Angiotensin Receptor Type 2 Activation Induces Neuroprotection and Neurogenesis After Traumatic Brain Injury

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Published online: 24 June 2014

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Abstract Angiotensin II receptor type 2 (AT₂) agonists have been shown to limit brain ischemic insult and to improve its outcome. The activation of AT₂ was also linked to induced neuronal proliferation and differentiation *in vitro*. In this study, we examined the therapeutic potential of AT₂ activation following traumatic brain injury (TBI) in mice, a brain pathology that displays ischemia-like secondary damages. The AT₂ agonist CGP42112A was continuously infused immediately after closed head injury (CHI) for 3 days. We have followed the functional recovery of the injured mice for 35 days post-CHI, and evaluated cognitive function, lesion volume, molecular signaling, and neurogenesis at different time points after the impact. We found dose-dependent improvement in functional recovery and cognitive performance after CGP42112A treatment that was accompanied by reduced lesion volume and induced neurogenesis in the neurogenic niches of the brain and also in the injury region. At the cellular/molecular level, CGP42112A induced early activation of neuroprotective kinases protein kinase B (Akt) and extracellular-regulated kinases 1/2 (ERK1/2), and the neurotrophins nerve growth factor and brain-derived neurotrophic factor; all were blocked by treatment with the AT₂ antagonist PD123319. Our results suggest that AT₂ activation after TBI promotes neuroprotection and neurogenesis, and may be a novel approach for the development of new drugs to treat victims of TBI.

Key Words: Traumatic brain injury · angiotensin receptor type 2 · neuroprotection · neurogenesis · CGP42112A · neurotrophins.

Introduction

Traumatic brain injury (TBI) remains a major cause of mortality and disability worldwide, followed by long-term physical and cognitive consequences. TBI-induced deleterious cerebral effects involve a complex of primary and secondary damage due to glutamate toxicity, oxidative stress, ionic and metabolic imbalance, inflammation, and ischemia [1]. In turn, this damage leads to necrotic and apoptotic cell death. Nonetheless, neuroprotective and repair mechanisms are correspondingly activated in response to injury to reduce brain damage [2]. Despite the acute need of effective pharmacological means to treat victims of TBI, pharmacotherapy remains poorly available and new drug targets are clinically necessary [3]. The ideal drug would not only enhance neuroprotection to reduce tissue damage, but would also promote repair mechanisms in the injured brain.

The renin–angiotensin system (RAS) was discovered more than a century ago [4]; over the years its profound impact on cardiovascular regulation has been well established. Seventy years after its discovery, the production and presence of all the components of the RAS within the adult brain, autonomous of the systemic RAS, was described and provided indications that RAS underlies more than cardiovascular control [5]. The classical active peptide, angiotensin II binds to 2 main receptors: angiotensin receptor type 1 (AT₁) and angiotensin receptor type 2 (AT₂). Both are members of the G protein-coupled family of transmembrane receptors. While AT₁ is predominantly distributed in various regions of the adult brain and is restricted to glia cells [6], AT₂ is extensively expressed in the fetal brain and its levels remain high mostly in neurons at

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specific regions of the adult brain related to cognitive, behavior, and locomotor control [7]. Studies from AT₂ knockout mice imply a developmental role of AT₂, as these mice suffer from perturbations in exploratory behavior and locomotor activity, alongside anxiety like behavior [8–10]. Most of the known effects of brain angiotensin II are mediated through the AT₁ signaling pathway that involves G_{q/11} coupling and mediates vasoconstriction [11]. It also leads to brain ischemia and inflammation, along with mood and memory alterations [12–15]. However, the AT₂ signaling pathway remains enigmatic as most of the known effects of the receptor are independent from G coupling mechanisms [16]. However, *in vitro* experiments have revealed that AT₂ activation involves subsequent activation of tropomyosin-related kinase receptor A (TrkA) and its downstream effectors p42/p44 mitogen-activated protein kinase [extracellular signal-regulated kinases (ERKs) 1 and 2] [17], as well as the release of nitric oxide (NO) and subsequent activation of protein kinase B (Akt) activation [18, 19]. Recently, Namsolleck et al. [20] suggested that activation of AT₂ increases the levels of brain-derived neurotrophic factor (BDNF) and TrkA/B mRNA in primary neurons. Moreover, accumulating evidence is shedding light on an important role for AT₂ in neuronal differentiation and neuronal stem cell proliferation [21–29]. Furthermore, *in vivo* studies point to a role of AT₂ in neuronal survival after ischemic stroke [30–32], axonal regeneration [33], and spinal cord injury [20], as well as in the regeneration of sciatic or optic nerve [33, 34]. However, despite the pathophysiological similarities between brain ischemia and TBI [1], whether AT₂ has a therapeutic potential after TBI has not, to the best of our knowledge, been the subject of any study to date. In this study, we investigated the effect of AT₂ activation using a selective agonist, CGP42112A, after TBI.

