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Role of Na/K-ATPase Non-enzymatic Signaling in Renal Proximal Tubule Sodium Transport

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ROLE OF NA/K-ATPASE NON-ENZYMATIC SIGNALING IN RENAL PROXIMAL TUBULE SODIUM TRANSPORT

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APPROVAL OF THESIS

We, the faculty supervising the work of Shreya Tapan Mukherji, affirm that the dissertation, *Role of Na/K-ATPase Non-Enzymatic Signaling in Renal Proximal Tubule Sodium Transport*, meets the high academic standards for original scholarship and creative work established by the Biomedical Research Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

This dissertation is dedicated to my advisor, the late Dr. Zijian Xie. I was extremely fortunate to have had a brilliant scientist, a pioneer, and a compassionate person like him as a mentor and I wish he were still here with us. I would also like to dedicate this dissertation to my parents and my sister- they have always been the wind beneath my wings.

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ABSTRACT

The renal proximal tubule (RPT) is the center stage for renal handling of Na^+, a potent determinant of systemic Na^+ and volume homeostasis in health and disease. In the RPT, through its classic ion-pumping function, Na^+/K^+-ATPase (NKA) generates the Na^+ gradient that drives apical Na^+ absorption, mostly through Na^+/H^+ exchanger (NHE3). In contrast, pharmacological evidence suggests that the activation of the NKA non-enzymatic signaling function, through Src and other signaling partners, triggers a cellular redistribution of NKA and NHE3 that decreases transcellular Na^+ flux in cultured RPT cells. However, the physiological relevance of the non-enzymatic function of NKA compared to its enzymatic function in the regulation of RPT Na^+ transport has not been directly studied. Thus, to address this, we developed a genetic approach in RPT cells and mice. Loss of NKA α1 in RPT cells *in vitro* and *in vivo* resulted in a prominent increase in transepithelial Na^+ transport coupled to an increased membrane abundance of apical NHE3 and basolateral Na/HCO_3^- cotransporter-1A (NBCe1A). Consistently, the hyper-absorptive phenotype observed in RPT-specific hypomorphic NKA α1 mice was rescued upon crossing with RPT-specific hypomorphic NHE3 mice, confirming the importance of NKA/NHE3 coupling. Consistent with previous pharmacological studies reported a role for Src in the natriuretic regulation by NKA receptor, rescue of RPT cells with wild-type but not Src-binding null NKA α1 restored NHE3 and NBCe1A to basal levels, indicative of a role for NKA/Src receptor function in the tonic inhibition of Na^+ transporters *in vitro*. *In vivo*, while female mice expressing Src-binding mutant NKA α1 in RPT exhibit increased NHE3 and RPT Na^+ transport, male mice exhibit no change. These observations are compatible with a sexual dimorphism in the NKA/Src mechanism of regulation of NHE3 and Na^+ transport in the RPT. Hence, NKA non-enzymatic signaling is a potent natriuretic mechanism of tonic inhibition of RPT Na^+ transport

by regulating key apical and basolateral Na^+ transporters. Remarkably, the natriuretic NKA signaling is also functionally dominant over its classically recognized anti-natriuretic ion-pumping role. NKA signaling therefore provides a long sought-after mechanism for the natriuretic action of endogenous NKA ligands such as cardiotonic steroids.

CHAPTER 1

INTRODUCTION

Hypertension, a condition of chronically elevated blood pressure of 140/90mmHg or more in humans, is one of the most common chronic diseases affecting over a billion people worldwide (Iqbal & Jamal, 2019; Lawes, Vander Hoorn, & Rodgers, 2008; Lloyd-Jones et al., 2010). Despite a wide prevalence of hypertension, a substantial portion of affected individuals does not achieve the target level of blood pressure control recommended by current guidelines (Tocci, Presta, Ferri, Redon, & Volpe, 2020). A major factor contributing to these poor outcomes is the gap in our understanding of the pathogenesis of hypertension. As many as 90% of the hypertensive cases are diagnosed as essential or idiopathic hypertension, i.e., hypertension caused from internal dysfunction of unknown cause (Iqbal & Jamal, 2019; Lawes et al., 2008; Lloyd-Jones et al., 2010). Interestingly, about half of the hypertensive cases are also classified as salt-sensitive hypertension making Na^+ and its systemic handling a major factor influencing the development of this chronic disease (Iqbal & Jamal, 2019; Lloyd-Jones et al., 2010; Sacks et al., 2001).

In 1909, Ernest Henry Sterling, in his landmark lecture series of 'The Fluids of the Body' stated that: "The function of the kidney is to keep the composition of the circulating fluid constant, and we can therefore alter the urine in any direction according to the nature of the changes we bring about in the composition of the body" (Starling, 1909). Decades later, Guyton and colleagues confirmed Sterling's foresighted postulates when they noted changes in urinary Na^+ and water excretion with changes in arterial pressure in intact animals and proposed a central role for the kidneys in the regulation of blood pressure, and as a corollary a key target for the pathogenesis of hypertension (Guyton, 1991; Guyton et al., 1972). The latter was confirmed by

kidney cross-transplantation studies in strains of hypertensive rats, in which an elevated blood pressure in the normotensive rat receiving the hypertensive donor kidney was observed (Bianchi, Fox, Di Francesco, Bardi, & Radice, 1973; Bianchi, Fox, Di Francesco, Giovanetti, & Pagetti, 1974; Churchill, Churchill, & Bidani, 1992).

Under normal physiological conditions, the kidney reabsorbs as much as 99% of the Na^+ in the filtered luminal fluid. Every thirty minutes, the kidney filters the entire plasma fluid and almost two-thirds of the Na^+ is bulk reabsorbed in the renal proximal tubule (RPT) which forms the earliest tubular segment of renal nephron. Studies in various cellular and animal models have shown that RPT has a major role in renal and systemic Na^+ handling and associated blood pressure homeostasis (Burnier, Bochud, & Maillard, 2006; Chiolero, Maillard, Nussberger, Brunner, & Burnier, 2000; Doris, 2000; McDonough, 2010b; Zhuo & Li, 2013). This homeostatic function requires the presence of many specific carriers to transport a large variety of substrates and their fine control by endogenous factors and hormones. The Na/K-ATPase (NKA) protein complex is expressed abundantly on the basolateral membrane of the polarized epithelial cells of the RPT. NKA has been at the forefront of studies to understand the mechanisms and regulations of RPT-mediated Na^+ transport, salt-sensing, and hypertension (Aperia, Bertorello, & Seri, 1987; Beach, Schwab, Brazy, & Dennis, 1987; De Wardener & Clarkson, 1985; De Wardener, Millett, Holland, MacGregor, & Alaghband-Zadeh, 1987; Doris, 2000; Katz, 1982; Maude, 1969; Nishi, Bertorello, & Aperia, 1992; Obando, Marín, Proverbio, & Proverbio, 1987; Pedemonte, Efendiev, & Bertorello, 2005b; Periyasamy et al., 2005; Rector Jr, 1983; Y. Zhang et al., 1998). In addition to its traditional ion-pumping function, the past few decades have seen an increase in awareness of the more recently identified non-enzymatic functions of NKA in studies spearheaded by independent groups worldwide (Aydemir-Koksoy,

Abramowitz, & Allen, 2001; Barwe et al., 2005; Haas, Askari, & Xie, 2000; Huang, Li, & Xie, 1997; Lee, Jung, Kim, & Guidotti, 2001; Miyakawa-Naito et al., 2003; Yan et al., 2013; L. Zhang, Zhang, Guo, & Wang, 2008). Specifically in the RPT, *in vitro* and pharmacological studies demonstrate NKA receptor-mediated regulatory function in Na^+ transport (Arnaud-Batista et al., 2012; H. Cai et al., 2008; Godinho et al., 2017; Liu et al., 2011). Even though several studies demonstrate NKA receptor-mediated inhibition of Na^+ transport in RPT cells, the physiological relevance of the non-enzymatic receptor function of NKA compared to its enzymatic function in the regulation of RPT Na^+ transport has not been extensively studied. Thus, considering the importance of RPT Na^+ absorption in human health and disease, we explored the importance of the NKA non-enzymatic signaling in the regulation of RPT Na^+ transport.

Renal Proximal Tubule: Structure and Function

Physiology and Pathophysiology

RPT is the earliest nephron tubular segment comprising of the proximal convoluted tubule followed by a proximal straight tubule (Wilhelm Kriz & Bankir, 1988; Wand Kriz & Kaissling, 2013; Zhuo & Li, 2013). It is responsible for most of the renal reabsorption with two-third of solutes, comprising of ions and organic nutrients, absorbed iso-osmotically (water and solutes in equal proportion) by the RPT. The proximal convoluted tubule forms the early S1 and S2 regions of the whole proximal tubule (Wilhelm Kriz & Bankir, 1988) and the region we are primarily referring to as RPT in this dissertation. It is in this S1 and S2 segments that most of the Na^+ in the proximal tubule is absorbed (Wand Kriz & Kaissling, 2013). Structurally, RPT has an optimal design for reabsorbing the bulk of the glomerular filtrate. It is characterized by leaky polarized epithelial cells with a thick apical brush border membrane which has microvilli

projections that increases the surface area for such bulk reabsorption (Wand Kriz & Kaissling, 2013; Zhuo & Li, 2013). The basolateral membrane of RPT comprises of extensive lateral invaginations and a closely associated large network of mitochondria that supports the high-energy demands of the basolateral expressed NKA (Wand Kriz & Kaissling, 2013). The active extrusion of Na^+ by NKA sets the electrochemical gradient for transporters expressed on the apical brush border membrane to couple Na^+ transport to that of specific solutes such as: glucose, sulfate, amino acids, phosphate, bicarbonate, and organic acids for their transcellular absorption. The leaky junctions between RPT cells gated by tight junction (TJ) proteins are a route of reabsorption for ionic solutes, like Na, K^+, Cl^-, Ca^{2+}, Mg^{2+}, along with water (Anderson & Van Itallie, 1995; Muto et al., 2010). As depicted in Figure 1, both transcellular transporters and paracellular junction proteins contribute to the bulk transport of solutes and water in RPT.

RPT functions are essential to renal Na^+ handling and systemic Na^+ and volume homeostasis. One such role is in the maintenance of glomerulotubular balance (GTB), a phenomenon by which changes in glomerular filtration rate (GFR) is accompanied by proportional changes in RPT absorption to minimize Na^+ wasting or prevent cessation of tubular excretion (Landwehr, Schnermann, Klose, & Giebisch, 1968). Dependent on GTB, any major fluctuations in GFR or RPT absorption influences Na^+ delivery to macula densa cells in distal tubule which then activates the tubuloglomerular feedback (TGF) mechanism to counteractively modulate GFR(Palmer & Schnermann, 2015). Although the molecular mechanisms of GTB are poorly understood, flow-dependent and mechanosensory changes are implicated in the adaptation of RPT Na^+ transport to GFR (Bartoli, Conger, & Earley, 1973; Du et al., 2004; Duan, Weinstein, Weinbaum, & Wang, 2010b; Palmer & Schnermann, 2015). Another function of RPT in Na^+ homeostasis involves its role in the mechanism of pressure natriuresis to restore systemic

homeostasis. Pressure natriuresis is the phenomenon of reduced Na^+ absorption and increased excretion in response to increased renal perfusion pressure, independent of GFR and TGB (Arendshorst, 1979; Arendshorst & Beierwaltes, 1979a). Depressed RPT Na^+ transport is the major contributing factor to this homeostatic response (Arendshorst & Beierwaltes, 1979b). Renal interstitial hydrostatic pressure (RIHP), nitric oxide (NO), and salt-sensing, all play a role in RPT's role in the pressure natriuresis response (Carlström, Wilcox, & Arendshorst, 2015; Hallow, Helmlinger, & Gebremichael, 2016), however the mechanisms are not completely understood. These functions are in addition to the modulation of RPT Na^+ absorption by numerous hormones, endogenous factors, and neuronal stimulation to maintain homeostasis (Aperia et al., 1987; Beach et al., 1987; Chiolero et al., 2000; Cogan, 1990; Cogan & Rector Jr, 1982; McKinney & Myers, 1980; T. Wang, Egbert Jr, Aronson, & Giebisch, 1998).

Despite the importance of RPT functions to the renal regulation of blood pressure, the distal nephron was traditionally attributed with the regulation of Na^+ and volume homeostasis through 'fine-tuning' and considered as the key pathological target for hypertension. Investigations in gene knockout animals along with clinical case studies have found the defects in Na^+ transport in the distal nephron to be associated with the rare monogenic and congenital forms of hypertension (Boscardin et al., 2018; Burnier et al., 2006; Lifton, Gharavi, & Geller, 2001; Rossier, Pradervand, Schild, & Hummler, 2002; Schultheis et al., 1998). However, several animal models and clinical studies, pertaining to the more prevalent polygenic hypertension, report an impaired and hyper-reabsorptive RPT associated with salt-sensitive hypertension (Bianchi et al., 1994; Burnier et al., 2006; Doris; Roos, Kirchner, Abernethy, & Langford, 1984; Thomas, Harris, &

Morgan, 1988). Thus, defining the molecular machineries involved in Na^+ sensing and absorptive functions of RPT is critical to our understanding of essential hypertension.

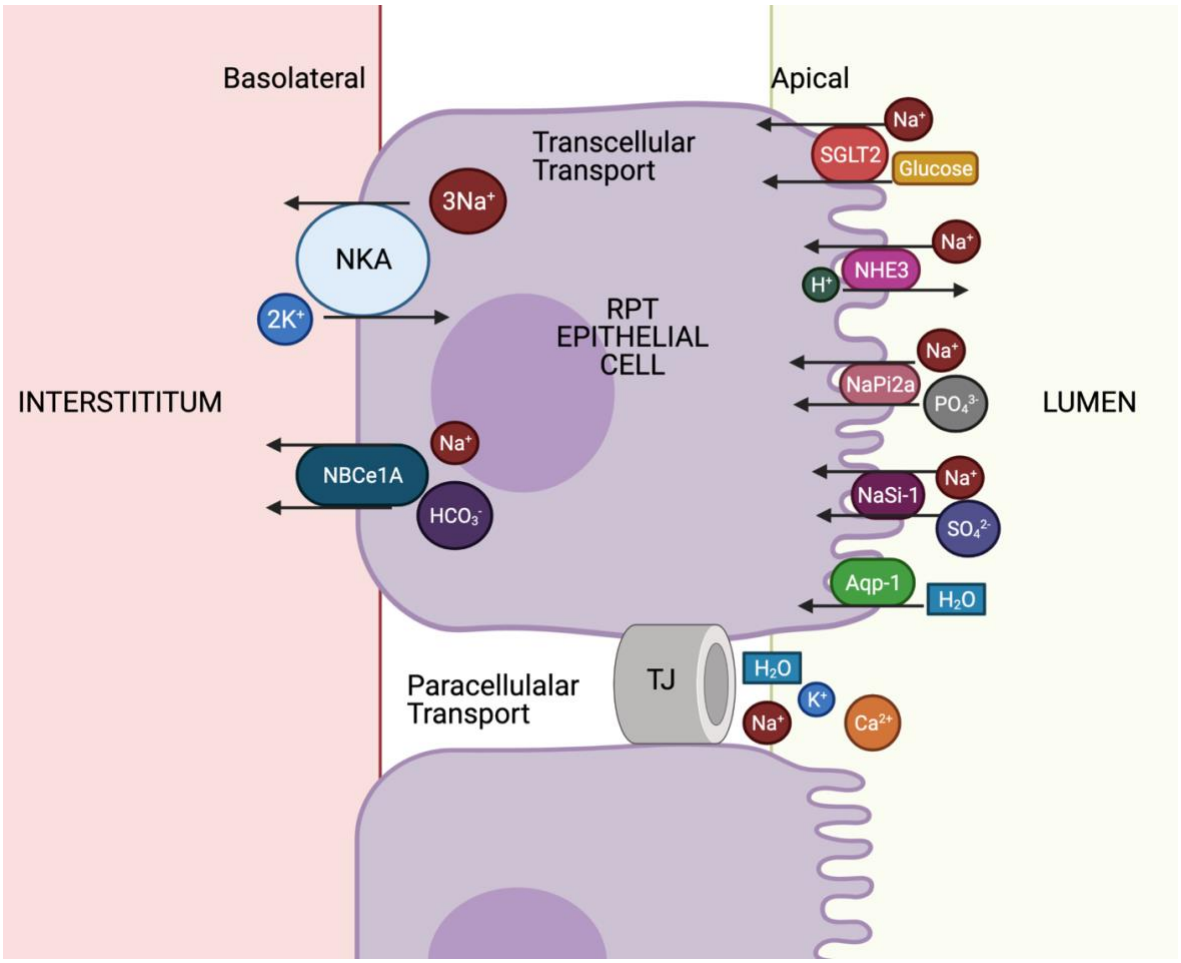

Figure 1: Transport of Solutes and water in RPT.
Scheme depicting several of the apical and basolateral transporters involved in transcellular transport along with the paracellular transport gated by tight junction (TJ) proteins to reabsorb solute and fluid in RPT. SGLT2: Na^+/Glucose cotransporter-2; NHE3: Na^+/H^+ exchanger-3; NaPi2a: Na^+/PO_4^{3-} cotransporter-2a; NaSi-1: Na^+/SO_4^{2-} cotransporter-1; Aqp-1: Aquaporin-1; NKA: Na^+/K^+-ATPase; NBCe1A: Na^+/HCO_3^- cotransporter-1A. Created by Biorender.

Sexual Dimorphism

The structure and function of the kidneys show sexual dimorphism in mammals (R. Hu, McDonough, & Layton, 2020; Q. Li, McDonough, Layton, & Layton, 2018; Veiras et al., 2017). In rodents, there are sex-dependent differences in the expression of renal transporters including a decrease in RPT transporters NHE3, Na^+/PO_4^{2-} (NaPi2a), and paracellular tight junction protein claudin-2, and an increase in distal tubule transporters $Na^+/K^+/Cl^-$ cotransporter (NKCC2) and Na^+/Cl^- cotransporter (NCC), in females compared to males (Veiras et al., 2017). This functionally translates to a decreased dependence on RPT-mediated bulk Na^+ absorption and higher Na^+ absorption in the distal tubule in females compared to males (Q. Li et al., 2018; Veiras et al., 2017). The sex-dependent differences could also be indicative of a tighter regulation of RPT absorption in the female rodents. The distinction in the distribution of Na^+ absorption across the nephron may be important to the sex-dependent differences in the flexibility and adaptability required to compensate for tubular defects.

Since RPT plays a crucial role in the homeostatic response to an elevated blood pressure, the sex-dependent differences may underlie a sexual dimorphism in systemic mechanisms of Na^+ and volume homeostasis. Indeed, women exhibit lower basal blood pressure and show decreased risk and severity of hypertension than men (Mozaffarian et al., 2016; Song, Ma, Wang, Chen, & Zhong, 2020). Therefore, elucidating the mechanisms underlying the sexual dimorphism in RPT Na^+ transport is vital to understanding the role of gender in pathogenesis of hypertension.

NKA as an Ion-Pump: Classic role in RPT Na^+ transport

Molecular composition and enzymatic functions of the renal NKA

The discovery of NKA in 1957 (Skou, 1957) by 1997 Nobel laureate Jens Skou was a defining moment in cellular physiology, which eventually paved the way for the identification

and characterization of the critical role of NKA enzymatic activity in RPT Na^+ reabsorption (Glynn, 1985; Gottschalk, 1962; Katz & Epstein, 1967; Maude, 1969; Ullrich et al., 1963; Whittembury, 1965; Windhager & Giebisch, 1961). A cornerstone of kidney transport machinery, NKA is a ubiquitous plasma membrane P-type ATPase protein that is highly conserved in eukaryotic cells. It is comprised of two subunits: catalytic α subunit (4 isoforms), and a membrane-trafficking and stabilizing β subunit (3 isoforms) (Blanco & Mercer, 1998). The kidney and the RPT mainly express a combination of α1 and β1 in the basolateral membrane, closely surrounded by mitochondria in the RPT cells. The catalytic α subunit, with a molecular mass of approximately 110 kDa, comprises of 10 transmembrane domains M1 to M10 with binding sites for Na^+ and ATP in the cytoplasmic domain and the binding sites for K^+ and cardiotonic steroids at the extracellular domain (Blanco & Mercer, 1998; Lingrel & Kuntzweiler, 1994; Mercer, 1993; Pressley, 1996). The glycosylated β glycoprotein subunit, with a molecular mass of 31.5 kDa, is functionally crucial for the efficient membrane trafficking of the α subunit, the expression of the enzyme on the plasma membrane, induction of tight junction assembly and polarity in epithelial cells, and in cell-cell adhesion function (Bab-Dinitz et al., 2009; Blanco & Mercer, 1998; D. C. Chow & Forte, 1995; McDonough, Geering, & Farley, 1990; Rajasekaran et al., 2001). The αβ complex forms the functional dimeric unit of sodium pump based on current evidence (Glynn, 1985; Glynn, 1993; Jørgensen, Håkansson, & Karlish, 2003; Skou & Esmann, 1992). FXYD proteins, which belong to family of single span membrane proteins characterized by the conserved FYXYD motif, bind to NKA to modulate its properties in a tissue-specific way but is not necessary to its pumping function itself. FXYD2, also referred to as γ, is expressed in the kidney with RPT cells expressing splice variant FYXYD2a. FXYD2 regulates the NKA enzymatic activity by decreasing Na^+ affinity and Vmax and the animal knockouts exhibit

increased renal cortical NKA activity but show no change in basal renal function (Arystarkhova & Sweadner, 2005; Jones et al., 2005).

NKA undergoes conformation changes on hydrolysis of ATP to pump $3Na^+$ out and $2K^+$ into the cell against their respect electrochemical gradients. Per the Albers-Post model (Figure 2)(Albers, 1967; Post, Hegyvary, & Kume, 1972), NKA and other P-type ATPases (Sachs & Munson, 1991) cycle through two main conformational states that can be either unphosphorylated (E1 and E2) or phosphorylated (E1P and E2P) and are characterized by their respective affinities for Na^+, K^+, and ATP, and by the accessibility of the cationic sites at the intracellular or extracellular sides of the membrane (Albers, 1967; Post et al., 1972). Na^+, ATP and Mg^{2+} bind to E1 on the intracellular side of the pump, promoting the phosphorylation of E1. After release of ADP, the exergonic trans-conformation of E1P to E2P occurs, which promotes the extracellular delivery and release of the occluded Na^+ followed by the binding of extracellular K^+. Binding of K^+ induces the dephosphorylation of E2P. Spontaneous reversion to E1 releases the occluded K^+ inside the cell, thereby completing one entire reaction cycle (Albers, 1967; Post et al., 1972). Because of its $3Na^+/2K^+$ stoichiometry, the pump is electrogenic, and generates a negative electric potential difference across cell membrane. By generating the electrochemical gradient of Na^+ and K^+, NKA not only maintains the membrane potential but also performs the vital function of cell volume regulation. Indeed, NKA's expression and activity are altered to adapt to hypertonic or hypotonic challenges to maintain the cell volume (Yordy & Bowen, 1993).

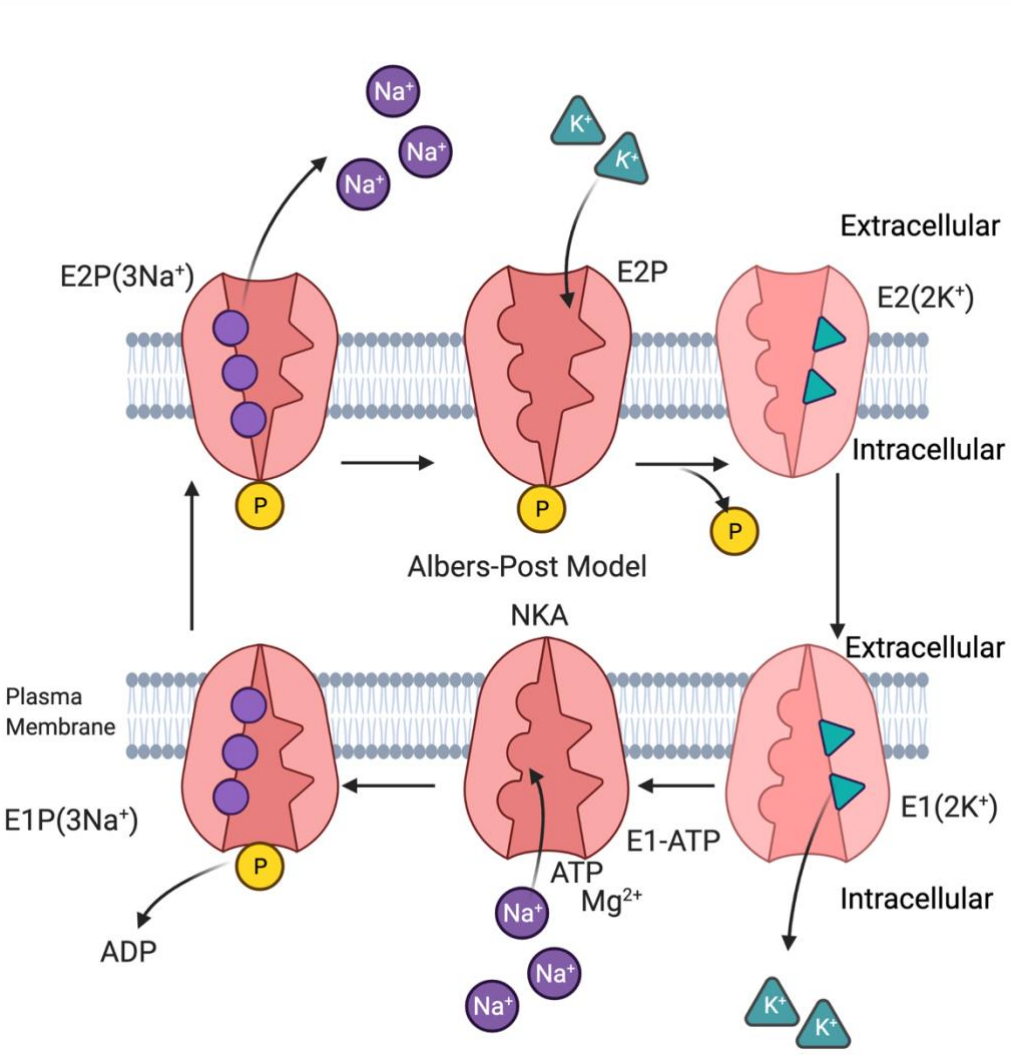

Figure 2: Albers-Post mechanism of active transport by NKA.
Schematic of active transport of $3Na^+$ and $2K^+$ by NKA through changes in its conformation as described by the Albers-Post mechanism. Encircled P indicates phosphorylation status of NKA conformation. Created by Biorender.

Transcellular Na^+ Transport in the RPT

As early as 1961, Windhager and Giebisch identified the active nature of transcellular Na^+ transport and postulated that it was the primary mechanism of Na^+ transport in the RPT based on early short circuit experiments on rat kidneys (Windhager & Giebisch, 1961). The kidney is a rich source of NKA, and the abundantly expressed basolateral NKA drives the active

transcellular Na^+ transport across renal epithelium including RPT cells (Gottschalk, 1962; Hook, 1969; Jørgensen, 1980; Maude, 1969; Schatzmann, Windhager, & Solomon, 1958). Most of our current knowledge on the role and contribution of NKA ion-pump in RPT Na^+ transport is based on early pharmacological studies using ouabain (Hook, 1969; Maude, 1969; Schatzmann et al., 1958). Like other cardiotonic steroid (CTS), ouabain is a natural and potent inhibitor of NKA. Ouabain binds extracellularly to NKA under its E2P conformation, decreasing its affinity for K^+ and competitively inhibits its enzymatic activity (Yatime et al., 2011). Various CTS have been isolated and identified endogenously in plants and animals, including in human beings (Bagrov, Fedorova, Austin-Lane, Dmitrieva, & Anderson, 1995; Hamlyn et al., 1991; Laredo, Hamilton, & Hamlyn, 1995).

In RPT, around 25-50 million NKA units are expressed in each cell (El Mernissi & Doucet, 1984; Jørgensen, 1980). However, the assessment of NKA ion-transport activity *in situ* with the ouabain-sensitive rubidium uptake indicates that the enzymatic pump is working at 20–30% of its maximum rate in intact cells (Cheval & Doucet, 1990; El Mernissi & Doucet, 1984; Féraille & Doucet, 2001). This proportion is consistent with measurements of intracellular Na^+ concentration and Na^+ affinity (Cheval & Doucet, 1990; El Mernissi & Doucet, 1984; Féraille & Doucet, 2001). The redundancy in expression of NKA has been proposed as an indicator of the non-enzymatic functions of NKA.

Na^+ transport across RPT epithelia is dynamically controlled by changing apical Na^+ entry coupled with basolateral Na^+ exit to increase or decrease absorption. Most of the luminal Na^+ along the early RPT is apically transported by the Na^+/ H^+ exchanger-3 (NHE3) in exchange for proton (Wu, Biemesderfer, Giebisch, & Aronson, 1996). As depicted in Figure 3, the electrochemical gradient generated by NKA drives the Na^+ absorption predominantly by

apical NHE3. Other Na^+-dependent transporters and paracellular pathway also contribute to the total transepithelial flux (Figure 1). The uptake of Na^+ in exchange for H^+ secretion by NHE3 also results in luminal bicarbonate (HCO_3^-) uptake by the RPT cells, which is transported along with Na^+ by basolateral Na^+/ HCO_3^- cotransporter-1A (NBCe1A). Other important apical Na^+ transporters also utilize the Na^+ gradient set by NKA to transport glucose (SGLT-2), phosphate (NaPi-2a), sulfate (NaSi-1), and amino acids and several organic acids, along with Na^+.

