

RESEARCH PAPER

Angiotensin receptor type 1 antagonists protect against neuronal injury induced by oxygen–glucose depletion

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BACKGROUND AND PURPOSE

Several clinical trials and *in vivo* animal experiments have suggested that blockade of angiotensin receptor type 1 (AT₁) improves ischaemic outcomes. However, the mechanism(s) underlying these effects has not been elucidated. Here, we have investigated the protective effects of pretreatment with AT₁ receptor antagonists, losartan or telmisartan, against ischaemic insult to neurons *in vitro*.

EXPERIMENTAL APPROACH

Primary rat neuron–astrocyte co-cultures and astrocyte-defined medium (ADM)-cultured pure astrocyte cultures were prepared. Ischaemic injury was modelled by oxygen–glucose depletion (OGD) and lactate dehydrogenase release after OGD was measured with or without AT₁ receptor antagonists or agonists (L162313), AT₂ receptor antagonist (PD123319) or agonist (CGP-42112A) pretreatment, for 48 h. Activity of glutamate transporter 1 (GLT-1) was evaluated by [³H]-glutamate uptake assays, after AT₁ receptor agonists or antagonists. Immunoblot and real-time PCR were used for analysis of protein and mRNA levels of GLT-1.

KEY RESULTS

AT₁ receptor agonists augmented OGD-induced cellular damage, which was attenuated by AT₁ receptor antagonists. AT₁ receptor antagonists also suppressed OGD-induced extracellular glutamate release, reactive oxygen species production and nitric oxide generation. GLT-1 expression and glutamate uptake activity were significantly enhanced by AT₁ receptor antagonists and impaired by AT₁ receptor agonists. AT₁ receptor stimulation suppressed both ADM-induced GLT-1 protein expression and mRNA levels. AT_{1b} receptor knock-down with siRNA enhanced GLT-1 expression. In postnatal (P1–P21) rat brains, protein levels of GLT-1 and AT₁ receptors were inversely correlated.

CONCLUSIONS AND IMPLICATIONS

Suppression of AT₁ receptor stimulation induced GLT-1 up-regulation, which ameliorated effects of ischaemic injury.

Abbreviations

ADM, astrocyte-defined medium; Ang II, angiotensin II; AT₁, angiotensin receptor type 1; AT₂, angiotensin receptor type 2; GLT-1, glutamate transporter 1; OGD, oxygen–glucose depletion; ROS, reactive oxygen species

Introduction

Stroke, an insult to the brain, is the second leading cause of death and a major cause of adult disability worldwide (Donnan *et al.*, 2008). Among a wide variety of risk factors for strokes, elevated arterial

blood pressure is notable. Even a modest blood pressure decrease (<5 mmHg) significantly reduces the frequency of stroke events (Lo *et al.*, 2003). In addition, compared with other anti-hypertensive therapies, such as calcium-channel blockers, angiotensin-converting enzyme (ACE) inhibitors or

angiotensin II receptor (AT₁) antagonists (receptor nomenclature follows Alexander *et al.*, 2009) significantly lower the risk of stroke and lead to a comparable reduction in blood pressure, based on reports from the HOPE trial (Yusuf *et al.*, 2000), the ACCESS study (Schrader *et al.*, 2003) and the MOSES study (Schrader *et al.*, 2005). The HOPE study demonstrated that ramipril, an ACE inhibitor, significantly reduced the rates of death, myocardial infarction and stroke in high-risk patients. In ACCESS, candesartan cilexetil, an AT₁ receptor antagonist, was reported to have beneficial effects in cerebral and myocardial ischaemia. The MOSES study compared an AT₁ receptor antagonist with a calcium channel blocker in secondary stroke prevention. In high-risk hypertensive stroke patients, an early normotensive and comparable blood pressure was achieved. The AT₁ receptor antagonist, eprosartan, was reported to be more effective than nitrendipine, a calcium channel blocker. Therefore, in addition to blood pressure control, there are distinct mechanisms for the protection against cerebrovascular and cardiovascular events with ACE inhibitors or AT₁ receptor antagonists that still need to be elucidated.

A separate renin–angiotensin system (RAS), independent of the peripheral RAS, exists in the vertebrate CNS (Phillips, 1987) and all the components of the classical RAS, including angiotensinogen, renin, ACE and angiotensin II (Ang II), are present in the brain (Dzau *et al.*, 1986). The brain RAS plays an important role in the regulation of neurogenic hypertension (Gyurko *et al.*, 1993), cerebrovascular fluid homeostasis (Paulson *et al.*, 1988) and sodium intake (Weisinger *et al.*, 1997). In recent years, clinical and experimental evidence has suggested that brain RAS is also involved in strokes (Maeda *et al.*, 1999; Inaba *et al.*, 2009), as well as in several other neurological diseases, such as epilepsy (Ramser *et al.*, 2005), Alzheimer's disease (AbdAlla *et al.*, 2009a,b; Kehoe *et al.*, 2009), Parkinson's disease (Joglar *et al.*, 2009), depression and stress disorders (Wright *et al.*, 2008). Radioimmunoassay and immunohistochemistry were used to demonstrate that brain Ang II was significantly increased at 24 h after transient middle cerebral artery occlusion (Kagiyama *et al.*, 2003).

The angiotensin receptor type 1 (AT₁) is commonly expressed in glia in the brain (Sumners *et al.*, 1991), whereas another subtype, angiotensin receptor type 2 (AT₂), is mostly present in neurons (Li *et al.*, 2005). AT₁ receptors mediate most of the physiological and pathological effects of Ang II, including the elevated angiotensin response after stroke (Phillips, 1987). In angiotensinogen-over-expressing mice, infarct size was enlarged after 1 h of transient middle cerebral artery occlusion. By

contrast, a much smaller lesion area of energy failure was reported in AT₁ receptor knock-out mice compared to the wild-type littermates (Walther *et al.*, 2002). However, several experimental studies have indicated that alternative over-stimulation of AT₂ receptors by Ang II after blocking AT₁ receptors might contribute to the neuroprotective effects of AT₁ receptor antagonists (Li *et al.*, 2005).

Glutamate, a principal neurotransmitter essential for neuronal synaptic activity in the CNS, is released in significant amounts during strokes (Dávalos *et al.*, 1997). Excessive elevation of extracellular glutamate mainly mediates excitotoxicity and causes neuronal cell death in stroke episodes (Beal, 1992). There is no enzymatic system available for metabolizing glutamate in the extracellular space, and the only way to maintain glutamate homeostasis is through glutamate uptake via the glutamate transporter. The glutamate transporter 1 (GLT-1) in rodents (also known as EAAT2 in humans), predominantly located on astrocytes, is responsible for up to 90% of the glutamate clearance from neuronal synapses in the adult CNS (Rothstein *et al.*, 1996; Danbolt, 2001; Beart and O'Shea, 2007). Reductions or dysfunctions of GLT-1 have also been documented in several other neurological disorders, including epilepsy (Ingram *et al.*, 2001), brain tumours (Münch *et al.*, 2001), Alzheimer's disease (Sasaki *et al.*, 2009), Huntington's disease (Liévens *et al.*, 2001) and amyotrophic lateral sclerosis (ALS) (Lin *et al.*, 1998). Therefore, pharmacological up-regulation of GLT-1 is pivotal for preventing neuronal cell death after strokes and in other neurological disorders.

A relationship between AT₁ receptors and GLT-1 expression remains to be elucidated. Both AT₁ receptors and GLT-1 proteins are mainly found in astrocytes in the CNS (Danbolt, 2001; Li *et al.*, 2005), so there may be an intrinsic association between astroglial AT₁ receptors and GLT-1. In the present study, we sought to clarify the mechanisms underlying the neuroprotective effect of AT₁ receptor antagonism and its possible involvement in GLT-1 regulation. In order to remove the influence of AT₁ receptors in cerebral microvessels or arteries (Phillips, 1987), primary neuron–astrocyte co-cultures and pure astrocyte cultures were prepared, and oxygen–glucose depletion (OGD) was used to provide an *in vitro* model of ischaemia.

Methods

Cell cultures

All animal care and experimental procedures in the present study were in strict accordance with the ethical guidelines of the Kyoto University Animal

Experimentation Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. Primary neuron–astrocyte co-cultures were obtained from fetal Wistar rats (Japan SLC, Shizuoka, Japan) at embryonic day 17.5 (E17.5) (Kihara *et al.*, 1997). Briefly, pregnant rats were given an anaesthetic dose of sodium pentobarbital (80 mg·kg⁻¹, i.p.), and fetal cerebral cortices were collected and placed in ice-cold Hank's balanced salt solution. The animals were killed by overdosing with sodium pentobarbital. After removal of the meninges, the cortices were dispersed into the same buffer containing 0.25 µg·mL⁻¹ trypsin (5 mL, 37°C, 20 min). Trypsin digestion was stopped by adding a fourfold volume of Eagle's minimal essential medium (EMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2.5 mM L-glutamine and 50 µg·mL⁻¹ kanamycin (growth medium). After gentle trituration, digested tissues were centrifuged (180× g, 4°C, 10 min). The cell pellets were resuspended in 20 mL of growth medium. After filtration through 100 µm nylon mesh, cells were seeded onto 0.5% (v/v) D-polyethyleneimine-coated plates at a density of 2.1 × 10⁵ cells·cm⁻². Cultures were incubated in a humidified atmosphere of 5% CO₂–95% air at 37°C. Only mature cultures (10–14 days *in vitro*) were used in this study.

