

Brain Renin–Angiotensin System Does It Exist?

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Abstract—Because of the presence of the blood–brain barrier, brain renin–angiotensin system activity should depend on local (pro)renin synthesis. Indeed, an intracellular form of renin has been described in the brain, but whether it displays angiotensin (Ang) I–generating activity (AGA) is unknown. Here, we quantified brain (pro)renin, before and after buffer perfusion of the brain, in wild-type mice, renin knockout mice, deoxycorticosterone acetate salt–treated mice, and Ang II–infused mice. Brain regions were homogenized and incubated with excess angiotensinogen to detect AGA, before and after prorenin activation, using a renin inhibitor to correct for nonrenin-mediated AGA. Renin-dependent AGA was readily detectable in brain regions, the highest AGA being present in brain stem (>thalamus=cerebellum=striatum=midbrain>hippocampus=cortex). Brain AGA increased marginally after prorenin activation, suggesting that brain prorenin is low. Buffer perfusion reduced AGA in all brain areas by >60%. Plasma renin (per mL) was 40× to 800× higher than brain renin (per gram). Renin was undetectable in plasma and brain of renin knockout mice. Deoxycorticosterone acetate salt and Ang II suppressed plasma renin and brain renin in parallel, without upregulating brain prorenin. Finally, Ang I was undetectable in brains of spontaneously hypertensive rats, while their brain/plasma Ang II concentration ratio decreased by 80% after Ang II type 1 receptor blockade. In conclusion, brain renin levels (per gram) correspond with the amount of renin present in 1 to 20 μL of plasma. Brain renin disappears after buffer perfusion and varies in association with plasma renin. This indicates that brain renin represents trapped plasma renin. Brain Ang II represents Ang II taken up from blood rather than locally synthesized Ang II. (*Hypertension*. 2017;69:00-00. DOI: 10.1161/HYPERTENSIONAHA.116.08922.) • **Online Data Supplement**

Key Words: angiotensin ■ brain ■ DOCA salt ■ lisinopril ■ mouse ■ olmesartan ■ prorenin ■ renin ■ SHR

Since the discovery of renin in the brain nearly 50 years ago,¹ numerous studies have proposed that a so-called brain renin–angiotensin system (RAS) exists. Given the presence of the blood–brain barrier, brain RAS activity should depend on the local synthesis of renin or prorenin (together denoted as (pro)renin) in the brain rather than uptake from blood. In support of this concept, an intracellular, nonsecreted form of renin (icREN) has been shown to occur exclusively in the brain. This renin isoform is derived from an alternative transcript of the renin gene, lacking the signal peptide and part of

the prosegment.^{2,3} To what degree this truncated prorenin truly generates angiotensin (Ang) I remains elusive. Lee-Kirsch et al² detected low Ang I–generating activity (AGA) levels in cell lysates of AtT20 cells transfected with icREN during incubation with excess angiotensinogen, but failed to demonstrate to what degree this AGA was renin-mediated (eg, by making use of a renin inhibitor). Peters et al⁴ showed increased AGA in cardiac homogenates of transgenic rats overexpressing icREN and were able to block this with the renin inhibitor CH732. Yet, unexpectedly, the AGA increase was observed only after

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prosegment removal with trypsin, in disagreement with the fact that truncated prorenin does not require prosegment removal to display activity.⁵ Moreover, icREN overexpression in the heart, if anything, resulted in effects that were unrelated to angiotensin formation.⁶

Deoxycorticosterone acetate (DOCA) salt treatment is widely believed to stimulate brain RAS activity. Confusingly, it lowers icREN expression, but increases the expression of the classical, secreted form of renin in brain tissue (sREN),⁷ possibly because icREN, via an unknown mechanism, inhibits sREN expression.⁸ Li et al^{9,10} proposed that DOCA salt selectively increases brain prorenin, which, in the absence of a prosegment-cleaving enzyme in the brain, requires interaction with the (pro)renin receptor to allow Ang I generation locally. The underlying assumption of this concept is that prorenin binding to the (pro)renin receptor results in a conformational change in the prorenin molecule, allowing it to display enzymatic activity without prosegment cleavage.¹¹ Yet, the low (nanomolar) affinity of the (pro)renin receptor implies that high prorenin levels are required for receptor binding,¹¹ for which there currently is no evidence.¹⁰

Given these uncertainties, in the present study, we set out to re-evaluate the occurrence of (pro)renin in the brain. We quantified brain (pro)renin in a wide variety of brain regions, derived from control mice, mice exposed to DOCA salt or Ang II, and renin-deficient mice. Under all conditions, a comparison was made with plasma (pro)renin, and the renin inhibitor aliskiren was applied in the assay to evaluate whether AGA was truly renin mediated. Mice were studied given the fact that their (pro)renin levels are several orders of magnitude higher than those in humans or rats, thereby, facilitating the detection of renin-dependent AGA, even in areas with low (pro)renin levels. To obtain a more complete understanding of the brain RAS, we also quantified brain angiotensinogen, and we studied the changes in brain angiotensin generation making use of brain stem tissue obtained from control spontaneously hypertensive rats (SHR) and SHR treated with the Ang II type 1 (AT₁) receptor blocker olmesartan or the angiotensin-converting enzyme inhibitor lisinopril for 4 weeks.

Methods

Mouse and Rat Studies

Renin, prorenin, and angiotensinogen were measured in plasma and brain regions (cerebellum, brain stem, cortex, hippocampus, midbrain, striatum, and thalamus) obtained from wild-type mice, mice treated with DOCA salt or Ang II, and renin-deficient mice (Ren1c^{-/-}), either without and with transcatheterial perfusion with PBS to wash away blood from the brain vasculature. Renin expression in brain tissue was also explored by using Ingenuity Pathway Analysis, and angiotensinogen synthesis was additionally studied in rat primary cortical astrocytes. Angiotensins were measured in plasma and brain stem tissue obtained from SHR treated with vehicle, lisinopril or olmesartan. For further details, see the Methods section in the [online-only Data Supplement](#).

Statistical Analysis

Data are expressed as mean±SEM. Univariate linear associations between plasma and brain renin levels were assessed by calculation of Pearson's coefficient of correlation. Differences between groups were evaluated by Student's *t* test or analysis of variance and

corrected for multiple testing by post hoc Bonferroni analysis when needed. *P*<0.05 was considered significant.

Results

Aliskiren Inhibits AGA in the Mouse Brain

Aliskiren identically inhibited AGA in mouse plasma (n=2), mouse kidney homogenate (n=2), and mouse brain homogenate (n=3; Figure 1). Its half maximal inhibitory concentration was in the nanomolar range, as has been reported before for mouse renin.^{12,13} These data suggest that AGA in mouse brain homogenates is caused by renin. All subsequent AGA measurements were performed both in the absence and presence of 10 μmol/L aliskiren to correct for nonrenin (ie, nonaliskiren-inhibitable) AGA.

Buffer Perfusion Reduces Mouse Brain Renin by >60%

Renin-dependent (ie, aliskiren-inhibitable) AGA was readily detectable in brain regions, the highest AGA being present in brain stem (>thalamus=cerebellum=striatum=midbrain>hippocampus=cortex; Figure 2; n=5/group). AGA increased in each individual brain region after prorenin activation, but only when analyzing all brain regions together by multivariate analysis of variance did this increase reach significance (*P*<0.05). Applying the prorenin activation procedure to 3 mouse brain homogenates (cortex, midbrain, and brain stem, respectively) to which recombinant human prorenin had been added yielded values in a renin immunoradiometric assay (211±12 pg/mL) that were similar to those when activating the same amount of recombinant human prorenin in buffer with aliskiren¹⁴ (169±6 pg/mL). This confirms that our prorenin activation procedure was appropriate. PBS perfusion of the mouse brain reduced AGA in all brain areas by >60% (Figure 2; *P*<0.01) and diminished the percentage of AGA that could be blocked by aliskiren (Table S2 in the [online-only Data Supplement](#)). These data suggest that blood removal predominantly washes away renin, but not nonrenin enzymes that are also capable of reacting with angiotensinogen. Plasma

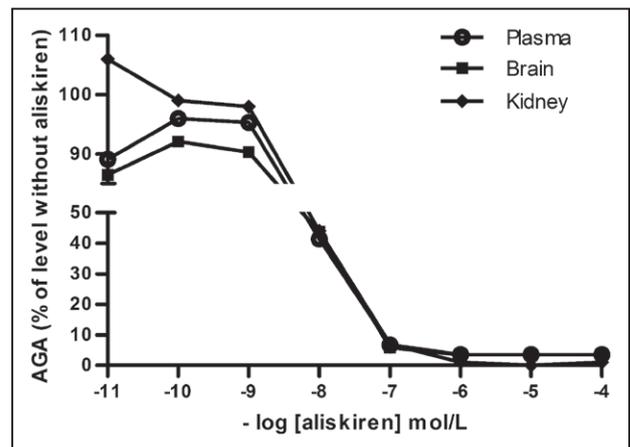


Figure 1. Concentration-dependent inhibition of angiotensin I-generating activity (AGA) by aliskiren in mouse plasma (n=2), mouse kidney homogenate (n=2), and mouse brain homogenate (n=3, representing pooled brain stem, cortex, and midbrain regions, respectively, from 3 to 4 mice each).

