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DISSERTATION

Pharmacological stimulation of the AT2 receptor activates endogenous neuro-protective and neuro-regenerative mechanisms via BDNF-mediated signaling

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Abstract (English)

The renin-angiotensin system (RAS) plays an important role in the initiation and progression of cardiovascular diseases. The detrimental actions of the AT1 receptor (AT1R) including cardiac and vascular hypertrophy, inflammation and apoptosis have been well established and described. Recently, several publications have suggested novel, protective actions of the RAS not only in the cardiovascular- but also in the nervous system. The so-called protective arm of the RAS includes AT2- and Mas receptors and is characterized by effects different from and often opposing those of the AT1R. In the current work, the neuro-protective and neuro-regenerative potential of AT2R stimulation was studied in detail. In an animal model of stroke, indirect stimulation of AT2R reduced stroke volume and promoted neuronal survival accompanied by improved sensomotoric functions. In animals subjected to spinal cord injury, AT2R stimulation ameliorated locomotor performance in a time-dependent manner suggesting involvement of neuro-regenerative processes. An extensive analysis of the tissue samples revealed elevated numbers of regenerating axons cranially and caudally from the lesion area in animals treated with an AT2R agonist. The underlying mechanisms were elucidated using gene expression analysis and morphological assays. The results showed for the first time that the neuro-protective and neuro-regenerative actions of AT2R are mediated through a defined neurotrophic pathway.

Abstract (German)

Entstehung und Progression kardiovaskulärer Erkrankungen werden maßgeblich durch das Renin-Angiotensin-System (RAS) beeinflusst. Viele der pathologischen Effekte des AT1-Rezeptors (AT1R), darunter Herz- und Gefäßhypertrophie, Inflammation und Zelltod, sind mittlerweile weitgehend untersucht. In mehreren kürzlich erschienenen Publikationen wurde jedoch beschrieben, dass dem RAS auch eine protektive Seite zukommt und dies nicht nur auf kardiovaskulärer Ebene, sondern auch im Nervensystem. Zum "protektiven Arm" des RAS zählen der AT2- und der MAS-Rezeptor, welche unterschiedliche, dem AT1R teilweise diametral entgegengesetzte Effekte vermitteln. In der vorliegenden Arbeit wurden die neuroprotektiven und neuroregenerativen Effekte einer direkten AT2R-Stimulation detailliert untersucht. Im Schlaganfall-Tiermodell konnte gezeigt werden, dass die indirekte Stimulation des AT2R zu einer Reduktion des Infarktareals führt und neuronalen Zelluntergang inhibiert, wobei sich dies auch in einer verbesserten sensomotorischen Leistung der Versuchstiere bemerkbar machte. Bei Versuchstieren mit Rückenmarksverletzung zeigte eine direkte AT2R-Stimulation in zeitabhängiger Weise einen positiven Einfluss auf deren Lokomotor-Funktion, was auf zu Grunde liegende neuroregenerative Prozesse schließen ließ. In ausführlichen Analysen von Gewebeproben konnte demonstriert werden, dass die mit einem AT2R-Agonisten behandelte Tiere in cranial und caudal der Läsion gelegenen Regionen eine vermehrte Anzahl an regenerierenden Axonen aufweisen. Zur Darstellung des zugrundeliegenden Mechanismus kamen Expressionsanalysen und morphologische Assays zum Einsatz. Die Ergebnisse zeigen zum ersten Mal, dass die neuroprotektiven und neuroregenerativen Effekte des AT2R durch definierte neurotrophe Signalwege vermittelt werden.

1. Introduction and aims

One of the oldest hormone systems, the renin-angiotensin system (RAS), has been traditionally linked to cardiovascular diseases. The circulating components of RAS, with the effector molecule angiotensin II (Ang II), regulate blood pressure and salt and water homeostasis. Two major receptor subtypes for Ang II have been identified: the angiotensin AT1 receptor (AT1R) and angiotensin AT2 receptor (AT2R). While the AT1R mediates various detrimental cellular processes including apoptosis and inflammation, the AT2R subtype displays different and often opposing effects (1).

All of the RAS components found in the central nervous system (CNS) can be synthesized locally and independently from peripheral precursors. In contrast to the AT1R, the AT2R is highly expressed during fetal development and is less abundant in adult tissue (2).

The current work focuses on two processes, neuro-protection and neuro-regeneration that might be, at least partially, mediated by AT2R. Neuro-protection can be defined as a process that directly prevents necrotic or apoptotic neuronal cell death (primary neuro-protection) or affords protection of myelin, axons, and neurons by e.g. anti-inflammation (secondary neuro-protection). Neuro-regeneration can be defined as a complex process restoring the interrupted neuronal connectivity and resulting in functional recovery. The underlying mechanisms may involve cell renewal, synaptic plasticity, regrowth of severed axons and sprouting of non-damaged neurons compensating the loss of a neighboring tract (3).

Several studies have suggested that the AT2R acts as a neuro-protective principle: The expression of AT2R is massively up-regulated in neuronal damage as demonstrated in animal models of stroke and of sciatic or optic nerve crush (4–6). Genetically modified animals lacking AT2R subjected to focal cerebral ischemia have larger infarcts as compared to the wild-type animals, mainly due to exacerbated inflammation and generation of reactive oxygen species (7). AT2R stimulation may prevent neuronal cell death *in vivo* as shown in an animal model of stroke (4). In addition to the anti-inflammatory and anti-apoptotic activities, the AT2R has been reported to have a neuro-regenerative potential. Stimulation of the AT2R induces axonal sprouting *in vivo* (5, 8) and neurite outgrowth *in vitro* (4). These observations have been made indirectly, using the natural ligand, Ang II, in combination with AT1R- or AT2R-blockers. However, using this strategy, it was difficult to single out the molecular mechanisms underlying the above described AT2R-mediated effects.

In the last decade, research on AT2R has been limited mainly due the absence of appropriate pharmacological tools such as selective agonists. The only available AT2R-

agonist, peptide CGP42112A, has impaired *in vivo* stability and partial antagonistic properties limiting its use for AT2R research (9). In 2004, the first non-peptide AT2R-selective agonist, termed compound 21 (C21), has been introduced (10). With a reasonable half-life (4 h in rats) and a bioavailability of 20-30%, C21 can serve as a research tool for studies of AT2R function *in vivo*.

The primary aim of this work was to elucidate the molecular mechanisms involved in the neuro-protective and neuro-regenerative actions of AT2R. It was hypothesized that both, the anti-inflammatory activity and neurotrophic pathways, are crucial for the beneficial effects mediated *via* AT2R. First, the anti-inflammatory action of the selective AT2R agonist C21 was tested *in vivo* in rats subjected to myocardial infarction and compared with AT1R blockade. The relevance of these effects for the central nervous system was studied *in vitro* in CNS-derived cells. Further, in an animal model of cerebral ischemia, it was tested whether treatment with the AT1R blocker, telmisartan, provided sufficient anti-inflammatory and neuro-protective potential *via* unopposed stimulation of the AT2R by the endogenous Ang II. The detailed molecular mechanisms underlying the neuro-protective and neuro-regenerative effects of AT2R were investigated *in vitro* using C21 and AT2R antagonists in primary neurons and astrocytes. The expression of various neurotrophins was screened with a PCR array and validated using TaqMan chemistry. The impact of AT2R stimulation on neuronal differentiation and a potential role of neurotrophic factors were investigated using in-house-developed *in vitro* differentiation assays. Finally, data obtained from the above mentioned experiments were validated *in vivo*, in an animal model of spinal cord injury treated with the AT2R agonist C21.

2. Methods

All of the animal experimental procedures complied with the *Guide for the Care and Use of Laboratory Animals* of the State Government of Berlin, Germany and were approved by the State Government of Berlin.

2.1. Myocardial infarction in rat [P1]

In Wistar normotensive rats, myocardial infarction (MI) was induced by permanent ligation of the left coronary artery. Starting 24 h after MI, the animals were treated with either vehicle (0.9% NaCl IP), candesartan (0.1 mg/kg per day IP), C21 (0.03 mg/kg per day IP) or with a combination of C21 and PD123319 (0.03 mg/kg and 3.0 mg/kg per day IP, respectively). After seven days of treatment, the animals were sacrificed and hearts were collected for gene expression analysis.

2.2. Cerebral ischemia in rat [P2]

Starting at five days prior the induction of cerebral ischemia, the male normotensive rats were treated with either telmisartan (0.5 mg/kg once daily) or with vehicle (0.9% NaCl once daily) subcutaneously. The chosen dose of telmisartan inhibits renin-angiotensin system sufficiently without changing arterial pressure or cerebral blood flow. Cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) for 90 minutes with subsequent reperfusion. In order to analyze the volume of cerebral ischemic tissue, T2-weighted magnetic resonance imaging (MRI) was performed 48 hours after MCAO. Neurological deficits were evaluated daily using 18-point Garcia neurological scoring system.

2.3. Re-innervation assay [P3]

To investigate re-innervation *in vitro*, slice co-culture of entorhinal cortex of a beta-actin-EGFP mouse combined with hippocampus of wild-type animals was used. After 48 h of treatment with C21 (1 µM) or with vehicle, the slices were fixed and the percentage of EGFP-positive axons growing into the hippocampus was calculated using MetaMorph software.

2.4. Cell-culture [P3-5]

In the current work, neuroblastoma × glioma hybrid cell-line NG108-15, primary embryonic neurons (E15) and postnatal astrocytes (P2) obtained from Wistar rats, wild-type C57BL-6/J and AT2R knock out (AT2R-KO) mouse were used.

For the evaluation of anti-inflammatory properties of C21 in CNS-derived cells, primary rat astrocytes were stimulated with lipopolysaccharide (10 µg/ml) and treated with either vehicle or C21 (1 µM). For the neurite outgrowth assay, NG108-15 cells were cultivated on the poly-L-lysine-coated 96-well plates and treated daily with increasing concentration of C21, ranging from 0.2 to 4 µM. For other assays, primary mouse neurons and astrocytes were stimulated daily with either vehicle or C21 (1 µM) and co-treated with either AT2R antagonist PD123319 (10 µM) or nonselective Trk-receptor inhibitor K252a (100 nM). The AT2R-KO derived cells served as negative control.

2.5. Gene expression analysis [P1, P3, P5]

The gene expression of pro-inflammatory cytokines IL-1β, IL-2 and IL-6 in peri-infarct zone of rat hearts was quantified with TaqMan real-time PCR.

For the *in vitro* analysis, primary neurons and astrocytes were pharmacologically stimulated for 24 h and 12 h respectively, as described in paragraph 2.4. The Mouse Neurotrophin and Receptors RT^2 Profiler PCR Array (SABiosciences/Qiagen, Germany) was used for the screening of AT2R-mediated expression of neurotrophins as well as genes involved in neuronal regeneration, survival, differentiation and cell growth. Differentially expressed genes were validated with TaqMan real-time PCR. Additionally, the expression of selected genes, including pro-inflammatory cytokines IL-6 and TNFα, neurotrophins BDNF and NGF, their receptors TrkA, TrkB and TrkC, anti-apoptotic Bcl-2 and neurite outgrowth and plasticity marker GAP43, was evaluated by SYBR Green quantitative real-time PCR (qPCR).