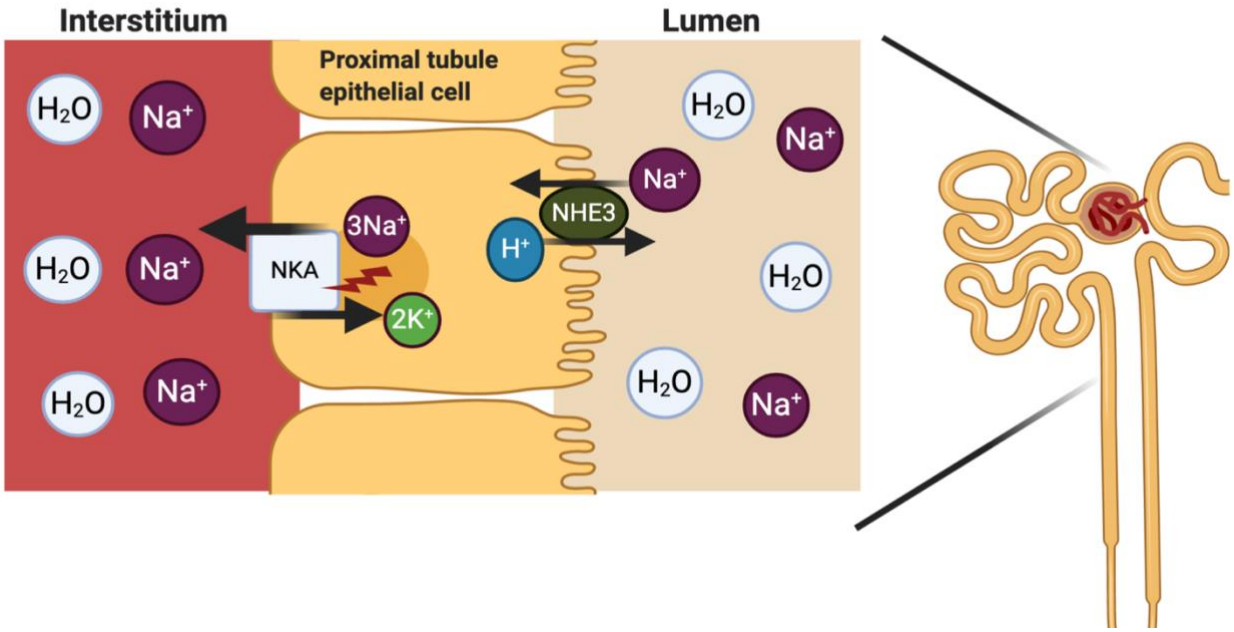

Figure 3: NKA enzymatic ion-pumping function in RPT Na^+ transport.
Basolateral pumping of $3Na^+$ out and $2K^+$ into the cell generates an electrochemical gradient that drives apical sodium transporters like NHE3 (predominant apical Na^+ transporter) to reabsorb Na^+ from the lumen down its concentration gradient. Lightning sign depicts the use of energy in the active transport of Na^+ by NKA.

Regulation of NKA Enzymatic Function

The importance of the regulation of Na^+ and fluid reabsorption by the RPT is well-illustrated by the many hormones, neurotransmitter, and endogenous factors that target RPT Na^+

transport. Elegant studies over the past few decades have identified dopamine, angiotensin-II, endogenous CTS, parathyroid hormone (PTH), epinephrine and norepinephrine, insulin, glucocorticoids, aldosterone, endothelin, NO, and shear stress to modulate RPT Na^+ transport (Table 1). Much of these natriuretic and anti-natriuretic factors regulate the RPT Na^+ absorption by targeting membrane abundance and activity of NKA and apical NHE3 by concerted but independent signaling pathways.

The mechanism of regulation of RPT Na^+ transport by the hormones dopamine and angiotensin-II has been the premise of a large number of studies aiming to understand the nuances of blood pressure regulation. Both hormones are synthesized locally by RPT cells (Kobori, Harrison-Bernard, & Navar, 2001; Soares-da-Silva, Fernandes, & Pinto-do-OA, 1994). Dopamine inhibits NKA activity through D1 receptor mediated activation of phosphoinositide 3-kinase IA (PI3K-IA) via protein kinase C-δ (PKCδ), as well as through the activation of cyclic adenosine monophosphate (cAMP)- protein kinase A (PKA) pathway. Conversely, angiotensin-II at lower 10^{-12}-10^{-10}M concentration stimulates NKA activity through AT_1 receptor-mediated activation of PKCβ that leads to phosphorylation of NKA at Ser-11 and 18 that increases NKA affinity for Na^+. Newer reports indicate that phosphorylation at Ser-938 of NKA along with Ser-18 increases membrane recruitment of NKA. Therefore, Na^+ excretion is bi-directionally regulated by dopamine and angiotensin-II.

Notably, Efendiev et al. (2003) demonstrated that opossum kidney (OK) cells treated simultaneously with saturating concentrations of dopamine and angiotensin-II depend on intracellular [Na^+] to determine the pathway controlling NKA activity. The authors suggest that an intracellular Na^+ sensor/s of yet unknown molecular identity may act as a master regulator of

the hormone-modulated Na^+ transport in the RPT (Efendiev, Budu, Cinelli, Bertorello, & Pedemonte, 2003).

Numerous preclinical and clinical studies, dating back as early as 50 years ago, have indicated a role for endogenous CTS in regulation of natriuresis. However, early inconsistencies in assay-based detection of endogenous CTS resulted in a period of disinterest in endogenous CTS and the questioning of its importance to human physiology. Several recent advances and discoveries including, advances in detection methodologies, the identification of NKA non-enzymatic functions, and studies in mice expressing NKA α1 transgene with increased ouabain sensitivity, have established endogenous CTS as an important physiological regulator. Subsequently, there is a renewed research interest to elucidate the mechanism of action underlying its natriuretic function in health and disease (Bagrov, Shapiro, & Fedorova, 2009; Haas, Wang, Tian, & Xie, 2002; Huang et al., 1997; Loreaux, Kaul, Lorenz, & Lingrel, 2008).

Factor	Effect on RPT NKA	Effect on RPT Na^+ transport	Reference
PTH	Inhibition	Inhibition	(Y. Zhang et al., 1999)
Norepinephrine	Stimulation	Stimulation	(Beach et al., 1987)
Aldosterone	Stimulation	Stimulation	(Salyer et al., 2013)
Nitric Oxide	Inhibition	Inhibition	(Mingyu Liang & Knox, 1999)
Insulin	Stimulation	Stimulation	(Féraille et al., 1999)
Endothelin	Inhibition	Inhibition	(Garvin & Sanders, 1991)
Fluid Shear Stress	Stimulation	Stimulation	(Duan, Weinstein, Weinbaum, & Wang, 2010a)
Endogenous marinobufagenin	Inhibition	Inhibition	(Periyasamy et al., 2005)

Table 1: Systemic and local factors regulating RPT NKA in Na^+ transport.

Non-Enzymatic Functions of NKA in RPT

Endogenous Cardiotonic Steroids: Effect on RPT and their role as the 'Third Factor'

British physician William Withering provided the first written scientific and medicinal account of digitoxin containing foxglove in 1795 (Withering, 2014). CTS specifically bind to extracellular sites on TM1-TM2, TM5-TM6, and TM7-TM8 loops of the alpha subunit of the NKA (Burns, Nicholas, & Price, 1996; Croyle, Woo, & Lingrel, 1997). Species and isoform-specific differences in sensitivity to CTS binding can be attributed to differences in amino acid sequences of these CTS binding sites. Indeed, R111Q and D122N mutations in the CTS binding sequence confers ouabain sensitivity to the resistant rat NKA α1(Jewell & Lingrel, 1991). The species-specific difference in CTS sensitivity was utilized to establish physiological importance of endogenous CTS (Loreaux et al., 2008; Wansapura, Lasko, Lingrel, & Lorenz, 2010).

Even prior to the discovery of NKA, Szent-Gyory in 1953 hypothesized that digitalis drugs substitute for compounds circulating endogenously in the human body- at that time referring to its function in cardiac function (Mullins, 1954). The high affinity and specificity of NKA for the plant-derived inhibitor ouabain further drove scientists to search for the existence of endogenous analogs.

In 1961, De Wardener elegantly demonstrated existence of a critical regulatory "Third factor" in determining renal Na^+ handling, in addition to aldosterone and GFR, when natriuresis by saline infusion was shown to be maintained even in presence of constant renal perfusion pressure and GFR (De Wardener, 1961). This striking finding was followed by a plethora of work in confirming and identifying the third factor (Buckalew, Martinez, & Green, 1970; Cort & Lichardus, 1963; De Wardener et al., 1971; Kramer & Gonick, 1974; Schrier, McDonald,

Marshall, & Lauler, 1968; Schrier, Verroust, et al., 1968). In the early 90s, Hamlyn et al. isolated a compound from the human plasma that was indistinguishable from the plant-derived ouabain and thereby named it endogenous ouabain (EO) (Hamlyn et al., 1991). The adrenal cortex and hypothalamus are both a source of the EO (Goto et al., 1996) (Laredo et al., 1995) (Hamlyn et al., 1991; Komiyama et al., 2001; Murrell et al., 2005). Adrenocorticotrophic hormone (ACTH), angiotensin-II, vasopressin, and phenylephrine, all stimulate EO synthesis, and ACTH-induced hypertension is associated with elevation in plasma levels of EO (Laredo, Shah, Lu, Hamilton, & Hamlyn, 1997; Shah, Laredo, Hamilton, & Hamlyn, 1999). Other CTS that have been isolated and identified endogenously in mammals include marinofugenin (MBG), and telecinobufagenin (TCG) (Komiyama et al., 2005).

EO does not induce natriuresis under basal conditions but arguably has a role in the adaptation to both Na^+ depletion and loading. While in one clinical study of 180 patients with untreated hypertension, plasma EO remained unchanged during two weeks of salt-loading but increased during salt-depletion, another study with 13 healthy men reported a tremendous 13-fold increase in EO after salt-loading and a 4-fold increase with salt-depletion (Manunta, Hamilton, & Hamlyn, 2006; Manunta et al., 2001). In mice, conferring ouabain sensitivity to NKA α1 augmented the natriuretic response to an acute salt load (Loreaux et al., 2008). Neutralization of endogenous NKA inhibitors with an anti-digoxin antibody fragment in the ouabain-sensitive mice, returned the Na^+ excretion rates to the level of the ouabain-resistant mice, re-enforcing a natriuretic role for EO in response to sodium-loading (Loreaux et al., 2008). Endogenous MBG is also elevated in rats and humans in response to salt loading, as well as in hypertension (Fedorova, Doris, & Bagrov, 1998; Gonick, Ding, Vaziri, Bagrov, & Fedorova, 1998; Periyasamy et al., 2005). In salt-loaded Sprague-Dawley rats, treatment with anti-MBG

antibody decreases urine Na^+ excretion along with substantial increase in RPT rubidium uptake and NKA enzymatic activity, indicating a role of endogenous MBG in the physiological adaptation of RPT to salt-loading (Periyasamy et al., 2005).

It is worth nothing that the plasma levels of EO, MBG, and TCG in humans are in the picomolar-nanomolar range in health and disease, a circulating concentration that is considerably lower than the range required for significant enzyme inhibition and subsequent changes of intracellular ion homeostasis (Hamlyn et al., 1991; Liu et al., 2002). However, CTS in the range of endogenous concentration can bind to NKA and activate its non-enzymatic functions. Despite numerous studies highlighting a physiological role of endogenous CTS in regulation of RPT Na^+ transport, investigation of its direct tissue-specific role and mechanism has been fraught with challenges due to the ubiquitous expression of its receptor NKA and the systemic distribution of endogenous CTS. There is a need for studies in transgenic mammalian models with RPT-specific alterations in NKA α1 sensitivity or expression to evaluate the specific physiological function of endogenous CTS in RPT Na^+ transport.

Discovery of NKA Receptor Function

In the late 1990s, a series of studies by Dr. Zijian Xie and collaborators uncovered a non-enzymatic signaling function of NKA, independent of the enzymatic ion-pump function, with a role in the regulation of cell growth and proliferation of neonatal rat cardiac myocytes (Haas et al., 2000; Huang et al., 1997). Subsequently, it became apparent that NKA interacts with membrane proteins and organizes cytosolic cascades of signaling complexes to regulate gene expression and cell function in a cell type-dependent manner. Binding of CTS at nanomolar concentration mirroring concentrations of circulation endogenous CTS modulate NKA interaction with neighboring proteins and activate protein kinase cascades, including but not

limited to, c-Src, ERK1/2, PI3K, caveolin-1, and PKC (Pierre & Xie, 2006; Z. Xie, 2003). Specifically, the signaling NKA resides with its partners in caveolae in the plasma membrane(Man Liang et al., 2007; H. Wang et al., 2004). Caveolae plays an essential role in the organization of the non-pumping pool of NKA and its assembly with other proteins as a receptor complex(Man Liang et al., 2007; H. Wang et al., 2004). In fact, NKA α1 binds to caveolin-1 through a conserved caveolin-binding motif which is necessary to its signaling function but not enzymatic function (X. Wang et al., 2020).

There is evidence that signaling is α–isoform specific (Madan et al., 2016; Tian et al., 2006; J. X. Xie et al., 2015). NKA/Src interaction is isoform specific to α1 and involves binding of NKA α1 to Src through two pairs of domain interactions (Tian et al., 2006). The second cytosolic domain (CD2) acts as a ligand to Src SH2 domain and is necessary for activation and targeting of Src (Tian et al., 2006). Specifically, the Y260 residue in CD2 of NKA α1 is the Src phosphorylation and binding site (Banerjee, Duan, & Xie, 2015). This interaction is constitutive and independent of NKA conformation and ouabain has no effect on the NKA/Src binding at this specific site (Tian et al., 2006). The binding to CD2 domain may function as a hinge to keep the activated Src bound to the signaling NKA for specific and robust signal transmission (Tian et al., 2006). On the other hand, Src kinase domain binds to a sequence labelled Naktide in the intracellular CD3 domain of NKA α1 in its E1 conformation (Z. Li et al., 2009; Tian et al., 2006; J. X. Xie et al., 2015). Binding of ouabain to NKA α1 stimulates a change of conformation from E1 to E2 which results in the freeing of the Src kinase domain from CD3 and subsequently the disinhibition of Src to activate downstream signaling molecules (Tian et al., 2006).

Active NKA/Src receptor complex also stimulates increase in reactive oxygen species (ROS) which in turn stimulates receptor activation to form a positive feedback loop (Yan et al.,

2013). The NKA/Src/ROS dysregulated loop promotes pathogenesis of diseases like obesity, steatohepatitis, and uremic cardiomyopathy (Sodhi et al., 2015; Sodhi et al., 2017; Sodhi et al., 2020). Moreover, through a well conserved LKK motif in its N-terminus, NKA binds and forms a signaling microdomain with inositol 1,4,5-triphosphate receptor (IP3R) which modulates Ca^{2+} release from intracellular stores (Miyakawa-Naito et al., 2003; S. Zhang et al., 2006). Ouabain mediated activation of NKA/IP3R signaling stimulates slow Ca^{2+} oscillations that activates nuclear factor kappa-B (NFkB) that inhibits apoptosis and promotes cell proliferation in renal cells derived from young rats (Aperia, 2012; Fontana, Burlaka, Khodus, Brismar, & Aperia, 2013; J. Li, Zelenin, Aperia, & Aizman, 2006). Thus, NKA receptor-mediated signaling activated by sub-inhibitory dose of ouabain regulates several cellular physiological functions, including, cell growth (Huang et al., 1997), proliferation (Haas et al., 2000), apoptotic threshold (J. Li et al., 2006), cell contact (Larre et al., 2010; Larre et al., 2006), and migration (Barwe et al., 2005).

In renal epithelial cells lacking 90% of NKA α1 after siRNA-mediated knockdown of α1, the cells retain 40% of cellular maximal enzymatic activity but have a diminished signaling capacity, consistent with the aforementioned concept of non-ion pumping NKA units detected in the RPT cells serving signaling receptor functions (Man Liang, Cai, Tian, Qu, & Xie, 2006; Man Liang et al., 2007). In addition to CTS, exogenous H_2O_2 (Y. Wang et al., 2014), ROS (Yan et al., 2013; Yan, Shapiro, Mopidevi, Chaudhry, Maxwell, Haller, Drummond, Kennedy, Tian, & Malhotra, 2016), intracellular [Na^+], and extracellular [K^+] (Ye et al., 2011) also serve as ligands to NKA receptor in RPT cells.

Coupled Regulation of NHE3 and NKA Membrane Trafficking

A number of *in vitro* and *in vivo* studies suggest that NKA α1 receptor function serves a regulatory role in RPT Na^+ transport. In LLC-PK1 cells, low doses of ouabain treatment stimulate coupled endocytosis of the basolateral NKA and the apical NHE3 and an overall decrease in transepithelial Na^+ flux, with no detectable change in intracellular [Na^+] (H. Cai et al., 2008; Liu et al., 2002). PP2 and wortmannin, inhibitors of Src family kinase and PI3K, respectively, suppress this action. This indicates a potential role for both signaling molecules in the NKA receptor-mediated regulation *in vitro* (H. Cai et al., 2008). The ouabain-mediated redistribution of NKA and NHE3 is not species-specific within mammals and stimulated endocytosis of the transporters in RPT cells from pig, humans, and cells expressing rat NKA (Yan et al., 2012). Another important characteristic of this regulation is the dependence on carbonylation modification of NKA at Pro224 for CTS to activate NKA receptor-mediated inhibition of NHE3 and the Na^+ flux *in vitro* (Yan, Shapiro, Mopidevi, Chaudhry, Maxwell, Haller, Drummond, Kennedy, Tian, Malhotra, et al., 2016).

Additionally, the administration of an antibody to MBG blocked the inhibition of NKA and suppressed the natriuresis seen with the high-salt diet *in vivo* (Periyasamy et al., 2005). Godinho et al. (2017) demonstrated that *in vivo* perfusion of sub-inhibitory doses of CTS bufalin, ouabain, telecinobufagin and marinobufagenin in rat kidney inhibits NHE3 activity and Na^+ transport (Arnaud-Batista et al., 2012; Godinho et al., 2017), consistent with *in vitro* findings (H. Cai et al., 2008). Inhibition of c-Src and MEK1/2-ERK1/2 in these CTS perfused rat kidneys, suppresses the effect of bufalin on NHE3 activity, highlighting their role in the regulatory pathway (Arnaud-Batista et al., 2012; Godinho et al., 2017). Therefore, CTS-mediated activation of NKA signaling inhibits RPT Na^+ transport, counteracting its enzymatic function.

In contrast to the previous identified hormone-mediated regulations, the proposed non-enzymatic regulatory function of NKA orchestrates not only concerted but also interdependent inhibition of two prominent Na^+ transporters to reduce absorption of Na^+ by RPT. Remarkably, NKA non-enzymatic signaling is activated by changes in intracellular [Na^+], with increased Na^+ concentration driving the E1/E2 conformational transition that leads to NKA/Src activation in renal cells, making it a prospective protein for salt sensing (Ye et al., 2011). The proposed NKA/NHE3 coupling mechanism could also have far-reaching fundamental ramifications, as it establishes a NKA-based mechanism for the regulation of RPT apical Na^+ reabsorption by CTS and non-CTS modulators of NKA that has remained vastly unexplored. With only 20-30% NKA engaged in enzymatic function in RPT (Cheval & Doucet, 1990; Féraille & Doucet, 2001), one can even speculate on a more functionally dominant role for the non-enzymatic regulatory function of the NKA receptor. The direct genetic evidence supporting a role for NKA signaling in mammalian physiology is still lacking. This is due, in part, to the ubiquitous expression of NKA α1 and the challenges of studying the non-enzymatic signaling separately from NKA's ATPase-mediated ion-pumping function because the latter is required for the survival of animal (Barcroft, Moseley, Lingrel, & Watson, 2004). One way of addressing this research gap is through the generation and study of animal models with RPT-specific alteration of NKA expression or function.

Paracellular Junction and Polarity

In the RPT, paracellular pathway contributes to atleast one-third of the total Na^+ reabsorption. Paracellular reabsorption depends on the transepithelial electrochemical force and tight junction (TJ) permeability. Madin-Darby canine kidney (MDCK) cells are one of the best characterized renal epithelial cell line, useful for study of tight junctions (Balkovetz, 2006;

Cereijido, Robbins, Dolan, Rotunno, & Sabatini, 1978; Gonzalez-Mariscal, De Ramirez, & Cereijido, 1985; Leighton, Estes, Mansukhani, & Brada, 1970; Taub & Saier Jr, 1979). MDCK-II cells, one of two strains of MDCK cells, exhibit properties of RPT epithelium, including a leaky and low transepithelial electrical resistance (TER) and expression of claudin-II (Balkovetz, 2006; Richardson, Scalera, & Simmons, 1981). Claudin-2 in the RPT forms paracellular channels with other protein that are selective for small cations like Na^+ and K^+, small anion like Cl−, as well as water (Enck, Berger, & Alan, 2018; Muto et al., 2010). While high concentrations of ouabain (1μM) trigger cell detachment, lower concentrations that do not affect the enzymatic activity increase TER and decrease the flux of neutral 3 kDa dextran (J_{DEX}), thereby modulating the permeability and sealing of the TJ in MDCK-II cells (Larre et al., 2010). The phenotype is accompanied by an increase in the protein expression and distribution patterns of claudins 1, 2, and 4, without affecting occludins (Larre et al., 2010). The changes in TER, J_{DEX}, and claudins all depend on c-Src activation and partially on ERK1/2 activation (Larre et al., 2010). Therefore, CTS through NKA/Src receptor modulate cell–cell contacts and paracellular ionic and non-ionic transport.

Polarity, another critical characteristic of epithelial cells to transport substances vectorially, is dependent on proper ciliogenesis. Ciliogenesis is an important determinant of apical polarity that contributes to apical transport of solutes. Renal cilia also serve as sensors that respond to changes in tubular flow to regulate transporters and cell transport. Ouabain-mediated activation of NKA signaling accelerates cilogenesis and modulates ciliary localization of claudin-2 in an ERK1/2-dependent manner in MDCK-II cells (Larre et al., 2011). Recent findings implicate cilia in mediating fluid shear stress sensitive mechano-transduction to regulate apical endocytosis in RPT epithelia (Duan et al., 2010a; Raghavan, Rbaibi, Pastor-Soler,

Carattino, & Weisz, 2014). Further studies can improve our understanding of the role of NKA signaling in RPT paracellular transport and ciliogenesis. Figure 4 illustrates a schematic of the regulatory functions indicated to be mediated by NKA non-enzymatic signaling in the RPT.

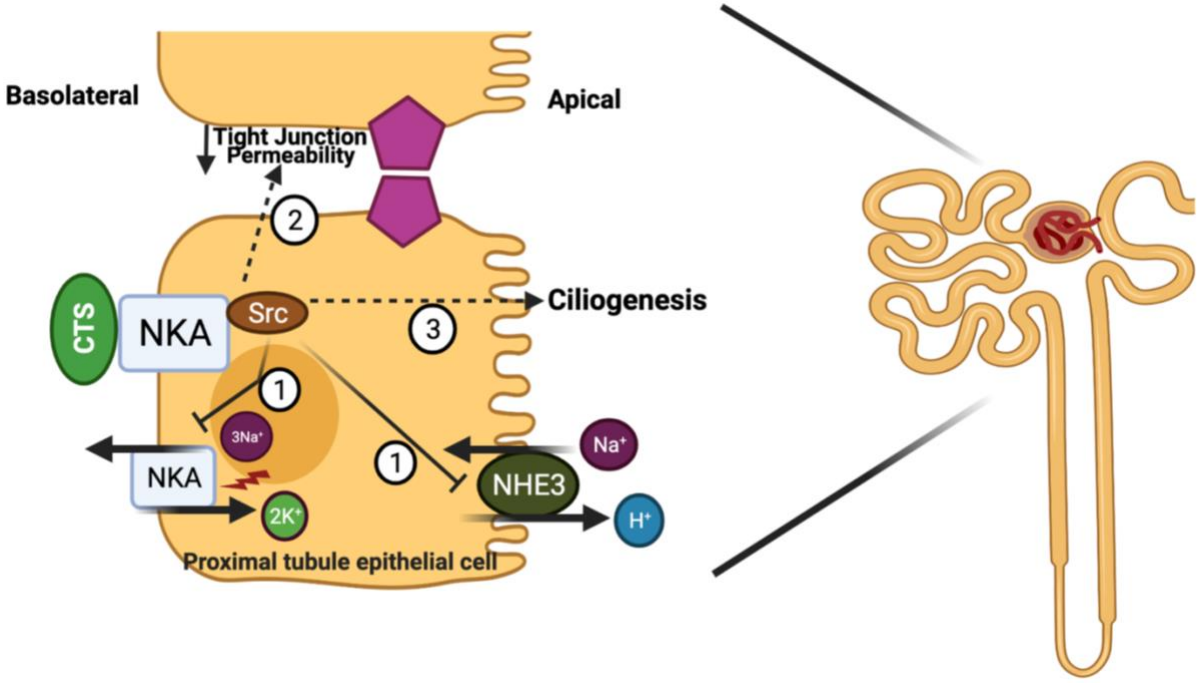

Figure 4: Non-enzymatic regulatory function of NKA in RPT Na^+ transport. NKA/Src signaling stimulates coupled endocytosis of NKA and NHE3 resulting in decreased Na^+ flux in RPT (1). NKA/Src signaling indicated to modulate expression of tight junction proteins to decrease the paracellular transport (2). Stimulation of NKA signaling indicated to promotes ciliogenesis (3).

Pathological importance of RPT NKA

Impairment of dopamine and angiotensin-II regulation of NKA enzymatic function

The natriuretic dopamine and anti-natriuretic angiotensin-II are both established as hormone regulators of RPT NKA and Na^+ transport, and in the regulation of blood pressure (Gurley, Allen, & Haase, 2008; M. Zhang et al., 2007). Dahl salt-sensitive rats exhibit a defective dopamine D1 receptor and adenylate cyclase coupling contributing to a lack of

inhibition of tubular NKA activity in response to salt and impaired natriuretic capacity in these rats (Nishi, Eklöf, Bertorello, & Aperia, 1993). A defect in dopamine receptor is also implicated in the development of salt-sensitive hypertension in C57Bl/6 mice (Escano et al., 2009). However, dopamine receptor agonists are limited to emergency use for severe hypertension and generally not prescribed for chronic essential hypertension (Murphy, Murray, & Shorten, 2001).

On the other hand, angiotensin-II levels are reportedly elevated in salt-sensitive hypertension and the chronic infusion of angiotensin-II in addition to high salt augments the increase in blood pressure in rats (Kobori, Harrison-Bernard, & Navar, 2002; Susic et al., 2011; Ying et al., 2012). The action of angiotensin-II on RPT Na^+ transport is indicated to have a role in the pathogenesis of salt-sensitive hypertension (Ramkumar & Kohan, 2013; Ying et al., 2012). Additionally, treatment of spontaneously hypertensive rats with angiotensin-II receptor (AT1R) blocker prevents the increase in renal NKA activity and in blood pressure (Queiroz-Madeira et al., 2010). Angiotensin-II receptor blockers are widely used for treatment of hypertension but recent clinical investigations have shown varying efficacy of drugs, development of resistance and complications in some patients despite being treated with one or more blockers (Bomback & Toto, 2009; Calhoun et al., 2008; Jorde et al., 2000).

With the recent identification of NKA natriuretic regulation *in vitro*, there is a need to revisit the molecular mechanisms involved in the dopamine-angiotensin-II axis of regulation, its impact on NKA receptor vs NKA enzyme, and test for possible interactions or cross-talk with NKA signaling function. Addressing these research gaps may pave the way to more efficient anti-hypertension therapeutics.

Elevated Endogenous CTS in Volume Expansion

Conflicting actions have been proposed for EO in the development and pathogenesis of hypertension. While some studies report that increase in blood pressure correlates directly with increased plasma EO concentration in humans (Manunta et al., 1999; Masugi, Ogihara, Hasegawa, & Kumahara, 1986; Pamnani et al., 1989), others found no change in plasma EO with Na^+ loading in hypertensive humans (Bernini, Paci, Sgrò, Moretti, & Salvetti, 1998; Manunta et al., 2001). Experimentally, there are differences in the study designs, including study population, and the routes of administration of salt challenge, which may all contribute to the conflicting findings. In Dahl salt-sensitive rats with salt-induced hypertension, a prominent model used in research on salt-sensitive hypertension, a transient increase in circulating EO precedes a sustained increase in circulating marinobufagenin after acute and chronic salt-loading in these animals (Fedorova, Lakatta, & Bagrov, 2000; Fedorova, Talan, Agalakova, Lakatta, & Bagrov, 2002). The pro-hypertensive effect of EO is often attributed to stimulation of vasoconstriction.

In contrast, MBG has been implicated in promoting natriuresis and decrease RPT Na^+ absorption in response to salt-loading, thereby playing an anti-hypertensive role (Periyasamy et al., 2005). Pharmacological agents targeting or blocking EO may stimulate vasodilation and improve blood pressure. However, as a trade-off if the increase in endogenous EO represents a physiological response to modulate a renal system unable to increase Na^+ excretion, blocking EO could decrease vascular resistance but produce adverse renal effects.

Polymorphisms Affecting NKA Trafficking and Regulation

Point mutations of adducin, a cytoskeletal protein critical for actin assembly, cell surface exposure of integrins, and cell signaling, is associated with genetic hypertension in the Milan hypertensive rats and in humans (Bianchi et al., 1994; Ferrandi et al., 1999). The ectopic expression of the hypertension-variant of human α-adducin in normal cells induces a

hypertensive phenotype in the cells. The mutation is also associated with dysregulated NKA/Src complex and exhibit an increase in interaction between α-adducin/Src in HK2 renal cells transfected with mutant α-adducin (Ferrandi et al., 1999). Notably, this effect is suppressed when the cells and the hypertensive rats with the mutant α-adducin are treated with rostafuroxin, a digitoxigenin derivative that selectively disrupts the binding to the cSrc-SH2 domain by mutant α-adducin and the ouabain-activated NKA (Ferrari, Ferrandi, Valentini, & Bianchi, 2006).

In humans, DNA-sequencing detected a 12-nucleotide long thymidine (12T) insertion(ins)/deletion(del) polymorphism within a poly-T sequence (38T vs 26T) in the NKA α1 gene (*ATP1A1)* 5'-regulatory region associated with a decreased risk of hypertension in a male Sardinian population (Herrera et al., 2015). However, the impact of this polymorphism at the tissue and cellular-level is poorly understood. The study in the Sardinian population suggests existence of mutations or variants in promotor, enhancer, or intron sequences, that may impact NKA expression or its interaction with proteins for non-enzymatic signaling.

Impaired Ouabain Mediated NKA/Src Signaling

Following their *in vitro* studies identifying active NKA receptor-mediated regulation of RPT Na^+ transport, Liu and collaborators reported the failure of both high salt and ouabain to exert a coordinated regulation of NKA and NHE3 membrane trafficking in RPT cells isolated from Dahl salt-sensitive rats (Liu et al., 2011). Moreover, ouabain/NKA mediated activation of Src and ERK was impaired in these salt-sensitive rats (Liu et al., 2011). This study highlighted the ligand-mediated RPT NKA/Src signaling in regulation of RPT Na^+ absorption, with its impairment leading to salt-sensitivity.

