Upregulated Angiotensin Ia Receptors in the Hypothalamic Paraventricular Nucleus Sensitize Neuroendocrine Vasopressin Release and Blood Pressure in a Rodent Model of Polycystic Kidney Disease

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Abstract
Introduction: Angiotensin (Ang) II signalling in the hypothalamic paraventricular nucleus (PVN) via Ang type-1a receptors (AT1R) regulates vasopressin release and sympathetic nerve activity – two effectors of blood pressure regulation. We determined the cellular expression and function of AT1R in the PVN of a rodent model of polycystic kidney disease (PKD), the Lewis polycystic kidney (LPK) rat, to evaluate its contribution to blood pressure regulation and augmented vasopressin release in PKD. Methods: PVN AT1R gene expression was quantified with fluorescent in situ hybridization in LPK and control rats. PVN AT1R function was assessed with pharmacology under urethane anaesthesia in LPK and control rats instrumented to record arterial pressure and sympathetic nerve activity. Results: PVN AT1R gene expression was upregulated in the PVN, particularly in corticotrophin-releasing hormone neurons, of LPK versus control rats. PVN microinjection of Ang II produced larger increases in systolic blood pressure in LPK versus control rats (36 ± 5 vs. 17 ± 2 mm Hg; p < 0.01). Unexpectedly, Ang II produced regionally heterogeneous sympathoinhibition (renal: −33%; splanchnic: −12%; lumbar: no change) in LPK and no change in controls. PVN pretreatment with losartan, a competitive AT1R antagonist, blocked the Ang II-mediated renal sympathoinhibition and attenuated the pressor response observed in LPK rats. The Ang II pressor effect was also blocked by systemic OPC-21268, a competitive V1A receptor antagonist, but unaffected by hexamethonium, a sympathetic ganglionic blocker. Discussion/Conclusion: Collectively, our data suggest that upregulated AT1R expression in PVN sensitizes neuroendocrine release of vasopressin in the LPK, identifying a central mechanism for the elevated vasopressin levels present in PKD.

Introduction
Angiotensin II (Ang II) is a neuropeptide critically involved in cardiovascular homoeostasis and hydromineral balance. Ang II acts on the Ang II type 1 receptor (AT1R)
within the central nervous system to stimulate water and sodium consumption, increase neuroendocrine secretion of arginine vasopressin (AVP), and modulate sympathetic outflow to the kidney and circulatory system. The hypothalamic paraventricular nucleus (PVN) is an essential node for regulating neuroendocrine and sympathetic outflow [1, 2] and a major site of central Ang II action [3–7]. Microinjection of Ang II into the PVN evokes pressor responses in the anaesthetized rat, and the amplitude of pressor response to PVN Ang II microinjection is increased following osmotic and haemodynamic stress [8, 9], and in heart failure [10], renovascular hypertension [11], and diabetes [12, 13]. As such, augmented Ang II signalling in the PVN may contribute to the development of cardiovascular-related disease via increased AVP release and sympathetic overactivity.

Significant research effort has been dedicated to characterizing the central distribution of AT1R expression to establish where and how Ang II acts within the PVN. Surprisingly, the AT1R has not been localized to AVP-synthesizing magnocellular neurons nor to parvocellular neurons with projections to the rostral ventrolateral medulla (RVLM) or spinal cord [5, 14–17] despite functional evidence that Ang II can directly excite presympathetic PVN neurons [18, 19]. A model of indirect stimulation of AVP and presympathetic neurons by Ang II has therefore been proposed, which is well supported by pre- and postsynaptic mechanisms antecedent to neuroendocrine effectors. Postsynaptic expression of the AT1R is consistently observed in corticotrophin-releasing hormone (CRH) neurons, a neuronal population that mediates neuroendocrine stress responses [14, 15, 20] partially via (CRH) neurons, a neuronal population that mediates neuroendocrine stress responses [14, 15, 20] partially via.

Material and Methods

Male Lewis and LPK rats (13–14 weeks of age) were obtained from the Animal Resource Centre, Murdoch, Western Australia, and group-housed in standard living conditions with a 12-h light/dark cycle and access to standard rodent chow and tap water ad libitum.

