Stimulation of Angiotensin Type 1A Receptors on Catecholaminergic Cells Contributes to Angiotensin-Dependent Hypertension

Nikola Jancovski, Jaspreet K. Bassi, David A. Carter, Yan-Ting Choong, Angela Connelly, Thu-Phuc Nguyen, Daian Chen, Elena V. Lukoshkova, Clement Menuet, Geoffrey A. Head, Andrew M. Allen

Abstract—Hypertension contributes to multiple forms of cardiovascular disease and thus morbidity and mortality. The mechanisms inducing hypertension remain unclear although the involvement of homeostatic systems, such as the renin–angiotensin and sympathetic nervous systems, is established. A pivotal role of the angiotensin type 1 receptor in the proximal tubule of the kidney for the development of experimental hypertension is established. Yet, other systems are involved. This study tests whether the expression of angiotensin type 1A receptors in catecholaminergic cells contributes to hypertension development. Using a Cre-lox approach, we deleted the angiotensin type 1A receptor from all catecholaminergic cells. This deletion did not alter basal metabolism or blood pressure but delayed the onset of angiotensin-dependent hypertension and reduced the maximal response. Cardiac hypertrophy was also reduced. The knockout mice showed attenuated activation of the sympathetic nervous system during angiotensin II infusion as measured by spectral analysis of the blood pressure. Increased reactive oxygen species production was observed in forebrain regions, including the subfornical organ, of the knockout mouse but was markedly reduced in the rostral ventrolateral medulla. These studies demonstrate that stimulation of the angiotensin type 1A receptor on catecholaminergic cells is required for the full development of angiotensin-dependent hypertension and support an important role for the sympathetic nervous system in this model. (Hypertension. 2013;62:00-00.) • Online Data Supplement

Key Words: blood pressure • brain • receptor, angiotensin, type 1 • tyrosine 3-monooxygenase

Hypertension is a key contributor to multiple cardiovascular diseases and thus morbidity and mortality worldwide.1 The renin–angiotensin system plays a pivotal role in the regulation of blood pressure (BP) through effects on fluid and electrolyte homeostasis, vascular constriction, and modulation of the autonomic nervous system.2 Overactivity of this system is implicated in the development and maintenance of hypertension, and antagonists of the angiotensin II (Ang II) type 1 receptor (AT1R) are a first-line option for treating hypertension.

Altered handling of fluid and electrolytes by the kidney plays an important part in hypertension,3,4 and part of the hypertensive response to Ang II is reliant on the expression of AT1R within the proximal tubule of the kidney.3,4 However, mice lacking the Ang II type 1A receptor (AT1AR) in the kidney show a partial hypertensive response to Ang II infusion,5 suggesting that AT1R in other regions plays a role.

Elevated sympathetic activity is observed in hypertensive people and experimental models of hypertension,6-11 and approaches to reduce this, such as renal nerve denervation, are effective antihypertensive therapies.12

Studies in experimental animals and humans show a strong correlation between the distribution of AT1R and central and peripheral cells of the sympathetic nervous system. Sympathetic postganglionic neurons, including adrenal medullary chromaffin cells, express AT1R and are stimulated by Ang II.13 Through presynaptic receptors, Ang II potentiates the release of noradrenaline from sympathetic nerve terminals.14 Many catecholamine-synthesizing neurons in sympathetic premotor regions of the brain, including the rostral ventrolateral medulla (RVLM), express AT1R.14-16

Given this interaction between AT1R-expressing catecholaminergic cells and the involvement of both the angiotensin and sympathetic systems in the development and maintenance of hypertension, we hypothesized that the deletion of AT1R from catecholaminergic cells would affect both basal BP and the development of hypertension in response to infusion of Ang II. This hypothesis was tested in a transgenic mouse model using...
Cre-lox approaches to delete AT$_1$R expression from tyrosine hydroxylase (TH)-expressing cells using a TH-Cre recombinase mouse line that has been extensively characterized.$^{17}$

**Methods**

Detailed descriptions of all experimental methods are included in the online-only Data Supplement.

**Results**

Through selective breeding of transgenic mice, we obtained littermate mice with (CAT-KO), and without (wild type [WT]), the deletion of AT$_1$R from all TH-expressing cells.

**Verification of Recombination**

The distribution of AT$_1$R and Ang II type 2 receptor was examined by in vitro autoradiography in the adrenal medulla and sympathetic ganglia, where catecholaminergic neurons are known to express these receptors. In WT mice, AT$_1$R was expressed in the adrenal medulla and cortex and in the thoracic and lumbar sympathetic ganglia (Figure 1A and 1B). The Ang II type 2 receptor was predominantly localized to the adrenal medulla (Figure 1A). Binding to AT$_1$R was not detected in the adrenal medulla and sympathetic ganglia of the CAT-KO animals (Figure 1A and 1B). There was no obvious change in the density of binding to AT$_1$R in the adrenal cortex or to Ang II type 2 receptor in the adrenal medulla of the CAT-KO animals.

The pressor response to microinjection of Ang II into the RVLM occurs through AT$_1$R localized on catecholaminergic C1 neurons.$^{15}$ Microinjection of glutamate was used to functionally identify the RVLM in anesthetized mice. No difference in pressor response was observed between CAT-KO and WT mice (Figure 1E; Figure S2A and S2B in the online-only Data Supplement). Microinjection of Ang II into the RVLM of WT mice induced an increase in BP identical to that observed by this laboratory in Agtr1A$^{fl/fl}$ (Fl) mice$^{15}$ (Figure 1D and 1E). This response to Ang II was significantly attenuated in CAT-KO mice (Figure 1C and 1E). Postmortem histological analysis confirmed that these Ang II injections were within the region of the C1 neurons (Figure S2C).

**Baseline Characteristics of the Mice**

We observed no difference between the CAT-KO and WT mice in any basal parameter measured, including multiple measures of activity and metabolism (Figures S3A–S3F and S4G), BP (Figure 2A, 2B, and 2D; Figure S4A and S4C), and heart rate (HR; Figure S4E and S4I), and any measure derived from cross-spectral analysis of the BP and HR signal (Table S1).

