Astrocytes Contribute to Angiotensin II Stimulation of Hypothalamic Neuronal Activity and Sympathetic Outflow

Javier E. Stern, Sookjin Son, Vinicia C. Biancardi, Hong Zheng, Neeru Sharma, Kaushik P. Patel

Abstract—Angiotensin II (AngII) is a key neuropeptide that acting within the brain hypothalamic paraventricular nucleus regulates neurohumoral outflow to the circulation. Moreover, an exacerbated AngII action within the paraventricular nucleus contributes to neurohumoral activation in hypertension. Although AngII effects involve changes in paraventricular nucleus neuronal activity, the precise underlying mechanisms, cellular targets, and distribution of AngII receptors within the paraventricular nucleus remain largely unknown. Thus, whether AngII effects involve direct actions on paraventricular neurons, or whether it acts via intermediary cells, such as astrocytes, is still controversial. To address this important gap in our knowledge, we used a multidisciplinary approach combining patch-clamp electrophysiology in presympathetic paraventricular neurons and astrocytes, along with in vivo sympathetic nerve recordings and astrocyte-targeted gene manipulations. We present evidence for a novel mechanism underlying central AngII actions, which involves astrocytes as major intermediary cellular targets. We found that AngII type I receptor mRNA is expressed in paraventricular astrocytes. Moreover, we report that AngII inhibited glutamate transporter function, increasing in turn extracellular glutamate levels. This resulted in the activation of neuronal extrasynaptic NMDA (N-methyl-d-aspartate) receptors, increased presympathetic neuronal activity, enhanced sympathoexcitatory outflow, and increased blood pressure. Together, our studies support astrocytes as critical intermediary cell types mediating brain AngII regulation of the circulation and indicate that AngII-mediated neuronal and sympathoexcitatory effects are dependent on a unique neuroglial signaling modality involving nonsynaptic glutamate transmission. (Hypertension. 2016;68:1483-1493. DOI: 10.1161/HYPERTENSIONAHA.116.07747.) • Online Data Supplement

Key Words: angiotensin ■ astrocyte ■ blood pressure ■ heart failure ■ hypothalamus

Angiotensin II (AngII) is a critical neuropeptide involved in cardiovascular and fluid homeostasis. In addition to its peripheral actions, AngII acts on central nervous system type I receptors to stimulate sympathetic and neuroendocrine outputs to influence cardiovascular function.1 Importantly, AngII-mediated neurohumoral activation is now recognized as a critical mechanism in neurogenic hypertension and heart failure.2-4

Within the central nervous system circuitry involved in sympathetic regulation, the paraventricular nucleus (PVN) of the hypothalamus is a major center mediating central AngII action.5,6 AngII within the PVN stimulates sympathetic activity, whereas blockade of PVN AT1 receptors reduced sympathetic activity under different conditions, including challenges to fluid balance.6,7 Moreover, AngII actions within the PVN have been implicated in sympathovagal activation during hypertension and heart failure.8,9 Sympathetic outflow from the PVN is largely determined by the activity of presympathetic neurons that project to the rostral ventrolateral medulla (RVLM) and preganglionic sympathetic neurons in the spinal cord.10,11 Both in vivo5 and in vitro12,13 studies compellingly showed that AngII stimulates PVN neuronal activity. However, reports on underlying mechanisms have been less consistent and included activation of a postsynaptic mixed cationic conductance,12 oxidative stress,14 and changes in presynaptic input activity.13 Moreover, recent studies showing that activation of both AngII and glutamate receptors within the PVN was shown to be necessary for maintaining blood pressure during water deprivation,15 whereas blockade of glutamate receptors abrogated the antihypertensive effect of AT1 receptor blockade within the PVN,16 support interactions between AngII and glutamate in the modulation of PVN activity. These studies altogether support the notion that AngII effects within the PVN are mediated by direct actions on PVN neurons. Still, a model that mechanistically explains these seemingly disparate mechanisms involving AngII actions in the PVN is missing.

We recently showed that in addition to transient synaptic glutamate actions, PVN neurons also display a persistent...
excitatory modality mediated by activation of extrasynaptic NMDA receptors by ambient glutamate levels. Importantly, we showed that the magnitude of this tonic modality is dictated by the activity of astrocyte glutamate transporters (GLT1) that actively buffer extracellular glutamate. On the basis of recent studies supporting the expression of AngII AT1 receptors in glial cells, and a contribution of astrocytes to AngII-driven sympathetic activity during heart failure, we tested the hypothesis that astrocytes are key intermediares for AngII actions in the PVN. Using a multidisciplinary approach combining patch-clamp recordings from presymptomatic PVN neurons and astrocytes, along with whole-animal sympathetic nerve recordings and astrocyte-targeted gene manipulations, we show that AngII inhibits astrocyte GLT1 activity, enhancing extrasynaptic glutamate excitatory tone, thus indirectly stimulating neuronal activity and sympathoexcitatory outflow from the PVN, and that this effects involve astrocyte-derived oxidative stress.

**Methods**

**Animals**

All the procedures were approved by Georgia Regents University and the University of Nebraska Institutional Animal Care and Use Committee. Details about strains are provided in the online-only Data Supplement.