Anatomical Experiments

Retrograde Tracing

Putative presympathetic PVN neurons with projections to the RVLM were identified using a retrograde tracing approach. Male Lewis and LPK animals (n = 4 per strain) were anaesthetized with isoflurane and administered carprofen (2.5 mg/kg s.c.) and cephazolin (50 mg/kg i.m.) for analgesia and antibiotic prophylaxis, respectively. With rats positioned in a stereotaxic apparatus, a small craniotomy was made in the occipital plate above the left RVLM. Antidromic mapping of the facial motor nucleus was used to identify the RVLM as previously described [33, 34]. Then 30 nL of cholera toxin subunit B (CTB)-555 (0.25% in saline; Invitrogen, MA, USA) was pressure injected 300 μm caudal and 300 μm ventral to the caudal pole of the facial motor nucleus (see online suppl. Fig. S1 for representative injection site; for all online suppl. material, see www.karger.com/doi/10.1159/000525337). These coordinates were optimized according to the distribution of spinally projecting RVLM neurons and their dendrites [34, 35]. After the surgery, the incision site was closed with surgical staples, and the animal was recovered. All animals received carprofen (2.5 mg/kg s.c.) and supplemental fluids on the first post-operative day and as required after that.

Fluorescent in situ Hybridization and Immunohistochemistry

The cellular distribution and expression level of AT1R mRNA was quantified in the PVN with RNAscope fluorescent in situ hybridization (FISH; Advanced Cell Diagnostics, CA, USA) in animals that received an RVLM injection of CTB-555 or no injection (n = 3–4 per strain). Animals were sacrificed 5 days after CTB-555 injection via cardiac exsanguination following sodium pentobarbital anaesthesia (100 mg/kg i.p.). Following immediate isolation of the brain, 25 μm thick coronal sections were obtained from fresh frozen tissue blocks using a cryostat, mounted onto Superfrost Plus slides (Thermo Fisher Scientific, MA, USA) and stored at −80°C. RNAscope FISH was performed as per the manufacturer’s instructions. On the day of the assay, slides were immersed in ice-cold 4% paraformaldehyde/0.1 M PB for 15 min and sequentially dehydrated with ethanol (50% for 5 min, 70% for 5 min, and 100% twice for 5 min). After air-drying the sections for ~10 min, a probe (Pre-treatment 4; Advanced Cell Diagnostics) was applied for 30 min at room temperature. After that, the sections were rinsed in phosphate-buffered saline (PBS) (twice for 2 min) and incubated for 2 h at 40°C in a negative control probe (DapB, a bacterial gene; Cat No. 320871), positive control probe (Cat No. 320891), or target probes against AT1R mRNA (Cat No. 422661-C1) and

PVN AT1R Increase BP via Vasopressin Release in PKD
either glial fibrillary acidic protein mRNA (GFAP, astrocyte marker; Cat No. 407881-C3), AVP mRNA (Cat No. 401421-C2), or CRH mRNA (Cat No. 318931-C3). Amplification steps were performed as per the manufacturer’s instructions using the AltA protocol. Slides were then coveslipped with Prolong Gold with DAPI (Thermo Fisher, Macquarie Park, NSW, Australia) or underwent immunohistochemistry procedures as follows.

Sections processed for FISH against GFAP mRNA were further processed with immunohistochemistry for GFAP protein to identify astrocyte somata robustly. Slides were washed in PBS (5 times for 2 min) and incubated in blocking solution (2% normal horse serum in Tris-PBS with 0.2% Tween) for 1 h at 4°C. Sections were then incubated in a solution containing a polyclonal rabbit anti-GFAP antibody (AB_477035, Sigma; 1:400 in blocking solution) for 14 h at 4°C. Slides were subsequently washed (PBS; 5 times for 2 min) and incubated for 3 h at room temperature in a solution containing Cy3-conjugated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch). After a final series of washes (PBS; 5 times for 2 min), sections were coveslipped with Prolong Gold with DAPI (Thermo Fisher).