Methods

Animals and Maintenance

The study was approved by the Institutional Animal Ethics Committee of the Hebrew University, and complied with the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996). Sabra mice, 9–10 weeks old, weighing 40–51 g were used. Animals were kept under a controlled temperature (24 °C ± 1 °C) and a 12 h light/12 h dark cycle. Food and water were provided *ad libitum*.

Induction of Closed Head Injury

Experimental closed head injury (CHI) was induced under isoflurane anesthesia using a modified weight drop device that was developed in our laboratory [35]. Briefly, after anesthesia,

a midline longitudinal incision was performed, exposing the skull. A Teflon-tipped cone (2 mm diameter) was placed 1–2 mm lateral to the midline in the midcoronal plane. The head was held in place and a 95-g weight was allowed a free-fall on the cone from a pre-established height, resulting in focal injury to the left hemisphere. Motor and neurobehavioral tests, as well histological manifestation of injury and protein markers of signaling pathway network neurogenesis, were measured as detailed below.

Drug Treatment

Mice were randomly allocated to treatment with vehicle (sterile isotonic saline), CGP42112A (Sigma Aldrich, St. Louis, MO, USA) an AT₂ agonist, which is a peptide with a short half-life [25], or PD 123319, an AT₂ antagonist [36, 37]. CGP42112A was delivered in 1 of 3 doses: 0.1, 1.0, or 10.0 ng/kg/min [31]. PD 123319 was delivered at 10 mg/kg/day. All treatments were delivered for 3 days post-CHI, using Alzet osmotic minipumps 1003D (Alzet, Cupertino, CA, USA) as described below (n=9).

Surgical Procedures

At the time of surgery and while anesthetized, the Alzet miniosmotic pumps were implanted into the right lateral ventricle as previously described [38]. The infusion was inserted using an Alzet brain infusion kit into appropriate coordinates (−0.3 mm anterior to bregma; +1 mm to midline; −2.5 mm depth) [39], and the treatment delivered for 3 days postinjury. Cannulas were implanted in a 25-gauge drilled hole. Sham controls received anesthesia, skin incision, and the 25-gauge hole in their skull without minipump infusion or the induction of trauma. For AT₂ antagonist PD 123319 (Tocris Bioscience, Bristol, UK) infusion Alzet miniosmotic pumps were transplanted subcutaneously. After recovery from anesthesia, the mice were given postoperative care in their cages with access to food and water *ad libitum*. Sham control mice underwent anesthesia and skin incisions.

BrdU Injections

From day 1 after injury or sham operation, and for 10 days, all animals received twice-daily intraperitoneal injections of the tracer 5-bromo-2-deoxyuridine (BrdU; 50 mg/kg, q12 h) to label dividing cells.

Neurobehavioral Evaluation

The functional status of the mice was evaluated according to the Neurological Severity Score (NSS) by an observer blinded to the treatment. The NSS is a 10-point scale assessing functional neurological status based on the presence of some

reflexes and the ability to perform motor and behavioral tasks such as beam walking, beam balance, and spontaneous locomotion [40]. Animals are awarded 1 point for failure to perform a task, that is scores increase with the severity of dysfunction. The NSS obtained 1 h post-CHI reflects initial injury severity. NSS values were measured at 1 h, once daily for the first 3 days postinjury and once weekly for 5 weeks postinjury (n=8–10 mice/group).

Novel Object Recognition Test

The object recognition test was performed 3 days after injury as previously described [41]. This is a sensitive and reproducible test for measuring cognitive abnormalities in this model, as was shown in several studies using neuroprotective drugs [38]. Mice were placed for a 1-h habituation period in an open glass aquarium-like transparent box, one at a time, in a sound-isolated room. The next day they were reintroduced into the box for 5 min with 2 identical clean plaster objects, placed in 2 different corners of the box. Four hours later, one of the objects was replaced with a new one of the same size and texture, and the mice were reintroduced for additional 5 min to the same cage. Time spent by the mouse in object exploration was recorded manually by a person blinded for the different treatments, and the cumulative time spent at each of the objects was recorded. Exploration of an object was defined as directing the nose to the object at a distance of 2 cm and/or touching it with the nose. The percentage of the total exploration time that the animal spent investigating the new object out of total exploration time was the measure of recognition memory (n=8–10 mice/group).

