was 11% small, 74% medium, and 15% large. Glu treatment for 4 hr increased the large nuclear area pool to 34% and reduced the medium pool to 52% (Fig. 3D). At 8–12 hr, the small pool was reduced to 7%, but the large pool remained at 35%. In contrast, 4 hr NH_4Cl treatment induced a dual change. The small nuclei pool increased to 22% (shrinkage) and the large pool to 28% (swelling; Fig. 3D). This distribution pattern remained unchanged at 8 hr. At 12 hr, the small nuclei pool was reduced significantly to 8%, and the overall nuclear area distribution resembled that under 12 hr Glu treatment (Fig. 3D). Therefore, the nucleus swells faster under Glu than NH_4Cl treatment.

Dosage Effects of Glu and NH_4Cl on Nuclear Size Change

We treated astrocytes in cultures for 8 hr with increasing doses of Glu (0.1, 0.2, 0.5, 1 and 2 mM; Fig. 3E) and NH_4Cl (1, 2, 5 and 10 mM; Fig. 3F). Glu at 0.5 mM could induce a significant nuclear area increase to 14.8% $\pm$ 1.4% (n = 384), and so did the higher doses such as 1 mM to 14.9% $\pm$ 1.3% (n = 434) and 2 mM to 17.9% $\pm$ 1.4% (n = 424). There is a slight but statistically insignificant increase in nuclear area at lower doses. NH_4Cl at 2 mM significantly increased nuclear

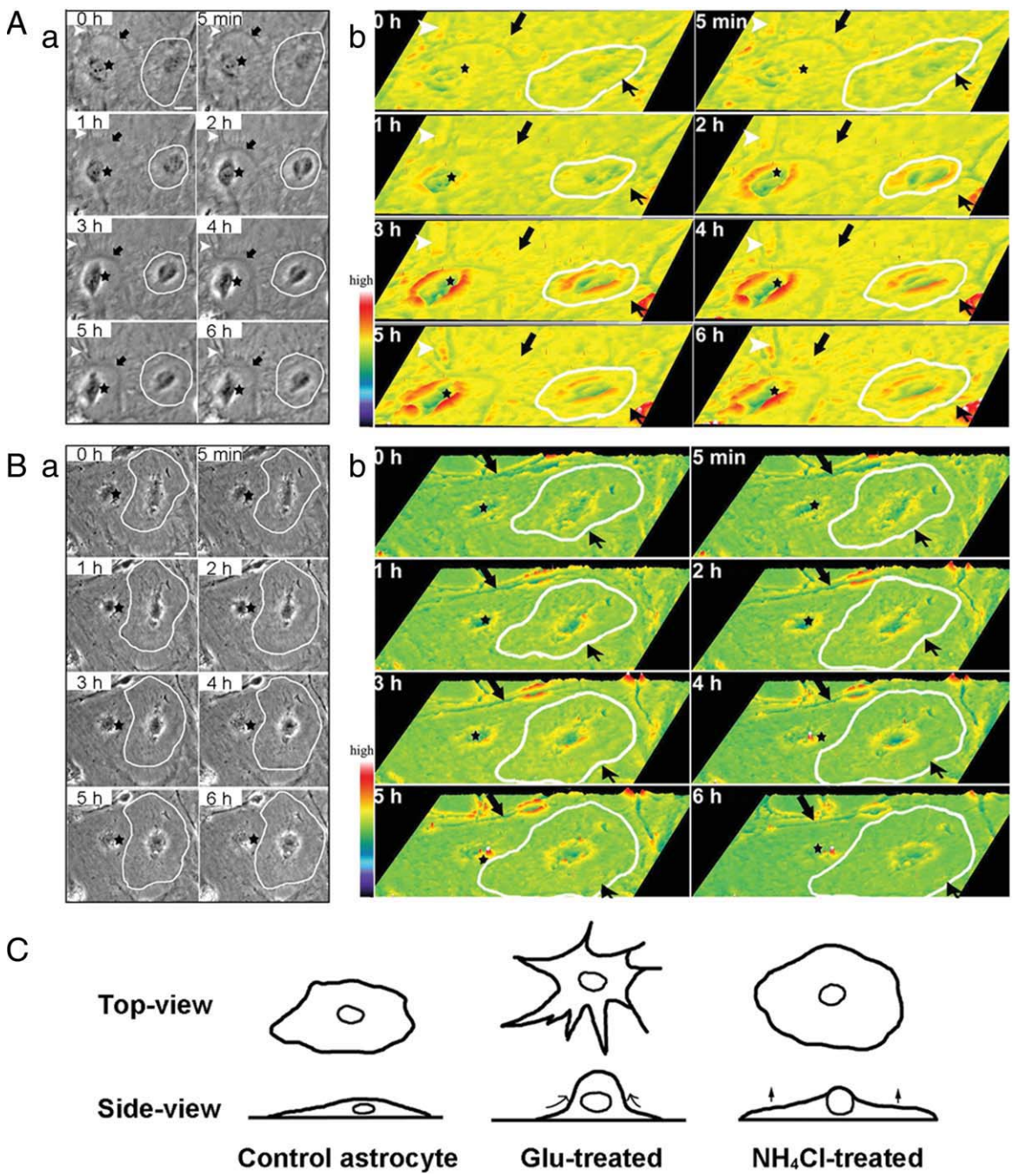

Fig. 1. Swelling effects of Glu and NH_4Cl on primary culture of astrocytes. Time-lapse phase-contrast microscopy recording for 6 hr (**A, B**). Aa: Glu-induced cytoplasmic swelling and nuclear swelling were observed in 5 min. Cell boundary (black arrow) and nucleus (black star) became visible. Cell processes (white arrowhead) appeared at the cell boundary. Astrocyte at right is outlined to highlight changes in cell area. Ba: NH_4Cl induced the nucleus (black star) to round up and become phase bright in 1 hr. The astrocyte at right is outlined to highlight changes in cell area. Ab, Bb: Intensity profile analysis of Aa and Ba. The color bar indicates light intensity and relative grayness. Ab: Under Glu treatment, the cell boundary (thick black arrow) and processes (white arrowhead) became red. The nuclear region (black star) swelled, and the extracellular space (thin black arrow) decreased. The cytoplasmic area is outlined. Bb: No height changes in the cell boundary under NH_4Cl treatment (thick black arrow). The nuclear region (black star) had increased in height by 1–5 hr. The extracellular space (thin black arrow) decreased. The cytoplasmic area is outlined. **C:** Diagrams to compare Glu- and NH_4Cl -induced swelling in astrocytes. Scale bars = 40 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