2.6. Neurite outgrowth assays *in vitro* [P3, P4]

AT2R-mediated neuritogenesis was evaluated in NG108-15 cell-line and in primary neurons. In NG108-15 cells, neurite outgrowth was evaluated microscopically 72 h after treatment with C21 as a percentage of cells harboring two or more neurites. The neuritogenesis was studied more extensively in primary neurons. After 48 h of stimulation, cells were labeled with monoclonal MAP-2 antibody followed by Cy3-coupled secondary antibody. The axon and plexus length and the number of branches were analyzed using ImageJ, a computer tool designed for quantification of microscopy images.

2.7. Spinal cord injury in mice [P3]

Spinal cord injury was induced after partial laminectomy, by the precise compression at thoratic level T8. Only in the first treatment strategy, a Gelfoam patch was placed on the top of perforated dura. Finally, the corticospinal tract tracing was performed by injection of biotinylated dextran amine (BDA) into the motor cortex.

Animals randomly assigned to the treatment groups were daily treated intraperitoneally with either vehicle or C21 (0.3 mg/kg bw) for four weeks.
Locomotor performance was assessed with the Basso Mouse Scale and a footprint analysis and documented with video camera.

2.8. Immunocytochemical, histological and immunohistochemical analysis [P2, P3, P5]

For the immunocytochemical staining of IL-6 and TNFα, rat astrocytes were fixed and incubated with primary antibody followed by Cy3-labelled secondary antibody.
For the tissue analysis, the brain and spinal cord tissues were cryopreserved and sectioned using cryostat. The staining of IL-6, TNFα and TrkB was performed with primary antibody followed by Cy3-labelled secondary antibody. Viable neurons were stained with NeuroTrace Fluorescent Nissl Stain. The fluorescent images were quantified by ImageJ. For the corticospinal tract tracing the BDA-positive nerve fibers were counted and the representative camera lucida drawings were made.

3. Results

3.1. AT2R stimulation and AT1R blockade act anti-inflammatory *in vivo* and *in vitro*

In order to prove the postulated anti-inflammatory properties of AT2R-stimulation, selective AT2R-specific stimulation with C21 was first tested *in vivo* in an animal model of myocardial infarction. The relevance of these effects for the central nervous system was then investigated *in vitro*, in primary rat astrocytes. Using an animal model of cerebral ischemia, it was tested whether the treatment with the AT1R blocker, telmisartan, provided sufficient anti-inflammatory potential *via* unopposed stimulation of the AT2R by the endogenous Ang II.

A. C21 reduces inflammation induced by myocardial infarction in rats

As compared to the sham animals, MI strongly elevated expression of pro-inflammatory IL-1β, IL-2 and IL-6 in peri-infarct zone of rat hearts [P1; Fig.6A-C]. Treatment with candesartan or with C21 reduced these cytokines to the level observed in sham-operated animals. The anti-inflammatory effect of C21 was absent in animals concomitantly treated with C21 and PD123319, revealing specificity of the AT2R-agonist.

B. C21 reduces inflammation in CNS-derived cells

In primary rat astrocytes, stimulation with lipopolysaccharide induced expression of IL-6 and TNFα as shown by real-time PCR [P5; Fig.2] and immunocytochemical staining [P5; Fig.3]. The production of both cytokines was significantly reduced in cells treated with C21, indicating strong anti-inflammatory activity also in CNS-derived cells.

C. Telmisartan reduces inflammation in a cerebral ischemia model

As compared to the sham-operated animals, MCAO elevated the number of IL-6- and TNFα-positive cells in periventricular penumbra [P2; Fig.4C-D]. Treatment with telmisartan significantly decreased the number of TNFα-positive cells, whereas only a non-significant trend was observed for IL-6 staining.

3.2. Treatment with telmisartan provides pronounced neuro-protection in an animal model of cerebral ischemia

It was hypothesized that the blockade of the AT1R provided neuro-protection *via*, at least partially, unopposed stimulation of the AT2R by the endogenous Ang II.

A. The AT1R blockade reduces neurological deficits

Following MCAO, impairment of sensomotoric performance was observed in vehicle-treated animals. The treatment with telmisartan significantly improved neurological outcome 24 h and 48 h after cerebral ischemia [P2; Fig.3D].

B. The AT1R blockade reduces infarct volume

As estimated by small-animal MRI two days after transient cerebral ischemia, animals treated with telmisartan had significantly smaller infarct volume as compared to the vehicle-treated group [P2; Fig.3C].

C. The AT1R blockade prevents neuronal cell loss and restores impaired expression of TrkB

Since injured neurons are less reactive for Nissl stain, the number of Nissl-positive cells in ischemic periventricular penumbra reflects the number of surviving cells protected from ischemic damage. In comparison to the sham-operated animals, MCAO induced neuronal cell loss in ipsilateral hemisphere [P2; Fig.4A-B] and reduced the number of TrkB-positive cells [P2; Fig.4E]. Treatment with telmisartan partially prevented neuronal cell loss and completely restored impaired TrkB expression.

3.3. Compound 21 induces neurite outgrowth *in vitro via* selective AT2R stimulation and promotes re-innervation in organotypic cultures

It was postulated, that AT2R-stimulation possess a neuro-regenerative potential mainly *via* neuritogenesis and could re-innervate target tissue.

A. C21, in dose-dependent manner, elevates the number of neurite-positive cells

The neurite outgrowth was investigated in NG108-15 cell-line. Stimulation with C21 for 3 days induced neurite outgrowth [P4; Fig.1]. As compared to the vehicle-treated cells, the dose of 2.0 μM significantly elevated the number of neurite-positive cells but also lower doses of C21 (0.4 and 1.0 μM) showed positive trends.

A. C21 induces AT2R-specific neurite outgrowth in primary neurons

Neurite outgrowth was determined in primary mouse neurons stimulated for 48 h with either vehicle or C21 (1 μM). C21 significantly induced neurite outgrowth in neuronal cultures obtained from wild-type animals that was absent in AT2R-KO neurons and in cells co-treated with the AT2R antagonist PD123319 [P3; Fig.8A-C]. This provides strong evidence for the specific binding of C21 to the AT2R.

B. C21 promotes re-innervation in a co-culture of entorhinal cortex and hippocampus

Treatment with C21 for 48 h elevated the number of EGFP-positive axons growing from the entorhinal cortex into the hippocampus, indicating that AT2R stimulation promotes re-innervation of a selected target area [P3; Fig.7A-D].

3.4. AT2R stimulation induces expression of neurotrophins and their receptors in primary neurons and astrocytes

Neurotrophins are well-known inducers of neuronal differentiation, possessing neuro-regenerative and neuro-protective potential. Therefore it was assumed that pronounced neurite outgrowth and re-innervation may be mediated by the neurotrophic factors.

A. Screening for neurotrophins regulated by AT2R

Using the Mouse Neurotrophin and Receptors RT2 Profiler PCR-Array, the expression of 84 genes involved in neuronal differentiation and neuro-protection was analyzed. In primary neurons treated with AT2R agonist, 9 genes were upregulated more than 1.5-fold as compared to the vehicle treated cells. From those only two, the brain-derived neurotrophic factor (BDNF) and the galanin-1 receptor (Gal1R) could be confirmed with TaqMan qPCR [P3; Fig.9A-B].

B. C21 induces expression of neurotrophins and its receptors in primary neurons and astrocytes

Based on the results obtained from the screening assay, the measurement of BDNF expression was extended to neutrophin receptors. In primary neurons or astrocytes treated with either vehicle or C21, the expression of BDNF, TrkA, TrkB and TrkC were analyzed with SYBR Green qPCR. Upon the AT2R-stimulation, all of the selected molecules, excluding TrkC, showed significantly increased expression as compared to the vehicle-treated cells [P3; Fig.10A-C and Fig.11A-C]. All of the observed effects were blocked with AT2R-antagonist, PD 123319 confirming the AT2R-specificity.

C. C21 induces expression of neurotrophic pathway target genes

Since AT2R stimulation elevated the expression of neurotrophic factors, the expression of their target genes was investigated in primary neurons and astrocytes. In cells stimulated with C21, an increased expression of anti-apoptotic Bcl-2 was found [P3; Fig.10D and Fig.11D]. Moreover, the stimulation of AT2R led to the significant upregulation of GAP 43 [P3; Fig.10E], a marker for neuronal differentiation and plasticity. Co-incubation with PD123319 completely abolished C21-mediated effects, indicating AT2R-specificity.

3.5. Neurotrophin signaling is crucial for C21-induced neurite outgrowth

The above described expression changes in neurotrophic pathway clearly showed that neurotrophins and their receptors are the target genes of the AT2R. However, the observation does not provide functional proof for the role of neurotrophins in C21-mediated neurite outgrowth. In the above described neurite outgrowth assay, a blockade of neurotrophin signaling with K252a should have abolished the C21-induced sprouting of neurites. As shown in Fig. 12 [P3], C21-induced neurite outgrowth was strongly reduced in

cells co-treated with K252a, indicating that the AT2R stimulation induces neurite outgrowth mainly *via* the neurotrophin pathway.

3.6. AT2R-stimulation reduces neurological deficits, promotes axonal plasticity and neuronal survival in an animal model of spinal cord injury

The data obtained from *in vitro* experiments elucidated the molecular mechanisms involved in AT2R-mediated morphological changes of neuronal cells. The spinal cord injury model would reveal the *in vivo* relevance of these effects. Animals were daily treated with either C21 (0.3 mg/kg BW) or vehicle over a period of 4 weeks. Another group received additional topical treatment with either C21 or vehicle immediately after compression of spinal cord. The neurological performance was scored using two different methods: Basso Mouse Scale (BMS) and foot-print analysis. At the end of the study, the spinal cord tissue was extensively analyzed using immunohistological staining.

A. C21 improves neurological outcome after SCI in mice

As shown with BMS, the C21-treated animals were characterized by improved neurological performance reaching 1.47 points difference at the end of the study as compared to the vehicle-treated group [P3; Fig.1C]. In the group receiving both systemic and local-treatment, no additional improvement was observed, however a better neurological performance in the first week of the study was apparent [P3; Fig.1A]. In the BMS subscore evaluating the paw position, animals treated with C21 showed continuous recovery over the whole study period that was not observed in the vehicle-treated animals [P3; Fig.1B and Fig.1D]. Another neurological evaluation, the foot-print analysis, was performed on day 21 [P3; Fig.2A]. There was no difference in base width of forelimbs and hindlimbs between treatment regimens [P3; Fig.2B]. However, the stride length of forelimbs and hindlimbs was significantly improved in C21-treated group [P3; Fig.2C-D].