In addition to regulating the predominant Na^+ transporters in RPT, Xiao et al (2018) demonstrated that the impairment of NKA/Src signaling is associated with pathogenesis of

hyperuricemia-induced renal tubular injury in human RPT epithelial cells (PTECs) and in experimentally-induced hyperuricemia rat models (Xiao et al., 2018). RPT is responsible for almost all renal urate transport and increased serum uric acid level is strongly associated with development of hypertension (Mazzali et al., 2001). Consistently, mice with PNx-mediated uremic cardiomyopathy, when treated with pNaktide to inhibit the NKA/Src/ROS amplification loop, improves cardiac function and attenuates the development of uremic cardiomyopathy (Liu et al., 2016a). Therefore, the NKA receptor function might be the key to addressing several unresolved research gaps with a lot of clinical relevance, such as the physiological importance of NKA in RPT Na^+ transport and salt-sensing, the role and mechanism of endogenous CTS in RPT as a natriuretic hormone, and the physiological relevance of RPT NKA functions in systemic sodium and volume homeostasis in health and disease.

A Natriuretic Role for NKA in RPT

If the history of NKA has taught us anything, it is that there is more to this ubiquitous multi-faceted protein than meets the eye. After its discovery, for a long time, NKA was solely appreciated for its role in maintaining the electrochemical gradient for the transepithelial Na^+ transport and was perceived as a housekeeping enzyme. Eventually, elegant studies revealed the dynamic nature of the pump and its regulation by endogenous hormones and factors to increase or decrease its activity to modulate Na^+ absorption. Recent discoveries have revealed even newer facets of this versatile protein. While its function as an ion-pump is vital, NKA also serves as a non-enzymatic receptor independent of its enzymatic function that inhibits Na^+ transport through interdependent inhibition of NKA and NHE3 in RPT, and shows promise as a salt-sensor in RPT-mediated regulation of systemic sodium and volume homeostasis. Thus, based on the current evidence, RPT NKA simultaneously serves two critical and opposing roles in Na^+

reabsorption: anti-natriuretic through its classic ATPase-driven ion transport function, and natriuretic through its more recently recognized receptor/signaling function. To date, the relative contributions of these two NKA functions to the net Na^+ reabsorption by RPT remains a fundamentally and therapeutically essential question that has been challenging to answer**.** Translationally, a clarification of the RPT NKA signaling in Na^+ overload and salt sensitivity could open a new field of pathophysiological and pharmacological studies and pave the way for novel therapeutics targeting hypertension. Therefore, to address the crucial research question of physiological relevance of NKA non-enzymatic function in RPT Na^+ transport, we performed an array of studies in genetically modified renal cells and mice with RPT-specific alteration of NKA α1.

CHAPTER 2

NA/K-ATPASE SIGNALING TONICALLY INHIBITS SODIUM ABSORPTION IN THE RENAL PROXIMAL TUBULE

A manuscript under review

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Abstract

Active ion transport by Na/K-ATPase (NKA) drives the transcellular reuptake of Na^+ in the renal proximal tubule (RPT). In addition, NKA has a Src kinase-dependent signaling function that downregulates Na^+ reuptake by other RPT ion transporters. To establish the importance of this dual function on Na^+ and water homeostasis, we examined the effect of genetic downregulation of NKA expression in RPT *in vitro* and *in vivo*. Silencing NKA in RPT cell monolayers resulted in a doubling of Na^+ transport, without increase in paracellular transport. Genetic suppression of RPT NKA in mice increased renal Na^+ absorption and markedly decreased daily urinary Na^+ and water excretion. These effects were attributable to the dismantling of the NKA-Src signaling axis, through uncoupling and redistribution of Src, which leads to unregulated plasmalemmal ion transporters. Hence, RPT NKA signaling is not only physiologically important, but predominates over the enzymatic function of NKA in the regulation of RPT Na^+ absorption

Introduction

The mammalian kidney maintains extracellular homeostasis amidst large qualitative and quantitative variations in dietary intake of solutes and water by processing >20% of the cardiac output every minute. Two-thirds of the filtered sodium, potassium, chloride, bicarbonate, phosphate, and water, as well as virtually all the filtered glucose and amino acids, are reabsorbed in the renal proximal tubule (RPT). Adequate modification of bulk absorption of salt and water in the RPT in response to physiological stimuli (e.g. increased renal perfusion pressure, high salt diet) is an independent determinant of blood pressure (BP), and is therefore highly consequential clinically (Joe & Shapiro, 2012; McDonough, 2010a; McDonough & Nguyen, 2015).

In the RPT, as in all Na^+-absorbing epithelia, Na/K-ATPase (NKA) is exclusively located in the basolateral membrane. In the renal nephron, the peritubular Na^+ gradient generated by NKA, which operates only at 20-30% of its maximum rate under basal conditions, is primarily dissipated by apical membrane transporters (Cheval & Doucet, 1990). This architectural organization results in a net transfer of Na^+ from the nephron lumen toward the interstitial space. The apical Na^+/H^+-exchanger-3 (NHE3, Slc9a3) mediates 80% of luminal Na^+ intake in the RPT, which is primarily coupled to H^+ extrusion, and secondarily linked to bicarbonate absorption. The basolateral Na^+/HCO_3^- cotransporter (NBCe1A, Slc4a4A), which transports all of the filtered bicarbonate, is directly coupled to Na^+ (Kurtz & Zhu, 2013; Sciortino & Romero, 1999).

To adjust Na^+ transport to physiological demand, apical Na^+ entry (NHE3), basolateral Na^+ exit (NKA and NBCe1A), or both, can be quickly modulated through post-translational mechanisms that include modulation of their transport properties and cellular redistribution. Regulatory pathways described to date suggest that apical and basolateral Na^+ transport systems are modulated through concerted but independent mechanisms (Féraille & Doucet, 2001; M. C. Hu et al., 2001; Kunimi et al., 2000; McDonough, 2010a; McKinney & Myers, 1980; Pedemonte et al., 2005b; Y. Zhang et al., 1999). For example, the natriuretic hormones dopamine and parathyroid hormone stimulate parallel PKC-mediated inhibition of NKA and cAMP-dependent inhibition of NHE3 and NBCe1A to decrease RPT Na^+ absorption (Aperia et al., 1987; Kunimi et al., 2000; McKinney & Myers, 1980; Pedemonte, Efendiev, & Bertorello, 2005a; Y. Zhang et al., 1999). An advantage of a concerted parallel control of Na^+ transporters is that it minimizes variations in intracellular ion concentrations, pH or cell volume that would otherwise alter numerous cellular functions.

In contrast, inhibition of RPT Na^+ absorption by the specific NKA ligands cardiotonic steroids (CTS) is a NKA/NHE3 coupled regulatory mechanism. Importantly, this regulatory mechanism is distinct from the natriuretic and diuretic effects observed in response to high concentrations of CTS, which cause a substantial (and physiologically irrelevant) inhibition of the basolateral absorption of Na^+ by NKA (Eliades, Pamnani, Swindall, & Haddy, 1991; Hook, 1969; Nechay, 1974). Rather, the NKA/NHE3 coupling operates at much lower concentration of CTS, and within the CTS range reported in human plasma, which makes this system physiologically relevant (Hamlyn & Blaustein, 2016; Hamlyn et al., 1991). Thus, circulating levels of CTS acutely downregulate apical NHE3 through a mechanism that is not associated with a sustained measurable change in intracellular [Na^+], but requires NKA redistribution to the intracellular membrane compartment prior to NHE3 internalization (Arnaud-Batista et al., 2012; H. Cai et al., 2008; Godinho et al., 2017; Yan et al., 2012). *In vitro* approaches exploiting well-known inter-species differences in NKA affinity for CTS have suggested that basolateral RPT NKA α1 is the isoform that mediates CTS-induced NHE3 distribution and inhibition of apical Na^+ influx (Loreaux et al., 2008; Yan et al., 2012). Those studies also suggest that the mechanism is Src-dependent, which is consistent with the CTS-induced activation of NKA α1/Src receptor mechanism.

This proposed NKA/NHE3 coupling mechanism could have far-reaching fundamental physiological and clinical ramifications, as it establishes a NKA-based mechanism for the regulation of RPT apical Na^+ absorption by CTS and non-CTS modulators of NKA, which has remained unexplored. Unfortunately, experimental limitations have hampered progress in this line of research to date. First and foremost, the observation that the proposed signaling mechanism can occur without measurable inhibition of NKA enzymatic activity limits the impact

of pharmacological approaches. As a result, it has been challenging to firmly establish NKA α1 as the molecular machinery that couples the exposure to low concentration of CTS to NHE3 inhibition. The need for a definitive demonstration has been further accentuated by recent studies that have challenged the classically recognized role of NKA as the specific receptor and mechanism of action of CTS (Arnaud-Batista et al., 2012; Banerjee et al., 2018; H. Cai et al., 2008; Godinho et al., 2017; Z. Li et al., 2009; Liu et al., 2002; Yan et al., 2012). Further, given the ubiquitous expression of NKA α1 and the systemic distribution of endogenous CTS, a specific functional impact on RPT Na^+ absorption has also been difficult to evaluate. Finally, NKA coupling to RPT Na^+ transporters other than NHE3 has not been explored.

To address those critical issues and establish the importance of NKA non-ion pumping function in RPT Na^+ handling, we used a genetic approach to suppress *Atp1a1*, the gene that encodes the α1 isoform of NKA, in RPT cells and in RPT-specific mouse models. Our findings are consistent with a model whereby NKA signaling exerts a tonic inhibition on Na^+ absorption by regulating key apical and basolateral Na^+ transporters. This action, which is lifted upon NKA genetic suppression in cells and *in vivo*, tonically counteracts the classic NKA enzymatic function. Strikingly, NKA/Src signaling is not only physiologically relevant, it is functionally dominant over NKA ion-pumping in the control of RPT Na^+ absorption. NKA signaling therefore provides a long sought-after mechanism for the natriuretic action of endogenous NKA ligands such as CTS.

Results

Knockdown of 90% NKA α1 redistributes key apical and basolateral Na^+ transporters and increases transepithelial Na^+ flux.

To assess the contribution of NKA signaling vs. NKA classic ion-pumping role in the regulation of transepithelial Na^+ transport, we used the PY-17 cell model. This model is derived from the porcine renal epithelial cell line LLC-PK1 and is the only mammalian cell system with a 90% knockdown of NKA α1, which is the only NKA α-isoform expressed in renal epithelial cells (Man Liang et al., 2006). Our initial characterization of PY-17 has shown that those cells lose 60% of cellular NKA enzymatic activity, which results in increased intracellular Na^+ concentration. Those studies also showed that signaling by the caveolar NKA signaling complex is lost in PY-17 (Man Liang et al., 2006; Man Liang et al., 2007). This was evidenced by a dysregulation of Src and other NKA signaling partners, an inability to activate in response to the CTS ouabain, and an abnormal cellular distribution of the protein partners (T. Cai et al., 2008; Yiliang Chen et al., 2009; Ying Chen et al., 2008; Man Liang et al., 2006).

In the present study, LLC-PK1 and PY-17 monolayers were grown on transwell membrane inserts. As shown in Figure 5A, total transepithelial $^{22}Na^+$ flux was doubled in PY-17 cells. We also observed that transepithelial electrical resistance (TER) was significantly increased in PY-17, excluding increased paracellular permeability as the mechanism for increased transepithelial Na^+ flux (Figure 5B). Western blot analysis confirmed the expected minimal expression of NKA α1 in PY-17 cells (Figures 5C-D), and further revealed that total NHE3 was unchanged (Figures 5C, 5F). This set of studies also revealed that phosphorylation of NHE3 at Ser552 (which mediates NHE3 internalization and inactivation (Kocinsky, Dynia, Wang, & Aronson, 2007; Kurashima et al., 1997)) was decreased by 50% (Figures 5C, 5E),

indicative of an increase in active membrane NHE3. Moreover, expression of the basolateral NBCe1A, the other major route of Na^+ efflux in RPT cells, was also significantly increased in PY-17 (Figures 5C, 5G). The higher transcellular Na^+ flux, activation of NHE3, and the upregulation of expression of NBCe1A upon genetic suppression of NKA α1 in PY-17 is consistent with the existence of a NKA-dependent mechanism that tonically tempers transcellular Na^+ influx in the RPT cells and therefore counteracts the NKA transport driven function that generates the steep transcellular Na^+ gradient.

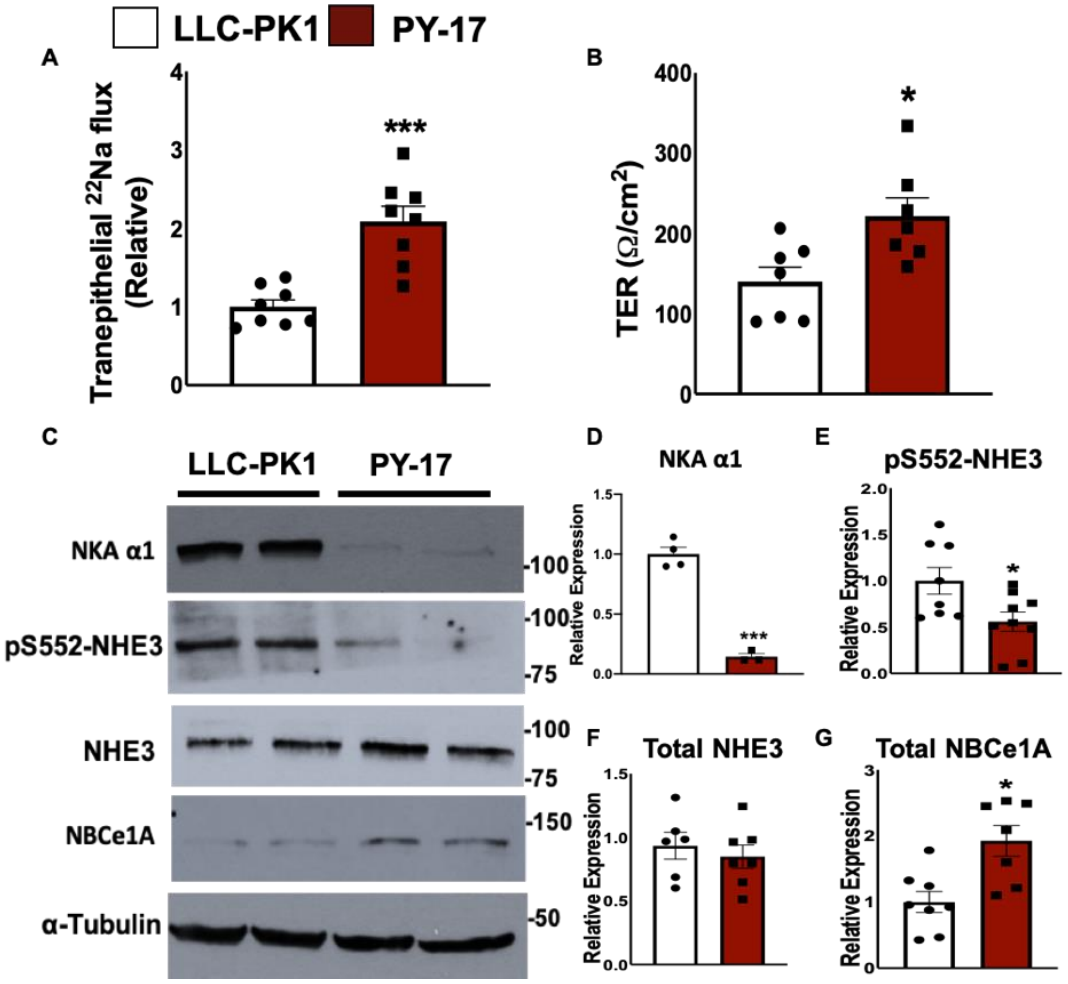

Figure 5: Genetic ablation of 90% of NKA α1 in renal epithelial cells leads to increased transepithelial Na^+ transport, decreased phosphorylation(ser552)-mediated inactivation of NHE3, and increased total NBCe1A.

(A) Transepithelial 22Na-flux (relative to average of control LLC-PK1 cells) and **(B)** Transepithelial Electrical Resistance (TER) were measured in polarized monolayers of NKA knockdown (PY-17) or parental wild type LLC-PK1 cells. Representative films from western blots for NKA α1, phosphoser552-NHE3, total NHE3, and total NBCe1A and their respective quantitation **(C-G)**. All quantifications were normalized to α-tubulin followed by normalization to average of control values per gel. Results are presented as mean ± SEM and data were analyzed by t-test. *p< 0.05 and ***p<0.001 versus LLC-PK1.

Genetic targeting of RPT NKA α1 in the mouse.

We generated floxed *Atp1a1* mice, designed to have exons 15-18 excised when crossed with a Cre mouse. The strategy of removing exons 15-18, summarized in Figure 6A, was selected to 1) disrupt the transmembrane loop between TM4-TM5, which is vital for ATPase activity and Src interaction, and 2) cause a frameshift leading to nonsense mRNA-mediated decay. The floxed A*tp1a1* mouse was crossed with the Na^+/glucose cotransporter 2-Cre mouse (SGLT2-Cre) (Rubera et al., 2004). Homozygous (RPT$\alpha1^{-/-}$) mice were viable. Body weight, food consumption, water intake, and body composition were not modified (Table 2). Western blot analysis indicated a decrease of about ~40% in total NKA α1 expression in renal cortex relative to RPT$\alpha1^{+/+}$ (control) littermates (Figures 7A-B), but not in the medulla (Figures 7C-D). To verify that this cortical decrease of NKA expression was exclusively attributable to the RPT, histological preparations were analyzed by immunofluorescence. As shown in Figure 7E, renal cortices stained with villin-1, a microvillar actin bundling protein specifically expressed in the RPT (magenta) and NKA α1 (red) were obtained from 4-month old males RPT $\alpha1^{+/+}$ and RPT $\alpha1^{-/-}$ mice. Quantitative analyses revealed a significant and specific decrease of 70% of NKA signal in the RPT of RPT$\alpha1^{-/-}$ mice (Figures 7E-F).

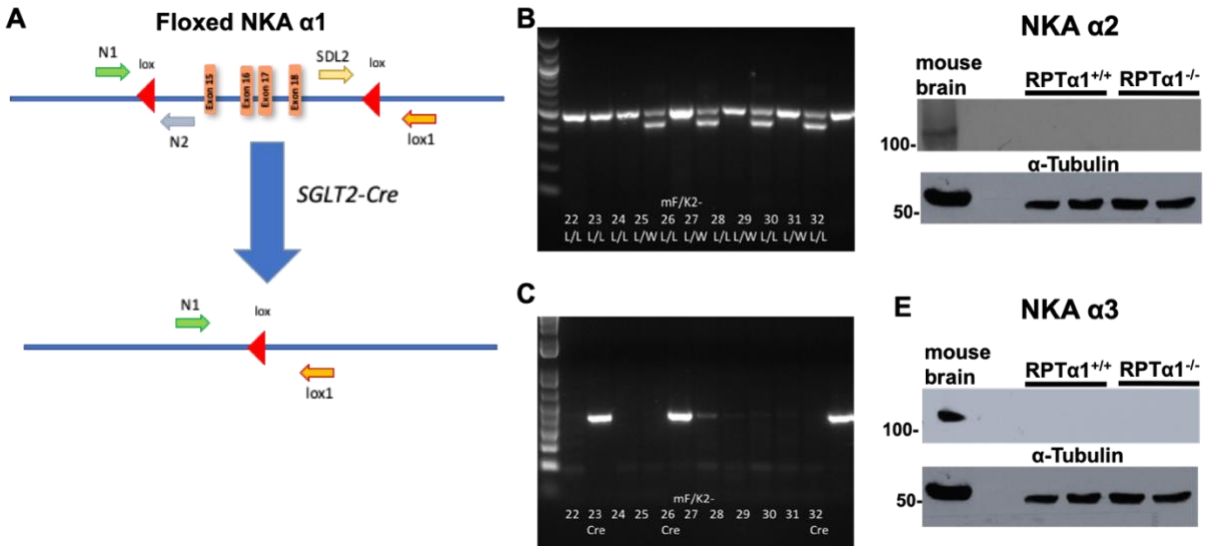

Figure 6: Proximal tubule-specific Atp1a1 ablation.
(A) Scheme for generation of RPT-specific *Atp1a1* knockout mouse model using a Cre/Lox approach. DNA genotyping for homozygous floxed NKA α1 **(B)** and heterozygous SGLT2-Cre **(C)**. Mice positive for homozygous floxed α1 and heterozygous SGLT2-Cre were grouped as RPTα1$^{-/-}$ and mice positive for homozygous floxed α1 but negative for SGLT2-Cre were grouped as controls RPTα1$^{+/+}$. (**D-E)** Representative blots of NKA α2 and α3 in RPTα1$^{-/-}$ and RPTα1$^{+/+}$ cortex with control mouse brain used as positive control (4-month male).

There was no compensatory expression of alternate NKA α-isoforms, α2 and α3, detected in kidney homogenates (Figures 6D-E). Such robust yet not complete reduction of RPT NKA α1 was not unexpected, since SGLT2 is only expressed in the S1 (major) and S2 (minor) segments. Additionally, SGLT2-Cre has been reported to produce a robust but not complete ablation of RPT transporters (H. C. Li et al., 2013). Hence, the RPTα1$^{-/-}$ mouse is a hypomorphic model with NKA α1 expression reduced to 30% in the RPT. Kidney histological analysis after periodic acid schiff (PAS) staining and hematoxylin and eosin (H&E) staining did not reveal any

gross structural alteration (Figures 8A-F). Glomerular number and area, RPT area, and number of cells per RPT in RPTα1$^{-/-}$ were comparable to those in control littermates (Figures 8A-E).

	RPTα1$^{+/+}$	RPTα1$^{-/-}$
Body Weight (g)	28±1	28±1
Daily water intake (g)	7±0.4	7±0.3
Daily food intake (g)	7±0.4	7±0.5
Body Composition:		
Fat (g)	2.1±0.5	2.7±1
Lean (g)	25±1	24±1
Total Water (g)	21±1	20±1
Free Water (g)	0.21±0.05	0.16±0.02

Table 2: Basal metabolic characteristics and Body Composition Analysis by EchoMRI. Values are mean ± SEM for n=10-12 (Metabolic Cage) and n=5-6 (EchoMRI) for 4-month male and female (1:1) mice. No significant difference was observed by Student's t-test.

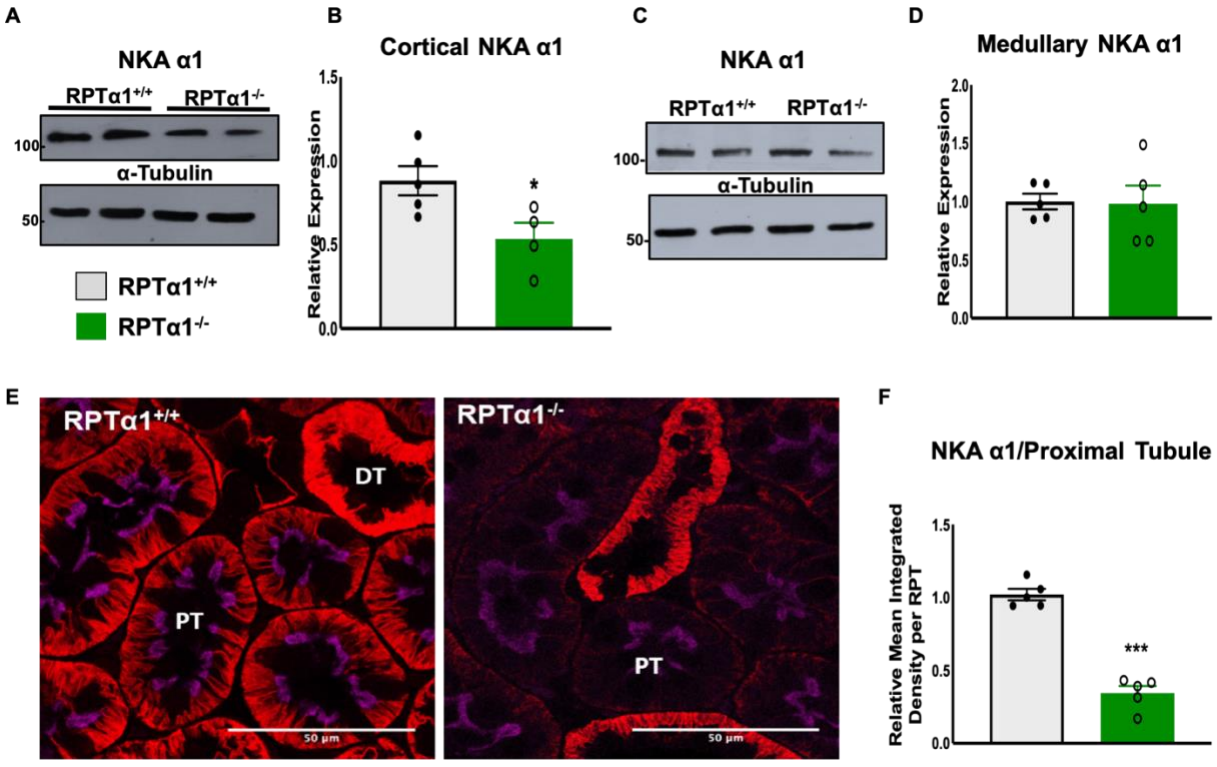

Figure 7: NKA α1 expression in the RPT α1 hypomorphic mouse.

Representative western blots and quantification of NKA α1 in renal cortex homogenates from

RPTα1$^{-/-}$ and RPTα1$^{+/+}$ control mice **(A-B)**. Representative western blots and quantification of NKA α1 in renal medulla homogenates from RPTα1$^{-/-}$ and RPTα1$^{+/+}$ control mice **(C-D)**. All quantifications were normalized to α-tubulin followed by normalization to the average of control values in each gel. Representative immunostaining of RPT for NKA α1 (red), RPT brush-border marker Villin-1(purple), in kidney cross-section and quantitation of NKA α1 in villin-1-stained RPT (n=5) for RPTα1$^{-/-}$ and RPTα1$^{+/+}$**(E-F).** Scale bar: 50 µm. Adult 4-month male RPTα1$^{-/-}$ and RPTα1$^{+/+}$ controls were used. Results are presented as mean±SEM and data were analyzed by t-test. *p< 0.05, ***p<0.001 versus RPTα1$^{+/+}$.

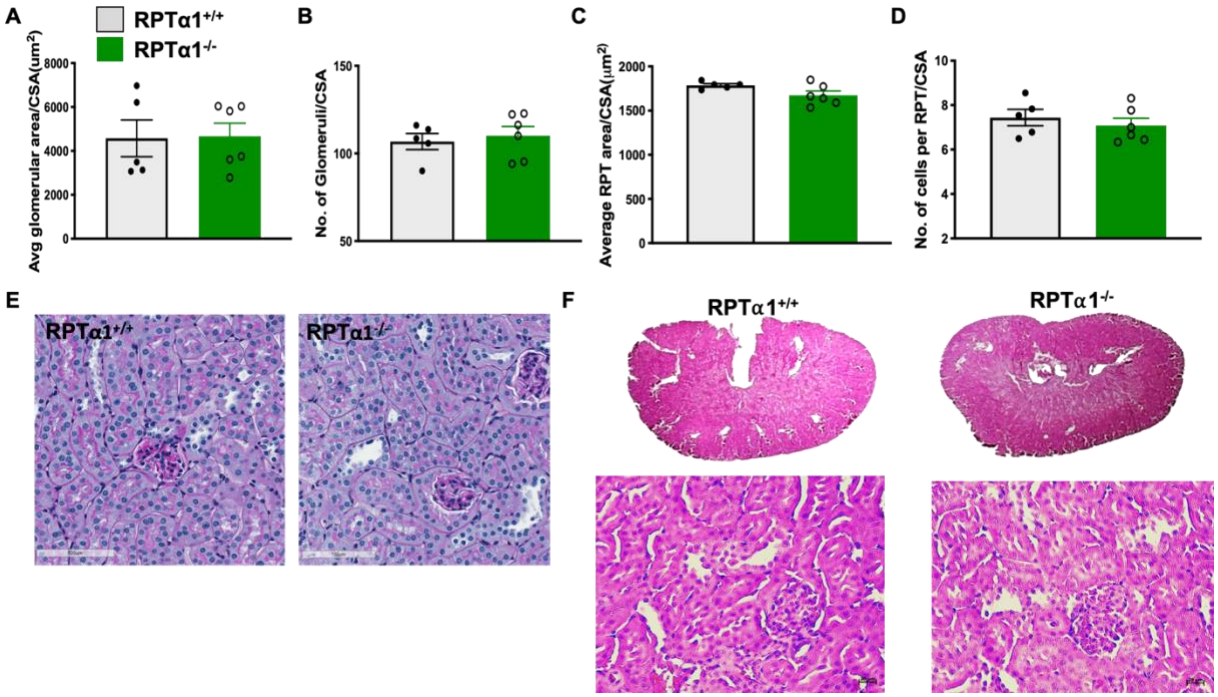

Figure 8: Morphometric and Histological Analysis of Kidney cross-sections by Periodic Acid Schiff and Masson's Trichrome staining in RPTα1$^{-/-}$.
(A-B) Number and average area of glomerulus per cross-section in RPTα1$^{-/-}$. **(C-D)** Average area and number of cells per proximal tubule per cross-section in RPTα1$^{-/-}$. **(E-F)** Representative images from PAS-stained and Masson's Trichrome stained cross-sections of kidney from

RPTα1$^{-/-}$ compared to RPTα1$^{+/+}$. Values are mean ± SEM and data were analyzed by Student's t-test. No significance difference was observed.

Increased Na^+ absorption in the hypomorphic RPTα1$^{-/-}$ mouse.

To test whether the hypomorphic RPTα1$^{-/-}$ mouse has a hyper-absorptive renal phenotype as predicted by our observations in PY-17 cells (Figure 5), a functional evaluation was performed in 4-month-old males and females. Mice were acclimated for 24h in metabolic cages prior to 24h of urine collection to determine daily urine and Na^+ output, and to analyze urine chemistry. As shown in Figures 9A-B and 10A-B, a robust and significant decrease of over 65% in daily urinary output and daily absolute Na^+ excretion was observed in the RPTα1$^{-/-}$ mouse of both sexes. SGLT2-Cre mice exhibited no change in body weight, water and food intake, or daily urine output (Table 3).

	Control	SGLT2-Cre
Body Weight (g)	28.4±0.7	28.5±0.1
Daily water intake (g)	6.7±0.2	6.9±0.9
Daily food intake (g)	5.1±0.2	4.7±0.2
Daily Urine Output (mL)	1.7±0.1	1.9±0.4

Table 3: Basal metabolic characteristics of SGLT2-Cre mice.
Values are mean ± SEM for n=3 4-month male mice. No significant difference was observed by Student's t-test.