Primary astrocyte cultures were obtained from postnatal day 1 (P1) Wistar rats. Briefly, cerebral cortices were collected, dissociated by gentle trituration with fire-polished glass Pasteur pipettes and centrifuged (180× g, 4°C, 10 min). Cell pellets were resuspended in EMEM supplemented with 10% (v/v) FBS and 50 µg·mL⁻¹ kanamycin (culture medium) and filtered through a 100 µm nylon mesh. Dissociated cells were seeded into 75 cm² tissue culture flasks and incubated in a humidified atmosphere of 5% CO₂–95% air at 37°C. Each flask culture contained 1.5 brains. Cells were grown 20–24 days until confluent. Glial cultures were shaken at 400 rpm (37°C, 10 min), and the supernatant was removed. After recovering in the culture medium (37°C, 2 h), glial cultures were shaken again at a lower speed (220 rpm, 37°C, 15 h) to dislodge the microglia and oligodendrocytes. Astrocyte cultures were detached with solution minimal essential medium containing 0.25 µg·mL⁻¹ trypsin (5 mL per flask, 37°C, 15 min). An equal volume of culture medium was added to stop the trypsin digestion. The suspension was centrifuged (180× g, 24°C, 10 min) and resuspended in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% (v/v) FBS and 50 µg·mL⁻¹ kanamycin [normal medium (NM)]. Astrocyte-defined medium (ADM)-cultured astrocytes were incubated

in DMEM (high glucose) supplemented with transferrin (50 µg·mL⁻¹), sodium selenite (5.2 ng·mL⁻¹), fibronectin (1.5 µg·mL⁻¹), heparan sulphate (0.5 µg·mL⁻¹), epidermal growth factor (EGF; 10 ng·mL⁻¹), basic fibroblast growth factor (bFGF; 5 ng·mL⁻¹), insulin (5 µg·mL⁻¹) and kanamycin (50 µg·mL⁻¹) (Miller *et al.*, 1995). The seeding density was 2.5 × 10⁴ cells·cm⁻². Cells presented in the culture were shown to be astrocytes with a purity of 97 ± 2% after characterization by immunostaining with a primary specific anti-glial fibrillary acidic protein (GFAP) antibody.

OGD

OGD experiments were performed using an Anaero-Pack System (Kawahara *et al.*, 2005). Briefly, neuron–astrocyte co-cultures used for OGD experiments were washed twice with 5% CO₂–95% N₂ bubbled glucose-free Earl's balanced salt solution (EBSS), and culture media were replaced with the same EBSS. Co-cultures were placed into a 5% CO₂–95% N₂-pre-flushed (at least 15 min) anaerobic chamber. Near-anaerobic conditions were achieved using deoxygenation reagents that catalytically reacted with residual oxygen in the well-sealed anaerobic chamber. The oxygen level was monitored by an anoxic indicator in the chamber according to the manufacturer's instructions. Within 1 h of incubation at 37°C, a near-anaerobic condition of <1% O₂ concentration and >5% CO₂ concentration was achieved. The exposure time to sub-lethal OGD was 90 min. The OGD was terminated by exchanging the medium with phenol red-free EMEM [10% (v/v) FBS, 50 µg·mL⁻¹ kanamycin], and then incubated in a normoxic incubator at 37°C (reperfusion). Control co-cultures, which were not depleted of oxygen, were kept in EBSS containing 5.5 mM glucose and in a humidified atmosphere of 5% CO₂–95% air at 37°C.

Assessment of cell viability

The amount of lactate dehydrogenase (LDH) released from damaged neuronal cells induced by OGD was measured using an MTX-LDH assay kit according to the manufacturer's instructions. Briefly, 25 µL of phenol red-free reperfusion medium was collected 24 h after OGD, added into a 96-well plate and then incubated with 75 µL of the reaction solution. After 30 min of incubation at 37°C, the reaction was terminated by adding 100 µL of the stop solution. The LDH value was spectrophotometrically measured at 570 nm with a Bio-Rad microplate reader (Hercules, CA, USA). The LDH activities in the control and experimental groups were normalized by subtraction of the basal LDH

amount of phenol red-free medium and plotted as the percentage of the LDH released from the control group.

Reactive oxygen species (ROS) assay

Intracellular generation of ROS was detected using a sensitive fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H₂DCFDA) according to the manufacturer's instructions. Briefly, cells cultured in a 96-well black-walled plate were incubated with CM-H₂DCFDA (10 μ M) diluted in EBSS in a humidified atmosphere of 5% CO₂-95% air (37°C, 1 h). After being washed twice with the same buffer without CM-H₂DCFDA, the cultures were recovered in phenol red-free EMEM without FBS for 15 min. The fluorescent value was measured using a WALLAC ARVO SXFL multilabel counter (excitation 485 nm/emission 538 nm). The ROS generation in the control and experimental groups was normalized by subtraction of the basal ROS amount in the medium and plotted as the percentage of the ROS amount of the control group.

Glutamic acid/glutamate release assay

Glutamic acid/glutamate release induced by OGD was measured using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit according to the manufacturer's instructions. Briefly, 50 μ L of supernatant of phenol red-free reperfusion medium was collected and added into a 96-well black-walled plate. After adding an equal volume of the enzymatic reaction solution containing 0.08 U·mL⁻¹ glutamate oxidase, 200 μ M L-alanine, 0.05 U·mL⁻¹ L-glutamate-pyruvate transaminase, 100 μ M 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) and 0.25 U·mL⁻¹ horseradish peroxidase (HRP), the mixed solution was incubated at 37°C for 1 h to generate a highly fluorescent product, resorufin (Mohanty *et al.*, 1997). The fluorescence was excited at 544 nm, detected at 590 nm and measured using a FLEX STATION spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). The extracellular glutamate level was detected as an increase in the resorufin fluorescence value. The fluorescence value was normalized by subtracting the basal glutamate level of the phenol red-free medium and plotted as the percentage of the glutamate released from the control groups.

Nitric oxide (NO) release assay

NO concentration induced by OGD was determined using the Nitrate/Nitrite Colorimetric Assay Kit according to the manufacturer's instructions. Briefly, at 48 h after OGD, 50 μ L of supernatant fluid of the phenol red-free reperfusion medium was

collected and added into a 96-well plate. After adding 25 μ L Griess Reagent 1 immediately followed by 25 μ L Griess Reagent 2, the plate was sealed with a plastic plate cover, gently shaken and incubated at 37°C for 15 min to allow colour development. The absorbance at 540 nm was measured using a Bio-Rad microplate reader. A nitrite standard curve was drawn for each experiment to calculate relative NO values. The NO release in the control and experimental groups was normalized by subtraction of the basal NO amount of the phenol red-free medium, and plotted as the percentage of NO release from the control group.

[³H]-glutamate uptake

Neuron-astrocyte co-cultures and astrocyte cultures grown on 24-well plates were prepared, and [³H]-glutamate uptake assay was performed as previously described (Nakagawa *et al.*, 2008). Briefly, cells were incubated with Na⁺ or Na⁺-free uptake buffer containing 100 μ M glutamate and 1 μ Ci·mL⁻¹ [³H]-glutamate for 10 min. The [³H]-glutamate uptake was stopped by washing with ice-cold Dulbecco's phosphate-buffered saline (DPBS). Cells were lysed with 100 μ L of 0.3% (v/v) Triton X-100 solution. Lysate (70 μ L) was used for radioactivity measurement with a liquid scintillation counter; a fraction of the remaining lysate was used for protein concentration determination. After subtraction of the basal [³H]-glutamate uptake ([³H]-glutamate uptake in Na⁺-free buffer), the [³H]-glutamate uptake in the control and experimental groups was normalized by protein concentration and plotted as the percentage of control [³H]-glutamate uptake values.

Immunocytochemistry

Primary cultures were plated on sterilized, 7 mm round glassware. After defined culture periods, cells were fixed with freshly prepared 4% (w/v) paraformaldehyde at room temperature for 15 min. Cells were incubated with the same buffer, which contained 5% (v/v) normal goat serum and 0.005% (w/v) saponin for blockage and permeabilization (room temperature, 30 min) and then incubated with either of two primary antibodies, mouse monoclonal anti-GFAP antibody (1:500) or rabbit polyclonal anti-GLT-1 antibody (1:500) diluted in DPBS [1% (v/v) goat normal serum, 0.005% (w/v) saponin, 4°C, overnight]. Cells were incubated (room temperature, 1 h) with two secondary antibodies, Cy2-conjugated AffiniPure goat anti-mouse IgG (H+L) or Cy3-conjugated AffiniPure goat anti-rabbit IgG (H+L), followed by cell nucleus staining with 4',6-diamidino-2-phenylindole (DAPI) (room temperature, 10 min). The immunostained cells

were mounted on glass coverslips in FluorSave reagent. After being dried, immunoreactive cells were visualized and photographed with an Olympus IX81 fluorescence microscope (Olympus Optical, Tokyo, Japan).

Western blot analysis

After being rinsed three times with ice-cold DPBS, cells were solubilized in lysis buffer containing 20 mM Tris-HCl, 2 mM Na₂EGTA, 25 mM 2-glycerophosphate, 2 mM dithiothreitol, 1% (v/v) Triton X-100, 1% (v/v) protease inhibitor cocktails and 1% (v/v) phosphatase inhibitor cocktails (on ice, 30 min). The cell lysates were collected by gentle scraping and centrifuged at 16 100× *g* for 20 min at 4°C. The total protein in supernatant fluids in each sample was determined using the Bio-Rad Protein Assay kit. The prepared samples were stored at -20°C until analysed. For the brain sample, the cortex was collected, homogenized mechanically and then processed in the same way as above.