B. C21 promotes axonal plasticity after SCI in mice

As demonstrated *in vitro*, stimulation of AT2R induces neurite outgrowth in primary cell cultures and promotes axonal growth in entorhinal-hippocampal co-cultures. The relevance of this effect for the improved neurological performance after SCI was studied using corticospinal tract tracing. At the beginning of the study, biotinylated dextran amine (BDA) was injected into the motor cortex, which is known to be transported anterogradely. The BDA-positve corticospinal tract fibers were evaluated in tissue samples at the end of the study period. In animals treated with C21, more BDA-positive axons were found caudal to the site of injury as demonstrated by the Camera lucida-like drawings [P3; Fig.4A-B] and microscopy [P3; Fig.4C-E]. The statistical quantification of labeled axons at 0.5, 2.0 and 5.0 mm caudal to the lesion center and between the end of CST and the lesion center

showed significantly higher numbers in C21-treated animals as compared to the vehicle-treated ones (P3; Fig.5A). Moreover, the number of BDA-positive fibers in individual animals positively correlated with BMS score, suggesting functionality of these fibers [P3; Fig.5B].

C. C21 induces expression of neurotrophin receptor TrkB and promotes neuronal survival after SCI in mice

As shown *in vitro*, AT2R stimulation of AT2R led to the enhanced neurite outgrowth in neurotrophin/neurotrophin receptor-dependent manner. To confirm this finding *in vivo*, TrkB expression in spinal cord tissue was evaluated. In animals treated with C21, significantly increased immunoreactivity of TrkB within the injured and in peri-lesional tissue was observed [P3; Fig.13A-B]. Since neurotrophins not only promote axonal growth but also inhibit apoptosis of injured neurons, a staining for viable neurons was performed. As compared to the vehicle, C21 enhanced signal intensity of NeuroTrace Fluorescent Nissl stain, indicating higher neuronal survival within injured area [P3; Fig.13C- D].

4. Discussion

The cardiovascular action of the renin-angiotensin system has been studied extensively in the last 50 years, associating RAS with a variety of physiological actions such as blood pressure regulation, water and ion homeostasis as well as with pathological processes including kidney and heart failure, cardiac hypertrophy, atherosclerosis and stroke (2). Most of the detrimental effects of RAS including inflammation and apoptosis were clearly linked to the AT1 receptor that led to the development of AT1-receptor blockers (ARBs). Together with other cardiovascular drugs interfering with RAS, including angiotensin-converting enzyme (ACE) inhibitors and direct renin inhibitors, ARBs became first-choice drugs for the treatment of hypertension. However, AT1R is not the only receptor of RAS, since the effector peptide, angiotensin II, binds with similar affinity also to another subtype of angiotensin receptor, the AT2 receptor (AT2R). In the late 80's, AT2R was discovered by two independent research teams. It is a member of seven-transmembrane domain G-protein-coupled receptor family and exhibits only 33% amino acid homology to AT1R (11). The effects mediated by AT2R are often opposing to AT1R; they have recently been attributed to the "protective arm of RAS" (9).

The protective role of AT2R in pathophysiological conditions of central nervous system was postulated by several authors (summarized in: 2,11). The AT2R is widely expressed in fetal tissues, while after birth it is restricted to certain tissues including endothelium, adrenal glands, heart, uterus, retina and CNS (2,11). However, in neuronal damage, the expression of AT2R is massively up-regulated as demonstrated in animal models of stroke as well as sciatic or optic nerve crush (4–6). The protective function of AT2R in CNS may cover anti-inflammation, neuro-protection and neuro-regeneration.

It is well accepted that the inflammatory processes and generation of reactive oxygen species following cerebral ischemia or neuronal injury largely contributes to neuronal degeneration and apoptosis. In the present work, it could be demonstrated in cerebral ischemia model that the blockade of AT1R with telmisartan reduced expression of pro-inflammatory cytokines in peri-infarct zone. However, using only AT1R-blockers, it could not be clarified whether the anti-inflammatory action of telmisartan is attributed to the blockade of AT1R or to AT2R *via* unopposed stimulation by the endogenous Ang II. Therefore, the anti-inflammatory potential of AT2R was tested using the selective agonist, compound 21. In animals subjected to myocardial infarction, C21 reduced the expression of pro-inflammatory cytokines to a similar extent as the AT1R-blocker candesartan. The relevance of the anti-inflammatory activity of C21 for the CNS was confirmed *in vitro* in primary rat astrocytes. This is in agreement with previously published *in vitro* study where the TNFα-induced IL-6

expression was investigated (12). AT2R stimulation by either C21 or Ang II under concomitant AT1R blockade with irbesartan reduced expression of IL-6 mainly *via* inhibition of nuclear factor NFκB.

In addition to the anti-inflammatory activity of AT2R, neuro-protection was also attributed to the receptor. Neuro-protection, as a treatment option that prevents or slows down the loss of neurons, was demonstrated here in animal models of cerebral ischemia and spinal cord injury. Treatment with the AT1R blocker, telmisartan, prevented from neuronal loss in peri-infarct zone in animals subjected to middle cerebral artery occlusion. This was in agreement with a previously published study involving an animal model of stroke, where in the central administration of AT2R-agonist CGP42112A increased neuronal survival in the infarcted region (13). In the current work, a more specific strategy for the evaluation of AT2R-mediated neuro-protection was used in a spinal cord injury model. It could be shown that C21 promotes neuronal survival in injured spinal cords. The underlying molecular mechanisms can have a multiple nature including anti-inflammation but also induction of neurotrophins as will be discussed below.

In the mid 90's, neurite outgrowth was attributed to the AT2R (2) serving later as a research tool for pharmacological drug development but also indicating a neuro-regenerative potential. In 1998, it was shown for the first time that AT2R stimulation provided neurotrophic-like action in the CNS of adult animals (5). Rats subjected to the optic nerve lesion and treated locally with Ang II showed outgrowth of axon bundles within the proximal optic nerve that was AT2R-dependent since it could be inhibited by an AT2R-antagonist. The neuro-regenerative potential of AT2R was demonstrated first *in vivo* in a sciatic nerve crush model in rats. Ang II not only increased axonal diameters and promoted remyelination *via* AT2R but also improved functional recovery as shown by increased toe spread distance (parameter for motor-function) and improved the foot reflex withdrawal reaction (parameter of sensomotoric function) (8). In the present work, it could be demonstrated that specific stimulation of AT2R with C21 led to an elevated neurite outgrowth *in vitro*. Similar effects had previously been shown by stimulation with Ang II under concomitant blockade of AT1R (4). Using the favourable pharmacological properties of C21, the animals were treated systemically, and the impact of the direct AT2R stimulation on axonal growth was evaluated *in vivo*. In mice subjected to spinal cord injury, C21 elevated the number of regenerating axons cranially and caudally from the lesion area. The number of regenerating fibers positively correlated with improved locomotor performance indicating functionality of these fibers. To reveal the molecular mechanisms underlying AT2R-mediated neuro-protection and neuro-regeneration, an extensive analysis of published studies was performed. As a most prominent candidate, the neurotrophin pathway was chosen.

The neurotrophins are proteins involved in the development of the central nervous system, neuronal survival and regeneration in neurodegenerative diseases and in acute injuries including stroke and spinal cord injury. The family of classical neurotrophic factors contains nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) that selectively bind their receptors (14). Neurotrophin receptors belong to two distinct classes of receptors: tumor necrosis factor receptor superfamily (TNFRSF) with $p75^{NTR}$ and receptor tyrosine kinase family (RTK) with TrkA, TrkB and TrkC. The Trk and p75 receptors form homo- and heterodimers that determine the ligand specificity and variety of effects mediated by the neurotrophin-receptor complex (14). In addition to their role in embryo- and organogenesis, neurotrophins regulate synaptic function and plasticity, and promote neuronal survival, axon and dendrite growth and glial differentiation. Using the neurotrophin and receptor PCR array followed by validation with TaqMan PCR, elevated expression of BDNF, TrkA and TrkB could be detected in primary neurons stimulated with C21. The functional proof for the role of neurotrophins in C21-mediated neurite outgrowth was provided by a neurite outgrowth assay where treatment with unselective Trk inhibitor completely abolished the AT2R-mediated neurite outgrowth. In the tissue obtained from cerebral ischemia- and spinal cord injury-study, elevated TrkB immunoreactivity was observed in animals treated with telmisartan or C21, respectively. To my knowledge, this is the first evidence clearly linking the neurotrophic pathway with AT2R in the context of neuro-protection and neuro-regeneration.

In addition to the molecular mechanisms involved in neuro-regeneration, pharmacological stimulation of AT2R also provides an attractive approach for therapeutic intervention in CNS injuries. Several strategies for the delivery of BDNF to the CNS are known. In an animal model of stroke, intraventricular injection with BDNF fused with the collagen-binding domain led to reduced neurological severity scores, improved brain perfusion and reduced the number of apoptotic cells in the ischemic boundary zone (15). In other studies, neurotrophic factors were delivered to the damaged target tissue using a virus-mediated gene transfer strategy. All of the strategies, however interesting for the research, do not provide any therapeutic option for humans. The unique combination of anti-inflammatory, neuro-protective and neuro-regenerative properties in one molecule targeting AT2R could be beneficial for the treatment of stroke, spinal cord- and acute brain-injury and for diseases characterized by BDNF deficiency including Rett syndrome.

To summarize, the current work demonstrates clearly that selective AT2R stimulation provokes anti-inflammatory, neuro-protective and neuro-regenerative effects. Furthermore, the molecular mechanisms underlying the neuro-regenerative actions of the AT2R were elucidated pointing to a pivotal role of the neurotrophic pathway. Future work should address the molecular anti-apoptotic mechanisms mediated by AT2R. The cross-talk between AT2R

and other receptors of the renin-angiotensin system, including the Mas-receptor, could provide further information regarding signaling and clarify the complexity of the beneficial effects mediated by the “protective RAS”. Moreover, novel AT2R agonists with a higher lipophilicity enabling better penetration into the CNS might provide stronger therapeutic effects with a lower drug dosage.

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6. Personal contribution (Anteilserklärung an den erfolgten Publikationen)

Pawel Namsolleck hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Kaschina E, Grzesiak A, Li J, Foryst-Ludwig A, Timm M, Rompe F, Sommerfeld M, Kemnitz UR, Curato C, Namsolleck P, Tschöpe C, Hallberg A, Alterman M, Hucko T, Paetsch I, Dietrich T, Schnackenburg B, Graf K, Dahlöf B, Kintscher U, Unger T, Steckelings UM., Angiotensin II type 2 receptor stimulation: a novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction?, Circulation, 2008

Beitrag im Einzelnen:
The PhD student contributed to the molecular analysis of heart tissue with the focus on pro-inflammatory cytokines.

Publikation 2: Thoene-Reineke C, Rumschüssel K, Schmerbach K, Krikov M, Wengenmayer C, Godes M, Mueller S, Villringer A, Steckelings U, Namsolleck P, Unger T., Prevention and intervention studies with telmisartan, ramipril and their combination in different rat stroke models, PLoS One, 2011

Beitrag im Einzelnen:
The PhD student contributed to the planning and performing of the intervention study, histological analysis of the brain tissue and writing of the manuscript together with the first and the last authors.

Publikation 3: Namsolleck P, Boato F, Schwengel K, Paulis L, Matho K, Geurts N, Thöne-Reineke C, Lucht K, Seidel K, Hallberg A, Dahlöf B, Unger T, Hendrix S, Steckelings UM., AT2-receptor stimulation enhances axonal plasticity after spinal cord injury by upregulating BDNF expression, Neurobiology of Disease, 2013

Beitrag im Einzelnen:
The PhD student contributed to the planning of the study equally with the second and two last authors. The experimental part and data analysis were performed mainly by the first and second author. The PhD student was involved in writing of the manuscript.

