The Calcineurin Inhibitor Cyclosporine A Activates the Renal Na-(K)-Cl Cotransporters via Local and Systemic Mechanisms

vorgelegt von
Dipl.-Ing.
Katharina Ilse Blankenstein

von der Fakultät III – Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades
Doktor der Ingenieurwissenschaften
– Dr.-Ing. –
genehmigte Dissertation

Promotionsausschuss:
Vorsitzender: Prof. Dr. Lorenz Adrian
Erster Gutachter: Prof. Dr. Sebastian Bachmann
Zweiter Gutachter: Prof. Dr. Roland Lauster
Dritte Gutachterin: Prof. Dr. Vera Meyer

Tag der wissenschaftlichen Aussprache: 12. Juli 2019

Berlin 2019

Statement on the originality of the data

Herewith I declare that I prepared the Ph.D. thesis ***'The Calcineurin Inhibitor Cyclosporine A Activates the Renal Na-(K)-Cl Cotransporters via Local and Systemic Mechanisms'*** on my own and with no other sources and aids than quoted.

Abstract

The Calcineurin Inhibitor Cyclosporine A Activates the Renal Na-(K)-Cl Cotransporters via Local and Systemic Mechanisms

The two calcineurin inhibitors (CNIs) cyclosporine A (CsA) and tacrolimus are potent immunosuppressive drugs that have become indispensable in the post-transplantational therapy and in the treatment of autoimmune diseases. Immunosuppression via CNIs is achieved by inhibition of the calcineurin phosphatase and thus blocking the signaling pathway of NFAT (nuclear factor of activated T cells), thereby inhibiting T cell immune response. However, chronic use of CNIs is complicated by serious side effects which particularly affect the kidney, reflected by a large number of patients who develop electrolyte disorders, hypertension and renal failure under CNI treatment. The mechanisms of CNI-induced salt retention and hypertension are complex, involving not only intraepithelial but also systemic effects. Since calcineurin inhibition is associated with activation of the renal Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) and the Na^+-Cl^- cotransporter (NCC) that are expressed in the thick ascending limb (TAL) and the distal convoluted tubule (DCT) of the nephron, respectively, it has been suggested that the calcineurin phosphatase is involved in the regulation of the salt transporters. Interestingly, previous studies have demonstrated that CsA increases both NKCC2 and NCC activity, whereas tacrolimus merely stimulates NCC function. This study aimed to unravel the local and systemic mechanisms underlying calcineurin inhibition that lead to salt retention and hypertension.

We found that key proteins involved in calcineurin inhibition and the activation of NKCC2 and NCC are ubiquitously expressed along TAL and DCT. These results indicate that the distinct effects of CsA and tacrolimus on the activation of the salt transporters do not arise from differential expression of these key proteins, so that systemic mechanims might play a role. Thus, we sought to differentiate between local and systemic effects of CsA treatment by applying short- and long-term treatment protocols *in vivo* and *in vitro*. We analyzed physiological changes induced by CsA treatment and studied the role of endocrine factors such as the hormone arginine vasopressin (AVP). We show that CsA-induced activation of NKCC2 and NCC and their activating kinases chiefly occurs via post-translational modification by phosphorylation/dephosphorylation reactions. We further found that CsA-induced activation of NKCC2, but not NCC, requires AVP signaling. In a pilot study on NCC knockout mice we show that CsA treatment, unlike tacrolimus, might induce high blood pressure regardless of NCC activity. These findings provide evidence for the major role of NKCC2 in CsA-induced hypertension.

In summary, the results of this study demonstrate that CsA-induced activation of NKCC2 and NCC and their activating kinases chiefly occurs at the post-translational level via increased phosphorylation. In DCT cells, local calcineurin inhibition is sufficient to induce NCC activation, whereas NKCC2 activation appears to require additional stimulation by AVP. Our data further suggest a pivotal role of NKCC2 in CsA-induced hypertension. Altogether, this study contributes to a better understanding of CNI-induced salt retention and hypertension and can help improve blood pressure control.