Since this decrease in urinary excretion was not associated to a parallel increase in body mass over the course of the study (Figure 10C), we investigated water excretion through other measurable known routes of excretion in mice. As shown in Figures 10D-F, those studies revealed that water loss through expired air and fecal matter compensated for the decreased loss through urine in RPTα1$^{-/-}$ mice.

Given the high reliance on RPT Na^+ transporters for Na^+ handling in males relative to females (Veiras et al., 2017), the following set of studies characterizes RPT function in males and excludes females to limit confounding factors due to sexual dimorphism. Serum and urine chemistry panels, including blood urea nitrogen (BUN), albumin and creatinine (Tables 4&5) did not indicate that glomerular and/or tubular dysfunction was associated to this drastic reduction of diuresis in RPT$\alpha 1^{-/-}$ male mice, which is consistent with normal renal morphology and histology reported in Figure 8. Despite the substantial decrease in diuresis, urine chemistry values were within the normal range in RPT$\alpha 1^{-/-}$ mice. The panel presented in Table 4 indicated an absence of change in urinary concentrations for most electrolytes (Na^+, K^+, Cl^-, and Ca^{2+}), with modest but significant decreases in urine pH and glucosuria detailed in the next paragraph (Table 4). Remarkably, although excretion was drastically reduced, blood homeostasis for all metabolites and electrolytes tested (Table 5), along with glucose and insulin tolerance (Figures 10G-J) remained unchanged in RPT$\alpha 1^{-/-}$ mice.

The glomerular filtration rate (GFR), measured by determining FITC-sinistrin clearance, was unchanged in RPT$\alpha 1^{-/-}$ mice (Figures 9C-D). On the other hand, RPT$\alpha 1^{-/-}$ exhibited reduced hematocrit (45.9±0.8% vs 48.5±0.5 in RPT$\alpha 1^{+/+}$), a 40% decrease in serum aldosterone, and a 40% increase in serum atrial natriuretic peptide levels (Figures 9E-G), which are generally observed in mice under increased systemic sodium levels and increased blood pressure (Graudal, Hubeck-Graudal, & Jürgens, 2012; Loreaux et al., 2008; Merrill, Ebert, Skelton, & Cowley Jr, 1989; Simchon et al., 1989; Yatabe et al., 2010). Hence, although extra-renal excretion routes (including expired air and fecal) appear to provide a remarkably efficient compensatory mechanism to allow the 4-month-old RPT$\alpha 1^{-/-}$ mouse to maintain homeostasis under basal laboratory conditions, early manifestation of sodium overload is present in those animals.

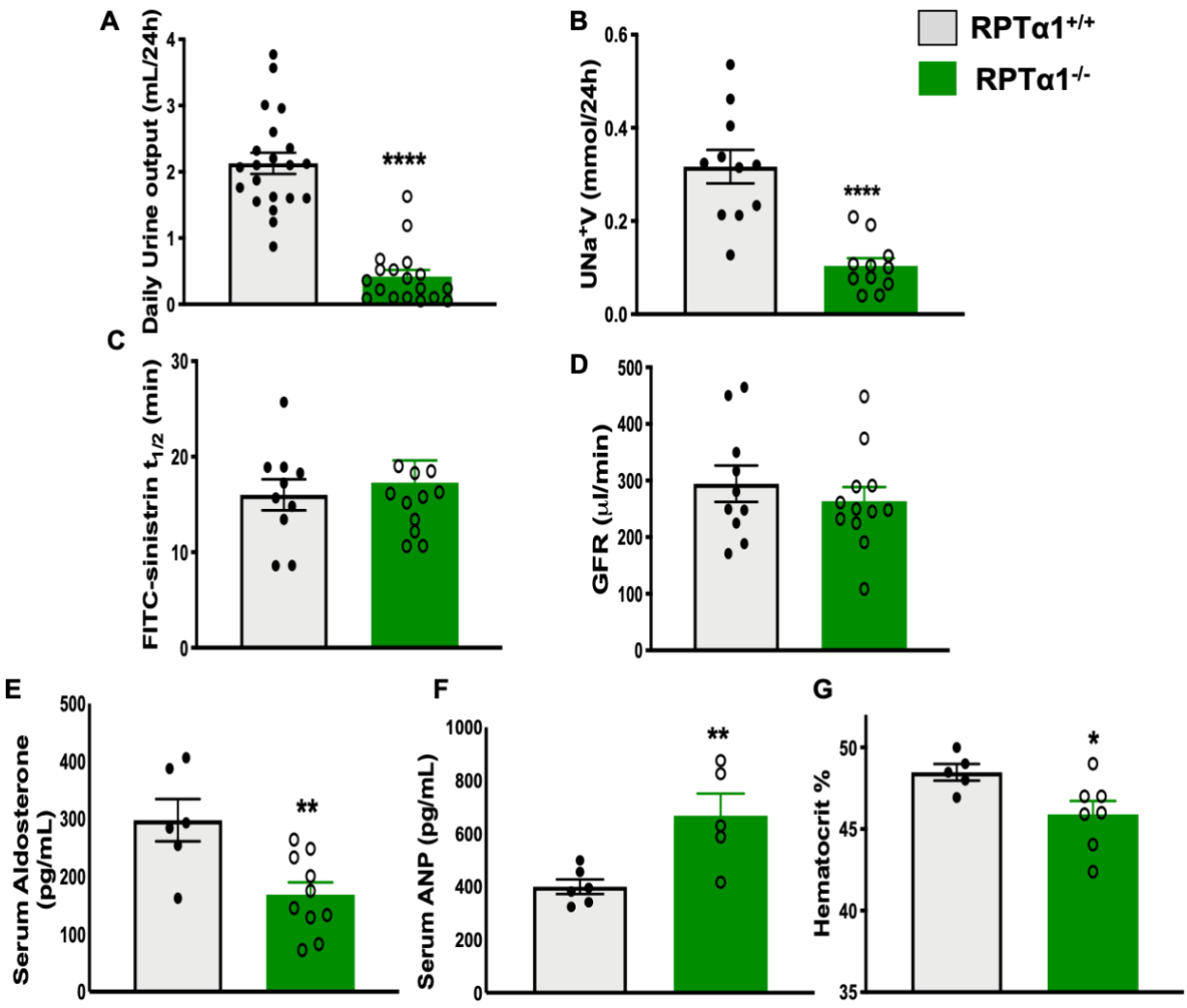

Figure 9: Renal sodium handling in RPTα1$^{-/-}$.
Four-month-old male and female mice were used in a 1:1 ratio to assess daily urine output **(A)** and absolute Na^+ excretion (UNa^+V) expressed as UNa^+V = Urine [Na^+] (mmol/mL) x Urine volume (mL/24h) **(B)**. Transcutaneous measurement of FITC-sinistrin clearance $t_{1/2}$ in 4-month old male mice, used to calculate GFR (µL/min), is shown normalized to mouse body weight. GFR (µL/min) = (14616.8/FITC-Sinistrin $t_{1/2}$)*BW/100 (**C-D).** Serum aldosterone **(E)**, Serum atrial natriuretic peptide (ANP) **(F),** Hematocrit **(G)** in 4-month-old male mice of the indicated

genotype. Results are presented as mean ± SEM. Data were analyzed by t-test. *p < 0.05, **p<0.01, and ****p<0.0001 versus RPTα1$^{+/+}$

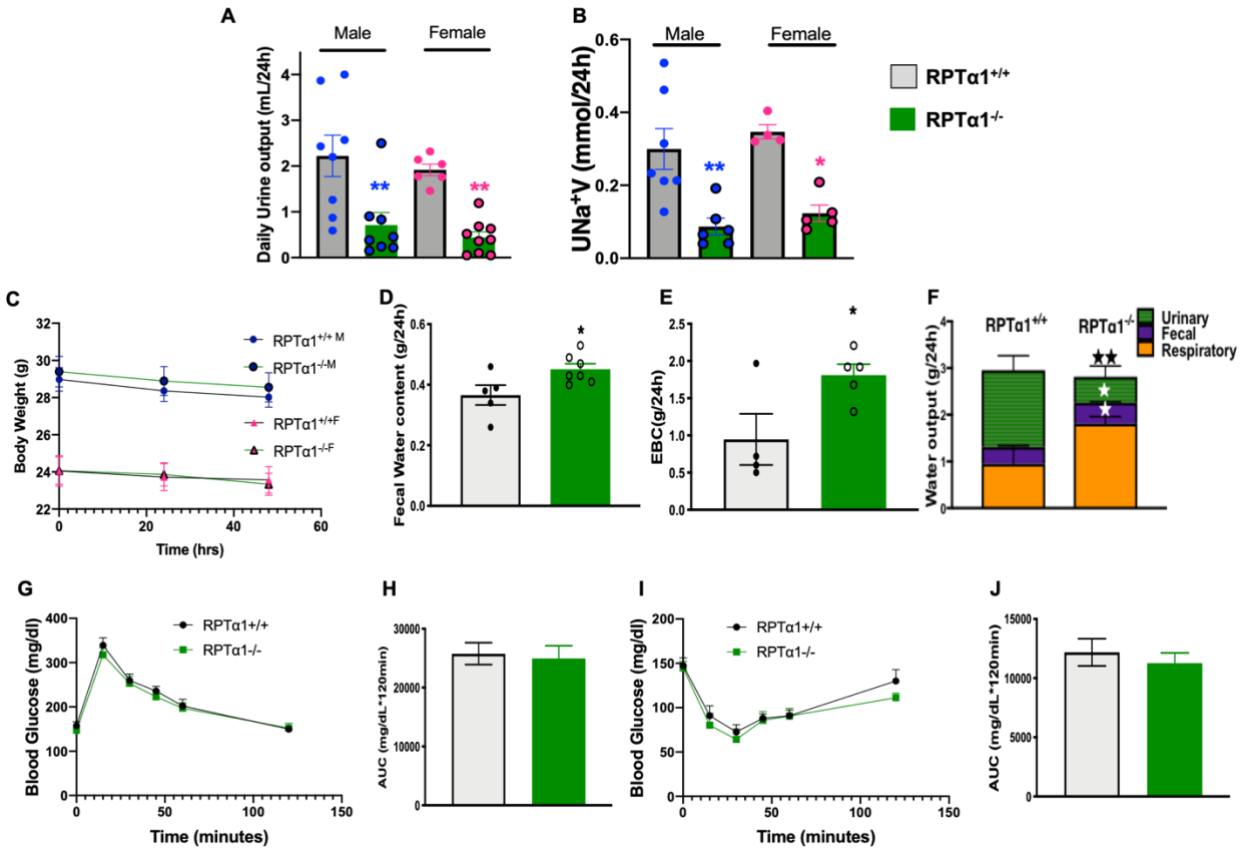

Figure 10: Urine and Na$^+$ output, Water excretion through alternate routes and Glucose and Insulin tolerance tests in RPTα1$^{-/-}$.

Daily urine output **(A)** and Absolute Na$^+$ excretion (UNa$^+$V) expressed as UNa$^+$V = Urine [Na$^+$] (mmol/mL) x Urine volume (mL/24h) **(B)** in male and female RPTα1$^{-/-}$ and controls. **(C)** Body weight measured during 48hr metabolic cage analysis of RPTα1$^{-/-}$ and controls (M for Males and F for Females) **(D)** Fecal water loss measured by 72hrs drying of feces collected by metabolic cage. **(E)** Respiratory water loss measured as exhaled breath condensate (EBC). **(F)**Total water loss through urine (orange), fecal (purple), and respiratory (green) in RPTα1$^{-/-}$ (n=4-7, 4-month male and female) **(G-H)** Glucose tolerance test curve and the quantitation of area under the curve (n=10-14, 4-month male) **(I-J)** Insulin tolerance test curve tolerance test curve and the

quantitation of area under the curve (n=5-6, 4-month male). Values are mean ± SEM and data were analyzed by one-way ANOVA with a Tukey multiple comparisons test (A-C) or Student's t-test (D-J). **p<0.01, ***p<0.005 vs RPTα1$^{+/+}$, blue **<p0.001 vs Male RPTα1$^{+/+}$, and pink *p<0.05 and **<p0.001 vs Female RPTα1$^{+/+}$.

	RPTα1$^{+/+}$	RPTα1$^{-/-}$
Sodium (mEq/L)	217±10, n=11	202±20, n=9
Potassium (mEq/L)	413±30, n=8	346±50, n=6
Chloride (mEq/L)	300±20, n=11	290±30, n=9
Creatinine (mg/dL)	53±3, n=12	53±5, n=10
Phosphorus (mg/dL)	159±20, n=12	135±10, n=9
Calcium (mg/dL)	21±4, n=5	14±5, n=6
Magnesium (mg/dL)	48±7, n=7	60±7, n=6
Glucose (mg/dL)	127±20, n=7	72±10*, n=6
pH	6.4±0.1, n=7	6.1±0.05*, n=7

Table 4: Urine chemistry panel of RPTα1$^{-/-}$ and control RPTα1$^{+/+}$ mice.
Values are mean±SEM in 4-month male mice. *p<0.05 vs RPTα1$^{+/+}$.

	RPTα1$^{+/+}$	RPTα1$^{-/-}$
Sodium (mEq/L)	148±1	148±2
Potassium (mEq/L)	5.07±0.1	4.86±0.1
Chloride (mEq/L)	118±1	116±2
Creatinine (mg/dL)	0.12±0.04	0.14±0.04
Phosphorus (mg/dL)	8.2±1	9.3±1
Calcium (mg/dL)	8.8±0.3	8.9±0.2
Fasting glucose (mg/dL)	131±7	128±10
Albumin(g/dL)	2.4±0.1	2.4±0.1
BUN (mg/dL)	39±4	36±6
Total protein (g/dL)	4.6±0.2	4.5±0.2

Table 5: Serum chemistry panel of RPTα1$^{-/-}$ and control.
Values are means±SEM in 4-month male mice (n=5-6/genotype). No significant difference was observed.

Increased RPT Na^+ absorption and upregulation of Na^+ transporters in the RPTα1$^{-/-}$ mouse mirror the *in vitro* mechanism.

The reduction of natriuresis following NKA reduction in the RPT S1/S2 segments of the RPT$\alpha1^{-/-}$ mouse is consistent with a reduced absorption of Na^+ by the RPT. Although it is at odds with the phenotype solely predicted based on NKA enzymatic function (less basolateral NKA ion-pumping should decrease Na^+ absorption by the RPT), it is consistent with the predicted phenotype based on a model that includes NKA signaling function (less basolateral NKA signaling should increase NHE3-mediated absorption). In fact, the net increase in Na^+ absorption upon NKA suppression suggests that signaling is functionally dominant under physiological conditions. Remarkably, this was also predicted by the increased transcellular Na^+ flux in RPT cells with genetic suppression of NKA in PY-17 (Figure 5). Consequently, we set out to test this hypothesis by assessing whether 1) cellular redistribution of apical and basolateral Na^+ transporters occur in the RPT of RPT$\alpha1^{-/-}$ mice as observed in PY-17 and 2) whether those changes are associated with functional evidence of hyper-absorption in the RPT of the RPT$\alpha1^{-/-}$ mouse.

As shown in Figures 11A-C, a significant decrease of over 50% in NHE3 phosphorylation at Ser-552 without change in total NHE3 was observed by western blot in the renal cortex of RPT$\alpha1^{-/-}$ mice. As predicted by decreased phosphorylation of NHE3 (i.e., decreased inactivation), a 2-fold increase in membrane expression of NHE3 was also detected (Figures 11D, 11F). This cellular redistribution of NHE3 was accompanied by a 60% decrease in urine lithium clearance and decreased serum lithium clearance in RPT$\alpha1^{-/-}$ mice (Figures 11G-I). The proposed molecular mechanism implies a causative link between RPT NHE3 redistribution to the apical membrane and increased absorption in the RPT$\alpha1^{-/-}$ mouse. Specifically, that NHE3 activation drives increased Na^+ absorption in the RPT upon removal of 70% of RPT NKA.

Consistent with this model, the hyper-absorptive phenotype of the RPTα1$^{-/-}$ mouse was not observed in the double RPTα1$^{-/-}$/RPTNHE3$^{-/-}$ mouse obtained by crossing RPTα1$^{-/-}$ with Floxed NHE3 mice (H. C. Li et al., 2013; X. C. Li et al., 2018), using the strategy shown in Figure 12A. Like RPTα1$^{+/+}$NHE3$^{+/+}$, RPTα1$^{-/-}$NHE3$^{-/-}$ mice were born with the expected Mendelian frequency and equal male: female ratio. They had no gross change in appearance, body weight, or food and water intake (Table 6), and the expected drastic reduction of NHE3 and NKA was observed by western blot in renal cortex homogenates (Figures 12E-G) but not in renal medulla homogenates (Figures 12H-J). As shown in Figures 11J-K, urine volume and urinary Na^+ content were significantly greater in the RPTα1$^{-/-}$NHE3$^{-/-}$ mice compared to the RPTα1$^{-/-}$ mice, undistinguishable from their control littermates with normal expression of NKA and NHE3 (RPTα1$^{+/+}$NHE3$^{+/+}$).

In addition to NHE3 modulation, a 2-fold increase in NBCe1A membrane expression was detected in the RPTα1$^{-/-}$ renal cortex (Figures 11E-F). This increase is consistent with the increased expression noted in the PY-17 model (Figure 5), but perhaps more importantly it is corroborated functionally by a modest but significant decrease in urine pH detected in RPTα1$^{-/-}$ (Table 4). Indeed, a decreased urine pH is predicted by the combined increase of apical NHE3 (increased H^+ excretion) and basolateral NBCe1A (increased $NaHCO_3$ absorption). A significantly decreased glucosuria (Table 4), still within the normal range of values reported for laboratory mice (Green, 1966) but unexpected given the already reduced glucose excretion in the RPTα1$^{-/-}$ mouse, was further indicative of a possible direct or indirect effect on apical Na^+/glucose co-transporter 2 (SGLT2, Slc5a2).

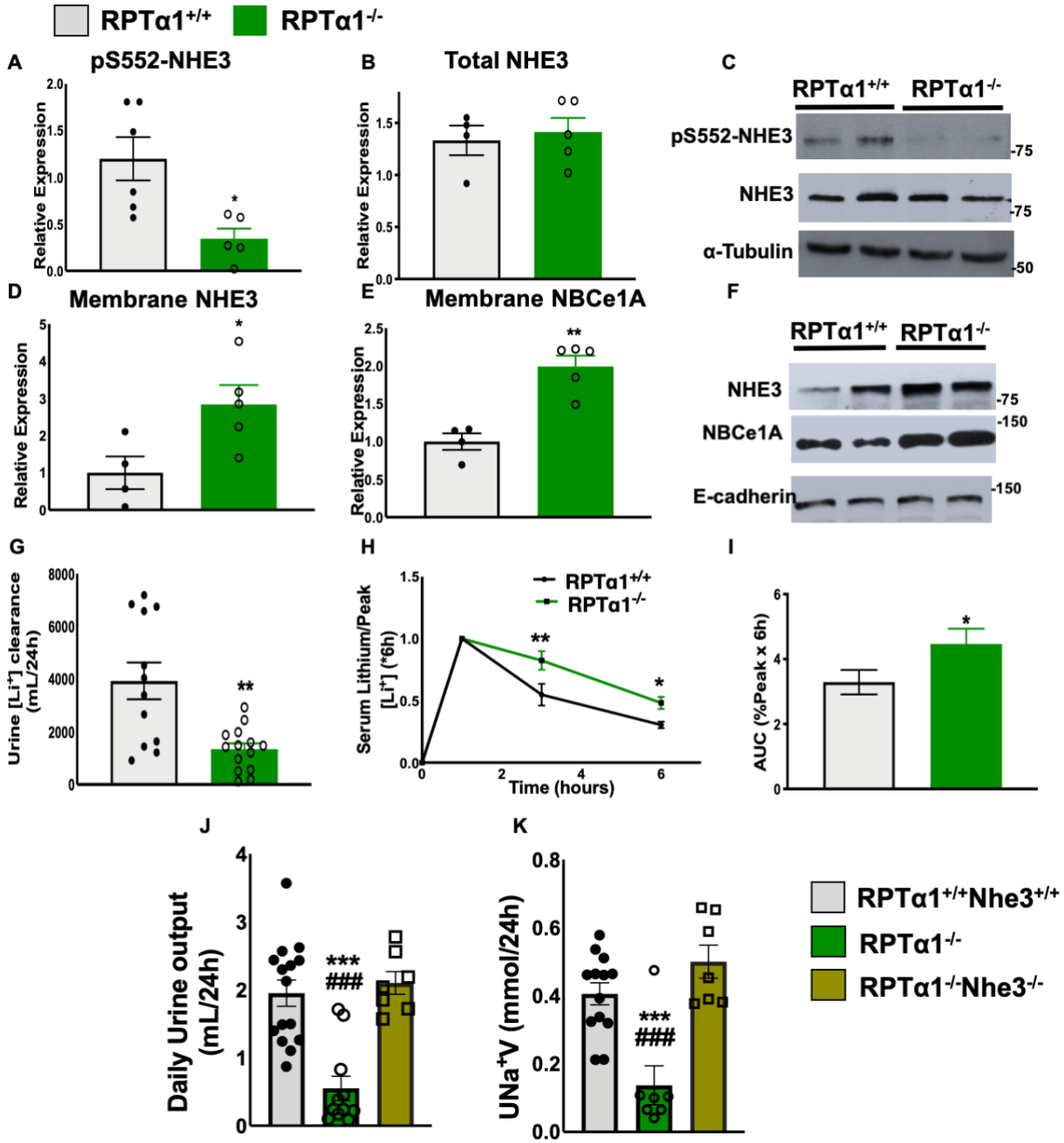

Figure 11: RPT sodium transporters and sodium reabsorption in RPTα1-/-.

Protein expression and corresponding representative film from western blots for phospho^{ser552}-NHE3 and total NHE3 in RPTα1$^{+/+}$ and RPTα1$^{-/-}$ mouse renal cortex homogenates. All quantifications were normalized to α-tubulin followed by normalization to average of control values**(A-C)**. Protein expression and corresponding representative films from Western blots for

NHE3 and NBCe1A in membranes isolated from renal cortex of RPTα1$^{+/+}$ and RPTα1$^{-/-}$ mouse kidneys All quantifications were normalized to E-Cadherin (membrane protein marker) followed by normalization to average of control values **(D-F)**. Urine Lithium clearance as a functional marker for RPT Na^+ reabsorption function in 4-month male mice of the indicated genotype (n=11-14) **(G)**. (**H-I)** Serum lithium clearance curve and quantification of area under curve (AUC). Curve and AUC normalized to peak dose in serum measured at 1hr (n=6-8, 4-month male). **(J)** Daily urine output measured in 4-month male mice using metabolic cage and (**K)** Absolute Na^+ excretion (UNa^+V) RPTα1$^{-/-}$NHE3$^{-/-}$ mice compared to RPTα1$^{-/-}$ and controls (RPTα1$^{+/+}$NHE3$^{+/+}$). UNa^+V = Urine [Na^+] (mmol/mL) x Urine volume (mL/24h). Results are presented as mean ±SEM and data were analyzed by t-test (A-I) or one-way ANOVA with a Tukey multiple comparisons test (J-K). *p<0.05, **<p<0.01, and ***p<0.001 vs RPTα1$^{+/+}$or RPTα1$^{+/+}$NHE3$^{+/+}$, ### p<0.001 vs RPTα1$^{-/-}$NHE3$^{-/-}$.

	RPTα1$^{+/+}$NHE3$^{+/+}$	RPTα1$^{-/-}$NHE3$^{-/-}$
Body Weight (g)	31.1 ± 1	32.5 ± 0.9
Daily water intake (g)	7.3 ± 0.5	6.6 ± 0.2
Daily food intake (g)	5.35 ± 0.4	4.92 ± 0.2

Table 6: Basal metabolic characteristics of RPTα1$^{-/-}$NHE3$^{-/-}$mice.
Values are mean±SEM for 4-month male mice (n=6-7/genotype). No significant difference was observed by Student's t-test.

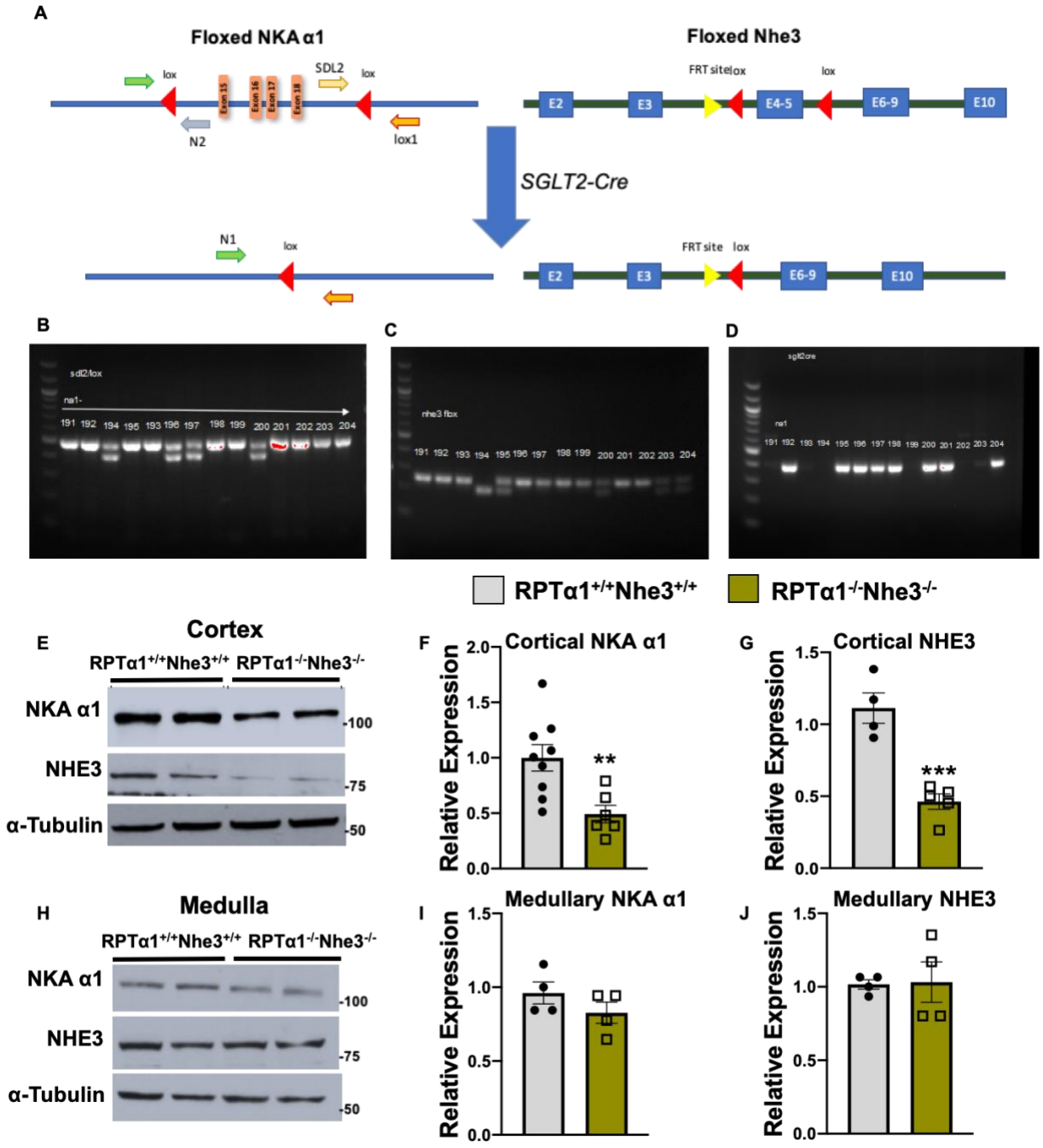

Figure 12: Proximal-tubule specific Atp1a1 and NHE3 ablation.
Scheme for generation of RPT-specific ablation of *Atp1a1* and NHE3 in mouse using Cre/Lox system **(A)**. DNA genotyping for homozygous floxed NKA α1 **(B)**, homozygous floxed NHE3 **(C),** and heterozygous SGLT2-Cre **(D)**. Mice positive for homozygous floxed α1 and

homozygous floxed NHE3 and heterozygous SGLT2-Cre were grouped as RPTα1$^{-/-}$NHE3$^{-/-}$ and mice positive for homozygous floxed α1 and homozygous floxed NHE3 but negative for SGLT2-Cre were grouped as controls RPTα1$^{+/+}$NHE3$^{+/+}$. Representative western blots and quantification of NKA α1 and NHE3 in renal cortex homogenates from RPTα1$^{-/-}$NHE3$^{-/-}$ and RPTα1$^{+/+}$NHE3$^{+/+}$ control mice **(E-G)**. Representative western blots and quantification of NKA α1 and NHE3 in renal medulla homogenates from RPTα1$^{-/-}$NHE3$^{-/-}$ and RPTα1$^{+/+}$NHE3$^{+/+}$ control mice **(H-J)**. All quantifications were normalized to α-tubulin followed by normalization to the average of control values in each gel. Values are mean ±SEM and data were analyzed by Student's t-test. **p<0.01, ***p<0.001 vs RPTα1$^{+/+}$NHE3$^{+/+}$

Dysregulation of Src kinase in the proximal tubules of the hypomorphic RPTα1$^{-/-}$ mouse.

We next turned our attention to the underlying signaling mechanism that could modulate Na^+ absorption. Regulation of NHE3 and Na^+ influx in RPT cells by CTS has been shown to be Src-dependent (H. Cai et al., 2008; Godinho et al., 2017; Liu et al., 2011; Yan et al., 2012). CTS are widely viewed as specific ligands of NKA, which suggests that disruption of the NKA α1/Src receptor mechanism, as first described by Z. Xie (Haas et al., 2000; Haas et al., 2002; Man Liang et al., 2006; Tian et al., 2006; Z. Xie, 2003), could be the critical event that led to NHE3/NBCe1A activation and subsequent decrease in urine excretion.