**Retrograde Tracing**

Presynaptic RVLM-projecting PVN neurons (PVN-RVLM) were identified by injecting rhodamine beads unilaterally into the brain stem region containing the RVLM. The location of the tracer was verified histologically. A sample of an RVLM injection is shown in Figure S1 in the online-only Data Supplement. Animals were used for 3 to 4 days after surgery.

**Patch-Clamp Electrophysiology**

Conventional, whole-cell patch-clamp recordings from PVN-RVLM neurons were obtained from acute hypothalamic slices (250 μm thick). The internal solution in patch pipettes (3–7 mol/L) contained in mmol/L: 140 potassium glutamate, 5 EGTA, 10 HEPES, 10 KCl, 0.9 MgCl₂, 0.5 CaCl₂, 4 MgATP, 0.3 NaGTP, and 20 phosphocreatine (Na⁺). Recordings were obtained with an Axopatch 700A amplifier (Axon Instruments, Foster City, CA). For current-clamp recordings, the mean firing rate was analyzed in segments of 2 minutes before, during, and after bath-applied drugs.

**Microinjections Into the PVN**

Rats were anesthetized with α-chloralose (70–140 mg/kg, IP) and urethane (0.75–1.5 g/kg, IP). The co-ordinates of the right PVN with reference to bregma were calculated as being 1.5 mm posterior, 0.4 mm lateral, and 7.8 mm ventral to the dura. A needle (0.2 mm OD) connected to a microsyringe (0.5 μL) was lowered into the PVN, and various drugs (50–100 nL) injected. Two days after injection, the rats were used for functional and immunohistological experiments. Representative examples and summary of injection locations into the PVN are shown in Figures S2 and S3.

**Renal Sympathetic Nerve Activity and Arterial Pressure Measurements**

Rats were anesthetized with α-chloralose (70–140 mg/kg, IP) and urethane (0.75–1.5 g/kg, IP). The left renal nerve was isolated, and the electric signal was recorded with the PowerLab (AD Instruments, Co) to monitor renal sympathetic nerve activity (RSNA). The changes in integration and frequency of the nerve discharge were expressed as a percentage from basal value. Responses of mean arterial pressure (MAP) and heart rate were expressed as the absolute difference between the basal value and the value after each dose of a drug.

**Western Blotting**

PVN punches were lysed in 50 μL lysis buffer (10 mmol/L Tris, 1 mmol/L EDTA, 1% SDS, and 0.1% Triton X-100 containing complete protease inhibitor cocktail from BaculoGold) for 20 minutes at 4 °C, and samples subjected to electrophoresis. Protein content of lysates was estimated using the bicinchoninic acid method with bovine serum albumin as a standard (Pierce, Rockford, IL). An enhanced chemiluminescence substrate (Pierce Chemical, Rockford, IL) was used to visualize the signals, which were detected by Worklab digital image system. Image J (NIH) was used to quantify the signal. GAPDH was used as the housekeeping gene.

**Hypothalamic Astrocyte Isolation**

Enriched astrocytes were isolated from the hypothalamus using Percoll density gradient. The resulting homogenate was centrifuged, and the cell pellet was resuspended in 70% isotonic Percoll. Cell counting and morphological analysis was performed using a handheld automatic cell counter (Scepter instrument; EMD Millipore, Billerica, MA). Standard immunofluorescence was performed to characterize the population collected. To evaluate whether AT1a receptor mRNA was expressed in the collected astrocytes, we performed real-time polymerase chain reaction (PCR). β-Actin was used as housekeeping gene. Formation of PCR product was monitored in real time using the 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). DNA bands were visualized through an ethidium bromide–treated 2% agarose gel electrophoresis. A PCR product band of 285 bp for GLT1, 193 bp for AT1a, and 61 bp for β-actin were expected.

**Statistical Analysis**

All values are expressed as mean±SEM and passed a test for normality (D’Agostino-Pearson test). Differences between groups were determined using paired or unpaired t tests and were considered significant at P<0.05. All statistical analyses were conducted using GraphPad Prism 5.00 (GraphPad Software).

**Results**

**AngII Increases PVN Presymptomatic Neuronal Firing Activity and Sympathetic Outflow in a Glutamate-Dependent Manner**

Bath-applied AngII (0.5 μmol/L) increased PVN-RVLM firing activity (≈430%; P<0.01; n=13), an effect that was largely blocked after glutamate receptor blockade (100 μmol/L AP5 [amino-5-phosphonopentanoate]+10 μmol/L DNQX [6,7-dinitroquinoxaline-2,3-dione]; P=0.01 versus AngII alone; Figure 1A through 1C). Moreover, AngII directly microinjected into the PVN (0.2 nmol) induced a significant increase in RSNA, an effect blunted by a previous microinjection of the NMDA receptor blocker AP5 (2 nmol; P<0.05; n=5; Figure 1D). As previously reported, microinjection of AngII by itself increased RSNA, arterial pressure, and heart rate (data not shown).

