Association of the novel, non-AT$_1$, non-AT$_2$ angiotensin binding site with neuronal death

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List of nonstandard abbreviations: AngII, angiotensin II; SI-AngII, Sar1, Ile8 angiotensin II; RAS, renin angiotensin system; ZD7155, 5,7-Diethyl-3,4-dihydro-1-[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-1,6-naphthyridin-2(1H)-one hydrochloride; PD123319, 1-[[4-(Dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid difluoroacetate; PCMB, p-chloromercuribenzoate; PCMPS, p-chloromercuri-benzenesulfonate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; GSH, reduced glutathione; GSSG, oxidized glutathione; HBSS, Hank's Buffered Salt Solution; EBSS, Earle's Balanced Salt Solution; DMEM, Dulbecco's Modified Eagle Medium; DNAse, deoxyribonuclease; DAPI, 4',6-diamidino-2-phenylindole; GFAP, Glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor molecule 1; E14, embryonic day 14; P1, postnatal day 1.
Abstract

Recently, we discovered a non-AT₁, non-AT₂ angiotensin binding site in rodent and human brain membranes which, based on its pharmacological/biochemical properties and tissue distribution, is different from angiotensin receptors and key proteases processing angiotensins. In this study, the novel angiotensin binding site was localized to a specific brain cell type using radioligand receptor binding assays. Our results indicate that the novel binding site is expressed in mouse primary cortical neuronal membranes but not in primary cortical astroglial and bEnd.3 brain capillary endothelial cell membranes. Whole cell binding assays in neurons showed that the binding site faces the outer side of the plasma membrane. Consistent with our previous observations, the novel binding site was unmasked by sulfhydryl reagent p-chloromercuribenzoate (PCMB). This effect had a bell-shaped curve and was reversed by reduced glutathione, indicating that function of the binding site might be regulated by redox state of the environment. Density of the novel binding site measured by saturation binding assays was significantly increased in neuronal membranes of cells challenged in four in vitro models of cell death (oxygen-glucose deprivation, sodium azide-induced hypoxia, NMDA and hydrogen peroxide neurotoxicity). Additionally, our in vivo data from developing mouse brains showed that the density of the novel angiotensin binding site changes similar to the pattern of neuronal death in maturating brain. This is the first time that evidence is provided on the association of the novel angiotensin binding site with neuronal death, and future studies directed towards understanding of the function(s) of this protein are warranted.
Brain renin-angiotensin system (RAS) is one of the independently regulated, local angiotensin systems most known for its role in cardiovascular and hydromineral balance regulation both in health and disease (McKinley, et al., 2003; Cuadra, et al., 2010). Although substantial progress in understanding of these functions of the brain RAS has been made over the past decades, many questions remain unanswered regarding the precise nature and role of the system (Karamyan and Speth, 2007a; Speth and Karamyan, 2008; Saavedra, 2005; Phillips and de Oliveira, 2008). For example, almost all key components of the brain RAS including renin and its recently identified receptor, angiotensinogen, angiotensin converting enzyme and its human homolog (ACE2), angiotensin II (AngII) and its classical receptors are found not only in brain areas involved in cardiovascular regulation but also in regions playing a role in neurogenesis, plasticity and memory, cognition and analysis of new information, reward and sensation of pleasure, motor coordination, etc. (McKinley, et al., 2003; Phillips and de Oliveira, 2008; Lazartigues, 2009). Thus, it is believed that the role of brain RAS is not exclusively associated with cardiovascular and hydromineral regulation, but a number of other processes/functions are regulated by this system (McKinley, et al., 2003; Phillips and de Oliveira, 2008; Lazartigues, 2009).

Recently, in the course of radioligand binding studies of brain AngII receptors, we discovered a novel, non-AT₁, non-AT₂ binding site for angiotensins in rat, mouse and human brain membranes (Karamyan and Speth, 2007b; Karamyan, et al., 2008b; Karamyan, et al., 2008a). This binding site is insensitive to blockade by specific type 1 (AT₁) and type 2 (AT₂) angiotensin receptor antagonists and is present in the brains of mice lacking these and Mas (the hypothesized receptor for angiotensin (1-7)) receptors (Karamyan and Speth, 2007b; Karamyan,
Moreover, the novel angiotensin binding site is not present in rodent tissues abundant with angiotensin receptors, such as liver, adrenal and kidney (Karamyan and Speth, 2007b; Karamyan, et al., 2008a), and is abundantly distributed in the rat brain including nuclei involved in both cardiovascular and non-cardiovascular functions (Karamyan and Speth, 2008). Lastly, a unique feature of the novel angiotensin binding site is that it is unmasked (i.e., able to bind angiotensins) in the presence of optimal concentrations of organomercurial sulfhydryl reagents p-chloromercuribenzoate or p-chloromercuri-benzenesulfonate (PCMB and PCMPS). This effect is reversed by disulfide-reducing reagents dithiothreitol and 2-mercaptoethanol, indicating involvement of cysteine residues (thiol groups) in unmasking and function of the novel angiotensin binding site (Karamyan and Speth, 2007b). Currently, the identity of this protein is unknown and we only have information about the molecular size and some biochemical properties of this protein (Karamyan, et al., 2010), and efforts are being made towards its identification.

