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Nedd4-2 up-regulation is associated with ACE2 ubiquitination in hypertension

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Aims

Angiotensin-converting enzyme 2 (ACE2) is a critical component of the compensatory renin–angiotensin system that is down-regulated during the development of hypertension, possibly via ubiquitination. However, little is known about the mechanisms involved in ACE2 ubiquitination in neurogenic hypertension. This study aimed at identifying ACE2 ubiquitination partners, establishing causal relationships and clinical relevance, and testing a gene therapy strategy to mitigate ACE2 ubiquitination in neurogenic hypertension.

Methods and results

Bioinformatics and proteomics were combined to identify E3 ubiquitin ligases associated with ACE2 ubiquitination in chronically hypertensive mice. *In vitro* gain/loss of function experiments assessed ACE2 expression and activity to validate the interaction between ACE2 and the identified E3 ligase. Mutation experiments were further used to generate a ubiquitination-resistant ACE2 mutant (ACE2-5R). Optogenetics, blood pressure telemetry, pharmacological blockade of $GABA_A$ receptors in mice expressing ACE2-5R in the bed nucleus of the stria terminalis (BNST), and capillary western analysis were used to assess the role of ACE2 ubiquitination in neurogenic hypertension. Ubiquitination was first validated as leading to ACE2 down-regulation, and Neural precursor cell-expressed developmentally down-regulated protein 4-2 (Nedd4-2) was identified as a E3 ligase up-regulated in hypertension and promoting ACE2 ubiquitination. Mutation of lysine residues in the C-terminal of ACE2 was associated with increased activity and resistance to angiotensin (Ang)-II-mediated degradation. Mice transfected with ACE2-5R in the BNST exhibited enhanced GABAergic input to the paraventricular nucleus (PVN) and a reduction in hypertension. ACE2-5R expression was associated with reduced Nedd4-2 levels in the BNST.

Conclusion

Our data identify Nedd4-2 as the first E3 ubiquitin ligase involved in ACE2 ubiquitination in Ang-II-mediated hypertension. We demonstrate the pivotal role of ACE2 on GABAergic neurons in the maintenance of an inhibitory tone to the PVN and the regulation of pre-sympathetic activity. These findings provide a new working model where Nedd4-2 could contribute to ACE2 ubiquitination, leading to the development of neurogenic hypertension and highlighting potential novel therapeutic strategies.

Keywords Autonomic regulation • Sex differences • GABA • Sympathetic drive • Blood pressure

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1. Introduction

Angiotensin-converting enzyme 2 (ACE2) is a member of the renin–angiotensin system (RAS), primarily involved in the conversion of the vasoconstrictor octapeptide angiotensin (Ang)-II to the vasodilatory heptapeptide Ang-(1-7). Decreases in cellular ACE2 levels have been associated with cardiovascular diseases like hypertension and diabetes, while overexpression of ACE2 has been shown to have beneficial effects in preventing these diseases.[1,2] Our group previously identified several post-translational mechanisms mediated by the Ang-II type 1 receptor (AT_1R), involved in ACE2 down-regulation and their contribution to the development of neurogenic hypertension, including ACE2 shedding[3] and internalization followed by degradation in lysosomes.[4,5] Importantly, we observed that this latter mechanism might be associated with ubiquitination induced by elevated Ang-II levels.[4] Despite recent reports identifying murine double minute 2 (MDM2) and S-phase kinase-associated protein 2 (Skp2) as important E3 ligases for ACE2 ubiquitination in the lung,[6,7] there is a general gap in knowledge regarding ACE2 ubiquitination and its implications, notably for cardiovascular diseases such as hypertension.

Ubiquitination is a general post-translational modification of proteins consisting of the addition of ubiquitin, a 76-amino-acid polypeptide, to substrate proteins, leading to either degradation in proteasomes or digestion in lysosomes. The latter is typical for plasma membrane proteins and is of utmost importance in the regulation of cellular signaling. Ubiquitin is covalently attached by its C-terminus to specific lysine residues on the target protein,[8,9] a process mediated by the sequential action of three types of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases.[10] Ubiquitin is first activated by E1 in an adenosine triphosphate (ATP)-dependent manner and then transferred to a cysteine residue at the active site of E2. Finally, the E3 ligase directly or indirectly catalyzes the covalent attachment of ubiquitin to the target protein.[10] The human genome encodes two E1 enzymes, about 60 different E2 enzymes, and more than 600 E3 ubiquitin ligases. Ubiquitin can be attached as single or multiple residues to the substrate protein, resulting in mono- or poly-ubiquitination, respectively, and is essential for controlling the expression level of substrate proteins.

The brain RAS contributes to the regulation of cardiovascular function, and overactivation of this system, notably in hypertension, is well known to lead to enhanced pre-sympathetic activity of glutamatergic neurons in forebrain nuclei, such as the paraventricular nucleus (PVN).[11] We previously observed that ACE2 is expressed on GABAergic neurons and a lack of ACE2 leads to impaired inhibitory input to the PVN, leading to enhanced sympathetic activity to peripheral end organs.[12] The activity of pre-sympathetic PVN neurons is under the influence of GABAergic input originating from outside the PVN. The bed nucleus of the stria terminalis (BNST) is a region rich in GABAergic neurons known to regulate cardiovascular parameters, notably during stress.[13] Our group previously reported that ACE2 is expressed in the BNST[14] and more recently suggested that it contributes to the inhibitory tone to the PVN.[12] However, in addition to the lack of knowledge with regard to ACE2 ubiquitination partners, there is also limited information on the impact of ubiquitination, notably within the compensatory RAS, on the regulation of sympathetic activity and the development of neurogenic hypertension. To address these gaps in knowledge, we aimed to identify E3 ligases regulating ACE2 ubiquitination and investigated this mechanism in the context of neurogenic hypertension.

In the present study, we identified Neural precursor cell-expressed developmentally down-regulated protein 4-2 (Nedd4-2) as an E3 ubiquitin ligase targeting lysine residues in the carboxy tail of ACE2. Following *in vitro* and *in vivo* validation of Nedd4-2 interaction with ACE2, we used a ubiquitination-resistant ACE2 mutant for site-specific gene therapy to the brain to highlight the detrimental role of constitutive ACE2 ubiquitination in the activity of inhibitory GABAergic neurons and demonstrated that this mechanism contributes to the development of neurogenic hypertension.

2. Methods

The authors declare that all supporting data are available within the article and in Supplementary material online. A detailed Methods section is available in Supplementary material online.

Experiments were conducted in adult C57BL6/J and Vgat-IRES-cre mice (10–12 weeks old, 20–25 g; Jackson Laboratory, Bar Harbor, ME, USA) from both sexes. Mice were housed in a temperature- (~25°C) and humidity-controlled facility under a reversed 12 h dark/light cycle, fed standard mouse chow (Envigo, iOS Teklab Extruded Rodent Diet 2019S, Huntingdon, UK) and water *ad libitum*. Mice were anaesthetized with isoflurane (2%) through nose inhalation for the duration of all surgeries and with avertin (200 µg/g) for euthanasia. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Louisiana State University Health Sciences Center (#3540) and the Southeast Veterans Healthcare System (#620) Institutional Animal Care and Use Committees in accordance with the 'Principles of Laboratory Animal Care by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals' (National Institutes of Health Publication No. 86-23, revised 1996). Cardiac samples from patients were obtained from the Medical College of Wisconsin (IRB #PRO00010828) and Duke University (IRB #PRO00005621) from organs not suitable for transplant. Informed consent forms were obtained prior to experiments, and all procedures conform to the principles outlined in the Declaration of Helsinki.

3. Results

3.1 Ubiquitination reduces ACE2 expression and activity

We previously reported that Ang-II activation of AT_1R leads to a reduction in ACE2 plasma membrane localization and total cellular expression levels, possibly through ubiquitination.[4] To confirm that this mechanism is indeed critical for the regulation of ACE2 expression and activity, we first assessed ACE2 ubiquitination levels by co-immunoprecipitation in HEK293T cells transfected with ACE2, AT_1R, and ubiquitin (*Figure 1A*). As expected, Ang-II treatment (100 nM, 4 h) involves AT_1R-dependent ACE2 ubiquitination, as this effect was blocked by the AT_1R antagonist losartan and was not observed in cells not transfected with AT_1R (*Figure 1A*, right). This suggests that AT_1R activation is required for Ang-II-mediated ubiquitination of ACE2, extending our previous observations that ACE2 is not internalized by Ang-II in cells not expressing AT_1R.[4,5] In addition, overexpression of ubiquitin resulted in reduced ACE2 activity (*Figure 1B*). The seemingly contrasting observations that in cells transfected with ubiquitin, treatment with Ang-II did not further decrease ACE2 activity (*Figure 1B*) might suggest that ACE2 ubiquitination can occur both constitutively and in the presence of Ang-II.