Zusammenfassung

Der Calcineurin-Inhibitor Cyclosporin A Aktiviert die Renalen Na-(K)-Cl-Kotransporter mittels Lokaler und Systemischer Mechanismen

Die Calcineurin-Inhibitoren (CNI) Cyclosporin A (CsA) und Tacrolimus sind hochwirksame immunsuppressive Substanzen, die in der Posttransplantationstherapie und in der Behandlung verschiedener Autoimmunerkrankungen unverzichtbar geworden sind. Die durch CNI ausgelöste Immunsuppression wird über die Inhibition der Calcineurinphosphatase vermittelt. Dies führt zur Blockade des NFAT (nuclear factor of activated T cells)-Signalwegs und somit zur Inhibierung der T-Zell-Immunantwort. Allerdings wird die chronische CNI-Gabe durch ernsthafte Nebenwirkungen erschwert, die insbesondere die Niere betreffen. Dies spiegelt sich in der großen Anzahl an Patienten wieder, die unter CNI-Behandlung Störungen im Elektrolythaushalt, Bluthochdruck und Nierenversagen entwickeln. Die Mechanismen, die zu CNI-induzierter Salzretention und Bluthochdruck führen, sind komplex und umfassen neben intraepithelialen auch systemische Effekte. Weil die Calcineurin-Inhibition mit einer Aktivierung der renalen Na^+-K^+-$2Cl^-$- (NKCC2) und Na^+-Cl^--Kotransporter (NCC) in Zusammenhang steht, die jeweils in der dicken aufsteigenden Henle-Schleife (TAL) und dem distalen Konvolut (DCT) des Nephrons exprimiert sind, vermutet man, dass die Calcineurinphosphatase in die Regulation der Salztransporter involviert ist. Interessanterweise haben frühere Studien gezeigt, dass CsA die Aktivität von NKCC2 und NCC steigert, während Tacrolimus nur die NCC-Funktion stimuliert. Die vorliegende Arbeit hatte daher zum Ziel, die lokalen und systemischen Mechanismen der Calcineurin-Inhibition aufzudecken, die zu Salzretention und Bluthochdruck führen.

Die von uns untersuchten Schlüsselproteine, die in die Calcineurin-Inhibition und die Aktivierung von NKCC2 und NCC involviert sind, zeigten sich entlang dem TAL und dem DCT ubiquitär exprimiert. Diese Ergebnisse wiesen darauf hin, dass die unterschiedliche Wirkung von CsA und Tacrolimus hinsichtlich der Aktivierung der Salztransporter nicht auf einer differentiellen Expression dieser Schlüsselproteine beruht. Stattdessen könnten systemische Mechanismen eine Rolle spielen. Deshalb untersuchten wir im nächsten Schritt lokale und systemische Effekte der CsA-Behandlung mittels Kurz- und Langzeitbehandlungen am *in vivo*- und *in vitro*-Modell. Hierbei ermittelten wir die durch CsA induzierten physiologischen Änderungen und untersuchten die Rolle endokriner Faktoren wie dem Hormon Arginin-Vasopressin (AVP). Wir konnten zeigen, dass die CsA-induzierte Aktivierung von NKCC2 und NCC sowie ihrer regulierenden Kinasen hauptsächlich auf

posttranslationaler Modifizierung via Phosphorylierungs-/Dephosphorylierungsreaktionen beruht. Außerdem zeigen wir, dass für die CsA-induzierte Aktivierung von NKCC2 die Stimulation durch AVP notwendig ist, während für die Aktivierung von NCC die lokale Calcineurin-Inhibierung ausreichend ist. In einer Pilotstudie an NCC-Knockout-Mäusen liefern wir zudem Hinweise dafür, dass CsA, im Gegensatz zu Tacrolimus, trotz Abwesenheit von NCC zu Bluthochdruck führt. Diese Ergebnisse deuten auf eine entscheidende Rolle von NKCC2 in der Entstehung von CsA-induziertem Bluthochdruck hin.

Zusammengefasst demonstriert die vorliegende Arbeit, dass die CsA-induzierte Aktivierung von NKCC2 und NCC sowie ihrer regulierenden Kinasen hauptsächlich auf der posttranslationalen Ebene über eine Hochregulation der Phosphorylierung abläuft. In DCT-Zellen genügt für die Aktivierung von NCC die lokale Calcineurin-Inhibition, während die NKCC2-Aktivierung in TAL-Zellen einer zusätzlichen Stimulation durch AVP bedarf. Unsere Ergebnisse lassen auf eine Schlüsselrolle von NKCC2 bei CsA-induziertem Bluthochdruck schließen. Insgesamt tragen diese Daten zu einem besseren Verständnis von CsA-induzierten Störungen des Elektrolythaushalts und Hypertonie bei und können einen Beitrag zur Verbesserung der Behandlung von Bluthochdruck liefern.