**Angiotensin-Dependent Hypertension**

**BP, HR, and Activity**

In WT mice, Ang II infusion increased BP at day 5, and this reached a maximal level at day 13 (Figure 2A; Figure S4A). The BP remained elevated until day 21 before gradually returning to baseline by day 25. The CAT-KO mice also showed an increase in BP in response to Ang II infusion, but this was delayed in onset and reduced in magnitude (BP onset: WT day 5±0.5; CAT-KO day 7±0.7; $P<0.001$; maximum BP: WT 153±2.3 mm Hg; CAT-KO 137±3.5 mm Hg; $P<0.01$; Figure 2A,2C; Figure S4A). Apart from an initial delay in the BP response to Ang II infusion, female mice showed

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**Figure 1.** Assessment of angiotensin (Ang) II type 1A receptor (AT$_1$R) expression and function in catecholaminergic cells. Photomicrographs of x-ray films displaying $^{125}$I-[Sar$_1$, Ile$_8$] Ang II binding in the adrenal gland (A) and thoracic ganglion (B) of wild-type (WT) and CAT-KO mice. T depicts total receptor binding that occurs in all layers of the adrenal cortex and medulla and throughout the thoracic ganglion. L is binding in the presence of Ang II type 1 receptor (AT$_1$R) antagonist losartan and shows Ang II type 2 receptor (AT$_2$R) in the adrenal cortex and medulla, with background levels in the thoracic ganglion. PD is binding in the presence of AT$_2$R antagonist PD123319, which shows AT$_1$R expression throughout the adrenal cortex and medulla and thoracic ganglion of the WT. Expression of AT$_1$R is absent in the adrenal medulla and thoracic ganglion of the CAT-KO mouse. Scale bar, 200 µm. Ratemeter records of arterial pressure (AP), mean arterial pressure (MAP), and heart rate (HR) recorded from an anesthetized CAT-KO (C) and WT (D) mouse. The arrow denotes the time when 50 nL of Ang II (1 mmol/L solution) was injected unilaterally into the rostral ventrolateral medulla (RVLM). Scale bar, 20 s. E, Grouped data (n=4 for each genotype) showing the MAP response to these microinjections into the RVLM. Data are means±SEM. ***$P<0.001$, c indicates cortex of the adrenal gland; m, medulla of the adrenal gland; and ns, nonsignificant difference.
an identical response to the male mice (Figure S4B). During a 24-hour period, at day 13 the BP in both strains showed normal circadian entrainment and was significantly higher in the WT at all time periods (Figure 2E; Figure S4D).

In contrast to the change in BP, the HR response to Ang II infusion was minimal (Figure S4I), and there was no difference between the WT and CAT-KO mice (Figure S4F). Physical activity in the telemetered mice showed normal nocturnal entrainment and no difference between groups (Figure S4G and S4H).

**Cardiac Hypertrophy**

At the completion of the Ang II infusion period, there was an increased heart-to-body weight ratio in WT and Fl mice compared with noninfused littermates (Table S2). In contrast, there was no increase in the heart-to-body weight ratio of Ang II–infused CAT-KO mice (Table S2).

**Spectral Analysis**

Most parameters examined were not different between WT and CAT-KO mice either at baseline or after Ang II infusion (Table S1). Total power in the HR and mean arterial pressure spectra was increased in WT after Ang II infusion, with an increase being observed in the midfrequency band (0.3–0.5 Hz), suggesting an increased sympathetic influence on the circulation during Ang II infusion (Figure 3B; P<0.05). An increase in midfrequency HR or mean arterial pressure power did not occur in the CAT-KO mice during Ang II infusion (Figure 3A). After Ang II infusion, the midfrequency mean arterial pressure power was significantly elevated in WT compared with CAT-KO (P<0.05) mice.

**Brain Reactive Oxygen Species Production**

We examined reactive oxygen species (ROS) production by dihydroethidium fluorescence in several brain regions. No activation...
of ROS production was observed in noninfused CAT-KO or WT mice (Figure 4; Figure S5B). After Ang II infusion for 13 days, increased ROS production occurred in the subformical organ and hypothalamic paraventricular nucleus of both CAT-KO and WT mice (Figure 4A and 4B; Figure S5B). Increased ROS production was also observed in the RVLM of Ang II–infused WT mice, but this was markedly attenuated in CAT-KO mice (P<0.001 compared with Ang II–infused WT; Figure 4C; Figure S5B). Despite the dramatic decrease, a small increase in ROS production remained in the RVLM of Ang II–infused WT mice, but this was markedly attenuated in CAT-KO mice (Figure 4C; P<0.01). At this level of the medulla, the Ang II–induced increase in dihydroethidium fluorescence is confined to the region of the RVLM and does not occur in neighboring regions (Figure S5A).

Discussion
We characterized a unique mouse line in which the expression of AT1R is deleted from TH-expressing cells. This deletion was verified by loss of AT1R binding in sympathetic ganglia and adrenal medulla, as well as loss of a functional response to Ang II in the RVLM. At baseline, we observed no effect of this deletion. Subcutaneous infusion of a low dose of Ang II increased BP in both groups, but the increase was significantly delayed in onset (Discussion in the online-only Data Supplement) and reduced in magnitude in the CAT-KO mice. In WT mice, Ang II–dependent hypertension was associated with increased sympathetic activity as evidenced by increased power in the midfrequency band of the mean arterial pressure and HR spectra and activation of ROS production in key brain regions involved in the regulation of sympathetic activity. The CAT-KO mice have an attenuated sympathetic activation in response to Ang II and showed reduced ROS production in the RVLM. Overall, in Ang II–dependent hypertension, loss of AT1R on catecholaminergic neurons leads to a reduced hypertensive response, which is accompanied by attenuated cardiac hypertrophy. We conclude that direct effects of Ang II, potentially on both central and peripheral neurons involved in generating sympathetic activity, contribute to the development of Ang II–dependent hypertension.