The importance of ubiquitination in regulating ACE2 expression and activity is further supported by specific inhibition of ubiquitination and deubiquitination. PYR-41, a cell-permeable inhibitor of E1 ubiquitin-activating enzymes (50 µM for 2 h), did not affect basal ACE2 activity or expression (*Figure 1C* and *D*). In contrast, pre-treatment with PR-619, a broad-spectrum reversible inhibitor of cysteine-reactive deubiquitinating enzymes (20 µM, 6 h), reduced both ACE2 activity (*Figure 1C*) and protein expression (*Figure 1D*) to levels similar to those achieved by Ang-II treatment, suggesting that deubiquitination is critical to maintaining ACE2 expression and activity. To ensure that these results are not dependent on the amount of plasmids transfected, we repeated this experiment in Neuro2A cells (*Figure 1E*) and confirmed that ACE2 deubiquitination is a constitutive process that maintains ACE2 expression. Together, these data suggest that ubiquitination and deubiquitination are critical mechanisms for the regulation of ACE2 expression.

3.2 Nedd4-2 is up-regulated in hypertension

A multipronged discovery strategy was then implemented to identify E3 ubiquitin ligases interacting with ACE2 in neurogenic hypertension. A predictive bioinformatic analysis, to identify key E3 ubiquitin ligases involved in ACE2 ubiquitination, revealed that Nedd4-2 (also known as NEDD4L) has the highest confidence level for interaction with ACE2 (*Figure 2A*) after MDM2, another E3 ligase previously reported to ubiquitinate ACE2.[6]

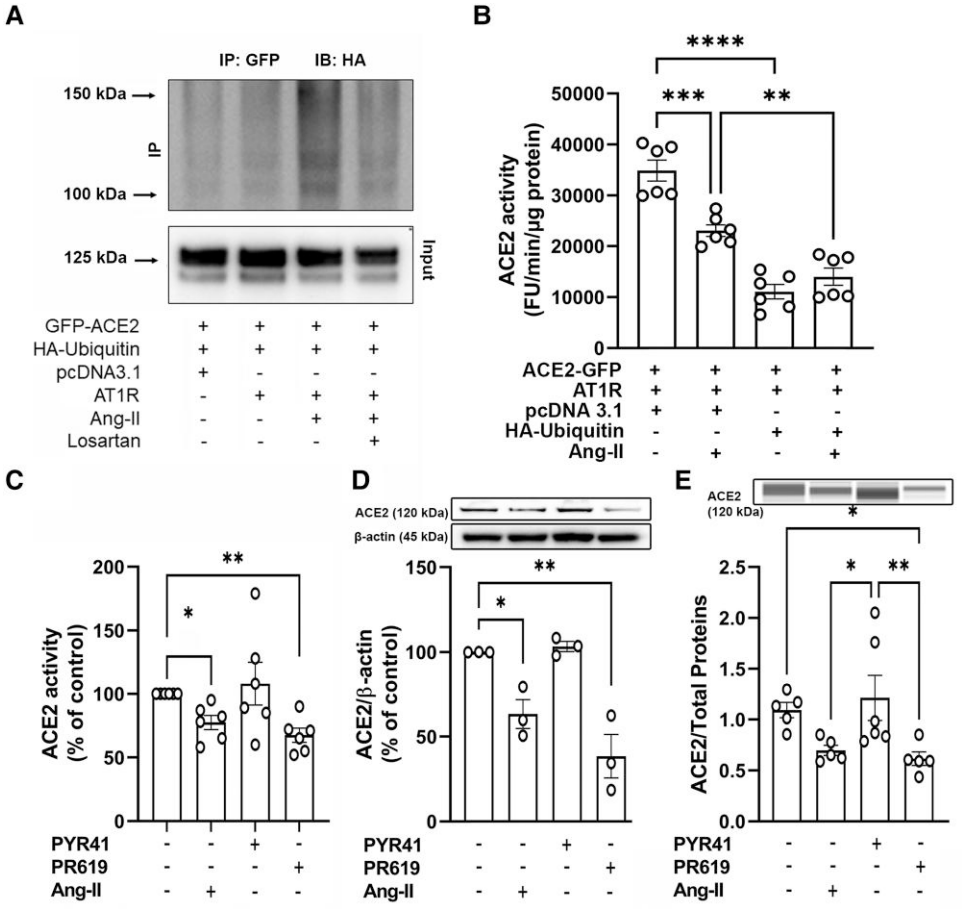


Figure 1 Ubiquitination reduces ACE2 expression and activity. (*A*) Representative blot showing the levels of ubiquitination of ACE2 in control HEK293T cells before and after Ang-II treatment. The first two lanes are from cells transfected with GFP-tagged ACE2, AT_1R, HA-tagged ubiquitin, and pcDNA3.1, and the last two lanes are from cells transfected for 48 h, serum-starved for 24 h, and subsequently treated with (left) Ang-II (100 nM for 4 h) or Ang-II (100 nM) and losartan (1 µM) for 4 h. GFP was then immunoprecipitated from the cells, and immunoblotting was performed against HA, as described in the Methods section. (*B*) ACE2 activity in HEK293T cells transfected with pcDNA3.1 or HA-tagged ubiquitin (in duplicate from three separate transfections, $n = 6$). (*C*) ACE2 activity ($n = 6$) and (*D*) expression ($n = 3$) normalized to β-actin in HEK293T cells transfected with pcDNA3.1 and treated with PYR-41 (50 µM for 2 h), a cell-permeable inhibitor of E1 ligases, PR-619 (20 µM, 6 h), a broad-spectrum reversible inhibitor of cysteine-reactive deubiquitinases, or Ang-II (100 nM). (*E*) ACE2 expression in Neuro2A cells treated with the same drugs ($n = 5$–6). Two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

Four potential Nedd4-2 recognition motifs were identified within the ACE2 amino acid sequence, including two in the C-terminal, next to multiple lysine residues (*Figure 2B*). To identify changes in E3 ligase expression in neurogenic hypertension, a parallel proteomic analysis was performed using hypothalamic samples isolated from mice infused with Ang-II. Again, Nedd4-2 was highlighted as a positive hit with potential changes in expression affected by both sex and hypertension (*Figure 2C*). To validate this finding and determine tissue specificity, Nedd4-2 and ACE2 protein expression levels were assessed in various organs affected by neurogenic hypertension. Basal ACE2 expression was higher in the brain (*Figure 2D*) and heart (*Figure 2E*) of males, but Nedd4-2 was not different between sexes in these tissues. In the kidney, however, lower Nedd4-2 levels in females were associated with higher ACE2 expression (*Figure 2F*). Ang-II-mediated neurogenic hypertension, which was more pronounced in males than in females (see Supplementary material online, *Table S1*), was associated with a strong Nedd4-2 up-regulation, mostly in males, and a parallel reduction in ACE2 levels in all tissues. However, while ACE2 was reduced in Ang-II-infused females, this was only associated with an up-regulation of Nedd4-2 in the heart, suggesting that ACE2 down-regulation in females might be independent of Nedd4-2.

Based on the above lack of tissue specificity and similarities between ACE2 and Nedd4-2 expression in both the brain and the heart, we further examined the clinical relevance of the Nedd4-2/ACE2 relationship in cardiac samples (*Figure 2G*) from normotensive and hypertensive donors (see Supplementary material online, *Table S2*). Surprisingly, Nedd4-2 up-regulation in hypertensive patients and the parallel reduction in ACE2 expression were only observed in the left ventricle of African American males, with no difference in Caucasian donors (see Supplementary material online, *Figure S1*). While Nedd4-2 up-regulation was also observed in hypertensive African American females, it was not associated with a reduction in ACE2 levels (*Figure 2G*), pointing again to the lack of involvement of Nedd4-2 in ACE2 ubiquitination in females.

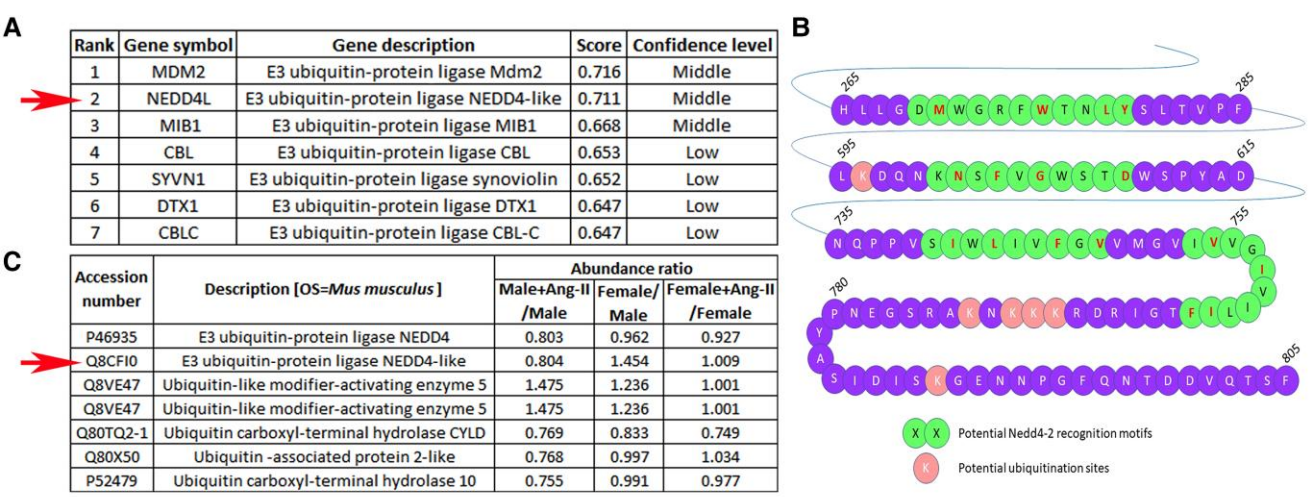
Together, these data suggest that hypertension is associated with an up-regulation of Nedd4-2 and a parallel decrease in ACE2 expression, predominantly in males. Based on the lack of correlation between ACE2 and Nedd4-2 expression in female mice and patients, ensuing *in vivo*