Pharmacological specificity of the novel angiotensin binding site was studied in detail both in rodent and human brain membranes and was not in the scope of the current study (Karamyan and Speth, 2007b; Karamyan, et al., 2008b; Karamyan, et al., 2008a). The purpose of this study was to locate the novel angiotensin binding site to specific brain cell type(s) using mouse brain cultures, to estimate the density of the binding site in hosting cell type(s), and to get more clues on the unmasking mechanism(s) of this protein. Additionally, we provide evidence (in vitro and in developing brain tissue) pointing to an association of the novel angiotensin binding site with neuronal death.
Methods

Animals. Timed pregnant CD-1 female mice (Charles River) were used for isolation of primary brain cultures. For developmental studies we used CD-1 mouse embryos and in house-born pups of both genders. All animals were maintained in 12 h light/dark cycle and fed ad libitum. Animal procedures were carried out using a protocol approved by Texas Tech University Health Sciences Center IACUC.

Cell cultures. Mouse primary cortical neurons were isolated and cultured according to Mattson et al. (1995) with some modifications. Briefly, cerebral cortices were obtained from E16 embryos (CD-1 mice, Charles River) and dissected in Hank’s balanced salt solution without Ca^{2+} and Mg^{2+} supplemented with 10 μg/ml gentamycin (HBSS). Dissected pieces of cortices (free of meninges) were digested in trypsin (+DNAse in HBSS) for 15 min at room temperature, neutralized with trypsin inhibitor, and washed three times with HBSS. Dissociated cell suspensions were transferred into 100 mm Petri dishes or 6-well plates (0.5-0.6 x 10^4 per cm^2 surface area) coated with polyethylenimine and cultured in Neurobasal medium (Invitrogen) supplemented with 1.3 mM L-glutamine, 25 μg/ml gentamicin and 2% B27 (Invitrogen), at 37°C in a humidified atmosphere of 5% CO₂ in air. After overnight incubation half of the medium was replaced with fresh Neurobasal medium (+supplements), and the cells were cultured for 12 more days without renewal of the medium (also referred as ‘old medium’ group). Unless otherwise mentioned, all our experiments utilized neurons grown in these conditions. Primary cortical neurons isolated by this approach yielded ~95 % pure neuronal cultures determined by immunocytochemical analysis using specific neuronal (anti-neuron specific β III tubulin antibody; Abcam), astrocyte (anti-GFAP antibody; Cell Signaling) and nuclear (DAPI; Invitrogen) markers.
Mouse primary cortical astroglial cells were cultured as described in (Keller, et al., 1996). Briefly, cerebral cortices were obtained from one day-old CD-1 mice, dissected and treated as detailed above for primary neurons. Dissociated cell suspensions were transferred into 75 mm² cell culture flasks and cultured in DMEM (with high glucose, sodium pyruvate and L-glutamine) supplemented with 10% fetal bovine serum and penicilline/streptomycin (100 U/ 0.1 mg per ml, respectively), at 37°C in a humidified atmosphere of 5% CO₂ in air. Confluent cultures (~10 days) were subcultured in 100 mm Petri dishes for another 5-7 days and collected upon reaching confluency. The purity of astroglial cultures (>95 %) was estimated by immunocytochemical analysis using specific astrocyte (anti-GFAP antibody; Cell Signaling), microglial (anti-Iba1 antibody; Abcam) and nuclear (DAPI; Invitrogen) markers.

bEnd.3 mouse brain capillary endothelial cell line was purchased from American Type Culture Collection (ATCC) and maintained according to the vendor’s recommended protocol. The choice of a cell line over primary culture was reasoned by very small harvesting volumes of primary brain capillary endothelial cells from mice and the need of large amounts of membranes for receptor binding assays. bEnd.3 cells are one of few cell lines commonly used for in vitro studies of blood brain barrier functions (Huppert, et al., 2009).

On the day of collection, all cells were washed twice by phosphate buffered saline, scraped and kept frozen in -80°C before their use in radioligand binding experiments.

