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Erasmus MC
Universitair Medisch Centrum Rotterdam

Brain Renin-Angiotensin System: Does It Exist?

Bibi S. van Thiel[1,2,3], Alexandre Góes Martini[1], Luuk te Riet[1,2], David Severs[1,4], Estrellita Uijl[1,4], Ingrid M. Garrelds[1], Frank P.J. Leijten[1], Ingrid van der Pluijm[2,3], Jeroen Essers[2,3,5], Fatimunnisa Qadri[6], Natalia Alenina[6,7], Michael Bader[6-10], Ludovit Paulis[11,12], Romana Rajkovicova[11], Oliver Domenig[13,14], Marko Poglitsch[13] and A.H. Jan Danser[1]

[1]Division of Vascular Medicine and Pharmacology, Department of Internal Medicine; [2]Department of Vascular Surgery; [3]Department of Molecular Genetics; Cancer Genomics Center Netherlands [4]Division of Nephrology and Transplantation, Department of Internal Medicine; [5]Department of Radiation Oncology, Erasmus MC, Rotterdam, The Netherlands; [6]Max Delbrück Center, Berlin, Germany; [7]DZHK (German Center for Cardiovascular Research), partner site Berlin, Germany; [8]Berlin Institute of Health (BIH), Berlin, Germany; [9]Charité - University Medicine, Berlin, Germany; [10]Institute for Biology, University of Lübeck, Lübeck, Germany; [11]Institute of Pathophysiology, Faculty of Medicine, Comenius University; [12]Institute of Normal and Pathophysiological Physiology, Slovak Academy of Sciences, Bratislava, Slovak Republic;
[13]Attoquant Diagnostics and [14]Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria

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Erasmus University Rotterdam

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 40x to 800x 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 I type 1 receptor blockade. In conclusion, brain renin levels (per gram) correspond with the amount of renin present in 1 to 20 μL 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.

INTRODUCTION

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 al[2] 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 al[4] 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 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 (AT_1) receptor blocker olmesartan or the angiotensin-converting enzyme inhibitor lisinopril for 4 weeks.

MATERIAL AND 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 deoxycorticosterone acetate (DOCA)-salt or angiotensin II, and renin-deficient mice ($Ren1c^{-/-}$), either without and with transcardial 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: Fig.1). The half maximal inhibitory concentration (IC50) 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.

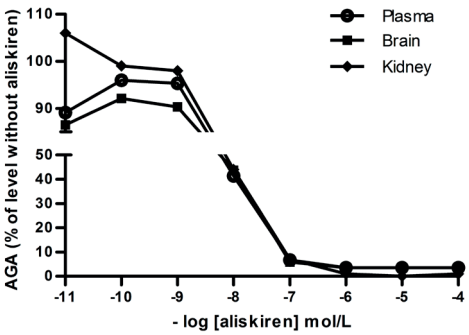

Figure 1. Concentration-dependent inhibition of angiotensin I-generating activity (AGA) by aliskiren in mouse plasma (n=2), mouse kidney homogenate (n=2) and mouse brain homogenate (n=3, representing pooled brain stem, cortex, and midbrain regions, respectively, from 3 to 4 mice each).

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=midb rain >hippocampus=cortex: Fig.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 (IRMA; 211±12 pg/mL) that were similar to those when activating the same amount of recombinant human prorenin in buffer with aliskiren[14] (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% (Fig. 2; P<0.01) and diminished the percentage of AGA that could be blocked by aliskiren (Table S2). These data suggest that blood removal predominantly washes away renin, but not

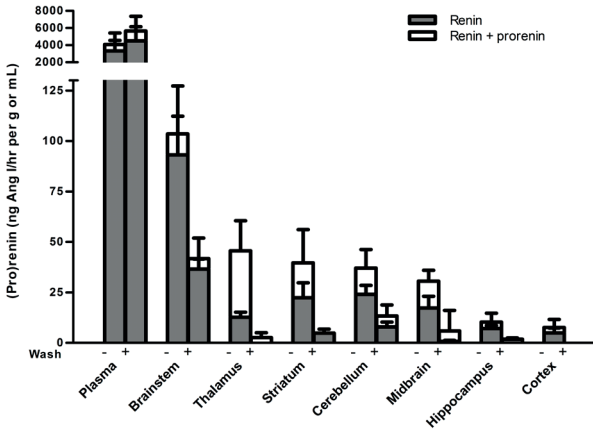

Figure 2. Renin and total renin (=renin+prorenin) levels in plasma and brain regions of mice before and after buffer perfusion (wash) of the brain. Data are mean±SEM of n=5. Multivariate analysis of variance (ANOVA) showed that total renin levels were higher than renin levels (P<0.05) and that buffer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

nonrenin enzymes that are also capable of reacting with angiotensinogen. Plasma renin (expressed per milliliter of plasma) was 40x to 800x 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 (Fig. 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 (Fig. 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 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 μmol/L aliskiren.

