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1	Kidney Dopamine D1-like Receptors and Angiotensin 1-7 Interaction Inhibits Renal
2	Sodium Transporters
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20 Abstract

21 The role of dopamine D1-like receptors (DR) in the regulation of renal sodium transporters, 22 natriuresis, and blood pressure is well established. However, the involvement of Angiotensin 23 (Ang) 1-7—Mas receptor (MasR) in the regulation of sodium balance and blood pressure is not clear. This study aims to investigate the hypothesis that Ang 1-7 could regulate sodium 24 25 homeostasis by modulating renal dopamine system. Sprague Dawley rats were infused with saline alone (vehicle) or saline with Ang 1-7, Ang 1-7 antagonist A-779, DR agonist SKF38393, 26 27 and antagonist SCH23390. Infusion of Ang 1-7 caused significant natriuresis and diuresis when 28 compared to saline alone. Both natriuresis and diuresis were blocked by A-779 and SCH23390. SKF38393 caused a significant, SCH23390 sensitive, natriuresis and diuresis and A-779 had no 29 effect on SKF38393 response. Concomitant infusion of Ang 1-7 and SKF38393 did not show a 30 cumulative effect when compared to either agonist alone. Treatment of renal proximal tubules 31 32 with Ang 1-7 or SKF38393 caused a significant decrease in Na/K-ATPase and Na/H-Exchanger 33 (NHE) 3 activity. While SCH23390 blocked both Ang 1-7 and SKF38393 induced inhibition, DR response was not sensitive to A-779. Additionally, Ang 1-7 activated protein kinase (PK) G, 34 enhanced tyrosine hydroxylase activity via serine-40 phosphorylation and increased renal 35 36 dopamine production. These data suggest that Ang 1-7 via PKG enhances tyrosine hydroxylase activity which increases renal dopamine production and activation of DR and subsequent 37 natriuresis. These studies provide evidence for a unidirectional functional interaction between 38 39 two G protein-coupled receptors to regulate renal sodium transporters and induce natriuresis. Keywords: Na/K-ATPase, Na/H-Exchanger, Natriuresis, Renal Tubules 40

42 Introduction

It is well established that renal dopamine system plays a pivotal role in maintaining body fluid 43 44 and electrolyte balance and long term blood pressure regulation especially during sodium replete condition (17, 40, 53). Renal dopamine activates D1-like receptors (DR) and inhibits tubular 45 sodium transporters such as Na/K-ATPase and Na/H-Exchanger (NHE) 3 and causes natriuresis 46 47 and diuresis (10, 23, 36, 51). It is believed that the renal dopaminergic system is a local independent system that acts in an autocrine or paracrine manner (2, 3, 12). The renal dopamine 48 49 production is dependent upon the tubular uptake of L-3,4-dihydroxyphenylalanine (L-DOPA) and enzymatic activity of aromatic L-amino acid decarboxylase (AADC) which converts L-50 DOPA to dopamine (2, 3, 12). The proximal tubules exhibit a high concentration of AADC and 51 are considered the primary source of renal dopamine (3, 12, 13, 17). It has been demonstrated 52 that renal specific deletion of AADC in mice leads to the development of hypertension and salt 53 sensitivity (57). Although, it has been postulated that other transporters belonging to solute 54 55 carrier superfamily which include basolateral organic cation transporters (OCT1, OCT2, and OCT3) and apical transporters (OCTN1, OCTN2, and OCTN3) also play a role in dopamine 56 transport, their impact on sodium regulation is not clear (30, 31, 55). It is widely perceived that 57 58 L-DOPA, freely filtered by the glomerulus, is transported into proximal tubules mainly by sodium-independent L-amino acid transporter (LAT) 2 making it a rate-limiting step in 59 dopamine synthesis (3, 11, 27, 45). However, recent studies show that an alternative pathway 60 involving tyrosine hydroxylase an enzyme which converts tyrosine to L-DOPA, a rate-limiting 61 step in neuronal dopamine synthesis, could be contributing to tubular dopamine production (52). 62 63 Renal Dopamine interacts with Angiotensin (Ang) II receptors, both type 1 and type 2 to

regulate sodium balance and blood pressure (15, 35, 40, 41, 46, 50). While the interaction with