Acknowledgements

I would like to thank Prof. Dr. Sebastian Bachmann for giving me the opportunity to work in his department and for supporting me at all stages of this thesis.

I am grateful to PD Dr. Kerim Mutig for his support, his feedback and inspiring ideas on our work.

I am also very thankful to Prof. Dr. Roland Lauster, my second supervisor, for his mentoring throughout my academic career and for broadening the research perspective.

My sincere thanks goes to Dr. David Ellison for the time working in his group, a fruitful collaboration and always exciting discussions.

Additionally, I want to thank all the members of the Sebastian Bachmann laboratory for their support and the positive working environment but also for the great time we had outside the lab.

Finally, my profound gratitude goes to my family and friends who constantly support and encourage me.

Contents

List of Figures

List of Tables

Abbreviations

ATPase	**A**denosine **Tri**p**h**osphatase
AVP	**A**rginine **V**aso**P**ressin
BW	**B**ody**W**eight
cAMP	**C**yclic **A**denosine **M**onophosphate
CCT	**C**onserved **C**-**T**erminal
CD	**C**ollecting **D**uct
cDNA	**c**omplementary **D**esoxyribonucleic **A**cid
CnA	**C**alci**n**eurin Catalytic Subunit **A**
CnB	**C**alci**n**eurin Regulatory Subunit **B**
CNI	**C**alcineurin **I**nhibitor
CNT	**C**onnecting **T**ubule
COX-2	**C**yclo**o**xygenase **2**
CsA	**C**yclosporine A
CUL3	**C**ullin-**3**
Cy	**Cy**anine
CypA	**Cy**clo**p**hilin **A**
CypB	**Cy**clo**p**hilin **B**
DCT	**D**istal **C**onvoluted **T**ubule
DDAVP	1-**D**esamino-8-**D**-**A**rginine **V**asopressin
DIG	**Dig**oxygenin
ENaC	**E**pithelial **N**atrium **C**hannel
ER	**E**ndoplasmatic **R**eticulum
FHHt	**F**amilial **H**yperkalemic **H**yper**t**ension
Fkbp12	**FK**506-**b**inding **p**rotein-12

GFR	**G**lomerular **F**iltration **R**ate
KLHL3	**K**elc**h**-**L**ike Protein **3**
KO	**K**nock**o**ut
mDCT	**m**ouse **D**istal **C**onvoluted **T**ubule
mRNA	**m**essenger **R**ibo**n**ucleic **A**cid
NaCl	**N**atrium **C**hloride
NCC	**N**atrium **C**hloride **C**otransporter
NFAT	**N**uclear **F**actor of **A**ctivated **T** Cells
NKCC2	**N**atrium **K**alium **C**hloride **C**otransporter 2
OSR1	**O**xidative **S**tress-**R**esponse Kinase **1**
PBS	**P**hosphate **B**uffered **S**aline
PKA	**P**rotein **K**inase **A**
qPCR	**Q**uantitative **P**olymerase **C**hain **R**eaction
RAAS	**R**enin **A**ngiotensin **A**ldosterone **S**ystem
raTAL	**ra**t medullary **T**hick **A**scending **L**imb
RNA	**R**ibo**n**ucleic **A**cid
RT-PCR	**R**everse **T**ranscription-**P**olymerase **C**hain **R**eaction
SLC	**S**o**l**ute **C**arrier
SPAK	**S**te20-related **P**roline **A**lanine-rich **K**inase
TAL	**T**hick **A**scending **L**imb
TCR	**T** **C**ell **R**eceptor
WNK	**W**ith **N**o Lysine **K**inase
WT	**W**ild**t**ype

Chapter 1

Introduction

1.1 Calcineurin Inhibitors - Dealing with Hypertension

Immunosuppressants have become indispensable in the post-transplantational therapy to prevent acute and chronic graft rejection and improve long-term survival of the patients. They are also widely prescribed for the treatment of several autoimmune diseases such as rheumatoid arthritis, psoriasis and atopic dermatitis. One of the most commonly used class of immunosuppressive drugs are the two calcineurin inhibitors (CNIs) cyclosporine A (CsA) and tacrolimus. Especially after solid organ transplantation, CNIs provide a powerful tool for reducing morbidity and mortality ([1], [2], [3]).