A

Rank	Gene symbol	Gene description	Score	Confidence level
1	MDM2	E3 ubiquitin-protein ligase Mdm2	0.716	Middle
2	NEDD4L	E3 ubiquitin-protein ligase NEDD4-like	0.711	Middle
3	MIB1	E3 ubiquitin-protein ligase MIB1	0.668	Middle
4	CBL	E3 ubiquitin-protein ligase CBL	0.653	Low
5	SYVN1	E3 ubiquitin-protein ligase synoviolin	0.652	Low
6	DTX1	E3 ubiquitin-protein ligase DTX1	0.647	Low
7	CBLC	E3 ubiquitin-protein ligase CBL-C	0.647	Low

C

Accession number	Description [OS=*Mus musculus*]	Abundance ratio		
		Male+Ang-II /Male	Female/ Male	Female+Ang-II /Female
P46935	E3 ubiquitin-protein ligase NEDD4	0.803	0.962	0.927
Q8CFI0	E3 ubiquitin-protein ligase NEDD4-like	0.804	1.454	1.009
Q8VE47	Ubiquitin-like modifier-activating enzyme 5	1.475	1.236	1.001
Q8VE47	Ubiquitin-like modifier-activating enzyme 5	1.475	1.236	1.001
Q80TQ2-1	Ubiquitin carboxyl-terminal hydrolase CYLD	0.769	0.833	0.749
Q80X50	Ubiquitin -associated protein 2-like	0.768	0.997	1.034
P52479	Ubiquitin carboxyl-terminal hydrolase 10	0.755	0.991	0.977



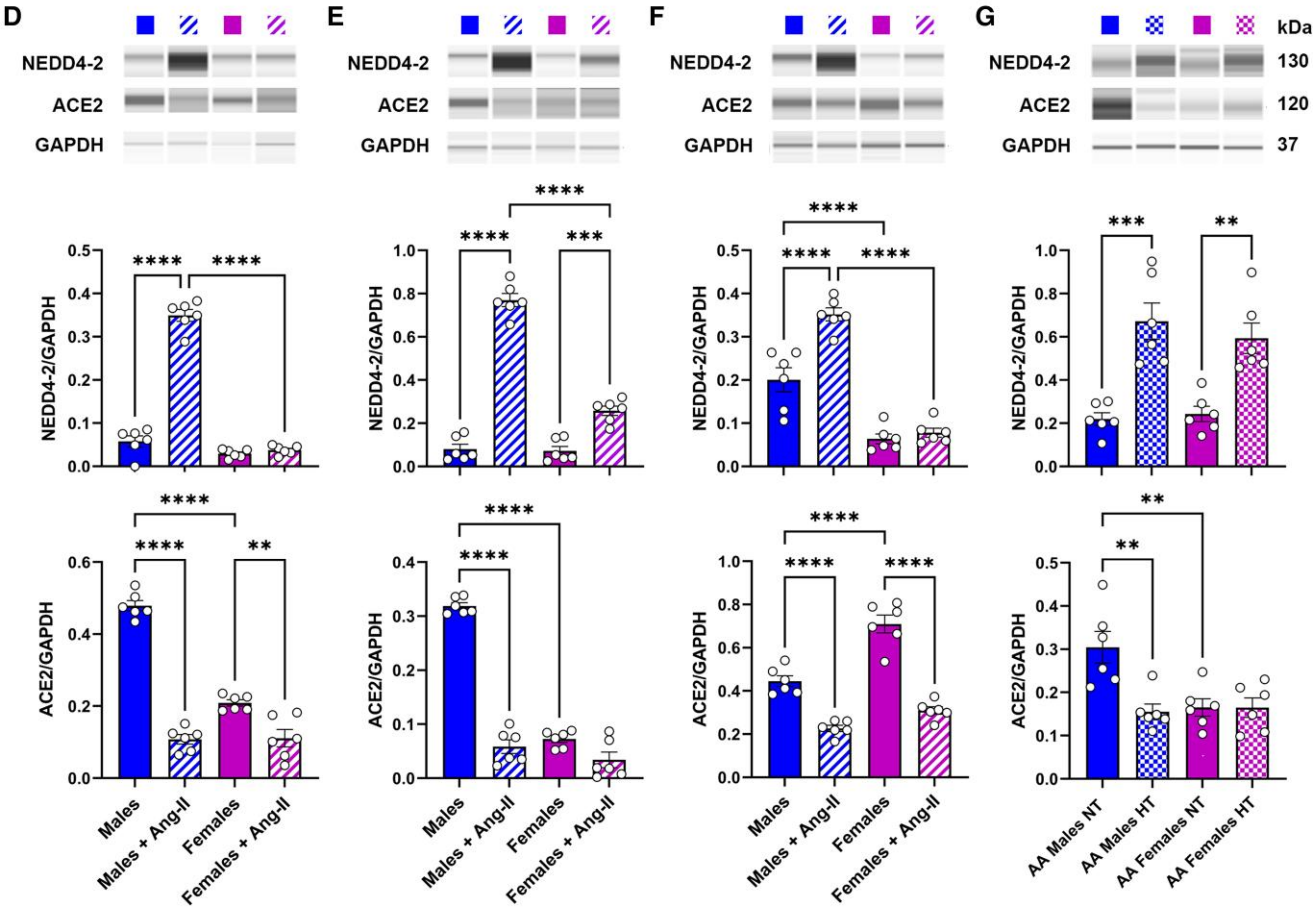


Figure 2 Predicted ACE2-interacting Nedd4-2 is up-regulated in hypertension. (*A*) Bioinformatic analysis (UbiBrowser v1.0) showing E3 ubiquitin ligases predicted to interact with ACE2 based on E3 recognition motifs, network loops, and enriched gene ontology pairs. (*B*) Identification of four potential Nedd4-2 (also known as NEDD4L) recognition motifs (green) in the amino acid sequence of ACE2. Close-by lysine residues (K) are highlighted as potential ubiquitination sites. (*C*) Changes in E3 ubiquitin ligase abundance identified by proteomic analysis from the hypothalamus of normotensive and Ang-II-infused mice from both sexes. Representative pictures and capillary western analysis of Nedd4-2 (130 kDa) and ACE2 (120 kDa) expression in the mouse ($n = 6$/group) brain (*D*), heart (*E*), kidney (*F*), and cardiac left ventricles ($n = 6$/group) from African American donors (*G*) normalized to GAPDH (~37 kDa). Two-way ANOVA followed by Tukey's test for multiple comparisons. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

experiments, targeting Nedd4-2-mediated ubiquitination of ACE2, were conducted only in males.

3.3 ACE2 is ubiquitinated by Nedd4-2

The next set of experiments aimed at establishing a direct relationship between ACE2 and Nedd4-2. Transfection of HEK293T cells with wild-type (WT) Nedd4-2 significantly decreased basal ACE2 activity, and this was not further reduced by Ang-II treatment (*Figure 3A*). In contrast, transfection with a catalytically inactive Nedd4-2 mutant with a serine mutated to cysteine (C/S) in the HECT domain of the protein[15] had no effect on basal ACE2 activity. In addition, this catalytically inactive form prevented Ang-II-induced ACE2 down-regulation (*Figure 3A*). Overexpression of WT Nedd4-2 also reduced cellular ACE2 levels, which was not observed in cells transfected with the catalytically inactive Nedd4-2 mutant (*Figure 3B*). To further establish the direct relationship between ACE2 and Nedd4-2, independent of transfection, in non-cancerous cells, primary human aorta endothelial cells (HAEC) that naturally express both ACE2 and Nedd4-2 were used. As with HEK293T (see Supplementary material online, *Figure S2*) and Neuro2A cells,[4] HAEC exposed to Ang-II exhibited ACE2 internalization and resulted in increased co-localization with intracellular Nedd4-2 (*Figure 3C*). In these cells, Ang-II-mediated up-regulation of Nedd4-2 was also associated with a reduction in ACE2 levels (*Figure 3D*). Pre-treatment with Nedd4-2 siRNA significantly knocked down the expression of the E3 ubiquitin ligase and resulted in a restoration of ACE2 levels that were no longer reduced by Ang-II treatment (*Figure 3D*). Together, these data establish causality between Nedd4-2 up-regulation and ACE2 down-regulation, confirming that Nedd4-2 is required for Ang-II-mediated ACE2 ubiquitination.