Consistent with this hypothesis, sucrose-density-based cellular fractionation studies revealed a significant redistribution of residual NKA α1 and Src kinase outside the light caveolar-rich domains in the renal cortex of RPTα1$^{-/-}$ mice (Figures 13A-B), akin to the redistribution observed in PY-17 cells (Figures 13C-D). Using triple immunofluorescence labeling in renal cortical sections (villin: magenta; phospho^{Tyr419}Src: green; NKA: red), we

further observed a diffuse intracellular signal for activated phospho^{Tyr419}Src in villin-positive RPTα1$^{-/-}$ tubules with reduced colocalization of phospho^{Tyr419}Src with NKA (Pearson's correlation coefficient, PCC=-0.05±0.03 vs 0.4±0.02 in RPTα1$^{+/+}$) which markedly differed from its clustered membrane distribution in RPTα1$^{+/+}$ (Figure 13E). For comparison, immunolabeling of NKA (red) and phospho^{Tyr419}Src (green) was performed in parental (LLC-PK1) and NKA knockdown (PY-17) pig RPT cells. As shown in Figure 13F, a diffuse intracellular redistribution of phospho^{Tyr419}Src away from the plasma membrane and decreased colocalization with membrane NKA (PCC=0.5±0.03 vs 0.9±0.01 in LLC-PK1) and comparable to the redistribution in the RPTα1$^{-/-}$ was observed. Collectively, these observations support a role for NKA/Src receptor complex in regulation of NHE3, NBCe1A and RPT Na^+ transport.

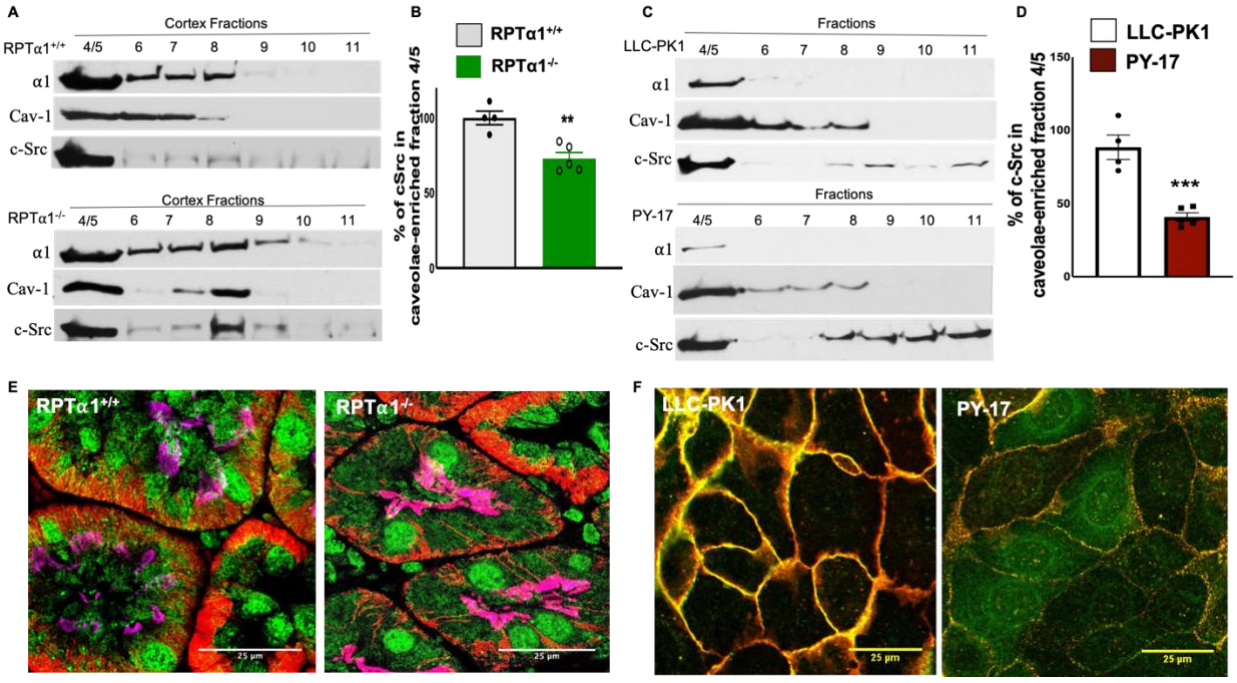

Figure 13: Loss of NKA α1 leads to dismantlement of NKA/Src receptor complex in RPT cells.
(A-B) Representative western blot for NKA α1, caveolin-1, and c-Src in caveolin-enriched fractions (4/5) together with fractions 6-11 obtained after sucrose-density fractionation in renal

cortex from RPTα1$^{+/+}$ and RPTα1$^{-/-}$ and the respective quantitation of percentage of signals from caveolin-enriched fraction against total signal. **(C-D)** Representative western blot for NKA α1, caveolin-1, and c-Src in caveolin-enriched fractions (4/5) together with fractions 6-11 obtained after sucrose-density fractionation in LLC-PK1 and PY-17 cells and the respective quantitation of percentage of signals from caveolin-enriched fraction against total signal. **(E)** Representative immunostaining for NKA α1 (red), phospho^{Tyr419}-Src (green), villin-1**(**magenta**)** in kidney cross-sections from RPTα1$^{+/+}$ and RPTα1$^{-/-}$. Scale bar: 25µm. **(F)** Representative immunostaining for NKA α1 (red) and phospho^{Tyr419}-Src (green) in LLC-PK1 and PY-17 cells. Scale bar: 25µm. Results are presented as mean±SEM and data were analyzed by t-test. **p< 0.05 versus RPTα1$^{+/+}$ and ***p<0.001 versus LLC-PK1.

NKA α1/Src receptor function causes tonic inhibition of NHE3 and NBCe1A.

To functionally challenge our hypothesis of NKA α1/Src-mediated tonic inhibition of NHE3 and NBCe1A in RPT cells, we utilized a loss-of-function/gain-of-function approach. The strategy was based on recent advances in our understanding of the structural basis of NKA/Src receptor function and its NKA isoform-specificity. It relies on the fact that the two known critical Src binding sites (the NaKtide sequence and Y260 (Banerjee et al., 2018; Lai et al., 2013; Z. Li et al., 2009; Yu et al., 2018)) required for adequate NKA α1/Src-mediated signaling are specific to the mammalian NKA α1 polypeptide sequence, the only NKA α isoform expressed in RPT cells. Critically, the Y260 residue is absent in the NKA α2 isoform, which does not support Src-dependent signaling (Banerjee et al., 2018). Table 7 summarizes key features of the cell lines used in the set of studies shown in Figure 14. All these lines were obtained by rescuing PY-17 cells with WT or selected mutant forms of rat NKA α1 or α2. The AAC-19 line, expressing the WT rat NKA α1, was the Src-signaling-enabling control for the Y260A line, a Src-signaling-null

rat α1 mutant line (Table 7) (Banerjee et al., 2018; Lai et al., 2013; J. X. Xie et al., 2015; Yu et al., 2018). In the reciprocal gain-of-function experiment, the LX-α2 line expressing WT rat NKA α2 (Src-signaling null) was the control for LY-a2, a mutant rat α2 that was engineered to contain both Src binding sites (Table 7). Importantly, enzymatic NKA activity was identical in all those cell lines, which allowed direct comparison of Na^+ transporter regulation.

When compared to AAC-19 cells, Y260A cells had a ~50% decrease in inhibitory ser552 phosphorylation of NHE3 and no change in total NHE3 (Figures 14A-B, 14D). Conversely, a 50% increase in phosphorylation-mediated inactivation and no change in total NHE3 was observed in LY-a2 cells compared to LX-α2 (Figures 14E-F, 14H). Finally, total NBCe1A expression was increased by 2-fold in Y260A cells compared to AAC-19 cells (Figures 14C-D), and decreased by 2-fold in LY-a2 cells compared to LX-α2 cells (Figures 14G-H). Taken together, these data indicated that NKA/Src signaling is required for tonic inhibition of NHE3 and NBCe1A by NKA α1.

Collectively, these results provide genetic evidence for tonic inhibition of RPT Na^+ transport by NKA α1/Src receptor signaling in RPT cells. This novel regulatory pathway physiologically counteracts NKA-dependent enzymatic Na^+ absorption as summarized in Figure 15.

Cell Line	NKA Isoform	Y260	NaKtide sequence	NKA/Src signaling	NKA ion-pumping (% of LLC-PK1)
LLC-PK1	Pig α1	253 RGIVV**Y**TGDRT 263	413 S**A**TWLALSRIAGLCNRAVF**Q** 432	Enabled	100
PY-17	10% Pig α1	253 RGIVV**Y**TGDRT 263	413 S**A**TWLALSRIAGLCNRAVF**Q** 432	Disabled	40
AAC-19	Rat α1	255 RGIVV**Y**TGDRT 265	415 S**A**TWFALSRIAGLCNRAVF**Q** 434	Enabled	100
Y260A	Mutant Rat α1	255 RGIVV**A**TGDRT 265	415 S**A**TWFALSRIAGLCNRAVF**Q** 434	Disabled	100
LX-α2	Rat α2	253 RGIVI**A**TGDRT 263	413 S**P**TWTALSRIAGLCNRAVF**K** 432	Disabled	100
LY-a2	Mutant Rat α2	250 RGIV**VY**TGDRT 260	410 S**A**TW**F**ALSRIAGLCNRAVF**Q** 429	Enabled	100

Table 7: Amino acid sequences of the Src binding sites of the WT rat α1, loss-of-function signaling mutant α1 NKA, WT rat α2, and gain-of-function signaling mutant α2 NKA expressed by the AAC-19, Y260A, LX-α2, and LY-a2 cell lines.
Underlined letters indicate residues that have been changed in mutant α1 and α2, while bold underlined letters indicate residues that differ from wild-type rat α1.

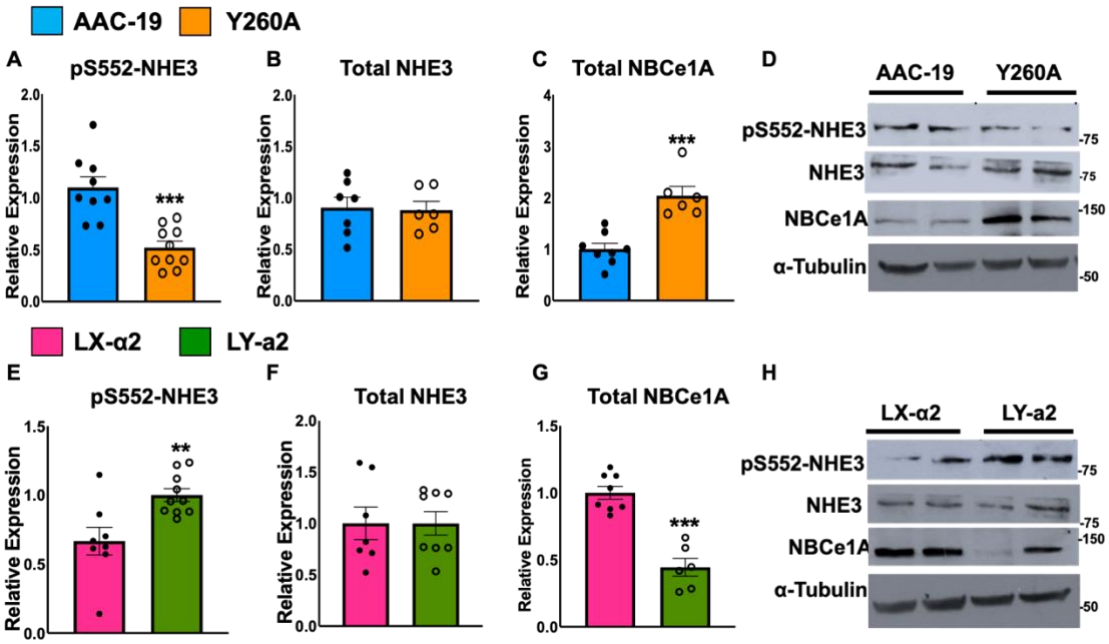

Figure 14: Loss of Src-binding in NKA α1 reduces NHE3 inhibitory phosphorylation at ser552 and increases NBCe1A in RPT cells.

Representative films from Western blots for phospho^{ser552}-NHE3, NHE3 and NBCe1A with respective quantification **(A-C).** AAC-19 (control) and Y260A, and **(D-F)** LX-α2 and LYα2 cells. All quantifications were normalized to α-tubulin followed by normalization to average of control values. Results are presented as mean ± SEM and data were analyzed by Student's *t*-test. **p< 0.01, ***p<0.001 versus AAC-19 or versus LX-α2.

Discussion

The renal proximal tubule is the center stage for physiological and pathological changes associated with sodium balance and volume homeostasis. Distinctive architectural features and cellular properties underlie unique regulatory responses of this highly permeable epithelium to local and systemic cues. These regulatory mechanisms, some of which remain incompletely understood, are considered of primordial importance for the understanding and management of

hypertensive disorders (Joe & Shapiro, 2012; McDonough, 2010b; McDonough & Nguyen, 2015).

Using a genetic approach, the present studies uncovered a potent natriuretic pathway controlled by a familiar ion pump in the RPT, the Na/K-ATPase (NKA). Arguably, NKA makes quite an unlikely candidate as a molecular counteractor of Na^+ absorption. In fact, NKA is universally recognized as the very enzymatic machinery that drives avid absorption of Na^+ along the RPT (Figure 15A). With hindsight, it could also be argued that NKA has evolved as a perfect molecular entity to sense intracellular Na^+ and coordinate the cellular response to temper absorption by the RPT. Indeed, we have learned that a Src-regulatory capability was acquired selectively by the mammalian α1 isoform of the NKA's catalytic subunit (Banerjee et al., 2015; Man Liang et al., 2006; Madan et al., 2016; J. X. Xie et al., 2015; Yu et al., 2018).

Evidence in RPT cells have suggested that this NKA-centered mechanism downregulates cell surface expression of apical NHE3 and basolateral NKA upon binding of a NKA ligand such as the cardiotonic steroid (CTS) ouabain (H. Cai et al., 2008; Godinho et al., 2017; Liu et al., 2004; Oweis et al., 2006; Yan et al., 2012). In the present study, genetic targeting of NKA in RPT cells *in vitro* and *in vivo* further indicates that this NKA/Src mechanism also downregulates the basolateral NBCe1A, which could be secondary to NHE3 reduction. To our knowledge, this is the first description of a mechanism that links NKA, NHE3, and NBCe1A in an interdependent pathway. It contrasts with regulatory pathways of RPT Na^+ handling described to date, which involve coordinated but independent regulation of NHE3, NBCe1A, and NKA [9, 59-61]. For example, the natriuretic hormones dopamine and parathyroid hormone have been shown to stimulate parallel PKC-mediated inhibition of NKA and cAMP-dependent inhibition of NHE3 and NBCe1A to decrease RPT Na^+ absorption (Aperia et al., 1987; Kunimi et al., 2000;

McKinney & Myers, 1980; Pedemonte et al., 2005b; Y. Zhang et al., 1999). Importantly, the increase of NHE3/NBCe1A and transcellular Na^+ in RPT cells monolayers indicates that the NKA/Src-induced suppression of Na^+ transporters exerts its effect tonically, and that this tonic inhibition is lifted upon genetic suppression (Figure 5) or loss-of-function mutation (Figure 14). As shown in Figure 15, we propose that NKA/Src provides a regulatory mechanism to temper NKA-driven transcellular Na^+ absorption and is activated by CTS.

The fundamental role of this NKA/Src mechanism was revealed by the hyper-absorptive phenotype of the hypomorphic RPT$\alpha1^{-/-}$ mouse. With no morphological or biochemical indication of renal failure, and no indication of change in the GFR, a striking characteristic in this model was that oliguria was not associated with major changes in blood or urine composition. Consequently, and although extra-renal routes of fluid excretion provided a remarkably efficient compensatory mechanism, evidence of systemic sodium loading and extracellular volume expansion were noted, including decreased serum aldosterone and a reduced hematocrit. Hence, with 2/3 of NKA units depleted and an overall neuro-hormonal status favoring further reduction of NKA-mediated Na^+ absorption, the RPT of the RPT $\alpha1^{-/-}$ mouse still reabsorbed copious amounts of Na^+. This is consistent with the hypothesis that NKA/Src signaling leads to a loss of intracellular Na^+ sensing in the RPT, which in turn leads to an aberrant absorptive response. Indeed, it has been suspected for decades that intracellular Na^+ modulates the response to natriuretic and anti-natriuretic hormones in the RPT as the first step mechanism to control Na^+ absorption (Efendiev et al., 2003). As an example, dopamine-induced inhibition of NKA ion transport function in RPT cells is sensitized by increased intracellular Na^+ concentrations (Efendiev et al., 2003). The NKA/Src dependent signaling is NKA conformation-dependent (Ye et al., 2013; Ye et al., 2011), and therefore a viable Na^+ sensor to

sensitize/desensitize RPT response to natriuretic and anti-natriuretic modulators. This sensor may also play a role in the relatively low sensitivity of RPT Na^+ absorption to hormonal signals in response to changes in dietary Na^+ compared to other nephron segments, and consequently maintain a steady delivery of Na^+ to the distal tubule regardless of dietary intake in the healthy kidney (Ellison, Velazquez, & Wright, 1989; Malnic, Klose, & Giebisch, 1966; Palmer & Schnermann, 2015; Vallon et al., 2002).

The RPT NKA/Src mechanism uncovered by this genetic approach provides a long sought-after receptor mechanism to explain the natriuretic effect of endogenous CTS, which has first been proposed more than 50 years ago. Endogenous CTS, then referred to as 'third factor', were first identified as a crucial regulatory natriuretic factor by DeWardner (De Wardener, 1961; De Wardener et al., 1971) and later isolated from circulating human plasma at subnanomolar concentrations (Hamlyn et al., 1991). Their mechanism of action has remained debated, particularly their action on NKA, as those concentrations are well below the concentration necessary to inhibit the enzymatic NKA ion pumping activity (Liu et al., 2002). Further, the ubiquitous expression of NKA α1 has made it difficult to assign CTS effects to a specific organ system. Nonetheless, the first genetic evidence for a physiological role of endogenous CTS came from an elegant study of the Lingrel lab, in which increased affinity of NKA α1 in mice led to an increased natriuretic response and decreased Na^+ absorption without change in GFR in response to acute saline load (Loreaux et al., 2008). Our observations in the RPT$\alpha1^{-/-}$ are consistent with these findings, and further suggest that renal NKA α1/Src is the necessary and sufficient receptor mechanism that mediates CTS-induced natriuresis.

This has significant clinical implications. Indeed, increased levels of endogenous CTS have been reported in conditions such as volume expansion, pre-eclampsia, essential

hypertension, primary aldosteronism, and end-stage renal disease (Bagrov et al., 1995; Bagrov & Shapiro, 2008; Fridman et al., 2002; Gonick et al., 1998; Lopatin et al., 1999). The ability of CTS to cause vasoconstriction has led many to postulate that these hormones contribute to the development of the hypertensive state, despite their natriuretic properties. In contrast, experimental models have indicated that an intact diuretic response to CTS is important for low salt sensitivity of blood pressure. For example, in RPT cells from Dahl salt-sensitive rats, CTS-induced coupled inhibition of NKA and NHE3 is impaired. This may support increased RPT Na^+ absorption as observed in the RPT$\alpha 1^{-/-}$ mouse, and contribute to elevated blood pressure in these rats (Liu et al., 2011). Likewise, in chronically NaCl-loaded Sprague-Dawley rats, renal excretion of the CTS marinobufagenin (MBG) is significantly elevated, and anti-MBG antibody reduce natriuresis and restore sodium pump activity in the renal cortex (Periyasamy et al., 2005). Consistent with an effect of MBG on the RPT NKA α1/Src mechanism described in the present study, it was shown that MBG exhibited its natriuretic effects via internalization of the sodium pump in the RPT. Through RPT NKA/Src, endogenous CTS released during high salt conditions may therefore favor the excretion of excessive salt and ultimately contribute to the maintenance of normal BP. Our approach using tissue-specific genetic targeting of the NKA receptor may serve as a tool to explore whether endogenous CTS, acting on renal NKA α1, are indeed a positive factor in the defense against hypertension.

A few aspects of these findings will require additional investigations. Indeed, in addition to revealing a potent NKA/Src natriuretic signaling in the RPT, the data suggest that the loss of NKA pump activity allows NBCe1A to functionally assume the role of an "auxillary Na^+ pump" in the RPT. It should also be noted that our investigation primarily focused on RPT NHE3, NKA and NBCe1A. Other RPT transporters such as SGLT2 and Na^+/Pi cotransporter (NaPi2) may

also be regulated by the NKA/Src network. In RPTα1$^{-/-}$ mice, a modest modulation of acid-base balance and absorption of glucose was detected without significant effect on GTT or ITT under basal conditions. This is consistent with compensatory mechanisms by other nephron segments, but could lead to functional imbalances in disease stages. Additionally, the decreased diuresis and natriuresis were noted in a cohort of 4-month-old RPT α1 mice that included males and females in a 1:1 ratio. Additional studies were performed exclusively in males of that age group, and we therefore cannot generalize all our findings to the female sex given the increasing evidence of renal sexual dimorphism (R. Hu et al., 2020; Q. Li et al., 2018; Veiras et al., 2017).

Based on this study, a better understanding of the dual antagonistic roles of NKA in the RPT has become increasingly important, as both clinical and experimental evidence suggests that a dysregulation of RPT NKA function by CTS and other regulators of renal Na^+ absorption may occur in salt-sensitive hypertension.

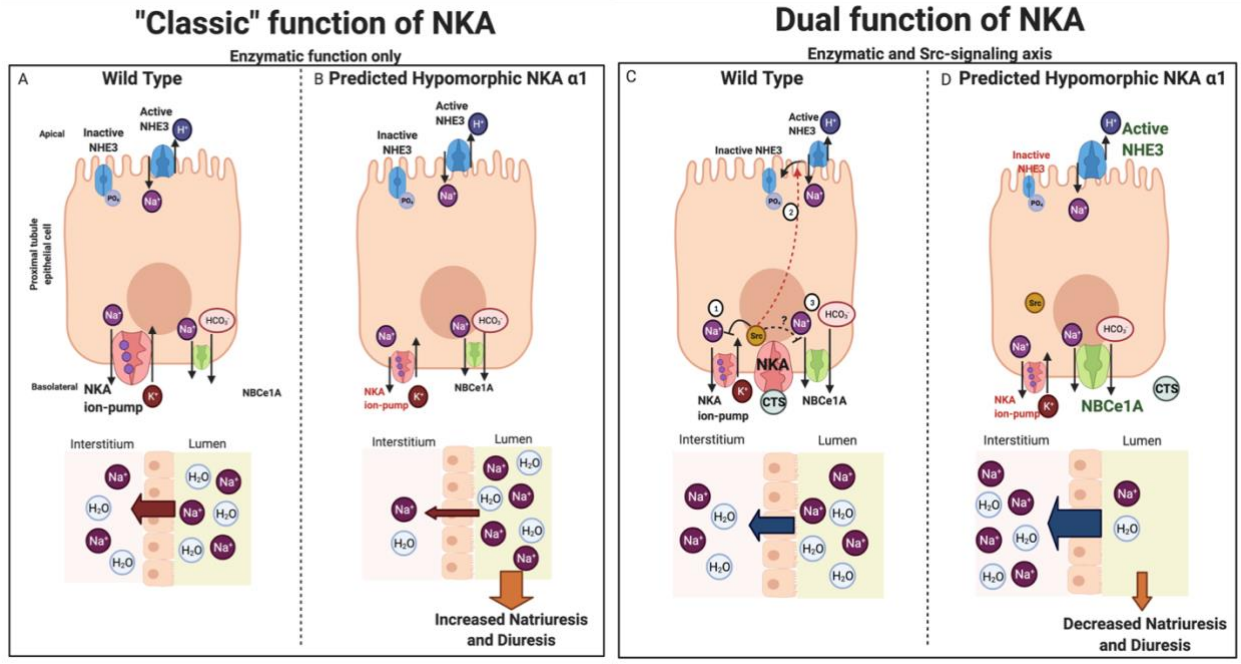

Figure 15: Scheme depicting Classic vs Dual function of NKA in RPT Na^+ reabsorption and corresponding predicted phenotype of the hypomorphic RPTα1$^{-/-}$ mouse.

The classical role of basolateral NKA in RPT Na^+ transport is that of solely an enzymatic ion-pump that drives the Na^+ gradient and transport. Apical transporter NHE3 is the major route for Na^+ reabsorption in the RPT, with basolateral NBCe1A playing a more modest role **(A)**. Based on this classical role, a knockdown of NKA α1 in RPT would be expected to result in hypo-reabsorption of Na^+ and water, and increased natriuresis and diuresis in the hypomorphic NKA α1 mice **(B)**. According to the dual function model of NKA, NKA/Src receptor-mediated signaling plays a counteractive role over its enzymatic function such that stimulation of NKA/Src signaling by endogenous ligands such as cardiotonic steroids (CTS) leads to internalization of basolateral NKA ion-pump (1), stimulates phosphorylation-mediated inactivation of apical NHE3 prior to internalization (2), and decreases membrane expression of basolateral NBCe1A directly or secondary to NHE3 reduction (3)- leading to tonic inhibition of RPT Na^+ reabsorption **(C)**. Based on the dual functions of NKA, a loss of NKA/Src receptor function in the hypomorphic NKA α1 mouse would be expected to result in a dismantled NKA/Src receptor complex leading to an increased membrane expression of RPT NHE3 and NBCe1A, and thereby promoting reabsorption of Na^+ and water **(D)**. The hyper-reabsorptive phenotype of the hypomorphic RPT$\alpha1^{-/-}$ mice indicate that actually NKA/Src receptor is functionally dominant over the classic enzymatic NKA function in RPT Na^+ reabsorption. **CTS**: cardiotonic steroids; **NKA**: Na/K-ATPase; **NBCe1A**: NaHCO3 cotransporter 1A; **NHE3**: NaH exchanger 3. *Created with BioRender.com*

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Materials and Methods

Reagents.

The polyclonal anti-Na/K-ATPase α1 antiserum NASE, polyclonal anti-Na/K-ATPase α2, and polyclonal anti-Na/K-ATPase α3 used for tissue western blots were raised in rabbits and were generous gifts from Drs. T. Pressley and P. Artigas at Texas Tech University Health Sciences Center (Pressley, 1992). The monoclonal anti-Na/K-ATPase α1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa was used for cellular western blot analysis. The monoclonal Anti-Na/K-ATPase α1 from Millipore (#05-369) was used for immunocytochemistry. Anti-NHE3 (Millipore, catalogue #AB3085MI) and anti-phosphorylated NHE3 at serine 552 (Santa Cruz Biotechnology, Catalogue #sc-516102) were selected based on previous literature (Kurashima et al., 1997). Anti-NBCe1A chicken antibody was developed by Dr. Michael Romero (Mayo Clinic) and gifted to us for use. Anti-α-tubulin antibody (sigma, catalogue #T5168) and anti-E-cadherin (Cell Signaling, Catalogue # 3195S) were used as a loading control for total and membrane lysates, respectively. The c-Src antibody and caveolin-1 antibody for western blot were both from Cell Signaling #2108S and #3267, respectively). The polyclonal anti-Tyr(P)418-Src antibody from Invitrogen(#44-660G) was used for immunocytochemistry. Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit (R&D systems, Catalogue# HAF008), anti-chicken (Invitrogen, Catalogue# SA1-72064) and anti-mouse (Santa-Cruz biotechnology Inc., Catalogue# sc-516102). $^{22}Na^{+}$ was obtained from PerkinElmer (Catalogue # NEZ081100UC)

Cell Culture.

The parental LLC-PK1 cells were purchased from ATCC. Mutant cell lines used in this work were derived from LLC-PK1 cells. The generation of Na/K-ATPase α1 knockdown PY-17 cells from LLC-PK1 has been described in details (Liang et al., 2006). PY-17 cells express about 8% of α1 Na/K-ATPase in comparison to LLC-PK1 cells, and do not express other isoforms of Na/K-ATPase.

Using the well-established protocol of knockdown and rescue, we have generated several stable cell lines (Lai et al., 2013; Xie et al., 2015; Yan et al., 2013). The generation and characterization of the α1-rescued AAC-19 cells, α1 mutant-rescued Y260A, α2-rescued LX-α2, and α2 mutant-rescued cells LY-a2 used in this study have been reported (Banerjee et al., 2018; Lai et al., 2013; J. X. Xie et al., 2015; Yu et al., 2018). After cells reached 95-100% confluence, they were serum-starved overnight and used for experiments. All cell lines were cultured in DMEM plus 10% FBS with 1% penicillin/streptomycin. All cells were grown in 100mms dishes and serum-starved at 90-100% confluency for 24hrs before isolation of protein for biochemical analysis, unless otherwise stated.

Transepithelial ^{22}Na flux.

To measure active transepithelial $^{22}Na^+$ flux, a transepithelial $^{22}Na^+$ transport assay was performed in LLC-PK1 and PY-17 monolayers (grown on Costar Transwell culture filter inserts, filter pore size: 0.4 μm, Costar, Cambridge, MA) as previously described (H. Cai et al., 2008). Briefly, transepithelial $^{22}Na^+$ flux (apical to basolateral) was determined by counting radioactivity in the basolateral aspect at 1 h after $^{22}Na^+$ (1uCi/mL) addition on the apical part of the transwell insert.

Measurement of Transepithelial electrical Resistance (TER).

To measure paracellular permeability, TER was measured in confluent monolayers of serum-starved LLC-PK1 and PY-17 (grown on Costar Transwell culture filter inserts, filter pore size: 0.4 μm, Costar, Cambridge, MA). EVOM2 (Epithelial Volt Ohm Meter; World Precision Instruments) along with STX-2 probe (World Precision instruments) were utilized for the TER measurements. Final values were obtained by subtracting the resistance of the bathing solution and an empty support, and then dividing the surface area of insert. Results are expressed as ohms per square centimeter.

Immunocytochemistry.

Cells were cultured on glass coverslips until 80% confluency was achieved. After being fixed with Methanol and washed with 1x PBS, the cells were permeabilized for 10 min with 0.1% Triton X-100 in PBS. The cells were washed again with 1xPBS and blocked with 5% horse serum in PBS for 30mins. Primary antibodies for NKA α1 and p-Src were diluted to 1:100 in 2% BSA in PBS and coverslips were incubated with antibodies overnight at 4°C. After three washes with PBS, AlexaFluor 555/488–conjugated antibodies were added and incubated for 1hr at room temperature. Samples were washed and mounted onto slides using VectorMount mounting media. Cells were visualized using Leica confocal SP5 microscope equipped with a 63x/1.3 oil objective. To avoid the cross-talk between the two fluorescent dyes, we used the sequential method featured by the Leica confocal microscope to acquire the images for measuring colocalization of the two proteins.

Animals.