Publikation 4: Steckelings UM, Rompe F, Kaschina E, Namsolleck P, Grzesiak A, Funke-Kaiser H, Bader M, Unger T., The past, present and future of angiotensin II type 2 receptor stimulation, Journal of Renin-Angiotensin-Aldosterone System, 2010

Beitrag im Einzelnen:
The PhD performed the experiments presented in Fig.1 and was involved in writing of the manuscript (It is a review article with own, previously unpublished data).

Publikation 5: Steckelings UM, Larhed M, Hallberg A, Widdop RE, Jones ES, Wallinder C, Namsolleck P, Dahlöf B, Unger T., Non-peptide AT2-receptor agonists, Current Opinion in Pharmacology, 2011

Beitrag im Einzelnen:
The PhD performed the experiments presented in Fig.2 and Fig.3 and was involved in writing of the manuscript (It is a review article with own, previously unpublished data).

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Unterschrift des Doktoranden/der Doktorandin

7. Publications included in the work

7.1. Publication 1 [P1]

Journal: Circulation. 2008 Dec 9;118(24):2523-32

Title: Angiotensin II type 2 receptor stimulation: a novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction?

Authors: Kaschina E, Grzesiak A, Li J, Foryst-Ludwig A, Timm M, Rompe F, Sommerfeld M, Kemnitz UR, Curato C, Namsolleck P, Tschöpe C, Hallberg A, Alterman M, Hucko T, Paetsch I, Dietrich T, Schnackenburg B, Graf K, Dahlöf B, Kintscher U, Unger T, Steckelings UM.

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7.2. Publication 2 [P2]

Journal: PLoS One. 2011;6(8):e23646

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Authors: Thoene-Reineke C, Rumschüssel K, Schmerbach K, Krikov M, Wengenmayer C, Godes M, Mueller S, Villringer A, Steckelings U, Namsolleck P, Unger T.

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Prevention and Intervention Studies with Telmisartan, Ramipril and Their Combination in Different Rat Stroke Models

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Abstract

Objectives: The effects of AT1 receptor blocker, telmisartan, and the ACE inhibitor, ramipril, were tested head-to head and in combination on stroke prevention in hypertensive rats and on potential neuroprotection in acute cerebral ischemia in normotensive rats.

Methods: Prevention study: Stroke-prone spontaneously hypertensive rats (SHR-SP) were subjected to high salt and randomly assigned to 4 groups: (1) untreated (NaCl, n = 24), (2) telmisartan (T; n = 27), (3) ramipril (R; n = 27) and (4) telmisartan +ramipril (T+R; n = 26). Drug doses were selected to keep blood pressure (BP) at 150 mmHg in all groups. Neurological signs and stroke incidence at 50% mortality of untreated SHR-SP were investigated. Intervention study: Normotensive Wistar rats were treated s.c. 5 days prior to middle cerebral artery occlusion (MCAO) for 90 min with reperfusion. Groups (n = 10 each): (1) sham, (2) vehicle (V; 0,9% NaCl), (3) T (0,5 mg/kg once daily), (4) R (0,01 mg/kg twice daily), (5) R (0,1 mg/kg twice daily) or (6) T (0,5 mg/kg once daily) plus R (0,01 mg/kg twice daily). Twenty-four and 48 h after MCAO, neurological outcome (NO) was determined. Forty-eight h after MCAO, infarct volume by MRI, neuronal survival, inflammation factors and neurotrophin receptor (TrkB) were analysed.

Results: Stroke incidence was reduced, survival was prolonged and neurological outcome was improved in all treated SHR-SP with no differences between treated groups. In the acute intervention study, T and T+R, but not R alone, improved NO, reduced infarct volume, inflammation (TNFα), and induced TrkB receptor and neuronal survival in comparison to V.

Conclusions: T, R or T+R had similar beneficial effects on stroke incidence and NO in hypertensive rats, confirming BP reduction as determinant factor in stroke prevention. In contrast, T and T+R provided superior neuroprotection in comparison to R alone in normotensive rats with induced cerebral ischemia.

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Introduction

Several recent clinical trials have demonstrated the efficacy of angiotensin AT1 receptor blockers (ARBs) and angiotensin-converting enzyme inhibitors (ACEI) in primary and secondary prevention of stroke in patients with hypertension and/or high cardiovascular risk [1–6]. Whereas the antihypertensive actions of these drugs are thought to contribute to their stroke-preventing features, the question as to potential blood pressure-independent neuroprotective effects of renin-angiotensin-system (RAS) inhibitors under cerebral ischemia still remains open, especially since clinical drug trials on stroke, for obvious reasons, usually address stroke incidence alone and rarely issues of neuroprotection.

ACE inhibitors and angiotensin receptor antagonists both target the RAS, though in different ways. ACE inhibitors never block the RAS completely. Even when ACE is inhibited by more than 90%, there will still be some angiotensin II production by other enzymes that compensate for the lack of ACE activity. Angiotensin receptor antagonists, on the other hand, feature a more specific mechanism since they selectively block the angiotensin receptor subtype-1 (AT1), which is responsible for most of the deleterious effects of

angiotensin II, and expose the unblocked angiotensin receptor subtype-2 (AT2) to increased angiotensin II concentrations. Published data suggest that the AT2 receptor can counterbalance the effect of the AT1 receptor *in vitro* as well as *in vivo* [7]. It has also been speculated that combining ACE inhibition and AT1 receptor blockade could have advantages over each therapeutic principle alone [8] but with respect to neuroprotection in cerebral ischemia, this hypothesis has not yet been tested.

In the present study, we addressed two questions: First, in a well established hypertensive animal model with cerebrovascular disease and a high incidence of stroke, the salt-fed stroke-prone spontaneously hypertensive rat (SHR-SP), we asked whether a similar inhibition of the RAS as evidenced by equal blood pressure reductions by ACE-inhibition, AT1 receptor blockade, or their combination would engender comparable effects on stroke incidence and stroke-related morbidity and mortality (prevention study).

Second, in normotensive rats, we evaluated the potential blood pressure-independent neuroprotective effects and mechanisms of subantihypertensive treatment regimens involving comparable blockade of ACE or AT1 receptors or combined treatment with both of these principles (acute intervention study).

We used telmisartan as ARB and ramipril as ACEI since these drugs have recently been used alone and in combination in a large clinical trial involving stroke prevention in patients with high cardiovascular risk [6]. In the rat, both drugs have been demonstrated to cross the blood-brain barrier with telmisartan having a higher propensity to enter the brain than ramipril [9,10].

Materials and Methods

Animals and treatment

Rats were kept in a SPF (specific pathogen free) barrier under standardized conditions with respect to temperature and humidity, and were housed on a 12 h light/12 h dark cycle in groups of 4–5 with food and water *ad libitum*. Animal housing, care, and applications of experimental procedures were approved by State Office of Health and Social Affairs Berlin, Germany (project IDs G0034/06 and G0088/04).

The human end point was set by a neurological score of 3.

Prevention study

Male SHR-SP (n = 104), aged 7 weeks, were obtained from Charles River Laboratories (Sulzfeld, Germany). Baseline measurements (MRI, blood pressure by tail cuff method) and neurological score were performed at the age of 9 weeks. Subsequently, all rats were switched to a high salt diet (RMH-TM rat chow; protein 22.5%; fat 4.8%; potassium 0.85%; sodium 0.40%; Hope Farms, Sniff, Soest, Germany) and 1% NaCl (170 mmol/L) in the drinking water in order to accelerate the appearance of cerebrovascular events [11]. With the beginning of the high salt diet, SHR-SP were randomly assigned to four different treatment groups: group 1 served as control (untreated; $n = 24$), group 2 treated with the angiotensin AT1 receptor blocker telmisartan in the drinking water (initial dose T; 1.23 mg/kg bwt; $n = 27$), group 3 treated with the ACE-inhibitor ramipril in drinking water (initial dose R; 1.06 mg/kg bwt; $n = 27$), and group 4 was treated with a combination of telmisartan and ramipril in the drinking water (initial dose T; 0.25 mg/kg bwt plus R, 0.25 mg/kg bwt; n = 26) (Figure S1A). Individual drug doses were selected on the basis of achieving equal blood pressure (150 mmHg) throughout the protocol (Figure 1A).

Systolic blood pressure was measured weekly in trained, conscious and pre-warmed rats by tail cuff method using a model from ADInstruments with PowerLab software [12]. Rats were screened daily for neurological disorders by the scoring system of Garcia et al. [13]. The protocol was terminated at 50% mortality in the untreated SHR-SP group (Figure 1A–D).

Acute intervention study

In a pilot study, male normotensive Wistar rats (160–200 g, HARLAN Winkelmann, Borchen, Germany) were randomly divided in several groups (Ang I+0,9% NaCl; Ang I+R 0.01 mg/kg; Ang I+T 0.05+R 0.01; Ang II+0,9% NaCl; Ang II+T 0.05 mg/kg, Ang II T 0.05+R 0.01; n: 5 per group) to determine the appropriate treatment doses of the comparators (Figure 2A–B; Figure S2A–B).

Based on the above dose finding, further animals were randomly allocated to five different treatment groups (n = 10 each): (1) sham-operated, vehicle (V; 0.9% NaCl once daily) treated, (2) stroke V (0.9% NaCl once daily) treated, (3) stroke T (0.5 mg/kg once daily) treated, (4) stroke R (0.01 mg/kg twice daily) treated, (5) stroke R (0.1 mg/kg twice daily) treated or (6) stroke T (0.5 mg/kg once daily) plus R (0.01 mg/kg twice daily). Subcutaneous (s.c.) drug treatment was begun 5-days prior to MCAO [14] (Figure S1B).

Femoral artery and vein catheter in acute intervention study

Two days before blood pressure recordings (pilot study) and three days after initiation of treatment (main study), polyethylene catheters (PP-50) were inserted through the femoral artery into the abdominal aorta and in the femoral vein. The arterial catheter was used for blood pressure measurement via a transducer using the software PowerLab by ADInstruments (Spechtbach, Germany). The femoral vein catheter was used for intravenous (i.v.) injections of angiotensin II (Ang II; 50 ng/kg bwt) and angiotensin I (Ang I; 150 ng/kg bwt), respectively [15].

Determination of equipotent doses of telmisartan, ramipril and their combination in vivo for acute intervention study

On day two after catheterization, five animals per group were placed into individual cages and connected via the arterial catheter to a transducer for hemodynamic measurements and, via the venous line, to a microsyringe for small volume injections. After baseline blood pressure recordings for 30 min, animals received an intravenous (i.v.) bolus injection of Ang II or Ang I, respectively. Rats were then treated s.c. with NaCl solution, telmisartan or ramipril or their combination at different doses. The pressor responses to i.v. bolus injections of Ang II (telmisartan group or combination group) or Ang I (ramipril group or combination group) were recorded at time intervals of 5, 15, 30, 60 and 90 min and 3, 6 and 10 hours after s.c. vehicle, telmisartan or ramipril or their combination.