3.4 Ubiquitination-resistant ACE2 exhibits enhanced activity

Since Nedd4-2 has 13 known and 2262 predicted high-confidence substrates in the mouse, including ACE (see Supplementary material online, *Table S3*), we focused our targeting strategy on ACE2 ubiquitination rather than Nedd4-2 itself. Based on the predicted interaction of Nedd4-2 with the ACE2 carboxy tail (*Figure 2B*), we next designed an ACE2 mutant resistant to Nedd4-2 ubiquitination. Ubiquitination takes place primarily at lysine sites embedded in the intracellular part of the protein.[8–10] Human ACE2 has a short intracellular C-terminus consisting of 43 amino acids, among which five lysine residues constitute putative ubiquitination sites. These residues are also located next to one of the four predicted Nedd4-2 recognition motifs (*Figure 4A*). To determine the role of these lysine residues in the regulation of ACE2 expression levels and activity, we generated mutants in which lysine residues were substituted by arginine, another positively charged amino acid that cannot be ubiquitinated.[16] The first five mutants had a single lysine mutated to arginine (*Figure 4A*), while the sixth mutant (Δ6 or ACE2-5R) included all five mutations. Basal ACE2 activity in transfected HEK293T cells was significantly increased in all mutants (*Figure 4B*). ACE2-5R displayed an enzymatic activity comparable to other ACE2 mutants, indicating that lysine residues from the C-terminus play redundant roles in Ang-II-induced ACE2 ubiquitination. Furthermore, all mutants, including ACE2-5R, were resistant to Ang-II-mediated degradation (*Figure 4B* and *C*). Transfection with hACE2-5R resulted in a four-fold increase in ACE2 activity (*Figure 4B*), independent of any increase in ACE2 expression (*Figure 4C*), despite using an antibody targeting amino acids 392–744 that do not overlap with the point mutations. These data suggest that hACE2-5R not only is resistant to Ang-II-mediated ubiquitination but also exhibits enhanced activity compared to native ACE2. Accordingly, ACE2-5R was selected for *in vivo* targeting of ACE2 ubiquitination and packaged into a commercial adeno-associated viral delivery system (*Figure 4D*).

3.5 ACE2 is expressed on GABAergic neurons

Having generated a new gene therapy tool, the next set of experiments aimed at validating the injection site for optimum targeting of ACE2 ubiquitination. Our previous work showed that ACE2 is expressed in the BNST and suggested that expression on GABAergic neurons in the BNST might contribute to an inhibitory input to PVN excitatory neurons.[12,14] To validate the BNST as an appropriate region for ACE2-5R injection, we first verified that (i) GABAergic neurons in the BNST are expressing ACE2 and (ii) these GABAergic neurons project an inhibitory tone to the PVN. Injection of a cre-dependent AAV-ChR2-eYFP in the BNST of Vgat-cre mice (*Figure 5A*) resulted in the expression of eYFP fluorescence selectively on GABAergic neurons, and ACE2 co-localization confirmed the enzyme expression on these inhibitory neurons (*Figure 5B*). Importantly, ACE2 immunoreactivity was only detected on GABAergic cell bodies and was undetectable on the outer edge of the BNST where GABAergic neurons are absent. To verify that these GABAergic neurons are involved in blood pressure (BP) regulation, unilateral photoactivation of neuronal cell bodies in the dorsal BNST (*Figure 5C*) was performed in anesthetized Vgat-cre mice using a blue LED light (473 nm, 1–10 Hz, 1 min). This resulted in a time- and frequency-dependent reduction in both mean BP (~16 mmHg) and heart rate (HR) (~25 b.p.m.) that immediately returned to baseline once the stimulation ceased while shining a green LED light (532 nm) over the dorsal BNST produced no response. To further confirm that these GABAergic neurons regulate BP via inhibition of PVN neurons, the same protocol was used to stimulate GABAergic projections to the PVN (*Figure 5D*). The blue light produced a similar reduction in mean BP and HR following direct stimulation of the BNST. Importantly, the timeframe of these responses suggests an autonomic rather than a hormonal pathway. Finally, to verify that these GABAergic inhibitory projections terminate in the PVN, photostimulations of BNST GABA neurons were repeated following the blockade of $GABA_A$ receptors in the PVN. Bicuculline injections into the PVN (*Figure 5E*) prevented the reduction in BP and HR. Together, these data confirm that ACE2 is expressed on GABAergic neurons in the BNST and that these neurons exert an inhibitory tone to PVN excitatory neurons involved in BP regulation. This set of experiments confirms that the BNST is an appropriate target region to further study ACE2 ubiquitination and its role in neurogenic hypertension.

3.6 ACE2-5R enhances the GABAergic inhibitory tone

To assess the impact of ACE2 ubiquitination in the BNST in the development of neurogenic hypertension, C57BL6/J male mice were injected bilaterally with AAV-hACE2-5R or AAV-GCaMP-6S as the control virus. Six weeks were allowed for viral expression before chronic infusion with Ang-II to induce neurogenic hypertension. In normotensive mice, hACE2-5R expression resulted in a significant reduction in baseline mean BP during the resting phase (93 ± 3 vs. 108 ± 4 mmHg, $P < 0.05$) but not the active phase (107 ± 4 vs. 117 ± 3 mmHg, $P = 0.08$) of the nychthemeral cycle, without affecting HR (*Figure 6A*). The lack of HR changes contrasts with the optogenetic data showing pronounced bradycardia resulting from a forced stimulation of BNST GABAergic neurons devoid of any regulatory influence (*Figure 5C*). In conscious mice, however, baroreflex regulation is likely to have buffered this bradycardia. The reduction in BP in the hACE2-5R group did not persist, and only bradycardia was observed in both active and resting normotensive mice (without Ang-II infusion) by the end of the infusion (*Figure 6A* and *B*). Ang-II infusion resulted in a significant rise in mean BP, associated with bradycardia. Expression of hACE2-5R in Ang-II-infused mice resulted in a reduction in BP, as early as the second week of infusion, which was restricted to the active phase and abolished the circadian fluctuations of BP. To determine the impact of ACE2 ubiquitination on the GABAergic inhibitory tone to the PVN, BP was recorded in anaesthetized mice following blockade of $GABA_A$ receptors in the PVN. Removal of GABAergic input to the PVN following bilateral bicuculline injections resulted in an immediate rise in BP that was exacerbated in hACE2-5R mice infused with Ang-II (*Figure 6C*). This suggests that prevention of ACE2 ubiquitination in the BNST resulted in enhanced GABAergic input to the PVN, capable of blunting the development of hypertension. Analysis of protein expression in the