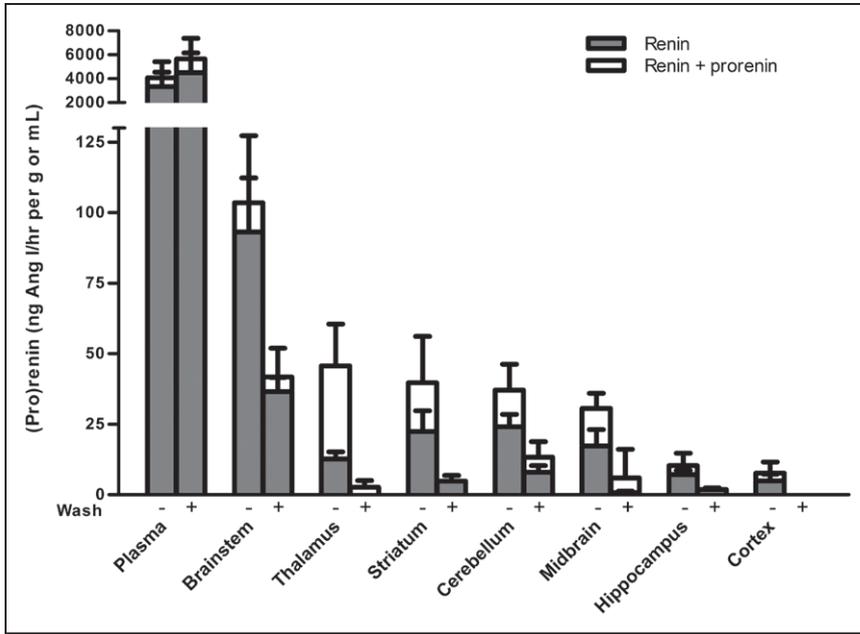


Figure 2. Renin and total renin (=renin+prorenin) levels in plasma and brain regions of mice before and after buffer perfusion (wash) of the brain. Data are mean±SEM of n=5. Multivariate analysis of variance (ANOVA) showed that total renin levels were higher than renin levels ($P<0.05$) and that buffer perfusion reduced renin by >60% in all regions ($P<0.01$).

renin (expressed per milliliter of plasma) was 40× to 800× higher than brain renin (expressed per gram of tissue) and, as expected, identical in mice that were exposed to buffer perfusion. Plasma prorenin levels were of the same order of magnitude as plasma renin levels, as demonstrated earlier in mice.¹⁵

Comparable Reductions in Brain and Plasma (Pro)Renin After DOCA Salt Treatment, Ang II Infusion, and Renin Deficiency

DOCA salt (n=6) and Ang II (n=7) suppressed plasma renin versus wild-type (n=6) mice, and parallel decreases were observed for brain stem, midbrain, and cortex renin

(Figure 3), although significance was not reached in all cases. Nevertheless, brain renin levels (expressed per gram of tissue) correlated significantly with plasma renin levels (expressed per milliliter of plasma) in all 3 brain regions (Figure 4). The different slopes may reflect the different blood content of each brain region. Plasma prorenin levels were comparable to plasma renin levels, and prorenin activation in brain regions nonsignificantly increased brain AGA. Plasma renin and prorenin were undetectable in *Ren1c*^{-/-} mice (n=4), and renin (ie, aliskiren-inhibitable AGA) was also undetectable in the 3 brain regions obtained from *Ren1c*^{-/-} mice. However, low levels of aliskiren-inhibitable

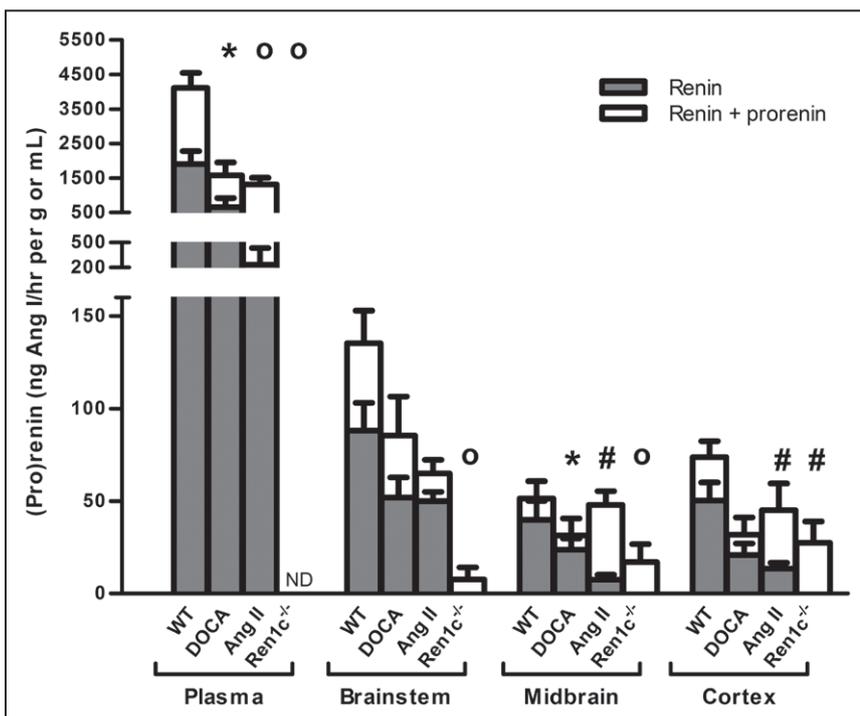


Figure 3. Renin and total renin (=renin+prorenin) levels in plasma and brain regions of untreated mice (wild-type [WT]), mice treated with deoxycorticosterone acetate (DOCA) salt, mice infused with angiotensin (Ang II), and *Ren1c*^{-/-} mice. Data are mean±SEM of n=4 to 7. Differences in renin levels were assessed by 1-way analysis of variance (ANOVA), followed by correction for multiple testing by post hoc Bonferroni analysis. * $P<0.05$, # $P<0.01$, ° $P<0.001$ vs WT.

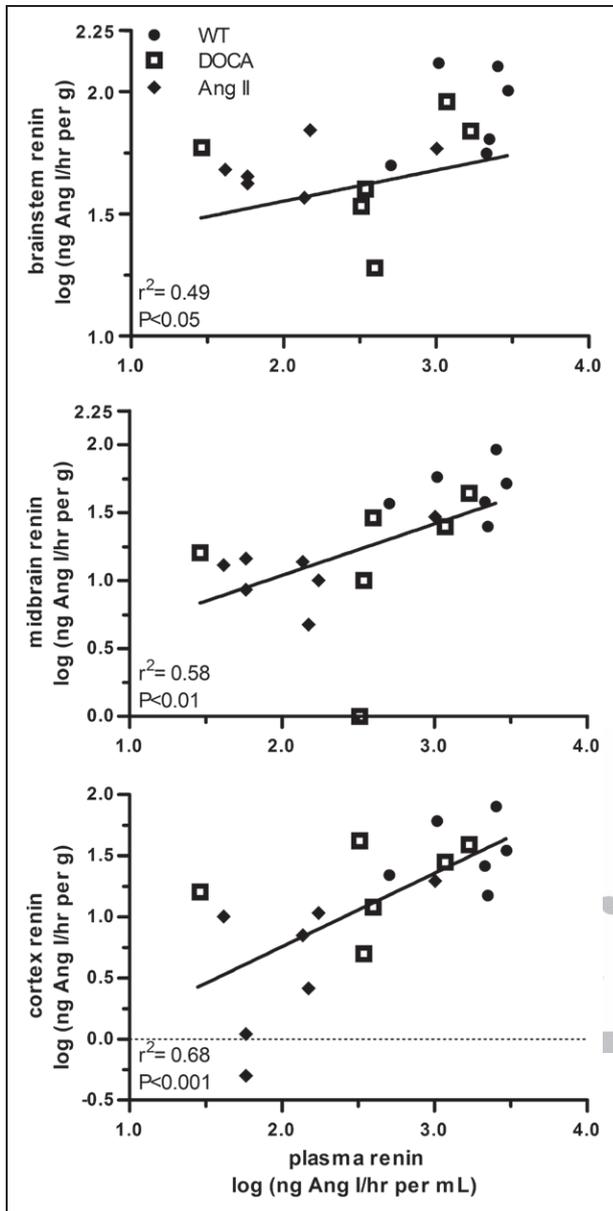


Figure 4. Relationship between renin in plasma and renin in 3 different brain regions in untreated mice (wild-type [WT], $n=6$), mice treated with deoxycorticosterone acetate (DOCA) salt ($n=6$), and mice infused with angiotensin (Ang II; $n=7$).

AGA were present in brain stem (1 out of 4), midbrain (3 out of 4), and cortex (2 out of 4) after prorenin activation in the *Ren1c^{-/-}* mice. Because this cannot represent prorenin, these data imply that our prorenin activation procedure occasionally activated a renin-like enzyme, capable of reacting with angiotensinogen, the activity of which can be blocked by 10 $\mu\text{mol/L}$ aliskiren.