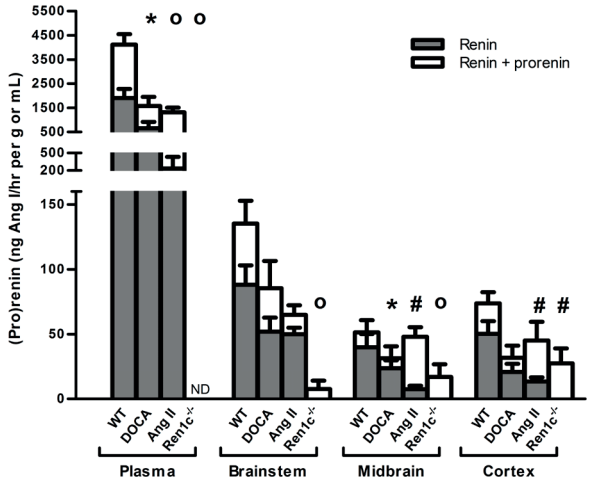

Figure 3. Renin and total renin (=renin+prorenin) levels in plasma and brain regions of untreated mice (wild-type [WT]), mice treated with deoxycorticosterone acetate (DOCA) salt, mice infused with Ang II, and Ren1c$^{-/-}$ mice. Data are mean±SEM of n=4 to 7. Differences in renin levels were assessed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

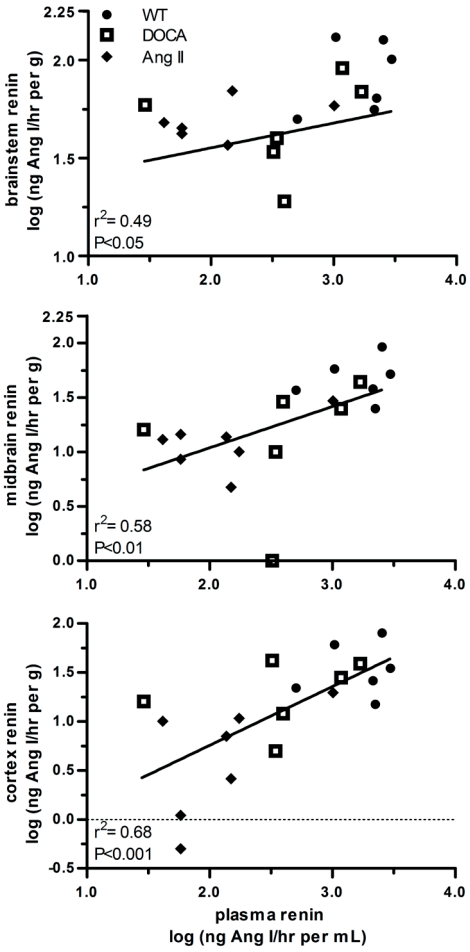

Figure 4. Relationship between renin in plasma and renin in 3 different brain regions in untreated mice (wild-type [WT], n=6), mice treated with deoxycorticosterone acetate (DOCA) salt (n=6), and mice infused with angiotensin (Ang) II (n=7).

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 (Fig. 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 datasets 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 (Fig. 5), at C_t 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).

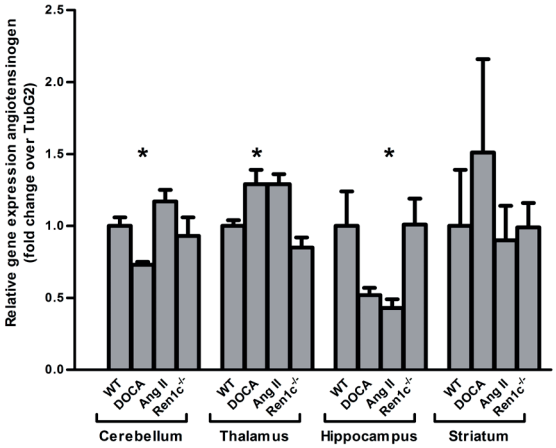

Figure 5. Angiotensinogen mRNA expression in different brain regions in untreated mice (wild-type [WT]), mice treated with deoxycorticosterone acetate (DOCA) salt, mice infused with angiotensin (Ang) II, and Ren1c$^{-/-}$ mice. Data, presented as fold change over TubG2 relative to WT levels, are mean±SEM of n=3-6. 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.

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 ≈25% of the Ang II levels in blood plasma (Table S3; Fig. 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 ≈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 AT_1 receptor blockade, the brain/plasma ratio of Ang II decreased by ≈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 ≈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.