Bolus i.v. injections of Ang II or Ang I, five, 15, 30, 60 and 90 min after s.c. treatment with vehicle elicited consistent increases in mean arterial pressure of about 40 mmHg. Subcutaneous injection of telmisartan prior to Ang II dose- and time-dependently attenuated the pressor responses to the peptide. At 0.1 mg/kg bwt, telmisartan was not effective, whereas at 0.5 mg/kg bwt, telmisartan completely inhibited the pressor responses to Ang II within 30 minutes of s.c. administration. Based on these findings, a telmisartan dose of 0.5 mg/kg bwt was determined to be used in further experiments (Figure S2A).

The corresponding dose of ramipril, established by the same criteria as above (with exception that Ang I instead of Ang II was

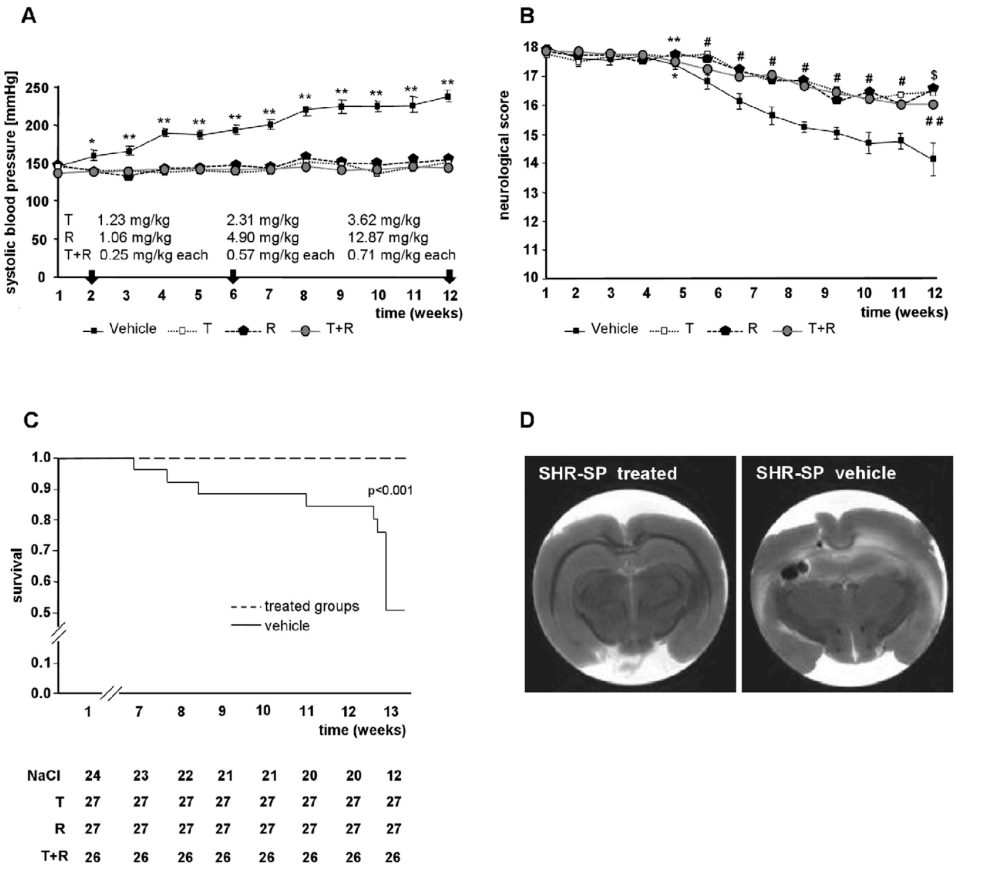

Figure 1. Prevention study SHR-SP. A) Development of systolic blood pressure in hypertensive treated and untreated rats measured by tail cuff method. Results area expressed as mean SEM. *$p<0.05$ or **$p<0.01$ significant differences between treatment groups and vehicle. B) Effect of vehicle, telmisartan, ramipril or telmisartan plus ramipril supplemented in drinking water on neurological deficits in salt-loaded, stroke-prone spontaneously hypertensive rats. *$p<0.05$ telmisartan treated group; **$P<0.01$ ramipril treated group vs vehicle group, 13 weeks of age. $^{\#}p<0.01$ telmisartan, ramipril and telmisartan + ramipril treated groups vs vehicle group, 14–20 weeks of age. $^{\#\#}p<0.05$ telmisartan + ramipril treated group; $^{\$}p<0.01$ telmisartan, ramipril treated groups vs vehicle group, 21 weeks of age. C) Survival rate in salt-loaded, stroke prone spontaneously hypertensive rats (SHR-SP). Nine week-old SHR-SP were treated with vehicle, telmisartan, ramipril or telmisartan plus ramipril supplemented in drinking water. Figures under the curve show number of surviving animals. Mortality was compared among groups using Kaplan Meier analysis of survival followed by log-rank test. *$p<0.001$; telmisartan, ramipril and telmisartan + ramipril treated groups vs vehicle group. D) Representative picture of SHR-SP with vehicle and stroke and with treatment without stroke.
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used as the i.v. pressor agent) was 0.01 mg/kg bwt. This dose blocked the pressor responses to i.v. Ang I by 90–100% from 30 to 90 min (Figure S2B).

The inhibition of the respective Ang II- and Ang I pressor responses with the above doses of telmisartan and ramipril after 3, 6 and 10 hours are shown in Figure 2A. Both compounds were inhibitory for 10 hours. Thereafter, ramipril lost effectiveness faster than telmisartan (Figure 2B). Based on these findings, a treatment protocol with twice daily s.c. injections was established for ramipril to guarantee continuous 24–hour RAS blockade *in vivo*.

Since inhibition of the brain RAS inside the blood-brain barrier (BBB) has been demonstrated to be neuroprotective under ischemic conditions [14–18] and since peripherally administered ramipril, at the dose established above, may penetrate the BBB less readily than telmisartan [9,10], we introduced an additional group of rats pre-treated s.c. with ramipril at 0.2 mg/kg bwt (ramipril high dose) to allow for better comparison between drugs.

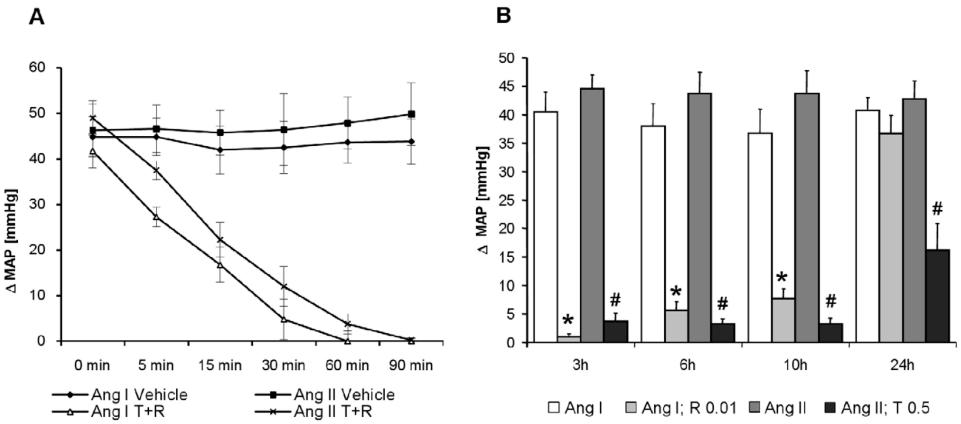

Figure 2. Intervention study. A) Effects of combination of ramipril (0.01 mg/kg bw) and telmisartan (0.5 mg/kg bw) or vehicle administered subcutaneously on the pressor responses to i.v. injected angiotensin I (150 ng/kg bw) or angiotensin II (50 ng/kg bw; n = 7). B) Duration of the pressor responses of selected doses of ramipril (0.01 mg/kg bwt) and telmisartan (0.5 mg/kg bwt) administered subcutaneously induced by angiotensin I (150 ng/kg bwt) and angiotensin II (50 ng/kg bwt) injected intravenously (n = 7 per group); *: $p<0.05$ vs. ANG II; #: $p<0.05$ vs. ANG I.
doi:10.1371/journal.pone.0023646.g002

Middle cerebral artery occlusion with reperfusion

Five days after the beginning of treatment, focal cerebral ischemia was induced by right middle cerebral artery occlusion (MCAO) with subsequent reperfusion as described previously [16,19]. Under general anaesthesia with chloralhydrate 400 mg/kg, the right cervical carotid bifurcation was exposed through a midline neck incision. A 4–0 silicon-coated nylon monofilament (Prolene, Ethicon GmbH, Norderstedt, Germany) was gently inserted through the proximal external carotid artery (ECA) into the internal carotid artery up to middle cerebral artery offshoot. After 90 minutes, the filament was withdrawn into the stump of the ECA to allow reperfusion (Figure S3A). Sham-operated rats underwent the same surgical procedures except that the occluding monofilament was not inserted. Cerebral blood flow (CBF) was monitored during surgical intervention with a probe attached to the skull above the supply territory of the MCA (2 mm caudal to bregma, 6 mm lateral to midline) by Laser-Doppler flowmetry (Periflux system 5000, PERIMED, Stockholm, Sweden). The procedure was considered successful, when an over 75% drop in CBF was observed after MCAO (Figure S3B). BP was measured before, during and after MCAO with atrial catheter (Figure 3A). Body weight was investigated before and after surgery (Figure 3B).

T2-weighted Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed 48 hours after MCAO on a 7 T Bruker scanner (Pharmascan 70/16 AS, Bruker Biospin, Ettlingen, Germany) in isoflurane anaesthesia. Cerebral ischemic areas were visualized with a T2-weighted; fat suppressed 2D turbo spin-echo sequence (TR 5218.7 ms; TE_{eff} 65.2 ms, RARE factor 8 and 6 averages). 35 axial slices with a slice thickness of 0.5 mm and no interslice distance were positioned to cover the whole brain. The field of view (FOV) was 3.5×3.5 cm and the matrix was 256×256 resulting in an inplane resolution of 137 µm. Calculation of lesion volume was carried out with the program Analyze 5.0 (Analyze Direct, Inc.; Lenexa USA) [13,19] (Figure 3 C).

Neurological score

Neurological evaluation was accomplished by a blinded observer 24 h and 48 h after MCAO using the 18-point neurological scoring system of Garcia et al [13,20]. This neurological score will test for spontaneous activity, motor impairments and sensorial function. Severe impairments were graded 0 or 1, and no observed deficits were graded 3 (Figure 3D).

Blood parameters

Blood samples were taken by retrobulbar puncture to determine oxygen- and carbon dioxide-partial pressure, pH, hematocrit, glucose-, sodium- and potassium concentration before and after MCAO (Table S1). Data were quantified using a RADIOMETER ABL 555 SERIES (Radiometer medical A/S, Copenhagen, Denmark).

Nissl staining

Rat brains were coronally sectioned into slices of 7 µm thickness in a cryostat and, subsequently, fixed in acetone for 5 min. For the staining of viable neurons sections were incubated with Neuro-Trace (diluted 1:50 in PBS) followed by DAPI staining (both from Molecular Probes, Eugene, Oregon, USA) according to the manufacturer's instructions. The area for counting the positively stained neurons was determined by comparing HE-stained sections and MRI sections of the animals of the experiment to choose an area of approx. 1 mm^2 in the periventricular penumbra in all animals. In addition, the corresponding area in the contralateral hemisphere was investigated. Cells were counted by a blinded investigator (Figure 4A).