Renin Expression in the Brain

Renin (secreted+intracellular), sREN, or icREN mRNA expression levels were undetectable in all brain regions in wild-type, DOCA salt-treated, and Ang II-infused mice. Primer specificity for renin (secreted+intracellular) and sREN was validated by measuring renal renin expression in *Ren1c^{-/-}* mice (Figure S1B). In the Ingenuity Pathway Analysis tissue

expression data sets, renin expression was found in cerebellum, hypothalamus, and pituitary, but only in 3 out of 9 different data sets examined (data not shown).

Despite Angiotensinogen Expression, Angiotensinogen Protein Is Undetectable in Mouse Brain and Rat Astrocytes

Mouse plasma contained detectable levels of angiotensinogen (28 ± 5 pmol/mL). Angiotensinogen mRNA expression was observed in different brain regions (Figure 5), at C_i values of 23 (cerebellum), 22 (thalamus), 25 (hippocampus), and 27 (striatum) versus ≈ 18 in the liver. Brain expression levels changed inconsistently after DOCA salt, Ang II, and renin deficiency: an increase was observed in the thalamus after DOCA salt ($P < 0.05$), while decreases occurred in the cerebellum after DOCA salt ($P < 0.05$) and in the hippocampus after Ang II ($P < 0.05$). Yet, angiotensinogen protein was undetectable (< 1 pmol/g) in mouse cortex and brain stem ($n=4$ of each). Angiotensinogen was also undetectable in the medium of cultured rat astrocytes (< 0.3 pmol/mL), cultured for 24, 48, 72, or 96 hours ($n=4$ for each condition), or the accompanying cell lysates (< 0.3 pmol/mg protein).

Angiotensins in the SHR Brain With and Without RAS Blockade

Ang I, Ang-(1-7), and Ang-(2-8) were below detection limit in brain tissue of untreated SHR ($n=6$), while Ang II could be detected in the rat brain at levels corresponding with $\approx 25\%$ of the Ang II levels in blood plasma (Table S3; Figure 6). Ang I and Ang-(2-8), but not Ang-(1-7), were detectable in plasma in untreated SHR. Brain Ang-(1-7) and Ang-(2-8) remained undetectable after olmesartan ($n=6$) or lisinopril ($n=4$), while Ang I became detectable in the rat brain after both types of RAS blockade ($P < 0.001$ for both). Because plasma Ang I increased ≈ 20 -fold after olmesartan and lisinopril ($P < 0.001$ for both), it could be calculated that during both types of RAS blockade, brain Ang I levels corresponded with $\approx 1\%$ of the Ang I levels in plasma. Olmesartan increased brain Ang II ≈ 5 -fold ($P < 0.001$) and plasma Ang II ≈ 25 -fold ($P < 0.001$), so that after AT₁ receptor blockade, the brain/plasma ratio of Ang II decreased by $\approx 80\%$ ($P < 0.05$). Lisinopril decreased plasma Ang II by $> 90\%$ ($P < 0.001$) and diminished brain Ang II to undetectable levels ($P < 0.001$). Lisinopril also decreased plasma Ang-(2-8) to undetectable levels and greatly increased plasma Ang-(1-7), while olmesartan increased both plasma Ang-(2-8) and plasma Ang-(1-7). Taken together, given that brain Ang I levels correspond with $\approx 1\%$ of the circulating Ang I levels, 10 μL plasma per gram brain tissue is sufficient to explain the entire brain Ang I content. Brain Ang II levels, relative to plasma Ang II levels, are higher, suggesting either local synthesis or an active uptake mechanism. The massive decrease in the Ang II brain/plasma ratio after olmesartan supports the latter.

Discussion

The present study confirms that renin-dependent AGA can be detected in virtually every region of the mouse brain. Yet, as compared with plasma, brain renin levels were

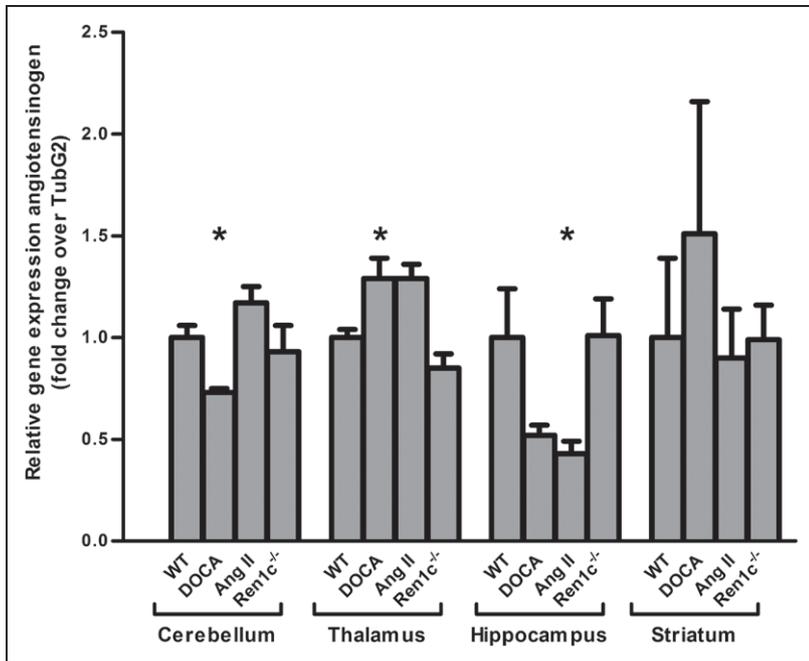


Figure 5. Angiotensinogen mRNA expression in different brain regions in untreated mice (wild-type [WT]), mice treated with deoxycorticosterone acetate (DOCA) salt, mice infused with angiotensin (Ang II), and Ren1c^{-/-} mice. Data, presented as fold change over TubG2 relative to WT levels, are mean±SEM of n=3 to 6. Differences were assessed by 1-way analysis of variance (ANOVA), followed by correction for multiple testing by post hoc Bonferroni analysis. *P<0.05 vs WT.

low, corresponding with the amount of renin in 1 to 25 μL blood plasma per gram brain tissue (≈0.1%–2.5% [v/v]). This volume mimics the amount of blood plasma in various brain regions determined with tritiated inulin or Evans blue dye.^{16,17} Moreover, perfusing the brain with PBS prior to the collection of the various regions reduced brain renin uniformly by >60%. Had local renin synthesis occurred in one or more specific brain regions, the washout percentage should have been much lower in these regions, similar to the fact that in the kidney, one cannot wash away stored renin,^{18,19} while this does happen in nonrenin producing organs like the heart.²⁰ Furthermore, DOCA salt, like Ang II, reduced circulating renin and, contrary to our expectations, did not increase brain prorenin. In fact, if anything, both DOCA salt and Ang II lowered brain renin in parallel with plasma renin. Aliskiren-inhibitable AGA was entirely absent in the brain of Ren1c^{-/-} mice, supporting the validity of our brain renin measurement. Taken together, our data do not support the presence of kidney-independent (pro)renin synthesis in the brain, nor the concept that this occurs particularly in the DOCA salt model. In fact, brain renin levels

are so low that the accumulation of renin at brain tissue sites outside the blood compartment seems unlikely. This greatly differs from other organs (eg, the heart), where renin diffuses freely into the interstitium or binds to a receptor, thereby reaching tissue levels that are, on a gram basis, at least as high as the renin levels in blood plasma (on an mL basis).^{20–22} Clearly, the presence of the blood–brain barrier prevents such distribution.

Prorenin activation resulted in modest AGA increases in all brain regions, and significance for this increase was only obtained by analyzing all regions together. Applying recombinant human prorenin to brain homogenates prior to the prorenin activation procedure (on the basis of acid activation²²) confirmed that this procedure resulted in complete prorenin activation. However, small rises in aliskiren-inhibitable AGA were also observed in brain homogenates from Ren1c^{-/-} mice after their exposure to acid. Because Ren1c^{-/-} mouse brain tissue cannot contain prorenin, this implies that the brain contains a nonrenin proenzyme, which is activated by acid exposure and which is capable of cleaving Ang I from angiotensinogen in an aliskiren-inhibitable manner. A

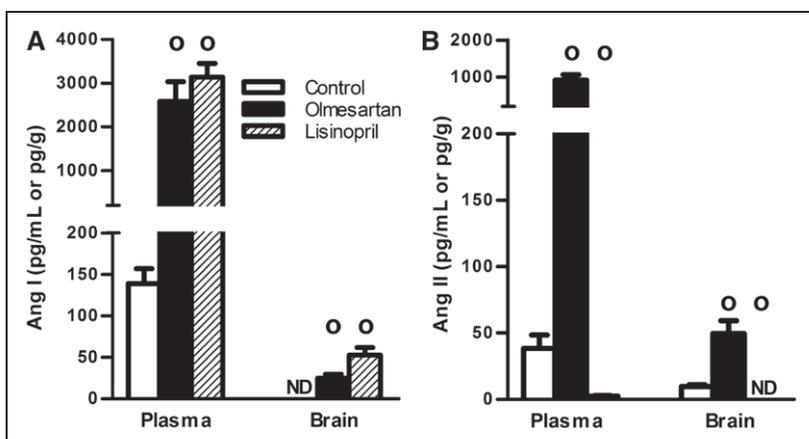


Figure 6. Angiotensin (Ang) I and II levels in plasma and brain of SHR treated with vehicle (control), olmesartan, or lisinopril. Data are mean±SEM of n=4 to 6. Differences were assessed by 1-way analysis of variance (ANOVA), followed by correction for multiple testing by post hoc Bonferroni analysis. °P<0.001 vs control. ND indicates not detectable.