65	Ang II type 1 receptor is antagonistic in that dopamine suppresses Ang II-mediated
66	antinatriuretic pathway, the interaction with type 2 receptors is synergistic as DR stimulation
67	induces natriuresis via Ang type 2 receptor (15, 35, 40, 41, 46, 50). However, little is known
68	about the interaction of renal dopamine with Ang 1-7-Mas receptor (MasR) system. The role of
69	Ang 1-7 in renal sodium is controversial, with some studies suggesting antinatriuretic effect
70	while others showing natriuretic effect (21, 37, 38, 43). It has been shown that Ang 1-7 can
71	activate serine/threonine protein kinases and inhibits renal proximal tubular sodium transporters
72	however, the mechanism remains unclear (14, 34). It is reported that Ang 1-7 activates protein
73	kinase (PK) G and PKG has been shown to stimulate tyrosine hydroxylase by phosphorylating
74	the enzyme at serine-40 (22, 26). Activation of tyrosine hydroxylase converts tyrosine to L-
75	DOPA, a rate limited step in dopamine synthesis. Interestingly, both PKG and tyrosine
76	hydroxylase are highly expressed in epithelial cells (1, 16, 20, 29, 52). Therefore, here we test
77	the hypothesis that Ang 1-7-MasR signaling could increase renal dopamine production via
78	tyrosine hydroxylase stimulation which will activate renal DR and induce natriuresis and
79	diuresis.
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86 Experimental procedure

87 Materials

- Angiotensin (Ang) 1-7 (Asp-Arg-Val-Tyr-Ile-His-Pro), A-779 (D-Ala7-Ang-(1-7)-a selective 88 Ang 1-7 antagonist, ouabain—a Na/K-ATPase inhibitor, S3226 (3-[2-(3-guanidino-2-methyl-3-89 oxo-propenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydro-chloride)-a 90 Na/H-Exchanger (NHE) 3 inhibitor, KT5823-a protein kinase (PK) G inhibitor, HBH (3-91 Hydroxybenzylhydrazine dihydrochloride)—an aromatic L-amino acid decarboxylase (AADC) 92 inhibitor, (3-Iodo-L-tyrosine)—a tyrosine hydroxylase inhibitor, KT5720—a protein kinase (PK) 93 A inhibitor, monoclonal anti-tyrosine hydroxylase antibody (catalog number: T2928), anti-94 phospho-tyrosine hydroxylase (pSer⁴⁰) antibody (catalog number: SAB4503789), secondary 95 96 antibodies and other analytical grade chemicals, unless otherwise mentioned, were purchased from Millipore Sigma (St. Louis MO, USA). Protein kinase G-1 antibody (catalog number: 97 3248) was purchased from Cell Signaling (Danvers, MA). 98 Surgical procedure for Ang 1-7 renal response 99 Eight-week-old male Sprague Dawley rats (Harlan, Indianapolis, IN) were group housed and had 100 free access to water and normal rodent diet. All the experimental procedures were approved by 101 IACUC. Surgeries were performed as detailed before (39), briefly, rats were anesthetized with 102 isoflurane/oxygen mixture and blood pressure was measured by catheterizing the left carotid 103 artery with a solid state pressure transducer connected to power lab (ADI Colorado Springs CO, 104 USA). For drug administration, the left jugular vein was catheterized with PE50 and for urine 105 collection, a midline abdomen incision was made to catheterize urinary bladder. Throughout the 106 107 surgery, the animals were infused with normal saline (1% body wt ml/h) and blood pressure and
- 108 heart rate were continuously monitored. To determine the effect of Ang 1-7 on sodium and water

excretion rats were infused with saline alone or saline with Ang 1-7 (1 µg·kg⁻¹·min⁻¹) or 109 SKF38393 (1 µg·kg⁻¹·min⁻¹). A-779 (100 µg·kg⁻¹), SCH23390 (100 µg·kg⁻¹) or KT5823 (10 110 $\mu g \cdot k g^{-1}$) were administered intravenously as a single bolus dose immediately prior to the 111 initiation of Ang 1-7 or SKF38393 infusion. The procedure consisted of a 40 min stabilization 112 period after the surgery followed by a 45 min collection of urine in the absence of drugs (saline 113 alone) followed by another 45 min period urine collection during drug infusion. Urine and 114 plasma sodium concentration was measured by atomic absorbance spectroscopy (Perkin Elmer 115 AA400) and creatinine levels were measured by creatinine analyzer (model 2, Beckman, CA). 116 117 Urinary volume was measured by Rainin electronic pipet (Mettler-Toledo Rainin, Oakland CA, USA). Glomerular filtration rate (GFR, ml/min) was calculated based on the clearance of 118 creatinine, and fractional excretion of sodium (FE_{Na}, %) was calculated based on the clearance of 119 sodium and creatinine. Urinary dopamine was measured by HPLC-mass spectrometry as detailed 120 before (6). 121