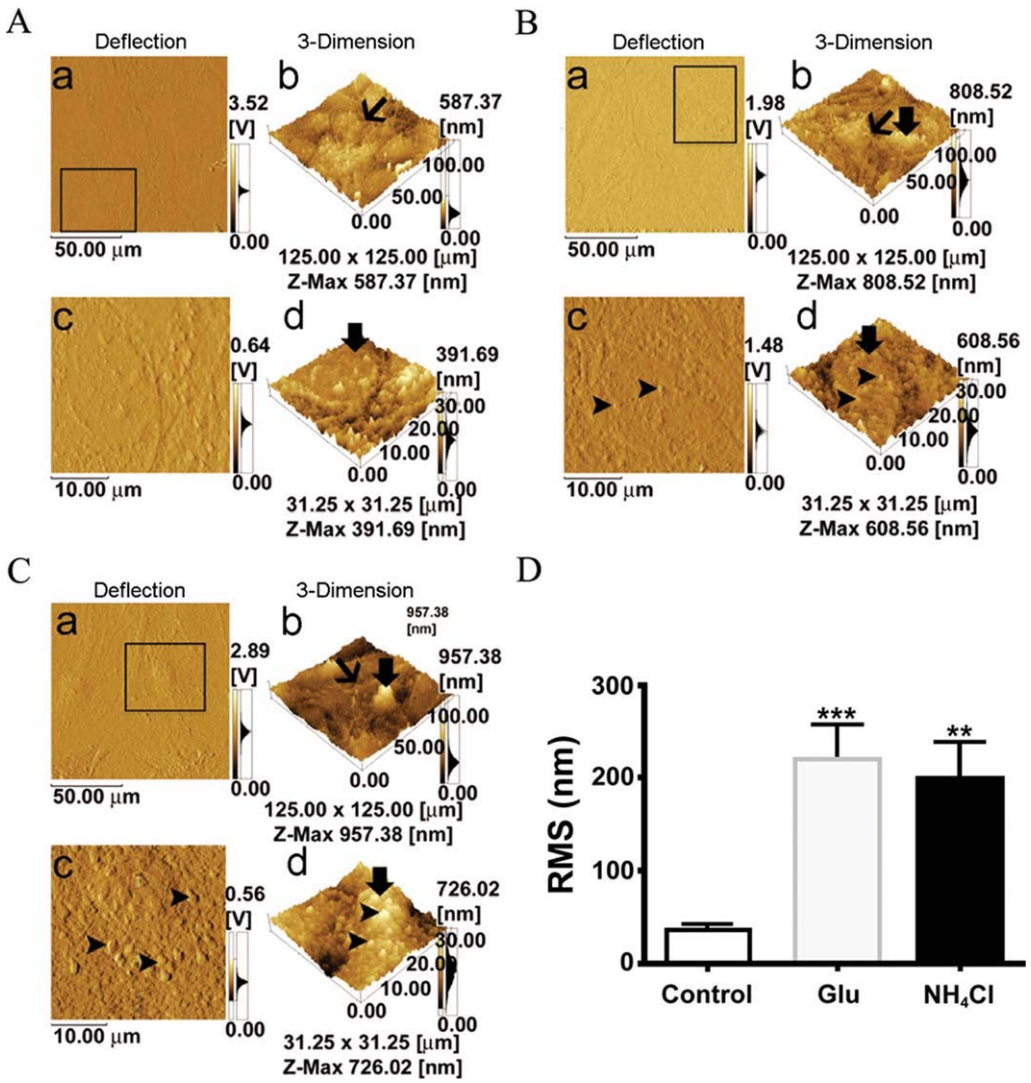

Fig. 2. Nanochanges of the astrocytic membrane following 12 hr of treatment with Glu or NH_4Cl were analyzed by AFM. Deflection and three-dimensional views are shown in two resolutions. AFM images in two different views: deflection (a, c) and three dimensions (b, d). The boxed area in a corresponds to the area in b, viewed at a higher resolution in c and d, respectively. **A:** Control astrocytes. The membrane appeared smooth. The cell boundary (thin arrow) and nuclear area (thick arrow) could not be clearly distinguished. **B:** Glu-treated astrocytes. The membrane was rougher, with peak-like structures (arrowheads). The cell boundary (thin arrow) and nuclear area (thick arrow) became clearer. **C:** NH_4Cl-treated astrocytes. The membrane appeared rougher. Arrowheads indicate membrane protrusions, "tubers." The nuclear area (thick arrow) protruded above the cell body. The cell boundary (thin arrow) was less distinct. **D:** Comparison of membrane roughness of astrocytes under Glu and NH_4Cl treatment. Both Glu and NH_4Cl significantly increased membrane roughness by sevenfold. $^{**}P < 0.01$, $^{***}P < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

area to 7.5% ± 1.4% (n = 433) and 5 mM to 6.2% ± 1.2% (n = 439).

Response of Nuclei Isolated From Astrocytes to Glu and NH_4Cl Treatment

We used nuclei acutely isolated from astrocytes in culture to elucidate the direct effect of Glu (1 mM) and NH_4Cl (5 mM) on nuclear size change. The average area of isolated nuclei was measured to be ~69.5 μm^2, considerably smaller than the ~155.9 μm^2 in intact cells (Fig. 4A). Areas of the isolated nuclei did not change significantly during the 12 hr control incubation (data not shown). We tested the integrity and viability of these isolated nuclei by treatment with hypotonic solution (150 Osm; Highland et al., 1991; Mladenovic et al., 1995; Lionetto et al., 2005). The nuclear area increased to 122.0% ± 4.9% (n = 260) at 4 hr and to 109.7% ± 2.7% (n = 289) at 8 hr under hypotonic treatment (Fig. 4B). However, this swelling response subsided 16 hr after isolation (data not shown).