possible candidate is procathepsin D. Indeed, renin inhibitors, at high micromolar concentrations, do inhibit cathepsin D.²³ Our difficulty to demonstrate prorenin in the brain is reminiscent of earlier studies in organs not synthesizing prorenin themselves, like the heart.²² Obviously, blood plasma contains prorenin, and thus, some prorenin should be detected in the blood-containing homogenates derived from such tissues. Yet only under conditions where circulating prorenin levels were greatly elevated, like in heart failure in humans, did we reliably detect prorenin in cardiac tissue.²⁴ In mice, in contrast to humans, circulating prorenin levels are relatively low (versus renin), making it even more difficult to show a rise in AGA on top of already low renin-mediated AGA. A further complicating factor is that tissue homogenization per se may result in (partial) prorenin activation. In summary, given the presence of prorenin in blood plasma, brain homogenates should minimally contain the amount of prorenin present in a few microliter of blood. The rises in AGA after prorenin activation are consistent with this view, but should still be interpreted with caution given the fact that nonrenin enzymes also came into play after acid activation. Brain-selective prorenin rises, for example, after DOCA salt, were not observed. This implies that prorenin-(pro)renin receptor interaction is unlikely to occur in the mouse brain, particularly after DOCA salt (which lowers brain (pro)renin), although it may obviously occur after intracerebroventricular infusion of pharmacological prorenin doses into the brain.¹⁰

Most, if not all, studies on brain renin relied on the detection of renin mRNA in the brain, either under normal conditions or after deleting/overexpressing sREN or icREN. Deleting sREN in neurons or glia did not affect blood pressure, heart rate, water intake, or metabolic rate,²⁵ while preservation of icREN did not compensate for the consequences of whole-body sREN deficiency (hypotension, renal defects, and lethality).²⁶ Surprisingly, brain-selective deletion of icREN even caused neurogenic hypertension, possibly because icREN inhibits sREN.⁸ These data seem to argue against icREN as an Ang I-generating enzyme. Yet, overexpressing either human icREN or sREN in astrocytes, if combined with human angiotensinogen, resulted in Ang II-dependent hypertension and an increase in drinking volume.²⁷ Because icREN under the latter conditions was not detectable in cerebrospinal fluid, it was concluded that this phenomenon involved intracellular Ang II formation. We attempted to detect renin mRNA, using either specific assays for sREN or icREN or a nonspecific assay that detects both sREN and icREN. Under no condition were we able to show renin (secreted+intracellular), sREN, or icREN gene expression in any of the different regions of the brain: the expression level was below the detection threshold of the reverse transcriptase polymerase chain reaction assay, even with the use of the highly sensitive Taqman probes. The specificity of our renin primers was validated by making use of the kidneys of Ren1c^{-/-} mice. Of course, poor renin expression in the brain has been noted before.²⁸⁻³⁰ Because of the technical limitations inherent in any reverse transcriptase polymerase chain reaction assay, we could not load >100 ng of total RNA. Our results, therefore, indicate that if renin is expressed in the

brain, its expression is >2¹⁸-fold lower than that in the kidney (no signal after 40 cycles, with renin detection in the kidney at C_t=22). The Ingenuity Pathway Analysis expression data sets confirm this view. Yet, Kubo et al³¹ observed a blood pressure drop after intraventricular renin antisense injection in SHR. In their hands only 1 of 3 tested antisense oligonucleotides acted hypotensive, and this response was accompanied by a 20% drop in renin mRNA (detected after 45 cycles of reverse transcriptase polymerase chain reaction). These authors did not measure renin levels in brain or plasma and were unable to rule out antisense leakage to the kidney. Therefore, these data cannot be taken as definitive proof for the existence of an independent brain RAS.

The mouse RAS differs from the human RAS, in that the circulating renin levels in mice are ≤1000-fold higher (on a nanogram Ang I/mL hour basis) than in humans. As a consequence, circulating angiotensinogen levels in mice are far below K_m range, as confirmed in the present study. Nevertheless, despite these differences, mouse angiotensin levels in blood and tissue are comparable to those in humans, rats, and pigs.³²⁻³⁵ We attempted to measure angiotensinogen in the mouse brain, both at the mRNA and protein level. Although we did observe angiotensinogen mRNA expression in different regions of the brain, in full agreement with previous work,^{36,37} expression was ≤500-fold lower than in the liver. Under no condition were we able to detect angiotensinogen protein in the brain. Given the detection limit of our assay (1 pmol/g), this implies that brain angiotensinogen, if present, occurs at levels (per gram of tissue) that are <3% of the levels in plasma (per milliliter of plasma). Such low levels have been reported before in the rat brain, as well as in human and rat cerebrospinal fluid,^{16,38-40} and thus, our data entirely agree with the literature. Clearly, mice, given their low angiotensinogen levels, are not the optimal species to study brain angiotensinogen. As astrocytes are assumed to be the source of brain angiotensinogen,^{41,42} we additionally studied angiotensinogen synthesis by rat primary cortical astrocytes, but again failed to detect any angiotensinogen. Nevertheless, data from Schink et al.⁴⁰ do support the functional presence of angiotensinogen in the rat brain. These authors artificially elevated renin in the brain by either intracerebroventricular renin infusion (in Sprague-Dawley rats) or by making use of transgenic hypertensive rats overexpressing mouse Ren2. The responses to both approaches (drinking and blood pressure reduction, respectively) were greatly diminished after lowering brain angiotensinogen by brain-selective expression of an antisense RNA against angiotensinogen mRNA.

Finally, given our observation that brain renin is confined to the plasma compartment, while brain angiotensinogen is extremely low (if not also confined to the plasma compartment), an urging question is what degree local angiotensin generation truly occurs in the brain. We, therefore, collected brain stem tissue (ie, the brain region with the highest renin level) from SHR under control conditions and during RAS blockade with olmesartan or lisinopril. Rats rather than mice were used here because at identical angiotensin levels in both species, the larger rat brain stem would allow a more reliable quantification of angiotensins. Without treatment,

brain Ang I was undetectable, while Ang II occurred at levels that were $\approx 25\%$ of the levels in plasma (per gram tissue weight). This contrasts with other organs where Ang II is usually much higher than in plasma, while Ang I is easily detectable.^{43–46} RAS blockade induced the usual rise in Ang I levels in plasma, and now brain Ang I became detectable, however, at only 1% (v/v) of its plasma levels. It seems reasonable to assume that also in the untreated animals, brain Ang I levels were in the 1% range of plasma Ang I and, therefore, too low to be detected with our assays. If so, this implies that under all conditions, brain Ang I at most represented the amount of Ang I that is inherently present in brain tissue because it contains a small amount ($\approx 1\%$) of blood.¹⁶ Lisinopril decreased brain Ang II to undetectable levels, while olmesartan reduced the brain/plasma Ang II ratio by $>80\%$. The latter finding suggests that, normally, circulating Ang II accumulates in brain tissue via binding to AT₁ receptors. Such uptake occurs in multiple organs⁴⁷ and facilitates the intracellular accumulation of Ang II.⁴⁸ Without receptors (ie, in AT receptor-deficient mice), tissue Ang II levels drop dramatically,⁴⁹ suggesting that tissue Ang II levels do not originate intracellularly. If Ang II binding to AT₁ receptors is the only source of Ang II in the brain, one would expect angiotensin metabolites that do not (or only with low affinity) bind to this receptor to be undetectable in the brain. This is indeed what we observed for both Ang-(2–8) and Ang-(1–7). An olmesartan-induced reduction in brain Ang II levels was also observed in Dahl-sensitive hypertensive rats, albeit in the absence of an effect on blood pressure.⁵⁰ Clearly, therefore, the changes observed in brain Ang II are blood pressure independent.