To further characterize the mechanism underlying the glutamate-dependent AngII effect within the PVN, we performed in vitro voltage-clamp experiments. Bath-applied AngII (0.5 μmol/L) evoked a slow-developing inward current in PVN-RVLM neurons (ΔIholding −18.9±4.8 pA; P<0.01; n=7), which also corresponded with an increase in RMS noise (Δ 0.60±0.2 pA; P<0.05; Figure 2A through 2C). The AngII-mediated inward current persisted in the presence of
the Na⁺ channel blocker tetrodotoxin (0.5 µmol/L; ΔIholding −17.6±2.4 pA; P<0.01; n=5) but was largely blocked after glutamate receptor blockade, which shifted Iholding and reduced RMS noise beyond the baseline control levels (Figure 2A through 2C; P<0.05 for Iholding and RMS; n=7). Conversely, fast synaptic inward currents (representing a mix of glutamate and GABA PSCs [gamma-aminobutyric acid postsynaptic currents]) were not affected by AngII (PSC frequency: artificial cerebrospinal fluid: 5.6±1.6 Hz; AngII: 6.7±1.8 Hz; n=7; P>0.2). In the presence of the AT1 receptor blocker losartan (10 µmol/L), AngII failed to induce a change in Iholding or RMS (P>0.5; n=7; Figure 2D and 2E).

AngII-Driven PVN Presympathetic Neuronal Activity and Sympathoexcitatory Outflow Is Prevented by Blockade of Astrocyte GLT1

The glutamate-dependent, AngII-mediated slow inward current in PVN-RVLM neurons resembled the inward current that we recently reported in supraoptic nucleus (SON) and PVN neurosecretory neurons after blockade of astrocyte GLT1, which lead to a build-up of extracellular glutamate, subsequently activating an NMDA receptor–mediated inward shift in Iholding and increased RMS noise.17,19 Similar to neurosecretory neurons, selective GLT1 blockade dihydrokainic acid (DHK, 300 µmol/L) induced in PVN-RVLM neurons an inward shift in Iholding (ΔIholding −28.4±5.7 pA; P<0.01; n=6; Figure 3A), membrane depolarization (ΔVm=27.9±3.8 mV; P<0.01), and firing activity (Δfiring=1.9±1.1 Hz; P<0.05; n=4; Figure 3B), effects that as we previously described17 were almost completely blocked by glutamate receptor blockade (data not shown).

Microinjections of DHK (100 pmol) into the PVN increased RSNA, MAP, and heart rate (n=6). These effects were completely blocked by simultaneous injections with the NMDA receptor blocker AP5 (2 nmol; P<0.01 for RSNA and MAP and P<0.05 for heart rate; Figure 3C through

Figure 1. Angiotensin II (AngII)–driven presympathetic paraventricular nucleus (PVN) neuronal activity and sympathoexcitatory output requires functional glutamate receptors. A, Bath-applied AngII (0.5 µmol/L) increases the firing activity of a presympathetic PVN neuron, an effect blocked by glutamate receptor antagonism. B, Representative segments of firing activity for each recording condition in A are shown at an expanded time scale. C, Summary data showing mean firing activity in each recording condition (n=13). D, Summary data showing mean percent changes in renal sympathetic nerve activity (RSNA) evoked by AngII (0.2 nmol) in control conditions or in the presence of the NMDA receptor blocker AP5 (amino-5-phosphonopentanoate; 2 nmol, n=5). *P<0.05
These results indicate that astrocyte GLT1 transporters tonically restrain ambient glutamate levels, limiting in turn glutamate excitatory actions on PVN-RVLM neurons, as well as glutamate-mediated sympathetic outflow from the PVN.

We found that in the presence of DHK, AngII failed to induce an inward shift in $I_{\text{holding}}$ (P > 0.1; n=6; Figure 4A and 4B) and increases in RSNA and MAP (P < 0.01 for RSNA and P < 0.05 MAP; n=9; Figure 4C through 4E). Taken together, these studies suggest that the excitatory effects of AngII on PVN-RVLM neurons and sympathetic responses were mediated indirectly via inhibition of glial GLT1 transporter activity.

**AngII Inhibits Astrocyte GLT1 Activity**

To directly test whether AngII modulated astrocyte GLT1 function, we obtained patch-clamp recordings from PVN astrocytes (n=16). Astrocytes were identified by their small rounded appearance, absence of action potentials, and a characteristic linear current–voltage relationship (range: −120 mV to +100 mV; reversal potential, −87.3±2.5 mV; Figure 5B, inset). Their identity was confirmed by intracellular staining, unveiling a typical astrocytic morphology, including thin and extended processes (Figure 5A). Astrocyte GLT1 currents ($I_{\text{GLT1}}$) were activated by puffing glutamate (5 mmol/L; 250 ms duration; 3=5 pounds per square inch) onto the patched astrocytes in the presence of ionotropic and metabotropic glutamate receptor antagonism (AP5 + DNQX).
glutamate receptor blockers. Glutamate evoked an inward current whose magnitude was partially but significantly reduced by the GLT1 blocker DHK (300 µmol/L; 40% inhibition; P<0.05; n=7; Figure 5B and 5C), as previously reported in astrocytes elsewhere. Importantly, AngII (0.5 µmol/L) significantly diminished both the peak and the area of the evoked I_{GLT} (=35% inhibition; P<0.001 and P<0.02 for peak and area; n=10; Figure 5D and 5E). A similar AngII-mediated inhibition of I_{GLT} was observed in a small subset of astrocytes recorded from the SON (n=4, data not shown).