**Image Capture and Analysis**

Z stack images (1.1 µm step size, 9–11 optical sections) were captured at x20 magnification using an Axio Imager epifluorescence microscope (Zeiss, Germany) and used to generate maximal intensity projection images for all analyses. The LED power and camera exposure time were constant for all experiments and optimized using sections hybridized with positive and negative control probes, such that there was a low level of fluorescence in sections hybridized with the DapB negative control probe.

With the RNAscope platform, bright puncta correspond to single RNA molecules [36]. We quantified individual AT1R mRNA puncta using the spot detection plugin in Icy software [37], with a sensitivity threshold optimized with sections that were hybridized with negative and positive control probes. This approach easily distinguished bright, positive control puncta from low-intensity background puncta. This plugin was used to automatically quantify the total PVN AT1R mRNA density bilaterally in one to four sections between −1.4 and −2.0 mm rostral of Bregma per animal and to identify AT1R mRNA puncta localized to the somata of astrocytes, AVP neurons, and CRH neurons, which were then manually counted. A cell with two or more AT1R mRNA puncta was defined as AT1R-expressing. Detection of total AT1R mRNA density and AT1R mRNA in astrocytes was performed bilaterally in two sections for each animal. Detection of AT1R mRNA in AVP neurons and CRH neurons was performed bilaterally in a single section between −1.6 and −1.9 mm rostral of Bregma. Detection of AT1R mRNA in CTB neurons was performed ipsilateral to the injection site in one or two sections.

**Physiological Experiments**

**Surgical Preparation**

Urethane was used to induce (1.3 g/kg i.p.) and maintain (65–130 mg/kg i.p. or i.v.) anaesthesia. The femoral artery and vein were cannulated to measure arterial pressure and administration of intravenous drugs and fluids (Hartmann’s solution 5 mL/kg/h), respectively. A tracheostomy was performed, and the animal artificially ventilated with oxygen-enriched room air adjusted to maintain arterial pH at 7.4 ± 0.05 and pCO2 at 40 ± 5 mm Hg. Animals were paralysed with cisatracurium (6 mg/kg i.v. for induction and 6 mg/kg/h i.v. for maintenance). Body temperature was assessed with a rectal thermometer and maintained at 37 ± 0.5°C with a heating mat and infrared lamp. For sympathetic nerve recordings, a dorsal flank incision was made to access the retroperitoneal cavity and expose the left kidney. As previously described [32, 38], the left greater splanchnic nerve, a branch of the left renal nerve, and lumbar sympathetic nerve were isolated for recording. Nerves were cut at their distal end, immersed in paraffin oil, and mounted onto a silver bipolar electrode.

**Drugs**

Drugs for PVN microinjection were 10 mM Ang II dissolved in 0.1 M PBS [39] (Abcam, Melbourne, VIC, Australia), and 500 mM losartan, an AT1R antagonist dissolved in PBS [39]; Abcam), which either contained fluorescent blue (1/2,000; Thermo Fisher Scientific, Waltham, CA, USA) or red polystyrene beads (1/1,000; Sigma-Aldrich) in the drug solution for validation of the microinjection site. Drugs administered intravenously included the ganglionic blocker hexamethonium (8 mg/kg in saline [40]; Sigma-Aldrich, Australia) and the selective V1a antagonist OPC-21268 (3 mg/kg in 20% DMSO-saline [41]; Sigma-Aldrich).

**PVN Microinjection**

Animals were positioned in a stereotaxic apparatus with the head in the flat-skull position. A small burr-hole was made on the dorsal surface of the skull to permit intracranial access for PVN microinjections. Following a stabilization period of at least 30 min, animals received a bilateral PVN microinjection (100 nL/side) of vehicle (PBS) or losartan. After microinjection, blood pressure, heart rate (HR), and sympathetic nerve activity (SNA) were recorded continuously for >15 min. Immediately after that, animals received a unilateral 50 nL PVN microinjection of Ang II. In a further cohort of LPK rats, a series of pharmacological experiments examined the effects of PVN Ang II pressor response following pre-treatment with the ganglionic blocker hexamethonium and pre-treatment or post-treatment with the selective V1a antagonist OPC-21268. These drugs were administered intravenously 5–10 min before or following the PVN Ang II microinjection. At the termination of the experimental protocol, all animals were euthanized with potassium chloride (3 M, 1 mL i.v.), brains removed, post-fixed in 10% neutral-buffered formalin for >12 h and cut at 75 µm with a vibrating microtome to identify the anatomical location of each injection site relative to the rat brain atlas [42]. The distribution of unilateral Ang II microinjection sites is shown in online Supplementary Figure S2.