Cell lysates (10 µL) from each sample fraction were loaded for sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene difluoride membranes. The membranes were incubated in buffer containing 5% (w/v) non-fat milk (room temperature, 1 h) for non-specific binding blockage, and then incubated with rabbit polyclonal anti-GLT-1 (1:500), rabbit polyclonal anti-AT₁ receptor (1:500) or mouse monoclonal anti-GAPDH (1:100 000) diluted in PBS-Tween buffer (4°C, overnight). The membranes were then incubated with HRP-conjugated secondary antibodies. Immunoreactive specific protein bands were visualized with an enhanced chemiluminescence detection system, and exposed to X-ray film. Densitometric analysis was performed using ImageJ 1.36b.

RNA interference

Stealth/siRNA duplex oligoribonucleotides (Agtr1b-RSS34217) for AT_{1b} RNA interference and Stealth RNAi Negative Control Duplexes (cat. no. 12935-112) were purchased from Invitrogen, Tokyo, Japan. The rat AT_{1b} RNA interference sequence was 5'-AUA CGU UUC GGU AGA UGA CGG CUG G-3'. The RNA interference studies were performed using a Targefect-siRNA transfection kit according to the manufacturer's instructions. Briefly, 200 pmol of dsRNA was used to form 1 mL of the transfection complex solution. Astrocytes grown on a six-well plate were incubated with the transfection complex solution at 37°C for 2 h. One millilitre of DMEM (20% FBS) was added and incubated for 10 h

(37°C). Astrocytes were recovered in NM for 2 h and then incubated with ADM or used for further treatments.

Real-time PCR

Total RNA was extracted from cells grown on six-well plates with an ISOGEN kit (Nippon Gen, Tokyo, Japan) according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically and the purity was confirmed by the relative absorbance of OD₂₆₀ versus OD₂₈₀. After being treated with DNase inhibitors, 2 µg of RNA was used to generate cDNA in a reverse transcription-PCR reaction using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). Real-time PCR (RT-PCR) was performed for the amplification of rat GLT-1 and rat cyclophilin A using a Thermal Cycler Dice Real Time System TP850 (Takara Bio Inc.) (Wang *et al.*, 2009). RT-PCR was carried out on a total volume of 25 µL containing 100 ng cDNA template, 0.2 µM of the primers and 12.5 µL of the SYBR II Premix EX Taq solution (2×). The primers used were: rat GLT-1 forward, 5'-GTT CAA GGA CGG GAT GAA TGT CTT A-3', and reverse, 5'-CAT CAG CTT GGC CTG CTC AC-3'; and rat cyclophilin A forward, 5'-GGC AAA TGC TGG ACC AAA CAC-3', and reverse, 5'-AAA CGC TCC ATG GCT TCC AC-3'. PCR amplification was conducted under the following protocol: step 1: 10 s at 95°C; step 2: 40 cycles of 5 s at 95°C and 30 s at 60°C; and step 3: 15 s at 95°C, 30 s at 60°C and 15 s at 95°C. Each experiment was conducted three times and each sample was run in triplicate. For quantitative analysis, amplifications were performed under the same conditions with five quantities (100, 20, 4, 0.8 and 0.16 ng) of each cDNA template mixture using the primer of GLT-1 and cyclophilin A respectively. Relative standard curves were generated by plotting the cycle threshold (CT) values versus the log of the cDNA concentration respectively. Based on the CT values obtained from each sample, the relative amounts of target genes GLT-1 or cyclophilin A in the control and experimental groups were calculated using the standard curves with the software provided by Takara Thermal Cycler Dice Real Time System. Each sample was run in triplicate and normalized to the relative amplification of rat cyclophilin A.

Statistical analysis

Values are expressed as the mean ± SE of at least three independent experiments. Differences between means were determined with a one-way ANOVA followed by a pairwise comparison by using

the GraphPad Prism Software 4.03. Differences were considered statistically significant when the *P* value was less than 0.05.

Materials

The following reagents were used: L-[³H]-glutamate from GE Healthcare (Buckinghamshire, UK); 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) and Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit from Invitrogen (Carlsbad, CA, USA); Stealth/siRNA duplex oligoribonucleotides (Agr1b-RSS34217) and Stealth RNAi Negative Control Duplexes (cat. no. 12935-112) from Invitrogen Japan; transferrin from Roche (Basel, Switzerland); CGP-42112A, L162313, PD123319 and mouse monoclonal anti-GFAP antibody from Sigma (St Louis, MO, USA); EGF and bFGF from Peprotech (Rocky Hill, NJ, USA); Ang II from Bachem (Torrance, CA, USA); losartan from LKT Laboratories (St Paul, MN, USA); telmisartan as a kind gift from Boehringer Ingelheim (Ingelheim, Germany); lactacystin and FluorSave reagent from Calbiochem (San Diego, CA, USA); AnaeroPack System from Mitsubishi Gas Chemical (Tokyo, Japan); MTX-LDH assay kit from Kyokutou Pharmaceutical Industry (Tokyo, Japan); Nitrate/Nitrite Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI, USA); rabbit polyclonal anti-AT₁ receptor; rabbit polyclonal anti-GLT-1 (cat. no. sc-15317) from Santa Cruz (Delaware, CA, USA); mouse monoclonal anti-GAPDH from Ambion

(Austin, TX, USA); DAPI from Molecular Probes (Eugene, OR, USA); ISOGEN kit from Nippon Gen; PrimeScript 1st Strand cDNA Synthesis Kit and SYBR II Premix EX Taq Kit from Takara Bio Inc.; and Targefect-siRNA transfection kit from Targeting Systems (San Diego, CA, USA).

Results

Treatment with AT₁ receptor antagonists reduces OGD-induced neuronal cell death

To investigate the *in vitro* protective effects of AT₁ receptor antagonists on neuron–astrocyte co-cultures, an AnaeroPack system was used to perform the OGD experiments in the present studies. First, we carried out a time-course study to determine the exposure time needed for OGD insults. The LDH release was increased in a time-dependent manner after OGD (Figure 1A). A 90 min OGD treatment induced moderate cell death (approximately twofold increase of LDH) after 24 h reperfusion. By microscopic observation, only neuronal loss was found; astrocyte death was not observed in our OGD system based on the TUNEL staining (data not shown). No LDH release was detected during OGD. Neuronal cell death induced by 90 min OGD was rescued by NMDA antagonist MK801 (10 μM). In contrast, a 120 min OGD treatment was lethal to neurons. Treatment with MK801 (10 μM) after the 24 h reperfusion served as a posi-

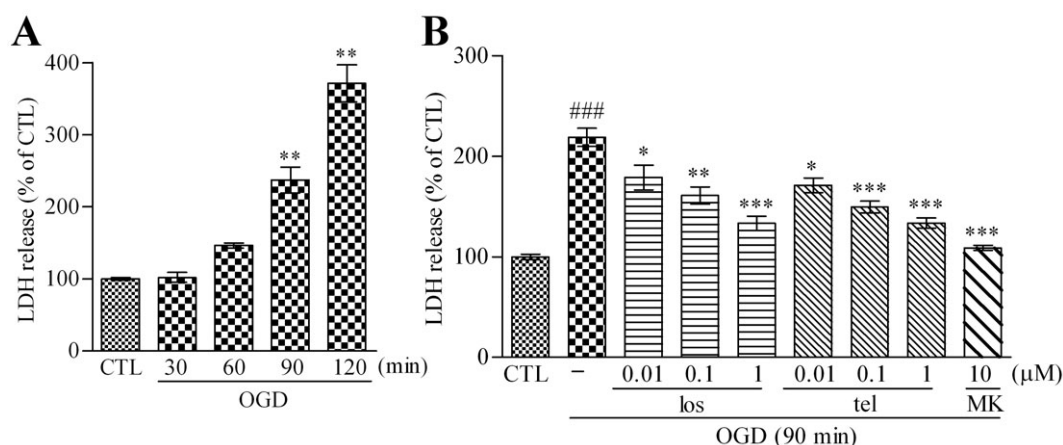


Figure 1

AT₁ receptor antagonist pretreatment reduces OGD-induced LDH release from neuron–astrocyte co-cultures. (A) OGD injury using the AnaeroPack system induced LDH release from neuron–astrocyte co-cultures in a time-dependent manner. Data represent mean \pm SE, *n* = 6 for each group. ***P* < 0.01 versus control; ANOVA followed by Dunnett's multiple comparison test. (B) A 48 h pretreatment with the AT₁ receptor antagonist, losartan (0.01–1 μM) or telmisartan (0.01–1 μM), reduced LDH release induced by 90 min OGD in a concentration-dependent manner. A 24 h post-treatment of MK801 (10 μM) also reduced the LDH release after 90 min OGD. Data represent mean \pm SE, *n* = 7 for each group. ###*P* < 0.001 versus control; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus OGD; ANOVA followed by Newman–Keuls multiple comparison test. los, losartan; tel, telmisartan; MK, MK801.

tive control in the present studies, totally attenuating LDH release in our OGD system (Figure 1B). This result was further confirmed with another NMDA receptor antagonist, AP5 (data not shown). These results indicated that glutamate-mediated excessive excitatory neurotoxicity after OGD insult was primarily responsible for neuronal cell death in our system.

Losartan, a surmountable/non-competitive AT₁ receptor antagonist, and telmisartan, an insurmountable/non-competitive AT₁ receptor antagonist (Timmermans, 1999) were used in our experiments. A 48 h pretreatment with losartan or telmisartan significantly reduced OGD-induced LDH release in neuron–astrocyte co-cultures in a concentration-dependent manner (0.1–1 μM) (Figure 1B). In our preliminary experiments, a shorter treatment with AT₁ receptor antagonists (24 h) did not exert a significant protective effect (data not shown). The AT₁ receptor antagonist treatment might be involved in protection against the glutamate-mediated excitatory neurotoxicity after OGD.