Lesion Volume

Thirty-five days after injury, mice from the saline-treated group and from the 1 ng/kg/min CGP42112A-treated group were deeply anesthetized and perfusion-fixed with 4 % paraformaldehyde. Their brains were frozen-sectioned at 10 μ m. Brain slices 200 μ m apart between bregma +1.78 mm and bregma –2.54 mm were obtained. Sections were stained with Giemsa stain-modified solution (1:1; Fluka, Sigma-Aldrich) and were photographed using a stereoscope Stemi SV11 (Zeiss, Jena, Germany) equipped with a digital camera (Coolpix 4500; Nikon, Tokyo, Japan). The volume of injured tissue was measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The damaged tissue volume was calculated as previously described by dividing the volume of the injured left hemisphere by that of the right, nonlesioned hemisphere [38]. The results are expressed as a percentage of hemispheric tissue (n=8–10 mice/group).

Western Immunoblotting

Mice from the saline- and 10 ng/kg/min CGP42112A-treated groups were sacrificed at 24 or 72 h after injury and surgical procedure (n=6–9/group). PD 123319-treated mice were sacrificed after 72 h. After decapitation, brains were rapidly removed and frontal segments (40–60 mg) from the left, injured hemispheres were separated and frozen at –80 °C until analysis. Sample preparation was performed as previously described [42]. After homogenization in a buffer containing sucrose 0.25 M, Tris 20 mM (pH 7.6), MgCl₂ 1.5 mM, glycerol 10 %, ethylenediaminetetraacetic acid 1 mM, samples were centrifuged at 5000 g for 10 min and supernatants were stored at –80 °C until analysis. Protein concentration was determined using a Pierce BSA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Equal protein samples (40 μ g) were separated on 10 % sodium dodecyl sulfate polyacrylamide gels with 4.5 % sodium dodecyl sulfate stacking gels and electrotransferred onto 0.2 μ m nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Blots were probed with anti-nerve growth factor (NGF) 1:200 (Almone Labs, Jerusalem, Israel), anti-pAkt (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-tAkt (1:1000; Cell Signaling Technology), anti-BDNF (1:500), anti-TrkA (1:1000; Abcam, Cambridge, UK), anti-TrkB (1:1000; Abcam), anti-AT₂ (1:1000; Santa Cruz, Dallas, TX, USA), anti-p-ERK (1:1000; Santa Cruz) and anti-t-ERK (1:1000; Santa Cruz). Anti- β -actin antibody (1:1000; Cell Signaling Technology) was used to confirm equal protein loading. Appropriate peroxidase-coupled IgG (1:10,000; Jackson ImmunoResearch, Soham, UK) was used for secondary incubations. Reactive bands were visualized using the enhanced chemiluminescence system (Biological Industries, Beit Haemek, Israel). The optical density of reactive bands was quantified using Tina software (Raytest, Straubenhardt, Germany) and protein levels were expressed as the optical density of the examined factor relative to β -actin in the same lane.

Immunohistochemistry

Evenly spaced (200 μ m) slices, between bregma +1.78 mm and bregma –2.54 mm, were counted for each brain of mice in the saline- and 1 ng/kg/min CGP42112A-treated groups. In each slice, all the relevant fields from subventricular zone (SVZ), dentate gyrus, and from entire area surrounding the lesion were counted. As the lesion core had already liquefied by 35 days after TBI, the area forming the outer boundary of the brain represents the border zone that survived. This area mainly included the cortex and subcortical regions. Brain slices were double-stained for immunohistochemical evaluation using fate-specific antibodies that included anti-BrdU (2 μ g/ml; Calbiochem, Darmstadt, Germany) and for anti-NeuN (1:750; Merck Millipore, Darmstadt, Germany) or glial

fibrillary acidic protein (1:1000; Dako, Glostrup, Denmark). Dylight 488 (1:300; Abcam) and NorthernLights 557 (R&D Systems Emeryville, CA, USA) conjugates were used as secondary antibodies. In order to avoid positive artifacts, UltraCruz mounting medium (Santa Cruz) containing 46-diamidino-2-phenyl indole was used to visualize nuclei.

Statistical Analysis

For statistical analyses, we used commercially available computer software (SigmaStat 2.03; Systat Software, San Jose, CA, USA). Treatments were the independent variables and the outcomes of the TBI parameters were the dependent variables. Significance was tested using one way analysis of variance, followed by the Student–Newman–Keuls post-test method. For NSS calculation, Mann–Whitney analysis was used. p -Values < 0.05 were considered significant for all comparisons. Data are expressed as mean $\pm$ SEM.