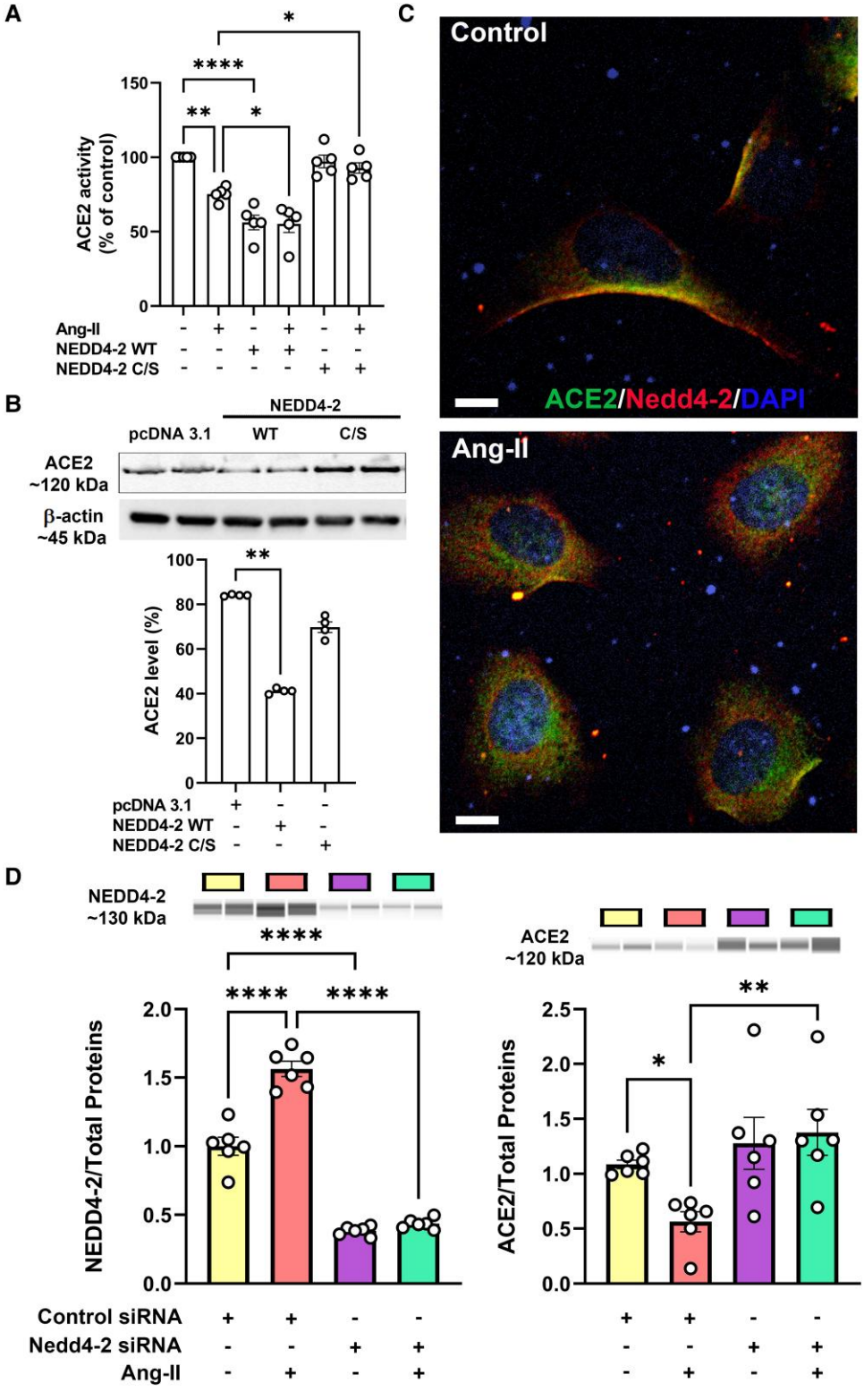


Figure 3 ACE2 is ubiquitinated by Nedd4-2. (*A*) Impact of WT Nedd4-2 and its catalytically inactive mutant (C/S) transfection on ACE2 activity in HEK293T cells exposed to Ang-II ($n = 5$). (*B*) Modulation of ACE2 cellular levels by Nedd4-2 in HEK293T cells ($n = 4$). (*C*) Subcellular localization of ACE2 and Nedd4-2 in HAEC in the absence (control) or presence of Ang-II (100 nM for 4 h). (*D*) Representative pictures and capillary western analysis of Nedd4-2 and ACE2 expression in HAEC treated with Ang-II in the presence or absence of Nedd4-2 siRNA. Two-way ANOVA followed by Tukey's test for multiple comparisons, except for panel (*B*) (one-way ANOVA followed by Bonferroni's test). Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Scale bars are 10 μm.

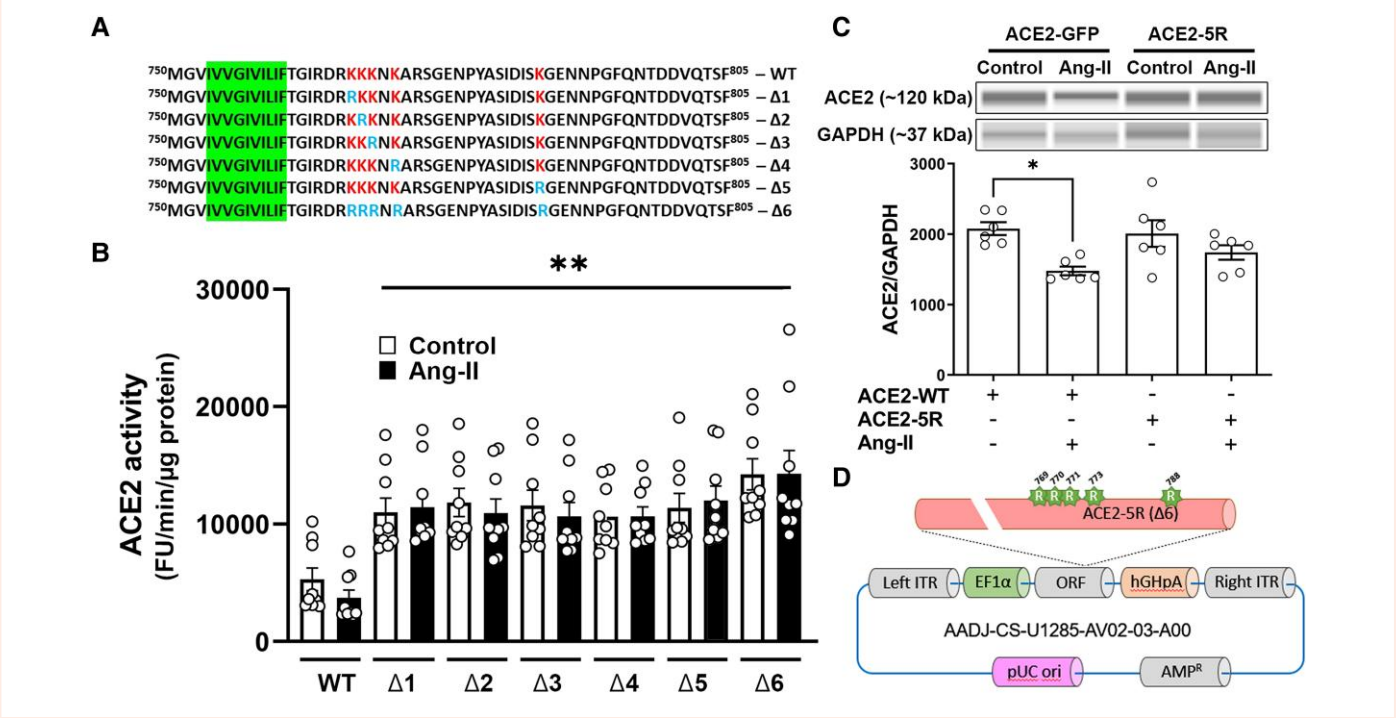


Figure 4 Ubiquitination-resistant ACE2. (*A*) Amino acid sequence of the native ACE2 C-terminus and the six mutants (Δ) where lysine residues were replaced by arginine. The distal predicted Nedd4-2 recognition motif is highlighted in green. (*B*) ACE2 activity for WT and hACE2 mutants in the absence and presence of Ang-II (100 nM, 4 h). (*C*) Representative western electrophoresis and quantified data showing the impact of Ang-II (100 nM, 4 h) on total cellular levels of ACE2 ($n = 6$) in HEK293T cells transfected with WT ACE2 or ACE2-5R. All cells were transfected with AT_1R plasmids. (*D*) Schematic of the hACE2-5R (Mutant 6) adeno-associated viral vector. Two-way ANOVA. Statistical significance: $^*P<0.05$ and $^{**}P < 0.01$ vs. WT.

BNST confirmed that Ang-II infusion was associated with increased Nedd4-2 (*Figure 6D*) and a concomitant reduction in ACE2 levels (*Figure 6E*), while AT_1R, Mas1 receptor (Mas1R), and MDM2 were not changed. Surprisingly, hACE2-5R injection in normotensive mice resulted in a reduction in ACE2. Although this was concomitant to Nedd4-2 (*Figure 6D*) and MDM2 (*Figure 6H*) up-regulation, these E3 ligases are unlikely responsible since their target lysine residues were mutated. This was also associated with a down-regulation of AT_1R and an up-regulation of Mas1R, suggesting that hACE2-5R enhanced activity had effectively compensated for the reduction in ACE2 expression. In hypertensive mice, although Nedd4-2 up-regulation was not significantly affected by hACE2-5R, ACE2 was significantly increased and AT_1R was reduced (*Figure 6F*), while Mas1R (*Figure 6G*) expression was not affected and MDM2 was similar to baseline levels (*Figure 6H*). These data suggest that, unlike ACE2, hACE2-5R is resistant to hypothalamic E3 ligases and able to blunt the development of neurogenic hypertension by enhanced processing of Ang-II levels.

4. Discussion

Although impaired ACE2 activity has been extensively shown to be associated with cardiovascular diseases, including hypertension, the precise mechanisms responsible remain largely unknown.[2–4] In this study, we identify Nedd4-2 as a mediator of ACE2 ubiquitination and we propose a novel cellular mechanism behind the development of neurogenic hypertension. Specifically, we demonstrate that Ang-II mediates Nedd4-2 up-regulation in experimental hypertension, which in turn promotes ACE2 ubiquitination and degradation. Nedd4-2 up-regulation was also observed in hypertensive patients. Knockdown of Nedd4-2 expression or activity prevented Ang-II-mediated down-regulation of ACE2. Finally, we validated these findings in a neurogenic hypertension model and showed that expression of an ACE2 ubiquitination-resistant mutant resulted in an enhanced inhibitory input to pre-sympathetic PVN neurons and a reduction in the development of hypertension.