Immunofluorescent staining

For staining of intracellular cytokines TNFα and IL-6, sections were blocked with 5% donkey serum (PAN Biotech GmbH, Aidenbach, Germany) and with 10% donkey serum for staining of TrkB, both diluted in PBS. Subsequently, the blocking buffer was removed and sections were incubated with primary antibodies

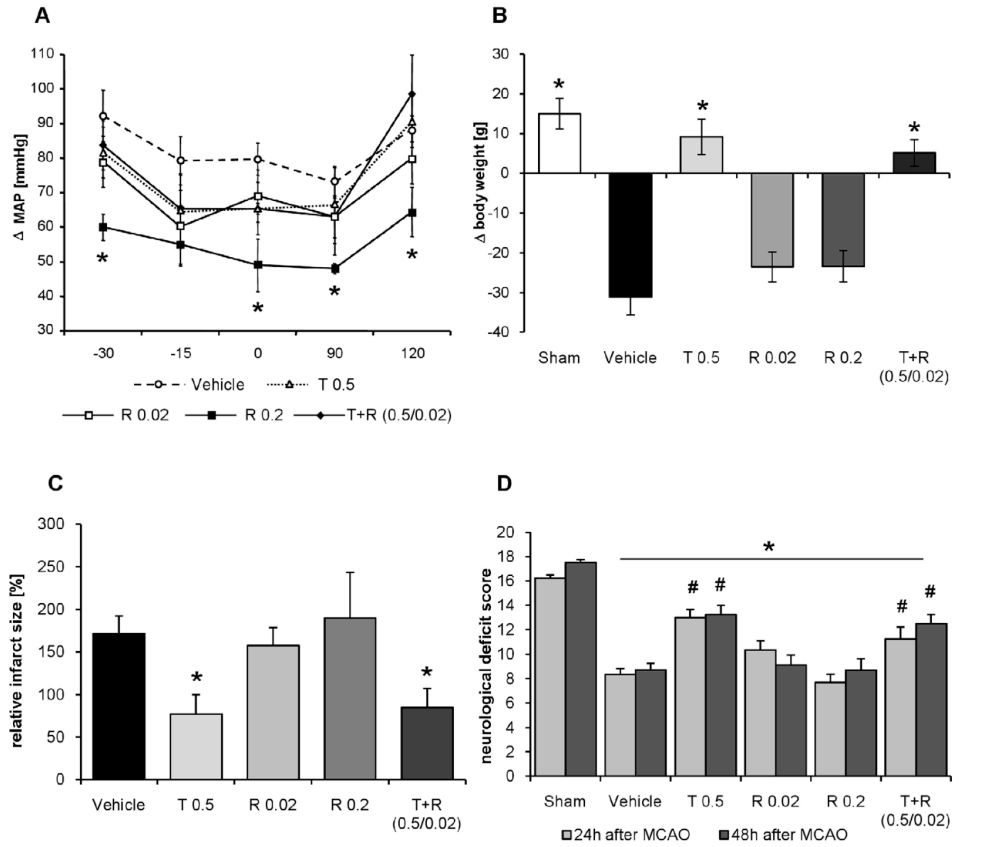

Figure 3. Intervention study. A) Effect of 5 day pretreatment with telmisartan (0.5 mg/kg bwt), ramipril (0.02 mg/kg bwt and 0.2 mg/kg bwt) and their combination (T 0.5 mg/kg bwt, R 0.02 mg/kg bwt) administered subcutaneously on mean arterial blood pressure (mmHg) before, during and after MCAO. Baseline (−30), initiation of anesthesia (−15), start of MCAO (0), 90 min. of occlusion (+90) and 30 min. of reperfusion (+120) (n = 7 per group); *: $p<0.05$ vs. Vehicle. B) Effects of 5 day pretreatment with ramipril, telmisartan and their combination administered subcutaneously at established doses on body weight gain 48 h after MCAO. Columns from the left: sham, vehicle, telmisartan 0.5 mg/kg (T 0.5), ramipril 0.02 mg/kg (R 0.02), ramipril 0.2 mg/kg (R 0.2) and combination of telmisartan 0.5 mg/kg and ramipril 0.02 mg/kg (T+R 0.5/0.02), n = 7 per group. *$p<0.05$ vs. vehicle C) Effect of 5 day pretreatment with ramipril, telmisartan and their combination on infarct volume. Columns from the left: vehicle, telmisartan 0.5 mg/kg (T 0.5), ramipril 0.02 mg/kg (R 0.02), ramipril 0.2 mg/kg (R 0.2) and combination of telmisartan 0.5 mg/kg and ramipril 0.02 mg/kg (T+R 0.5/0.02), n = 7 per group. *$p<0.05$ vs. stroke/vehicle D) Effect of 5 day pretreatment with ramipril, telmisartan and their combination on neurological deficit score 24 h (bright bars) or 48 h (dark bars) after MCAO. Columns from the left: sham, vehicle, telmisartan 0.5 mg/kg (T 0.5), ramipril 0.02 mg/kg (R 0.02), ramipril 0.2 mg/kg (R 0.2) and combination of telmisartan 0.5 mg/kg and ramipril 0.02 mg/kg (T+R 0.5/0.02), n = 7 per group. *$p<0.05$ vs. sham; #: $p<0.05$ vs. vehicle.
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(Santa Cruz Biotechnology Inc., Heidelberg, Germany; all diluted 1:50 in blocking buffer) at 4°C, overnight. After incubation, primary antibodies were rinsed from sections three times with PBS. Cy3-conjugated secondary antibodies (1:100 in 5% donkey serum in PBS, Chemicon, Schwalbach/Ts, Germany) were added and allowed to incubate for 1 hour at room temperature. The secondary antibodies were rinsed three times with PBS, and the sections were treated with 10 ng/ml DAPI for 15 min at room temperature. After washing for three times with PBS, coverslips were mounted on slides with mounting medium (Dako Cytomation, Hamburg, Germany). A Leica DM IRE2 microscope (Leica Microsystems, Wetzler, Germany) was used to generate the images. The counting area was determined by comparing HE-stained section and MRI sections of all animals of this experiment to choose an area (approximately 1 mm^2 for the Nissl staining and 0.4 mm^2 for the immunostaining) that was part of the penumbra in all animals. In this experiment, this area was in a periventricular location. In addition, the corresponding area in the contralateral hemisphere was investigated. Cells were counted by a blinded investigator (Figure 4B–E).

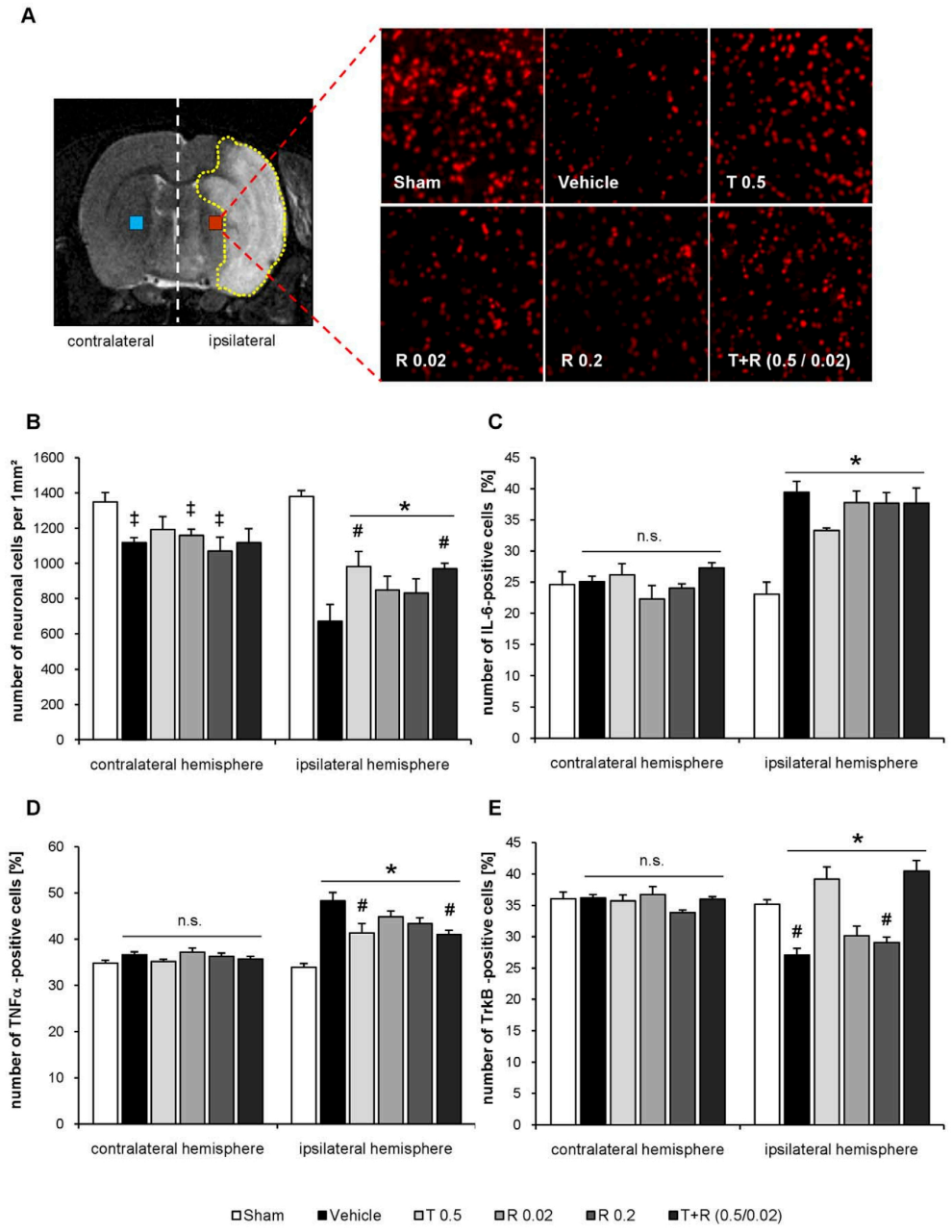
A
contralateral
ipsilateral
Sham
Vehicle
T 0.5
R 0.02
R 0.2
T+R (0.5 / 0.02)
B
number of neuronal cells per 1mm²
contralateral hemisphere
ipsilateral hemisphere
C
number of IL-6-positive cells [%]
n.s.
D
number of TNFα -positive cells [%]
E
number of TrkB -positive cells [%]
Sham
Vehicle
T 0.5
R 0.02
R 0.2
T+R (0.5/0.02)