Results

CGP42112A Improves Motor and Cognitive Functional Outcome After TBI

Neurological deficits were measured at predetermined time points after TBI using the NSS scale (Fig. 1A). For the first 2 weeks no significant difference among the groups was prominent; however, at 3 weeks and up to 5 weeks postinjury, a dose-dependent effect of the drug was noted, with no effect of the lower dose of 0.1 ng/kg/min CGP 42112A. While the saline-treated group showed no further recovery, mice treated with the higher doses of CGP 42112A retained their recovery rate for 2 more weeks. When compared with saline treatment, the impairment of motor function of both 1 ng/kg/min- and 10 ng/kg/min CGP 42112A-treated groups was dramatically decreased between 3 and 5 weeks postinjury ($p < 0.01$). The experiment was stopped 5 weeks postinjury, after all groups showed no further changes in NSS scores.

Cognitive function was evaluated using the novel object recognition test at 3 days postinjury (Fig. 1B). Both 1 ng/kg/min CGP 42112A-treated group ($p = 0.03$) and 10 ng/kg/min CGP 42112A-treated group ($p = 0.02$) performed better in the test than the saline-treated group, with longer exploration times of the novel object.

CGP42112A is Neuroprotective After TBI and does not Alter the Expression of Receptors

To evaluate whether AT_2 stimulation also leads to a reduction of tissue loss we evaluated the lesion volumes of the injured mice at 35 days postinjury. Lesion volumes were calculated

Fig. 1 Activation of angiotensin receptor type 2 (AT_2) improves traumatic brain injury (TBI) outcome. (A) CGP42112A- (0.1/1.0/10.0 ng/kg/min) or saline-treated mice were subjected to TBI, and neurobehavioral outcome was evaluated using the neurological severity score (NSS) at 1 h postinjury for initial disability assessment and at multiple time points thereafter. CGP42112A treatment led to a dose-dependent decrease of the neurobehavioral deficits. *10 ng/kg/min CGP42112A versus saline-treated mice, $p < 0.01$; **1 ng/kg/min CGP42112A versus saline-treated mice, $p < 0.01$, as determined with the Mann–Whitney test ($n = 8–10$ /group). (B) Mice were subjected to the novel object recognition test 3 days after TBI. CGP42112A treatment led to dose-dependent longer exploration times of the novel object. * $p < 0.05$ versus saline-treated mice, determined using one-way analysis of variance ($n = 8–10$ /group)

after Giemsa staining (Fig. 2). A 3-fold reduction of lesion volume was noted in the 1 ng/kg/min CGP42112A-treated group compared with saline-treated mice ($p < 0.05$).

To investigate molecular pathways associated with neuroprotection in mice treated with CGP42112A, we examined the expression levels of 2 kinases, Akt and ERK, both of which are active in their phosphorylated state. At 24 h postinjury, CGP42112A induced phosphorylation of both ERK (Fig. 3A) and Akt (Fig. 3B) compared with saline treatment ($p < 0.05$ for both). At 72 h postinjury, no further phosphorylation was observed. The AT_2 antagonist PD12319 significantly reduced the level of phosphorylation of both kinases ($p < 0.01$). No changes in total ERK and Akt were noted at the same times (Fig. 3C, D). Moreover, at 24 h postinjury, 1 ng/kg/min CGP42112A induced the expression levels of two

Fig. 2 Activation of angiotensin receptor type 2 (AT₂) reduces lesion volume after traumatic brain injury (TBI). Mice were sacrificed 35 days after TBI, and evenly separated 10- μ m slices were stained with Giemsa. Reduced lesion volume was measured in 1 ng/kg/min GCP42112A-treated mice. * $p < 0.05$ vs saline-treated mice determined using one-way analysis of variance ($n = 8$ –10/group)

neurotrophins—NGF ($p < 0.01$; Fig. 4A) and BDNF ($p < 0.05$; Fig. 4B)—while at 72 h there was spontaneous increase in NGF levels, and CGP42112A treatment had no further effect. No difference in BDNF expression levels between the saline and drug treatment was found at 72 h. The antagonist

PD12319 reduced BDNF protein levels at 72 h postinjury ($p < 0.01$; Fig. 4B) and blocked the spontaneous elevation of NGF seen in saline-treated mice ($p < 0.01$; Fig. 4A). Treatment with 1 ng/kg/min CGP42112A did not affect the expression levels of the neurotrophin receptors TrkA (Fig. 4C) and TrkB (Fig. 4D), nor that of AT₂ receptors (Fig. 4E). However, PD12319 reduced the receptor levels at of TrkA ($p < 0.05$; Fig. 4C) and TrkB ($p < 0.05$; Fig. 4D) 72 h after injury, while no significant reduction in AT₂ levels was found (Fig. 4E).