**Radioligand binding assays.** \(^{125}\text{I}-\text{Sar}^1-\text{Ile}^8-\text{AngII} (^{125}\text{I}-\text{SI}-\text{AngII})\) was purchased from the Peptide Radioiodination Service Center of the University of Mississippi and American Radiolabeled Chemicals. Receptor binding studies in neuronal membranes were carried out using established procedures (Karamyan and Speth, 2007b;Karamyan, et al., 2008a) except that sonication (instead of homogenization) was used for preparation of membranes, and ZD7155
(instead of losartan; Tocris) was used to block the AT$_1$ angiotensin receptors. Unless otherwise mentioned, 10 μM final assay concentration of PCMB was used in all our binding studies. The protein concentration was determined by the method of Bradford using bovine serum albumin as standard. Whole-cell binding assays on neurons were carried out in 6-well plates in 50 mM Tris-HCl assay buffer (pH 7.2) containing 140 mM NaCl, 100 μM EDTA and 100 μM o-phenanthroline (Demaegdt, et al., 2008) in 800μl final volume. $^{125}$I-SI-AngII was used at ~0.6 nM concentration ± 10 μM AngII for non-specific binding, in the presence of 1 μM ZD7155 (an AT$_1$ angiotensin receptor antagonist), 10 μM PD123319 (an AT$_2$ angiotensin receptor antagonist) and 10 μM final assay concentration of PCMB. After 1 hr incubation and 3-time wash with 1 ml phosphate buffered saline, cells were dissolved in 750 μl of 1 M NaOH for 30 min at 24°C and transferred into cell culture tubes. The wells were washed with 250 μl water, transferred into corresponding cell culture tubes and radioactivity was measured in a gamma-counter (Wizard$^2$ 2470, Perkin Elmer).

In the reversal experiments utilizing glutathione analogs, neuronal membranes or whole neurons were first pre-treated with PCMB, and then after at least 5 min, one of the glutathione analogs and the radioligand were added onto the membranes or cells. The specific binding of the radioligand was estimated in the same way as in the regular binding experiments.

In a set of experiments, primary neurons were treated with glutathione biosynthesis inhibitor L-buthionine-(S,R)-sulfoximine (150 μM for 24 hr), and specific binding of $^{125}$I-SI-AngII was estimated in the presence or absence of PCMB in neuronal membranes (in the presence of AT$_1$ and AT$_2$ angiotensin receptor antagonists).

*In vitro models of cell death.* Primary cortical neurons utilized in this set of experiments were grown in conditions where 1/3 of the Neurobasal medium (+ supplements) was refreshed
twice a week. Four well-established in vitro models of neuronal death were used in our study. For oxygen-glucose deprivation/re-oxygenation (model of ischemia-reperfusion injury (Abbruscato, et al., 2004)) 12 in vitro-day-old neurons were deprived of oxygen and glucose in Earle's balanced salt solution without glucose (EBSS) in oxygen-free N2/CO2 (95%/5%) atmosphere (hypoxia chamber; Billups-Rothenberg) at 37°C for 3 hr. Thereafter, the medium was replaced by Neurobasal medium (+ supplements) and 24 hr later the cells were collected for binding assays. NMDA neurotoxicity (model of excitotoxicity (Hewett, et al., 2000)) was carried out in the following way. Briefly, 12 in vitro-day-old neurons were treated with 0.5 mM NMDA in HBSS for 30 min (at 37°C in a humidified atmosphere of 5% CO2 in air), followed by replacement of medium with Neurobasal medium (+ supplements). After 24 hr the cells were collected for binding assays. Sodium azide neurotoxicity (model of hypoxic injury (Marino, et al., 2007)) was induced similar to NMDA neurotoxicity except that 3 mM sodium azide was used. Hydrogen peroxide neurotoxicity (model of oxidative injury (Whittemore, et al., 1995)) was performed similar to NMDA and sodium azide, except that 30 µM hydrogen peroxide was used.

**Cell viability assays.** Alamar Blue (indicator of metabolic activity of cells (White, et al., 1996)), lactate dehydrogenase (indicator of plasma membrane integrity (Hewett, et al., 2000)) and MTT (indicator of mitochondrial function) assays were used to estimate viability of neurons challenged in the above mentioned models. Measurements were performed 24 hr after challenging neurons (grown in 12-well plates) in the in vitro models, according to manufacturers’ recommended protocols (Alamar Blue – BioSource, Invitrogen; lactate dehydrogenase /Cytotoxicity Detection Kit, LDH/ – Roche Diagnostics; MTT – Sigma /In vitro Toxicology Assay Kit, MTT based/).
Statistical analyses. Determination of B_{max} (fmole of radioligand bound per mg protein), K_{d} and IC_{50} values were carried out using one-site saturation (Y = B_{max} * X / (K_{d} + X)) and competition (Y = Bottom + (Top – Bottom) / (1 + 10^{(X – LogIC_{50})})) binding models of Prism software (Graphpad Software, San Diego, CA). Values reported were significantly different from zero and are presented as mean ± S.E.M. K_{i} value for AngII was determined using the Cheng-Prusoff equation: K_{i} = IC_{50} / (1 + H / K_{d}), where H is the radioligand concentration and K_{d} is the affinity of the radioligand. K_{on} and K_{off} values, and association and dissociation half-times were calculated using one-phase association (Y = Y_{0} + (Plateau – Y_{0}) * (1 – exp(–K * X))) and dissociation (Y = (Y_{0} – Plateau) * exp(–K * X) + Plateau) models of Prism software.