**Figure 3.** Blockade of astrocyte glutamate transporters (GLT1) increases firing activity of presympathetic neurons and sympathoexcitatory outflow from the paraventricular nucleus (PVN). A, Bath-applied dihydrokainic acid (DHK, 300 µmol/L) induced a slowly developing inward shift in I_{holding} (left) in a presympathetic PVN neuron. The mean I_{holding} before and after DHK is shown in right (n=6). B, Bath-applied DHK (300 µmol/L) induced a slowly developing membrane depolarization and evoked firing discharge in a presympathetic PVN neuron. The mean change (Δ) in Vm and firing rate evoked by DHK is shown in right (n=4). C, Representative sample showing that DHK microinjections directly into the PVN of an anesthetized rat (100 pmol) increased heart rate (HR), arterial pressure (AP), mean arterial pressure (MAP), and renal sympathetic nerve activity (RSNA; the integrated [int] and raw traces are shown). Left, Continuous traces. Right, Segments of traces at expanded time scale. D, Representative traces showing that the DHK effect was prevented by the simultaneous microinjection of AP5 (amino-5-phosphonopentanoate, 2 nmol) into the PVN. Left, Continuous traces. Right, Segments of traces at expanded time scale. E, Summary data showing changes in mean RSNA (% of basal value), MAP (Δ mmHg), and HR (Δ beats/min) evoked by DHK or DHK+AP5 (n=6). *P<0.05; **P<0.01; and ***P<0.001.
Figure 4. Blockade of astrocyte glutamate transporters (GLT1) occludes angiotensin II (AngII)–mediated stimulation of presympathetic neuronal activity and sympathoexcitatory outflow from the paraventricular nucleus (PVN). A, Sample of dihydrokainic acid (DHK)–induced inward shift in \( I_{\text{holding}} \). Note that in the presence of DHK, AngII (0.5 µmol/L) failed to induce any further shift in \( I_{\text{holding}} \). Lower traces show expanded segments of the trace above. B, Summary data showing mean \( I_{\text{holding}} \) values in each recording condition (n=6). C, Representative sample showing that AngII microinjections directly into the PVN of an anesthetized rat (200 pmol) increased heart rate (HR), arterial pressure (AP), mean arterial pressure (MAP), and renal sympathetic nerve activity (RSNA; the integrated [int] and raw traces are shown). Left, Continuous traces. Right, Segments of traces at expanded time scale. D, Representative traces showing that the AngII effects were occluded by the simultaneous microinjection of DHK (100 pmol) into the PVN. Left, Continuous traces. Right, Segments of traces at expanded time scale. E, Summary data showing changes in mean RSNA (% of basal value), MAP (Δ mmHg), and HR (Δ beats/min) evoked by DHK or DHK+AP5 (n=6). AP5 indicates amino-5-phosphonopentanoate. *P<0.05; **P<0.01; and ***P<0.001.
AT1 Receptor mRNA Is Expressed in SON and PVN Astrocytes

We performed real-time PCR for AT1 receptor mRNA in isolated hypothalamic astrocytes (Figure 6A and 6B). The enriched astrocyte cell population had a mean cell body diameter of 6.96±0.05 µm and showed positive immunostaining for the astrocyte-specific markers GLT1 and GFAP (glial fibrillary acidic protein; Figure 6D and 6E). PCR performed on the isolated hypothalamic astrocytes showed amplification product for both GLT1 and AT1 receptor mRNAs (Figure 6C). Conversely, no amplification was detected for IBA1 mRNA (ionized calcium binding adaptor molecule; a marker for microglia cells) or for vasopressin mRNA, an abundant neuronal neuropeptide widely expressed in the hypothalamus. Similar results were observed in 6 independently performed studies.

Discussion

We present evidence for a novel mechanism underlying central AngII modulatory actions on neuronal activity and sympathetic outflow from the PVN, which involves astrocytes as major intermediary cell types. Specifically, we show that AngII inhibits astrocyte GLT1 function, leading to increased ambient glutamate levels and subsequent activation of a sustained, extrasynaptic excitatory modality in presympathetic neurons.

AngII Enhances Tonic Extrasynaptic Glutamate Function by Inhibiting the Activity of Astrocyte GLT1

A growing body of evidence supports that astrocytes regulate neuronal function and neurohumoral outflows from the SON and PVN. Astrocytes affect neuronal function partly via the release of gliotransmitters, including ATP, d-serine, and NO. Moreover, via the expression of selective neurotransmitter uptake transporters, including the GLT1, astrocytes influence the time course and concentration of neurotransmitters in the extracellular space. We recently showed that glutamate in the extracellular space in the PVN and SON evokes a persistent form of excitation that involves activation of extrasynaptic NMDA receptors, whose magnitude is determined by the activity of GLT1 expressed in astrocyte processes that...
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enwrap SON/PVN neurons. Thus, we found that either pharmacological blockade of GLT1 or activity-dependent removal of astrocyte processes from the vicinity of neurons induced a sustained NMDA-mediated inward current and increased firing activity. These studies indicate that the basal activity of astrocyte GLT1 tonically influence neuronal excitability in the PVN. However, to what extent astrocyte GLT1 and the associated glutamate extrasynaptic modality are amenable to direct modulation by critical cardiovascular-related signals remains unknown.