CGP42112A Induces Proliferation and Neurogenesis After TBI

As AT₂ was suggested to induce cell proliferation and differentiation *in vitro*, we investigated these effects in the injured mice. The number of newborn cells was evaluated by counting BrdU-positive cells and that of new mature neurons by counting double-labeled cells with BrdU and NeuN. Cells in 3 brain regions of interest were counted: the dentate gyrus and the SVZ of the ipsilateral hemisphere, and the cortical region surrounding the injury core. In the dentate gyrus (Fig. 5), after treatment with CGP42112A, significantly more BrdU-positive cells ($p < 0.05$; Fig. 5B), as well as more BrdU and NeuN double-positive cells, were counted ($p < 0.01$; Fig. 5C). Similar results were obtained from the SVZ (Fig. 6), where CGP42112A treatment resulted in more BrdU-positive cells ($p < 0.01$; Fig. 6B) and more BrdU and NeuN double-labeled cells ($p < 0.05$; Fig. 6C). In the region surrounding the injury, CGP42112A treatment did not significantly increase the number of BrdU-labeled cells (Fig. 7A–C); however, it significantly increased the double-stained newborn neurons in the injured region after TBI ($p < 0.05$; Fig. 7A, D), while decreasing the number of double-stained newborn astrocytes ($p < 0.05$; Fig. 7B, E).

Discussion

This study is the first to examine the therapeutic effects of AT₂ activation after TBI. It is also, to the best of our knowledge, the first to demonstrate *in vivo* that AT₂ mediates neurogenesis in brain pathology. A beneficial effect of AT₂ was manifested in behavioral tests that elucidated improved cognitive function and sustained favorable motor recovery after the injury, and in histological examination that revealed attenuated lesion volume after the activation of AT₂. We suggest that induced neurotrophin signals of NGF and BDNF lead to enhanced Akt and ERK1/2 phosphorylation, which results not only in neuroprotection, but also in increased neuronal proliferation and differentiation.

Fig. 3 Activation of angiotensin receptor type 2 (AT_2) induces the phosphorylation (p) of protein kinase B (Akt) and extracellular signal-regulated kinase (ERK). Mice were subjected to traumatic brain injury (TBI), and after treatment either with saline, CGP42112A (CGP; 10 ng/kg/min) or PD123319 (PD; 10 mg/kg/day), were sacrificed 24 or 72 h post-TBI. (A) CGP42112A induced an early increase in the

phosphorylation of (A, C) ERK and in (B, D) Akt with no changes in their total levels, while PD123319 treatment resulted in attenuated phosphorylation levels of both proteins. (E) Representative blots. [#] $p < 0.05$ versus saline-treated mice at 24 h; ^{**} $p < 0.05$ versus saline-treated mice at 72 h, determined using one-way analysis of variance ($n = 6-9$ /group)

CGP42112A Induces Improved Motor Recovery and Cognitive Function

The recovery of neurobehavioral function of mice treated with CGP42112A after TBI was significantly greater than that of their saline-treated littermates. The effect was dose-dependent and only reached significance by 21 days post-TBI. This study also suggests that activation of AT_2 attenuated the cognitive impairment typically seen after TBI. In the novel object recognition test, mice treated with CGP42112A explored the novel object for a longer length of time, indicating preserved memory function. This effect was dose-dependent, with the lowest dose being no more effective than saline. Interestingly, this significant effect was noted 3 days after injury, whereas the effect on the composite NSS only reached significance at 3 weeks. The NSS

and the novel object recognition test are 2 distinguished behavioral tests that examine different physiological abilities. However, these data may imply that the activation of AT_2 is sufficient to induce a cognitive effect, whereas the motor effects may involve more complex processes. It is possible that cognitive function is more affected by induced neuroprotection than the motor performance that is more influenced by the delayed neuroregeneration. Moreover, it has previously been shown that after TBI the hippocampal neurons are extremely vulnerable, which may also underlie the differential effect of PD123319 on cognitive and motor function [43]. The effect of AT_2 activation on cognitive function has been the focus of many recent studies. Angiotensin II receptor type 1 blockers are associated with attenuated progression of Alzheimer's disease and dementia compared with angiotensin converting enzyme inhibitors,