Comparison of B_{max} and K_{d} values obtained from binding assays in membrane preparations of neurons challenged in in vitro models of cell death, and in developing brains was carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. The results of cell viability assays were compared in the same way. Values reported are mean ± S.E.M.
Results

The expression of the novel angiotensin binding site in membrane preparations of mouse primary cortical neurons was tested by radioligand receptor binding assays using $^{125}$I-Sar$^1$-Ile$^8$-AngII ($^{125}$I-SI-AngII) in the presence of specific AT$_1$ and AT$_2$ angiotensin receptor antagonists ZD7155 and PD123319, respectively. First, 150 µM final assay concentration of PCMB (similar to our previous studies in rodent and human brain membranes) was applied to unmask the binding site in neuronal membranes (Figure 1a). Next, the optimal range of PCMB concentrations for unmasking of the binding site in primary neuronal membranes was determined. Specific binding of $^{125}$I-SI-AngII (10 µM AngII displaceable) as a function of PCMB concentrations had a bell-shape, with optimal concentrations of PCMB ranging from 10 - 100 µM (Figure 1b). Based on these results, 10 µM PCMB was used in all our subsequent experiments. Under the same experimental conditions, unlabeled AngII showed ~100 nM affinity (K$_i$ value) for the novel angiotensin binding site in neuronal membranes (Figure 1c). Therefore, 10 µM final concentration of AngII was used to displace $^{125}$I-SI-AngII and determine its non-specific binding in subsequent binding experiments.

To properly identify the specific radioligand binding and estimate the density of the novel binding site, correlation of the membrane/receptor concentration with the extent of specific binding of the radioligand was determined (Hoffman and Lefkowitz, 1980). Specific binding of $^{125}$I-SI-AngII (10 µM AngII displaceable) to the novel angiotensin binding site was linear to the neuronal membrane protein concentrations from 50 to 300 µg/ml in the assay medium (data not shown). For this reason, 200-300 µg/ml neuronal membranes were used in our future experiments.
Association rate of $^{125}$I-SI-AngII ($\sim 0.6$ nM) to the novel angiotensin binding site in neuronal membranes displayed a half-time of $\sim 15$ min at $24^\circ$C, reaching a steady-state at $\sim 90$ min (Figure 2a). The dissociation half-time of $^{125}$I-SI-AngII from the binding site was $\sim 76$ min at $24^\circ$C (Figure 2b), and the calculated kinetic dissociation constant ($K_d$) was $0.2$ nM. Notably, the kinetic dissociation constant calculated from these experiments was similar to the equilibrium dissociation constant obtained from saturation binding studies (Figures 3).

The $B_{\text{max}}$ value ($128 \pm 6$ fmol/mg protein) for the novel angiotensin binding site in primary cortical neuronal membranes was calculated from saturation binding experiments conducted at steady state conditions (2 hr incubation at $24^\circ$C), based on the results of time-course association experiments (Figure 2a). The equilibrium dissociation constant ($K_d$) for $^{125}$I-SI-AngII was $0.65 \pm 0.1$ nM. There was no significant saturable binding of the radioligand to the classical $\text{AT}_1$ and $\text{AT}_2$ angiotensin receptors in these neuronal membranes (data not shown).

Saturation binding experiments in membrane preparations obtained from mouse primary cortical astroglial cells and bEnd.3 mouse brain capillary endothelial cell line did not show any significant saturable binding of the radioligand under the same experimental conditions (Figure 3).

High-affinity binding of $^{125}$I-SI-AngII to the novel angiotensin binding site was also observed in intact primary cortical neurons using both PCMB and its sulfonic acid derivative p-chloromercuri-benzenesulfonate (PCMPS) (Figure 4). Similar to membrane preparations, there was no binding to the classical $\text{AT}_1$ and $\text{AT}_2$ angiotensin receptors in these cells (Figure 4).

Figure 5 summarizes the effects of reduced and oxidized glutathione, and their structural analog $\gamma\text{Glu}-\text{Abu}-\text{Gly}$ (cysteine residue is replaced with 2-aminobutyric acid, Abu; Phoenix
Pharmaceuticals) on the unmasking effect of PCMB in primary neuronal membranes (Figure 5a) and whole neurons (Figure 5b).

Depletion of intracellular levels of reduced glutathione by its biosynthesis inhibitor L-buthionine-(S,R)-sulfoximine in neurons did not unmask the novel binding site in neuronal membranes, and presence of PCMB was still required for high-affinity binding of $^{125}$I-SI-AngII (data not shown).