No consistent changes were observed in HR or activity at baseline or during Ang II infusion in either WT or CAT-KO mice. Using spectral analysis, we also examined baroreceptor sensitivity and frequency power at multiple bands. None of these measures were altered either between strains or by Ang II infusion. The difference in sympathetic activation between WT and CAT-KO mice supports a role for Ang II interacting with the autonomic nervous system in the development of Ang II–dependent hypertension. Ang II–dependent hypertension in mice requires activation of neurons in the lamina terminalis and is associated with increased ROS production in key central cardiovascular nuclei. It is proposed that blood-borne Ang II acts in a circumventricular organ, most likely the subformical organ, to stimulate a multisynaptic pathway regulating sympathetic activity, in which brain-derived Ang II acts as a gain modulator. The first link in this chain where AT1R is expressed in TH neurons is the RVLM. In CAT-KO mice, ROS production occurs in several forebrain regions but is attenuated in the RVLM. The RVLM is a key node where supramedullary neurons synapse to modulate sympathetic vasomotor activity. Although we were unable to demonstrate loss of receptor binding within the RVLM or other central nervous system sites (see the online-only Data Supplement for further discussion), we did demonstrate functional loss of the response to microinjection of Ang II into the RVLM. Hence, we propose that stimulation of RVLM C1 neurons by Ang II plays a role in the development of Ang II hypertension.

**Figure 4.** Reactive oxygen species (ROS) production in brain during angiotensin II (Ang II) infusion in CAT-KO mice. The schematic coronal brain diagrams indicate the brain region examined: (A) subformical organ (SFO); (B) hypothalamic paraventricular nucleus (PVN); and (C) rostral ventrolateral medulla (RVLM). The bar graphs show grouped data (means±SEM) of ROS production, measured by dihydroethidium (DHE) fluorescence in noninfused wild-type (WT; n=4) and CAT-KO (n=4) and Ang II–infused WT (n=6) and CAT-KO (n=5) mice. These data are average fluorescence values at each brain region measured directly from the images with no manipulation. **P<0.01, ***P<0.001. ns indicates nonsignificant difference.
Both sympathetic postganglionic neurons and adrenal medullary chromaffin cells express TH and AT1AR. Ang II directly stimulates sympathetic postganglionic neurons, and this effect is enhanced in experimental hypertension, leading to elevated renal sympathetic nerve activity. Our data suggest that these neurons might also represent a key cell type where sustained Ang II infusion stimulates sympathetic activity to promote hypertension. Although a strong link between Ang II and stimulation of sympathetic nerve activity in many animal models of hypertension is accepted, most of these studies have concentrated on the link between systemic Ang II acting via the blood–brain barrier–deficient circumventricular organs such as the subfornical organ. The current observations indicate a direct effect of Ang II on catecholaminergic cells in the development of angiotensin-dependent hypertension.

This discussion has concentrated on sympathetic premotor neurons of the RVLM, sympathetic ganglia, and adrenal medullary chromaffin cells as sites of TH and AT1AR expression. Many other cells coexpress both these components and might contribute to any differences observed in our CAT-KO mice. There is considerable overlap in the distributions of AT1AR and catecholaminergic neurons throughout the neuraxis, including in the area postrema, caudal ventrolateral medulla, nucleus of the solitary tract, locus coeruleus, substantia nigra pars compacta, and several hypothalamic nuclei. Using a transgenic mouse expressing green fluorescent protein under the control of the AT1AR promoter, we confirmed cellular colocalization with catecholamine markers in RVLM. In addition to these central nervous system sites, transient expression of TH occurs in the developing heart, in cells destined to become pacemaker, and in cells of the conduction system. Expression of TH has also been reported in cells of the exocrine pancreas; non-neuronal, possibly immune cells of the duodenum; T lymphocytes; adipocytes; and skin keratinocytes. The association of each of these cell types with AT1AR expression has not been examined, and the role of Ang II and AT1AR in these cells in the angiotensin-induced hypertensive response is beyond the scope of this study.

Previous studies have used the same approach to induce cell-selective deletion of AT1AR. The deletion of AT1AR from the proximal tubules of the kidney decreased basal BP and reduced the hypertensive response to Ang II infusion. In contrast, the deletion of AT1AR from T lymphocytes and vascular smooth muscle cells of large arteries does not affect either basal BP or the hypertensive response to Ang II infusion. Our results are the first to demonstrate a significant role in the development of Ang II–dependent hypertension for AT1AR expressed in cells outside the kidney.

Using renal cross-transplantation approaches with kidneys from WT and AT1AR KO mice, Crowley et al also demonstrated the critical role of renal AT1AR in the development of Ang II–dependent hypertension and that this was largely as a result of altered sodium reabsorption. Mice with only renal AT1AR showed a delayed hypertensive response to Ang II infusion at 1000 ng/kg per minute but a maximal response similar to WT. Mice with AT1AR expressed in all organs except the kidney showed a dramatically attenuated hypertensive response although BP still increased. This experimental approach results in renal denervation of all cohorts, and so the involvement of altered sympathetic activity in the development of the hypertension cannot be determined. Clearly, the renin–angiotensin and the sympathetic systems interact closely to modulate sodium homeostasis.

Our data support the conclusion that angiotensin-dependent hypertension requires activation of the sympathetic nervous system in concert with direct renal tubular effects of Ang II, resulting in altered sodium and water handling by the kidney.

**Perspectives**

These results support the view that activation of the sympathetic nervous system is a key component of Ang II–dependent hypertension. Recent clinical studies showing decreased BP in drug-resistant hypertensive patients after radiofrequency lesions of the renal nerves support this view. Indeed, multiple therapeutic strategies designed to decrease sympathetic drive, including stimulation of baroreceptor afferents, are being tested in clinical trials for treatment of hypertension. Previous studies of Ang II–dependent hypertension have shown that Ang II acts via the subfornical organ to increase BP, presumably through increased sympathetic activity. We demonstrate that this activation of sympathetic efferent activity also requires the activation of AT1AR expressed by catecholaminergic cells most likely within the brain, as evidenced by the decreased ROS production in RVLM and in the periphery of the sympathetic ganglia. Clearly, Ang II–dependent hypertension requires activation of AT1AR in multiple cells throughout the body.

**Acknowledgments**

We acknowledge the assistance of Dr Kate Murphy and Prof Gordon Lynch with the metabolism studies.

**Sources of Funding**

This work was supported by the Australian National Health and Medical Research Council (No. 1029396, No. 1007451) and the Australian Research Council (DP1094301).

**Disclosures**

None.

**References**


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Acknowledgments

Sources of Funding

Disclosures

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Novelty and Significance

What Is New?