Mice with floxed endogenous NKA α1 isoform developed by the Kansas University Medical Center Core were crossed with Sglt2.iCre mice initially developed by Dr. Michel Tauc (Rubera et al., 2004) and obtained through the European Mouse Mutant Archive (EMMA) repository

(https://www.infrafrontier.eu/resources-and-services/access-emma-mouse-resources). Mice homozygous for floxed α1 (α1flox/α1flox) and heterozygous for Sglt2iCre (Sglt2iCre/WT) were bred with α1flox/α1flox Sglt2WT/WT mice (Figure 6A), resulting in litters of α1flox/flox-Sglt2iCre/WT (RPTα1$^{-/-}$) mice with α1flox/α1flox Sglt2WT/WT controls (RPTα1$^{+/+}$) (H. C. Li et al., 2013; Rubera et al., 2004). The mice generated were assessed for the presence of heterozygous SGLT2-Cre and homozygous floxed *Atp1a1* by PCR on genomic DNA extracted from tail clips collected from bred mice (Figures 6B-C).

Mice with heterozygous floxed endogenous NHE3 were generously provided by Dr. M. Soleimani at University of Cincinnati and were crossed with the previously generated α1flox/flox-Sglt2iCre/WT (RPTα1$^{-/-}$) mice resulting in litters of α1flox/flox-NHE3flox/flox-Sglt2iCre/WT (RPTα1$^{-/-}$NHE3$^{-/-}$) mice with α1flox/α1flox-NHE3flox/flox-Sglt2WT/WT controls (RPTα1$^{+/+}$NHE3$^{+/+}$). The mice generated were assessed for the presence of heterozygous SGLT2-Cre, homozygous floxed *Atp1a1*, and homozygous floxed Nhe3 by PCR on genomic DNA extracted from tail clips collected from bred mice (Figures 12B-D).

RPTα1$^{-/-}$, RPTα1$^{-/-}$NHE3$^{-/-}$ and their control littermates were housed in 12-hour light and dark cycles at constant temperature and humidity. All animal procedures were approved by the Marshall University Institutional Animal Care and Use Committee.

Metabolic Cage analysis.

Mice were housed individually in metabolic cages (manufactured by TechniPlast) for two consecutive days including 24hrs for acclimatization and the following 24hrs for measuring food and water intake, and urine output. Feces collected over 24hrs were weighed, dried over 72hrs, and weighed again to calculate the loss on drying to measure fecal water excretion.

Urine and Serum analysis.

Urine collected over 24hrs using metabolic cages was sent to Idexx Laboratories, Inc. for analysis of sodium, potassium, calcium, magnesium, phosphorus, creatinine, pH and glucose. Serum isolated from collected blood was sent to Idexx for analysis of sodium, potassium, calcium, magnesium, phosphorus, creatinine, and BUN. Aldosterone concentration was measuring in serum using Aldosterone Elisa kit (Cayman chemicals, Catalogue #501090) as per the manufacturer's instructions. Atrial natriuretic peptide concentration was measured in serum using Atrial Natriuretic Peptide EIA kit (Sigma, Catalogue#RAB0385). For Hematocrit measurement, tail vein was clipped and whole blood samples were collected by using a Heparinized Microhematocrit Capillary Tube (Produce number: 22-362-566, Fisherbrand, Pittsburgh, PA, USA) and then centrifuged for 5 min in a Unico Micro-Hematocrit Centrifuge (Model: C-MH30, Dayton, NJ, USA).

Measurement of Exhaled Breath Condensate (EBC).

We measured the water in the expired air as described (Wilhelm, Frydrychova, & Vizek, 1999). Conscious mice were individually placed in a body box [volume of 3L] for one hour with positive air in-flow at the rate of 500mL/min. The outlet of the box was connected to a stoppered glass flask that was submerged in dry ice to condense water in the expired air. The weight of the exhaled breath condensate was measured to assess the amount of water in exhaled air over 1 hour.

Transcutaneous FITC-sinistrin decay to measure glomerular filtration rate (GFR) in conscious mice.

The procedure was performed as previously described (Scarfe et al., 2018). In short, the transcutaneous device (MediBeacon GmbH, Germany) was fixed to the depilated skin on the back of adult mice using a double-sided adhesive patch (Lohmann, Neuwied, Germany).

Transcutaneous measurement started with background reading for 5 min. before 7.5mg/100g BW FITC-Sinistrin (Fresenius Kabi, Linz, Austria; diluted in 0.9% saline; Braun, Melsungen, Germany) was administered I.V. via retro-orbital route. Animals were allowed to fully recover and move freely until transcutaneous measurement was stopped after 90 minutes. Using a 1-compartment model, the half-life of FITC-Sinistrin was calculated from the transcutaneous measured kinetic by the MediBeacon software.

Urine and Serum Li^+ Clearance tests.

To characterize proximal tubular function, we injected mice subcutaneously with 10 mmol/kg of LiCl (Huls et al., 2007; Wood, Goodwin, De Souza, & Green, 1986). After collecting blood from all mice before injection, blood was collected at selected time-points of peak 1hr, half-life 3hrs, and 6hrs after injection (Huls et al., 2007). To reduce influence of variations in injection volume, all values were normalized to peak 1hr serum [Li^+] concentration. For urine clearance test, mice were acclimatized in metabolic cage for 24hrs before injecting mice subcutaneously with 10mmol/kg of lithium chloride and placing them back in the individual metabolic cages. Urine was collected over 24hrs and blood was collected at 24hrs after injection. Serum was isolated from all collected blood and stored in -20 °C. [Li^+] concentrations were determined in serum and urine by using Contra-300 model of atomic absorption spectrophotometer (Analytik Jena, Upland, CA).

Tissue Collection.

Mice were anaesthetized with 50mg/kg pentobarbital administered vid intra-peritoneal (I.P.) injection. Tissues were dissected and weighed. Kidneys used for western blot analysis were flash frozen in liquid nitrogen then stored at -80 until later use. Kidneys used for histological analysis were fixed in 10% neutrally buffered formalin for 24 hours then stored in 70% ethanol

until they were embedded in paraffin blocks. The outer darker cortical region rich in glomerular and proximal and distal tubules was identified and demarcated from the lighter inner medullary region for all histological assessments.

Immunohistochemistry.

Kidneys were collected and then washed twice with ice-cold PBS, fixed with 10% neutrally buffered formalin for 24 hours, then stored in 70% ethanol until they were embedded in paraffin blocks. Cross-section of the mid-region of the kidney were immunostained for Periodic acid schiff by Wax-it Histology Services Inc. (Vancouver, Canada) to analyze structural and morphometric changes in kidney. Additional sections were stained for NKA α1 and Villin-1, and NKA α1 and phosphoY418-Src by Wax-it, Inc. The samples were examined on a Leica confocal SP5 microscope under 100x oil-immersion lens. The images were processed with the Leica Application Suite advanced fluorescence (LAS/AF) suite (Leica microsystems, Wetzlar, Germany) and FIJI ImageJ for quantification and to construct figures. Proximal tubules were selected as regions of interest (ROI) based on staining of proximal tubule marker villin-1 (stained purple). They were then manually, randomly and blindly annotated and the integrated density of NKA α1 fluorescent staining and adjacent background per ROI was measured by the ImageJ software (from National Institute of Health). Measurements obtained were corrected for background per ROI and normalized to average of control values. Colocalization was determined by examination of merged images. For the colocalization described, the 488 nm Argon and 561 nm diode lasers were used, as well as the 633nm He-Ne laser, to see the expression of phospho^{Tyr418}Src, NKA, and Villin-1 respectively. Colocalization of phospho^{Tyr418}Src and NKA was quantified using coloc2 plugin in FIJI ImageJ.

Morphometric tissue analysis.

Images of kidney sections stained with periodic acid schiff were processed by Wax-it, Inc. and images were captured with digital whole slide scanning. Aperio ImageScope software was used to determine the cross-sectional area of each glomerulus and proximal tubule (F. Chow, Ozols, Nikolic-Paterson, Atkins, & Tesch, 2004; Gotoh et al., 2010; Kotelevtsev et al., 1999). Every glomerulus was counted to determine the average number of glomerulus per section (n=3 sections/sample). In each section, 200 proximal tubules were randomly and blindly selected in the cortical region and manually analyzed for cross-sectional area and number of cells per tubule, (n=3 sections/sample).

Purification of Caveolin-rich Membrane Fractions.

Caveolin-rich membrane fractions were obtained via sucrose gradient fractionation as described previously (Yiliang Chen et al., 2009). Briefly, cells were washed with ice-cold phosphate-buffered saline and cells were scraped and ground tissue was suspended in 2 ml of 500 mM sodium carbonate, pH 11.0. The cell lysates were homogenized by a Potter-Elvehjen homogenizer (three 6-s bursts) and subjected to sonication using Branson digital sonifier (three 40-s bursts). The homogenates were then adjusted to 45% sucrose by addition of 2 ml of 90% sucrose in MBS (25 mM MES, 0.15 M NaCl, pH 6.5) and placed at the bottom of ultracentrifuge tubes. The ultracentrifuge tubes were then loaded with 4 ml of 35% sucrose and 4 ml of 5% sucrose (both in MBS containing 250 mM sodium carbonate) and centrifuged at 39,000 rpm for 17 h in an SW40 rotor (Beckman Instruments). Twelve gradient fractions of 1 ml were collected from the top to the bottom. Among the fractions, fraction 4 and 5 were combined and diluted with 4 ml of MBS containing 250mM sodium carbonate, then centrifuged at 40,000 rpm in a Beckman type 70 rotor for 1 h. The pellet was resuspended in 250 µL of MBS and is considered as caveolin-enriched caveolar fraction.

Western Blot.

Kidney cortex was separated from medulla on ice, as described (Veiras et al., 2017). Cortex was ground into fine powder with a mortar and pestle and homogenized in ice-cold radioimmunoprecipitation (RIPA) buffer (0.25% sodium deoxycholate, 1% NP-40, 1mM EDTA, 1mM PMSF, 1mM sodium orthovanadate, 1mM Sodium fluoride, 150mM NaCl, 50mM Tris-HCl, pH 7.4 and 1% protease inhibitor cocktail) with a Potter-Elvehjen homogenizer. Homogenates were rotated for 15mins and centrifuged at 14,000xg for 15min, supernatants were collected, and protein content was measured using DC protein assay kit from BioRad (catalogue number 500-0114 and 500-0113). For membrane protein analysis, kidney cortex was homogenized consistently with Mem-PER kit extraction reagents containing protease inhibitor cocktail (Fisher Catalogue #PI89842) as per manufacturer's instructions. Isolated membrane protein was assessed for concentration using DC protein assay kit from BioRad (catalogue number 500-0114 and 500-0113). Sucrose-gradient fractions were loaded by equal volume. For the rest, equal amounts of protein of each sample were loaded, separated by SDS-PAGE, and transferred to nitrocellulose membranes.

Membranes probed for NKA α1, phosphorylated ser552-NHE3, NHE3, NBCe1A, c-Src, caveolin-1 were blocked in 5% milk in TBST, then primary antibodies diluted in blocking buffer were added overnight at 4degree Celsius. Nitrocellulose and PVDF membranes were visualized with SuperSignal West Pico Chemiluminescent Substrate (Fisher Cat# PI34080) or in case of weak signal with Enhanced Luminal Reagent (Perkin Elmer Cat# NEL103001EA) using radiographic film. Densitometry quantifications was performed using ImageJ software.

Glucose Tolerance Test.

Fasting blood glucose was measured from the tail vein using a OneTouch Ultra glucometer after a 6 hour fast. A 20% glucose solution was administered via intraperitoneal injection at a dose of 2 g/kg BW. Blood glucose was measured from the tail vein at 15, 30, 45, 60, 90, and 120 min. post injection. The area under the curve was calculated to determine glucose tolerance.

Insulin Tolerance Test.

Fasting blood glucose was measured from the tail vein using a OneTouch Ultra glucometer after a 6 hour fast. Insulin (0.15 units/mL) was administered via intraperitoneal injection at a dose of 0.75 units/kg BW. Blood glucose was measured from the tail vein at 15, 30, 45, 60, 90, and 120 min. post injection. The area under the curve was calculated to determine insulin resistance.

Statistical Analysis.

Statistical analysis was performed using GraphPad Prism (GraphPad Sofware, San Diego, California USA). All data presented are mean ± S.E.M, and statistical analysis was performed using the Student's t test. When more than two groups were compared, one-way ANOVA was performed prior to post-hoc comparison of individual groups using Tukey's multiple comparison test. Significance was accepted at $p<0.05$.

CHAPTER 3

RENAL PROXIMAL TUBULE-SPECIFIC ALTERATION OF THE NKA/SRC RECEPTOR COMPLEX IN THE MOUSE: EVIDENCE FOR SEXUAL DIMORPHISM

Introduction

The regulation of Na^+ transport in the renal proximal tubule (RPT) is vital to systemic Na^+ balance and blood volume homeostasis. As two-third of Na^+ absorbed in the RPT, modulation of the transport in this region also has pathological implications.

Na^+ /K^+-ATPase (NKA) expressed on the basolateral membrane of RPT cells serves dual counteractive functions- the anti-natriuretic enzymatic ion-pump generating the Na^+ gradient for absorption of Na^+ by apical transporters like NHE3, and the natriuretic regulatory receptor complex that when activated results in interdependent inhibition of NKA, NHE3 and Na^+/HCO_3^- cotransporter-1A(NBCe1A), and transepithelial Na^+ flux (Figure 5, Figure 11). The RPT-specific NKA α1 hypomorphic mice (RPTα1$^{-/-}$) exhibit increase in RPT-mediated Na^+ reabsorption in both male and female mice (Figures 9-10). Notably, the natriuretic non-enzymatic function of NKA shows functional dominance over the anti-natriuretic NKA enzymatic function as evidenced by studies in the RPT-specific hypomorphic NKA α1 mouse (RPTα1$^{-/-}$, Figure 11). The restoration of urine and Na^+ output in the RPT-specific NKA α1 and NHE3 double hypomorphic mice (RPTα1$^{-/-}$NHE3$^{-/-}$) confirmed the importance of NHE3 in the NKA α1-mediated regulation of Na^+ absorption by RPT (Figure 11). However, the specific molecular mechanisms involved in the potent NKA receptor-mediated tonic inhibitory function , which has been linked to the NKA/Src functions *in vitro*, has not been tested *in vivo*.

NKA binds and interacts with Src kinase to form a receptor signaling complex. GST pull down assays that demonstrate NKA interacts with Src via two domain- domain interactions (Z.

Li et al., 2009; Tian et al., 2006). The second cytosolic domain (CD2) acts as a ligand to Src SH2 domain and is necessary for activation and targeting of Src (Banerjee et al., 2015; Tian et al., 2006). Specifically, the Y260 residue in CD2 of NKA α1 is the Src phosphorylation and binding site (Banerjee et al., 2015). Binding at this site is constitutive and independent of NKA conformation and thereby ouabain has no effect on this specific interaction (Banerjee et al., 2015; Tian et al., 2006). The binding to CD2 domain may function as a hinge to keep the activated Src bound to the signaling NKA for specific and robust signal transmission (Banerjee et al., 2015; Tian et al., 2006). Phosphorylation of the Y260 residue of NKA α1 (pY260) by Src is also necessary for the binding of Src and the associated signaling transduction (Banerjee et al., 2018). Renal epithelial cells with knockdown of endogenous NKA α1 and rescue of expression with Y260A mutant NKA α1 exhibit a significant decrease in Src-binding and show a loss of ouabain-mediated activation of signaling molecules downstream to Src, like ERK and AKT (Banerjee et al., 2018). EGF and ouabain treatment of LLC-PK1 cells resulted in increased pY260 in time and dose-dependent manner (Banerjee et al., 2018). Thus, pY260 represents a feature of Src regulation relevant to NKA receptor function. On the other hand, Src kinase domain binds to nucleotide binding domain of the cytoplasmic NKA α1 (CD3) in its E1 conformation (Z. Li et al., 2009; Tian et al., 2006; J. X. Xie et al., 2015). Binding of ouabain to NKA α1 stabilizes the conformation of NKA α1 in E2, which results in the freeing of the Src kinase domain from CD3 and subsequently the disinhibition of Src to activate downstream signaling molecules (Z. Li et al., 2009; Tian et al., 2006). The identification of the 20 amino acid sequence in the N domain that binds Src kinase resulted in development of the cell-permeable peptide pNaktide that inhibits Src-kinase both *in vitro* and *in vivo* (Z. Li et al., 2009; Z. Li et al., 2011; Liu et al., 2016b; Sodhi et al., 2015; Sodhi et al., 2017).

Previous pharmacological studies have proposed Src-mediated signaling in the NKA non-enzymatic inhibition of RPT NHE3 and Na^+ transport (H. Cai et al., 2008; Godinho et al., 2017). Notably, like RPT$\alpha1^{-/-}$, renal epithelial cells expressing Src-binding mutant Y260A NKA $\alpha1$ show a 50% decrease in phosphorylation-mediated inactivation of NHE3 compared to the wild-type NKA $\alpha1$ expressing AAC-19 cells (Figure 14), implicating NKA/Src in the regulation of NHE3. While, RPT$\alpha1^{-/-}$ display a renal cortical redistribution of c-Src from the caveolar to non-caveolar fractions in absence of NKA $\alpha1$ (Figure 13), the specific physiological importance of NKA/Src receptor-mediated regulation in NHE3 in RPT Na^+ transport is yet to be elucidated.

Based on previous reports (H. Cai et al., 2008; Godinho et al., 2017) and our *in vitro* studies in the genetically modified cells (Figure 14), we hypothesized that a loss of NKA/Src interaction and binding in RPT will lead to a loss of tonic inhibition of Na^+ transport and decrease natriuresis in both male and female mice as it did in the RPT$\alpha1^{-/-}$. To test the proposed NKA/Src mechanism, we utilized a genetic approach to suppress the endogenous *Atp1a1*, the gene that encodes NKA $\alpha1$, and rescue the expression of NKA $\alpha1$ with exogenous wild-type or Y260A Src-signaling null mutant NKA $\alpha1$ in RPT-specific mouse model. Our investigation suggests a sexual dimorphism in the NKA/Src mechanism.

Materials and Methods

Reagents.

The polyclonal anti-Na/K-ATPase $\alpha1$ antiserum NASE, used for tissue western blots were raised in rabbits and were generous gifts from Drs. T. Pressley and P. Artigas at Texas Tech University Health Sciences Center (Pressley, 1992). Anti-phospho-Y260- Na/K-ATPase $\alpha1$-Assay Biotech (Fremont, CA) was used as described (Banerjee et al., 2018). Anti-NHE3 (Millipore, catalogue #AB3085MI) and anti-phosphorylated NHE3 at serine 552 (Santa Cruz

Biotechnology, Catalogue #sc-516102) were selected based on previous literature (Kurashima et al., 1997). Anti-α-tubulin antibody (sigma, catalogue #T5168) and anti-E-cadherin (Cell Signaling, Catalogue # 3195S) were used as a loading control for total and membrane lysates, respectively. Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit (R&D systems, Catalogue# HAF008) and anti-mouse (Santa-Cruz biotechnology Inc., Catalogue# sc-516102).

Animals.

Mice with floxed Neo-STOP cassette upstream of wild type (WT) NKA α1 or Y260A mutant NKA α1 construct targeted at the Rosa26 locus on chromosome 6 were generated and obtained through a contract with inGenious Targeting Laboratory, Inc. (Stony Brook, NY) and bred with previously generated RPTα1$^{-/-}$ mice with homozygous floxed endogenous NKA α1 isoform, homozygous Rosa26-WT or Rosa26-Y260A mutant *Atp1a1*, and heterozygous for Sglt2.iCre mice. The mice generated were assessed for the presence of the SGLT2-Cre transgene, homozygous floxed *Atp1a1*, and homozygous Rosa26-WT or Rosa26-Y260A NKA α1 by PCR on genomic DNA extracted from mouse tail clips.

All animals were housed in 12-hour light and dark cycles at constant temperature and humidity. All animal procedures were approved by the Marshall University Institutional Animal Care and Use Committee.

Metabolic Cage analysis.

Mice were housed individually in metabolic cages (manufactured by TechniPlast) for two consecutive days, including 24 hours for acclimatization and the following 24 hours for measuring food and water intake, and urine output.

Urine and Serum analysis.

Urine collected over 24 hours using metabolic cages was sent to Idexx (North Grafton, MA, USA) for analysis of sodium, potassium, calcium, magnesium, phosphorus, creatinine, pH and glucose. Serum isolated from blood collected from mesenteric artery of the four month mice was sent to Idexx for analysis of sodium, potassium, calcium, magnesium, phosphorus, creatinine, and blood urea nitrogen (BUN).

Serum Lithium Clearance tests.

To characterize RPT function, we injected mice subcutaneously with 10 mmol/kg of lithium chloride(Huls et al., 2007; Koomans, Boer, & Mees, 1989; Wood et al., 1986). After collecting blood from all mice before injection, blood was collected at selected time-points of peak one hour, half-life three hours, and six hours after injection. To reduce the influence of variations in injection volume, all values were normalized to peak 1hr serum lithium concentration. Serum was isolated from all collected blood and stored in -20 °C. Lithium concentrations were determined in serum by using Contra-300 model of atomic absorption spectrophotometer (Analytik Jena, Upland, CA).

Tissue Collection.

Mice were anaesthetized with 50mg/kg pentobarbital administered via intra-peritoneal (I.P.) injection. Tissues were dissected and weighed. Kidneys used for western blot analysis were flash frozen in liquid nitrogen then stored at -80 until later use. Kidneys used for histological analysis were fixed in 10% neutrally buffered formalin for 24 hours then stored in 70% ethanol until they were embedded in paraffin blocks. The outer darker cortical region rich in glomerular and proximal and distal tubules was identified and demarcated from the lighter inner medullary region for all histological assessments.

Immunohistochemistry.

Kidneys were collected and then washed twice with ice-cold PBS, fixed with 10% neutrally buffered formalin for 24 hours, then stored in 70% ethanol until they were embedded in paraffin blocks. Cross-section of the mid-region of the kidney were immunostained for Periodic acid schiff by Wax-it, Inc (Vancouver, Canada) to analyze structural and morphometric changes in kidney. Additional sections were stained for NKA α1 and Villin-1, by Wax-it, Inc. The samples were examined on a Leica confocal SP5 microscope under 100x oil-immersion lens. The images were processed with the Leica Application Suite advanced fluorescence (LAS/AF) suite (Leica microsystems, Wetzlar, Germany)

Morphometric tissue analysis.

Images of kidney sections stained with periodic acid schiff (PAS) were processed by Wax-it, Inc. and images were captured with digital whole slide scanning. Aperio ImageScope software was used to determine the cross-sectional area of each glomerulus and proximal tubule (F. Chow et al., 2004; Gotoh et al., 2010; Kotelevtsev et al., 1999). Every glomerulus was counted to determine the average number of glomerulus per section (n=3 sections/sample). In each section, 200 proximal tubules were randomly and blindly selected in the cortical region and manually analyzed for cross-sectional area and number of cells per tubule, (n=3 sections/sample).

Cortical Membrane preparations and Western Blotting.

Kidney cortex was separated from medulla on ice, as described (Veiras et al., 2017; Yang et al., 2018). To isolate membrane protein, the kidney cortex was homogenized with Mem-PER kit extraction reagents containing protease inhibitor cocktail (Fisher Catalogue #PI89842) as per manufacturer's instructions. Isolated membrane protein was assessed for concentration using the DC protein assay kit from BioRad (catalogue number 500-0114 and 500-0113). Equal amounts

of protein of each sample were loaded, separated by SDS-PAGE, and transferred to nitrocellulose membranes.

Membranes probed for pY260 NKA α1, total NKA α1, NHE3, and loading control E-Cadherin were blocked in 3% or 5% milk in TBST, then primary antibodies diluted in blocking buffer were added overnight at four degrees Celsius. Nitrocellulose membranes were visualized with SuperSignal West Pico Chemiluminescent Substrate (Fisher Cat# PI34080) or in case of weak signal with Enhanced Luminal Reagent (Perkin Elmer Cat# NEL103001EA) using radiographic film. Densitometry quantifications was performed using ImageJ software.

Statistical Analysis.

Statistical analysis was performed using GraphPad Prism (GraphPad Sofware, San Diego, California USA). All data presented are mean ± S.E.M, and statistical analysis was performed using the Student's t test. When more than two groups were compared, one-way ANOVA was performed prior to post-hoc comparison of individual groups using Tukey's multiple comparison test. Significance was accepted at $p<0.05$.

Results

Generation of mouse with RPT-specific expression of WT NKA α1 in RPTα1$^{-/-}$

We generated transgenic mice containing a floxed Neo-STOP cassette upstream of wild type (WT) NKA α1 construct targeted at the Rosa26 locus on chromosome 6 and floxed endogenous *ATP1a1* on chromosome 3. As illustrated in Figure 16, upon crossing with SGLT2-Cre mice, RPT-specific expression of *Cre Recombinase* removes the exons 15-18 of *ATP1a1* causing frameshift leading to nonsense mRNA-mediated decay and the Neo-STOP cassette, which allows knockout of endogenous NKA α1 and the expression of the exogenous WT NKA α1 in the RPT (RPTα1^{WT}).

Homozygous RPTα1^{WT} were viable. Body weight, food consumption, and water intake, were not modified in male and female RPTα1^{WT} (Tables 8 & 9). Renal cortices stained with villin-1, a microvillar actin bundling protein specifically expressed in the RPT (magenta) and NKA α1 (red) were obtained from four month old male and female RPTα1^{WT} and their controls (RPTα1^{control}) (Figures 17A-B). Western Blot showed a visible rescue in the expression of total NKA α1 expression in renal cortex of RPTα1^{WT} relative to RPTα1^{control} in both male and female mice, in contrast to the decrease observed in the cortex of male and female RPTα1$^{-/-}$ mice compared to their controls (RPTα1$^{+/+}$) (Figures 17 C-D), also assessed in Figure 7. Thus, the visibly comparable expression of total NKA α1 in RPTα1^{WT} suggests a successful rescue of NKA α1 expression in the RPT of these mice.

Generation of mouse with RPT-specific expression of Src-binding mutant Y260A NKA α1 in RPTα1$^{-/-}$

Expression of Y260A mutant NKA α1 in pig renal epithelial cells results in loss of Src-binding to NKA and the associated non-enzymatic signal transduction, but not the enzymatic ion-pumping function *in vitro* (Banerjee et al., 2018). Using the strategy depicted in Figure 17 for rescue of WT *Atp1a1*, we generated mice with RPT-specific expression of exogenous Y260A NKA α1 Src-binding mutant (RPTα1^{Y260A}).

Homozygous RPTα1^{Y260A} were viable. Body weight, food consumption, and water intake, were not modified in male and female RPTα1^{Y260A} (Table 10 &11). The rescue of NKA α1 was confirmed by immunostaining of renal cortices obtained from four month old male and female RPTα1^{Y260A} and their respective controls (RPTα1^{control}) with villin-1, marker for RPT (magenta), and NKA α1 (red) (Figures 18A-B). Western blot analysis indicated a decrease of

about 50% of cortical pY260 NKA α1 with no change in total NKA α1 in both male and female RPTα1^{Y260A} mice compared to their controls, confirming rescue of NKA α1 expression with the mutant Y260A NKA α1 in membranes in the RPT (Figures 18C-H).

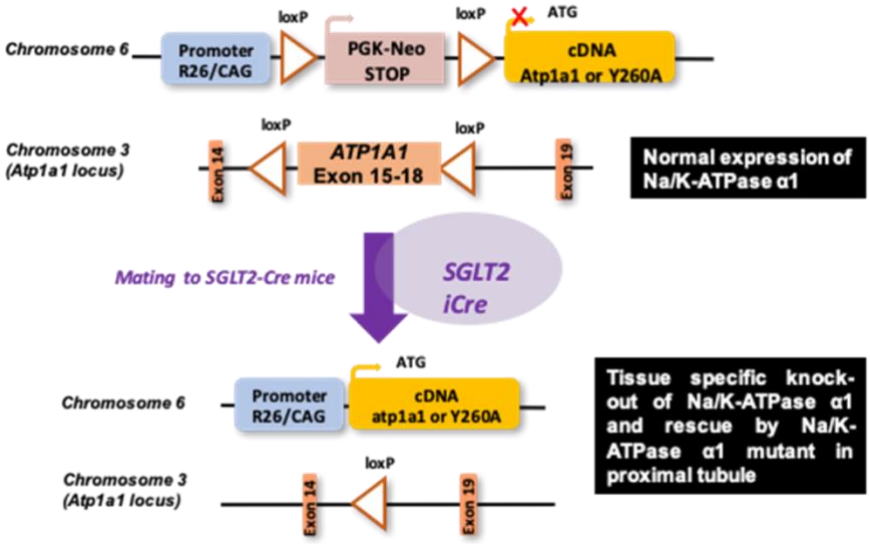

Figure 16: Strategy for RPT-specific Wild-type and Y260A signaling mutant Atp1a1 rescue in mouse.
Strategy for Cre-inducible knockout on Chromosome 3 of floxed Exons 15-18 of endogenous gene for NKA α1(Atp1a1) and rescue with exogenous WT or mutant (Y260A) NKA α1 on chromosome 6 at Rosa26 locus. Expression of Cre Recombinase is driven by the SGLT2 promoter which is tissue specific to RPT S1/S2 segments in the kidney.