The density of the non-AT$_1$, non-AT$_2$ angiotensin binding site was also determined in membrane preparations obtained from primary cortical neurons (in the presence of 1 µM ZD7155, 10 µM PD123319 and 10 µM PCMB) challenged in four well-established in vitro models of neuronal death; oxygen-glucose deprivation/re-oxygenation (model of ischemia-reperfusion injury (Abbruscato, et al., 2004)), NMDA neurotoxicity (model of excitotoxicity (Hewett, et al., 2000)), sodium azide neurotoxicity (model of hypoxic injury (Marino, et al., 2007)) and hydrogen peroxide neurotoxicity (model of oxidative injury (Whittemore, et al., 1995)). As presented in Figure 6, the density ($B_{max}$ value) of the novel binding site was significantly increased in neuronal membranes of cells challenged in all four in vitro models. Also, the density of the novel angiotensin binding site was significantly increased in neurons from the ‘old medium’ group (Figure 6), where the medium was not refreshed throughout the culturing period (see “Material and methods” section). Notably, the averaged $K_d$ values did not differ significantly between the groups, and the neurotoxic insults alone did not unmask the binding site in neuronal membranes (presence of PCMB was required for unmasking).

Viability of neurons was measured 24 hr after challenging the cells in the above-mentioned models using lactate dehydrogenase, Alamar Blue and MTT assays. All assays indicated significantly decreased viability of neurons in all experimental groups (except the ‘old
medium’ group in the lactate dehydrogenase assay) compared with the ‘fresh medium’ group (Figure 7).

Saturation binding experiments in membrane preparations of mouse forebrains (in the presence of 1 µM ZD7155, 10 µM PD123319 and 150 µM PCMB), obtained at different stages of development, indicated that the density (B\text{max} value) of the novel angiotensin binding site gradually increases from E14 to P10 period, followed by a significant drop on P21 and a maintained level in 9-12-week old animals (Figure 8). The averaged K\text{d} values did not differ significantly between the groups.
Discussion

The initial purpose of this study was to locate the novel, non-AT\textsubscript{1}, non-AT\textsubscript{2} angiotensin binding site to specific cell type(s) using mouse brain cultures and radioligand receptor binding assays. Consistent with previous observations reported in rodent and human brain membranes, 150 µM final assay concentration of PCMB unmasked the binding site in neuronal membranes (Figure 1a). Concentration-effect curve for unmasking had a bell-shape, with optimal concentrations of PCMB ranging from 10 - 100 µM (Figure 1b). Notably, unmasking of the binding site (i.e., ability to bind angiotensins) by sulfhydryl reagents is one of the unique features of this protein (Karamyan and Speth, 2007b). Organomercurials and particularly PCMB, are the most specific sulfhydryl agents forming easily reversible mercaptide bond with thiol group (Rothstein, 1970). A probable explanation of our observations is that unmasking of the binding site is associated with interaction of PCMB with thiol group of cysteine(s) in this protein (Karamyan and Speth, 2007b). In other words, the state of reduced (–SH) and oxidized (–SR) thiols in the binding site likely determines the conformation at which this protein binds angiotensins with high affinity. Furthermore, the bell-shaped concentration-effect curve of PCMB indicates fine regulation of this process, as only a certain number of thiols need to be oxidized/modified in order the binding site is efficiently unmasked and able to bind angiotensins (Vyas, et al., 2002).

Saturation binding experiments at steady-state conditions indicated high affinity, saturable binding of \textsuperscript{125}I-SI-AngII to the binding site in neuronal membranes but not in membrane preparations of astroglial and bEnd.3 cells (Figure 3), suggesting that this protein is mainly expressed in neurons.
The presence of the binding site was confirmed in intact neurons using both PCMB and PCMPS, indicating that the protein is located on the plasma membrane and is accessible for its ligands in extracellular environment (Figure 4). Considering that PCMPS is a cell-impermeable analog (Rothstein, 1970; Vyas, et al., 2002), these data also indicate that the thiol group(s) involved in unmasking is facing the cell-surface.

Next, we performed experiments using glutathione (GSH) analogs to further confirm the involvement of thiol groups in the unmasking of the binding site. Addition of reduced GSH to neuronal membranes or intact neurons pre-incubated with PCMB prevented the unmasking with IC₅₀ value of 5 - 15 μM (Figure 5). Importantly, oxidized glutathione (GSSG) and γGlu-Abu-Gly (both lack the thiol group) did not reverse the unmasking effect of PCMB (Figure 5). These results confirm that unmasking of the binding site is specifically associated with modification of thiol group(s) in this protein. Considering that reduced GSH does not cross the plasma membrane (Skalska, et al., 2009), these results further suggest that the thiol group(s) responsible for unmasking faces the cell surface. Additionally, these results indicate that the process of unmasking is reversible and may be regulated upon changes in extracellular levels of GSH in the brain. In other words, physiological/high extracellular concentrations of GSH favor the masked state of the binding site, whereas decreased levels of GSH (e.g., following oxidative stress) favor its unmasking and function. Notably, extracellular/CSF concentrations of GSH in normal/healthy brain are in the range of 5 - 15 μM (opposite to mM intracellular concentrations), which change at different adaptive/pathophysiological conditions (Yang, et al., 1994; Lada and Kennedy, 1997). To further study the unmasking mechanism of the binding site, we used glutathione biosynthesis inhibitor L-buthionine-(S,R)-sulfoximine to deplete intracellular levels of reduced glutathione in
neurons. This treatment did not unmask the novel binding site in neuronal membranes, and presence of PCMB was still required for high-affinity binding of $^{125}$I-SI-AngII.