The activity of any specific protein, including ACE2, is critically dependent on cellular expression levels and its subcellular localization. Since its discovery two decades ago, many important findings have highlighted the multifunctional role of ACE2 within and outside the RAS. Indeed, ACE2 has been shown to modulate circulating levels of Ang-II and Ang-(1-7), which play crucial and opposing roles in the regulation of cardiovascular function.[17] The recent discovery that ACE2 is a cellular receptor for SARS-CoV-2, the coronavirus responsible for COVID-19 disease, has exponentially increased research aimed at understanding the mechanisms controlling its expression levels and subcellular localization. Both Ang-II and SARS-CoV-2 have been shown to induce AT_1R-dependent internalization of plasma membrane ACE2 followed by a decrease in its total cellular levels.[4,5] Based on our initial findings that ACE2 is ubiquitinated in basal conditions,[4] we hypothesized that this post-translational modification is responsible for lysosomal targeting of the enzyme in neurogenic hypertension. However, only a few studies have attempted to identify E3 ligases involved in ACE2 ubiquitination, and none have investigated this interaction in neurogenic hypertension. Our combined proteomic and bioinformatic approach highlighted Nedd4-2 as a highly likely E3 ligase participating in ACE2 ubiquitination, with four potential recognition sites, including one close to the C-terminal of ACE2 in a lysine-rich region.

Nedd4-2 belongs to the HECT-containing subfamily of E3 ligases and is involved in the regulation of cell proliferation, viral budding, and intracellular trafficking. These ligases modulate G protein-coupled receptor activity either through direct interaction or via binding to β-arrestin.[15,16] As ubiquitination takes place exclusively at intracellular lysine residues, we examined the five such residues localized in the intracellular C-terminus of ACE2, next to the Nedd4-2 recognition motif (*Figure 4*). Mutation of any of these

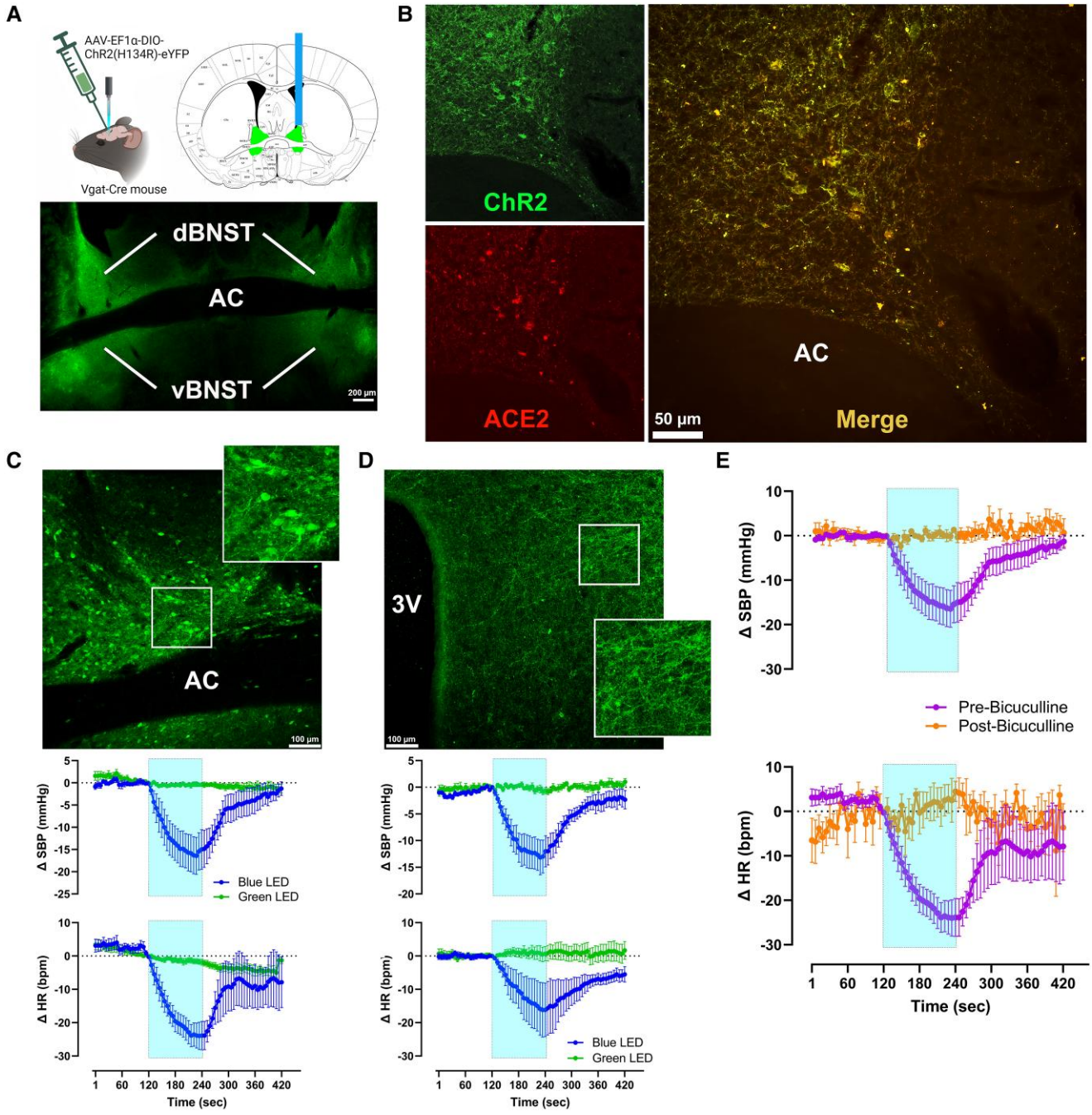


Figure 5 ACE2 is expressed on GABAergic neurons. (*A*) Schematic of the injection protocol and representative expression of AAV-ChR2-eYFP in the dorsal (dBNST) and ventral (vBNST) bed nucleus of the stria terminalis. Scale bar: 200 µm. (*B*) Immunohistochemistry pictures showing the co-localization of GABAergic neuron-targeted channelrhodopsin (ChR2) expression (green) with ACE2 (red). Photostimulations of ChR2 (10 Hz, 2 min, $n = 7$) with blue LED on GABAergic cell bodies in the BNST (*C*) and neuronal projections in the PVN (*D*) lead to an immediate reduction in systolic BP and HR, while green LED is ineffective. Inserts show magnifications (×2) of boxed regions. Scale bars: 100 µm. (*E*) Changes in systolic BP and HR initiated by photoactivation of ChR2 in the BNST are prevented by $GABA_A$ receptor blockade by bicuculline (1 mM) in the PVN. Abbreviations: 3V, third ventricle; AC, anterior commissure.

lysine residues led to enhanced enzymatic activity, clearly demonstrating that ACE2 is constitutively ubiquitinated and supporting the potential therapeutic benefit of using ubiquitination-resistant ACE2 to overcome hyperactivity of the RAS.

Nedd4-2 is a well-known E3 ubiquitin–protein ligase controlling cell surface expression of kidney epithelial Na^+ channels (ENaC), and impaired function of Nedd4-2 is associated with salt-sensitive hypertension.[18,19] Our data highlight another mechanism by which Nedd4-2 may contribute to alterations in cardiovascular function, namely by decreasing ACE2 expression levels and activity. This effect requires the enzymatic activity of Nedd4-2 as its catalytically inactive mutant[15] had no effect on Ang-II-induced ACE2 down-regulation (*Figure 3A* and *B*). Interestingly, Ang-II treatment enhanced