Glu treatment for 4 hr significantly increased the area of these isolated nuclei by 19.7% ± 2.3% (n =

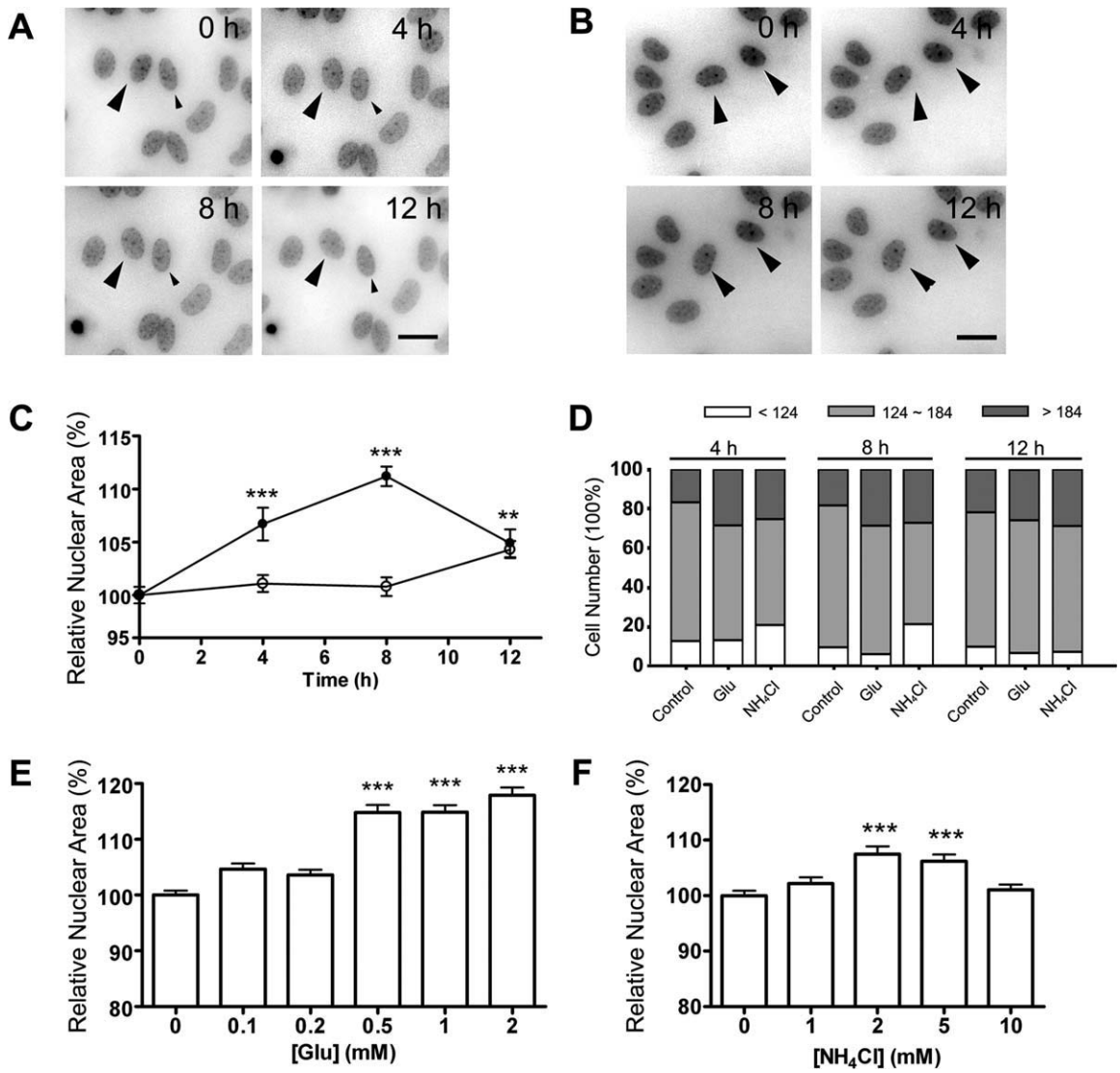

Fig. 3. Nuclear area changes after Glu or NH_4Cl treatments of astrocytes in culture. Fluorescent images of astrocytic nuclei treated with Glu (**A**) and NH_4Cl (**B**). Glu and NH_4Cl induced nuclear size changes (large arrowhead). Some nuclear size recovery was observed at 12 hr of Glu treatment (small arrowhead). **C:** Relative nuclear areas of control at each time point. Glu (solid circles) induced a significant increase in nuclear area at 4, 8 and 12 hr. NH_4Cl (open circles) did not increase nuclear area until 12 hr. **D:** Nuclear size distribution analysis. Nuclear sizes were categorized into small (<124 μm^2), medium (124–184 μm^2), and large (>184 μm^2). The numbers of nuclei in C were recounted, categorized, and analyzed by χ^2 test. **E:** Glu induced a dose effect on astrocytic nuclei. **F:** NH_4Cl induced a dose effect on astrocytic nuclei. Data are mean ± SEM from at least three batches of astrocyte culture and from three independent experiments; n = 426–1,538 for each time point. Significant differences from control are indicated by $^{**}P < 0.01$, $^{***}P < 0.001$ (Bonfferoni posttest). Scale bars = 20 μm.

391). The area remained swollen at 8 hr by 13.5% ± 1.8% (n = 378) and at 12 hr by 6.7% ± 4.5% (n = 372; $P > 0.05$). Even though the swelling was reduced with time, it was still significantly greater than in the control. However, the area of isolated nuclei under NH_4Cl treatment was significantly reduced at 4 hr by 19.2% ± 1.7% (n = 543), at 8 hr by 24.6% ± 0.9% (n = 882), and at 12 hr by 10.3% ± 2.7% (n = 186; Fig. 4B).

The modal values (values with the highest frequency) for nuclear area distributions allowed us to compare the differences between Glu and NH_4Cl effects on isolated nuclei (Fig. 4C). The modal values for nuclear area of the control remained at 60 μm^2 at 4 and 8 hr (Fig. 4C). Hypotonic and Glu treatments increased the modal values to 80 μm^2 at 4 hr and to 60 μm^2 at 8 hr; whereas NH_4Cl reduced the modal values to 40 μm^2 under all incubation conditions. These findings confirmed that Glu induced nuclear swelling and NH_4Cl induced shrinkage.