Perspectives

The absence of renin-dependent AGA in the brain outside the blood compartment implies that angiotensin generation in the brain, if occurring, does not involve renin. Brain prorenin levels, if anything, were even lower than brain renin levels and, therefore, like renin, at most represented the amount of prorenin expected in brain tissue based on its blood content. Selective brain prorenin upregulation, for example, after DOCA salt, could not be observed, arguing against the concept that DOCA salt-induced neurogenic hypertension involves prorenin–(pro)renin receptor interaction. Finally, the absence of Ang I in brain tissue outside the blood compartment (which contrasts sharply with the presence of Ang I in every other organ of the body) strongly suggests that there is no local Ang I generation in the brain. Apparently, therefore, nonrenin enzymes do not compensate for the absence of renin, assuming at least that brain angiotensinogen levels are of sufficient magnitude to allow independent Ang I generation at all. Only *ex vivo*, after prohormone-activating procedures, did we occasionally obtain evidence for a modest contribution of such nonrenin enzymes, but the *in vivo* relevance of these findings is questionable. Brain Ang II, therefore, seems to originate in the blood compartment. Of course, circulating Ang II will bind to brain AT receptors that are outside the blood–brain barrier (eg, in the circumventricular organ). Yet, it may also gain access to brain areas behind this barrier, for example, under conditions where blood–brain

barrier permeability is compromised, like in (DOCA salt) hypertension.^{51,52} In fact, Ang II itself may be responsible for disturbing the blood–brain barrier, thus, facilitating its own access to critical brain areas like the hypothalamus and brain stem. From this perspective, the brain RAS in reality represents circulating Ang II that accumulates in brain nuclei, possibly after it has (partially) broken down the blood–brain barrier. This Ang II subsequently activates sympatho-neurohumoral outflow, for instance, by upregulating reactive oxygen species.^{52,53} This is entirely different from other organs like heart, kidney, and vascular wall, where renin and angiotensinogen diffuse freely into the interstitial space, allowing local production of Ang I to occur.^{20,54,55} Blocking AT₁ receptors or enhancing Ang II degradation (eg, by angiotensin-converting enzyme 2) will prevent the effects of circulating Ang II in the brain, thereby explaining the success of intracerebroventricular application of losartan or brain-selective angiotensin-converting enzyme 2 overexpression in DOCA salt hypertension.⁵⁶

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Disclosures

None.

References

- Ganten D, Minnich JL, Granger P, Hayduk K, Brecht HM, Barbeau A, Boucher R, Genest J. Angiotensin-forming enzyme in brain tissue. *Science*. 1971;173:64–65.
- Lee-Kirsch MA, Gaudet F, Cardoso MC, Lindpaintner K. Distinct renin isoforms generated by tissue-specific transcription initiation and alternative splicing. *Circ Res*. 1999;84:240–246.
- Mercure C, Thibault G, Lussier-Cacan S, Davignon J, Schiffrin EL, Reudelhuber TL. Molecular analysis of human prorenin prosegment variants *in vitro* and *in vivo*. *J Biol Chem*. 1995;270:16355–16359.
- Peters J. Secretory and cytosolic (pro)renin in kidney, heart, and adrenal gland. *J Mol Med (Berl)*. 2008;86:711–714. doi: 10.1007/s00109-008-0328-0.
- Grobe JL, Xu D, Sigmund CD. An intracellular renin-angiotensin system in neurons: fact, hypothesis, or fantasy. *Physiology (Bethesda)*. 2008;23:187–193. doi: 10.1152/physiol.00002.2008.
- Wanka H, Staar D, Lutze P, Peters B, Hildebrandt J, Beck T, Bäumgen I, Albers A, Krieg T, Zimmermann K, Sczodrok J, Schäfer S, Hoffmann S, Peters J. Anti-necrotic and cardioprotective effects of a cytosolic renin isoform under ischemia-related conditions. *J Mol Med (Berl)*. 2016;94:61–69. doi: 10.1007/s00109-015-1321-z.
- Grobe JL, Rahmouni K, Liu X, Sigmund CD. Metabolic rate regulation by the renin-angiotensin system: brain vs. body. *Pflugers Arch*. 2013;465:167–175. doi: 10.1007/s00424-012-1096-9.
- Shinohara K, Liu X, Morgan DA, Davis DR, Sequeira-Lopez ML, Cassell MD, Grobe JL, Rahmouni K, Sigmund CD. Selective deletion of the brain-specific isoform of renin causes neurogenic hypertension. *Hypertension*. 2016;68:1385–1392. doi: 10.1161/HYPERTENSIONAHA.116.08242.
- Li W, Sullivan MN, Zhang S, Worker CJ, Xiong Z, Speth RC, Feng Y. Intracerebroventricular infusion of the (pro)renin receptor antagonist PRO20 attenuates deoxycorticosterone acetate-salt-induced hypertension. *Hypertension*. 2015;65:352–361. doi: 10.1161/HYPERTENSIONAHA.114.04458.
- Li W, Peng H, Mehaffey EP, Kimball CD, Grobe JL, van Gool JM, Sullivan MN, Earley S, Danser AH, Ichihara A, Feng Y. Neuron-specific (pro)renin receptor knockout prevents the development of salt-sensitive hypertension. *Hypertension*. 2014;63:316–323. doi: 10.1161/HYPERTENSIONAHA.113.02041.