122 Na/K-ATPase and Na/H-Exchanger activity

A separate group of rats was used to prepare renal proximal tubules as detailed in our previous 123 124 publications (6-8). Renal proximal tubules were incubated for 10 min at 37°C with Ang 1-7 (0.1 μmol/L), SKF38393 (0.1 μmol/L), A-779 (10.0 μmol/L), SCH23390 (10.0 μmol/L), KT5823 125 126 (1.0 µmol/L), HBH (100 µmol/L), and 3-Iodo-L-tyrosine (100.0 µmol/L). Na/K-ATPase activity 127 was determined by the method of Quigley and Gotterer (47) with slight modification as reported 128 earlier (6-8). The tubules were lysed by rapid freezing and thawing with liquid nitrogen and protein was assayed by using a BCA kit (Thermo Fisher). The lysed tubular suspension (0.1 mg 129 130 protein/ml) was used to assay ouabain (4 mmol/L)-sensitive Na/K-ATPase activity, using endpoint phosphate hydrolysis of ATP (4 mmol/L) (6-8). NHE3 activity was measured in proximal
tubular brush border membranes as detailed before (7).

133 Tyrosine hydroxylase and Protein Kinase G expression and activity

Tyrosine hydroxylase expression and phosphorylation were determined by ELISA or western 134 blotting according to our previously published standardized protocol (5, 9). Briefly, microplates 135 were coated with an antigen (equal amount of cell lysate protein) and incubated with anti-136 tyrosine hydroxylase or anti-phospho-tyrosine hydroxylase (pSer⁴⁰) antibody and quantitated by 137 ELISA (5). We also coated microplates with an anti-tyrosine hydroxylase or anti-phospho-138 tyrosine hydroxylase (pSer⁴⁰) antibody followed by incubation with an equal amount of cell 139 lysate protein followed by ELISA (5). Renal proximal tubular tyrosine hydroxylase activity was 140 measured as described by Baillien et at (4). Briefly, proximal tubules were homogenized in 141 potassium phosphate buffer (50 mmol/L, pH 6.0) and 80 mg (1mg/ml protein concentration) was 142 added to an assay mixture containing 25 mmol/L L-tyrosine, ferrous ammonium sulfate (10 143 mmol/L), catalase (3,200 units), ascorbic acid (1 mmol/L), tetrahydrobiopterin and 0.1 mmol/L 144 HBH dissolved fresh in a small volume of 0.01 mol/L HCl, 50 mmol/L potassium phosphate 145 146 buffer pH 6.0 without and with 3-Iodo-L-tyrosine (100 µmol/L). The mixture was incubated for 15 min at 37°C and the reaction was stopped by adding chilled 10% trichloroacetic acid and 147 148 DOPA was measured by HPLC-mass spectrometry as described by Haavik and Flatmark (28) 149 and detailed in our previous publication (6). PKG expression was determined by ELISA (5) and activity was measured as described by Fiscus and Murad (25) and detailed previously (9). Renal 150 proximal tubular PKA activity was determined as detailed by Corbin and Reimann (18) in the 151 152 absence and presence of forskolin (10 µmol/L), KT5720 (0.3 µmol/L) and KT5823 (1.0 µmol/L).