Figure 4. Intervention study. A) Typical MRI section showing infracted region (white area) from a Wistar rat 48 h after MCAO, the squares mentioned the counting area of the paraventricular penumbra in the ipsilateral and the correspondent area in the contralateral site. Representative pictures of Nissl staining of sham, vehicle, telmisartan 0.5 mg/kg (T 0.5), ramipril 0.02 mg/kg (R 0.02), ramipril 0.2 mg/kg (R 0.2) and combination of telmisartan 0.5 mg/kg and ramipril 0.02 mg/kg (T+R 0.5/0.02). B) Number of Nissl positive, viable neurons per mm^2 in the periventricular penumbra and the correspondent contralateral site 48 h after MCAO. Columns from the left: sham, vehicle, telmisartan 0.5 mg/kg (T 0.5), ramipril 0.02 mg/kg (R 0.02), ramipril 0.2 mg/kg (R 0.2) and combination of telmisartan 0.5 mg/kg and ramipril 0.02 mg/kg (T+R 0.5/0.02). *: $p<0.05$ vs. sham; #: $p<0.05$ vs. vehicle C) Relative number of IL-6 positive cells (% of all cells) in the periventricular penumbra and the correspondent contralateral site 48 h after MCAO. Columns from the left: sham, vehicle, telmisartan 0.5 mg/kg (T 0.5), ramipril 0.02 mg/kg (R 0.02), ramipril 0.2 mg/kg (R 0.2) and combination of telmisartan 0.5 mg/kg and ramipril 0.02 mg/kg (T+R 0.5/0.02), counting area 0.4 mm^2. *$p<0.05$ vs. sham D) Relative number of TNF-alpha positive cells (% of all cells) in the periventricular penumbra and the correspondent contralateral site 48 h after MCAO. Columns from the left: sham, vehicle, telmisartan 0.5 mg/kg (T 0.5), ramipril 0.02 mg/kg (R 0.02), ramipril 0.2 mg/kg (R 0.2) and combination of telmisartan 0.5 mg/kg and ramipril 0.02 mg/kg (T+R 0.5/0.02), counting area 0.4 mm^2. *$p<0.05$ vs. sham; #$p<0.05$ vs. vehicle E) Relative number of TrkB positive cells (% of all cells) in the periventricular penumbra and the correspondent contralateral site 48 h after MCAO. Columns from the left: sham, vehicle, telmisartan 0.5 mg/kg (T 0.5), ramipril 0.02 mg/kg (R 0.02), ramipril 0.2 mg/kg (R 0.2) and combination of telmisartan 0.5 mg/kg and ramipril 0.02 mg/kg (T+R 0.5/0.02), counting area 0.4 mm^2. *$p<0.05$ vs. sham; #$p<0.05$ vs. vehicle.
doi:10.1371/journal.pone.0023646.g004

Statistics

All data were analysed using SPSS 16.0 for Windows and presented as arithmetic mean ± standard error of the mean (SEM).

Analysis of variance by ANOVA-analysis followed post hoc tests (Scheffe'- or tukey-test). Differences between groups were compared by the Wilcoxon rank sum test for independent and the Wilcoxon signed rank sum test for dependant samples.

Survival was analyzed using Kaplan Meier analysis followed by log-rank test. Differences between two groups of interest were analyzed using the Mann-Whitney-U test. Results were considered significantly different at a value of $p<0.05$.

Results

Stroke prevention in SHR-SP

Systolic blood pressure in SHR-SP. Figure 1A shows systolic blood pressure (SBP) during treatment. SBP was comparable in all groups at the beginning of salt loading. The development of hypertension was prevented in rats treated with telmisartan, ramipril and their respective combination.

Course of treatment in SHR-SP. Figure 1A shows the course of treatment to achieve equal SBP between treatment groups (150 mmHg) during experiment. With the beginning of the high salt diet, SHR-SP were randomly assigned to four different treatment groups: group 1 served as control (untreated; $n = 24$), group 2 treated with the angiotensin AT1 receptor blocker telmisartan in the drinking water (initial dose T; 1.23 mg/kg bwt has to be raised up to 2.31 mg/kg bwt at week 6 and to 3.62 mg/kg bwt at the end of the study; $n = 27$), group 3 treated with the ACE-inhibitor ramipril in drinking water (initial dose R; 1.06 mg/kg bwt has to be raised up to 4.9 mg/kg bwt within week 6 and to 12.87 mg/kg bwt at the end of the study; $n = 27$), and group 4 was treated with a combination of telmisartan and ramipril in the drinking water (initial dose T; 0.25 mg/kg bwt plus R, 0.25 mg/kg bwt; has to be raised up to 0.57 mg/kg bwt each within week 6 and to 0.71 mg/kg bwt each at study end; $n = 26$). The dose of ramipril had to be increased considerably more to keep blood pressure at 150 mmHg than the dose of telmisartan. The respective doses of T and R in combination therapy were relatively low and ineffective as monotherapy.

Neurological score of SHR-SP. Figure 1B shows the development of the neurological deficits over time and indicates the number of animals remaining in each group at the corresponding time point. Neurological scores were comparable in all groups at the beginning of the protocol. The development of neurological deficits was decelerated in rats treated with telmisartan, ramipril or their combination. At 4 weeks, vehicle-treated animals began to develop neurological signs associated with stroke: piloerection, jumping, aggression, prostration, loss of body symmetry and convulsions. Rats treated with telmisartan, ramipril or their combination did not display such neurological signs until the end of the study.

Survival rate in SHR-SP. Survival rates of animals are summarized in Figure 1C. Fifty percent of the animals in the vehicle-treated group died within 13 weeks of salt loading. Telmisartan, ramipril and combination treatment equally improved survival: within the 13 week duration of the study, all animals in the treated groups survived.

Stroke verification in SHR-SP. The brains of dead SHR-SP rats and, after finishing the experiment, the brains of survived animals were analysed by MRI. All dead SHR-SP animals had suffered from stroke, and the survived animals showed no signs of oedema, ischemic or haemorrhagic lesions. See representative pictures Figure 1D.

Acute intervention study in normotensive rats

Mean arterial blood pressure during MCAO. Blood pressure was monitored before MCAO in conscious rats, and during and after MCAO/reperfusion under anaesthesia. Mean arterial blood pressure remained unchanged and not different from vehicle-treated controls under the established pre-treatment doses of telmisartan and ramipril (low dose) or their combination. In contrast, pre-treatment with ramipril at 0.2 mg/kg bwt (high dose) significantly reduced mean arterial blood pressure before, during and after MCAO (Figure 3A).

Blood parameters. Oxygen, carbon dioxide, pH, glucose, hematocrit, sodium, potassium showed no significant differences between groups during MCAO (Table S1).

Cerebral blood flow. During MCAO, all animals showed a significant reduction in CBF in comparison to baseline conditions. After withdrawal of the occluding filament, ipsilateral blood flow was restored to approximately 90% of baseline levels. Reductions in CBF during MCAO and during reperfusion were identical in vehicle- and drug-treated groups (Figure S3B).

Body weight. After stroke, body weight decreased significantly in the vehicle-treated group in comparison to sham operated animals. In the telmisartan and combination pre-treated groups, but not in the ramipril-pre-treated group, body weight was not reduced and remained in the range of the sham operated animals (Figure 3B).

Infarct volume. Cerebral ischemic areas were visualized by the MRI technique and normalized to body weight. On day 2 after MCAO, the size of ischemic lesions was 150 mm^3/100 g bwt. on average in vehicle pre-treated animals with MCAO. Telmisartan- and combination pre-treatment, but not ramipril

pre-treatment, significantly reduced the mean volume of the ischemic area (Figure 3C).

Neurological deficits. In all stroke groups, a significant reduction in the Garcia score was recorded 24 h and 48 h after MCAO compared to sham operated animals (Figure 3D). Neurological outcome was significantly improved in the telmisartan- and combination-treated group after 24 h as well as 48 h after MCAO. In the ramipril-treated groups the improvement did not reach statistical significance (Figure 3D).

Mortality. In accordance with the literature [21–23], mortality rate within the first 24 h after MCAO was between 28% and 30% in all stroke groups.

Neuronal cell loss. For histological assessment of neuronal damage in the periventricular penumbra, Nissl staining was performed 48 h after MCAO. In injured neurons, the Nissl substance breaks apart. Compared with sham operation the number of Nissl positive cells was markedly reduced in the ipsilateral penumbra after MCAO ($p<0.05$), as shown in Figure 4A. Neuronal loss of the ischemic periventricular penumbra was significantly improved in telmisartan- and combination- but not in ramipril pre-treated groups ($p<0.05$). The corresponding contralateral brain region showed a significant reduction of neuronal cells in stroke-vehicle and ramipril treated groups in comparison to sham operated animals ($p<0.05$) (Figure 4B).

IL-6 and TNF-α by Immunohistochemistry. The proinflammatory cytokines, IL-6 and TNF-α, were significantly increased 48 h after MCAO in comparison to sham operated animals.

In both telmisartan- and combination pre-treated groups, protein levels of TNF-α in the ipsilateral penumbra were significantly decreased in comparison to the stroke- vehicle group, whereas IL-6 remained unchanged in all pre-treatment groups. The expression of TNF-α and IL-6 in the corresponding contralateral brain region was not different from the one in sham operated animals (Figure 4C–D)

TrkB by Immunohistochemistry. The BDNF receptor, TrkB, was significantly down-regulated in the ipsilateral penumbra 48 h after MCAO in the vehicle-treated group in comparison to sham operated animals. Pre-treatment with telmisartan and the combination increased, on protein level, the expression of BDNF receptor TrkB in comparison to stroke-vehicle group. The expression of receptor TrkB in the corresponding contralateral brain region was not different from the one in sham operated animals (Figure 4E).

Discussion

Stroke is one of the leading causes of death and invalidity in the modern world [24]. Consequently, prevention and treatment of stroke have become major issues in experimental and clinical research. Compared to other antihypertensive drugs, angiotensin AT1-receptor blockers (ARBs) have been demonstrated to be effective in primary and secondary stroke prevention in several clinical studies [2–6,25] even when blood-pressure reduction was similar between different study arms.

In contrast, in The Profess trial on secondary stroke, AT1-receptor blockade did not confer additional benefit when compared to other stroke therapies. However, interpretation of these findings is limited by to the short observation period [26].

The ONTARGET trial compared the efficacy of the angiotensin receptor antagonist, telmisartan, the angiotensin-converting enzyme inhibitor, ramipril, and their combination in reducing cardio- and cerebrovascular risk [6]. The study population included patients with or without hypertension, with coronary artery disease, stroke, peripheral vascular disease or diabetes with end-organ damage but excluded patients with congestive heart failure. Telmisartan was not inferior to ramipril with respect to the primary endpoint including stroke incidence, and the combination of the two drugs was not superior to each one alone [6].

Concerning stroke prevention, the present results in salt-fed stroke-prone SHR are in line with those of ONTARGET. With doses adjusted to achieve equal antihypertensive actions, telmisartan, ramipril or their combination all prevented stroke incidence and stroke-related neurological deficits during the 13 weeks of observation, while untreated hypertensive controls had a mortality of 50% at the end and experienced stroke-related neurological deficits during the course of the study. Like in the ONTARGET trial, it thus appears that in hypertensive individuals, the blood pressure lowering drug actions determine stroke incidence with no difference between principles of RAS inhibition.

Interestingly, during the course of treatment, the dose of ramipril had to be increased considerably more than the corresponding dose of telmisartan to keep blood pressure at 150 mmHg, and the dose of each compound in combination therapy was much lower than the respective monotherapy doses.