Fig. 4 Activation of angiotensin receptor type 2 (AT₂) increases the protein levels of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Mice were subjected to traumatic brain injury (TBI) and then treated with saline, CGP42112A (CGP; 10 ng/kg/min) or PD123319 (PD; 10 mg/kg/day) and sacrificed 24 or 72 h post-TBI. CGP42112A induced an early increase in (A) NGF and in (B) BDNF; however, PD123319 treatment lowered the levels of both proteins.

implying a beneficial involvement of AT₂ [44]. AT₂ activation was correlated with enhanced cognitive function [28], while genetic depletion of AT₂ resulted in a worse cognitive outcome than in wild-type controls [45]. It was suggested that AT₂ enhances cognitive function via improving microcirculation and cerebral blood flow as a result of bradykinin and the production of NO [46, 47]. As secondary ischemia is one of the deleterious consequences after TBI [1], improved microcirculation could explain, in part, the improved cognitive and motor recovery following AT₂ activation. However, measuring

CGP42112A did not alter the expression levels of neurotrophin receptors (C) tropomyosin-related kinase receptor (Trk)A and (D) TrkB, whereas PD123319 decreased their levels. (E) The expression of AT₂ e was not effected by either treatment. (F) Representative blots. [#]*p*<0.05 versus saline-treated mice at 24 h; ^{**}*p*<0.05 versus saline-treated mice at 72 h, determined using one-way analysis of variance (n=6–9 per group)

cerebral blood flow was beyond the scope of this study. BDNF was also investigated, and its enhanced levels, which are associated with improved memory and cognitive function [48, 49], may underlie this effect after AT₂ activation following TBI.

CGP42112A Induces Neuroprotection: A Role for Neurotrophins Signaling

The observation of reduced lesion volume after CGP42112A treatment further reinforces the neuroprotective role of

Fig. 5 Activation of angiotensin receptor type 2 (AT_2) induces neurogenesis in the dentate gyrus. Mice were subjected to traumatic brain injury (TBI), treated with saline or CGP42112A (CGP; 1 ng/kg/min) for 3 days, and sacrificed 35 days post-TBI. Evenly sliced 10- μ m sections were incubated with anti-NeuN for mature neurons and with anti-5-bromo-2-deoxyuridine (BrdU) for newborn cells. (A) CGP42112A enhanced neurogenesis in the dentate gyrus (e–h) compared with saline-

treated mice (a–d). Stereological quantification of the fields was used to count the number of BrdU-positive cells (BrdU⁺). Results show (B) the average number of BrdU⁺ per field and (C) the average number of double-positive cells for NeuN and BrdU (BrdU⁺/NeuN⁺) per field. * $p < 0.05$ versus mice treated with saline, determined using one-way analysis of variance ($n = 6$ –7 per group)

CGP42112A. Furthermore, reduced lesion volume corresponded to induced Akt phosphorylation as early as 24 h after TBI, a neuroprotective event that spontaneously occurred, in the saline-treated mice, only 72 h postinjury. The altered Akt dynamics suggest that AT_2 activation induces early neuroprotection, which, in turn, may explain the reduced tissue damage and the delayed development of improved motor outcome. Akt is a widely studied mediator that has been shown to play a role in neuroprotection after TBI in our model, as well as in other TBI models [50–52]. Our findings are in accordance with previous *in vitro* studies that

nominated Akt as a potential downstream target of AT_2 activation [19, 29, 53, 54]. Furthermore, it has been suggested that AT_2 -induced Akt phosphorylation results in enhanced NO production [53, 55, 56], which may lead to cerebrovascular relaxation and improved blood flow after the injury. Indeed, reduced blood flow in a model of cerebral ischemia was observed in AT_2 -deficient mice ($Agtr2^{-/-}$) [57], which may imply that AT_2 mediates improved perfusion to the injured area.

We have also demonstrated a similar dynamics profile of ERK1/2 phosphorylation after TBI. CGP42112A treatment

Fig. 6 Activation of angiotensin receptor type 2 (AT₂) induces neurogenesis in the subventricular zone (SVZ). Mice were subjected to traumatic brain injury (TBI) then treated with saline or CGP42112A (CGP; 1 ng/kg/min) for 3 days, and sacrificed 35 days post-TBI. Evenly sliced 10-μm sections were incubated with anti-NeuN for mature neurons and with anti-5-bromo-2-deoxyuridine (BrdU) for newborn cells. (A) CGP42112A enhanced neurogenesis in the SVZ (e–h) compared with

saline-treated mice (a–d). Stereological quantification of the fields was used to count the number of BrdU-positive cells (BrdU⁺). Results show (B) the average number of BrdU⁺ per field and (C) the average number of double-positive cells for NeuN and for BrdU (BrdU⁺/NeuN⁺) per field. **p* < 0.05 versus mice treated with saline, determined using one-way analysis of variance (n = 6–7 per group). LV left ventricle

led to an early induction of ERK1/2 phosphorylation, which is related to neuroprotection after TBI [58, 59], and to reduced neuronal apoptosis [60]. The phosphorylation of ERK1/2 was the first described downstream effector of AT₂ in neuronal cell lines and is a key event for differentiation and the induction of neurite outgrowth [61, 62].