11. Batenburg WW, Lu X, Leijten F, Maschke U, Müller DN, Danser AH. Renin- and prorenin-induced effects in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor: does (pro)renin-(pro)renin receptor interaction actually occur? *Hypertension*. 2011;58:1111–1119. doi: 10.1161/HYPERTENSIONAHA.111.180737.
12. Wood JM, Maibaum J, Rahuel J, et al. Structure-based design of aliskiren, a novel orally effective renin inhibitor. *Biochem Biophys Res Commun*. 2003;308:698–705.
13. Lu H, Rateri DL, Feldman DL, Charnigo RJ Jr, Fukamizu A, Ishida J, Oesterling EG, Cassis LA, Daugherty A. Renin inhibition reduces hypercholesterolemia-induced atherosclerosis in mice. *J Clin Invest*. 2008;118:984–993. doi: 10.1172/JCI32970.
14. Batenburg WW, de Bruin RJ, van Gool JM, Müller DN, Bader M, Nguyen G, Danser AH. Aliskiren-binding increases the half life of renin and prorenin in rat aortic vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2008;28:1151–1157. doi: 10.1161/ATVBAHA.108.164210.
15. Mercure C, Prescott G, Lacombe MJ, Silversides DW, Reudelhuber TL. Chronic increases in circulating prorenin are not associated with renal or cardiac pathologies. *Hypertension*. 2009;53:1062–1069. doi: 10.1161/HYPERTENSIONAHA.108.115444.
16. Gregory TJ, Wallis CJ, Printz MP. Regional changes in rat brain angiotensinogen following bilateral nephrectomy. *Hypertension*. 1982;4:827–838.
17. Migliarini S, Pacini G, Pelosi B, Lunardi G, Pasqualetti M. Lack of brain serotonin affects postnatal development and serotonergic neuronal circuitry formation. *Mol Psychiatry*. 2013;18:1106–1118. doi: 10.1038/mp.2012.128.
18. Fraune C, Lange S, Krebs C, et al. AT1 antagonism and renin inhibition in mice: pivotal role of targeting angiotensin II in chronic kidney disease. *Am J Physiol Renal Physiol*. 2012;303:F1037–F1048. doi: 10.1152/ajprenal.00672.2011.
19. Lange S, Fraune C, Alenina N, Bader M, Danser AH, Frenay AR, van Goor H, Stahl R, Nguyen G, Schwedhelm E, Wenzel UO. Aliskiren accumulation in the kidney: no major role for binding to renin or prorenin. *J Hypertens*. 2013;31:713–719. doi: 10.1097/HJH.0b013e32835e226b.
20. de Lannoy LM, Danser AH, van Kats JP, Schoemaker RG, Saxena PR, Schalekamp MA. Renin-angiotensin system components in the interstitial fluid of the isolated perfused rat heart. Local production of angiotensin I. *Hypertension*. 1997;29:1240–1251.
21. Heller LJ, Opsahl JA, Wernsing SE, Saxena R, Katz SA. Myocardial and plasma renin-angiotensinogen dynamics during pressure-induced cardiac hypertrophy. *Am J Physiol*. 1998;274(3 pt 2):R849–R856.
22. Danser AH, van Kats JP, Admiraal PJ, Derkx FH, Lamers JM, Verdouw PD, Saxena PR, Schalekamp MA. Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension*. 1994;24:37–48.
23. Deinum J, Derkx FH, Danser AH, Schalekamp MA. Identification and quantification of renin and prorenin in the bovine eye. *Endocrinology*. 1990;126:1673–1682. doi: 10.1210/endo-126-3-1673.
24. Danser AH, van Kesteren CA, Bax WA, Tavenier M, Derkx FH, Saxena PR, Schalekamp MA. Prorenin, renin, angiotensinogen, and angiotensin-converting enzyme in normal and failing human hearts. Evidence for renin binding. *Circulation*. 1997;96:220–226.
25. Xu D, Borges GR, Davis DR, Agassandian K, Sequeira Lopez ML, Gomez RA, Cassell MD, Grobe JL, Sigmund CD. Neuron- or glial-specific ablation of secreted renin does not affect renal renin, baseline arterial pressure, or metabolism. *Physiol Genomics*. 2011;43:286–294. doi: 10.1152/physiolgenomics.00208.2010.
26. Xu D, Borges GR, Grobe JL, Pelham CJ, Yang B, Sigmund CD. Preservation of intracellular renin expression is insufficient to compensate for genetic loss of secreted renin. *Hypertension*. 2009;54:1240–1247. doi: 10.1161/HYPERTENSIONAHA.109.138677.
27. Lavoie JL, Liu X, Bianco RA, Beltz TG, Johnson AK, Sigmund CD. Evidence supporting a functional role for intracellular renin in the brain. *Hypertension*. 2006;47:461–466. doi: 10.1161/01.HYP.0000203308.52919.dc.
28. Bader M, Ganten D. It's renin in the brain: transgenic animals elucidate the brain renin angiotensin system. *Circ Res*. 2002;90:8–10.
29. Lippoldt A, Fuxe K, Luft FC. A view of renin in the brain. *J Mol Med (Berl)*. 2001;79:71–73.
30. Saavedra JM. Brain angiotensin II: new developments, unanswered questions and therapeutic opportunities. *Cell Mol Neurobiol*. 2005;25:485–512. doi: 10.1007/s10571-005-4011-5.
31. Kubo T, Ikezawa A, Kambe T, Hagiwara Y, Fukumori R. Renin antisense injected intraventricularly decreases blood pressure in spontaneously hypertensive rats. *Brain Res Bull*. 2001;56:23–28.
32. Roksnoer LC, van Veghel R, de Vries R, Garrelds IM, Bhaggoe UM, Friesema EC, Leijten FP, Poglitsch M, Domenig O, Clahsen-van Groningen MC, Hoorn EJ, Danser AHJ, Batenburg WW. Optimum AT1 receptor-nephrilysin inhibition has superior cardioprotective effects compared with AT1 receptor blockade alone in hypertensive rats. *Kidney Int*. 2015;88:109–120. doi: 10.1038/ki.2015.107.
33. Campbell DJ, Duncan AM, Kladis A. Angiotensin-converting enzyme inhibition modifies angiotensin but not kinin peptide levels in human atrial tissue. *Hypertension*. 1999;34:171–175.
34. van Kats JP, Danser AH, van Meegen JR, Sassen LM, Verdouw PD, Schalekamp MA. Angiotensin production by the heart: a quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation*. 1998;98:73–81.
35. Klotz S, Burkhoff D, Garrelds IM, Boomsma F, Danser AH. The impact of left ventricular assist device-induced left ventricular unloading on the myocardial renin-angiotensin-aldosterone system: therapeutic consequences? *Eur Heart J*. 2009;30:805–812. doi: 10.1093/eurheartj/ehp012.
36. Davissou RL, Yang G, Beltz TG, Cassell MD, Johnson AK, Sigmund CD. The brain renin-angiotensin system contributes to the hypertension in mice containing both the human renin and human angiotensinogen transgenes. *Circ Res*. 1998;83:1047–1058.
37. Thomas WG, Sernia C. Immunocytochemical localization of angiotensinogen in the rat brain. *Neuroscience*. 1988;25:319–341.
38. Ito T, Eggena P, Barrett JD, Katz D, Metter J, Sambhi MP. Studies on angiotensinogen of plasma and cerebrospinal fluid in normal and hypertensive human subjects. *Hypertension*. 1980;2:432–436.
39. Schelling P, Müller S, Clauser E. Regulation of angiotensinogen in cerebrospinal fluid and plasma of rats. *Am J Physiol*. 1983;244:R466–R471.
40. Schinke M, Baltatu O, Böhm M, Peters J, Rascher W, Bricca G, Lippoldt A, Ganten D, Bader M. Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen. *Proc Natl Acad Sci U S A*. 1999;96:3975–3980.
41. Intebi AD, Flaxman MS, Ganong WF, Deschepper CF. Angiotensinogen production by rat astroglial cells *in vitro* and *in vivo*. *Neuroscience*. 1990;34:545–554.
42. Milsted A, Barna BP, Ransohoff RM, Brosnihan KB, Ferrario CM. Astrocyte cultures derived from human brain tissue express angiotensinogen mRNA. *Proc Natl Acad Sci U S A*. 1990;87:5720–5723.
43. van Kats JP, Chai W, Duncker DJ, Schalekamp MA, Danser AH. Adrenal angiotensin: origin and site of generation. *Am J Hypertens*. 2005;18:1104–1110. doi: 10.1016/j.amjhyper.2005.02.005.
44. van Kats JP, Schalekamp MA, Verdouw PD, Duncker DJ, Danser AH. Intrarenal angiotensin II: interstitial and cellular levels and site of production. *Kidney Int*. 2001;60:2311–2317. doi: 10.1046/j.1523-1755.2001.00049.x.
45. Campbell DJ, Kladis A, Duncan AM. Nephrectomy, converting enzyme inhibition, and angiotensin peptides. *Hypertension*. 1993;22:513–522.
46. Campbell DJ, Kladis A, Duncan AM. Effects of converting enzyme inhibitors on angiotensin and bradykinin peptides. *Hypertension*. 1994;23:439–449.
47. van Kats JP, de Lannoy LM, Danser AHJ, van Meegen JR, Verdouw PD, Schalekamp MA. Angiotensin II type 1 (AT1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half-life *in vivo*. *Hypertension*. 1997;30(1 pt 1):42–49.
48. van Kats JP, van Meegen JR, Verdouw PD, Duncker DJ, Schalekamp MA, Danser AH. Subcellular localization of angiotensin II in kidney and adrenal. *J Hypertens*. 2001;19(3 pt 2):583–589.
49. van Esch JH, Gemhardt F, Sterner-Kock A, Heringer-Walther S, Le TH, Lassner D, Stijnen T, Coffman TM, Schultheiss HP, Danser AH, Walther T. Cardiac phenotype and angiotensin II levels in AT1a, AT1b, and AT2 receptor single, double, and triple knockouts. *Cardiovasc Res*. 2010;86:401–409. doi: 10.1093/cvr/cvq004.
50. Pelisch N, Hosomi N, Ueno M, Nakano D, Hitomi H, Mogi M, Shimada K, Kobori H, Horiuchi M, Sakamoto H, Matsumoto M, Kohno M, Nishiyama A. Blockade of AT1 receptors protects the blood-brain barrier and improves cognition in Dahl salt-sensitive hypertensive rats. *Am J Hypertens*. 2011;24:362–368. doi: 10.1038/ajh.2010.241.
51. Biancardi VC, Son SJ, Ahmadi S, Filosa JA, Stern JE. Circulating angiotensin II gains access to the hypothalamus and brain stem during hypertension via breakdown of the blood-brain barrier. *Hypertension*. 2014;63:572–579. doi: 10.1161/HYPERTENSIONAHA.113.01743.
52. Biancardi VC, Stern JE. Compromised blood-brain barrier permeability: novel mechanism by which circulating angiotensin II signals to sympatho-excitatory centres during hypertension. *J Physiol*. 2016;594:1591–1600. doi: 10.1113/JP271584.

53. Wang HW, Huang BS, White RA, Chen A, Ahmad M, Leenen FH. Mineralocorticoid and angiotensin II type 1 receptors in the subformal organ mediate angiotensin II - induced hypothalamic reactive oxygen species and hypertension. *Neuroscience*. 2016;329:112–121. doi: 10.1016/j.neuroscience.2016.04.050.
54. de Lannoy LM, Danser AH, Bouhuizen AM, Saxena PR, Schalekamp MA. Localization and production of angiotensin II in the isolated perfused rat heart. *Hypertension*. 1998;31:1111–1117.
55. Schalekamp MA, Danser AH. Angiotensin II production and distribution in the kidney: I. A kinetic model. *Kidney Int*. 2006;69:1543–1552. doi: 10.1038/sj.ki.5000303.
56. Xia H, de Queiroz TM, Sriramula S, Feng Y, Johnson T, Mungrue IN, Lazartigues E. Brain ACE2 overexpression reduces DOCA-salt hypertension independently of endoplasmic reticulum stress. *Am J Physiol Regul Integr Comp Physiol*. 2015;308:R370–R378. doi: 10.1152/ajpregu.00366.2014.

Novelty and Significance

What Is New?

- Brain renin levels parallel plasma renin levels in a variety of hypertension models and in fact are as high as can be expected on the basis of the presence of blood in brain tissue.
- Because the latter was also true for brain prorenin, this implies that neither renin nor prorenin contributes to angiotensin I production in the brain.

What Is Relevant?

- Brain angiotensin II originates in the circulation, and the brain renin–angiotensin system activation that has been claimed to occur under pathological conditions (hypertension, deoxycorticosterone acetate salt) most likely represents a compromised blood–brain barrier, allowing circulating angiotensin II access to brain regions behind the blood–brain barrier.

Summary

Quantifying brain (pro)renin in wild-type mice, renin-deficient mice, deoxycorticosterone acetate salt–treated mice, and angiotensin II–infused mice revealed that changes in brain renin paralleled those in plasma renin, that brain renin disappeared after buffer perfusion of the brain, and that brain renin and prorenin levels were as high as expected based on the presence of (pro)renin in blood in brain tissue. Angiotensin I was undetectable in the brain, while angiotensin II type 1 receptor blockade reduced the brain/plasma angiotensin II concentration ratio by 80%. In conclusion, (pro)renin-mediated angiotensin I production in the brain is unlikely, and brain angiotensin II, therefore, represents angiotensin II sequestered from blood via angiotensin II type 1 receptor binding.



Hypertension

Brain Renin–Angiotensin System: Does It Exist?

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The brain renin-angiotensin system: does it exist?