We report here that AngII evoked a slowly developing inward current in presympathetic neurons that was sensitive to both AT1 receptor and glutamate receptor blockade, supporting a contribution of glutamate to AngII excitatory

Figure 6. AT1a receptor mRNA expression in isolated hypothalamic astrocytes. A, Astrocytes were isolated from rat brain hypothalami using a Percoll density gradient. Note the enriched astrocyte cell layer at the 35/50 interphase, which was clearly differentiated from the enriched microglia cell layer at the 50/75 interphase. B, A distribution histogram of extracted cell diameter showing the astrocyte cell population (arrow), with a peak diameter at ≈ 7 µm is shown to the right. C, Ethidium bromide–stained gel showing real-time polymerase chain reaction (PCR) product amplification of glutamate transporters (GLT1; 285 bp); AT1a (193 bp), and β-actin (61 bp) in the extracted astrocyte cell population (n=3 rats). D1, Sample of an isolated astrocyte cell population (TOTO, blue, arrowheads) that displayed GLT1-positive staining (D2, red). D3 shows the overlap of TOTO and GLT1. The white color displays pixels containing both signals (colocalization, ImageJ plugin). In D4, only those pixels showing colocalization of TOTO and GLT1 are displayed. D5, GFAP (glial fibrillary acidic protein) staining (green) in the same isolated astrocyte population. D6 shows the overlap of TOTO and GFAP. The white color displays pixels containing both signals (colocalization, ImageJ plugin). In D7, only those pixels showing colocalization of TOTO and GFAP are displayed. E1 and E2, Images in D4 and D7 are shown at an expanded scale. Scale bars=10 µm.
actions. The interaction between AngII and glutamate could occur at different loci, including presynaptically.\textsuperscript{13} However, the fact that blockade of GLT1 abrogated AngII effects suggests that AngII inhibited active astrocyte glutamate uptake, leading to a progressive increase in ambient glutamate levels and activation of extrasynaptic glutamate receptors.\textsuperscript{17}

To more conclusively demonstrate a functional interaction between AngII and astrocyte GLT1 activity, we directly assessed GLT1 function in patched PVN astrocytes. Glutamate uptake is electrogenic and can be quantitatively assessed as an current.\textsuperscript{23} We found that the GLT1-mediated inward current (\(I_{\text{GLT}}\)) in PVN astrocytes was inhibited by AngII, blunting the glutamate-buffering capacity of PVN astrocytes. These results are thus consistent with and provide more mechanistic evidence for the AngII-evoked, glutamate-mediated increased firing activity in presympathetic neurons. The functional significance of the AngII effect on PVN astrocytes is further highlighted by our in vivo studies showing a contribution of PVN astrocytes to the AngII-evoked increase in sympathetic nerve activity and blood pressure. Our results are also consistent with a recent study showing that targeted deletion of astrocyte AT1 receptors diminished sympathoexcitation in mice with heart failure.\textsuperscript{22}

Although previous studies demonstrated the effects of AngII on PVN neuronal activity and sympathetic outflow, whether these involved direct actions on presympathetic neurons or alternatively targeted intermediary cells was not systematically assessed. Taken together, our in vitro and in vivo studies collectively support an indirect, astrocyte-dependent effect of AngII on PVN presympathetic neurons and sympathoexcitatory outflow, results that are consistent with earlier work demonstrating a lack of AT1 receptor expression in spinal cord–projecting and medulla-projecting PVN neurons.\textsuperscript{30} A caveat to take into account is that the RVLM is a cellularly heterogeneous nucleus,\textsuperscript{31} in which PVN projections likely innervate RVLM neurons other than those involved in regulating sympathetic outflow to the circulation. Thus, it is possible that our in vitro recordings included functionally diverse PVN-RVLM neuronal populations. Nevertheless, our in vivo studies do support that the basic mechanisms described in vitro play an important role in regulating cardiovascular-related sympathoexcitatory outflow.

An important question that remains to be more conclusively determined, however, is whether AngII exerted a direct effect on PVN astrocytes to inhibit GLT1 function or whether it involved other intermediary cells. Our studies showing the expression of AT1 receptor mRNA in an isolated hypothalamic astrocyte cell population would support the possibility of a direct AngII action. Moreover, the fact that the effects of AngII on the astrocyte-dependent extrasynaptic tonic glutamate current in PVN-RVLM neurons persisted in the presence of tetrodotoxin would argue against AngII, evoking an activity-dependent release of another intermediary signal from a different neuronal source. Admittedly, however, the cellular distribution of AT1 receptors in the brain is still controversial, with studies supporting\textsuperscript{20,21} or arguing against\textsuperscript{32,33} the presence of AT1 receptors in non-neuronal cells. Moreover, the specificity of commercially available antibodies raised against the AT1 receptor is questionable.\textsuperscript{34} Yet, numerous studies, including our own, have shown AT1 receptor–mediated actions on astrocytes and microglial cells.\textsuperscript{20,35} Conversely, there is more consensus supporting increased AT1 receptor expression in glial cells in pathological conditions, including inflammation, hypertension, and heart failure.\textsuperscript{22,36}