We treated these isolated nuclei with various dosages of Glu (Fig. 4D) and NH_4Cl (Fig. 4E) for 4 hr. Similarly to nuclei in intact astrocytes, the area of

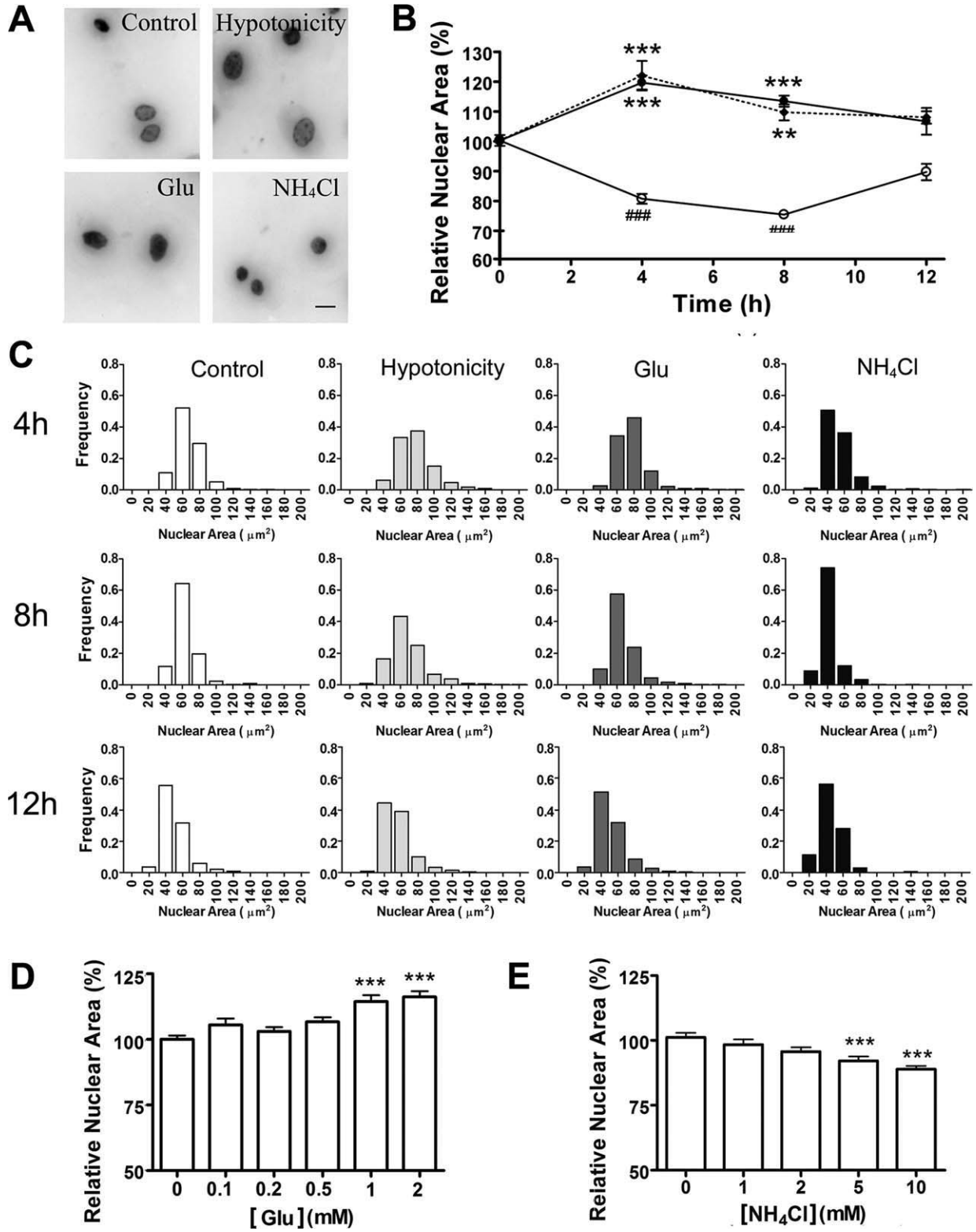

Fig. 4. Changes in isolated nuclei under Glu or NH_4Cl treatment. Isolated nuclei from astrocytes in culture were treated with 1 mM Glu or 5 mM NH_4Cl. Nuclei were stained with Hoechst 33342. **A:** Fluorescent images of isolated nuclei after 8 hr treatments. **B:** Swelling under hypotonic (150 Osm; lozenges), Glu (solid circles), and NH_4Cl (open circles) treatment for 4, 8, and 12 hr. **C:** Nuclear size frequency distribution of isolated nuclei from astrocytes under hypotonic, Glu, and NH_4Cl treatment for 4, 8, and 12 hr. **D:** Dose effect of Glu on isolated nuclei from astrocytes. **E:** Dose effect of NH_4Cl on nuclei isolated from astrocytes. Significant differences from control are indicated by $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$. Significant decreases from control are indicated by $^{\#\#\#}P < 0.001$ (Bonferroni posttest). Scale bar = 10 μm.

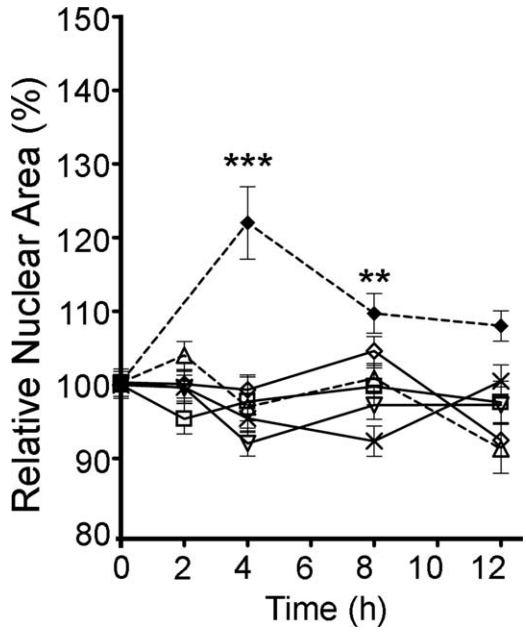

Fig. 5. Nuclear area changes under glutamine, taurine, aspartate, glycine, and ATP treatments. Nuclei isolated from astrocytes in culture were treated with hypotonic solution (solid lozenges), 1 mM glutamine (upward-pointing triangles), 1 mM taurine (open lozenges), 1 mM aspartate (downward-pointing triangles), 1 mM glycine (squares), and 1mM ATP (crosses). Nuclei were fixed and stained with Hoechst 33342 after treatments. Data are mean ± SEM from at least two batches of cultures and two independent experiments; n = 88–437 for each time point. Significant increases from control are indicated by $^{**}P < 0.01$, $^{***}P < 0.001$ (Bonferroni posttest).