The great treatment success achieved with CNIs since their market entry more than 20 years ago resulted in increasing and longer administration of the drugs. However, chronic CNI treatment is complicated by serious side effects. One of the most severely affected organs is the kidney; virtually all patients suffer from renal toxicity and some develop renal failure ([4], [5], [6]). Elevated blood pressure and electrolyte disorders are typical early symptoms after initiation of CNI therapy ([7], [8]). Since the pathogenetic mechanims of CNI side effects are complex, search for optimal treatment options has been challenging so far. Diuretics are commonly used to reduce sodium and water retention in order to control hypertension, but diuretic resistance or adverse effects such as potassium wasting complicate the anti-hypertensive treatment ([9], [10]).

CNIs suppress the immune response of the body via inhibiting the calcineurin signaling pathway inside T lymphocytes. Immune response in a T cell is induced by antigen binding to its T cell receptor. This increases intracellular Ca^{2+} levels and activates the Ca^{2+}-calmodulin-dependent calcineurin phosphatase. Calcineurin subsequently dephosphorylates the transcriptional factor NFAT (nuclear factor of activated T cells) promoting its nuclear translocation. Transcriptionally active NFAT then induces the expression of a large number of immunomodulatory genes encoding cytokines such as interleukin 2 and interleukin 4 (Figure 1.1). Inhibition of this pathway using CNIs successfully suppresses the T cell immune response ([11], [12], [13], [14]).