- Transgenic mice lacking the expression of angiotensin II (Ang II) type 1A receptor (AT1R) in catecholaminergic cells have normal basal characteristics but ameliorated hypertension in response to long-term Ang II infusion.
- This is associated with decreased sympathetic activity and reduced reactive oxygen species production in the rostral ventrolateral medulla.

What Is Relevant?

- We demonstrate that the expression of AT1R in cells outside the kidney is necessary for the full expression of Ang II–dependent hypertension.

Summary

The deletion of AT1R from catecholaminergic cells delays the onset of Ang II–dependent hypertension and reduces the maximal increase in blood pressure. This is associated with decreased sympathetic activity, reactive oxygen species production in the rostral ventrolateral medulla, and cardiac hypertrophy. The results show a key role for AT1R activation of catecholaminergic neurons in the development of Ang II–dependent hypertension.
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Hypertension. published online September 3, 2013;

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2013/09/03/HYPERTENSIONAHA.113.01474

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STIMULATION OF ANGIOTENSIN TYPE 1A RECEPTORS ON CATECHOLAMINERGIC CELLS CONTRIBUTES TO ANGIOTENSIN-DEPENDENT HYPERTENSION

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Supplemental Materials and Methods

Animals
Mice with a conditional Agtr1A allele (Agtr1A\textsuperscript{fl/fl}) were obtained from Dr T. Coffman’s laboratory at Duke University and bred as homozygotes (Fl) on a C57Bl/6 background.\textsuperscript{1} Mice with expression of cre-recombinase (Cre) under the control of the tyrosine hydroxylase (TH) promoter (TH-IRES-Cre\textsuperscript{+/–}) were obtained from Dr T. Ebendal’s laboratory via the European Mutant Mouse Consortium and maintained on a C57Bl/6 background.\textsuperscript{2} Both strains were bred in the Biological Resources Facility of the University of Melbourne, Australia to obtain Fl;TH-IRES-Cre\textsuperscript{+/–} and Fl;TH-IRES-Cre\textsuperscript{−/−} mice. These were bred to derive experimental mice with selective deletion of the AT1AR from TH-expressing cells (CAT-KO) and controls (WT). In most cases experiments were performed on WT and CAT-KO littermates. Prior to experimentation all mice were genotyped using standard PCR protocols. Experiments were performed in 3-6 month old CAT-KO, WT or Fl mice that were housed in standard cages on a 12:12 h light dark cycle with lights on between 6 am and 6 pm. Animals had free access to standard chow and water. The experimental procedures were approved by the University of Melbourne Animal Ethics and Experimentation Committee, and mice were handled in accordance with the guidelines of National Health and Medical Research Council of Australia, Code of Practice for the Care and Use of Animals for Scientific Purposes.

Genotyping Agtr1A\textsuperscript{fl/fl}; TH-IRES-Cre loci
Prior to experimentation all mice were genotyped. For genotyping, mouse tails were digested in Proteinase K lysis buffer (100 mmole/L Tris.Cl pH 8.5, 5 mmole/L EDTA, 0.2% (w/v) SDS and 200 mmole/L NaCl) containing 0.2mg/mL Proteinase K (Sigma Aldrich, St. Louis, MO) overnight at 55°C. Digested samples were spun down to remove undigested debris and genomic DNA was precipitated from the supernatant by adding equal volume of iso-propanol. The DNA pellet was washed with 70% (w/v) ethanol, air-dried and resuspended in TE buffer. For TH-IRES-Cre genotyping, PCR was performed using the following primers: TH-Fw 5’-CGG CGC TCC TTA GAG GGG GT-3’; TH-Rv 5’-GGG CAG GAG GAA TGC AGG ACC-3’ and Cre-UD 5’-GAT ACC TGG CCT GGT CTG-3’. The PCR reaction used 0.2 \mu moles/L of each primer, 50-100 ng DNA and a Taq DNA polymerase kit (New England Biolabs Inc, Ipswich, MA) following the manufacturer’s instructions. The thermocycler conditions were: initial denaturation at 95°C for 30 s; 35 cycles of 95°C for 30 s, 56°C for 1 min and 68°C for 1 min; final extension at 68°C for 5 min. PCR products were separated on 2% (w/v) agarose gel. The wildtype allele and TH-IRES-Cre allele amplified as 270bp fragment and 410bp fragments respectively. For Agtr1A\textsuperscript{fl/fl} genotyping, PCR was performed using the following primers: Agtr1A Fw 5’-CCG CGC TCC TTA GAG GGG GT-3’ and Agtr1A Rv 5’-AGG GGC GGT AGG AGA GCG-3’. PCR reaction was setup using 0.2 \mu moles/L of each primer, 50-100 ng DNA and Phusion High-Fidelity DNA polymerase kit (Finnzymes, Vantaa, Finland) following the manufacturer’s instructions. The thermocycler conditions were: initial denaturation at 98°C for 30 s; 35 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 1 min; final extension at 72°C for 5 min. PCR products (5 \mu L) were digested with Sall (site found in the loxP sequence). Both undigested and digested samples were separated on 2% (w/v) agarose gel. The wildtype allele (digested and undigested) showed a
2.5kb fragment. The Agtr1A floxed allele showed a 2.3kb fragment undigested and 1.5kb and 0.7kb fragments after digestion.

Assessment of basal metabolic parameters
Whole body metabolism and locomotor activity were measured using an Open Field Metabolic Chamber (Columbus Instruments) as described in detail elsewhere (Figure S1). Eight CAT-KO and 8 WT mice were acclimatized for 3 h before data were collected every 10 min over a 6 h light period and a 6 h dark period. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were used to calculate the respiratory exchange ratio (RER). VO2 and VCO2 were also used to calculate the total fat and carbohydrate (CHO) oxidation rates, as described previously. Fat and CHO oxidation rates were converted to kilocalories (9 kcal.g⁻¹ for fat; 4 kcal.g⁻¹ for CHO) and summed to calculate total energy expenditure. Fat and CHO oxidation as a percentage of total energy expenditure were also calculated. During the entire data collection period, mice received food and drinking water ad libitum.