isolated nuclei increased by 13.9% ± 2.5% (n = 369) at 1 mM and by 15.7% ± 2.2% (n = 429) at 2 mM Glu, although the area was reduced significantly by 7.8% ± 1.6% (n = 304) at 5 mM and by 11.1% ± 1.3% (n = 429) at 10 mM NH_4Cl.

Effects of Glutamine, Aspartate, Taurine, ATP, and Glycine on Nuclear Size

We treated isolated nuclei from astrocytes with 1 mM glutamine, 1 mM aspartate, 1 mM taurine, 1 mM ATP, and 1 mM glycine and measured the changes in area of these nuclei as a function of time of treatment. These treatments will allow us to elucidate whether the responses of astrocytic nuclei to Glu and NH_4Cl are specific. Interestingly, we did not find any significant changes in nuclear area under these treatments (Fig. 5).

Glu and NH_4Cl Effects on Nuclear Size in Other Cell Types

We examined whether Glu and NH_4Cl could induce similar nuclear responses in microglia in primary cultures and cell lines such as C6, HEK 293, and Hep G2. Both intact cells and their acutely isolated nuclei were treated with Glu and NH_4Cl, and the changes in nuclear area were measured. Surprisingly, Glu and NH_4Cl did not induce any detectable changes in nuclear size in any of these cells or their isolated nuclei (Fig. 6A–H).

DISCUSSION

This study investigated astrocytic swelling under treatments with Glu and NH_4Cl. We observed a nuclear size change in astrocytes under these treatments that is concomitant with but independent of the swelling of cytoplasm. We also investigated these nuclear responses to Glu and NH_4Cl with nuclei isolated from astrocytes. Isolated nuclei under hypotonic treatments became swollen, suggesting that these nuclei were intact and capable of undergoing significant swelling. We found that Glu induces a direct swelling on isolated astrocyte nuclei, whereas NH_4Cl induces shrinkage. These effects of Glu and NH_4Cl appeared to be astrocyte specific, insofar as we could not observe similar nuclear swelling with compounds closely related to Glu or in many other cell types.

These nuclear swelling responses to Glu and NH_4Cl in astrocyte were further confirmed in our AFM studies. The AFM observations showed that cytoplasm and nuclei swelled at different rates. AFM also illustrated membrane changes in astrocyte under Glu and NH_4Cl treatments. These changes were reflected by an increase in roughness of the cell membrane. Glu treatment induced dense, finger-like peak structures (Fig. 2Bc, d, arrowheads), whereas NH_4Cl treatment induced large, tuber-like structures (Fig. 2Cc, d, arrowheads) on the plasma membrane. We have previously reported that arachidonic acid could induce changes on astrocyte membrane as observed under AFM (Xu et al., 2009). Others have reported that peaks resulted from the anchoring cytoskeleton and that tubers were from stiff membrane proteins protruding from the membrane surface (Viitala and Jarnefelt, 1985; Nowakowski et al., 2001). Some studies showed that membrane changes observed under AFM may be related to cytoskeleton rearrangement (Weissmuller et al., 2000; Nowakowski et al., 2001). The cytoskeleton rearrangement was also shown to involve cell/nuclear volume changes (Pedersen et al., 2001; Morris, 2002), ion transportation (Ahmed et al., 2000), and water homeostasis via aquaporins (Nicchia et al., 2005). These changes in membrane surface morphology after Glu and NH_4Cl treatments might be an important index to reveal the cellular response to specific treatments and are worthy of further investigation.

During neuronal activity, astrocytes are responsible for removing most of the Glu from the synapse (Yu and Hertz, 1982; Danbolt, 2001; Hertz and Zielke, 2004; Yang et al., 2008). Astrocytes internalize extracellular Glu via Glu transporters on their cytoplasmic membrane accompanied by a translocation of water molecules to induce an osmotic response (MacAulay et al., 2001, 2002; Yang et al., 2008). Internalized Glu reacts with ammonia to form Gln by glutamine synthetase in the well-known Glu-Gln-Glu cycle (Yu and Hertz, 1982; Hertz et al., 1999; Hertz and Zielke, 2004; McKenna,