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Short title: Brain renin-angiotensin system revisited

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SUPPLEMENTAL INFORMATION

METHODS

All animal experiments were performed under the regulation and permission of the Animal Care Committee, conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8523, revised 1985).

Brain renin, prorenin and angiotensinogen levels in wild-type mice, mice treated with DOCA-salt or angiotensin II, and renin-deficient mice

Male and female C57BL/6J mice (wild-type, WT) were obtained by in-house breeding or purchased from Charles River (Sulzfeld, Germany). Mice (age 3-4 months) were either untreated, treated with deoxycorticosterone acetate (DOCA)-salt (150 mg, 60-day release pellet [Innovative Research of America, Sarasota, USA]) for 4 weeks, or infused with Ang II (490 ng/kg/min by osmotic minipump [Alzet, model 2004, DURECT, Cupertino, USA]¹) for 2 weeks. Ren1c homozygous null mice (Ren1c^{-/-}; 3 females and 1 male) were generated as described before (C57BL/6J background)² and sacrificed at the age of 3-6 months. All mice were housed under standard laboratory conditions (temperature 23±1°C, 12-hour light-dark cycle) and maintained on standard chow (Special Diets Services, Essex, UK) with ad libitum access to tap water. DOCA-salt-treated mice had ad libitum access to 0.15 mol/L (0.9%) NaCl solution. At the end of the treatment period, mice were sacrificed with an overdose of isoflurane and blood was collected by cardiac puncture into EDTA-coated tubes. Blood was centrifuged at 4600 rpm for 10 minutes and plasma was stored at -80°C until analysis. Five untreated mice were perfused transcardially with PBS to wash away blood from the brain vasculature. Brains were rapidly removed from all mice and the desired regions (cerebellum, brainstem, cortex, hippocampus, midbrain, striatum and thalamus) were dissected, frozen in liquid nitrogen, and stored at -80°C until analysis.

Brain and plasma angiotensin levels in spontaneously hypertensive rats

Male 10-week old SHR (Janvier Labs, Le Genest St. Isle, France) were treated with either vehicle (tap water), the ACE inhibitor lisinopril (15 mg/kg body weight; Sigma-Aldrich, Darmstadt, Germany) or the AT₁ receptor blocker olmesartan (10 mg/kg body weight, Daiichi Sankyo Co., Ltd., Japan) once daily per gavage for four weeks. Animals were housed under standard laboratory conditions (temperature 23±1°C, 12-hour light-dark cycle), they were fed a standard pellet diet (1.8% NaCl; Velaz, Prague, Czech Republic), and drank tap water ad libitum. At the end of treatment period, the animals were sacrificed in terminal isoflurane anesthesia (2-3%) by being bled out. Blood was collected in the presence of an inhibitor cocktail containing ethylenediaminetetraacetic acid (EDTA), pepstatin A, p-hydroxymercuribenzoic acid, phenanthroline and specific inhibitors for renin and aminopeptidases to a final concentration of 5% v/v (Attoquant Diagnostics, Vienna, Austria) and immediately cooled on ice.³ Plasma was isolated by centrifugation at 4°C and frozen at -80°C until analysis. Brains were sampled via skull trepanation followed by removal of the hemispheres and cerebellum. The brainstem was isolated, rapidly frozen in liquid nitrogen and preserved at -80°C until analysis.

Angiotensinogen synthesis by rat primary cortical astrocytes

Rat primary cortical astrocytes (Invitrogen, Thermo Fisher, Waltham, USA) were grown in 6-well plates (Corning Incorporated, Corning, USA) in DMEM medium (85% Dulbecco's Modified Eagle Medium containing high glucose 4.5 g/L, 15% FCS (Gibco, Thermo Fisher) and L-glutamine (600 mg/L; Flow Lab, UK)) in an incubator at 37°C and 5% CO₂ until confluency. Medium and cells were collected after 24, 48, 72 or 96 hours. For the determination of angiotensinogen, the culture medium was removed and stored at -80°C, while cells were gently washed with PBS, lysed with RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton

x-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA), and frozen at -80°C until analysis.

Measurement of renin, prorenin and angiotensinogen

Mouse plasma renin and prorenin were measured as described before.⁴ Brain tissue was homogenized in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, and the homogenates were used to measure renin, total renin (i.e., renin plus prorenin), and angiotensinogen. AGA was measured by enzyme-kinetic assay in the presence of excess sheep angiotensinogen, both without and with the renin inhibitor aliskiren (10 pmol/L-10 µmol/L).⁵ Total renin was measured identically after conversion of prorenin to renin by acidification.⁵ Angiotensinogen was measured in mouse plasma, mouse brain homogenate, rat astrocyte cell culture medium and astrocyte cell lysate (after its centrifugation at 8000 x g for 10 min) as the maximum quantity of Ang I that was generated during incubation with excess recombinant rat renin.⁶

LC-MS/MS based quantification of angiotensin metabolites

Plasma was thawed on ice, and samples were spiked with 200 pg of stable isotope-labeled internal standards for Ang I, Ang II, Ang-(1-7) and Ang-(2-8). Following C18-based solid-phase-extraction, samples were subjected to LC-MS/MS analysis using a reversed-phase analytical column (Acquity UPLC® C18, Waters, Milford, USA) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters) in MRM mode. Internal standards were used to correct for peptide recovery of the sample preparation procedure for each angiotensin metabolite in each individual sample. Angiotensin peptide concentrations were calculated considering the corresponding response factors determined in matrix calibration curves, on condition that integrated signals exceeded a signal-to-noise ratio of 10. Brain tissue samples were grinded under liquid nitrogen (pestle and mortar) and the resulting frozen tissue powder was rapidly dissolved in ice cold 6 mol/L guanidine hydrochloride supplemented with 1 % (v/v) TFA at a concentration of 100 mg tissue/mL.⁷ Resulting homogenates were spiked with 200 pg of stable isotope-labelled internal standard for each angiotensin metabolite analyzed and subjected to solid phase based peptide extraction and subsequent LC-MS/MS analysis. The lower limits of quantification for Ang I, Ang II, Ang-(1-7) and Ang-(2-8) in plasma were 2.1, 0.9, 1.9 and 1.1 pg/mL, and in brain 8.1, 6.7, 12.7 and 8.0 pg/g tissue, respectively.

Renin and angiotensinogen expression

Total RNA was isolated from kidney and liver tissue, using Tri Reagent (Sigma-Aldrich, Darmstadt, Germany), and from brain tissue (cerebellum, brainstem, cortex, hippocampus, midbrain, striatum and thalamus), using the RNeasy Lipid kit (Qiagen, Venlo, The Netherlands). RNA concentration was quantified using micro-spectrophotometry (NanoDrop Technologies, Wilmington, USA). Single stranded complementary DNA was synthesized using the genomic DNA-free total RNA using the Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. Quantitative real-time PCRs were conducted in 10 µL and 100 ng of cDNA, using the CFX384 Touch™ Real-time PCR detection system (BioRad, Hercules, USA), followed by measurement using either IQ™ SYBR® Green Supermix (BioRad) or Taqman probes (IDT, Coralville, USA). The exon-exon junction spanning oligonucleotide primers for qPCR were designed with NCBI (Primer-BLAST). Primer sequences and GenBank accession numbers for the sequences used to design the primers are listed in Table S1. Regarding mouse renin, primers were designed to selectively detect sREN and icREN, as well as to detect renin independently of its signal peptide and prosegment ('secreted + intracellular renin'; Figure S1A). The following cycling conditions were used [95°C for 3 min, (95°C for 3 sec, 60°C for 25 sec) × 40 cycles] for the KAPA SYBR® FAST qPCR Master Mix, [95°C for 5 min, (95°C for 10 sec, 59-72°C for 40 sec) × 40 cycles] for the IQ™ SYBR® Green Supermix, and [95°C

for 3 min, (95°C for 15 sec, 60°C for 1 min) × 45 cycles] for the Taqman assay. Expression levels in kidney, liver and brain tissue were normalised to the housekeeping genes B2M and TubG2, respectively. The $2^{-\Delta\Delta C_t}$ method was used for relative quantification of gene expression.

Renin expression in brain tissue was also explored by using Ingenuity Pathway Analysis (IPA). In IPA, tissue expression datasets were created based on expression annotations from the GNF Body Atlas (expression calls were made from microarray data from dissection of healthy, adult, untreated C57/BL6 mouse tissues). Data was also available in Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1133>). Published⁸ cases where a gene was marked "Present" in a particular tissue (based upon the Affymetrix MAS5 Absence/Presence call) were used as evidence of mRNA expression, and were incorporated into the tissue expression dataset for that particular tissue. The criteria used to define a gene as expressed in the body atlas data in IPA correspond to an average concentration of 3 transcripts per cell (10 transcripts per million). Additional mRNA expression calls were derived from findings in the Ingenuity Knowledge Base that describes observations of mRNA expression in normal, healthy, adult mammalian tissue (human, mouse, rat and mammalian orthologs). Only findings where high quality mRNA detection methods were used (e.g. Northern Blots, quantitative RT-PCR, etc.) were included. Additional information is provided at the following link: <http://ingenuity.force.com/ipa/IPATutorials?id=kA250000000TN5CCA>.