153 Statistical analysis

154	Differences between means were evaluated by using ANOVA followed by post-hoc Newman-
155	Keuls multiple test. $P < 0.05$ was considered statistically significant. For in vivo experiments
156	(drug infusion), 10-12 rats were used in each group, and for ex vivo experiments involving
157	proximal tubules, 6-8 rats were used in each group. Experiments involving biochemical analysis
158	were performed in quadruplicate. ELISA was performed in quintuplicate i.e. at least 5 wells were
159	used for a single sample.
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172 **Results**

Effect of Ang 1-7 on urine flow, urinary sodium excretion, fractional excretion of sodium, and
urinary dopamine excretion

Intravenous administration of Ang 1-7 significantly increased urine flow (UF), urinary sodium 175 excretion (UNa), and fractional excretion of sodium (FENa) when compared to saline infusion 176 alone (fig 1A-C). Ang 1-7 had no effect on GFR (fig 1D). Ang 1-7 –mediated increases in UV, 177 UNa, and FENa were abolished by Ang 1-7 antagonist A-779 and DR blocker SCH23390 (fig 178 2A-C). SKF38393 -mediated natriuresis and diuresis was blocked by SCH23390 but was 179 insensitive to A-779 (fig 2A-C). Concomitant administration of Ang 1-7 and SKF38393 did not 180 have a cumulative effect on natriuresis or diuresis when compared to Ang 1-7 or SKF38393 181 182 alone (fig A-C). A-779 per se had no effect while SCH23390 alone or in combination with A-779 reduced UF, UNa, or FENA but the difference was not statistically significant when 183 compared to saline (data not shown). Ang 1-7 and SKF38393 had no effect on blood pressure as 184 compared to saline (mean arterial pressure, mmHg, saline— 107.33 ± 5.36 , Ang 1-7— $101.03 \pm$ 185 6.01, SKF38393—105.65 \pm 4.88). A-779 and SCH23390 also had no significant effect on blood 186 pressure (data not shown). 187

188 Ang 1-7 administration caused a profound increase in urinary dopamine excretion as compared

to saline (dopamine pmol/min, saline— 4.63 ± 0.41 , Ang 1-7— $8.91 \pm 0.71^*$, *P < 0.05 vs. saline).

190 The increase in dopamine production was blocked by A-779 and KT5823 but was insensitive to

191 SCH23390 (Ang 1-7+A-779—5.32 \pm 0.51, Ang1-7+KT5823—5.03 \pm 0.44, Ang 1-

192 7+SCH23390—9.1 \pm 0.89^{*}, **P*<0.05 vs. saline). In the absence of Ang 1-7, SKF38393,

193 SCH23390, A-779 and KT5823, per se, had no significant effect on basal dopamine excretion

194 (data not shown).

195 *Effect of Ang 1-7 on renal sodium transporters*

196 Incubation of renal proximal tubules with Ang 1-7 or SKF38393 inhibited Na/K-ATPase and

- 197 NHE3 activity significantly as compared to saline (fig 3A,B). The inhibitory effect of Ang 1-7
- 198 on sodium transporters was sensitive to both A-779 and SCH23390 (fig 3A,B). However,
- 199 SCH23390 and not A-779 blocked DR –mediated inhibition of sodium transporters. Concomitant
- incubation of proximal tubules with Ang 1-7 and SKF38393 did not have a cumulative inhibitory
- effect on the sodium transporters (fig 3A,B). A-779 and SCH23390 had no effect on basal Na/K-
- 202 ATPase (nmol pi/min/mg protein, saline—225.33 \pm 16.36, A-779—239.29 \pm 21.22,
- SCH23390—241.69 \pm 19.89) and NHE3 activity (²²Na⁺ nmol/mg protein/min, saline—5.3 \pm
- 204 0.42, A-779–4.9 \pm 0.39, SCH23390–5.1 \pm 0.44).
- 205 Effect of Ang 1-7 on protein kinase G and tyrosine hydroxylase expression and activity
- Ang 1-7 had no effect on PKG expression in renal proximal tubules (PKG-1 (α/β), OD at 450
- nm; saline— 0.67 ± 0.11 , Ang 1-7— 0.74 ± 0.13). However, Ang 1-7 significantly increased
- 208 proximal tubular PKG activity which was blocked by A-779 and PKG inhibitor KT5823 but was
- insensitive to SCH23390 (fig 4A). KT5823 could inhibit PKA activity at higher concentration.
- As illustrated in figure 4B, the concentration of KT5823 (1.0 µmol/L) used in the present study
- had no effect on PKA activity whereas KT5720, a more specific PKA inhibitor, reduced both
- basal activity and forskolin-induced activation of PKA. Ang 1-7 did not affect tyrosine
- 213 hydroxylase expression (OD at 450 nm; saline— 0.83 ± 0.17 , Ang 1-7— 0.91 ± 0.13), figure
- 4C—lower panel and supplement figure 1 [URL: <u>https://figshare.com/s/49cecaa9c0fe7a7c6949</u>
- 215 DOI: 10.6084/m9.figshare.9201893]. However, Ang 1-7 significantly increased tyrosine
- hydroxylase serine-40 phosphorylation (fig 4C—upper panel, fig 4D and supplement fig 2,
- 217 [URL: https://figshare.com/s/49cecaa9c0fe7a7c6949 DOI: 10.6084/m9.figshare.9201893]) and