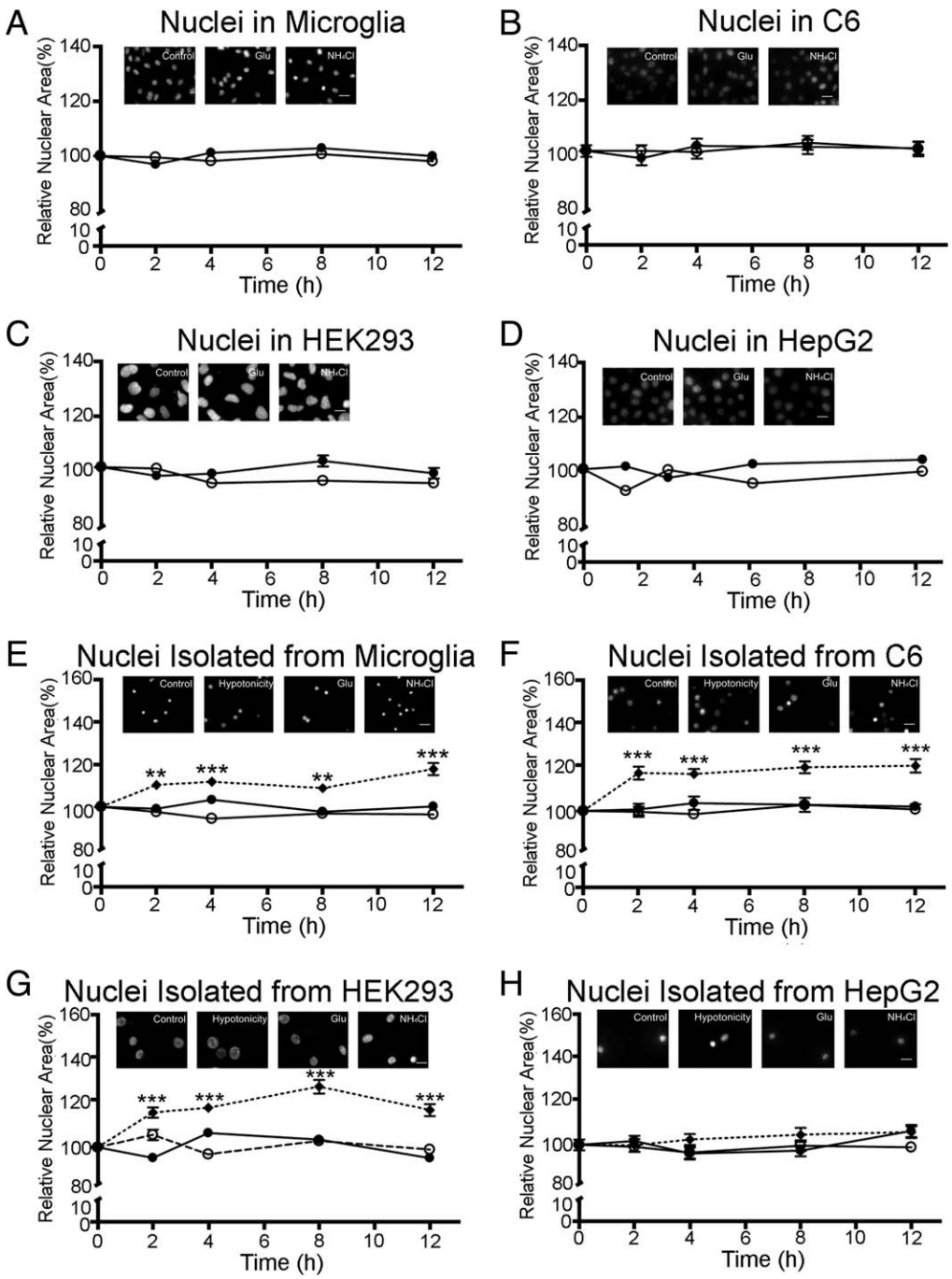

Fig. 6. Nuclear area changes in other cell types under Glu and NH_4Cl treatments. **Insets** are fluorescent images of nuclei at 4 hr of treatment. **A–D:** Nuclear area changes in intact microglia, C6, HEK 293, and Hep G2 in cultures. Nuclei in these four types of cells did not display swelling when treated with 1 mM Glu (solid circles) or 5 mM NH_4Cl (open circles) during the 12 hr treatment period. **E–H:** Area changes of nuclei isolated from microglia, C6, HEK 293, and Hep G2 treated with hypotonic solution (lozenges), 1 mM Glu (solid circles), or 5 mM NH_4Cl (open circles). Data are means ± SEM from at least two batches of cultures and two independent experiments; n = 76–1,294 for each time point. Significant increases from control are indicated by $^{**}P < 0.01$, $^{***}P < 0.001$ (Bonferroni posttest). Scale bar = 10 μm.

2007; Yang et al., 2008). Glu can also be metabolized into CO_2 through the TCA cycle (Yu and Hertz, 1982; McKenna et al., 1996; Yang et al., 2008). The activities of these two metabolic pathways control the intracellular Glu concentration in astrocytes. Although the tested concentration (1 mM) of Glu appeared to be relatively high, it was still within the range (2 μM to 1 mM) of Glu concentrations reported for the synaptic cleft

(Meldrum, 2000). This concentration has been used in other in vitro experiments (Koyama et al., 1991; O'Connor et al., 1993). Glu treatment increases nuclear sizes to a peak at 8 hr, and then they decrease at 12 hr. This accords well with the changes of Glu concentrations in astrocytes (Fig. 3C). Experiments on isolated nuclei confirmed that nuclear swelling was a direct effect of Glu.

Astrocytic membranes are permeable to NH_3. NH_4^+ enters astrocytes through specific cotransporters, such as the NH_4^+-Cl^- cotransporter, and other NH_4^+ high-affinity channels (Nagaraja and Brookes, 1998; Marcaggi and Coles, 2001; Marcaggi et al., 2004; Winkler, 2006). Several reports have shown a significant decrease in Glu and an increase in Gln in brain under high-ammonia conditions (Brusilow and Traystman, 1986; Hazell and Butterworth, 1999; Felipo and Butterworth, 2002; Norenberg et al., 2005). This might be caused by the Glu-Gln-Glu cycle forming Gln from Glu in the cytoplasm, a unique feature of astrocytes to detoxify ammonia in the brain. However, the increase in Gln was not responsible for the swelling observed under NH_4Cl treatment (Jayakumar et al., 2006; Albrecht et al., 2010). Our observation also showed that Gln and other related compounds did not induce significant swelling or shrinking of nuclei isolated from astrocytes (Fig. 5). Therefore, NH_4Cl treatment elicited a nuclear response entirely different from that of Glu treatment in astrocytes. Intact astrocytes exhibited nuclear shrinking and swelling under NH_4Cl treatment (Fig. 3D). The shrinkage may be due patially to the lowering of intracellular Glu as a result of binding with the internalized ammonia to form Gln and due patially to the direct effect of NH_4Cl on nuclei. This indicates that the early NH_4Cl-induced nuclear shrinkage observed in intact astrocytes probably was due to a combined effect. The later nuclear swelling observed in intact astrocytes under NH_4Cl treatment was in agreement with nuclear swelling observed in astrocytes under electron microscopy under clinical conditions such as hyperammonemia (Willard-Mack et al., 1996). This nuclear swelling observed in intact astrocytes probably was due to other metabolic events.