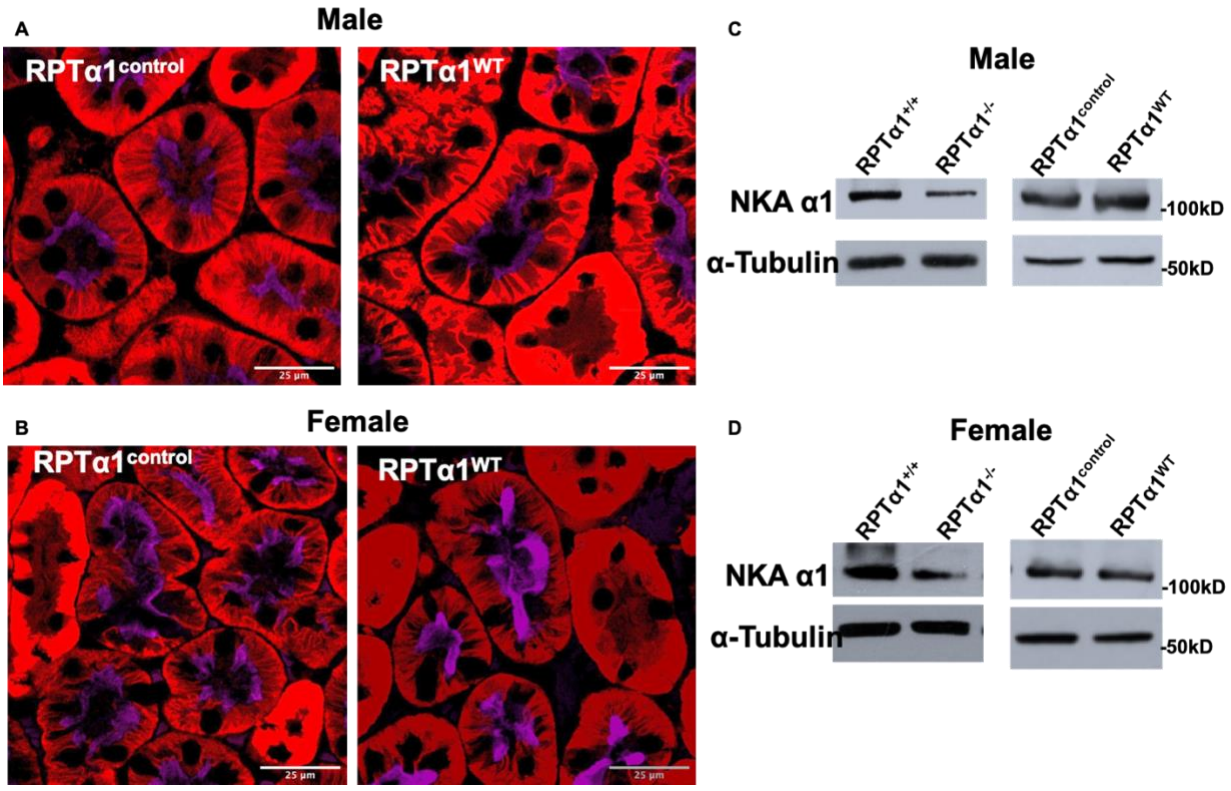

Figure 17: Generation of RPT-specific Wild-type *Atp1a1* rescue mouse model.
(A-B) Immunofluorescence for NKA α1 (red) and RPT brush-border marker villin-1(magenta) of kidney cross-sections from RPTα1^{WT} and RPTα1^{control} male and female mice. Scale bars: 25 µm. **(C-D)** Representative western blots for total NKA α1 along with loading control α-Tubulin for male and female RPTα1$^{-/-}$, RPTα1^{WT}, and their respective control mice (n=3-5).

	RPTα1^{control}	RPTα1^{WT}
Body Weight (g)	34±2.0	30±0.8
Water intake (g)	6.7±0.7	7.6±0.3
Food intake (g)	6.3±0.5	6.4±0.4

Table 8: Basal metabolic characteristics of male RPTα1^{WT} mice.
Values are mean±SEM for four month male and mice (n=5/genotype). No significant difference was observed between RPTα1^{WT} and their respective sex-matched controls by Student's t-test.

	RPTα1^{control}	RPTα1^{WT}
Body Weight (g)	25±1.4	25±0.7
Water intake (g)	8.6±1.0	7.6±0.6
Food intake (g)	7.1±0.5	6.1±0.5

Table 9: Basal metabolic characteristics of female RPTα1^{WT} mice.
Values are mean±SEM for four month female mice (n=7/genotype). No significant difference was observed between RPTα1^{WT} and their respective controls by Student's t-test.

	RPTα1^{control}	RPT α1^{Y260A}
Body Weight (g)	30±2.0	32±1.0
Water intake (g)	6.6±0.3	7.0±0.4
Food intake (g)	6.1±0.6	5.5±0.4

Table 10: Basal metabolic characteristics of male RPTα1^{Y260A} mice.
Values are mean±SEM for four month male mice (n=6-7/genotype). No significant difference was observed between RPTα1^{Y260A} and their respective controls by Student's t-test.

	RPTα1^{control}	RPTα1^{Y260A}
Body Weight (g)	25±1.0	25±0.6
Water intake (g)	7.9±0.5	8.1±0.4
Food intake (g)	5.2±0.3	6.0±0.4

Table 11: Basal metabolic characteristics of female RPTα1^{Y260A} mice.
Values are mean±SEM for four month female mice (n=6-7/genotype). No significant difference was observed between RPTα1^{Y260A} and their respective controls by Student's t-test.

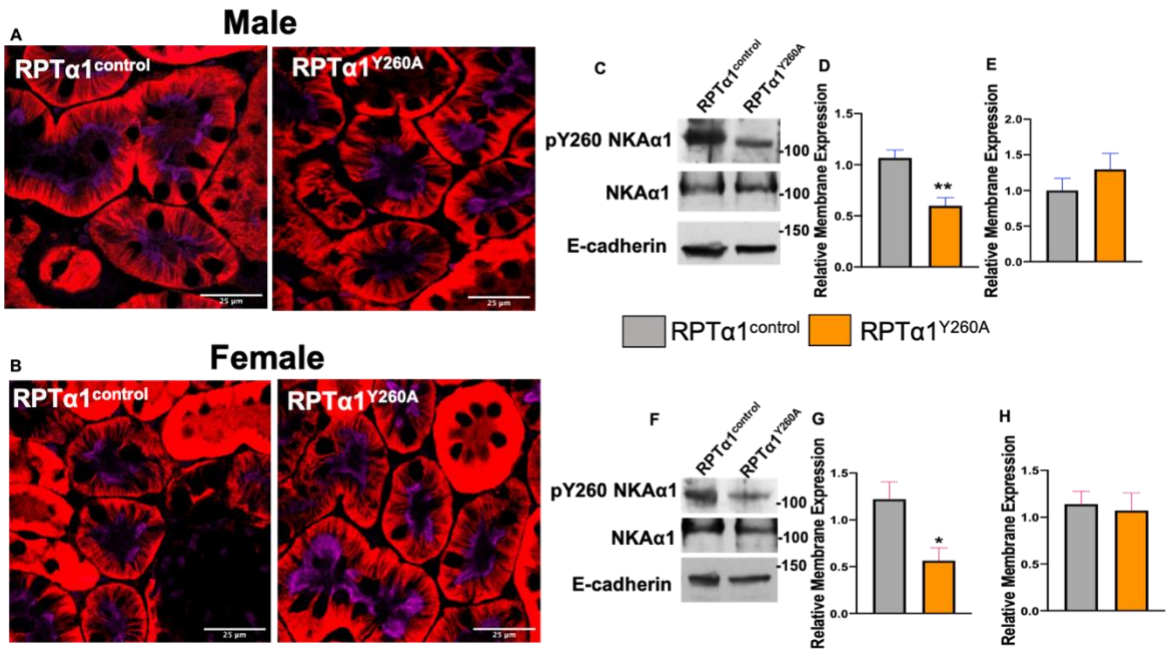

Figure 18: Generation of RPT-specific Y260A signaling mutant NKA α1 rescue mouse model.
(A-B) Immunofluorescence for NKA α1 (red) and RPT brush-border marker villin-1(magenta) of kidney cross-sections from male and female RPTα1^{Y260A} and RPTα1^{control} mice. Scale bars: 25 µm. Representative western blots and quantification of pY260 and total NKA α1 in renal cortex homogenates from male **(C-E)** and female **(F-H)** RPTα1^{Y260A}, RPTα1^{WT} and RPTα1^{control} control mice. All quantifications were normalized to α-tubulin followed by normalization to the average of control values in each gel. Values are mean ± SEM of n=3-4 and data were analyzed by Student's t-test.

RPT expression of mutant Y260A NKA α1 reveals sexual dimorphism in NKA/Src regulation of Na^+ absorption

To test whether the Src-binding mutant rescue RPTα1^{Y260A} mice have a hyper-reabsorptive renal phenotype compared to WT NKA α1 rescue RPTα1^{WT} mice, as expected based on our observations in AAC-19 and Y260A cells (Figure 14), male and female mice with RPT-specific WT NKA α1 rescue, mutant Y260A NKA α1 rescues, and their respective controls

were functionally evaluated. The mice were acclimated for 24 hours in metabolic cages followed by 24 hours urine collection to assess daily urine and Na^+ output, and urine chemistry. In both male and female $RPT\alpha1^{WT}$ mice, expression of exogenous WT NKA α1 in $RPT\alpha1^{-/-}$ resulted in the restoration of daily urine and absolute Na^+ output to values comparable to controls (Figures 19A-D).

However, in mice expressing Src-signaling null Y260A mutant NKA α1, we observed a striking sexual dimorphism in the urinary phenotype. While female $RPT\alpha1^{Y260A}$ exhibited a robust and significant 60% decrease in urine and 40% decrease in absolute Na^+ output, male $RPT\alpha1^{Y260A}$ showed insignificant change in their daily urine and Na^+ output compared to $RPT\alpha1^{control}$ mice (Figures 19E-H).

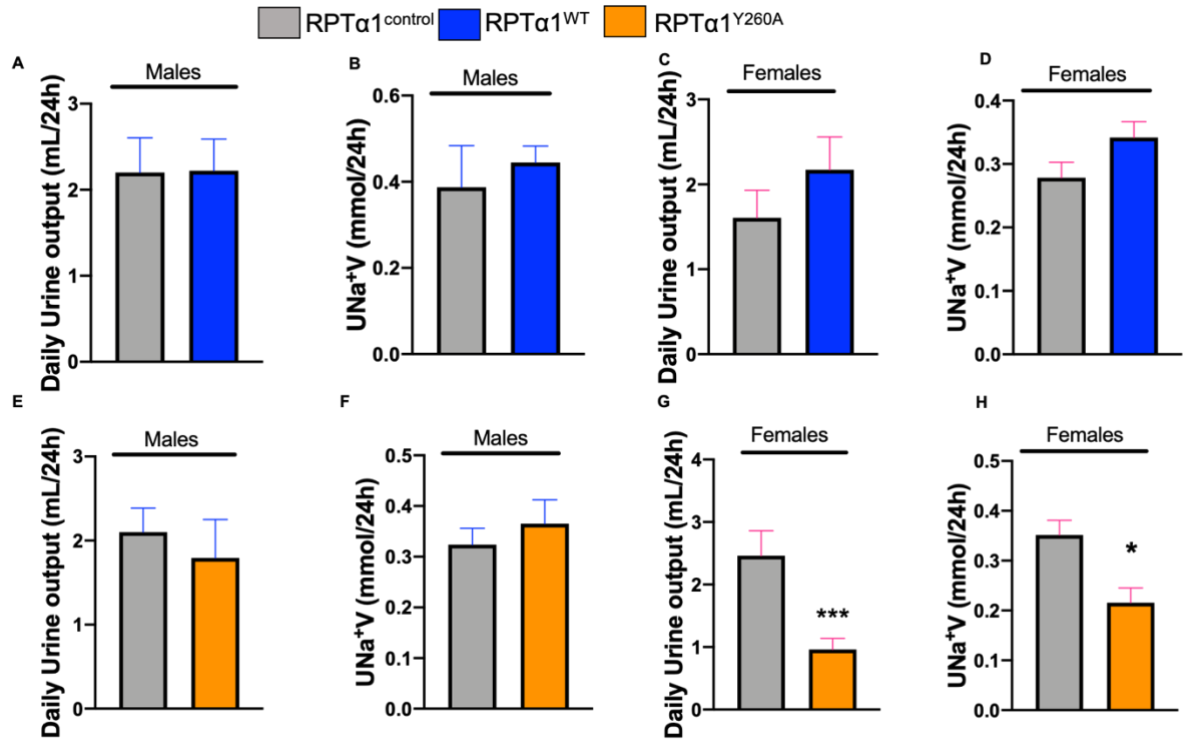

Figure 19: Rescue with Src-binding Y260A NKA α1 mutant decreases natriuresis and diuresis in sex-dependent manner.

Four month old mice were used to assess daily urine output and absolute Na^+ excretion (UNa^+V) expressed as UNa^+V = Urine [Na^+] (mmol/mL) x Urine volume (mL/24h) in RPTα1^{WT} male **(A-B)** and female **(C-D)** mice, and RPTα1^{Y260A} male **(E-F)** and female **(G-H)** mice. Results are presented as mean ± SEM for n=5-11. Data were analyzed by t-test. *p<0.05, ***p<0.001 vs RPTα1^{control}.

Despite the significant decrease in natriuresis in females, urinary and serum concentrations for most electrolytes, including Na^+, K^+, Cl^-, and Ca^{2+}, were within the normal range (Tables 12-19). The reduced urine and Na^+ output in females is consistent with the proposed prevalent role of NKA/Src receptor function in the tonic inhibition of RPT Na^+ reabsorption *in vitro* and *in vivo*, but the absence of hyper-reabsorptive phenotype in male RPTα1^{Y260A} reveals a sexual dimorphism in the dependence on NKA/Src mediated inhibitory mechanism.

	RPTα1^{control}	RPTα1^{WT}
Sodium (mEq/L)	204±18	190±13
Potassium (mEq/L)	448±29	460±21
Chloride (mEq/L)	291±17	297±16
Creatinine (mg/dL)	46.0±4.2	41.8±3.6
Phosphorus (mg/dL)	126.0±20.0	90.8±7.4
Calcium (mg/dL)	8.34±1.5	8.44±1.5
Magnesium (mg/dL)	64.0±9.8	45.3±7.0

Table 12: Urine chemistry panel of male RPTα1^{WT} and their controls (RPTα1^{control}). Values are mean ± SEM in four month male mice. No significant difference was observed between RPTα1^{WT} and their controls by Student's t-test.

	RPTα1^{control}	RPTα1^{WT}
Sodium (mEq/L)	163±20	178±21
Potassium (mEq/L)	496±110	421±53
Chloride (mEq/L)	290±73	267±31
Creatinine (mg/dL)	42.8±8.4	40.7±4.5
Phosphorus (mg/dL)	100.0±14.0	75.7±7.2
Calcium (mg/dL)	10.2±1.40	7.8±0.54
Magnesium (mg/dL)	61.0±15	80.8±24

Table 13: Urine chemistry panel of female RPTα1^{WT} and their controls (RPTα1^{control}). Values are mean ± SEM in four month female mice. No significant difference was observed between RPTα1^{WT} and their respective controls by Student's t-test.

	RPTα1^{control}	RPTα1^{Y260A}
Sodium (mEq/L)	159±8.8	163±14.0
Potassium (mEq/L)	371±24	384±36
Chloride (mEq/L)	244±17	245±19
Creatinine (mg/dL)	39.4±2.6	37.6±2.8
Phosphorus (mg/dL)	112±19.0	106±7.3
Calcium (mg/dL)	8.01±0.54	7.92±0.99
Magnesium (mg/dL)	53.1±4.3	49.6±4.6

Table 14: Urine chemistry panel of male RPTα1^{Y260A} mice and their controls (RPTα1^{control}). Values are mean ± SEM in four month male mice. No significant difference was observed between RPTα1^{Y260A} and their controls by Student's t-test.

	RPTα1^{control}	RPTα1^{Y260A}
Sodium (mEq/L)	170±14.0	195±8.1
Potassium (mEq/L)	395±42	518±45
Chloride (mEq/L)	252±25	313±17
Creatinine (mg/dL)	41.3±4.2	49.7±5.1
Phosphorus (mg/dL)	118±10	166±31
Calcium (mg/dL)	9.24±1.1	11.60 ±1.3
Magnesium (mg/dL)	60.8±3.6	79.2±19.0

Table 15: Urine chemistry panel of female RPTα1^{Y260A} mice and their controls (RPTα1^{control}).
Values are mean ± SEM in four month female mice. No significant difference was observed between RPTα1^{Y260A} and their controls by Student's t-test.

	RPTα1^{control}	RPTα1^{WT}
Sodium (mEq/L)	153±3	152±3
Potassium (mEq/L)	4.9	6.2±0.2
Chloride (mEq/L)	112±0.6	112±2
Creatinine (mg/dL)	0.1	0.12±0.02
Phosphorus (mg/dL)	6.2±0.07	7.8±0.8
Calcium (mg/dL)	9.1±0.3	8.5±0.7
Albumin (g/dL)	2.7±0.07	2.7±0.03
BUN (mg/dL)	26±2	28±2
Total protein (g/dL)	4.5±0.1	4.6±0.1

Table 16: Serum chemistry panel of male RPTα1^{WT} and their controls (RPTα1^{control}).
Values are mean ± SEM in four month male mice. No significant difference was observed between RPTα1^{WT} and their controls by Student's t-test.

	RPTα1^{control}	RPTα1^{WT}
Sodium (mEq/L)	157±3	154±4
Potassium (mEq/L)	5.5±0.3	5.5±0.7
Chloride (mEq/L)	112±5	112±1
Creatinine (mg/dL)	0.1±0.06	0.13±0.03
Phosphorus (mg/dL)	7.9±2	7.5±0.6
Calcium (mg/dL)	9.8±0.4	9.3±0.2
Albumin (g/dL)	2.8±0.05	2.9±0.06
BUN (mg/dL)	25±1	29±3
Total protein (g/dL)	4.6±0.2	4.8±0.2

Table 17: Serum chemistry panel of female RPTα1^{WT} and their controls (RPTα1^{control}).
Values are mean ± SEM in four month female mice. No significant difference was observed between RPTα1^{WT} and their controls by Student's t-test.

	RPTα1^{control}	RPTα1^{Y260A}
Sodium (mEq/L)	152±3	155±2
Potassium (mEq/L)	8.2±2.0	5.6±0.3
Chloride (mEq/L)	111±1	108±2
Creatinine (mg/dL)	0.13±0.03	0.13±0.03
Phosphorus (mg/dL)	8.1±0.9	8.3±0.8
Calcium (mg/dL)	9.1±0.2	9.6±0.0
Albumin (g/dL)	2.7±0.09	2.6±0.03
BUN (mg/dL)	22±0.7	32±2.0*
Total protein (g/dL)	4.6±0.20	4.5±0.09

Table 18: Serum chemistry panel of male RPTα1^{Y260A} mice and their controls (RPTα1^{control}).
Values are mean ± SEM in four month male mice. Data analyzed by Student's t-test. *$p<0.05$ vs RPTα1^{control}.

	RPTα1^{control}	RPTα1^{Y260A}
Sodium (mEq/L)	150±2	150±3
Potassium (mEq/L)	5.5±0.9	4.1±0.4
Chloride (mEq/L)	110±0.9	111±1.0
Creatinine (mg/dL)	0.13±0.03	0.17±0.03
Phosphorus (mg/dL)	7.8±0.4	7±1.0
Calcium (mg/dL)	9.3±0.06	8.7±0.20*
Albumin (g/dL)	2.8±0.03	2.7±0.06
BUN (mg/dL)	28±1	26±3
Total protein (g/dL)	4.6±0.07	4.5±0.20

Table 19: Serum chemistry panel of female RPTα1^{Y260A} mice and their controls (RPTα1^{control}).
Values are mean ± SEM in four month female mice. Data analyzed by Student's t-test. *$p<0.05$ vs RPTα1^{control}.

Increased RPT Na^+ absorption in female RPTα1^{Y260A} mice

The reduction of natriuresis in female RPTα1^{Y260A} is consistent with the recently identified functionally dominant NKA non-enzymatic signaling function in RPT Na^+ transport. Consequently, we set out to functionally confirm the role of RPT Na^+ transport in the observed hyper-reabsorption in these mice. Histological analysis after periodic acid schiff (PAS) staining did not reveal any gross structural alteration in the kidneys of female RPTα1^{Y260A}, including RPT area and number of cells per RPT (Figures 20A-C), excluding major histological defect as a cause for the observed oliguria in females. Measurement of serum Li^+ clearance, a marker for RPT Na^+ transport function, was significantly reduced in female RPTα1^{Y260A} mice confirming the role of increased RPT Na^+ absorption in the observed reduced natriuresis (Figures 20D-E).

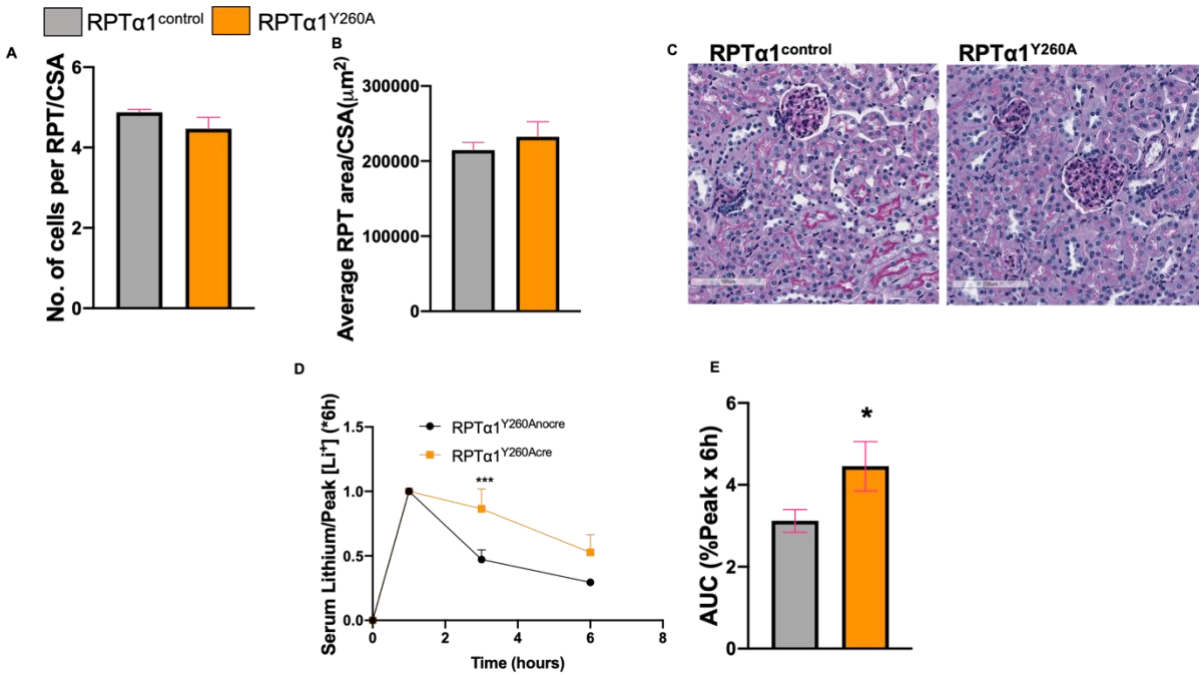

Figure 20: Female RPTα1^{Y260A} mice exhibit increased RPT Na^+ absorption.
(A-B) Average area and number of cells per proximal tubule per cross-section in female RPTα1^{Y260A} and RPTα1^{control}. **(C)** Representative images from PAS-stained cross-sections of kidney from female RPTα1^{Y260A} compared to RPTα1^{control}. **(D-E)** Serum lithium clearance curve and quantification of area under curve (AUC) in female RPTα1^{Y260A} and RPTα1^{control}. Curve and AUC normalized to peak dose in serum measured at 1hr (n=3-5, four month females). Results are presented as mean ± SEM and data were analyzed by Student's t-test. *p<0.05, ***p<0.001 vs female RPTα1^{control}.

Regulation of NHE3 by NKA/Src in male and female

Apical NHE3 expression and trafficking is a major influencer of Na^+ absorption by the RPT. The expression and membrane localization of NHE3 contributes to the sex differences in RPT Na^+ absorption, with female rodents exhibiting decrease expression of membrane NHE3 and Na^+ transport in the RPT, under basal conditions (Veiras et al., 2017). As NKA-mediated tonic inhibition of RPT Na^+ transport is dependent on the inhibition of NHE3 as confirmed by RPTα1$^{-/-}$NHE3$^{-/-}$ mice (Figure 11), we determined the role of NHE3 in the sexual dimorphism

observed in RPTα1^{Y260A} mice. Consistent with the sex-difference in the urinary phenotype, we found more than 50% increase in the membrane abundance of NHE3 in female RPTα1^{Y260A} compared to female RPTα1^{WT} as well as their controls, while there was no change in the males (Figure 21).

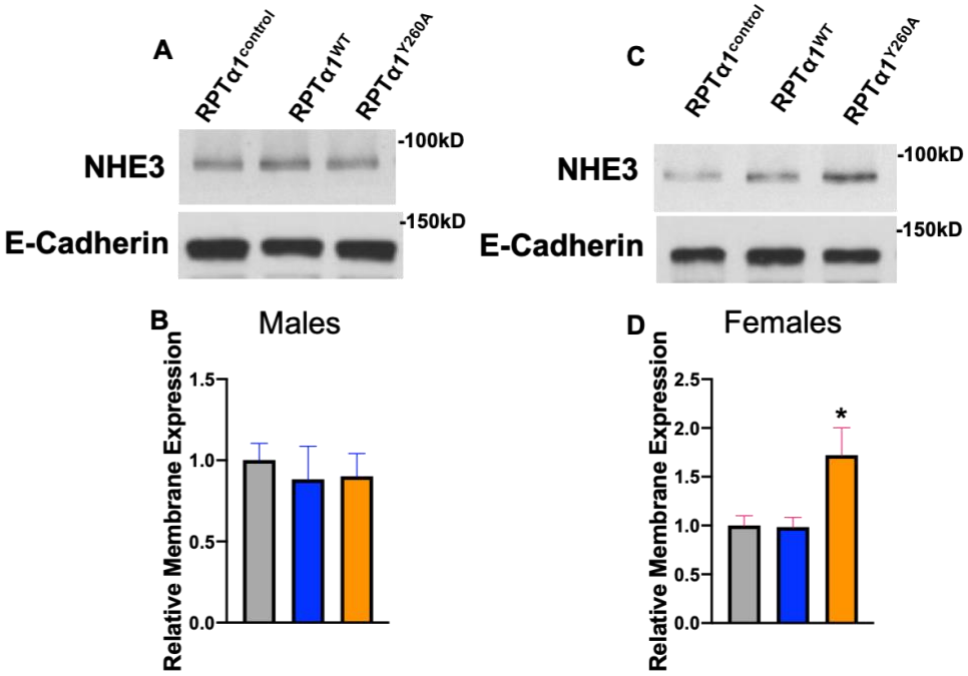

Figure 21: Membrane abundance of NHE3 in Male and Female RPTα1^{Y260A}. Representative western blot and quantification of NHE3 in isolated membrane homogenates from renal cortex of males (**A-B**, n=4) and females (**C-D**, n=4-5) RPTα1^{Y260A} mice compared to their respective controls. All quantifications were normalized to E-cadherin followed by normalization to the average of male in each gel. Results are presented as mean ± SEM and data was analyzed by one-way ANOVA with a Tukey multiple comparisons test. *p<0.05 vs RPTα1^{control}.

Sexual dimorphism in phosphorylation of Y260 residue of NKA α1 in renal cortex of C57Bl/6 mice under basal conditions.

The Y260 residue of NKA α1 is a Src-specific phosphorylation and binding site and the pY260 is a feature of Src regulation by NKA receptor (Banerjee et al., 2018). To assess whether the sex-differences in RPTα1^{Y260A} mice also indicate a difference in NKA/Src binding and regulation at basal level, we determined the protein expression of pY260 NKA α1, total NKA α1, as well as NHE3 in the membrane protein isolated from the RPT-rich renal cortex of 16-week male and female wild-type C57Bl/6 mice. We observed a threefold increase in the expression of pY260 NKA α1 with no change in the total NKA α1 in females compared to males, indicating a higher proportion of NKA α1 is phosphorylated and bound by Src and engaged in the regulation of Src in females compared to males (Figures 22A-D). Simultaneously, as reported in previous studies, we found a decrease in the membrane expression of NHE3 in female compared to male mice under basal conditions (Figures 22A, 22E).

Collectively, this set of data indicates a sexual dimorphism in NKA/Src-mechanism of inhibition of Na^+ transport and NHE3 in the RPT of mice.

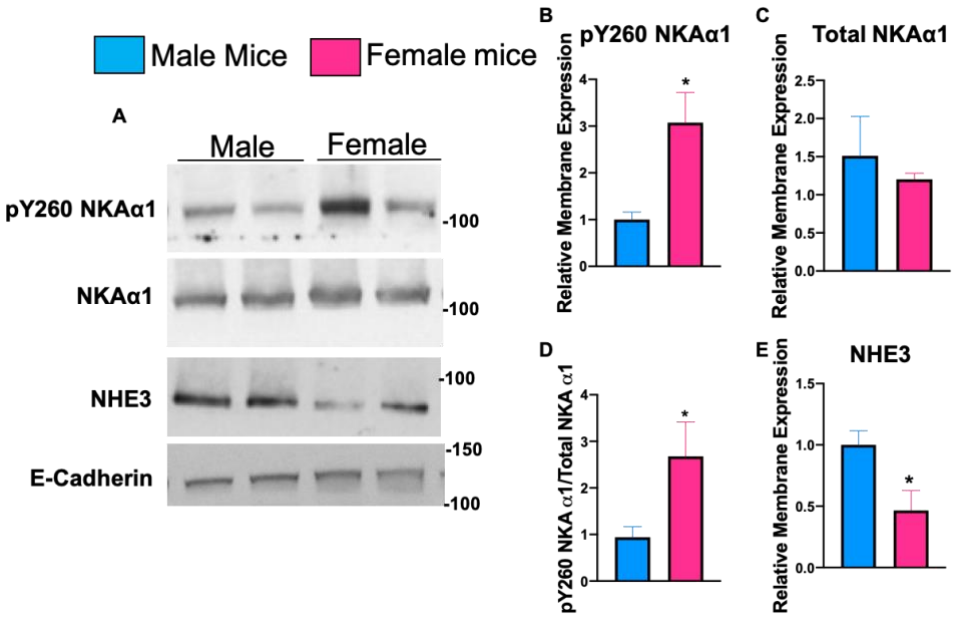

Figure 22: C57Bl/6 female mice exhibit increased phosphorylation of Src-binding Y260 residue of NKA α1 under basal conditions.
Representative western blots **(A)** and quantification of pY260 NKA α1 **(B)**, total NKA α1 **(C)**, the ratio of pY260 to total NKA α1 **(D)**, and NHE3 **(E)** in isolated membrane homogenates from renal cortex of male and female wild-type mice under basal condition. All quantifications were normalized to E-cadherin followed by normalization to the average of male values in each gel. Results are presented as mean ± SEM for n=4-5 and data was analyzed by Student's t-test. *p<0.05 vs male C57Bl/6 mice.

Discussion

An increasing number of studies report that the differences between sexes in physiology extend beyond the reproductive system (Karp et al., 2017). Women have lower basal blood pressure than men, have lower risk of developing hypertension, and are protected from cardiovascular and renal disease relative to men (Mozaffarian et al., 2016; Song et al., 2020). The wider prevalence of hypertension and cardiovascular diseases in men compared to women has shed light on clear sex-dependent differences in physiological adaptations and tissue regulation. Kidney structure and physiology exhibits notable sexual dimorphism in mice, rats, and humans (R. Hu et al., 2020; Q. Li et al., 2018; Sabolić et al., 2007; Veiras et al., 2017). Distinctive renal transporter expressions and their regulations between females and male nephron underlie the physiological and potentially pathophysiological difference in renal Na^+ handling and blood pressure regulation. The female nephron exhibits lower dependence on renal proximal tubule (RPT)-mediated bulk Na^+ absorption and exhibit higher absorption in the distal tubule compared to males that demonstrate a higher RPT-mediated absorption of Na^+ (Veiras et al., 2017). Investigations of molecular machineries involved in the regulation of RPT Na^+ transport

is of primordial importance to the development of targeted management of hypertension and gender has an important role to play in these investigations.