activity (fig 4E), both of which were blocked by A-779 and KT5823 while SCH2339	0 had no
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- effect (fig 4C-E). Tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine had no effect on Ang 1-7-
- 220 mediated serine-40 phosphorylation (fig 4C—upper panel, fig 4D) but reduced basal tyrosine
- 221 hydroxylase activity and abolished Ang 1-7-induced stimulation (fig 4E). The basal tyrosine
- hydroxylase activity was not affected by A-779, SCH23390 or KT5823 (data not shown).
- 223 Effect of protein kinase G, tyrosine hydroxylase and dopamine decarboxylase inhibition on Ang
- 224 *1-7-mediated inhibition of renal sodium transporters*
- Exposure of proximal tubules to PKG, tyrosine hydroxylase, and AADC inhibitors KT5823, 3-
- 226 Iodo-L-tyrosine and HBH respectively had no effect on basal Na/K-ATPase (fig 5A) or NHE3
- 227 activity (data not shown). However, inhibition of PKG, tyrosine hydroxylase and AADC
- abolished Ang 1-7 –mediated regulation of both Na/K-ATPase and NHE3 (fig 5B,C). Inhibitors
- of PKG, tyrosine hydroxylase, and AADC failed to abolish SKF38393-induced inhibition of
- 230 Na/K-ATPase or NHE3 (data not shown).
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239 Discussion

The present study demonstrates that Ang 1-7—MasR signaling increases renal dopamine production by stimulating PKG—tyrosine hydroxylase activity. Moreover, the increased dopamine production via DR inhibits renal sodium transporters Na/K-ATPase and NHE3 and induces natriuresis and diuresis in response to Ang 1-7—MasR stimulation. Our results suggest that Ang 1-7—MasR signaling regulates renal sodium excretion by modulating local dopamine production.

Ang 1-7, a relatively newer member of the renin-angiotensin-aldosterone system, is 246 enzymatically generated directly from Ang 1 and Ang II by neprilysin and angiotensin-247 converting enzyme 2 respectively or indirectly by angiotensin-converting enzyme 2 -mediated 248 conversion of Ang I to Ang 1-9, which is converted to Ang 1-7 by neprilysin or angiotensin-249 converting enzyme (48, 56). Proximal tubules are exposed to circulating Ang 1-7 as well as Ang 250 1-7 from the glomerular filtrate. In addition, kidneys are exposed to locally generated Ang 1-7 251 explaining the higher renal vs. circulating Ang 1-7 levels. The role of Ang 1-7, unlike Ang II, in 252 kidney electrolyte regulation is not clear. While earlier reports suggested anti- natriuretic and 253 diuretic properties of renal Ang 1-7 in rats, recent reports show that Ang 1-7 deficit could 254 contribute to Ang II-mediated sodium and water retention and subsequent increase in blood 255 pressure (32). The exact mechanisms for these discrepancies are not known, however, the 256 variability in animal model, experimental site (ex vivo vs. in vivo) and drug administration route 257 could be possible causes for the variable outcome of Ang 1-7 effect on renal sodium regulation. 258 In here, we found that acute Ang 1-7 administration caused robust natriuresis and diuresis 259 260 without affecting GFR or blood pressure suggesting a tubular effect. As expected, the intravenous infusion of SKF38393 caused significant natriuresis and diuresis. Interestingly, the 261

effects of Ang 1-7 were abolished by both Ang 1-7 antagonist and DR blocker however, the
effect of DR agonist was insensitive to Ang 1-7 antagonist. These data suggest that Ang 1-7—
MasR –mediated sodium excretion involves DR activation while the natriuretic response to
SKF38393 is independent of Ang 1-7—MasR signaling. These findings are novel as they
identify a unidirectional, as opposed to mutual, interaction between two G protein-coupled
receptors to increase renal sodium excretion.