Acute blood pressure and heart rate measurements in anesthetized mice
For acute arterial pressure recording, mice were initially anesthetized with inhalation of 4% isoflurane, and during initial surgery the anesthesia was maintained by 1.5-2.0% isoflurane via a nose cone. Four CAT-KO and 4 WT male mice were tracheotomised and artificially ventilated (SAR-830-P ventilator, CWE Inc., USA) with oxygen, and the end-tidal CO2 was maintained at 3.5–4.5% (MicroCapStar, CWE Inc., USA) and. Body temperature was kept at ~37°C with a heating pad (TC-1000 Temperature controller, CWE Inc.). The left carotid artery was cannulated with heat stretched polyethylene tubing (0.96 mm OD, 0.58 mm ID) for arterial pressure recording as previously reported. Arterial pressure was continuously monitored with a BP transducer (Neurolog System Digitimer Ltd, Hertfordshire, England) coupled to a data acquisition system (Cambridge Electronic Design Ltd (CED), UK) and the signal was saved for further analysis. Spike2 (CED, UK) software was used to derive mean arterial pressure (MAP) and HR from the BP pulse.

L-Glutamate and Ang II microinjections into the RVLM of anesthetized mice
The response to unilateral microinjections of L-glutamate (Sigma Chemical Co. USA) and Ang II (AusPep, Melbourne, Australia) in the RVLM was examined as described previously. In most mice independent injections were made into each side of the brain with at least 30 min elapsing between injections of Ang II. In brief, mice were anesthetized with isoflurane (4% for induction followed by 1.8-2% for maintenance of surgical anesthesia. Following carotid artery cannulation, mice were placed in a stereotaxic frame (Benchmark Stereotaxic Instruments, My Neurolab, St. Louis, MO, USA) and the skull was adjusted to be in a horizontal position between bregma and lambda. Injections were made through glass micropipettes made from capillary tubing (World Precision Instruments, Sarasota, FL, USA) with tip diameter 20-50 µm. For anatomical identification of the RVLM, microinjections of 10 nL (10 mmol/L solution) of L-glutamate were performed. The RVLM was determined as site where L-glutamate induced brief pressor response. Following L-glutamate, Ang II 50 nL (1 mmol/L solution) was injected bilaterally using the same coordinates (usual coordinates from lambda: anterior/posterior –1.7 mm; medial/lateral +1.2 mm; and dorsal/ventral –5.3 mm). Ang II was co-administered with 0.5% rhodamine-labeled microspheres (Invitrogen, Victoria, Australia) for histological verification of
the injection site. At the completion of the experiment, mice were killed by overdose of isoflurane and the brains were removed for post mortem analysis.

**Measurement of cardiovascular function by radiotelemetry**

Telemetry devices (Data Sciences International, MN, USA) were employed to record cardiovascular parameters in freely-moving mice as previously described. Stable recordings for the entire protocol, including 29 days post osmotic minipump implantation, were obtained in 4 WT and 4 CAT-KO male mice. As the maximal change in BP occurred at day 13 post Ang II infusion further experiments were concluded at day 14 post minipump implantation in 5 CAT-KO male; 3 CAT-KO female; 5 WT male and 3 WT female mice. Briefly, 30 min before the induction of anesthesia the mice received a subcutaneous injection of Carprofen (0.5 mg/100 g, i.p.; Norbrook Pharmaceuticals, KS, USA). Mice were anesthetized with inhalation of isoflurane (4%), and during the surgery the anesthesia was maintained with spontaneous breathing of 1.5 – 2.5% isoflurane in 100% O2 at a flow rate of 1 l/min via a nose cone. After determination of a surgical plane of anesthesia, a midline neck incision was made and the gel filled catheter from the telemetry probe inserted into the left carotid artery. The catheter was tied in place with surgical silk (5/0) and the body of the transmitter placed in a subcutaneous pocket made along the animal’s flank between the forelimb and the hind limb. After probe implantation, the skin incision was sutured and mice were allowed to recover for 10 days. After recovery, baseline BP and HR were recorded on a daily basis between 9:30 am and 12:30 pm for 4 consecutive days. The same cardiovascular parameters were recorded every second day after implantation of an osmotic minipump to infuse Ang II (see below). Twenty-four h recordings were also taken during the pre-infusion period and at day 13 after commencement of the Ang II infusion, in order to assess circadian variability and autonomic activity. Pulsatile arterial pressure was recorded and sampled at 1 kHz using an analog-to-digital data acquisition card (PCI-8024E; National Instruments, NSW, Australia). The beat-to-beat mean arterial pressure (MAP) and HR were detected and analyzed using a program developed at the Baker IDI Institute and written in Labview (National Instruments, NSW, Australia).9 Data are presented as average values over a 2 h period (10:30-12:30) during the day (Figure 2A, Figure S2A,B,I) or an hourly average throughout a 24 h cycle (Figure 2D-E, Figure S2C-H).

**Spectral analysis of blood pressure and heart rate**

Spectral analysis was performed on beat-to-beat data for MAP and HR in order to provide a surrogate measure of autonomic activity and baroreceptor reflex function. Measurements were made during periods with very low locomotor activity during the same night-time (between 21:00 and 1:00) and day-time (between 9:00 and 13:00) periods in individual mice. Measurements were made during the pre-infusion period and day 13 of Ang II infusion. The power and cross-power spectra were calculated for multiple overlapping (by 50%) segments of MAP and HR using Fast Fourier transform. The average value of the cross-spectral gain used as the estimate of the baroreflex sensitivity of the mid-frequency band (0.3-0.5 Hz). Baroreflex slope was considered acceptable if the coherence between MAP and HR across several overlapping segments in the analysed frequency band was >0.4. The mid-frequency band of the MAP power spectra was used as an index of sympathetic activity, whilst the mid and high frequency power of the HR spectra was considered to reflect vagal input to the heart.
Chronic angiotensin II infusions

Osmotic minipumps (model 1004, ALZET, CA, USA) containing Ang II (500 ng/kg/min) dissolved in 0.9% saline were implanted subcutaneously in all mice. Under isoflurane anesthesia, the mouse was shaved between the scapulae and an incision made to implant the osmotic minipump under the skin. Following implantation of the pump, the wound was closed with two Michel clips, and mice were placed individually in cages for recovery. This was performed in all mice included in this study.