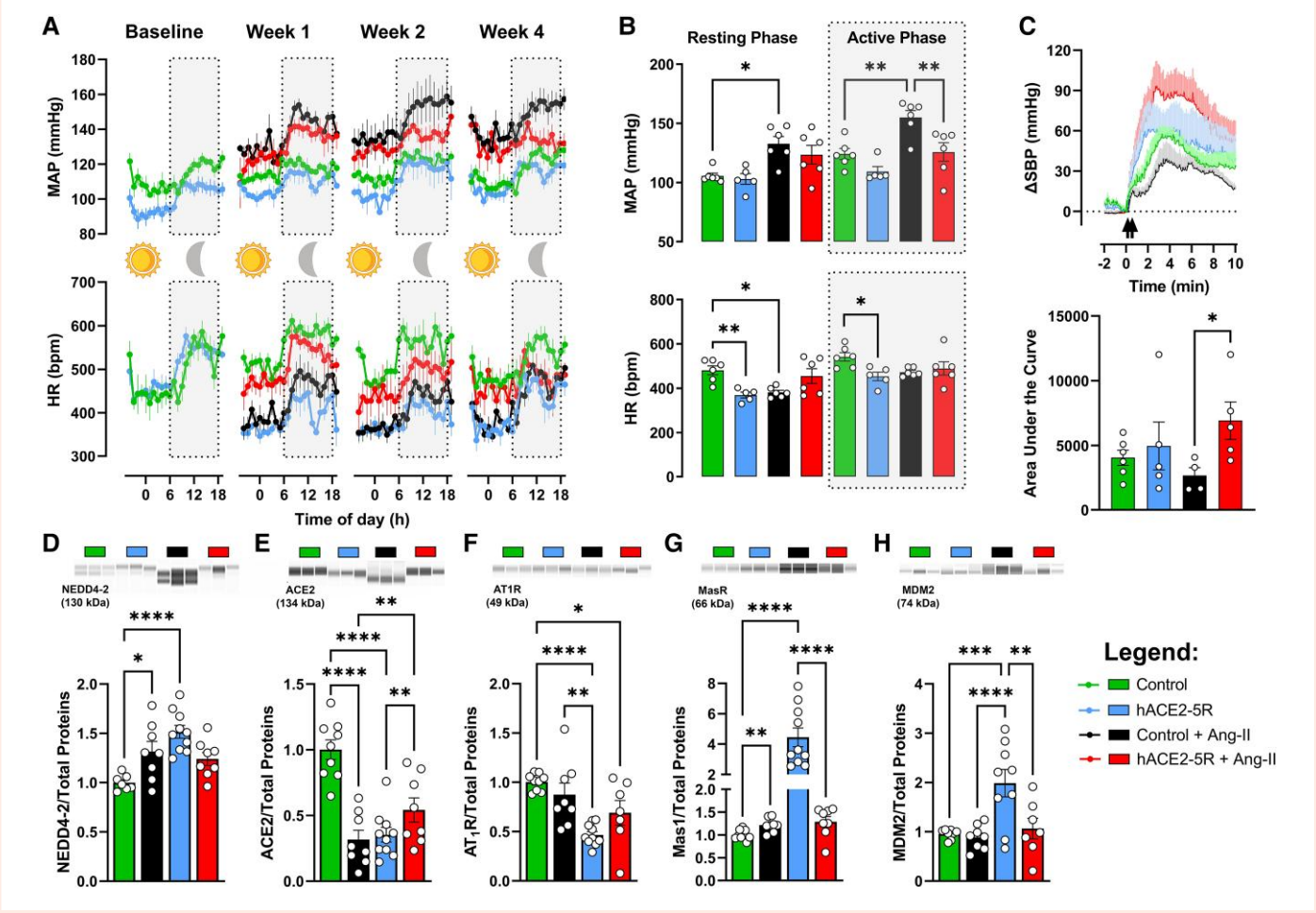


Figure 6 Ubiquitination-resistant ACE2-5R blunts neurogenic hypertension. Twenty-four-hour recording of BP and HR traces (*A*) and average data at 4 weeks (*B*) show that BNST expression of hACE2-5R blunted the development of Ang-II-mediated hypertension in mice during the active phase (grey box; $n = 5–6$/group). The resting and active phases are indicated by the sun and moon symbols, respectively. Cumulative traces and the area under the curve (*C*) show that PVN blockade of $GABA_A$ receptors with bicuculline (1 mM) mediates an enhanced systolic pressure response in Ang-II-infused mice expressing ACE2-5R in the BNST ($n = 4–6$/group). Arrows indicate bicuculline bilateral injections. Capillary western analysis of Nedd4-2 (*D*) (top band), ACE2 (*E*), AT_1R (*F*), Mas1R (*G*), and MDM2 (*H*) expression in the BNST normalized to total proteins ($n = 7–10$/group). Two-way ANOVA followed by Tukey's test for multiple comparisons. Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

co-localization between ACE2 and Nedd4-2, suggesting that AT_1R stimulation promotes ACE2 ubiquitination, at least partially, through Nedd4-2. Whether or not these effects are limited only to Nedd4-2 out of the large family of E3 ubiquitin ligases remains to be determined.

While ACE2 expression is known to be tissue-dependent and generally higher in males than females,[20] our findings highlight previously unreported sex differences in Nedd4-2 protein expression, with enhanced up-regulation in hypertensive males across tissues and limited up-regulation in females, restricted to the heart. These intriguing data suggest that females have signaling mechanisms that prevent Nedd4-2 up-regulation. This finding together with other supportive data we recently encountered is currently under investigation to further understand the crosstalk between sex hormones and Nedd4-2 expression in female mice. The sex-specific differences in Nedd4-2 expression are consistent with previous reports of Nedd4-2 gene polymorphisms associated with hypertension in males and the influence of oestrogens on reduced Nedd4-2 gene expression in the brain of females.[21,22] Due to its role in ENaC ubiquitination, Nedd4-2 has been studied extensively in the context of salt-sensitive hypertension, which is thought to be more prevalent in females than males.[23] In addition, polymorphisms of Nedd4-2 have been associated with hypertension in African Americans and other ethnic groups.[24–26] Accordingly, it is thought that mutation or a lack of Nedd4-2 results in elevated BP due to reduced ENaC ubiquitination. Our study differs from these observations. Indeed, Ang-II-mediated hypertension, which is associated with salt retention, was unequivocally associated with Nedd4-2 up-regulation (*Figures 2* and *6D*), consistent with the increase also observed in the nodose ganglion of spontaneously hypertensive rats.[27] This up-regulation paralleled a reduction in ACE2 levels in male mice (*Figures 2D–F* and *6E*) and African Americans (*Figure 2G*). While our *in vitro* knock-down experiments established causality between high Nedd4-2 and low ACE2 expression (*Figure 3*), it is unclear at this time why these findings did not extend to Caucasians (see Supplementary material online, *Figure S2*). Although African Americans are more susceptible to salt-sensitive hypertension,[24,25] it is likely that the patients' medications, notably those interfering with the RAS, also contributed to this observation. Unfortunately, the limited clinical information available from these donors did not allow for more in-depth analysis, and further studies are warranted in a larger patient population to investigate whether the up-regulation of Nedd4-2 selectively in African Americans was related to ethnicity or dependent on prescribed medications.