The specific source of the AngII that could activate the astrocyte-mediated stimulation of presympathetic neuronal activity within the PVN is at present unknown but could include subfornical organ-PVN projections that use AngII as a neurotransmitter,\textsuperscript{37,38} or locally produce AngII, given that all the renin–angiotensin system components have been shown to be present within the PVN.\textsuperscript{1} Moreover, we recently showed that circulating AngII could leak into the PVN because of breakdown of the blood–brain barrier integrity during hypertension.\textsuperscript{39}

An important question that remains open is the precise signaling pathway by which AngII influences astrocyte GLT1 function. Superoxide is a key mediator of central AngII actions, contributing to AngII pressor and sympathoexcitatory effects and to exacerbated sympathoactivation in hypertension and heart failure.\textsuperscript{40,41} Notably, a previous study showed that reactive oxygen species inhibited glutamate transport activity in cortical astrocytes, leading to excitotoxicity.\textsuperscript{42} Thus, future studies will be needed to determine whether AngII-induced reactive oxygen species production in PVN astrocytes contributed to GLT1 inhibition.

**Perspectives**

It has long been established that AngII is a critical neuropeptide that acting within the central nervous system participates in the central regulation of cardiovascular function and contributes to exacerbated sympathetic activation in hypertension. However, the precise cellular targets and underlying mechanisms mediating central AngII actions remain largely unknown. We provide here evidence for a novel mechanism by which AngII modulates neuronal activity and sympathetic outflow. Contrary to the prevailing notion that AngII directly targets central nervous system neurons, our study supports astrocytes and extrasynaptic glutamate signaling as key intermediaries and novel signaling mechanisms by which AngII stimulates presympathetic neuronal activity and sympathetic outflow from the PVN. We think this is novel and fundamental information that will contribute to obtain a more comprehensive understanding of pathophysiological mechanisms contributing to AngII-dependent hypertension.

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**Disclosures**

None.
References


What Is New?

- We demonstrate for the first time that effects of angiotensin II (AngII) on presympathetic neuronal activity in the paraventricular nucleus and sympathetic regulation by these neurons are largely mediated by AngII actions on local astrocytes. We also demonstrate for the first time that AngII can modulate glutamate excitatory signaling by directly modulating astrocyte glutamate transporters.

What Is Relevant?

- The present study unveils a novel cellular target and mechanism of action for AngII in the brain that involves a unique neuroglial signaling modality. This provides a mechanistic foundation for the development of novel glial-targeted therapies for the treatment of AngII-dependent hypertension.

Novelty and Significance

Summary

The present studies support astrocytes as novel cellular targets underlying brain AngII regulation of the circulation and indicate that AngII-mediated neuronal and sympathoexcitatory effects are dependent on a unique neuroglial signaling modality mediating nonsynaptic glutamate transmission.
Astrocytes Contribute to Angiotensin II Stimulation of Hypothalamic Neuronal Activity and Sympathetic Outflow

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ASTROCYTES CONTRIBUTE TO ANGIOTENSIN II STIMULATION OF HYPOTHALAMIC NEURONAL ACTIVITY AND SYMPATHETIC OUTFLOW

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Running Title: AngII inhibits astrocyte glutamate transporters

Supplemental Information

Supplemental Experimental Procedures.
Animals: For in vitro studies, male Wistar rats (180-220 g) were purchased from Harlan Laboratories (IN, USA). For in vivo studies, male Sprague-Dawley rats weighing (250 and 300 g) were obtained from SASCO Breeding Laboratories (Omaha, NE). In all cases, rats were housed in rooms with constant temperature (22-24°C) under 12-12 hour light-dark cycle and given free access to normal chow and water ad libitum. All the procedures used in this study were carried out and approved in agreement with Georgia Regents University and the University of Nebraska Institutional Animal Care and Use Committee guidelines.

Retrograde tracing: Hypothalamic, presympathetic rostral ventrolateral medulla (RVLM)-projecting paraventricular nucleus (PVN) neurons (PVN-RVLM) were identified by injecting rhodamine beads unilaterally into the brainstem region containing the RVLM as previously described. Rats were anesthetized (isoflurane (4%) and a stereotaxic apparatus was used to pressure inject 350 nL of rhodamine-labeled microspheres (Lumaflor) into the RVLM (starting from bregma, 12 mm caudal along the lamina, 2 mm medial lateral, and 8 mm ventral). In general, RVLM injection sites were centered at Bregma level -11.8 – 12.1 mm and ventrally located with respect to the nucleus ambiguous. The location of the tracer was verified histologically. A sample of an RVLM injection is shown in Fig.S1. Injections located either more rostral or lateral to the targeted area did not result in PVN labeling, and these animals were discarded from the study. Animals were used for electrophysiology 3–5 days after surgery.