However, stroke incidence, as much as it is part of the combined endpoint in many cardiovascular drug trials, constitutes a relatively crude measure of drug-induced brain protection since it is mainly related to the vascular, but not to the neuronal part of the stroke issue. The latter also includes potential neuroprotective drug effects limiting stroke extension and reducing post-ischemic neuronal damage.

In this regard, a number of animal studies have suggested that the beneficial effects of ARBs in stroke include a blood pressure–independent element of neuroprotection, and some of the underlying molecular mechanisms have already been described [14–18,26–29]. With respect to the ACE inhibitors, the experimental and clinical literature presents rather equivocal data on blood pressure related- or unrelated effects in stroke [1,11,20,30].

In the second part of the present study, we therefore compared potential blood pressure-independent neuroprotective effects of systemic pre-treatment with telmisartan, ramipril and their combination after focal brain ischemia induced by MCAO with reperfusion in normotensive rats. General criteria of comparative dose definition were equi-potency with respect to persistent blockade of the peripheral RAS in vivo (inhibition of the pressor responses to intravenous Ang I and AngII, respectively) and absence of blood pressure lowering effects before, during and after focal brain ischemia.

Given these experimental conditions, pre-treatment with telmisartan alone and with the combination of telmisartan and ramipril proved to be more effective than ramipril in reducing stroke volume, loss of body weight and neurological deficits. Combination treatment with telmisartan plus ramipril seems to be not superior to telmisartan alone.

These findings are in line with those of a previous experimental head-to-head comparison involving candesartan and ramipril [20] and with most of the animal studies on potential neuroprotective effects of RAS inhibitors [14–18,20,27–29]. Since cerebral blood flow was reduced during MCAO to the same extent with no differences between vehicle-treatment and drugs, mechanisms other then those merely related to brain perfusion have to be responsible for differences in neuroprotection between ARBs and ACE inhibitors.

One simple explanation for our findings could be that ARBs in general, and telmisartan or candesartan in particular [9,31], have a higher propensity to cross the blood-brain barrier (BBB) than

ACE inhibitors, eg. ramipril, [10] and to interfere with the brain RAS, i.e. inhibit the actions of non-vascular AT1 receptors and stimulate the actions of unopposed AT2 receptors inside the BBB [9]. Indeed, it has been demonstrated that AT2 receptors inside the brain contributes to the neuroprotective effects (reduced infarct volume and neurological deficits, improved neuronal survival) of central AT1 receptor blockade after MCAO [18], which is also underlined by the severe outcome after MCAO in AT2 receptor KO mice [29] and smaller lesion area and much larger penumbra in AT1 receptor KO mice [28]. Under this assumption, combined treatment with telmisartan and ramipril would reflect the central effects of telmisartan but afford no further benefits by ACE inhibition beyond AT1 receptor blockade.

Further mechanisms of neuroprotection that may be related to central AT1 receptor blockade include the neurotrophin receptor TrkB which showed an enhanced expression under telmisartan and combination therapy in the penumbra of ischemic brain tissue. Analyzing BDNF- and TrkB knockout mice, this neurotrophin system has been identified to be essential for the development and survival of several distinct neuronal populations *in vitro* and also *in vivo* [32–34]. We observed an exclusive increase of the TrkB protein in the telmisartan- and combination group compared to the other groups with brain ischemia. This finding, reflecting an intensified interaction of BDNF with its specific receptor, TrkB, points to a local, molecular mechanism of neuroprotective actions which may have contributed to the reduced neuronal loss in the ischemic penumbra under telmisartan- and combination treatment as observed in the present study.

Conclusion

In salt-fed SHR-SP equi-antihypertensive treatment with telmisartan, ramipril or their combination completely prevented neurological deficits and stroke occurrence pointing to blood pressure reduction as the leading mechanism in stroke prevention. Conversely, in acute stroke intervention in normotensive rats, sub-antihypertensive pre-treatment with telmisartan and combination treatment was significantly more effective than ramipril alone in terms of neuroprotection. The combination of telmisartan and ramipril seems to be not superior to telmisartan alone in this experimental setting.

Supporting Information

Figure S1 Study design. A) Prevention study Baseline measurements were performed at the age of 9 weeks as described in Material and Methods. Subsequently, all rats were switched to a high salt diet and randomly assigned to four different treatment groups. The initial drug doses are indicated. Individual drug doses were selected on the basis of achieving equal blood pressure (150 mmHg) throughout the protocol. Several parameters were estimated in various time-points as indicated in the figure. B) Intervention study In a pilot study, the appropriate treatment doses of the drugs were determine as described in Material and Methods. Based on the dose finding, animals were randomly allocated to five different treatment groups. Several parameters were estimated in various time-points as indicated in the figure.
(TIF)

Figure S2 Pilot study. A) Effects of different doses of telmisartan or vehicle administered subcutaneously on the pressor responses to intravenously injected angiotensin II (50 ng/kg bw n = 7 per group). B) Effects of different doses of ramipril or vehicle administered subcutaneously on the pressor responses to intravenously injected angiotensin I (150 ng/kg bw; n = 7).
(TIF)

Figure S3 Intervention study. A) Magnetic resonance angiography of the rat brain from a ventral view before MCAO, during occlusion and after reperfusion on cerebral perfusion. On the left: middle cerebral artery before cerebral ischemia was induced; in the middle: during occlusion is the MCA in the circle not visible; on the right: the apparent MCA after reperfusion. B) Changes in the rCBF in the zone of ischemia before, during and after occlusion of the middle cerebral artery for 90 minutes and during the reperfusion period in rats treated subcutaneously with vehicle (white bars; n = 12), or telmisartan (light grey bars; n = 13) or ramipril (grey bars; n = 11) or combination of telmisartan and ramipril (black bars; n = 8) on 5 consecutive days before the induction of ischemia. rCBF values (mean ±SD) are expressed as the percentage of baseline values recorded before occlusion of the middle cerebral artery. In all groups the reduction of rCBF during occlusion was significant reduced *$p<0.05$.
(TIF)

Table S1 Arterial blood oxygen, carbon dioxide, pH values, glucose, haematocrite, sodium and potassium concentration before middle cerebral artery occlusion in rats treated subcutaneously with vehicle (V), telmisartan (T = 0.5 mg/kg), or ramipril (R1 = 0.01 mg/kg bw; R2 = 0.1 mg/kg bw) or combination telmisartan and ramipril (T = 0.5 mg/kg bw and R1 = 0.01 mg/kg bw).
(TIF)

Author Contributions

Conceived and designed experiments: CT-R KR KS MK PN AV US TU. Performed the experiments: CT-R KR KS MK PN CW SM. Analyzed the data: CT-R KR KS MK PN MG SM. Contributed reagents, materials, analysis tools: SM AV. Wrote the manuscript: CT-R TU. Satistical analysis of data: MG. Manuscript correction: CT-R PN US TU.

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7.3. Publication 3 [P3]

Journal: Neurobiology of Disease. 2013; 51:177-191

Title: AT2-receptor stimulation enhances axonal plasticity after spinal cord injury by upregulating BDNF expression.

Authors: Namsolleck P, Boato F, Schwengel K, Paulis L, Matho K, Geurts N, Thöne-Reineke C, Lucht K, Seidel K, Hallberg A, Dahlöf B, Unger T, Hendrix S, Steckelings UM.

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7.4. Publication 4 [P4]

Journal: Journal of Renin-Angiotensin-Aldosterone System. 2010 Mar;11(1):67-73

Title: The past, present and future of angiotensin II type 2 receptor stimulation.

Authors: Steckelings UM, Rompe F, Kaschina E, Namsolleck P, Grzesiak A, Funke-Kaiser H, Bader M, Unger T.

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7.5. Publication 5 [P5]

Journal: Current Opinion in Pharmacology. 2011 Apr;11(2):187-92

Title: Non-peptide AT2-receptor agonists.

Authors: Steckelings UM, Larhed M, Hallberg A, Widdop RE, Jones ES, Wallinder C, Namsolleck P, Dahlöf B, Unger T.

http://dx.doi.org/10.1016/j.coph.2010.11.002

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http://dx.doi.org/10.1016/j.coph.2010.11.002

8. Curriculum vitae

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht"

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht"

9. Publication list

AT2R in Neuroprotection and Neuroregeneration. Namsolleck P, Culman J, Unger T. Book chapter in: *The Protective Arm of the Renin Angiotensin System: Functional Aspects and Therapeutic Implications*, 1st ed., April 2015; 57–65. Academic Press, Elsevier.

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Direct angiotensin AT2-receptor stimulation attenuates T-cell and microglia activation and prevents demyelination in experimental autoimmune encephalomyelitis in mice. Valero-Esquitino V, Lucht K, Namsolleck P, Monnet-Tschudi F, Stubbe T, Lucht F, Ebner F, Brandt C, Danyel L, Villela D, Paulis L, Thoene-Reineke C, Liu M, Dahlöf B, Hallberg A, Sumners C, Unger T, Steckelings UM. *Clin Sci (Lond).* 2015 Jan;128(2):95-109.

Role of cardiovascular risk factors in the etiology of dementia. Namsolleck P, Schmerler P, Unger T. *MMW - Fortschritte der Medizin* 2014;156:83–86.

Aldosterone synthase inhibitors in cardiovascular and renal diseases. Namsolleck P and Unger T. *Nephrol. Dial. Transplant*. 2014;29 Suppl 1:i62-i68

AT2 receptor and tissue injury: Therapeutic implications. Namsolleck P, Recarti C, Foulquier S, Unger T. *Curr Hypertens Rep,* 2014 16:416

AT2-receptor stimulation enhances axonal plasticity after spinal cord injury by upregulating BDNF expression. Namsolleck P, Boato F, Schwengel K, Paulis L, Thöne-Reineke C, Lucht K, Seidel K, Hallberg A, Dahlöf B, Unger T, Steckelings UM. *Neurobiology of Disease*. 2013; 51:177-191.

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Non-peptide AT2-receptor agonists. Steckelings UM, Larhed M, Hallberg A, Widdop RE, Jones ES, Wallinder C, Namsolleck P, Dahlöf B, Unger T. *Curr Opin Pharmacol.* 2011 Apr;11(2):187-92.

The past, present and future of angiotensin II type 2 receptor stimulation. Steckelings UM, Rompe F, Kaschina E, Namsolleck P, Grzesiak A, Funke-Kaiser H, Bader M, Unger T. *J Renin Angiotensin Aldosterone Syst.* 2010 Mar;11(1):67-73.

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Impact of cereal fibre on glucose-regulating factors. Weickert MO, Moehlig M, Koebnick C, Osterhoff M, Ristow M, Namsolleck P, Holst JJ, Rudovich N, Rochlitz H, Spranger J, Pfeiffer AFH. *Diabetologia* 2005 Nov;48(11):2343-53

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10. Declaration of academic honesty (Eidesstattliche Versicherung)

„Ich, Pawel Namsolleck, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Pharmacological stimulation of the AT2 receptor activates endogenous neuro-protective and neuro-regenerative mechanisms via BDNF-mediated signaling“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.icmje.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.
Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.
Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

_____
Unterschrift

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