The induction of both Akt and ERK1/2 may be a result of the enhanced levels of the neurotrophins NGF and BDNF. To the best of our knowledge, this is the first report to show that the activation of AT₂ induces the expression of both BDNF

and NGF *in vivo*. The levels of these neurotrophins were elevated 24 h after injury compared with saline-treated controls. NGF protein levels in the injured cortex were validated using enzyme-linked immunosorbent assay and were in agreement with the results obtained from the Western blot (data not shown). Both NGF and BDNF are strongly linked to neuroprotection and neuroregeneration (for a review see [63]), partly via the induction of Akt and ERK1/2 signaling [60, 63, 64]. Furthermore, both NGF and BDNF were shown to promote the elevation of Akt-mediated hypoxia inducible

◀ **Fig. 7** Activation of angiotensin receptor type 2 (AT₂) induces neurogenesis at the expense of astrogenesis in the injured region (inj). Mice were subjected to traumatic brain injury (TBI) then treated with saline or CGP42112A (CGP; 1 ng/kg/min) for 3 days, and sacrificed 35 days post-TBI. Evenly sliced 10- μ m sections were incubated with anti-NeuN for mature neurons and with anti-5-bromo-2-deoxyuridine (BrdU) for newborn cells. (A) CGP42112A enhanced neurogenesis in the region surrounding the injury (e–h) compared with saline-treated mice (a–d). (B) CGP42112A reduced astrogenesis in the region surrounding the injury (e–h) compared with saline-treated mice (a–d). Stereological quantification of the fields was used to count BrdU-positive cells (BrdU⁺). Results show (C) the average number of BrdU⁺ per field, (D) the average number of double-positive cells for NeuN and for BrdU (BrdU⁺/NeuN⁺) per field, and (E) the average number of double-positive cells for glial fibrillary acidic protein (GFAP) and for BrdU (BrdU⁺/GFAP⁺) per field. **p* < 0.05 versus mice treated with saline, determined using one-way analysis of variance (n = 6–7 per group)

factor 1 α [65, 66], which was recently described as an indispensable factor for the spontaneous recovery after TBI in our model [67].

Interestingly, the inhibition of AT₂ by PD123319 reduced the expression levels of both neurotrophins (NGF, BDNF) and their prosurvival downstream effectors (Akt and ERK1/2), and prevented the spontaneous elevation of all 4 proteins at 72 h after injury. This observation may also imply a role for AT₂ in the spontaneous neuroprotection process. Moreover, the administration of CGP42112A did not affect TrkA and TrkB receptor levels (NGF and BDNF receptors, respectively), while PD123319 did reduce their levels, which may indicate another prospective of the regulatory effect of AT₂ on neurotrophin signaling. Our results are not in full agreement with a recently published study that demonstrated induced mRNA levels of BDNF, TrkA, and TrkB after the activation of AT₂ both in primary neurons and astrocytes, as well as induced TrkB protein expression levels in injured spinal cord. However, one should remember that brain neurons differ from spinal cord neurons in TrkB expression and distribution [68]. In the present study we did not observe any changes in the expression level of AT₂ as a result of the injury. An intriguing recent study challenges these results and claims that the commonly used AT₂ antibodies lack specificity; this challenges not only our results, but also existing data regarding AT₂ protein expression after brain injury [69].

CGP42112A Treatment Post-TBI Induces Neurogenesis

An intriguing observation in this study is the timeframe for improved neurobehavioral recovery after CGP42112A treatment. Only by 21 days were the beneficial effects of the treatment noted in the NSS test. The delayed onset of these beneficial effects suggests that AT₂ mediates not only neuroprotection, but also neurorepair processes. According to the adult neurogenesis timeline described by Zhao et al. [70],

newborn neurons start to reach maturity and express NeuN at 14–28 days after their birth. That timeline fits the neurobehavioral improvement that was accelerated from 21 days postinjury after CGP42112A treatment shown in this investigation. Further improvement of treated mice is seen up to 5 weeks postinjury corresponding to integration timeframe of new neurons [70].