Measurement of Superoxide production in the brain

ROS production in the brain cardiovascular centers was measured according to previously established protocols. At the end of the experiments, male mice that had been implanted with osmotic minipumps WT (n=6); CAT-KO (n=5) and non-infused WT (n=4) and CAT-KO (n=4) controls were killed by isoflurane overdose, the brains were removed and immediately frozen in iso-pentane equilibrated with dry ice. Thirty µm coronal sections were cut on the cryostat, immediately mounted on microscope slides and then stored at -80°C. Alternate sections were stained with thionin to identify the regions for measurement. Prior to the ROS measurement, corresponding sections at defined anatomical regions were thawed at room temperature, rehydrated with 0.1 mol/L phosphate-buffered saline (PBS), and treated with dihydroethidium (DHE; 1 µmol/L)(Sigma Chemicals, MO, USA) protected from light. The sections were washed once again with PBS and the fluorescence was visualized as previously described. Image J picture analysis software (version 1.45s-NIH) was used to quantify DHE fluorescence.

In vitro autoradiography

In vitro autoradiography was employed to measure AT1R and AT2R binding site distribution and density using standard procedures. The density and distribution of AT1R and AT2R binding was examined in various tissues from male CAT-KO (n=4) and WT (n=4) mice. Briefly, Ang II receptor binding density was determined using 125I-[Sar1, Ile8] Ang II as a radioligand. Adrenal glands and thoracic and lumbar ganglia from CAT-KO and WT mice were removed and snap-frozen in iso-pentane equilibrated with dry ice and stored at -80°C. Coronal sections from the adrenal glands or longitudinal sections from the ganglia were cut (20 µm) on the cryostat (-18°C), desiccated at 4°C and stored at -80°C. For incubation, sections were equilibrated to room temperature before being pre-incubated in 10 mmol/L PO4 buffer containing 2.0% bovine serum albumen (BSA) and 0.4 mmol/L bacitracin at room temperature for 20 min. Sections were divided into four groups and the first set was incubated for 1 hour at room temperature in incubation buffer containing 125I-[Sar1, Ile8] Ang II (~90 pmol/L) to determine total receptor binding. The other groups were incubated in the incubation buffer containing either 10 mmol/L losartan (to show AT2R), 10 mmol/L PD123319 (to show AT1R), or 1 mmol/L Ang II (non-specific binding). Sections were washed in cold buffer (4x1 min) and then dried overnight at room temperature. Sections were apposed to X-ray film along with 125I-radioactivity standards for 3 weeks in a lead protected case. After three weeks, the film was developed and the images scanned using a microcomputer image analysis system to determine receptor binding Scion Image Software (Scion Corp, Frederick, MD, USA).

Measurement of cardiac hypertrophy

At the end of the experiment, male mice that had been infused with Ang II were weighed and sacrificed by isoflurane overdose. Hearts were immediately removed and weighed after carefully
clearing away blood vessels, atria, fat and connective tissue. Cardiac mass was expressed as a ratio of heart weight (mg) to body weight (g) as described previously.\textsuperscript{15}

**Statistical analysis**

All grouped data are presented as mean ± standard error of the mean (SEM). The statistical tests applied are described in the supplementary methods section. All longitudinal BP, HR and activity data 24 h BP and activity measures were analysed by two way repeated measures analysis of variance (ANOVA) comparing genotype and time, using SigmaPlot version 11.2 (Systat Software Inc. CA, USA). Post hoc analysis employed the Holm-Sidak method. Assessment of the time of BP increases was made by examining the day when BP increased 10 mmHg over baseline average in each animal and compared between WT and CAT-KO by t-test. Comparison of RVLM responses to glutamate or Ang II; maximal change in BP to Ang II infusion; DHE fluorescence; and cardiac index were analysed by one or two way ANOVA as appropriate followed by the Holm-Sidak method for all pairwise multiple comparison. Cardiovascular data from the spectral analysis of BP was analysed by a split-plot repeated-measures ANOVA where between group sums of squares due to change over time or genotype were used to compare between groups. Individual between-group Bonferroni-adjusted contrasts were made to compare values on different days. Values were considered significant at $p<0.05$.  

Supplementary Discussion

Verification of the recombination and deletion of AT1AR expression has been performed using receptor binding and functional responses to Ang II. Loss of AT1AR expression from the sympathetic ganglia and adrenal medulla is obvious (Figure 1). We have shown functional deletion of the AT1AR from RVLM as microinjection of Ang II into this nucleus does not alter blood pressure. As we were examining a loss of response we verified that the injections were made in a region where glutamate increased blood pressure and we also verified the injection site anatomically (Figure S2). Together these observations verify the conditional knockout.

Despite attempts we were unable to anatomically demonstrate loss of AT1AR expression within a CNS site in the CAT-KO mice. There are several reasons for this. We cannot detect the receptor using immunohistochemistry as there are no selective antibodies. We have performed in vitro autoradiography, as shown for the adrenal medulla and sympathetic ganglia, but have difficulty detecting any change between CAT-KO and WT. This is not surprising as, unlike humans, cats and rabbits, AT1R binding in the ventrolateral medulla is extremely low in rodents. In receptor binding it is very difficult to convincingly detect deletion of levels that are already close to background. We have also looked in the NTS where an AT1AR-GFP mouse also indicates an association with TH-positive cells. However we cannot detect a change here. Again examination of the relevant information makes this less surprising – the majority of AT1R in the NTS are on vagal afferent terminals (60-70%) and so we would be looking at loss of binding on a small remnant component – we can’t detect that. The other prominent TH containing nuclei, such as the locus coeruleus, also have low AT1R levels mixed in with AT2R in the mouse. The TH-expressing neurons of the PVN do not express AT1R (according to the AT1AR-GFP mouse (unpublished observation)). We could perform quantitative PCR on punches of RVLM but these suffer from contamination due to vascular or glial receptor expression.