Patch clamp electrophysiology: Whole-cell patch-clamp recordings from retrogradely-labeled PVN neurons were obtained as previously described. Acute hypothalamic slices (250 μm thick, cut with a
Leica vibrosilcer VT1200 were bathed (~2ml/min⁻¹) with an oxygenated (95% O₂–5% CO₂) and warmed (35 °C) artificial cerebrospinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, 20 d-glucose, 0.4 ascorbic acid, 2 CaCl₂, and 2 pyruvic acid; pH 7.4; 295 mOsm). Patch pipettes (3–7 MΩ) were pulled from thin-walled (1.5 mm od, 1.17 mm id) borosilicate glass (G150TF-3, Warner Instruments, Sarasota, FL) on a horizontal Flaming/Brown micropipette puller (P-97, Sutter Instruments, Novato, CA). The internal solution contained in mM: 140 potassium gluconate, 5 EGTA, 10 HEPES, 10 KCl, 0.9 MgCl₂, 0.5 CaCl₂, 4 MgATP, 0.3 NaGTP, and 20 phosphocreatine (Na⁺); pH was adjusted to 7.2–7.3 with KOH, and the osmolality was 285 mOsm. Recordings were obtained with an Axopatch 700A amplifier (Axon Instruments, Foster City, CA) using a combination of fluorescence illumination and infrared differential interference contrast (IR-DIC) videomicroscopy. The voltage output was digitized at 16-bit resolution, 10 kHz and was filtered at 2 kHz (Digidata 1440A, Axon Instruments). Data were discarded if the series resistance was not stable throughout the entire recording (>20% change). For current-clamp recordings, the mean firing rate was analysed in segments of 2 min before, during and after bath-applied drugs. Patched astrocytes were filled with lucyfer yellow and pseudo colored green for display. Astrocyte glutamate transporter currents were recorded in the presence of the ionotropic glutamate receptor blockers: AP5 (100 µM) and DNQX (20 µM) before and after AngII application (0.5 µM, either bath-applied or puffed into the recorded astrocyte).

Microinjections into the PVN. Rats were anesthetized with α-chloralose (70-140 mg/kg, i.p) and urethane (0.75-1.5 g/kg, i.p). Rats were placed in a stereotaxic apparatus. An incision was made on the midline of the scalp to expose bregma. The coordinates of the right PVN with reference to bregma were calculated as being, 1.5 mm posterior, 0.4 mm lateral, and 7.8 mm ventral to the dura. 30 mins after the surgery, a needle (0.2 mm OD) that was connected to a microsyringe (0.5 µl) was lowered into the PVN and various drugs (50 – 100 nl) injected as previously described 3, 4. At the completion of the experiment, monastral blue dye (2 % Chicago blue, 30 nl) was injected into the brain for histological verification. For GFAP-SOD2 plasmid transfection, a similar procedure was used. In this case, a 200 nl solution of pcDNA3-GFAPP-SOD2 plasmid (kind supplied by Dr. Giffard from Stanford University) mixed with the cationic lipid Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was microinjected into the PVN. The plasmid-lipid complexes were prepared by gently mixing plasmid with the same volume of Lipofectamine 2000 and incubating at room temperature (25 °C) for 45 min. Two days after injection, the rats were used for functional and immunohistological experiments. The transfection efficiency (SOD2 expression) was determined using Western blot. No damage to the neurons within the PVN was observed from light microscopic evaluation.

Renal sympathetic nerve activity and arterial pressure measurements. Rats were anesthetized with α-chloralose (70-140 mg/kg, i.p) and urethane (0.75-1.5 g/kg, i.p). The femoral vein was cannulated for administration of supplemental anesthesia and 0.9 % saline. The femoral artery was cannulated for recording mean arterial pressure (MAP) and heart rate (HR). The left renal nerve was isolated and the electrical signal was recorded with the PowerLab (ADInstruments, CO) to monitor RSNA as described before 3, 4. Basal nerve activity was determined at the beginning of the experiment, and background noise was determined by nerve activity recorded at the end of the experiment (after the rat was euthanized). The nerve activity during the experiment was calculated by subtracting the background noise from the recorded value. The changes in integration and frequency of the nerve discharge were expressed as a percentage from basal value. Responses of MAP and HR
were expressed as the absolute difference between the basal value and the value after each dose of a drug.

**Western blotting.** PVN punches from GFAP-SOD2 plasmid injected and sham-injected rats were lysed in 50 µl lysis buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 0.1% Triton X-100 containing complete protease inhibitor cocktail from BaculoGold) for 20 min at 4°C. Samples were homogenized on ice using a sonicator (GraLab 545) for 5-10 s and subjected to electrophoresis. Protein content of lysates was estimated using the bicinchoninic acid method with bovine serum albumin as a standard (Pierce, Rockford, IL). Samples were adjusted to normalize the concentration of total protein using 1% SDS, and then equal volumes of 2 X 4% SDS sample buffer were added. The protein (20-30 µg), mixed with SDS-PAGE buffer, was loaded and fractionated on a polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Non-fat dry milk (5% w/v) in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20) was used to block membrane at ambient temperature for 1h. Then the membrane was incubated with rabbit Anti-Mn-SOD (Millipore) primary antibody overnight at 4°C overnight, followed by the corresponding peroxidase-conjugated secondary antibody for 1h. An enhanced chemiluminescence substrate (Pierce Chemical, Rockford, IL) was used to visualize the signals, which were detected by Worklab digital image system. Image J (NIH) was used to quantify the signal. GAPDH was used as the housekeeping gene.