**Data Acquisition and Analysis**

Arterial blood pressure was recorded via a pressure transducer, with the signal acquired using a CED 1401 plus (Cambridge Electronic Designs Ltd., Cambridge, UK). HR and systolic blood pressure were extracted from the arterial pressure waveform. SNA signals were amplified and band-pass filtered (30–1,000 Hz; Bioamplifier from CWE Inc., Ardmore, PA, USA) sampled at 5 kHz and digitized with a CED 1401 plus. All physiological data were captured and analysed using Spike2 software (Cambridge Electronic Designs Ltd.). SNA waveforms were rectified, smoothed (1-s constant), and normalized to the average activity 60 s before PVN microinjection (baseline), setting this as 100% and the level of SNA following euthanasia to 0%. All variables were averaged in 60 s bins before and after PVN injection. The quality of nerve recordings is illustrated in online supplementary Figure S3.
Statistical Analysis

Data are expressed as mean ± SEM unless otherwise stated. Statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). A mixed-effects model was used to compare the effect of strain and Bregma level on the density of AT1R mRNA in the PVN. Strains differences in the distribution of AT1R mRNA in specific cell groups were compared with an unpaired Student’s t test. Bonferroni’s correction was used for all multiple comparisons. Two-way ANOVA with Tukey’s multiple comparisons was used to determine if the drug response differed between strain or treatment groups. A one-way repeated measures ANOVA with Dunnett’s multiple comparisons was used to determine if a drug significantly influenced resting systolic blood pressure, HR, or SNA in each strain. Statistical significance was set at \( p \leq 0.05 \).

Results

Upregulated Ang 1A Receptor mRNA in the PVN of LPK Rats

FISH was used to detect and quantify the levels of AT1R mRNA in the PVN of LPK and Lewis animals.
(Fig. 1a, b). AT1R mRNA was more abundant in LPK than Lewis animals, evidenced by the greater density of AT1R mRNA puncta observed in LPK (Fig. 1a, b). Total AT1R mRNA density in the PVN region spanning −1.4 to −2.1 mm Bregma was 94% greater in LPK compared to Lewis rats (Fig. 1c; $p < 0.05$ for overall AT1R density) and was influenced by the rostrocaudal level of the PVN ($F_{3,10} = 9.052, p = 0.003$) being significantly greater in the LPK

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### Table 1. Quantification of AT1R mRNA in different PVN cell types in LPK and Lewis rats

| Parameter                  | LPK              | Lewis             | $p$ value |
|----------------------------|------------------|-------------------|-----------|
| **RVLM-projecting neurons**|                  |                   |           |
| Cells counted per animal    | 25 (17–33)       | 41 (13–71)        |           |
| AT1R + CTB+ (% CTB)         | 0                | 0                 |           |
| AT1R puncta per AT1R + CTB+ | –                | –                 |           |
| **Astrocytes**             |                  |                   |           |
| Cells counted per animal    | 163 (157–171)    | 140 (137–143)     |           |
| AT1R + GFAP+ (% GFAP)       | 3 (3–4)          | 2 (0–5)           | 0.387     |
| AT1R puncta per AT1R + GFAP+| 2.4 (2.1–2.6)    | 2.4 (2.0–2.8)     | 0.772     |
| GFAP mRNA density (puncta/nm$^2$)| 37.8 (35.4–40.0)| 34.5 (17.1–62.1) | 0.773     |
| **AVP neurons**            |                  |                   |           |
| Cells counted per animal    | 71 (34–105)      | 155 (127–175)     |           |
| AT1R + AVP+ (% AVP)         | 8 (4–12)         | 0 (0–1)           | 0.038     |
| AT1R puncta per AT1R + AVP+| 3.9 (2.9–5.0)    | –                 |           |
| **CRH neurons**            |                  |                   |           |
| Cells counted per animal    | 111 (101–132)    | 114 (97–141)      | <0.001    |
| AT1R + CRH+ (% CRH)         | 82 (76–92)       | 41 (40–41)        |           |
| AT1R puncta per AT1R + CRH+| 5.7 (4.8–6.9)    | 3.0 (2.7–3.2)     | 0.013     |