Using pure neuron cultures (Appendix S1), OGD-induced or glutamate-induced cell death was not prevented by 48 h pretreatment with telmisartan (Figures S1 and S2). In contrast, exposure of neuron–astrocyte co-cultures to glutamate also caused cell death, and 48 h pretreatment with telmisartan significantly reduced glutamate-induced LDH release (Figure S3). These data suggested that astrocytes were necessary for the neuroprotective effect.

Pretreatment is essential for the neuroprotective effects of AT₁ receptor antagonists against OGD

The protective effects of pretreatment and post-treatment with AT₁ receptor antagonists were compared. Our data showed that telmisartan (1 μM) did not show any neuroprotective effects in 24 h AT₁ receptor antagonist post-treatment during the reperfusion period (Figure 2A). Next, the involvement of AT₂ receptors in the AT₁ receptor antagonist-induced neuroprotective effect was examined. Simultaneous treatment with an AT₂ receptor antagonist, PD123319 (10 μM), did not abolish the neuroprotective effect of telmisartan (1 μM) (Figure 2B). Furthermore, AT₂ receptor stimulation with CGP-42112A (1 μM) alone did not show any neuroprotective effect. Simultaneous treatment with CGP-42112A (1 μM) and telmisartan (1 μM) did not show any enhancement of AT₁ receptor antagonist-induced protection against OGD (Figure 2C). Even in a higher concentration of

CGP-42112A (3–30 μM), no protective effect was observed (Figure 2D). Therefore, our results suggested that the AT₁ receptor antagonism is essential for the AT₁ antagonist-induced neuroprotective effect against OGD and that AT₂ receptor stimulation did not affect the OGD injury.

Based on these data, we hypothesized that AT₁ receptor stimulation might augment OGD-induced cellular damage. For the continuous stimulation of AT₁ receptors, a non-peptidergic AT₁ receptor agonist, L162313, was selected for the experiments, because it is characterized by a rather slow, but more sustained AT₁ receptor agonist activity (Perlman *et al.*, 1995). Ang II (100 μM) or L162313 (10 μM) was added alone or together with 48 h pretreatment with telmisartan (1 μM). The results showed that pretreatment with Ang II or L162313 evoked increased LDH release after OGD, which was attenuated by simultaneous AT₁ receptor antagonism with telmisartan (1 μM) (Figure 3A,B). In addition, treatment with MK801 (10 μM) during the 24 h reperfusion significantly attenuated Ang II- or L162313-enhanced OGD-induced LDH release (Figure 3C,D).

Altogether, these data suggested that pre-exposure to AT₁ receptor antagonism and/or stimulation clearly affected the responses of neuronal cells to OGD.

Treatment with AT₁ receptor antagonists reduces OGD-induced glutamate release, ROS generation and NO release

As shown in Figures 1B and 3C,D, MK801 protected neuronal cells against OGD, implying that glutamate release following OGD insult was the main mechanism in our OGD-induced neuronal cell death model. To clarify this mechanism, we evaluated the extracellular glutamate concentration after OGD. A 90 min exposure to OGD induced significant glutamate release in neuron–astrocyte co-cultures. A 48 h pretreatment with AT₁ receptor antagonists, losartan or telmisartan, significantly reduced OGD-induced glutamate release in a concentration-dependent manner (0.01–1 μM) (Figure 4A). Extracellular glutamate release was one of the earliest events encountered with ischaemic injury, and subsequent ROS generation and NO release were important for glutamate-mediated neuronal cell death (Tamura *et al.*, 1992). We evaluated ROS and NO levels after OGD according to the previous reports (Lo *et al.*, 2003; Rossi *et al.*, 2007). The OGD-induced ROS generation was also significantly suppressed by 48 h pretreatment with losartan (0.01–1 μM) or telmisartan (0.01–1 μM) in neuron–astrocyte co-cultures (Figure 4B). The NO release was

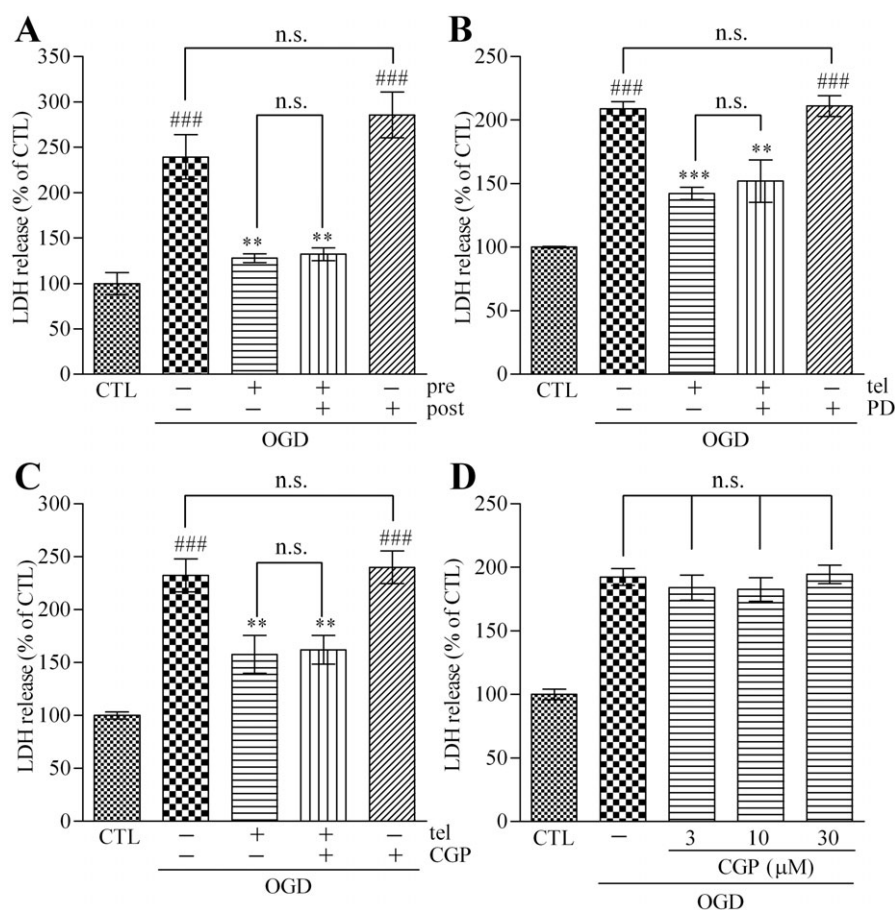


Figure 2

Pre-blockade of AT₁ receptors is essential for the neuroprotective effect of AT₁ receptor antagonism against OGD. (A) A 24 h post-treatment with telmisartan (1 μM) did not show neuroprotective effects. A 48 h pretreatment plus 24 h post-treatment with telmisartan (1 μM) did not enhance the neuroprotective effect of 48 h pretreatment of telmisartan (1 μM). (B) Simultaneous 48 h pretreatment of the AT₂ receptor antagonist, PD123319 (10 μM), did not abolish the neuroprotective effect of telmisartan (1 μM) against the effects of 90 min OGD. PD123319 (10 μM) alone did not affect the OGD-induced LDH release. (C) A 48 h pretreatment with the AT₂ receptor antagonist, CGP-42112A (1 μM), alone did not show neuroprotective effects against 90 min OGD. A simultaneous 48 h pretreatment with CGP-42112A (1 μM) did not enhance the neuroprotective effect of telmisartan (1 μM). (D) Higher concentration treatment of CGP-42112A (3–30 μM) did not exert neuroprotective effects. Data represent mean ± SE, n = 6 for each group. ###P < 0.001 versus control; **P < 0.01, ***P < 0.001 versus OGD; ANOVA followed by Newman-Keuls multiple comparison test. n.s., not significant; tel, telmisartan; PD, PD123319; CGP, CGP-42112A; pre, pretreatment; post, post-treatment.

significantly increased when measured at the end of the 48 h reperfusion after OGD. A 48 h pretreatment of losartan (0.01–1 μM) or telmisartan (0.01–1 μM) significantly suppressed this OGD-induced NO release (Figure 4C).

These results suggested that decreasing the concentration of extracellular glutamate after OGD might be the neuroprotective mechanism of AT₁ receptor antagonists.

Treatment with AT₁ receptor antagonists increases GLT-1 expression and glutamate uptake in neuron–astrocyte co-cultures

The GLT-1, an astroglial protein, is mainly responsible for removal of extracellular glutamate in the

CNS (Danbolt, 2001). Therefore, we investigated whether AT₁ receptor antagonists could affect GLT-1 expression in neuron–astrocyte co-cultures.

A 48 h treatment with telmisartan (1 μM) significantly up-regulated the protein level of GLT-1. After a longer-term treatment (4 or 6 days), GLT-1 was more profoundly up-regulated (data not shown). In contrast, a 48 h treatment with the AT₁ receptor agonist, L162313 (10 μM), significantly down-regulated the GLT-1 expression level in neuron–astrocyte co-cultures (Figure 5A).