**Hypothalamic Astrocyte Isolation:** Enriched astrocytes were isolated from the hypothalamus using Percoll density gradient as previously described. Briefly, animals were transcardially perfused with sterile saline. The hypothalamus was macro-dissected, mechanically homogenized with Dulbecco’s phosphate-buffered saline (dPBS) supplemented with 2% glucose and passed by a 40 µm cell strainer. The resulting homogenate was briefly centrifuged and the cell pellet was re-suspended in 70% isotonic Percoll. A discontinued Percoll density gradient (50, 35 and 0%) was layered over the 70% suspension and centrifuged at 1200 x g for 55 min. The resulting 50 and 35% interphase containing astrocytes was collected, washed in dPBS and re-pellet by centrifugation at 1000 x g for 15 min. The resulting pellet was resuspended in 100 µl of dPBS. Cell counting and morphological analysis was performed using a handheld automatic cell counter (Scepter instrument, EMD Millipore, USA). Standard immunofluorescence was performed in order to characterize the population collected. Cells were fixed in PFA 4% for 30 min and immunostained with the astrocyte marker glial fibrillary acidic protein (GFAP - anti-chicken, 1:200) and the astrocyte transporter GLT1 (anti-guinea pig, 1:200) in dPBS-Tween 0.05% and 5% horse serum overnight. Hoechst counterstaining (1 µg/ml) was performed after the complete immunohistochemistry reaction. To evaluate whether AT1a receptor mRNA was expressed in the collected astrocytes, we performed real time PCR experiments. cDNA was obtained from 50,000 cells/animal using Power SYBR green cells-to-Ct kit according to the manufacture instructions (Ambion, life technologies, USA). PCR amplification of cDNA was also performed using the same kit - 4µl of cDNA was added to a PCR cocktail with gene specific primer (200nM each of forward and reverse primer) and PCR cycling conditions set up as per manufacture instructions. The sequence of primers used were: 1) GLT1 [RefSeq: NM_001302089.1]: Forward 5'-CCATCCGAGGGAGCCAATAC-3'; Reverse 5'-CAAGCAGGCGATACCCAGC-3'; and 2) AT1a [RefSeq: NM_030985.4]: Forward 5'-CCGTCACTCCACCTCAA-3'; Reverse 5'-AACCCTCTGGTCTACGGC-3'. 3) IBA1 [RefSeq: NM_012967.1]: Forward 5'-TTCTTCTATTACCCCTG-3'; Reverse 5'-GGTGTCTCTTTCTTCTTGC-3'; 4) vasopressin (VP) [RefSeq: NM_016992.2]: Forward 5'-CCCTCACCTGTGCTCTGACTT-3'; Reverse 5'-GGGGCGATGGCTCAGTAGAC-3'; 5) β-actin [Genebank: NM_031144.3]: Forward 5'-
CACAGCCTGGATGGCTACGTA-3'; Reverse 5'-ACCCAGATCATGTTTGAGACCTT-3' was used as housekeeping gene. All primers were obtained from Integrated DNA Technologies (USA). Duplicate reactions were conducted for each experimental sample. Formation of PCR product was monitored in real time using the 7500 Fast Real time PCR System (Applied Biosystems, USA) and DNA bands were visualized through an ethidium bromide- treated 2% agarose gel electrophoresis.

Statistical Analysis: All values are expressed as mean± standard error mean (SEM), and passed a test for normality (D'agostino-Pearson test). Differences between groups were determined using paired or unpaired t tests, and were considered significant at p<0.05. All statistical analyses were conducted using GraphPad Prism 5.00 (GraphPad Software).

Supplemental References


Supplemental Figures

S1- Representative sample of a microinjection of rhodamine bead into the RVLM. Representative images showing of a rhodamine bead injection site in the RVLM, at various rostrocaudal levels in the ventral medulla (distances posterior to bregma (in mm): A: ~ -11.0; B, ~ -11.8 and C, ~ -13.8). Each panel shows a superposition of the same image taken in dark field and under fluorescent illumination. The asterisk in B is within the center of the injection site. 7, facial nucleus, 12, hypoglossal nucleus, NA, nucleus ambiguus. Scale bar: 500 µm. Vertical and horizontal arrows in B point dorsally and laterally, respectively.
S2: **A-C**, Schematic representations of serial sections from the rostral (-1.4) to the caudal (-2.1) extent of the region of the PVN. The distance (in mm) posterior to bregma is shown for each section. Filled circle represents the site of termination of an injection of DHK that is considered to be within the PVN region; "+" represents an injection of DHK with AP5. **D.** Histological photo showing the injection site (arrow) in the PVN of one rat. AH, anterior hypothalamic nucleus; f, fornix; 3V, third ventricle; OX, optic tract; SO, supraoptic nucleus.
S3: A-C Schematic representations of serial sections from the rostral (-1.4) to the caudal (-2.1) extent of the region of the PVN. The distance (in mm) posterior to bregma is shown for each section. Filled circle represents the site of termination of an injection of ANGII that is considered to be within the PVN region; "+" represents an injection of ANGII with DHK. D. Histological photo showing the injection site (arrow) in the PVN of one rat. AH, anterior hypothalamic nucleus; f, fornix; 3V, third ventricle; OX, optic tract; SO, supraoptic nucleus.