To identify the mechanisms for Ang 1-7-MasR and DR interaction in increasing sodium 268 excretion, we assessed the effect of Ang 1-7—MasR signaling on renal tubular sodium 269 270 transporters. The exposure of renal proximal tubules to Ang 1-7 or SKF38393 inhibited Na/K-ATPase and NHE3 activity. Interestingly, the Ang 1-7 –induced inhibition of sodium 271 transporters was sensitive to both A-779 and SCH23390 while DR effect was independent of 272 Ang 1-7 signaling. These data show that Ang 1-7—MasR inhibits renal tubular transporters via 273 DR activation and is in agreement with aforementioned in vivo finding. To find a link between 274 275 Ang 1-7—MasR and DR signaling as it relates to renal sodium regulation, we first measured urinary dopamine excretion. We found that Ang 1-7 treated rats exhibited a significant increase 276 in urinary dopamine level which was blocked by A-779 but insensitive to SCH23390 suggesting 277 278 that increased dopamine production involves Ang 1-7-MasR signaling but is independent of DR. Taken together, our data show that Ang 1-7—MasR stimulation increases renal dopamine 279 280 production which activates DR causing inhibition of Na/K-ATPase and NHE3 activity. The role 281 of Ang 1-7 in dopamine production and renal sodium regulation is conflicting. In contrast to our 282 data, Pawlak et al (44) reported that AT1R blockade is needed for Ang 1-7-mediated dopamine production in rat hypothalamus and Stragier et al (49) showed that conversion of Ang 1-7 to Ang 283 3-7 is responsible for dopamine production in rat striatum. Lara et al (33, 34) have shown that 284

Ang 1-7 via AT1R stimulates Na⁺-ATPase in adult pig renal tubules and inhibits Na/K-ATPase 285 in MDCK cells, however, the same group failed to observe Ang 1-7-mediated inhibition of 286 Na/K-ATPase in pig renal tubules (14). Consistent with our studies, DelliPizzi et al have shown 287 renal natriuretic effects of Ang 1-7 in rats (19). The exact mechanisms for these discrepancies are 288 not clear however the plausible explanation could be differences in animal model, central vs. 289 peripheral effect of Ang 1-7, water and sodium replete vs. deplete condition and local renin-290 angiotensin concentration. Nevertheless, we suggest that DR dependent inhibition of sodium 291 transporters could, in part, be responsible for Ang 1-7—MasR-mediated natriuresis and diuresis 292 293 as these transporters are responsible for more than 60% of transcellular proximal tubular sodium reabsorption (24, 54). 294

In neuronal cells, the rate-limiting step of dopamine synthesis involves tyrosine 295 hydroxylase -mediated conversion of tyrosine to L-DOPA which is decarboxylated to dopamine 296 by AADC (42). However, it is widely perceived that in kidney dopamine is locally synthesized 297 and involves apical uptake of filtered L-DOPA mainly via Lat 1 and Lat 2 (1-3, 11-13, 27, 45). 298 The involvement of transporters belonging to solute carrier superfamily have also been suggested 299 (30, 31, 55). However, the role of tyrosine hydroxylase, which is highly expressed in epithelial 300 301 cells, has not been fully assessed in renal dopamine synthesis (1, 20, 29, 52). The activity of tyrosine hydroxylase is highly regulated by serine phosphorylation involving various 302 serine/threonine kinases and phosphatases (22). While an increase in phosphorylation at serine-303 304 40 is known to activate the enzyme, the role of serine-19 or serine-31 is not clear (22). Herein, we found that Ang 1-7 had no effect on tyrosine hydroxylase expression but increased enzyme 305 serine-40 phosphorylation and activity. Ang 1-7 also increased PKG activity in renal tubules 306 without affecting PKG expression. Ang 1-7 mediated activation of both tyrosine hydroxylase and 307