Consistent with the results of GLT-1 protein regulation, [³H]-glutamate uptake was significantly increased after 48 h treatment with telmisartan (0.1–1 μM) and L162313 (1–10 μM) significantly

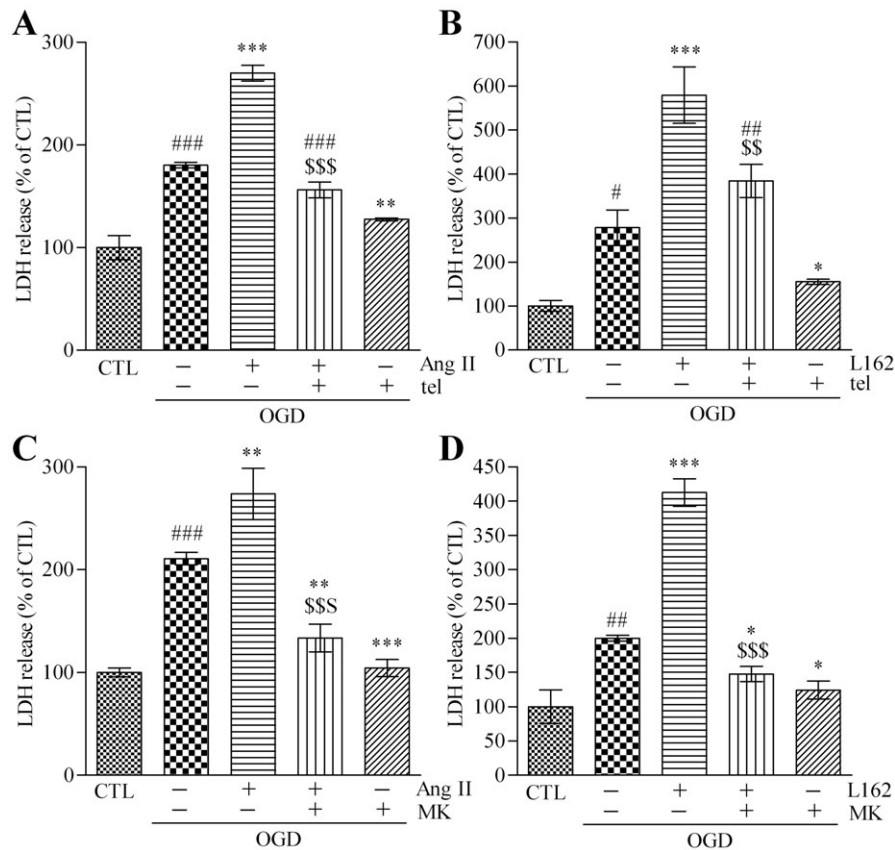


Figure 3

Pre-stimulation of AT₁ receptors augments OGD-induced neuronal cell damage. (A) A 48 h pretreatment with Ang II (100 μ M) increased LDH release induced by 90 min OGD, and it was attenuated by simultaneous treatment with telmisartan (1 μ M). (B) A 48 h pretreatment with the AT₁ receptor agonist, L162313 (10 μ M), increased the OGD-induced LDH release. Simultaneous treatment with telmisartan (1 μ M) decreased L162313 (10 μ M)-enhanced OGD-induced LDH release, and it was attenuated by 24 h post-treatment with telmisartan (1 μ M). (C) A 48 h pretreatment with Ang II (100 μ M) increased the 90 min OGD-induced LDH release, and it was attenuated by 24 h post-treatment with MK801 (10 μ M). (D) A 48 h pretreatment with L162313 (10 μ M) increased the OGD-induced LDH release which was significantly decreased by 24 h post-treatment with MK801 (10 μ M). Data represent mean \pm SE, $n = 6$ for each group. # $P < 0.05$; ### $P < 0.001$ versus control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus OGD; $^{ss}P < 0.01$, $^{sss}P < 0.001$ versus OGD plus Ang II (100 μ M) or OGD plus L162313 (10 μ M); ANOVA followed by Newman–Keuls multiple comparison test. tel, telmisartan; Ang II, angiotensin II; L162, L162313; MK, MK801.

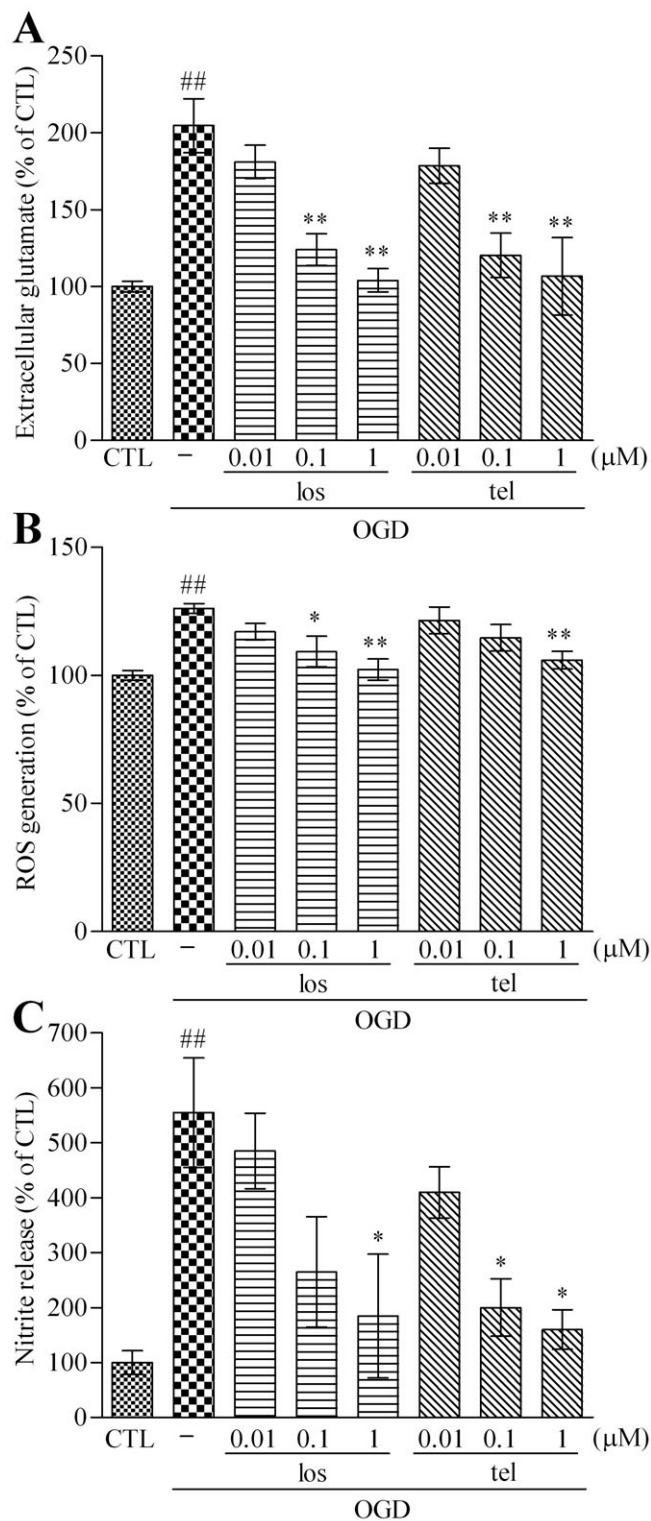
decreased [³H]-glutamate uptake in a concentration-dependent manner (Figure 5B). In addition, GLT-1 inhibitor, dihydrokainic acid (DHK), significantly abolished AT₁ receptor antagonist-induced [³H]-glutamate uptake (Figure 5B). DHK is a relatively selective GLT-1 inhibitor, although the effect was found at millimolar levels in our experiments. Other agents are available that would further clarify the relative contribution of GLT-1 (EAAT2) compared to that of another transporter, GLAST (EAAT1) in these cultures (Peacey *et al.*, 2009).

These results suggested that AT₁ receptors were involved in the regulation of GLT-1 expression and glutamate uptake. Thus AT₁ receptor antagonism reduced the extracellular glutamate elevation induced by OGD injury via up-regulation of GLT-1.

L162313 down-regulates GLT-1 expression in ADM-cultured astrocytes

Pure astrocyte cultures were prepared to investigate the mechanism of GLT-1 regulation in the absence of neuronal cells. Because astrocytes cultured in NM (DMEM + 10% FBS) express only very low levels of GLT-1 protein, ADM-cultured astrocytes were used in the following experiments. An ADM culture, a modification of G-5 supplement, is a well-established culture method suitable for inducing *in vitro* astrocyte maturation; this methodology provided us with a convenient approach for GLT-1 regulation research (Miller *et al.*, 1995; Vermeiren *et al.*, 2005).

After a 4 day culture with ADM, the typical flat polygonal morphology of astrocytes was significantly changed, showing a highly branched, stellate



morphology similar to the morphology of astrocytes *in vivo*. GLT-1 expression was significantly up-regulated after the 4 day ADM culture (Figure 6A,B). Simultaneous treatment with the non-peptidergic AT₁ receptor agonist, L162313, during the 4 days of ADM culture, caused significant

Figure 4

Pretreatment with AT₁ receptor antagonist reduces OGD-induced glutamate release, ROS generation and NO release. (A) OGD-induced extracellular glutamate release (from 413 ± 24 nM to 831 ± 122 nM) was reduced by 48 h pretreatment with losartan or telmisartan in a concentration-dependent manner (0.01–1 μ M). (B) A 48 h pretreatment with losartan (0.01–1 μ M) or telmisartan (0.01–1 μ M) reduced the OGD-induced ROS generation in a concentration-dependent manner. (C) OGD-induced NO release (from 267 ± 101 nM to 1480 ± 461 nM) was reduced by 48 h pretreatment with losartan or telmisartan in a concentration-dependent manner (0.01–1 μ M). Data represent mean \pm SE, $n = 3$ for each group. ## $P < 0.01$; ### $P < 0.001$ versus control; * $P < 0.05$, ** $P < 0.01$ versus OGD; ANOVA followed by Newman–Keuls multiple comparison test. los, losartan; tel, telmisartan.

down-regulation of the GLT-1 protein level in a concentration-dependent manner (0.1–3 μ M) (Figure 6B). A relatively high concentration of 3 μ M L162313 almost totally suppressed GLT-1 protein expression. No LDH release was detected with L162313 treatment, suggesting that no cell damage occurred during the treatment (data not shown).