In the present studies, we uncovered a remarkable sexual dimorphism in the natriuretic NKA/Src-mechanism of tonic inhibition of NHE3 and RPT Na^+ transport. Expression of Src-signaling null mutant Y260A NKA α1 in RPT of mice presented a RPT-mediated hyper-reabsorptive phenotype in females but not in males. The sex-difference in the urinary phenotype of RPTα1^{Y260A} mice was supported by increased membrane abundance of NHE3 in female renal cortex compared to both its control and female RPTα1^{WT} while in males there was no significant change. This sex-dependent presentation proposes difference in dependence on NKA/Src-mediated inhibition of RPT Na^+ transport as the regulatory mechanism contributing to the lower Na^+ absorption observed in female RPT compared to males in mice. The observed difference in phenotype presentation is consistent with the increased basal levels of Src-mediated phosphorylation of Y260 residue of NKA in females that may be an indicator of increased dependence on NKA/Src signaling function. The increased NKA/Src dependence may contribute to the reported increased inhibition of RPT NHE3 and Na^+ transport in females. Collectively, this study provides important evidence of sex-dependent difference in signaling mechanisms at the molecular level to impact function not only at the cellular level but potentially at the systemic level.

The lack of change in the RPT Na^+ transport in male RPTα1^{Y260A} is a contrast to the hyper-reabsorptive phenotype in male RPTα1$^{-/-}$. This may have several potential explanations which require exploration. First, we examined if the dimorphism is an artifact of the genetic model itself. However, as evidenced by comparable levels of downregulation in cortical pY260 and rescue of total NKA α1, we believe that to not be the case in these mice. Second, while

compensation through sex-dependent compensatory modulation of alternate RPT and distal Na^+ transporters could explain the unchanged natriuresis, we found that the membrane expression of NHE3 was unchanged in the male RPT$\alpha1^{Y260A}$ mice while it was increased in females. Thus, the sexual dimorphism is upstream to NHE3 regulation. Third, in its non-enzymatic signaling function, NKA α1 has been reported to interact and modulate a variety of crucial signaling proteins. In LLCPK1 cells, pharmacological inhibition of either c-Src or PI3K exhibits a loss of ouabain/NKA receptor-mediated inhibition of NKA and NHE3 (H. Cai et al., 2008). In RPT$\alpha1^{-/-}$ mice, both male and female mice exhibit the decreased natriuresis and Na^+ hyper-reabsorptive phenotype (Figures 9-10). However, the loss of NKA receptor leads to loss of interaction with all the protein interactions, including but not limited to, c-Src, PI3K, IP3R, PKC, caveolin-1, and cytoskeletal proteins (T. Cai et al., 2008; Ying Chen et al., 2008; Tian et al., 2006; L. Zhang et al., 2008). The contrast between male RPT$\alpha1^{-/-}$ and RPT$\alpha1^{Y260A}$ mice may also be a result of sex-dependent compensatory activation of other signaling partners or a sex-bias in receptor signaling. Sex-biased receptor signaling, wherein specific pathways engaged by ligand binding are determined by sex, has been demonstrated by receptor systems including several G-protein coupled receptors (Bangasser et al., 2010; Yagami, Tohkin, & Matsubara, 1990). NKA receptor exhibits biased signaling on binding of structurally distinct CTS ligands (Xu et al., 2021) that by affecting the conformation of NKA α1 influences the interaction of NKA with effector proteins, however the role of gender and sex hormones on this is unknown. Future investigation on the effect of Y260A mutation on the distribution of NKA and effector proteins in caveolar and non-caveolar fractions, and study of transgenic animals with mutations impacting NKA's interaction with other effector proteins, such as caveolin-1 or PI3K, in the RPT could be valuable to unraveling the mechanism of NKA/Src sexual dimorphism. Fourth, sex hormones could

influence NKA non-enzymatic inhibition of NHE3 and Na^+ absorption in the RPT. Testosterone upregulates NHE3 expression, potentially through the increased androgen receptor expression in RPT, in male mice (Harris, Lee, Verlander, & Weiner, 2020). Progesterone inhibits binding of ouabain to NKA α1 and ameliorates marinobufagenin (MBG)-induced collagen synthesis by cardiac fibroblast *in vitro* but its interaction with NKA at the physiological level is poorly understood (Drummond et al., 2013). RPT expresses both androgen and estrogen receptors to levels consistent with the respective gender (Davidoff, Caffier, & Schiebler, 1980; Harris et al., 2020; Takeda, Chodak, Mutchnik, Nakamoto, & Chang, 1990). Studies also report non-genomic binding and action of sex hormones on plasma membrane protein and receptors in non-renal tissues, including NKA (Farnsworth, 1970; Farnsworth, 1990; Nadal et al., 2000). There is a possibility for cross-talk between NKA receptor, sex hormone receptors and their respective ligands that collectively influence NKA's natriuretic function in the RPT. Thus, there are several possible explanations that on further exploration may help uncover the underlying cause of the observed sexual dimorphism in NKA/Src-mechanism of inhibition of NHE3 and Na^+ absorption in RPT.

In conclusion, this study highlights the importance of improving our understanding of the natriuretic mechanism of NKA signaling in the RPT and its potential impact on sex-based differences in renal physiology and potentially pathophysiology such as in the development salt-sensitive hypertension.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Functionally dominant natriuretic signaling through NKA α1 in RPT Na^+ transport

These studies revealed several new facets of NKA in RPT Na^+ transport. In the RPT-specific NKA α1 hypomorphic mouse model, we observed a significant increase in RPT Na^+ absorption with no change in GFR and decreased natriuresis and diuresis. This phenotype of increased RPT Na^+ absorption goes against expectations based solely on the loss of the classical NKA enzymatic activity, which would be expected to decrease RPT Na^+ reabsorption or result in mild or no change. Indeed, only 20-30% NKA is sufficient to support basal Na^+ reabsorption function (Cheval & Doucet, 1990). Although compensatory mechanisms of sodium transport in the paracellular pathway or distal segments may take place, they would be expected to restore homeostasis but not to increase sodium absorption above the controls. Thus, this remarkable phenotype reveals a critical physiological function of NKA as a non-enzymatic receptor in tonic inhibition of RPT Na^+ reabsorption.

Further, the non-enzymatic signaling-mediated natriuretic function of NKA α1 is not only physiologically relevant, but also functionally dominant over its anti-natriuretic enzymatic function. While the enzymatic function is indeed vital for cell survival and potentially plays a role in anti-natriuresis and balancing the natriuretic regulatory function, the classical ion-pump is not the dominant role of NKA α1 in the RPT. This non-enzymatic (i.e. signaling-mediated) inhibitory role for α1 in Na^+ transport regulation has been previously proposed in studies using different models, such as *in vitro* in LLC-PK1 cells treated with ouabain (H. Cai et al., 2008), mouse kidneys treated with CTS, and in the polygenic Dahl salt-sensitive rats (Liu et al., 2011). These studies while vital to our understanding of the pro-natriuretic regulation by NKA, did not

provide direct genetic, physiological, and tissue-specific evidence of the NKA non-enzymatic function. Therefore, this is the first genetic demonstration of a direct physiological relationship between NKA α1 and inhibition of RPT Na^+ transport.

Subsequently, the next step is to address the physiological importance of endogenous CTS as ligands to the NKA pro-natriuretic function in the RPT. We can utilize the established tissue-specific genetic rescue approach to achieve RPT-specific replacement of CTS-resistant α1 by CTS-sensitive WT or signaling-mutant α1 in mouse.

NKA α1 non-enzymatic regulation of NHE3 and NBCe1A.

In the hypomorphic RPTα1$^{-/-}$, the hyper-reabsorptive phenotype was accompanied by a decreased phosphorylation-mediated inactivation of NHE3 and its increased membrane expression, along with increased membrane abundance of basolateral NBCe1A (Figure 11). The importance of loss of NKA-mediated regulation of NHE3 in the observed hyper-reabsorption in RPTα1$^{-/-}$ was confirmed when ablation of NHE3 in the RPTα1$^{-/-}$ generated RPT-specific NKA α1 and NHE3 double hypomorphic mice (RPTα1$^{-/-}$NHE3$^{-/-}$) that restored urine and sodium output to values comparable to controls (Figure 11).

Na^+ transport and Acid-base balance by RPT

The inter-dependent regulation of NKA, NHE3 and NBCe1A potentially has implications in the uncoupling necessary for the RPT to carry out the reabsorption of bicarbonate and the secretion of acid. The uncoupling can be vital to establish acid-base balance in response to the acidic western diet without compromising the pressure and volume homeostasis. Previously, pH sensors have been postulated to stimulate the coupled changes in expression of NHE3 and NBCe1A. An early investigation supported a basolateral sensor in this response (L. K. Chen & Boron, 1995). While there have been reports of distinct molecular pathways in this mechanism,

activation of non-receptor tyrosine kinases Pyk2 followed by c-Src stimulates both NHE3 and NBCe1A in response to decreased intracellular pH (Espiritu, Bernardo, Robey, & Arruda, 2002; Preisig, 2007). Dahl salt-sensitive rats exhibit increased renal acid excretion and decreased intracellular pH which are hallmarks of development of metabolic acidosis (Batlle et al., 1993). The RPT cells from these salt-sensitive rats also demonstrate impaired CTS-activated NKA α1 inhibition of NHE3 which supports the increased RPT Na^+ absorption and blood pressure in these rats (Liu et al., 2011). The nuances of NKA α1-mediated interdependent inhibitory mechanism and its potential influence on the cellular response to intracellular pH require further investigations.

Molecular mechanism of NKA natriuretic function in RPT

Our findings in renal epithelial cells with loss of Src-binding NKA α1 mutation as well as the gain of Src-binding NKA α2 mutation indicated NKA/Src interaction is not only necessary for the phosphorylation-mediated inactivation of NHE3 but also inhibition of increased total expression of NBCe1A (Figure 14). To our knowledge, this is the first description of a Src-mediated mechanism that links NKA, NHE3, and NBCe1A in an interdependent pathway. This is a notable finding as the previously recognized regulatory pathways of RPT Na^+ handling such as PKA, PI3K, PKC play a role in the coordinated and parallel, but independent regulation of NHE3 and NBCe1A, and NKA (M. C. Hu et al., 2001; Kocinsky et al., 2007; Kurashima et al., 1997; Riquier-Brison, Leong, Pihakaski-Maunsbach, & McDonough, 2009).

The downstream signaling molecules involved in the NKA receptor mediated phosphorylation of (Ser-552)-NHE3 and subsequent decreased membrane abundance are yet unknown. The phosphorylation at ser552 of NHE3 is an established mechanism of inhibition by dopamine and in that pathway it involves activation of cAMP-PKA pathway (Kocinsky et al.,

2007; Kurashima et al., 1997). While c-Src, a non-receptor tyrosine kinase, does not directly phosphorylate a serine residue, it could activate intermediate signaling molecules to activate PKA via cAMP-dependent or independent mechanisms *in vitro* and *in vivo*. The cAMP-independent activation of PKA involves activation of endothelin-1 (Dulin, Niu, Browning, Richard, & Voyno-Yasenetskaya, 2001), a factor also indicated to activate NHE3 and NBCe1A, stimulated by Pyk2/c-Src pathway (Curthoys & Moe, 2014; Espiritu et al., 2002; Preisig, 2007). As part of our future studies, we can test the relevance of these signaling molecules in the NKA/Src regulation by utilizing *in vitro* genetic silencing approaches or specific inhibitors of cAMP and PKA to study its impact on basal and CTS-activated inhibition by NKA of NHE3.

Another factor proposed to play a role in the NKA/Src mechanism is reactive oxygen species (ROS). NKA/Src signaling activates ROS which in turn through direct carbonylation modification of Pro224 on NKA α1 activates NKA signal transduction (Y. Wang et al., 2014; Yan et al., 2013; Yan, Shapiro, Mopidevi, Chaudhry, Maxwell, Haller, Drummond, Kennedy, Tian, & Malhotra, 2016). Superoxide generated by NADPH-oxidase inhibits NHE3 in adult spontaneously hypertensive rats (Panico et al., 2009). Thus, physiological concentration of ROS could not only activate NKA signaling but also play an intermediary role in the signaling transduction *in vitro* and *in vivo* (Yan, Shapiro, Mopidevi, Chaudhry, Maxwell, Haller, Drummond, Kennedy, Tian, & Malhotra, 2016).

A third potential mechanism of modulation of NHE3 by NKA signaling is more relevant to our findings *in vivo* than *in vitro*. Intracellular [Na^+], through interaction with a yet to be established salt-sensing protein, modulates the sensitization or desensitization of molecular pathways that facilitate receptor recruitment of dopamine and angiotensin-II in RPT (Efendiev et al., 2003). Both dopamine and angiotensin-II are important hormonal regulators of natriuresis

and blood pressure through its action on RPT NHE3 and Na^+ absorption. NKA/Src signaling can also be activated by increased intracellular [Na^+] in renal epithelial cells (Ye et al., 2011). As reported previously (H. Cai et al., 2008) and shown in figure 14, the same NKA/Src signaling mechanism inhibits NHE3 and Na^+ transport. In human embryonic kidney cells (HEK293T), NKA forms a complex with D1 and D2 dopamine receptor and exhibits reciprocal regulation that influences dopamine receptor expression (Hazelwood, Free, Cabrera, Skinbjerg, & Sibley, 2008). While the findings in the RPT$\alpha1^{-/-}$ mouse establish NKA as a potent tonic inhibitory mechanism of Na^+ transport, they could also be an indicator of a master-regulator role for NKA as the long sought after salt-sensor. To test the role as a salt-sensor, future experiments need to be designed to study the impact of [Na^+] on NKA's pro-natriuretic function in RPT and the impact of signaling mutants on this interaction *in vitro* and *in vivo*.

Finally, as NKA receptor interacts with multiple proteins, we cannot discount the role of interaction with other signaling proteins in this regulatory mechanism by NKA $\alpha1$ *in vivo*. Indeed, when Src-signaling null Y260A NKA $\alpha1$ mutant is expressed in RPT of mouse, we see a loss of NKA-mediated regulation of Na^+ transport in females but not in males (Figure 20). This could indicate to a role for other signaling proteins that potentially compensates for absence of Src-mediated regulation or play a more predominant role in the NKA-mediated regulation in males.

Sexual Dimorphism in NKA/Src-mediated inhibition of RPT Na^+ absorption.

Sexual dimorphic variations are present in many aspects of biology and involve the structure and function of nearly every organ system in the mammalian body. Numerous studies have reported critical distinctions between male and female renal physiology which translates to the differences in risk of development and the severity of hypertension between men and women,

mainly premenopausal women (Mozaffarian et al., 2016; Song et al., 2020). In rats and mice, the most commonly used animal models for studies on renal physiology, females show decreased sodium transport in the RPT and an increased sodium transport in distal tubule compared to males. Consistently, in females, the expression of key Na^+ transporters in the RPT are reduced in total expression and/or reduced in their membrane localization while the transporter expression in the distal tubules is increased compared to males (Q. Li et al., 2018; Veiras et al., 2017). The physiological differences translate to females being less dependent on RPT for absorption of almost two-third of filtered Na^+ and an increased dependence on distal tubule improves the ability to adapt to systemic challenges, which include pregnancy, child birth, and breast-feeding (West, Sasser, & Baylis, 2016). Distinct transporter expression or its membrane abundance could be an indication of differences in mechanism of their regulation. NHE3, the major apical RPT Na^+ transporter, has a reduced membrane localization in females. Since NKA α1 mediated tonic inhibition of NHE3 is a potent mechanism of natriuretic regulation, differences in ligand-receptor-effector interactions could underlie the sex-based difference of NHE3.

In vitro studies in renal epithelial cells indicate Src to be a mediator in the NKA inhibition of NHE3 and NBCe1A. To test the relevance of this finding in physiology, we generated mice with RPT-specific hypomorphic endogenous NKA α1 and rescue of expression with exogenous WT or Src-signaling mutant NKA α1. In mice expressing the Src-signaling null Y260A mutant in their RPT, female mice exhibited hyper-reabsorption of Na^+ by their RPT due to a disrupted NKA/Src signaling while male mice showed no change in their daily urine and sodium output (Figure 20). Notably, female mice show increased phosphorylation of Y260 residue of NKA α1 that indicates an increased NKA/Src interaction and activity (Figure 22). The lack of change in male could be an indicator of sex-bias in signaling mechanism of NKA-

mediated inhibition of NHE3. NKA receptor interacts with multiple effector proteins that in turn activate a variety of downstream signaling pathways. Structurally distinct CTS on binding to NKA can influence the NKA/effector protein interaction leading to biased signaling. Further studies are needed to fully delineated the mechanisms underlying the sexual dimorphism in observed urinary phenotype of RPT$\alpha 1^{Y260A}$ mice.

While, we specifically investigated NHE3 and Na^+ absorption in this study, the sexual dimorphism in mechanism of NKA signaling could extend to RPT absorption of Na^+ via other RPT transporters and to absorption of other solutes through transcellular and paracellular pathways. RPT plays a crucial role in acid-base balance and in that ammonia metabolism is crucial to the net acid excretion. Renal ammonia metabolism is sexually dimorphic in mice and while the male and female mice generate a similar maximal ammonia excretion in response to an acid load, they use sex-specific pathways to achieve this response (Harris, Lee, Fang, Verlander, & Weiner, 2019). NKA non-enzymatic receptor regulates NBCe1A however the role of gender in this regulation is yet to be investigated.

Another important transporter with clinical importance in the RPT is the SGLT2 that primarily reabsorbs most of glucose filtered into the kidney. In rats, females exhibit higher SGLT2 expression and activity than males but whether these differences extend to humans is still unclear (Shepard, 2019). Interestingly, SGLT2 inhibitors (a drug of choice for treatment of type-II diabetes) exhibits greater adverse effects in females compared to male but the prevalence of type-II diabetes is higher in males compared to females (Kautzky-Willer & Harreiter, 2017). We observed a significant decrease in urinary glucose excretion in RPT$\alpha 1^{-/-}$ in males and females, however we cannot rule out sex-bias at the regulatory level like the bias in regulation of NHE3 and Na^+ transport in RPT$\alpha 1^{Y260A}$ mice.

Clinical implications.

Numerous preclinical and clinical studies have found association between endogenous CTS and changes in blood pressure in health and disease, but the study of the underlying mechanism of action has been fraught with challenges including the ubiquitous nature of its receptor NKA α1.The RPT NKA non-enzymatic regulatory mechanism uncovered by this genetic approach provides a long sought-after receptor mechanism to explain the natriuretic effect of endogenous CTS, which has first been proposed more than 50 years ago. With a wide prevalence of over a billion people globally affected by hypertension, half of which are considered as salt-sensitive hypertension, resolving the pathophysiological causes underlying this largely idiopathic disorder is the need of the hour. The identification of NKA as a regulator of Na^+ transport in RPT could pave the way for the development of novel treatments for salt-sensitive hypertension which target the NKA signaling axis, or revisiting targeted delivery of existing CTS drugs such as ouabain or digoxin that are capable of activating NKA signaling axis and thereby stimulating natriuresis.

Interestingly, in a clinical study in members of Framingham heart study, heterozygous and rare mutations in SLC12A3, SLC12A1, and KCNJ1 were shown to produce significant blood pressure reduction and protect participants from development of hypertension (Ji et al., 2008). These findings implicate other rare yet undiscovered alleles and variations in the prevention or development of hypertension. In light of the discovery of a functionally dominant pro-natriuretic role of NKA signaling, we should renew our investigations into variants of NKA α1 that may have protective effects or be associated with increased risk of hypertension.

The two transporters demonstrated to be inhibited by NKA α1-NHE3 and NBCe1A- also have functional importance in RPT-mediated acid-base balance by absorption of bicarbonate and

excretion of acid. Over the last few decades, investigations including genetic based studies have proposed that changes in the expression and function of the acid-base transporters can be involved in blood pressure dysregulation (Boedtkjer et al., 2011; Ehret et al., 2011; Luft, Zemel, Sowers, Fineberg, & Weinberger, 1990). There is reportedly an association between salt-sensitive hypertension and acidosis and salt-sensitive hypertensive animal models with acidosis observe acidemia before the onset of hypertension (Batlle et al., 1993; Lucas, Lacour, McCarron, & Drüeke, 1987). However, the underlying mechanism linking disturbed acid-base transport activity to hypertension is not clearly understood, partly due to gaps in our understanding of regulatory mechanisms involved in uncoupling of Na transport from acid-base transport in healthy physiology. With NKA signaling demonstrating an interdependent regulation of NKA, NHE3 and NBCe1A, it may hold clues to unraveling salt-sensing, pH sensing, and the mechanistic pathways balancing the two functions.

In RPT$\alpha1^{-/-}$, we also observed a decrease in glucose urinary excretion with no basal change in glucose and insulin tolerance which fits with systemic compensation in unchallenged conditions (Table 4, Figure 10). SGLT2, expressed in the RPT, is the primary glucose transporter in absorption of glucose in a Na^+-dependent manner in the kidney and SGLT2 inhibitors are used to treat type-II diabetes and are associated with reno-protective effects and decreased cardiovascular comorbidities (Wanner & Marx, 2018). However, the mechanism and regulation underlying these benefits are unclear. Thus, there is need to understanding the role and regulation of SGLT2 in metabolic and cardiovascular disorders. Since loss of RPT NKA receptor is associated with an increase in renal glucose absorption, NKA receptor in RPT should be investigated for its role in renal and systemic glucose homeostasis.

The findings in these studies could also improve our understanding of underlying mechanisms of conditions exhibiting overall defective RPT absorption like Fanconi syndrome. In the acquired Fanconi syndrome, an adult can develop the condition due to wide variety of reasons from consumption of chemotherapeutic agents like antivirals, to exposure to toxins, like lead poisoning, or even a honeybee sting (Keefe & Bokhari, 2020). There is no known cure and current treatment regimens are mainly limited to replacement of lost electrolytes and avoidance of dehydration. With RPT transport of all electrolytes primarily dependent on Na^+ transport, one can speculate on the primary and secondary effects of NKA-mediated regulations on the transport of all electrolytes by RPT and its potential therapeutic potential.

Finally, identification of a higher dependence on NKA/Src-mediated inhibition in females compared to males and a potential sex-bias in the regulation underscores the importance of studying sex-based differences in molecular physiologies. The identification of such distinctions could prove useful in improving sex-based targeted therapies with improved efficacy and minimized adverse effects.

Future studies

We have only begun to unravel the importance and mechanism of NKA-mediated regulation of Na^+ absorption by RPT. We have not investigated the impact of NKA ablation or expression of mutant NKA on other RPT transporters such as SGLT2 and Na^+/Pi cotransporter (NaPi2) which may also be regulated by the NKA receptor. Specific molecular mechanisms involved in NKA/Src mediated inhibition of NHE3 and NBCe1A in female and alternate molecular mechanism involved in NKA-mediated inhibition in males are yet to be defined. We also need to investigate the importance of this regulation in the modulation of blood pressure and in adaptation to changes in systemic sodium.

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APPENDIX A

LETTER FROM OFFICE OF RESEARCH INTEGRITY

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

June 29, 2020

Shreya Mukherji
Marshall Institute of Interdisciplinary Research (MIIR)

Dear Shreya:

This letter is in response to the submitted dissertation abstract entitled *"Role of Renal Proximal Tubule ATP1A1 in Sodium Handling."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #609 and #682. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director
Office of Research Integrity

APPENDIX B

ABBREVIATIONS

µg – micrograms; 1/1000000th of a gram

µL – microliter; 1/1000000th of a liter

µM – micromolar; 1/1000000th of a molar

µm – micrometer; 1/1000000th of a meter

AAC-19 – porcine renal epithelial cell line

ACTH – adrenocorticotrophic hormone

ADP – adenosine diphosphate

Akt – protein kinase B (see also: PKB)

ANOVA – analysis of variance

ANP – atrial natriuretic peptide

Aqp-1 – Aquaporin-1

AT1 receptor – receptor for angiotensin-II

ATCC – American Type Culture Collection

ATP – adenosine triphosphate

ATP1A1 – gene for Na/K-ATPase α1

AUC – area under the curve

BP – blood pressure

BUN – blood urea nitrogen

BW – body weight

Ca^{2+} – calcium

cAMP – cyclic adenosine monophosphate

cav-1 – caveolin-1

CD2 – second cytoplasmic domain

CD3 – third cytoplasmic domain

Cl^- – chloride

CSA – cross-sectional area

CTS – cardiotonic steroids

D1 receptor – subtype of dopamine receptor

D2 receptor – subtype of dopamine receptor

dL – deciliter; $1/100^{th}$ of a liter

DMEM – Dulbecco's modified Eagle's medium

EBC – exhaled breath condensate

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

EIA – enzyme immunoassay

EO – endogenous ouabain

ERK – extracellular regulated kinase

EVOM2 – epithelial volt ohm meter-2

FBS – fetal bovine serum

FIJI – FIJI is just ImageJ

FITC – fluorescein isothiocyanate

FXYD2 – gamma

g – gram

g/dL – gram/deciliter

GFR – glomerular filtration rate

GTB – glomerulotubular balance

GTT – glucose tolerance test

h – hour

H^+ – proton

HCO_3^- – bicarbonate

H&E – hematoxylin and eosin

HK2 – human kidney cells

H_2O – water

H_2O_2 – hydrogen peroxide

IP – intraperitoneal

IP3 – inositol 1,4,5-triphosphate

ITT – insulin tolerance test

I.V. – intra-venous

J_{DEX} – flux of neutral 3 kilodalton dextran

K^+ – potassium

kDa – kilodalton

LAS/AF – Leica Application Suite/Advanced Fluorescence

Li^+ – lithium

LiCl – lithium chloride

LX-α2 – LLC-PK1-derived cell line

LY-a2 – LLC-PK1-derived cell line

M– molar

mL – milliliter

MBG – marinobufagenin

MBS – MES-buffered saline

MDCK – Madin-Darby canine kidney cells

MEK – mitogen activated protein kinase kinase

MES – 2-(N-Morpholino)ethanesulfonic acid

Mg^{2+} – magnesium

mEq/L – milliequivalent/liter

mg/dL – milligram/deciliter

mg/g – milligram/gram

mg/kg – milligram/kilogram

MIIR – Marshall Institute for Interdisciplinary Research

min – minutes

mmHg – millimeters of Mercury

mM – millimolar; millimoles per liter (see also: mmol)

mRNA – messenger ribonucleic acid

Na/K-ATPase – sodium-potassium adenosine triphosphatase (see also: NKA)

Na^{+} – sodium

$^{22}Na^{+}$ – radioactive sodium

NaCl – sodium chloride

NADPH – reduced form of nicotinamide adenine dinucleotide phosphate

NaPi2a – sodium-phosphate cotransporter-2a

NaSi-1 – sodium-sulfate cotransporter-1

NBCe1A – sodium-bicarbonate cotransporter-1A

NC – normal chow

NCC – sodium-chloride cotransporter

NFkB – nuclear factor kappa B

NHE3 – sodium-proton exchanger-3

NIH – National Institute of Health

NKA – sodium-potassium adenosine triphosphatase (see also: Na/K-ATPase)

NKCC2 – sodium-potassium-chloride cotransporter-2

nm – nanometer

NO – nitric oxide

OD – optical density

OK – opposum kidney cells

PAS – periodic acid schiff

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

pg – picogram

PI3K – phosphoinositide 3-kinase

PKA – protein kinase A

PKC – protein kinase C

PMSF – phenylmethylsulfonyl fluoride

PNx – partial nephrectomy

PO_4^{3-} – phosphate

Pro – proline residue

pS552 – phosphorylation at serine residue 552

PTEC – proximal tubule epithelial cells

PTH – parathyroid hormome

PVDF – polyvinylidene difluoride

PY-17 – LLC-PK1-derived cell line

pY260 – phosphorylation at tyrosine-260 residue

Pyk2 – non-receptor protein tyrosine kinase-2

RIHP – renal interstitial hydrostatic pressure

RIPA – radioimmunoprecipitation assay

RNA – ribonucleic acid

ROI – region of interest

ROS – reactive oxygen species

rpm – rotations per minute

RPT – renal proximal tubule

RPT$\alpha1^{-/-}$ – renal proximal tubule-specific NKA $\alpha1$ hypormorphic mouse

RPT$\alpha1^{+/+}$– renal proximal tubule -specific NKA $\alpha1$ expressing mouse; control to RPT$\alpha1^{-/-}$

RPT$\alpha1^{control}$– renal proximal tubule -specific NKA $\alpha1$ expressing mouse; control to rescue mouse

RPT$\alpha1^{-/-}$NHE3$^{-/-}$– renal proximal tubule -specific NKA $\alpha1$ and NHE3 hypomorphic mouse

RPT$\alpha1^{+/+}$NHE3$^{+/+}$– renal proximal tubule -specific NKA $\alpha1$ expressing mouse; control to RPT$\alpha1^{-/-}$NHE3$^{-/-}$

RPT$\alpha1^{WT}$– renal proximal tubule -specific wild-type NKA $\alpha1$ rescue mouse

RPT$\alpha 1^{Y260A}$– renal proximal tubule -specific Src-binding mutant Y260A NKA $\alpha 1$ rescue mouse

RT – reverse transcriptase

RT-PCR – real-time polymerase chain reaction (see also: qPCR, qRT-PCR)

SDS-PAGE – sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SEM – standard error of the mean

Ser – serine residue

SGLT-2 – sodium-glucose cotransporter-2

siRNA – small-interfering ribonucleic acid

SO_4^{2-} – sulfate

Src – proto-oncogene tyrosine-protein kinase Src (see also: c-Src)

TBST – Tris-buffered saline

TCG – telecinobufagenin

TER – transepithelial electrical resistance

TGF – tubuloglomerular feedback

TJ – tight junction

Tris-HCl – tris(hydroxymethyl)aminomethane hydrochloride

Tyr – tyrosine residue

UNaV – Absolute sodium output

Vmax – maximum velocity of enzyme reaction

WT – wild-type

xg – times gravitational force

Y260A – LLC-PK1 derived cell line

APPENDIX C

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