308 PKG were sensitive to A-779 but independent of DR signaling. Additionally, Ang 1-7-MasR mediated serine-40 phosphorylation and activation were blocked by PKG inhibition. These data 309 provide strong evidence that Ang 1-7-MasR pathway increases dopamine production via 310 activation of tyrosine hydroxylase involving PKG. 311 To further substantiate the involvement of PKG-tyrosine hydroxylase in Ang 1-7-Mas 312 313 R mediated sodium excretion, we assessed Ang 1-7-induced inhibition of Na/K-ATPase and NHE3 in the absence and presence of PKG, tyrosine hydroxylase, and AADC inhibitors. We 314 315 found that Ang 1-7-MasR -mediated inhibition of renal sodium transporters was sensitive to PKG, tyrosine hydroxylase, and AADC inhibitors while these inhibitors had no effect on DR 316 signaling. Additionally, the infusion of PKG inhibitor abolished Ang 1-7-mediated increase in 317 urinary dopamine excretion. These data suggest that Ang 1-7-MasR -mediated renal sodium 318 regulation involves the modulation of local renal dopamine synthesis. 319 Limitations: The transport of renal tubular L-DOPA and tyrosine is complex and involves both 320 apical and basolateral, sodium-dependent and independent transporters. A detailed study is 321 warranted to identify the involvement of individual transporter in Ang 1-7-MasR signaling as it 322 relates to renal dopamine synthesis and sodium regulation. The investigation of these 323 transporters is beyond the scope of this study. 324 In conclusion, our data show that Ang 1-7-MasR invokes robust natriuresis and diuresis via 325 326 activation of renal DR. Mechanistically, Ang 1-7-MasR increased PKG activity, which activates tyrosine hydroxylase by serine-40 phosphorylation. The activation of tyrosine 327 hydroxylase leads to increased dopamine production and DR stimulation which in turn inhibits 328 329 renal sodium transporters Na/K-ATPase and NHE3. These phenomena lead to natriuresis and diuresis in response to Ang 1-7. While Ang 1-7—MasR –mediated renal sodium regulation is 330

331	dependent upon DR stimulation, SKF38393-DR -induced sodium excretion does not involve
332	Ang 1-7—MasR signaling. Taken together, this study provides a novel insight into the
333	complexity of renal sodium and dopamine regulation involving Ang 1-7-MasR and DR
334	interaction.
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337	Disclosures
338	No conflicts of interest, financial or otherwise, are declared by the author(s).
339	
340	Author Contributions
341	A.A.B. conception and design of research; A.A.B and A.D.D performed experiments and
342	analyzed data; A.A.B. interpreted results of experiments, prepared figures and drafted
343	manuscript; M.F.L. edited and revised manuscript; all authors approved the final version of the
344	manuscript.
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527 Figure legends

528 Figure 1. Effect of angiotensin (Ang) 1-7 on urine flow (UF), urinary sodium excretion (UNa), 529 fractional excretion of sodium (FENa), and glomerular filtration rate (GFR). Rats were infused 530 with saline and urine was collected for 45 min to establish a baseline. One group was continued on saline while the other group was administered Ang 1-7 in saline for 45 min. (A) Urine flow, 531 532 (B) urinary sodium excretion, (C) fractional excretion of sodium and (D) glomerular filtration rate were measured as detailed in material and method. *P < 0.05 vs. saline, repeated measures 533 ANOVA followed by Newman-Keuls post hoc test; n=10-12 rats. 534 Figure 2. Effect of angiotensin (Ang) 1-7 and dopamine D1-like receptor (DR) agonist 535 SKF38393 on urine flow (UF), urinary sodium excretion (UNa), and fractional excretion of 536 sodium (FENa). Rats were infused with saline alone or saline with Ang 1-7, SKF38393, Ang 1-7 537 antagonist A-779, and DR blocker SCH23390. After initiating drug infusion, urine was collected 538 for 45 to measure (A) Urine flow, (B) urinary sodium excretion, (C) fractional excretion of 539 sodium. *P < 0.05 vs. saline, 1-way ANOVA followed by Newman-Keuls post hoc test; n=10-12 540 541 rats. Figure 3. Effect of angiotensin (Ang) 1-7 and dopamine D1-like receptor (DR) agonist 542 SKF38393 on renal proximal tubular sodium transporters Na/K-ATPase and Na/H-Exchanger 543 (NHE) 3. Proximal tubules were incubated with Ang 1-7 and SKF38393 in the presence and 544 absence of Ang 1-7 antagonist A-779 and DR blocker SCH23390. Na/K-ATPase activity was 545 measured directly in lysed tubules (A), while NHE3 activity was measured in brush border 546 membranes isolated after incubation of tubules with desired drugs (B). P < 0.05 vs. saline, 1-way 547 548 ANOVA followed by Newman-Keuls post hoc test, n=6-8 rats. Na/K-ATPase and NHE3 were

sayed in quadruplicate from each rat.