Neurogenesis in the adult rodent brain occurs constantly in the SVZ lining the lateral ventricles [71], and in the subgranular zone (SGZ) of the hippocampal dentate gyrus [72]. The proliferation rate of neuronal precursor cells (NPCs) is dynamically altered by different brain pathologies, including TBI. Following injury, the proliferation rate of NPCs is increased and a shift of the migratory pathway of the newborn cells towards the lesion region or the striatum is seen [73–76]. Given this profile, using BrdU labeling we found that CGP2112A treatment enhanced the proliferation of NPCs in the SVZ of the left (ipsilateral) ventricle and in the SGZ of the left hippocampus (SGZ) after TBI. These results are in accordance with recent *in vitro* studies showing the proliferative effect of AT₂ on neuronal stem cells [29]. Furthermore, increased number of newborn mature neurons (double-labeled NeuN⁺/BrdU⁺ cells) in the SGZ and in the SVZ indicates enhanced neuronal differentiation after the administration of CGP2112A. These findings are in agreement with *in vitro* studies linking AT₂ with neuronal differentiation [30, 34, 61]. Li et al. [30] also found induced neurite outgrowth in AT₂-labeled neurons in the ischemic striatum, suggesting a possible role of AT₂ in neuronal differentiation *in vivo*.

While newborn neurons detected in the SGZ is an expected observation following TBI [73, 77], spotting mature neurons in the striatal SVZ was surprising. It is well accepted that SVZ-derived neuroblasts migrate in their immature form and differentiate only after the migratory period, and hence do not differentiate within the SVZ [76]. Nevertheless, we are not the first to observe this phenomenon, and BrdU⁺/NeuN⁺ were previously observed in murine SVZ [78]. Our observation may suggest that TBI alters the migration pathway and the differentiation fate of NPCs in the SVZ, and that this effect is enhanced by treatment with CGP42112A. Why migration is inhibited and/or differentiation occurs at early time point after migration initiation remains an enigma; however, it is possible that the increased BDNF levels in an early post-TBI phase may play a role in this process [79]. Theoretically, the migration of newborn neurons can be limited after TBI as the cell migration is guided partially by blood vessels, which are often compromised after TBI [70].

Interestingly, focusing on the injury region, no significant difference in the number of newborn BrdU⁺ cells was observed between saline- and CGP42112A-treated groups. However, more newborn neurons were counted in this region at the expense of newborn astrocytes after treatment with CGP2112A. As the cortex is not considered to be a neurogenic

region, one may assume that the vast majority of the newborn cells surrounding the injury migrated from the neurogenic niches and differentiate later mostly into astrocytes to create astrocytic scar [73–76]. It is important to mention existing evidence suggesting that bone marrow precursor cells can generate neurons [80], and as the blood–brain barrier is compromised after TBI [1, 35], we cannot exclude the possibility that newborn neurons seen in the lesion area are from hematopoietic origin. At this point in time we also cannot conclude whether more newborn cells were observed in the injury region owing to the fact that their primary proliferation and generation was induced in the SVZ, or that CGP42112A also promotes the migration of newborn neurons to the lesion region. The regulation of proliferation, differentiation, and migration of NPCs is the subject of many studies, but it remains to be elucidated. Our study suggests a potential involvement of AT₂ in each of these stages. The proliferative, survival, and prodifferentiation effects of CGP42112A may be attributed, at least in part, to the observed early elevation of the neurotrophins (e.g., BDNF and NGF) and their downstream effectors ERK1/2 and Akt [29, 70, 81–83]. Of note, as BrdU injections were given at early time points after TBI, when apoptotic events still take place [42], we cannot entirely exclude the possibility of false-positive labeling of neurons undergoing apoptosis. However, as the morphology of the labeled cells that did not correspond to the typical morphology of cells undergoing apoptosis, this option is highly unlikely.

Conclusions and Remarks

Taken together, this study implies that AT₂ activation after TBI enhances neuroprotection and neurogenesis, which lead to improved motor and cognitive function, and may be considered as a novel therapy for TBI insults. However, CGP42112A is unlikely to be used as a therapeutic agent owing to undesired pharmacokinetics properties (e.g., a peptide with a short half-life) [25, 27]. The nonpeptide AT₂ agonist compound 21, described by Wan et al. [84], has a more favorable pharmacokinetic profile and may be more applicable to clinical treatment for TBI victims. Future study is also required in order to determine the therapeutic window and to examine whether the beneficial effects of AT₂ activation are preserved when the agonist is administered at later time points after TBI.

Acknowledgments This study was supported, in part, by grants (to ES) from the Brettler Foundation at the School of Pharmacy, and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (AMRF).

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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