We were intrigued by the observation that the increase in blood pressure to Ang II infusion in the CAT-KO mice was not only decreased in maximal magnitude, but delayed in onset. The current view is that the neuronal component of Ang II-induced hypertension occurs later and is not responsible for the initial increase in BP. Direct data on Ang infusions and sympathetic activation is mostly derived from recordings in rabbits. These show that elevations in renal sympathetic activity are not observed in the early period of Ang II infusion (first 7 days) but become evident later (for example Guild et al. who used high salt in addition to Ang II infusion). Similar conclusions can be drawn from ganglion blockade experiments in rats. However, the interaction between Ang II and sympathetic nerve activity occurs at multiple levels from the central drive through to presynaptic modulation of noradrenaline release. Increased circulating Ang II in the presence of unaltered postganglionic sympathetic activity (the variable that is usually recorded) may well contribute to an increased neuroeffector response and the subsequent hypertension. This effect would be lost in the CAT-KO mice as they have lost their AT1AR in sympathetic postganglionic neurons. It is conceivable that increased systemic Ang II could interact with normal sympathetic postganglionic activity to affect the development of the hypertension in the early stages of this model.
Supplemental References


Supplemental Tables and Figures

Table S1. Average spectral power in mid frequency for coherence and gain, and low, mid and high frequency for MAP and HR in WT and CAT-KO mice at baseline and day 13 after Ang II infusion evaluated during the inactive period at night time (A) and day time (B)

<table>
<thead>
<tr>
<th>Night Time</th>
<th>WT</th>
<th>WT</th>
<th>CAT-KO</th>
<th>CAT-KO</th>
<th>WT</th>
<th>CAT-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Baseline</td>
<td>0.60 ± 0.02</td>
<td>0.63 ± 0.02</td>
<td>0.64 ± 0.02</td>
<td>0.66 ± 0.02</td>
<td>0.28</td>
<td>0.43</td>
</tr>
<tr>
<td>Day 13</td>
<td>15.16 ± 0.99</td>
<td>15.64 ± 0.77</td>
<td>15.78 ± 0.80</td>
<td>16.16 ± 1.26</td>
<td>0.09</td>
<td>0.77</td>
</tr>
<tr>
<td>Coherence</td>
<td>P</td>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gain</td>
<td>14.16 ± 0.11</td>
<td>15.31 ± 0.04</td>
<td>15.76 ± 0.80</td>
<td>16.16 ± 1.26</td>
<td>0.09</td>
<td>0.77</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>P</td>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Frequency</td>
<td>1.43 ± 0.11</td>
<td>1.84 ± 0.20</td>
<td>1.59 ± 0.13</td>
<td>1.82 ± 0.12</td>
<td>0.04</td>
<td>0.25</td>
</tr>
<tr>
<td>Mid Frequency</td>
<td>14.16 ± 0.99</td>
<td>15.64 ± 0.77</td>
<td>15.78 ± 0.80</td>
<td>16.16 ± 1.26</td>
<td>0.09</td>
<td>0.77</td>
</tr>
<tr>
<td>High Frequency</td>
<td>0.80 ± 0.10</td>
<td>1.19 ± 0.15</td>
<td>1.17 ± 0.13</td>
<td>1.30 ± 0.18</td>
<td>0.06</td>
<td>0.53</td>
</tr>
<tr>
<td>Total</td>
<td>3.78 ± 0.22</td>
<td>5.47 ± 0.53</td>
<td>5.08 ± 0.53</td>
<td>5.45 ± 0.42</td>
<td>0.02</td>
<td>0.58</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>P</td>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Frequency</td>
<td>195.0 ± 19.24</td>
<td>308.9 ± 39.62</td>
<td>278.0 ± 29.51</td>
<td>296.7 ± 53.25</td>
<td>0.03</td>
<td>0.73</td>
</tr>
<tr>
<td>Mid Frequency</td>
<td>94.2 ± 9.87</td>
<td>166.4 ± 21.22</td>
<td>131.7 ± 24.49</td>
<td>129.8 ± 22.42</td>
<td>0.02</td>
<td>0.85</td>
</tr>
<tr>
<td>High Frequency</td>
<td>359.4 ± 85.83</td>
<td>594.9 ± 43.98</td>
<td>581.2 ± 89.25</td>
<td>563.1 ± 72.75</td>
<td>0.20</td>
<td>0.50</td>
</tr>
<tr>
<td>Total</td>
<td>1218.6 ± 143.21</td>
<td>1822.9 ± 233.32</td>
<td>1462.7 ± 194.38</td>
<td>1507.3 ± 162.94</td>
<td>0.03</td>
<td>0.87</td>
</tr>
<tr>
<td>Day Time</td>
<td>WT</td>
<td>WT</td>
<td>CAT-KO</td>
<td>CAT-KO</td>
<td>WT</td>
<td>CAT-KO</td>
</tr>
<tr>
<td>B Baseline</td>
<td>15.90 ± 1.38</td>
<td>16.15 ± 1.33</td>
<td>14.07 ± 1.07</td>
<td>14.56 ± 1.71</td>
<td>0.00</td>
<td>0.81</td>
</tr>
<tr>
<td>Day 13</td>
<td>16.54 ± 0.77</td>
<td>15.78 ± 0.80</td>
<td>16.16 ± 1.26</td>
<td>0.09</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Coherence</td>
<td>P</td>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gain</td>
<td>15.16 ± 0.99</td>
<td>15.64 ± 0.77</td>
<td>15.78 ± 0.80</td>
<td>16.16 ± 1.26</td>
<td>0.09</td>
<td>0.77</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>P</td>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Frequency</td>
<td>1.43 ± 0.16</td>
<td>1.74 ± 0.18</td>
<td>1.57 ± 0.13</td>
<td>2.00 ± 0.20</td>
<td>0.21</td>
<td>0.11</td>
</tr>
<tr>
<td>Mid Frequency</td>
<td>14.16 ± 0.99</td>
<td>15.64 ± 0.77</td>
<td>15.78 ± 0.80</td>
<td>16.16 ± 1.26</td>
<td>0.09</td>
<td>0.77</td>
</tr>
<tr>
<td>High Frequency</td>
<td>0.80 ± 0.10</td>
<td>1.19 ± 0.15</td>
<td>1.17 ± 0.13</td>
<td>1.30 ± 0.18</td>
<td>0.06</td>
<td>0.53</td>
</tr>
<tr>
<td>Total</td>
<td>3.78 ± 0.22</td>
<td>5.47 ± 0.53</td>
<td>5.08 ± 0.53</td>
<td>5.45 ± 0.42</td>
<td>0.02</td>
<td>0.58</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>P</td>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Frequency</td>
<td>200.3 ± 25.18</td>
<td>312.6 ± 43.14</td>
<td>228.6 ± 17.91</td>
<td>309.0 ± 37.91</td>
<td>0.03</td>
<td>0.73</td>
</tr>
<tr>
<td>Mid Frequency</td>
<td>112.9 ± 15.39</td>
<td>149.3 ± 16.98</td>
<td>103.0 ± 12.89</td>
<td>142.9 ± 25.01</td>
<td>0.17</td>
<td>0.13</td>
</tr>
<tr>
<td>High Frequency</td>
<td>531.1 ± 97.21</td>
<td>497.9 ± 83.94</td>
<td>410.1 ± 49.06</td>
<td>354.6 ± 62.56</td>
<td>0.76</td>
<td>0.51</td>
</tr>
<tr>
<td>Total</td>
<td>1049.0 ± 146.18</td>
<td>1299.0 ± 195.31</td>
<td>894.7 ± 79.43</td>
<td>1064.3 ± 130.66</td>
<td>0.24</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Data are mean and standard error of the mean (SEM) indicating between animal variance (n=7 per group). P is probability for the difference between baseline and day 13 in the WT, P1 is probability for the difference between baseline and day 13 in the CAT-KO. Low frequency 0.08-0.3 Hz, Mid frequency 0.3-0.5 Hz, High frequency 0.5-1 Hz.
Table S2. Effects of systemic Ang II infusion (500ng/kg/min) on cardiac mass