Values are presented as mean (min–max). $p < 0.05$ obtained using a Student’s t test was considered significant. $n = 4$ per strain for CTB experiment, and $n = 3$ per strain for all other experiments. A hyphen indicates that data were not quantified due to the absence of AT1R puncta for the cell type. LPK, Lewis polycystic kidney rat; RVLM, rostral ventrolateral medulla; GFAP, glial fibrillary acidic protein; AVP, vasopressin; CRH, corticotrophin-releasing hormone.
PVN AT1R Increase BP via Vasopressin Release in PKD

at posterior Bregma levels (−1.6 to −2 mm Bregma, \( p < 0.05 \); Fig. 1d).

**LPK Rats Exhibit Upregulated Ang 1A Receptor Expression in CRH and AVP Neurons but Not in Glia or Putative Sympathetic Premotor Neurons**

Using FISH combined with retrograde neural tracing from the RVLM, we found that AT1R mRNA was absent from all examined RVLM-projecting PVN neurons in both strains (putative sympathetic premotor neurons; Fig. 2 and Table 1). AT1R mRNA was absent from virtually all (3 ± 0.5 out of 152 ± 2 cells; 98%) GFAP-positive PVN astrocytes (Fig. 3 and Table 1), the somata of which was demarcated by morphology, visualized with GFAP immunohistochemistry, and dense clustering of GFAP mRNA. AT1R mRNA was present in 8% of AVP neurons in LPK rats but was absent from all but a single neuron in the Lewis rat cohort (Fig. 4 and Table 1). CRH neurons abundantly expressed AT1R mRNA in the LPK and to a lesser extent in the Lewis control rat, such that a significantly higher proportion of CRH neurons expressed AT1R mRNA in the LPK (93 ± 14 out of 111 ± 10 cells assessed; 82%) versus Lewis (47 ± 5 out of 115 ± 13 cells assessed; 41%, \( p < 0.001 \); Fig. 4 and Table 1). AT1R transcript (“puncta”) count per neuron was also two-fold greater in the CRH neurons of LPK rats (Table 1).

**PVN Microinjection of Ang II in the LPK Generates a Heightened, Sustained Pressor Response and Renal Sympathoinhibition**

Next, we examined cardiovascular responses to Ang II (50 nL, 10 mM) microinjection into the PVN of LPK and Lewis rats. In the LPK, Ang II produced a large and sustained increase in blood pressure that peaked ∼5 min after the injection and remained elevated beyond the 15-min recording epoch examined (peak response 35 ± 5 mm Hg; online suppl. Fig. S3; Fig. 5a, b). Ang II in the PVN of LPK rats also caused regionally heterogeneous sympathoinhibition with larger falls in renal SNA (−26 ± 6% of baseline 15 min after injection; online suppl. Fig. 5a–d; Fig. 5c) than splanchnic SNA (−11 ± 7% of baseline 6 min after injection; online suppl. Fig. 5a–d; Fig. 5d) and no statistically significant effect on lumbar SNA or HR (Fig. 4f, 5e). In contrast, in Lewis control rats, Ang II produced a smaller magnitude pressor response (peak 15 ± 3 mm Hg) that recovered within 5 min and had no effect on resting renal and splanchnic SNA or HR (Fig. 5a–f).