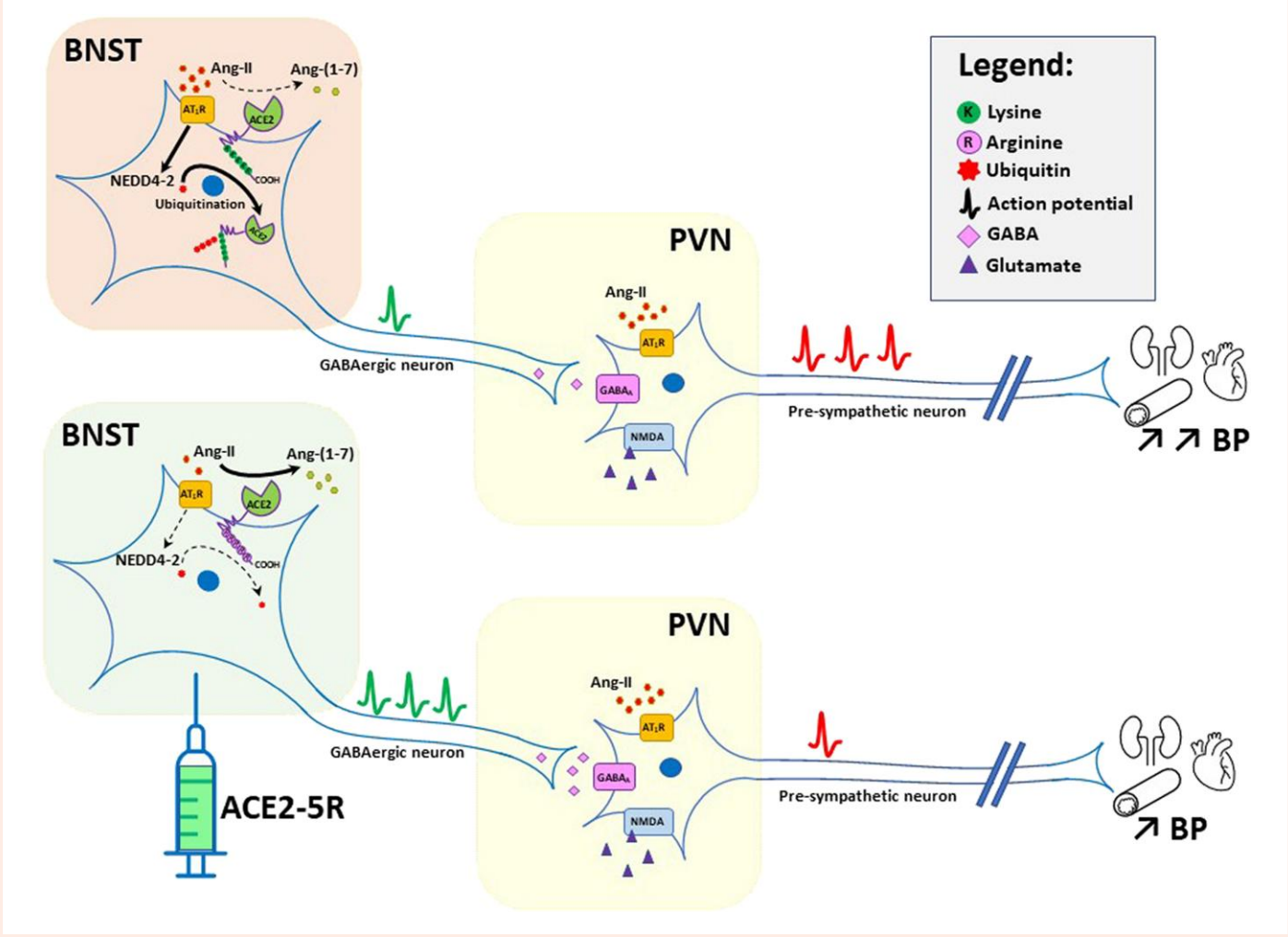


Figure 7 Working model. Schematic of the role of ACE2 ubiquitination in neurogenic hypertension. ACE2 is expressed on GABAergic neurons within the BNST where elevated Ang-II levels lead to Nedd4-2 up-regulation and ACE2 ubiquitination. Ubiquitinated ACE2 is internalized and degraded, preventing the conversion of Ang-II to Ang-(1-7). This is associated with a reduction in the GABAergic inhibitory input to pre-sympathetic neurons in the PVN, contributing to the development of neurogenic hypertension. BNST-targeted expression of a ubiquitination-resistant ACE2-5R mutant preserved ACE2 expression on the cell surface, leading to Ang-II conversion to Ang-(1-7), a reduction of Nedd4-2 expression, enhanced GABAergic inhibitory tone to the PVN, a and reduction in neurogenic hypertension.

We previously reported that ACE2 expression and activity are impaired in the brain during the development of neurogenic hypertension[3,28] and hypothesized that ACE2 ubiquitination could contribute to this down-regulation. The BNST is a neurochemically heterogeneous region, also known as the 'extended amygdala', which plays a role in cardiovascular responses to stress.[13] Most GABAergic neurons project to the parvocellular cluster of the PVN and provide an inhibitory tone to glutamatergic pre-sympathetic neurons. In addition, increased neuronal activity in the PVN has been reported following lesions of GABAergic cells within the anterior BNST,[29] suggesting that the BNST could differentially modulate autonomic responses to stressors. While stress-related cardiovascular responses involving baroreflex mechanisms have been reported following pharmacological activation or inhibition of the BNST,[13,30] the role of ACE2 ubiquitination in the BNST in the regulation of BP has never been investigated. Previous work from our group reported that ACE2 is expressed in the BNST and expression on GABAergic cell bodies could maintain an inhibitory input to PVN excitatory neurons, thus contributing to BP homeostasis.[12,14] Our new observations confirm the expression of ACE2 on GABAergic BNST neurons and that selective photostimulations of these cells result in a frequency- and time-dependent reduction in BP and HR that can be prevented by selective blockade of $GABA_A$ receptors in the PVN, further confirming direct projection from the BNST to the PVN. Although we also previously showed expression of ACE2 on vasopressinergic neurons,[12] the immediate responses following optogenetic stimulations suggest the involvement of the autonomic nervous system rather than hormonal responses. Importantly, we show that transfection of these GABAergic neurons in the BNST with a ubiquitination-resistant ACE2 mutant resulted in a significant reduction in baseline BP. This effect was exacerbated in hypertensive mice, notably during the active phase. These data confirm that ACE2 is constitutively ubiquitinated within the BNST, which may adversely affect the regulation of BP at baseline and in hypertensive conditions. Since ACE2 is widely expressed in the brain, we speculate that other ACE2-expressing GABAergic neurons projecting to the PVN could also regulate various physiological functions via similar mechanisms, thus warranting further investigation.

E3 ubiquitin ligases have previously been reported to affect GABAergic neurotransmission, leading to the modification of adaptor proteins and secondary signaling pathways, resulting in an increased or decreased

inhibitory tone.[31,32] The BNST is also known to contain AT_1R, and Ang-II regulates neuronal activity in this region.[30,33,34] Consistent with our observations, activation of AT_1R in the BNST during Ang-II-mediated hypertension up-regulated Nedd4-2, similar to that observed in HEK293T and endothelial cells, which in turn reduced ACE2 expression on the cell surface and likely affected the conversion of Ang-II to Ang-(1-7). On the other hand, prevention of ubiquitination in the BNST would likely result in enhanced processing of Ang-II, either blood-borne or locally generated, and formation of Ang-(1-7). Hypertension was not associated with significant changes in AT_1R (*Figure 6F*). Although the BNST is thought to lack AT_2R,[35] it contains Mas1R,[36] both of which can bind Ang-(1-7), and the up-regulation of Mas1R could be explained by a lack of Ang-(1-7) as a result of ACE2 ubiquitination and degradation. Interestingly, the up-regulation of Nedd4-2 and MDM2 following hACE2-5R expression in normotensive mice suggests that ACE2 ubiquitination is a tightly regulated mechanism and that targeting individual E3 ligases might not produce therapeutic benefits as feedback mechanisms would attempt to compensate for individual knockdowns.

Based on current and previous observations, a working model was designed (*Figure 7*) where Nedd4-2 could contribute to the excitatory activity of PVN neurons and downstream sympathetic activity in hypertension. Nedd4-2 has previously been shown to contribute to the activity of excitatory neurons, notably by affecting the expression of ion channels and cell surface proteins.[37] However, the role of Nedd4-2 in the brain is complex as it involves multiple and often opposite mechanisms. For example, a lack of Nedd4-2 ubiquitination, including in the brain, is known to be associated with an up-regulation of ENaC, resulting in salt-induced hypertension.[38] Conversely, Nedd4-2 knockdown also leads to reduced glutamate transporter ubiquitination and increased glutamate uptake,[39] possibly preventing glutamate neuroexcitability that is associated with reduced ACE2 activity.[40] Therefore, using recombinant hACE2-5R to control neurogenic hypertension might be a better therapeutic approach than interfering with Nedd4-2 expression.

There are some limitations associated with our study. First, our *in vivo* experiments did not specifically target Nedd4-2 in the brain but instead focused on the prevention of ACE2 ubiquitination in the BNST. Although this strategy ensured specificity regarding ACE2 ubiquitination, our data also support the possibility that in addition to Nedd4-2, the reduction in BP observed could be related to other E3 ligases targeting ACE2. However, while MDM2 and Skp2 have been reported to ubiquitinate ACE2,[6,7] they have not been studied outside the lung. Our data show that MDM2, which was previously shown to ubiquitinate ACE2 at ^{788}Lys, is not up-regulated by Ang-II infusion (*Figure 6H*), suggesting that it plays no part in our hypertension model. Second, although the reduction in ACE2 in the BNST appears to be compensated by the enhanced catalytic activity of ACE2-5R in neurogenic hypertension, the mechanism for this reduction in expression remains unknown. While ubiquitination is unlikely, ACE2 shedding[3] by ADAM17 is a possibility, and more work is needed to confirm this hypothesis. At the same time, assessment of hACE2-5R catalytic activity in the brain should be further investigated, ideally in a model with broader expression to overcome detection limitations. Finally, it is unclear how the Ang-II-mediated up-regulation of Nedd4-2 may have affected other targets of this E3 ligase, such as ENaC and Piezo2, and whether these targets contributed to the development of hypertension in our model.

In conclusion, we identified Nedd4-2 as a new E3 ligase targeting ACE2 in neurogenic hypertension. Nedd4-2 is up-regulated by elevated Ang-II levels and promotes ACE2 ubiquitination and degradation. Ubiquitination of ACE2 on GABAergic neurons located in the BNST results in a dampening of the inhibitory input to pre-sympathetic neurons in the PVN, thus contributing to the development of hypertension. Prevention of its ubiquitination allowed for preserved ACE2 compensatory activity in the BNST, leading to enhanced Ang-II conversion to Ang-(1-7), reduced up-regulation of Nedd4-2, and an increased inhibitory tone to the PVN, resulting in a dampening of neurogenic hypertension.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Authors' contributions

X.Y., C.M.F., and E.L. conceived and designed the experiments. M.M., B.O., M.E., N.L., C.B., I.G., J.J.G., S.S., J.X., and L.R. performed the experiments and statistical analysis. A.M.B., M.A.M-P., and D.E.B contributed to de-identified patient samples. X.Y., C.M.F., and E.L. wrote the manuscript. All authors contributed to manuscript revisions.

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Conflict of interest: None declared.

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Data availability

The authors declare that all supporting data are available within the article and in the data supplement.

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Translational perspective

While angiotensin-converting enzyme 2 (ACE2) conversion of angiotensin (Ang)-II to Ang-(1-7) is supposed to limit the overactivation of the renin–angiotensin system (RAS), the enzyme is down-regulated during the development of hypertension. As antihypertensive RAS blockers on the market only provide limited control of blood pressure among hypertensive patients, understanding the mechanisms responsible for this blunted compensation provides new possible targets for the treatment of hypertension. In this study, we show that Nedd4-2 up-regulation is associated with ACE2 ubiquitination, while prevention of this post-translational modification prevents the development of hypertension. Accordingly, targeting of ACE2 ubiquitination provides a new treatment strategy to reduce hypertension.