It is important to mention that reversible modification (oxidation/reduction) of thiol group of cysteine(s) (also known as reactive cysteines/thiols, cysteine/redox switches), is increasingly appreciated as an essential posttranslational modification of proteins (Jones, 2008; Janssen-Heininger, et al., 2008). Our results suggest that the novel angiotensin binding site is likely to be a similar redox-sensitive protein located on the plasma membrane of neurons, which depending on the redox state of the extracellular environment in the brain may be unmasked to interact with angiotensins.

The ubiquitous role of oxidative stress in cell death and redox-sensitivity of numerous proteins involved in this process is well documented (Calabrese, et al., 2010). To further investigate a possible association of oxidative stress with the binding site, we carried out experiments to estimate density of this protein in membrane preparations of dying neurons. Neurons challenged in four in vitro models of cell death as well as grown in conditions with limited availability of nutrients (‘old medium’) showed significantly increased density of the binding site (Figure 6). Importantly, the neurotoxic insults did not unmask the binding site in neuronal membranes, and presence of PCMB was required for unmasking. Parallel cell-viability assays indicated that viability of neurons was significantly decreased in all groups compared with the ‘fresh medium’ group (Figure 7). Notably, these data suggest that any stressor reaching the threshold to initiate cell death will likely result in up-regulation of the binding protein. Similar $B_{\text{max}}$ values between the experimental groups indicate that up-regulation of this protein is a regulated process with an upper limit independent of the potency of a stressor.
Lastly, we determined the density of the binding site in developing mouse brains. Our results indicated a gradual increase in \( B_{\text{max}} \) values in mouse forebrain membranes from E14 to P10 period, followed by a dramatic drop in P21 animals and similar levels in 9-12-week-old animals (Figure 8). Interestingly, the pattern of developmental changes in density of the binding site (Figure 8) is very similar to the occurrence of neuronal death in developing brain (Ferrer, et al., 1992; Naruse and Keino, 1995). The number of dead neurons in rat primary visual cortex is low at birth, which increases from P2 peaking at the end of the first week, and decreases during the second week followed by low numbers at the end of the first month (Ferrer, et al., 1992; Naruse and Keino, 1995). Clearly, these in vivo observations complement our in vitro data and support the association between neuronal cell death and the binding site. Moreover, these results clarify our previous observations in adult rat brain where distribution of the binding site was studied by in vitro receptor autoradiography (Karamyan and Speth, 2008). In the latter study, among brain regions with highest radioligand binding were olfactory bulb (highest compared with other brain regions), ventricle wall (throughout brain) and dentate gyrus. Interestingly, it was shown that the frequency of apoptosis in adult rat brain is up to 100 times higher in olfactory bulb, ventricle wall and dentate gyrus (in decreasing order) compared with other brain areas (Biebl, et al., 2000; Kuhn, et al., 2005).

Our results provide the first evidence on association of the novel angiotensin binding site with a (patho)physiological process, suggesting a potential role of this protein in neuronal cell death. While the exact function of the binding site is unknown, many features of this protein; e.g., high affinity and pharmacological specificity for physiologically relevant ligands, preservation in a number of species including humans, accessibility of ligands to the binding site from extracellular environment, direct association of this protein with neuronal death, strongly
favor the functional significance of this protein. This proposal is also supported by one of the primary concepts of functional genomics postulating that non-functional receptors/binding sites are evolutionarily discarded (Civelli, 1998).

Importantly, the link between AngII and oxidative processes is well documented. Numerous studies have established that reactive oxygen species are products of AngII signaling, via the AT\textsubscript{1} receptor (Griendling, et al., 1994; Zimmerman, et al., 2004). A direct role of redox-sensitive molecules in signaling pathways downstream of the AT\textsubscript{1} receptor (Tabet, et al., 2008), and in negative regulation of AT\textsubscript{1} receptor-mediated effects (Harrison and Sumners, 2009) were also demonstrated. Additionally, modulatory role of the AT\textsubscript{1} and AT\textsubscript{2} receptors in cell proliferation, differentiation, repair and apoptosis has been studied and debated in the last decades (Kaschina and Unger, 2003).

In summary, our study provides a number of important findings about the recently discovered non-AT\textsubscript{1}, non-AT\textsubscript{2} angiotensin binding site, which is primarily expressed in neurons facing the outer side of the plasma membrane. The unique pattern of unmasking of this binding site by specific sulfhydryl reagents and reversal of this effect by reduced GSH suggest that function of this protein likely depends on the redox state of the extracellular environment in the brain. In other words, it is hypothesized that the state of reduced and oxidized thiol group(s) in this protein determines its proper conformation for high-affinity binding of angiotensins. Lastly, evidence is provided for the first time \textit{(in vitro} and in developing brain) to support a potential role of the binding site in neuronal death.