**PVN Microinjection of Losartan, a Competitive AT1R Antagonist, Blocked Ang II-Mediated Renal Sympathoinhibition and Pressor Response in LPK Animals**

Following this, we examined the extent to which the sympathoinhibition and pressor effects of PVN Ang II were mediated via local AT1R signalling in LPK rats. Animals received a bilateral PVN microinjection of 500 mM losartan, a competitive AT1R antagonist, before unilateral microinjection of 50 nL, 10 mM Ang II. Ang II responses after losartan were compared to the previous dataset. The effect of PVN losartan on baseline haemodynamic parameters is shown in online supplementary Table S1 and in online supplementary Figure S4. PVN losartan significantly attenuated the magnitude and time course of the pressor response and prevented the Ang II-induced inhibition of renal SNA (Fig. 6a, c).
PVN Ang II Pressor Response Is Mediated by AVP Release and Vascular V1A Receptor Activation in the LPK

Given the absence of evidence for regional sympathoexcitation associated with PVN Ang II, we next sought to establish whether the Ang II-evoked pressor response observed in the LPK was mediated by the hypothalamic-pituitary release of AVP with actions via vascular V1A receptors. To confirm an AVP-dependent mechanism and
simultaneously rule out the sympathetic nervous system, we performed a series of three pharmacological experiments in LPK animals: (1) systemic administration of an antagonist for vascular V$_{1A}$ receptors, OPC-21268, before PVN Ang II microinjection (V$_{1A}$ blocker pre-treatment); (2) elimination of SNA by ganglionic blockade (hexamethonium, i.v.) before PVN Ang II microinjection; and (3) ganglionic blockade before PVN Ang II microinjection, followed by intravenous OPC-21268 to test whether Ang II pressor responses are reversible by subsequent systemic V$_{1A}$ receptor antagonism (V1A blocker post-treatment). The effect of OPC-21268 and hexamethonium on

Fig. 5. Grouped data for PVN microinjection of Ang II in LPK and Lewis rats. The broken line indicates the time point of unilateral Ang II injection, which produced (a, b) a sustained pressor response, a regionally heterogeneous sympathetic response (c–e), and a negligible change in HR (f), in the LPK compared to control Lewis rats. Data are shown as mean ± SEM. # Indicates two-way ANOVA $p < 0.05$ strain × time effect. $n = 9$ LPK and $n = 8$ Lewis for SBP and HR; $n = 9$ LPK and $n = 7$ Lewis for renal and splanchnic SNA; and $n = 8$ LPK and $n = 2$ Lewis for lumbar SNA. Two-way ANOVA was not performed on lumbar SNA due to lower sample size in the Lewis. A one-way ANOVA revealed that Ang II did not alter lumbar SNA in LPK rats ($p = 0.430$). SBP, systolic blood pressure.
the baseline haemodynamic parameters measured is shown in online supplementary Table S1 and in online supplementary Figure S5.

Systemic pre-treatment with the V1A antagonist OPC-21268 eliminated the Ang II pressor response (Fig. 7a). By contrast, PVN Ang II after ganglionic blockade produced pressor responses equivalent to those seen in the sympathetic-intact state (Fig. 7a): Ang II evoked a 45-mm Hg increase at peak ∼5 min after the injection and remained elevated beyond the 20-min epoch examined (Fig. 7a). The renal sympathoinhibitory response to PVN Ang II persisted in LPK animals pre-treated with systemic V1A blockade (Fig. 7b). Finally, systemic V1A blockade administered shortly after PVN Ang II completely reversed the Ang II pressor response in LPK animals treated with ganglionic blockade (Fig. 7c, d).

**Discussion**

This study demonstrates for the first time that in a rodent model of PKD: (1) AT1R expression is upregulated in CRH-producing PVN neurons; (2) activation of PVN AT1R differentially regulates neuroendocrine and sympathetic outflows, resulting in the release of AVP and select regional sympathoinhibition; and (3) AT1R regulation of AVP underlies the enhanced pressor responses to exogenous Ang II.