Glu mediates excitatory information to postsynaptic neurons and surrounding astrocytes. Our results indicated that, during excitation, astrocytes took up Glu and induced nuclear swelling. There are some reports indicating that nuclear swelling could induce gene expression, including transcription factors c-Jun and c-Fos (Finkenzeller et al., 1994; Sadoshima et al., 1996), ornithine decarboxylase (Lundgren, 1992; Benis and Lundgren, 1993), and tissue plasminogen activator (Lang et al., 1998). Hypertonicity has been shown to alter the karyotype (Uchida et al., 1987). However, whether nuclear swelling triggered by Glu in astrocytes could alter gene expression remains unknown.

This study revealed a specific relationship between Glu, NH_4Cl and nuclear size changes in astrocytes. Although the underlying mechanisms and subsequent effects of Glu-induced nuclear swelling are still unknown, our preliminary studies have demonstrated that nuclear swelling involves Glu entering the nucleus and requires unique aquaporin expression on astrocytic nuclear membrane (data unpublished). The lack of similar nuclear responses to Glu and NH_4Cl of intact and isolated nuclei from other cell types and cell lines reveals that this response is astrocyte specific. Therefore, Glu- and NH_4Cl-induced nuclear size changes in astrocytes could be considered a new addition to the list of cell specializations of astrocytes.

REFERENCES

Ahmed N, Ramjeesingh M, Wong S, Varga A, Garami E, Bear CE. 2000. Chloride channel activity of ClC-2 is modified by the actin cytoskeleton. Biochem J 352:789–794.

Albrecht J, Zielinska M, Norenberg MD. 2010. Glutamine as a mediator of ammonia neurotoxicity: a critical appraisal. Biochem Pharmacol 80:1303–1308.

Benis RC, Lundgren DW. 1993. Sodium-dependent co-transported analogues of glucose stimulate ornithine decarboxylase mRNA expression in LLC-PK1 cells. Biochem J 289:751–756.

Bennett MR, Farnell L, Gibson WG. 2009. P2X7 regenerative-loop potentiation of glutamate synaptic transmission by microglia and astrocytes. J Theor Biol 261:1–16.

Brusilow SW, Traystman R. 1986. Hepatic encephalopathy. N Engl J Med 314:786–787; author reply 787.

Brusilow SW, Koehler RC, Traystman RJ, Cooper AJ. 2010. Astrocyte glutamine synthetase: importance in hyperammonemic syndromes and potential target for therapy. Neurotherapeutics 7:452–470.

Butterworth RF. 2010. Altered glial–neuronal crosstalk: cornerstone in the pathogenesis of hepatic encephalopathy. Neurochem Int 57:383–388.

Danbolt NC. 2001. Glutamate uptake. Prog Neurobiol 65:1–105.

Dong Y, Liu HD, Zhao R, Yang CZ, Chen XQ, Wang XH, Lau LT, Chen J, Yu AC. 2009. Ischemia activates JNK/c-Jun/AP-1 pathway to up-regulate 14-3-3gamma in astrocyte. J Neurochem 109(Suppl 1):182–188.

Felipo V, Butterworth RF. 2002. Neurobiology of ammonia. Prog Neurobiol 67:259–279.

Finkenzeller G, Newsome W, Lang F, Haussinger D. 1994. Increase of c-jun mRNA upon hypo-osmotic cell swelling of rat hepatoma cells. FEBS Lett 340:163–166.

Gregorios JB, Mozes LW, Norenberg MD. 1985. Morphologic effects of ammonia on primary astrocyte cultures. II. Electron microscopic studies. J Neuropathol Exp Neurol 44:404–414.

Gunnarson E, Zelenina M, Axehult G, Song Y, Bondar A, Krieger P, Brismar H, Zelenin S, Aperia A. 2008. Identification of a molecular target for glutamate regulation of astrocyte water permeability. Glia 56:587–596.

Gunnarson E, Song Y, Kowalewski JM, Brismar H, Brines M, Cerami A, Andersson U, Zelenina M, Aperia A. 2009. Erythropoietin modulation of astrocyte water permeability as a component of neuroprotection. Proc Natl Acad Sci U S A 106:1602–1607.

Hansson E, Johansson BB, Westergren I, Ronnback L. 1994. Glutamate-induced swelling of single astroglial cells in primary culture. Neuroscience 63:1057–1066.

Haydon PG, Carmignoto G. 2006. Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev 86:1009–1031.

Hazell AS, Butterworth RF. 1999. Hepatic encephalopathy: an update of pathophysiologic mechanisms. Proc Soc Exp Biol Med 222:99–112.

Hertz L, Zielke HR. 2004. Astrocytic control of glutamatergic activity: astrocytes as stars of the show. Trends Neurosci 27:735–743.

Hertz L, Dringen R, Schousboe A, Robinson SR. 1999. Astrocytes: glutamate producers for neurons. J Neurosci Res 57:417–428.

Highland HN, Rao MV, Chinoy NJ, Shah VC. 1991. Analysis of the functional and nuclear integrity of human spermatozoa. Int J Fertil 36:43–47.

Jayakumar AR, Rao KV, Murthy CR, Norenberg MD. 2006. Glutamine in the mechanism of ammonia-induced astrocyte swelling. Neurochem Int 48:623–628.

Kimelberg HK, Rose JW, Barron KD, Waniewski RA, Cragoe EJ. 1989. Astrocytic swelling in traumatic-hypoxic brain injury. Beneficial effects of an inhibitor of anion exchange transport and glutamate uptake in glial cells. Mol Chem Neuropathol 11:1–31.

Koyama Y, Baba A, Iwata H. 1991. L-glutamate-induced swelling of cultured astrocytes is dependent on extracellular Ca^{2+}. Neurosci Lett 122:210–212.

Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D. 1998. Functional significance of cell volume regulatory mechanisms. Physiol Rev 78:247–306.

Lau LT, Yu AC. 2001. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. J Neurotrauma 18:351–359.

Lehmann C, Bette S, Engele J. 2009. High extracellular glutamate modulates expression of glutamate transporters and glutamine synthetase in cultured astrocytes. Brain Res 1297:1–8.