After 4 days of ADM culture, [³H]-glutamate uptake was significantly enhanced, and L162313 treatment abolished this ADM-induced increase in [³H]-glutamate uptake in a concentration-dependent manner (0.1–3 μ M) (Figure 6C). These results suggested that AT₁ receptor stimulation with L162313 down-regulated the expression of GLT-1 protein and glutamate uptake in ADM-cultured astrocytes.

L162313 reduces GLT-1 mRNA synthesis in ADM-cultured astrocytes

Because ubiquitination has been implicated in GLT-1 endocytosis and degradation (Sheldon *et al.*, 2008), and AT₁ receptor signalling contributes to the activation of the ubiquitin–proteasome system (Kurihara *et al.*, 2008), experiments using proteasome inhibitors were performed. Simultaneous treatment with the proteasome inhibitor, lactacystin (0.3 μ M), did not affect the decrease of GLT-1 induced by L162313 (Figure 7A).

In contrast, real-time PCR analysis revealed an enhancement of GLT-1 mRNA after a 4 day ADM culture, and simultaneous L162313 treatment abolished the ADM-induced up-regulation of GLT-1 mRNA in a concentration-dependent manner (0.1–3 μ M) (Figure 7B).

These results indicated that AT₁ receptor stimulation with L162313 influenced GLT-1 expression at a transcriptional level in ADM-cultured astrocytes.

AT₁ receptors are involved in GLT-1 regulation in astrocytes

To confirm that AT₁ receptors were actually involved in GLT-1 regulation, RNA interference experiments

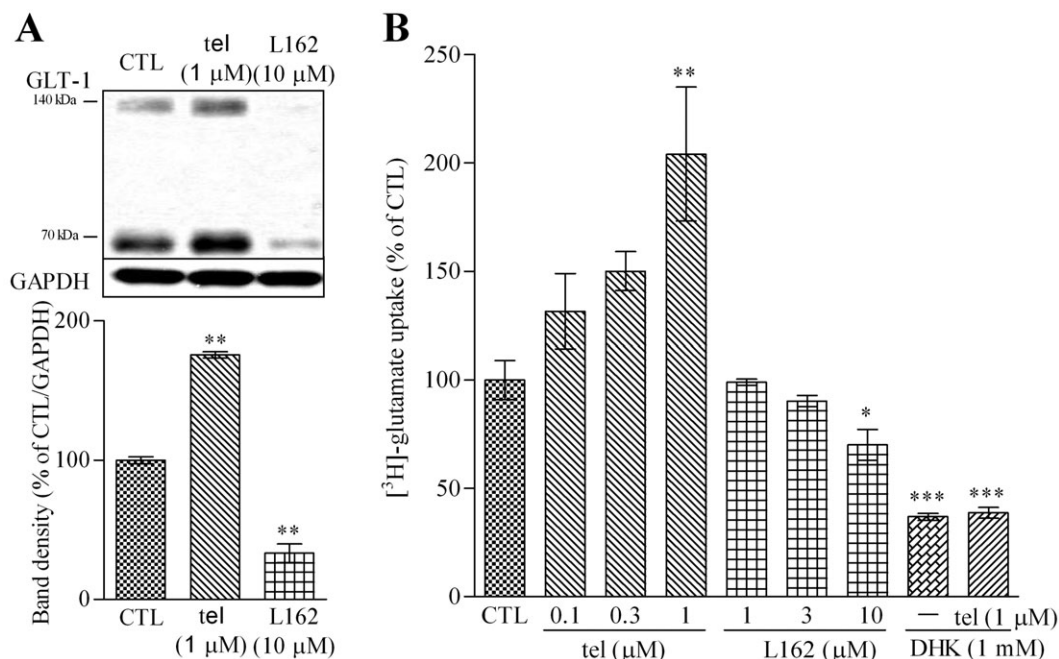


Figure 5

GLT-1 expression and glutamate uptake in neuron-astrocyte co-cultures are affected by AT₁ receptor antagonism or stimulation. (A) GLT-1 protein level was increased by 48 h treatment with telmisartan (1 μM) and was decreased by L162313 (AT₁ receptor agonist, 10 μM). (B) [³H]-glutamate uptake ability was enhanced by 48 h treatment with telmisartan and was reduced by L162313. DHK (GLT-1 inhibitor, 1 mM) significantly reduced telmisartan (1 μM)-induced enhancement of [³H]-glutamate uptake. Data represent mean ± SE, *n* = 3 for each group. **P* < 0.05, ***P* < 0.01 versus control; ANOVA followed by Dunnett's multiple comparison test. tel, telmisartan; L162, L162313.

targeting astroglial AT₁ receptors were performed. Although there are two sub-types of the AT₁ receptor, AT_{1a} and AT_{1b}, in the rodent brain RAS system, we selected knock-down of AT_{1b} receptors alone, as this has been reported to decrease the AT₁ receptors and responses to Ang II in rat CNS (Lu *et al.*, 1995). Stealth/siRNA duplex oligoribonucleotides (Agr1b-RSS34217) were delivered to the astrocytes for RNA interference with AT_{1b} receptors (Figure 8A). After a 4 day ADM culture, the AT₁ receptors in siAT_{1b}-treated astrocytes were approximately half of those in the negative control siRNA-treated group, according to the Western blot analysis (Figure 8C). At the same time, the GLT-1 protein level was significantly up-regulated in siAT_{1b}-treated astrocytes, and GLT-1 protein reduction by L162313 was significantly attenuated (Figure 8B). These results confirmed our hypothesis that AT₁ receptor signalling was involved in regulation of GLT-1 expression, at least during the ADM-induced astroglial maturation.

As previously reported, the expression of GLT-1 protein progressively increased to adult levels from the postnatal day 1 (P1) to postnatal day 21 (P21), and this period was almost equal to that of *in vivo* astrocyte maturation (Furuta *et al.*, 1997). Therefore, we hypothesized that AT₁ receptors might also be involved in astrocyte maturation *in vivo*. The

cortices from rats at P1, P7, P14 and P21 showed that GLT-1 was gradually increased (Figure 9A). Meanwhile, the AT₁ receptor level from P1 to P21 was significantly decreased with increased GLT-1 (Figure 9B,C).

Discussion

In the present study, we demonstrated that pretreatment with AT₁ receptor antagonists showed neuroprotective effects against OGD and that AT₁ receptor antagonist affected GLT-1 regulation in neuron-astrocyte co-cultures. A 48 h pretreatment with AT₁ receptor antagonists, losartan or telmisartan, significantly protected neuronal cells against OGD injury. Longer-term pretreatment facilitated the neuroprotective effects of the AT₁ receptor antagonists, whereas post-treatment did not reduce the neuronal cell damage induced by OGD.

The original design for the present OGD protocol was based on a stroke cascade: exaggerated glutamate release soon after cerebrovascular perfusion acts as an initiator of the events following a stroke. There was no LDH release during OGD, which models the characteristics of the ischaemic

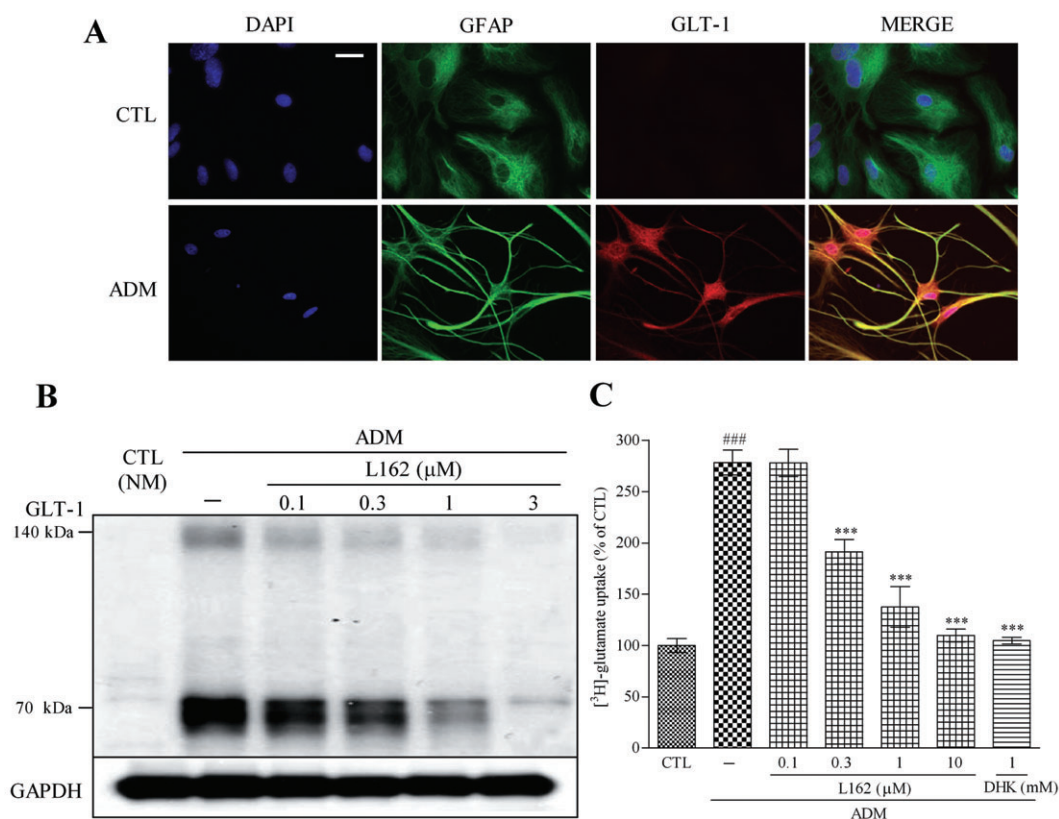


Figure 6

L162313 down-regulates GLT-1 expression in ADM-cultured astrocytes. (A) Astrocytes were immunostained with DAPI (blue), anti-GFAP (green) and anti-GLT-1 (red). Four-day incubation with ADM changed the morphology of astrocytes, leading to a highly branched, stellate shape comparison to the typical flat polygonal morphology in CTL (NM). GLT-1 expression was up-regulated according to the GLT-1 (red) immunostain. Scale bar: 30 μm. (B) Simultaneous 4 day treatment with L162313 (AT₁ receptor agonist) decreased GLT-1 expression induced by ADM culture. (C) Four-day ADM culture increased the [³H]-glutamate uptake ability. Simultaneous administration with L162313 (0.1–3 μM) attenuated the ADM-enhanced [³H]-glutamate uptake in a concentration-dependent manner. DHK (1 mM) significantly reduced ADM-induced enhancement of [³H]-glutamate uptake. Data represent mean ± SE, *n* = 3 for each group. ###*P* < 0.001 versus control; ****P* < 0.001 versus ADM; ANOVA followed by Newman–Keuls multiple comparison test. L162, L162313.

penumbra areas rather than the necrotic cores, and this factor should be taken into consideration when using our OGD protocol.