Statistical analysis

Data are expressed as mean±SEM. To determine the minimum number of animals needed for this study, we reasoned that, if renin is synthesized locally in certain brain areas, the majority of this renin (>50%) should not be washed away by buffer perfusion. Moreover, Grobe et al.⁹ have suggested that brain sREN expression doubles after DOCA-salt. On the basis of these 2-fold changes, at an SD of 40% (as observed in brainstem, see Results), with $\alpha = 0.05$ and $\beta = 80\%$, the minimum n-number is 3. Univariate linear associations between plasma and brain renin levels were assessed by calculation of Pearson's coefficient of correlation. Differences between groups were evaluated by Student's t-test or ANOVA, and corrected for multiple testing by post-hoc Bonferroni analysis when needed. $P < 0.05$ was considered significant. Statistical analysis was performed with IBM SPSS Statistics version 21.0 (IBM, Armonk, New York, USA).

REFERENCES

1. Batenburg WW, van den Heuvel M, van Esch JHM, van Veghel R, Garrelds IM, Leijten F, Danser AHJ. The (pro)renin receptor blocker handle region peptide upregulates endothelium-derived contractile factors in aliskiren-treated diabetic transgenic (mREN2)27 rats. *J Hypertens*. 2013;31:292-302.
2. Takahashi N, Lopez ML, Cowhig JE, Jr., Taylor MA, Hatada T, Riggs E, Lee G, Gomez RA, Kim HS, Smithies O. Ren1c homozygous null mice are hypotensive and polyuric, but heterozygotes are indistinguishable from wild-type. *J Am Soc Nephrol*. 2005;16:125-132.
3. Roksnoer LCW, van Veghel R, de Vries R, Garrelds IM, Bhaggoe UM, Friesema ECH, Leijten FPJ, Poglitsch M, Domenig O, Clahsen-van Groningen MC, Hoorn EJ, Danser AHJ, Batenburg WW. Optimum AT1 receptor-neprilysin inhibition has superior cardioprotective effects compared with AT1 receptor receptor blockade alone in hypertensive rats. *Kidney International*. 2015;88:109-120.
4. Fraune C, Lange S, Krebs C, et al. AT1 antagonism and renin inhibition in mice: pivotal role of targeting angiotensin II in chronic kidney disease. *Am J Physiol Renal Physiol*. 2012;303:F1037-F1048.

5. Danser AHJ, van Kesteren CAM, Bax WA, Tavenier M, Derkx FHM, Saxena PR, Schalekamp MADH. Prorenin, renin, angiotensinogen, and angiotensin-converting enzyme in normal and failing human hearts. Evidence for renin binding. *Circulation*. 1997;96:220-226.
6. Batenburg WW, Lu X, Leijten F, Maschke U, Müller DN, Danser AHJ. Renin- and prorenin-induced effects in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor: does (pro)renin-(pro)renin receptor interaction actually occur? *Hypertension*. 2011;58:1111-1119.
7. Campbell DJ, Kladis A, Duncan AM. Nephrectomy, converting enzyme inhibition, and angiotensin peptides. *Hypertension*. 1993;22:513-522.
8. Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci U S A*. 2004;101:6062-6067.
9. Grobe JL, Rahmouni K, Liu X, Sigmund CD. Metabolic rate regulation by the renin-angiotensin system: brain vs. body. *Pflugers Arch*. 2013;465:167-175.
10. Grobe JL, Xu D, Sigmund CD. An intracellular renin-angiotensin system in neurons: fact, hypothesis, or fantasy. *Physiology (Bethesda)*. 2008;23:187-193.

Table S1. Primer sequences and GenBank accession numbers.

Kidney and brain tissue (Mus musculus)		
<i>SYBR green assay</i>		
Beta-2 microglobulin (B2M)	CTCACACTGAATTCACCCCA GTCTCGATCCCAGTAGACGGT	>NM_009735.3
Tubulin gamma-2 chain (TubG2)	CAGACCAACCACTGCTACAT AGGGAATGAAGTTGGCCAGT	>NM_134028.2
Renin (secreted + intracellular)	AGCTACATGGAGAACGGGTC TTCCACCCACAGTCACCGAG	>NM_031192.3
Secreted renin (sRen)	GCACCTTCAGTCTCCCAACAC TCCCGGACAGAAGGCATTTTC	>NM_031192.3
Intracellular renin (icRen)	CCGGCTGCTTTGAAGATTTGAT ATGCCAATCTCGCCGTAGTA	-
Angiotensinogen (Agt)	ACCCCGAGTGGGAGAGGTTC GCCAGGCTGCTGGACAGACG	>NM_007428.3
<i>Taqman assay</i>		
Beta-2 microglobulin (B2M)	Assay ID-Mm.PT.58.10497647 (IDT) NM_009735(1)	
Tubulin gamma-2 chain (TubG2)	Assay ID-Mm.PT.58.41559687 (IDT) NM_134028(1)	
Renin (secreted + intracellular)	FW: TCAGCAAGACTGACTCCTGGC Rev: GCACAGCCTTCTTCACATAGC Probe: TCACGATGAAGGGGGTGTCTGTGGG	
Secreted renin (sRen)	FW: GCACCTTCAGTCTCCCAACAC Rev: TCCCGGACAGAAGGCATTTTC Probe: CCTTTGAACGAATCCC	
Intracellular renin (icRen)	FW: CCGGCTGCTTTGAAGATTTGAT Rev: CAGGTAGTTGGTGAGGACCAC Probe: TCACAAAGAGGCCTTCCTTGACCA	

Table S2. Percent inhibition of angiotensin I-generating activity by aliskiren in brain nucleus homogenates before and after buffer perfusion. Data are mean±SEM of n=5.

Brain nucleus	Before Buffer Perfusion		After Buffer Perfusion	
	Renin	Total Renin	Renin	Total renin
Brainstem	96±1	91±3	92±3	87±3
Thalamus	81±6	81±9	18±20	28±19
Cerebellum	86±4	92±2	73±12	54±18
Striatum	71±2	60±18	51±16	34±20
Midbrain	83±10	84±6	37±19	30±19
Hippocampus	49±6	48±22	47±15	13±13
Cortex	50±23	36±17	15±17	0±0

Table S3. Angiotensin (Ang) metabolites in SHR plasma and brain during treatment with placebo, olmesartan or lisinopril. Data are mean±SEM of n=4-6, and if undetectable, were based on the detection limit.

Treatment	Ang I (pg/mL or g)	Ang II (pg/mL or g)	Ang-(2-8) (pg/mL or g)	Ang-(1-7) (pg/mL or g)
Plasma				
Placebo	139±20	39±11	8±2	<2±0
Olmesartan	2579±497	911±171	223±57	9±2
Lisinopril	3142±362	2±1	<1±0	58±14
Brain				
Placebo	<8±1	9±2	<9±0	<13±1
Olmesartan	25±5	50±11	<8±0	<13±1
Lisinopril	53±10	<7±1	<8±0	<13±1

Figure S1A. Proposed pathway for secreted renin and intracellular renin. It is hypothesized that in the brain, a different mRNA, renin-b, is transcribed that result in a novel transcript lacking exon 1a. This renin isoform lacks the signal peptide and part of the prosegment, and is believed to remain intracellular. Adapted from Grobe et al.¹⁰

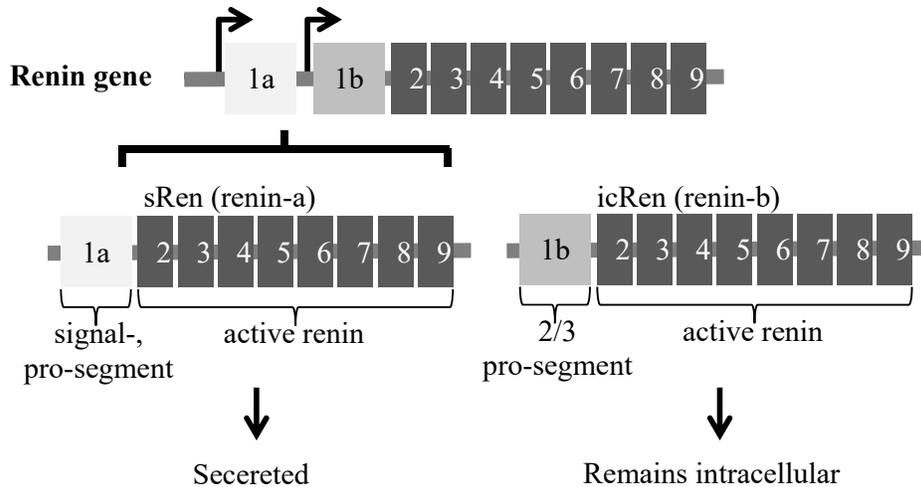


Figure S1B. Renin (secreted or intracellular), sRen and icRen mRNA expression in kidneys of wild-type (WT, n=6) and *Ren1c^{-/-}* mice (n=4). icRen was undetectable under all conditions, while renin (secreted + intracellular) and sRen were undetectable in kidneys of *Ren1c^{-/-}* mice. Ct values were generated with the SYBR Green assay.