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Body weight (g)</th>
<th>Heart weight (mg)</th>
<th>Heart weight to body weight ratio (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>24.5 ± 1.2</td>
<td>158.0±8.3</td>
<td>6.47± 0.2* †</td>
</tr>
<tr>
<td>AT1AR^{fl/fl}</td>
<td>27.2 ± 0.6</td>
<td>169.7±5.6</td>
<td>6.22± 0.1* †</td>
</tr>
<tr>
<td>CAT-KO</td>
<td>24.2 ± 1.1</td>
<td>129.2±5.2</td>
<td>5.34± 0.2</td>
</tr>
<tr>
<td>AT1AR^{fl/fl} (NI)</td>
<td>23.4 ± 0.9</td>
<td>122.0±6.7</td>
<td>5.20± 0.1</td>
</tr>
</tbody>
</table>

*P<0.05 vs. non infused, †P<0.05 vs. CAT-KO; NI-Non Infused. WT (n=9), AT1AR fl/fl (n=8), CAT-KO (n=9) and AT1AR fl/fl (NI) (n=4). Data are presented as mean ± SEM.
Figure S1. Genotyping of CAT-KO mice

Gel showing PCR analysis of tail tissue DNA from four mice (206,207,208,209). L-1kb ladder (promega), 430 bp-TH IRES Cre allele, 290bp- wild type allele.
Figure S2. Blood pressure response to L-glutamate microinjections in the RVLM of CAT-KO and WT mice

Ratemeter records of arterial pressure (AP), mean arterial pressure (MAP), and heart rate (HR) recorded from anesthetized CAT-KO (A) and WT (B) mice. The arrow indicates the time when 10nL of L-glutamate (10mmol/L solution) was microinjected into the RVLM. The scale bar represents 20 s. The photomicrograph shows the distribution of rhodamine-labeled microspheres that were co-injected with Ang II in the RVLM of a CAT-KO mouse taken under fluorescence illumination along with thionin staining of the same section taken under light illumination (C). Abbreviation: Amb-nucleus ambiguus.
Figure S3. Baseline metabolic characterization of WT and CAT-KO mice

Baseline metabolic parameters in WT and CAT-KO mice including volume of oxygen consumption (mL/kg/min) (A), carbon dioxide production (mL/kg/min) (B), CHO oxidation (g/min) (C), fat oxidation (g/min) (D), movement speed (cm/s) (E), and total energy expenditure (kcal/h) (F). There is no difference in the metabolic parameters between the two groups at baseline. Data are mean ± SEM.
Figure S4. Cardiovascular changes during development of angiotensin-dependent hypertension

Grouped data (mean±SEM) showing the mean arterial pressure (MAP) response recorded with radiotelemetry, before (B1-B4) and after infusion of Ang II (500ng/kg/min) in WT and CAT-KO mice (A). *P<0.05 or ** P<0.01- significant difference between WT and CAT-KO at the indicated time interval, #P<0.05 and ‡P<0.001- significant difference from baseline. Grouped data showing the systolic blood pressure response recorded with radiotelemetry before and after systemic infusion of angiotensin II (500ng/kg/min) in male (n=9) and female (n=3) WT mice and male (n=9) and female (n=3) CAT-KO mice (B). *P<0.05 and †P<0.01 indicate significant difference in male vs. female at the indicated time interval. Hourly averaged values depicting the circadian variation of mean arterial pressure (MAP) in WT and CAT-KO mice at baseline and day 13 after Ang II infusion. (C,D) Hourly averaged values depicting the circadian variation of heart rate (HR) (E,F) and activity (arbitrary units) (G,H) in WT and CAT-KO mice at baseline and day 13 after Ang II infusion. Grouped data showing the heart rate (HR) recorded with radiotelemetry before and after systemic infusion of angiotensin II (500ng/kg/min) in WT and CAT-KO mice (I). Data are mean ± SEM.
Figure S5. ROS production in key brain regions in response to Ang II infusion

A. Low power, light microscopic image of a thionin-stained, coronal section of mouse medulla at the level of the RVLM (central). Prior to staining the accompanying fluorescent images, showing DHE fluorescence, were taken of the boxed regions. These images are from an Ang II infused WT mouse. B. Fluorescence micrographs showing DHE fluorescence in the SFO, PVN and the RVLM of non-infused and Ang II infused WT and CAT-KO mice. Abbreviations: Amb-nucleus ambiguous; RVLM- rostral ventrolateral medulla; MVe- medial vestibular nucleus; Pr-
prepositus nucleus; Sp5I- spinal trigeminal nucleus, interpolar part; 3V-third ventricle. The scale bar represents 100µm in all images.