Lionetto MG, Giordano ME, De Nuccio F, Nicolardi G, Hoffmann EK, Schettino T. 2005. Hypotonicity induced K^+ and anion conductive pathways activation in eel intestinal epithelium. J Exp Biol 208:749–760.

Liva SM, de Vellis J. 2001. IL-5 induces proliferation and activation of microglia via an unknown receptor. Neurochem Res 26:629–637.

Lundgren DW. 1992. Effect of hypotonic stress on ornithine decarboxylase mRNA expression in cultured cells. J Biol Chem 267:6841–6847.

MacAulay N, Gether U, Klaerke DA, Zeuthen T. 2001. Water transport by the human Na^+-coupled glutamate cotransporter expressed in *Xenopus* oocytes. J Physiol 530:367–378.

MacAulay N, Gether U, Klaerke DA, Zeuthen T. 2002. Passive water and urea permeability of a human Na^+-glutamate cotransporter expressed in *Xenopus* oocytes. J Physiol 542:817–828.

Marcaggi P, Coles JA. 2001. Ammonium in nervous tissue: transport across cell membranes, fluxes from neurons to glial cells, and role in signalling. Prog Neurobiol 64:157–183.

Marcaggi P, Jeanne M, Coles JA. 2004. Neuron–glial trafficking of NH_4^+ and K^+: separate routes of uptake into glial cells of bee retina. Eur J Neurosci 19:966–976.

McKenna MC. 2007. The glutamate-glutamine cycle is not stoichiometric: fates of glutamate in brain. J Neurosci Res 85:3347–3358.

McKenna MC, Tildon JT, Stevenson JH, Huang X. 1996. New insights into the compartmentation of glutamate and glutamine in cultured rat brain astrocytes. Dev Neurosci 18:380–390.

Meldrum BS. 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130:1007S–1015S.

Mladenovic I, Micic S, Genbacev O, Papic N. 1995. Hypoosmotic swelling test for quality control of sperm prepared for assisted reproduction. Arch Androl 34:163–169.

Morris CE. 2002. How did cells get their size? Anat Rec 268:239–251.

Nagaraja TN, Brookes N. 1998. Intracellular acidification induced by passive and active transport of ammonium ions in astrocytes. Am J Physiol 274:C883–C891.

Nicchia GP, Srinivas M, Li W, Brosnan CF, Frigeri A, Spray DC. 2005. New possible roles for aquaporin-4 in astrocytes: cell cytoskeleton and functional relationship with connexin43. FASEB J 19:1674–1676.

Norenberg MD, Rao KV, Jayakumar AR. 2005. Mechanisms of ammonia-induced astrocyte swelling. Metab Brain Dis 20:303–318.

Nowakowski R, Luckham P, Winlove P. 2001. Imaging erythrocytes under physiological conditions by atomic force microscopy. Biochim Biophys Acta 1514:170–176.

Oberleithner H, Schneider SW, Albermann L, Hillebrand U, Ludwig T, Riethmuller C, Shahin V, Schafer C, Schillers H. 2003. Endothelial cell swelling by aldosterone. J Membr Biol 196:163–172.

O'Connor ER, Kimelberg HK, Keese CR, Giaever I. 1993. Electrical resistance method for measuring volume changes in monolayer cultures applied to primary astrocyte cultures. Am J Physiol 264:C471–C478.

Pedersen SF, Hoffmann EK, Mills JW. 2001. The cytoskeleton and cell volume regulation. Comp Biochem Physiol A Mol Integr Physiol 130:385–399.

Sadoshima J, Qiu Z, Morgan JP, Izumo S. 1996. Tyrosine kinase activation is an immediate and essential step in hypotonic cell swelling-induced ERK activation and c-fos gene expression in cardiac myocytes. EMBO J 15:5535–5546.

Schneider GH, Baethmann A, Kempski O. 1992. Mechanisms of glial swelling induced by glutamate. Can J Physiol Pharmacol 70(Suppl): S334–S343.

Uchida S, Green N, Coon H, Triche T, Mims S, Burg M. 1987. High NaCl induces stable changes in phenotype and karyotype of renal cells in culture. Am J Physiol 253:C230–C242.

Viitala J, Jarnefelt J. 1985. The red cell surface revisited. Trends Biochem Sci 10:392–395.

Wang H, Clapham DE. 1999. Conformational changes of the in situ nuclear pore complex. Biophys J 77:241–247.

Weissmuller G, Garcia-Abreu J, Mascarello Bisch P, Moura Neto V, Cavalcante LA. 2000. Glial cells with differential neurite growth-modulating properties probed by atomic force microscopy. Neurosci Res 38:217–220.

Willard-Mack CL, Koehler RC, Hirata T, Cork LC, Takahashi H, Traystman RJ, Brusilow SW. 1996. Inhibition of glutamine synthetase reduces ammonia-induced astrocyte swelling in rat. Neuroscience 71:589–599.

Winkler FK. 2006. Amt/MEP/Rh proteins conduct ammonia. Pflugers Arch 451:701–707.

Xu ZY, Liu HD, Lau LT, Yingge Z, Zhao R, Tong GL, Chan PH, Yu AC. 2009. Responses of astrocyte to simultaneous glutamate and arachidonic acid treatment. Neurochem Int 55:143–150.

Yang CZ, Zhao R, Dong Y, Chen XQ, Yu AC. 2008. Astrocyte and neuron intone through glutamate. Neurochem Res 33:2480–2486.

Yu AC, Hertz L. 1982. Uptake of glutamate, GABA, and glutamine into a predominantly GABA-ergic and a predominantly glutamatergic nerve cell population in culture. J Neurosci Res 7:23–35.

Yu AC, Lau LT. 2000. Expression of interleukin-1 alpha, tumor necrosis factor alpha and interleukin-6 genes in astrocytes under ischemic injury. Neurochem Int 36:369–377.

Zonta M, Carmignoto G. 2002. Calcium oscillations encoding neuron-to-astrocyte communication. J Physiol Paris 96:193–198.

Zwingmann C. 2007. Nuclear magnetic resonance studies of energy metabolism and glutamine shunt in hepatic encephalopathy and hyperammonemia. J Neurosci Res 85:3429–3442.