Extracellular glutamate levels after OGD were significantly reduced by pretreatment with AT₁ receptor antagonists. In addition, ROS and NO were mediators of the OGD-induced cell death, and these factors are generated after glutamate receptor stimulation. Attenuation of extracellular glutamate might result in the attenuation of ROS generation or NO release.

AT₁ receptor antagonists might cause alternative enhancement of AT₂ receptor stimulation by Ang II, and AT₂ receptor blockade could attenuate AT₁ antagonist-induced neuroprotective effects (Li *et al.*, 2005). Our co-cultures displayed both AT₁ and AT₂ receptor proteins (Figure S4). However, simultaneous treatment with an AT₂ receptor antagonist, PD123319, did not attenuate the protective effect of AT₁ antagonists against OGD in our system. Further-

more, an AT₂ receptor agonist did not exert neuroprotective effects as proposed. Involvement of AT₂ receptors in the neuroprotection seems therefore to be unlikely in our cultures.

To emphasize the crucial role of AT₁ receptors in regulation of tolerance to ischaemic injury, AT₁ receptor agonist (L162313 or Ang II) was given before OGD and resulted in increased neuronal cell damage. AT₁ receptor antagonism before exposure to OGD was necessary to show protection, and the involvement of AT₂ receptors would appear to be minimal in our OGD model. AT₁ receptor stimulation by Ang II might provide the cytotoxic or cell death-enhancing signals. As AT₁ receptors are mainly located on astrocytes, whereas AT₂ receptors are on neurons in the CNS, a possible role for AT₁ receptor antagonism in astroglial glutamate uptake is likely. Both GLT-1 protein levels and glutamate uptake were significantly enhanced in the neuron-

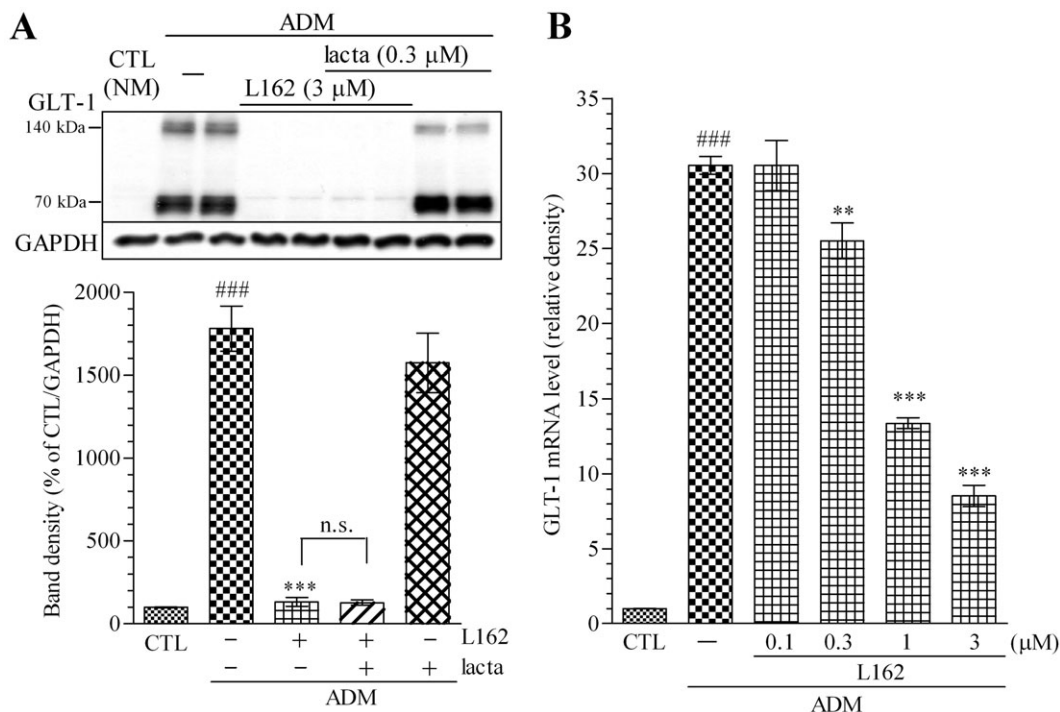


Figure 7

L162313 (AT₁ receptor agonist) reduces GLT-1 protein expression at a transcriptional level. (A) L162313 treatment decreased GLT-1 expression induced by 4 days of ADM culture. Simultaneous 4 day treatment with the proteasome inhibitor lactacystin (0.3 μM) did not attenuate L162313 (3 μM)-induced GLT-1 down-regulation in ADM-cultured astrocytes. (B) GLT-1 mRNA level was increased by ADM culture (4 days) and was decreased by L162313 (0.1–3 μM) to 8.5 ± 0.7 in a concentration-dependent manner. Data represent mean \pm SE, $n = 3$ for each group. ### $P < 0.001$ versus control; ** $P < 0.01$, *** $P < 0.001$ versus ADM; ANOVA followed by Newman–Keuls multiple comparison test. n.s., not significant; lacta, lactacystin; L162, L162313.

astrocyte co-cultures after treatment with AT₁ receptor antagonists. Furthermore, an AT₁ receptor agonist (L162313) impaired GLT-1 protein expression and glutamate uptake. From these findings, we could conclude that AT₁ receptor signalling in astroglial cells suppressed GLT-1 expression. However, degradation of GLT-1, mediated by the ubiquitin–proteasome system (Kurihara *et al.*, 2008), seems not to be involved in this suppression.

To confirm the AT₁ receptor-specific response, siRNA experiments targeting AT₁ receptors were subsequently performed. The ADM-induced increased GLT-1 protein level and glutamate uptake were significantly inhibited by L162313 treatment. AT₁ receptor knock-down attenuated the reduction of GLT-1 mediated by L162313. AT₁ receptor knock-down itself enhanced the protein level of GLT-1, indicating the existence of intrinsic AT₁ receptor agonists or spontaneous AT₁ receptor activation. However, agonist-independent or constitutive activity has not been reported for AT₁ receptors or most G protein-coupled receptors (GPCRs), except for thyroid-stimulating hormone and muscarinic receptors (de Gasparo *et al.*, 2000).

Several signal pathways were identified for manipulating GLT-1, including various growth factor-mediated pathways. The ADM used in the present study included a number of supplementary agents. EGF and bFGF were considered as being particularly responsible for astroglial morphological changes and GLT-1 up-regulation. In NM, morphologies of astrocytes are different from those found in neuron–astrocyte co-cultures. In contrast, stellate-shaped astrocytes cultured in ADM are similar to those found in the co-culture or brains *in vivo*. Removal of EGF and bFGF disabled ADM for astroglial maturation, resulting in the flat polygonal morphology (data not shown). In the present study, ADM was used for the induction of astrocyte maturation (Miller *et al.*, 1995), because expressing GLT-1 was considered one of the defining features of astroglial maturation.

Transcriptional regulation of GLT-1 is not fully understood. Blockade of central angiotensin AT₁ receptors improves neurological outcomes after brain ischaemia and reduces expression of AP-1 transcription factors. Transcription of apoptosis-related proteins might be suppressed by AT₁ receptor