551	Figure 4. Effect of angiotensin (Ang) 1-7 on protein kinase (PK) G and tyrosine hydroxylase
552	expression and activity. Renal proximal tubules were incubated with Ang 1-7 in the presence and
553	absence of Ang 1-7 antagonist A-779, DR blocker SCH23390, PKG inhibitor KT5823, and
554	tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine. Renal proximal tubular PKG activity (A) and
555	protein kinase A activity (B). Tyrosine hydroxylase (TH) expression and phosphorylation were
556	determined by western blotting (C) and ELISA (D). Tyrosine hydroxylase activity was
557	determined by HPLC-mass spectrometry (E). $*P < 0.05$ vs. saline, 1-way ANOVA followed by
558	Newman-Keuls post hoc test; n=6-8 rats. PKG and tyrosine hydroxylase activity was assayed in
559	quadruplicate while expression and serine-40 phosphorylation (ELISA) was performed in
560	quintuplicate from each rat.
561	Figure 5. Role of dopamine synthesis enzymes on angiotensin (Ang) 1-7 –induced inhibition of
562	sodium transporters Na/K-ATPase and Na/H-Exchanger (NHE) 3. Proximal tubules were
	F
563	incubated with Ang 1-7 and SKF38393 in the presence and absence of PKG inhibitor KT5823,
563 564	incubated with Ang 1-7 and SKF38393 in the presence and absence of PKG inhibitor KT5823, tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine, and aromatic L-amino acid decarboxylase
563 564 565	incubated with Ang 1-7 and SKF38393 in the presence and absence of PKG inhibitor KT5823, tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine, and aromatic L-amino acid decarboxylase (AADC) inhibitor 3-Hydroxybenzylhydrazine dihydrochloride (HBH). Na/K-ATPase activity
563 564 565 566	incubated with Ang 1-7 and SKF38393 in the presence and absence of PKG inhibitor KT5823, tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine, and aromatic L-amino acid decarboxylase (AADC) inhibitor 3-Hydroxybenzylhydrazine dihydrochloride (HBH). Na/K-ATPase activity was measured directly in lysed tubules (A and B), while NHE3 activity was measured in brush
563 564 565 566 567	incubated with Ang 1-7 and SKF38393 in the presence and absence of PKG inhibitor KT5823, tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine, and aromatic L-amino acid decarboxylase (AADC) inhibitor 3-Hydroxybenzylhydrazine dihydrochloride (HBH). Na/K-ATPase activity was measured directly in lysed tubules (A and B), while NHE3 activity was measured in brush border membranes isolated after incubation of tubules with desired drugs (C). * $P<0.05$ vs. saline,
563 564 565 566 567 568	incubated with Ang 1-7 and SKF38393 in the presence and absence of PKG inhibitor KT5823, tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine, and aromatic L-amino acid decarboxylase (AADC) inhibitor 3-Hydroxybenzylhydrazine dihydrochloride (HBH). Na/K-ATPase activity was measured directly in lysed tubules (A and B), while NHE3 activity was measured in brush border membranes isolated after incubation of tubules with desired drugs (C). * $P<0.05$ vs. saline, 1-way ANOVA followed by Newman-Keuls post hoc test, n=6-8 rats. Na/K-ATPase and NHE3
563 564 565 566 567 568 569	incubated with Ang 1-7 and SKF38393 in the presence and absence of PKG inhibitor KT5823, tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine, and aromatic L-amino acid decarboxylase (AADC) inhibitor 3-Hydroxybenzylhydrazine dihydrochloride (HBH). Na/K-ATPase activity was measured directly in lysed tubules (A and B), while NHE3 activity was measured in brush border membranes isolated after incubation of tubules with desired drugs (C). * $P < 0.05$ vs. saline, 1-way ANOVA followed by Newman-Keuls post hoc test, n=6-8 rats. Na/K-ATPase and NHE3 were assayed in quadruplicate from each rat.

Figure 1



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Figure 2

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Figure 4





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