Two important questions that are not clear from our study are the direct function of the binding site in neuronal death and the mechanism(s) of unmasking of this protein in (patho)physiological conditions without use of exogenous sulfhydryl compounds. These
questions warrant future studies and should provide insights into the way in which the novel binding site affects brain angiotensinergic activity and plays role in neuronal cell death.
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**Footnotes**

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Legends for Figures

Figure 1.

(a & b) Unmasking of the non-AT<sub>1</sub>, non-AT<sub>2</sub> angiotensin binding site in mouse primary cortical neuronal membranes. Specific binding (10 µM AngII displaceable) of <sup>125</sup>I-SI-AngII (~0.6 nM; 1 hr incubation at 24°C) was estimated in the presence of 1 µM ZD7155 (specific AT<sub>1</sub> angiotensin receptor antagonist), 10 µM PD123319 (specific AT<sub>2</sub> angiotensin receptor antagonist) and 150 µM (a; n = 2) or varying concentrations (b; n = 4 - 6) of PCMB.

(c) Representative competition binding analysis of <sup>125</sup>I-SI-Ang II binding in mouse primary cortical neuronal membranes (2 hr incubation at 24°C, based on kinetic and saturation binding studies provided in Figures 2 and 3). Specific binding of <sup>125</sup>I-SI-AngII (~0.6 nM) was estimated in the presence of 1 µM ZD7155, 10 µM PD123319, 10 µM PCMB and different competing concentrations of AngII.

Figure 2.

Representative association (a) and dissociation (b) kinetics of specific (10 µM AngII displaceable) <sup>125</sup>I-SI-AngII (~0.6 nM) binding to the novel angiotensin binding site in primary cortical neuronal membranes (in the presence of 1 µM ZD7155, 10 µM PD123319 and 10 µM PCMB; incubation at 24°C; n = 3).

Figure 3.

Representative saturation binding analyses of specific (10 µM AngII displaceable) <sup>125</sup>I-SI-AngII binding to the non-AT<sub>1</sub>, non-AT<sub>2</sub> angiotensin binding site in primary cortical neuronal and
astroglial, and bEnd.3 cell membranes (in the presence of 10 μM PD123319, 1 μM ZD7155; astroglial membranes contained double concentrations of blockers/ and 10 μM PCMB; 2 hr incubation at 24°C). In neuronal membranes $B_{\text{max}} = 94 \pm 3$ fmol/mg protein, $K_d = 0.35 \pm 0.05$ nM; in astroglial and bEnd.3 membranes $B_{\text{max}}$ and $K_d$ values are not significantly different from 0.

Figure 4.
Specific binding (10 μM AngII displaceable) of $^{125}$I-SI-AngII (~0.6 nM) in intact primary cortical neurons (1 hr incubation at 24°C; n = 6). Experimental groups: background, binding of the radioligand in the presence of specific AT$_1$ and AT$_2$ angiotensin receptor antagonists /1 μM ZD7155 and 10 μM PD123319, respectively/; AT$_1$ & AT$_2$, binding of the radioligand in the absence of specific AT$_1$ and AT$_2$ angiotensin receptor antagonists; non-AT$_1$, non-AT$_2$ (PCMB), binding of the radioligand in the presence of 10 μM PD123319, 1 μM ZD7155 and 10 μM PCMB; non-AT$_1$, non-AT$_2$ (PCMPS), binding of the radioligand in the presence of 10 μM PD123319, 1 μM ZD7155 and 10 μM PCMPS.

Figure 5.
Reversal of the unmasking effect of PCMB in primary neuronal membranes (a; n = 3) and whole neurons (b; n = 4 - 6) by reduced (GSH) and oxidized (GSSG) glutathione, and a structural analog of reduced glutathione, γGlu-Abu-Gly. Specific binding (10 μM AngII displaceable) of $^{125}$I-SI-AngII (~0.6 nM) was measured in the presence of 10 μM PD123319, 1 μM ZD7155, 10 μM PCMB and different concentrations of GSH, GSSG or γGlu-Abu-Gly (1 hr incubation at
24°C). Calculated IC$_{50}$ value for GSH is 5.3 ± 0.05 µM in neuronal membranes, and 16.3 ± 0.3 µM in whole neurons.