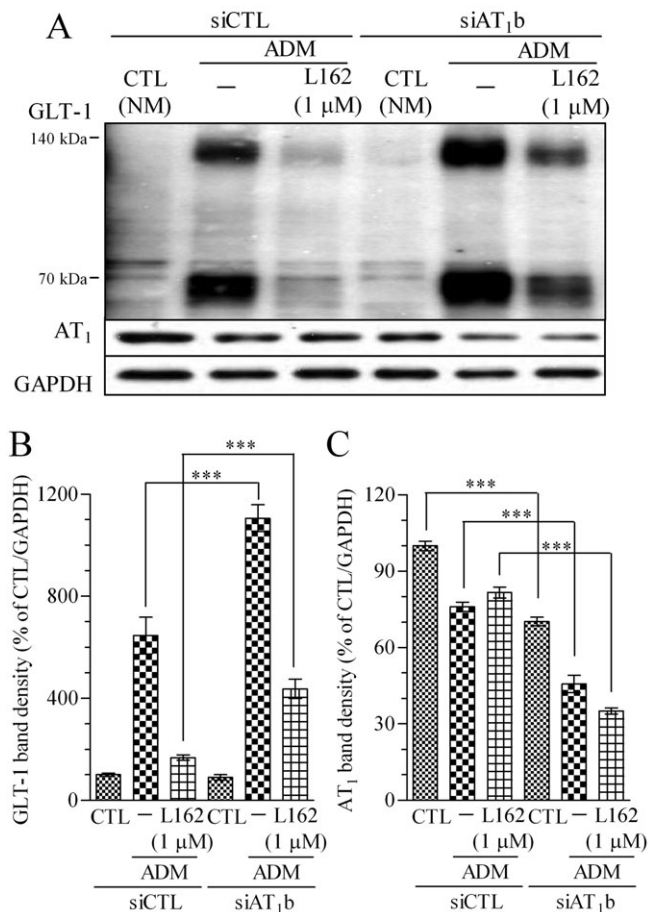


Figure 8

RNA interference experiments revealed that AT₁ receptors are involved in GLT-1 expression in ADM-cultured astrocytes. (A) Representative immunoblot data of GLT-1, AT₁ and GAPDH treated with or without RNA interference for AT_{1b} receptors in astrocytes. (B) Compared with GLT-1 in the control ADM treatment, GLT-1 in siAT_{1b}-treated astrocytes was increased. The diminishing ADM-induced GLT1 up-regulation by L162313 (AT₁ receptor agonist, 1 μM) was impaired with mRNA interference for AT_{1b} receptors (from 167.8 ± 10.3 to 436.8 ± 37.4). (C) The AT₁ receptor band density was down-regulated in CTL from 100 ± 1.9 to 70.2 ± 1.7 , in ADM from 76 ± 1.9 to 45.7 ± 3.4 and in ADM plus L162313 (1 μM) from 81.6 ± 2.1 to 35.1 ± 1.2 . Data represent mean \pm SE, $n = 3$ for each group. $***P < 0.001$; ANOVA followed by Newman-Keuls multiple comparison test. L162, L162313.

antagonism. In our data, however, mRNA levels of GLT-1 were increased by AT₁ receptor antagonism. We could assume that AT₁ receptor antagonism induces the up-regulation of a survival signalling pathway or that AT₁ receptor stimulation confers an inhibitory signal on GLT-1. It is not clear how AT₁ receptor signalling affects the GLT-1 promoter or if signals from another source apart from the angiotensin receptors up-regulate GLT-1. It was reported that Ang II exerts AT₁ receptor-mediated inhibition of adenylyl cyclase activity (de Gasparo *et al.*, 2000),

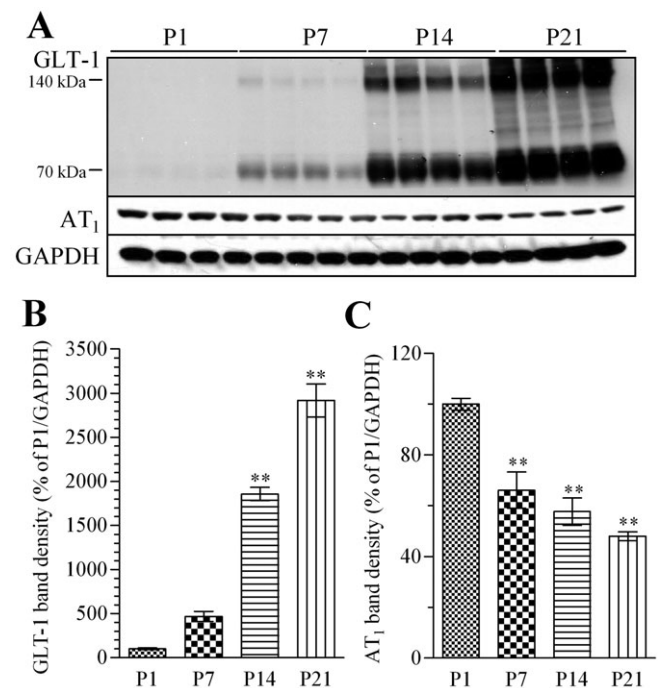


Figure 9

GLT-1 and AT₁ receptor levels in rat cortex on postnatal day 1 (P1), P7, P14 and P21. (A) Representative immunoblot data of GLT-1, AT₁ receptors and GAPDH of rat cortex at P1, P7, P14 and P21. (B) GLT-1 protein level increased progressively in cortices from P7, P14 and P21 rats (expressed as % of P1). (C) In the same samples, AT₁ receptor protein levels decreased progressively in P7, P14 and P21 rats (expressed as % of P1). Data represent mean \pm SE, $n = 4$ for each group. $**P < 0.01$ versus control; ANOVA followed by Dunnett's multiple comparison test.

and GLT-1 regulation might be cyclic AMP dependent (Thorlin *et al.*, 1998). AT₁ receptors might attenuate the inhibitory signal for the expression of GLT-1 via cyclic AMP regulation. Other candidate transcription factors binding to the GLT-1 promoter include nuclear factor-kappaB (NF-κB), peroxisome proliferator-activated receptor-gamma and kappa B-motif binding phosphoprotein (Zeleniaia *et al.*, 2000; Romera *et al.*, 2007; Lepore *et al.*, 2008). Growth factors in ADM would activate receptor tyrosine kinases (RTKs). In contrast, long-term GPCR activation could desensitize and down-regulate the RTK response. Pre-exposure of mesangial cells to 5-HT attenuated EGF-induced activation of multiple transcription factors, including NF-κB, CREB and E2-F (Grewal *et al.*, 2001). Therefore, sustained stimulation of AT₁ receptors, one of the GPCRs, might reduce the RTK response, resulting in the down-regulation of GLT-1 expression via the attenuation of a transcription factor, such as NF-κB. However, detailed mechanisms have not been fully elucidated.

The physiological functions of astroglial GLT-1 in the CNS are of crucial importance. Especially in strokes and in ALS, the levels of astroglial GLT-1 exert a major influence on the pathological progression. Enhanced GLT-1 expression level reinforces the tolerance to ischaemic injury *in vitro* and *in vivo* (Rothstein *et al.*, 2005; Romera *et al.*, 2007). Loss of function of GLT-1 has been implicated in the pathogenesis of ALS, resulting in damage of adjacent motor neurons (Rothstein *et al.*, 2005; Lepore *et al.*, 2008; Yang *et al.*, 2009). Other neurodegenerative diseases might also be influenced by GLT-1 function. Therefore, regulation of GLT-1 expression and function is critical in the treatment of neurological disorders mediated by glutamate excitotoxicity.

In conclusion, AT₁ receptor antagonists exert neuroprotective effects against the effects of OGD. The contribution of AT₂ receptor stimulation appears to be minimal, and blockade of AT₁ receptors leads to the up-regulation of GLT-1, resulting in a diminished OGD-induced increase in extracellular glutamate. AT₁ receptor signalling could suppress GLT-1 expression by influencing the levels of GLT-1 mRNA. Altogether, our results suggested that AT₁ receptor antagonists might be beneficial for the prevention of many neuronal diseases, including stroke.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Methods.

Figure S1 AT₁ receptor antagonist pretreatment has no neuroprotective effect against glutamate-induced neuronal cell death in primary neuron cultures. A 24-h exposure to glutamate (30 μM) induced a significant lactate dehydrogenase (LDH) release in primary neuron cultures, which was almost totally attenuated by simultaneous treatment with MK801 (NMDA receptor antagonist, 10 μM). A 48-h pretreatment with telmisartan (AT₁ receptor antagonist, 0.01–1 μM) did not reduce the glutamate-induced LDH release in primary neuron cultures. Data represent mean ± SE, *n* = 3 for each group. ****P* < 0.001 versus control; ###*P* < 0.001 versus glutamate; analysis of variance followed by Newman-Keuls multiple comparison test. n.s., not significant; CTL, control; Glu, glutamate; tel, telmisartan.

Figure S2 AT₁ receptor antagonist pretreatment has no neuroprotective effect against oxygen–glucose depletion (OGD)-induced neuronal cell death in primary neuron cultures. A 3-h OGD treatment induced a significant lactate dehydrogenase (LDH) release in primary neuron cultures, which was significantly attenuated by post-treatment with MK801 (NMDA receptor antagonist, 10 μM). A 48-h pretreatment with telmisartan (AT₁ receptor antagonist, 0.01–1 μM) did not attenuate the 3-h OGD-induced LDH release in primary neuron cultures. Data represent mean ± SE, *n* = 3 for each group. ###*P* < 0.001 versus control; ****P* < 0.001 versus OGD; analysis of variance followed by Newman-Keuls multiple comparison test. n.s., not significant; CTL, control; tel, telmisartan.

Figure S3 AT₁ receptor antagonist pretreatment attenuates glutamate-induced neuronal cell death in neuron-astrocyte co-cultures. A 24-h exposure to glutamate (100 μM) induced a significant lactate dehydrogenase (LDH) release in neuron-astrocyte co-cultures, which was significantly attenuated by simultaneous treatment with MK801 (NMDA antagonist, 10 μM). A 48-h pretreatment with telmisartan (AT₁ receptor antagonist, 0.01–1 μM) significantly reduced the glutamate-induced LDH release in a concentration-dependent manner in neuron-

astrocyte co-cultures. Data represent mean \pm SE, $n = 3$ for each group. ### $P < 0.001$ versus control; ** $P < 0.01$ and *** $P < 0.001$ versus glutamate; analysis of variance followed by Newman-Keuls multiple comparison test. tel, telmisartan; CTL, control; Glu, glutamate.

Figure S4 Both AT₁ receptor and AT₂ receptor are expressed in neuron-astrocyte co-cultures. Representative immunoblot data showed that after 10-day incubation with growth medium (Eagle's minimal essential medium supplemented with 10% (v/v) fetal bovine serum) AT₁ receptor and AT₂ receptor

were expressed in neuron-astrocyte co-cultures. Rabbit polyclonal anti-AT₂ receptor antibody and rabbit polyclonal anti-AT₁ receptor antibody used in the present study were obtained from Santa Cruz (Delaware, CA, USA).

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