Our observation that pressor responses to PVN Ang II microinjection were eliminated by prior systemic inhibition of V1A receptors, but not inhibition of sympathetic outflow, strongly suggests that PVN Ang II increases blood pressure by driving AVP release in both LPK and Lewis male rats. This is consistent with previous work showing that PVN Ang II increases the discharge of mag-
nocellular AVP neurons in vitro [43], and evokes systemic AVP release [3, 7]. Moreover, our data show that the pro-hypertensive actions of Ang II in the PVN are enhanced in PKD, as markedly exaggerated increases in arterial pressure were observed in response to PVN Ang II administration compared to control rats. The exaggerated Ang II pressor response in LPK animals is most likely due to greater quantile release of AVP from the neurohypophysis because we have previously shown that LPK rats have normal vascular reactivity to AVP [41]. It is impor-
tant to point out that our observations to date are limited to the male sex and future studies will need to examine whether AT1R-dependent Ang II sensitivity upregulates to a similar extent in female LPK rats.

Similar observations have been made in rat models of renovascular hypertension [11] and diabetes [12, 13]; however, in these disease models, the renal sympathoexcitatory action of PVN Ang II is also enhanced, in contrast to our observations in the LPK. The regional sympathoinhibition – most prominently in the renal SNA and lesser extent splanchic SNA – is most likely a direct response to PVN Ang II signalling because the sympathoinhibition persisted after V1A receptor blockade, which eliminated the Ang II pressor response and any effect of baroreflex-mediated sympathoinhibition. This patterned sympathoinhibitory response in the LPK may develop as the consequence of the chronic disturbances in fluid homeostasis that characterize PKD, where renal cysts impair urine concentrating capacity and promotes fluid depletion and plasma hypertonicity [44]. Thus, it is plausible that PVN microcircuits under the control of Ang II are (mal)adaptively altered in PKD to enhance vasopressin release, to restore water, and inhibit renal sympathetic tone, to minimize salt retention.

Our observations in the LPK are reminiscent of observations made in conscious sheep, whereby intracerebroventricular Ang II infusion produces an AT1R-dependent increase in blood pressure, baroreceptor-independent decrease in renal SNA, and increase in cardiac SNA, with no net effect on HR (presumably due to concurrent vagal activation) [45, 46]. As both the increase in blood pressure and reduction in renal SNA in response to intracerebroventricular Ang II in the sheep was dependent on the integrity of the lamina terminalis [47], it was concluded that the location of the AT1R responsible for this differential regulation of sympathetic outflow was the lamina terminalis and that the PVN acted as an integrator of input from the lamina terminalis that resulted in a selective reduction in renal SNA alongside an increase in cardiac SNA [48]. Our work extends these studies, adding a new perspective that suggests the actions of Ang II are localized to the PVN and that Ang II acts to facilitate the ongoing activity of PVN populations (likely CRH and AVP) that are regulated by inputs from the lamina terminalis.

The divergent effects of intracerebroventricular Ang II on blood pressure, renal SNA, and other sympathetic outflows mimic the effects of intracerebroventricular sodium administration, which produces an AT1R-dependent increase in blood pressure, AVP release and renal sympathoinhibition [48, 49], suggesting that the neural network recruited by sodium is perhaps the same as Ang II. Our observations in the LPK model suggest that in PKD, the sensitivity of these angiotensinergic, salt-sensing pathways is upregulated, leading to a greater propensity to drive AVP outflow, and consequently phasic increases in blood pressure and inhibition of renal sympathetic outflow when angiotensinergic tone is high. In the context of

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**Fig. 8.** Schematic representing the expression of AT1R and putative mode of action of Ang II under healthy conditions and in PKD. Under healthy conditions, AT1R on CRH or extra-PVN glutamatergic inputs results in the activation of AVP neurons that increase blood pressure via systemic AVP release. In PKD, AT1R expression is increased, notably on CRH-positive neurons. The increased AT1R expression results in an enhanced AVP-mediated increase in blood pressure. Additionally, activation of AT1R results in sympathoinhibition in PKD, possibly via a CRH-mediated activation of GABAergic interneurons in the PVN that inhibit presympathetic neurons that project to the RVLM or IML. IML, intermediolateral cell column. Created with BioRender.com.
PKD, this has significant clinical relevance as both blood pressure, and AVP levels are elevated in this disorder [41, 50]. AVP levels are strongly linked to cystogenesis [50], with a recent study showing that salt intake may accelerate renal disease progression in patients with PKD via salt-induced AVP release [51]. Further work is required to examine the central salt-sensing circuits in PKD and confirm their involvement in the overall progression of PKD.