**Figure 6.**
Density (B$_{\text{max}}$ values; binding of $^{125}$I-SI Ang II in the presence of 1 µM ZD7155, 10 µM PD123319 and 10 µM PCMB) of the non-AT$_1$, non-AT$_2$ angiotensin binding site in membrane preparations of primary cortical neurons from different experimental groups (n = 3 – 6; *p < 0.05; ***p < 0.001): ‘fr. med.’ – fresh medium, primary neurons were grown for 13 days in culture where 1/3 of the Neurobasal (+supplements) medium was refreshed twice a week (see “Materials and methods” section); ‘old med.’ – old medium, primary neurons were grown for 13 days in culture where 1/2 of the Neurobasal (+supplements) medium was refreshed only once on the next day of culturing; ‘NaN$_3$’ – 30 min exposure to 3 mM sodium azide (in EBSS w/o glucose)/24 hr recovery in Neurobasal (+supplements) medium; ‘NMDA’ – 30 min exposure to 0.5 mM NMDA (in EBSS w/o glucose)/24 hr recovery in Neurobasal (+supplements) medium; ‘OGD’ – 3 hr oxygen-glucose deprivation (in EBSS w/o glucose)/24 hr re-oxygenation in Neurobasal (+supplements) medium; ‘H$_2$O$_2$’ – 2 hr exposure to 30 µM hydrogen peroxide (in EBSS w/o glucose)/24 hr recovery in Neurobasal (+supplements) medium.

**Figure 7.**
Viability of primary cortical neurons in experimental groups measured by lactate dehydrogenase (LDH) (a; increased release of LDH indicates compromised integrity of cell plasma membrane), Alamar Blue (b; decreased reduction of Alamar Blue indicates attenuated metabolic activity of cells), and MTT (c; decreased formation of formazan dye indicates compromised mitochondrial
function of cells) assays (n = 3 – 6; *p < 0.05; **p < 0.01; ***p < 0.001; # significantly different from all other groups; & significantly different from ‘old med.’; Φ significantly different from ‘old med.’ and ‘NaN3’; in panel (b) all groups are significantly different from each other except ‘NMDA’ vs. ‘OGD’). See Figure 6 and “Materials and methods” section for detailed information on the experimental groups.

Figure 8.

Density (B_max values; binding of ^125^I-SI Ang II in the presence of 1 µM ZD7155, 10 µM PD123319 and 150 µM PCMB) of the non-AT_1, non-AT_2 angiotensin binding site in mouse forebrain membranes from E14 and E18 embryos; P1, P5, P10 and P21 pups, and 9 – 12 week-old adult animals (n = 3 – 5; a – significantly different from E14, b – significantly different from 9-12 week-old (9 – 12W), c – significantly different from P21, d – significantly different from E18).
Fig. 1b

![Graph showing specific binding (cpm) vs. Log [PCMB].](image-url)
Fig. 1c

![Graph showing the relationship between % specific binding and Log [Ang II]. The curve indicates a decrease in % specific binding as Log [Ang II] increases. The graph includes data points and a curve with the equation $K_i = 97.6 \text{ nM}$.]
Specific binding (fmol/mg protein)

Time (min)

$K_{on} = 0.045 \pm 0.004 \text{ nM}^{-1} \text{ min}^{-1}$

$T_{1/2} = 15.2 \text{ min}$
Fig. 2b

Specific binding (fmol/mg protein)

$K_{\text{off}} = 0.009 \pm 0.003 \text{ min}^{-1}$

$T_{1/2} = 76.28 \text{ min}$
Fig. 3

Graph showing specific binding (fmol/mg protein) against free \(^{125}\text{I}\)-SI-Ang II (nM) for different membrane types:
- Neuronal membranes
- Astroglial membranes
- bEnd3 membranes

The graph demonstrates a decrease in specific binding as the concentration of free \(^{125}\text{I}\)-SI-Ang II increases for all membrane types.
Fig. 4

The graph shows the specific binding (cpm) of different groups:
- non-AT₁, non-AT₂ (PCMPS)
- non-AT₁, non-AT₂ (PCMB)
- AT₁ & AT₂
- Background

The x-axis represents the specific binding (cpm) ranging from 0 to 25,000, while the y-axis is logarithmic, ranging from 1000 to 250,000. The data points are represented by different patterns and the error bars indicate the variability in the measurements.
Fig. 5b

Graph showing the inhibition of specific binding as a function of the logarithm of the concentration of GSH, GSSG, or γGlu-Abu-Gly. The percentage of specific binding is plotted on the y-axis against the log of the concentration on the x-axis. Different symbols represent different substances: GSH (circle), GSSG (diamond), and γGlu-Abu-Gly (triangle). The lines represent the theoretical curves fitting the data points.
Fig. 6

This graph shows the effect of different treatments on $B_{\text{max}}$ (fmol/mg protein). The treatments include fr. med., old med., NaN$_3$, NMDA, OGD, and H$_2$O$_2$. The results are indicated with asterisks: *** for significant increases and * for a significant decrease compared to the control group.
Fig. 7c

Relative formation of formazan (%)

- fr. med.
- old med.
- NaN₃
- NMDA
- OGD
- H₂O₂

Statistical significance indicated by:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- ***, ϕ
Fig. 8

[B_max (fmol/mg protein)]

- E14
- E18
- P1
- P5
- P10
- P21
- 9-12W

Legend:
- a, b
- a, b, c
- a, b, c, d