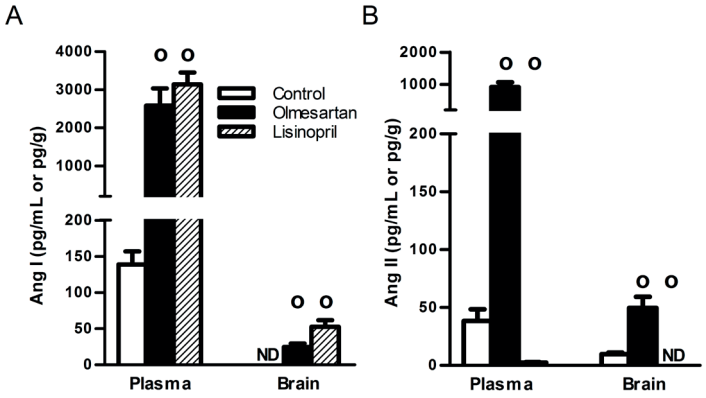

Figure 6. Angiotensin (Ang) I and II levels in plasma and brain of SHR treated with vehicle (control), olmesartan, or lisinopril. Data are mean±SEM of n=4 to 6. Differences were assessed by 1-way analysis of variance (ANOVA), followed by correction for multiple testing by post hoc Bonferroni analysis.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

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 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 and/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 activation[22]) 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 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 >2^{18}-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 al[31] 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 ≈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 (≈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 AT_1 receptors. Such uptake occurs in multiple organs[47] 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 AT_1 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 AT_1 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.

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.

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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 -80ºC 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 AT_1 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 scull 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% CO_2 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 deoxylcholate, 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 homog-

enate, 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). Published[8] 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/IPA Tutorials?id=kA250000000TN5CCAW.

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).

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Table S1. Primer sequences and GenBank accession numbers.

Kidney and brain tissue (Mus musculus)		
SYBR green assay		
Beta-2 microglobulin (B2M)	CTCACACTGAATTCACCCCCA 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)	ACCCCCGAGTGGGAGAGGTTC GCCAGGCTGCTGGACAGACG	>NM_007428.3
Taqman assay		
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.

	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

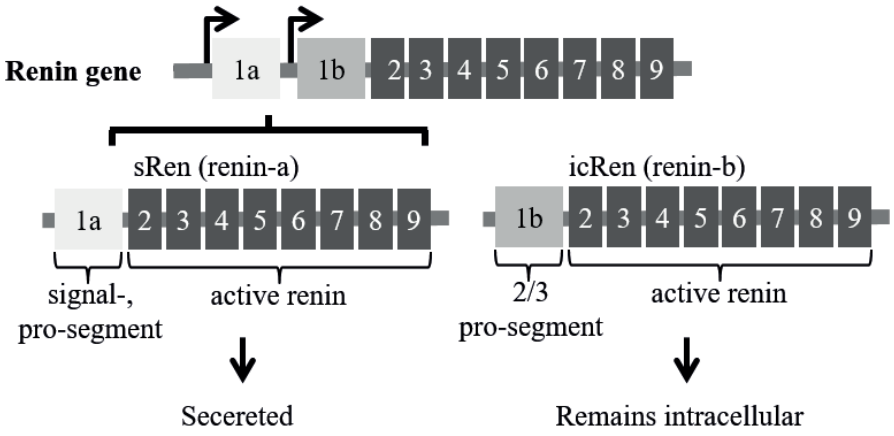

Figure S1A. Proposed pathway for secreted renin and intracellular renin. It is hypothesized that in the brain, a different mRNA, renin-b, is transcribed that result in a novel transcript lacking exon 1a. This renin isoform lacks the signal peptide and part of the prosegment, and is believed to remain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

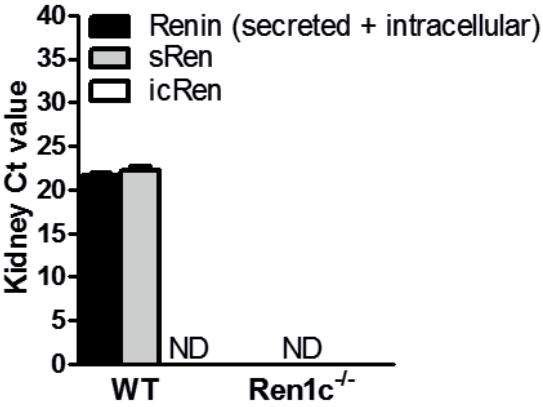

Figure S1B. Renin (secreted or intracellular), sRen and icRen mRNA expression in kidneys of wild-type (WT, n=6) and Ren1c$^{-/-}$ mice (n=4). icRen was undetectable under all conditions, while renin (secreted + intracellular) and sRen were undetectable in kidneys of Ren1c$^{-/-}$ mice. C_t values were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