The fact that the local actions of Ang II within the PVN can drive AVP release and significantly reduce renal SNA while simultaneously producing little to no change in other sympathetic outflows raises the question regarding the cellular location of the responsible AT1R. Our findings show that under healthy conditions, AT1R are only located postsynaptically on CRH neurons, although a limitation of the current study is the possibility that presynaptic AT1R could contribute to the effects seen [52–54]. However, determining the cellular location of AT1R is limited to techniques such as FISH due to the limited commercial availability of reliable AT1R antisera, precluding visualization of presynaptic AT1R.

The role of AT1R expressed on CRH neurons is incompletely characterized but offers an alternate explanation for the heightened AVP-mediated pressor response and renal sympathoinhibition observed in the LPK. Deletion of PVN AT1R (expressed on CRH neurons) results in a reduction in CRH mRNA levels, suggesting that PVN AT1R play an ongoing role in regulating CRH release [55]. Furthermore, the morphology of parvocellular CRH neurons is consistent with the ability to dendrodendrictically release CRH onto other hypothalamic nuclei [56]. It is plausible, therefore, that activation of AT1R on CRH neurons drives the dendrodendritic release of CRH that coordinates the activity of other populations of PVN neurons. Indeed, CRH1 receptors are also expressed by locally projecting GAD-67 and vGlut2 PVN interneurons which form monosynaptic connections with CRH neurons and other PVN neuronal populations (e.g., AVP neurons) [21]. Critically, while local CRH has net excitatory influence on the spiking of RVLM-projecting neurons under healthy conditions [22], activation of CRH1 receptor-expressing neurons can evoke IPSPs in preautonomic PVN neurons, potentially underpinning AT1R-dependent sympathoinhibition in PKD [21]. While CRH1 receptors are thought to be expressed predominantly on GABAergic interneurons within the PVN [57], specific stressors, particularly osmotic stress such as salt loading [58, 59] can induce CRH1 receptor expression on magnocellular AVP neurons within the PVN to drive AVP release. Therefore, an intra-PVN network may exist, illustrated in Figure 8, that couples the expression of AT1R on CRH neurons to the local release of CRH and a CRH1 receptor-dependent inhibition of preautonomic PVN neurons and activation of magnocellular AVP neurons. While this model requires further exploration, it may suggest the hypertension that occurs in PKD is in part due to osmotic-stress-induced actions of CRH locally within the PVN.

**Conclusion**

We have identified that PVN injected Ang II differentially regulates blood pressure and SNA in a rat model of PKD. Our findings support the hypothesis that Ang II transmission is enhanced in the PVN in PKD, reflected by an increased sensitivity of AVP-secreting neurons to Ang II and increased expression of AT1R in CRH neurons. This work has implications for the role of salt-induced regulation of AVP release in PKD and the overall progression of PKD.

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**Statement of Ethics**

Experiments were approved by the Macquarie University Animal Ethics Committee (ARA 2017/015, 2018/016) and performed according to The Australian Code of Practice for the Care and Use of Animals (8th Edition, 2013, updated 2021) and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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Author Contributions

Conor F. Underwood, Ann K. Goodchild, Jacqueline K. Phillips, and Cara M. Hildreth contributed to the conception and design of the work; Conor F. Underwood, Peter G.R. Burke, Natasha N. Kumar, Simon McMullan, and Cara M. Hildreth all contributed to the acquisition, analysis, and/or interpretation of data; Conor F. Underwood, Peter G.R. Burke, and Cara M. Hildreth drafted the work for publication; Conor F. Underwood, Peter G.R. Burke, Natasha N. Kumar, Ann K. Goodchild, Simon McMullan, Jacqueline K. Phillips, and Cara M. Hildreth revised the work critically for important intellectual contribution and approved the final version.

Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material.

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