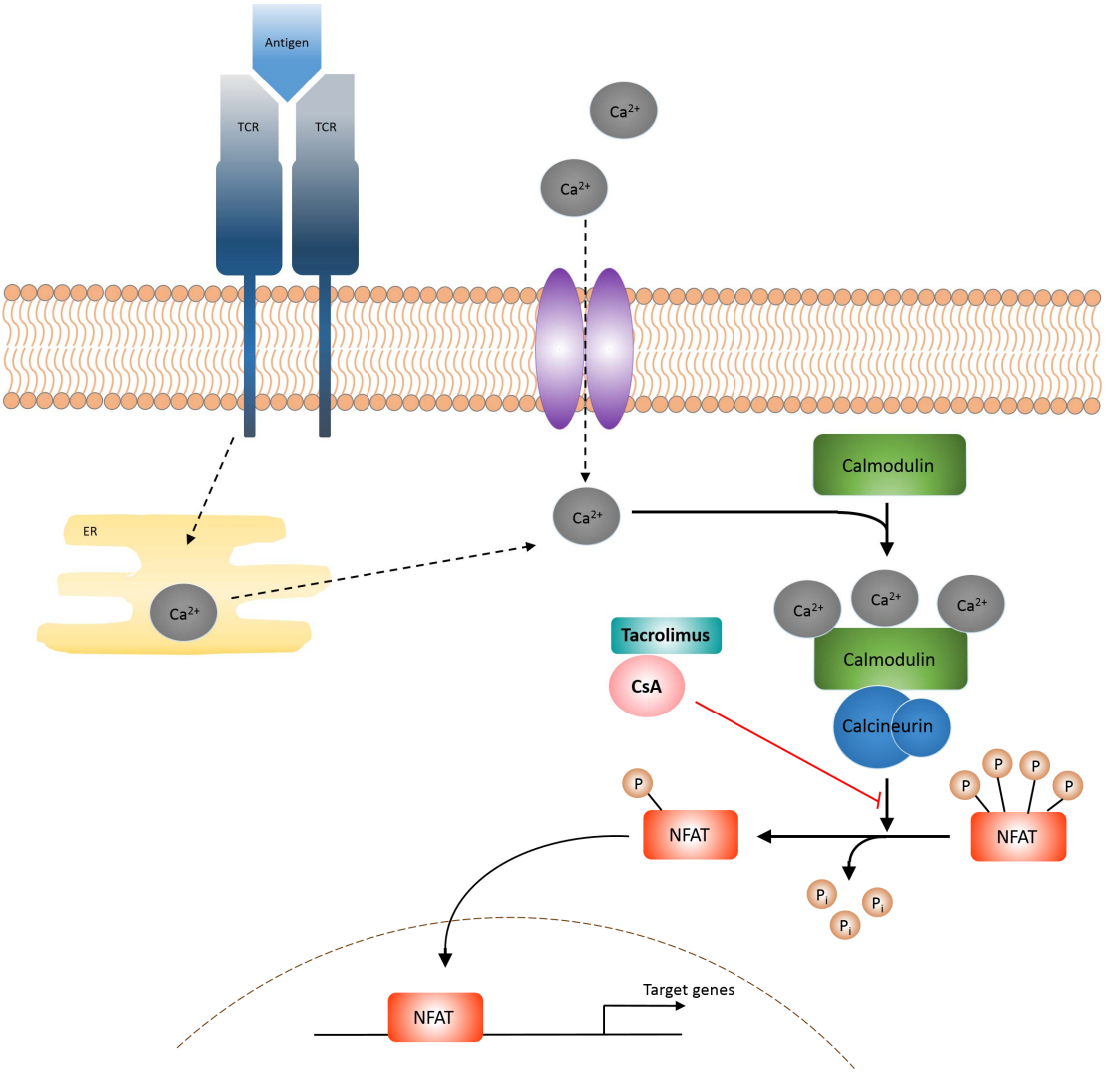

Figure 1.1. Mechanism of Immunosuppression in T Lymphocytes via Cyclosporine A and Tacrolimus Activation of a T cell through antigen binding of its T cell receptor (TCR) leads to increased intracellular Ca^{2+} levels and activation of the Ca^{2+}-calmodulin-dependent calcineurin phosphatase. NFAT (nuclear factor of activated T cells) is activated through dephosphorylation by calcineurin followed by nuclear translocation and induction of target gene expression such as IL-2 and IL-4. Cyclosporine A (CsA) and tacrolimus inhibit this pathway and thereby suppress T cell immune response. ER: Endoplasmatic Reticulum.

Recent research has focused on the pathogenesis of the renal adverse effects of CNIs; however, the precise molecular mechanisms remain to be completely understood. Numerous studies indicate that both intrinsic and extrinsic signals are responsible for the CNI-induced development of hypertension. Local mechanisms are reportedly based on activation of the sodium chloride cotransporters in the kidney distal nephron ([15], [16], [17], [18]). Additionally, endocrine signals such as arginine vasopressin (AVP) or the

renin-angiotensin-aldosterone-system (RAAS) may exacerbate the side effects of CNIs ([19], [20], [21]).
As chronically elevated blood pressure is a severe risk factor for the development of several cardiovascular conditions and ultimately leads to kidney failure, the clinical need for effective antihypertensive therapy has immensely increased in the last decades. Thus, in order to improve blood pressure control and prevent kidney damage, unraveling the mechanisms of CNI-induced hypertension has enormous clinical value ([22], [23], [24]).

1.2 Kidney Morphology and Functions

The kidney plays a key role in blood pressure control. It is responsible for filtration of the entire blood volume of the body. The filtration of blood in the kidney produces a large amount of primary urine. The subsequent reabsorption process in the renal tubules serves to preserve water, electrolytes and other useful components, whereas toxic substances are excreted with the final urine ([25]). By precise regulation of water and electrolyte homeostasis, the kidney is able to keep the renal blood filtration rate constant over a wide range of changes in perfusion pressure ([26], [27], [28]). This is achieved by its sophisticated morphology ([29]).

The parenchyma of the kidney is divided into the renal cortex and the renal medulla. The basic functional units of the kidney, the nephrons, span the cortex and the medulla. They are composed of the renal corpuscle, which is the initial filtering portion, and a segmented tubule ([30], [31], [32]). There are two sorts of a nephrons with different lenghts: the juxtamedullary and the cortical nephron with the latter possessing a longer loop of Henle and the hairpin reaching to the inner zone of the medulla. A renal tubule is composed of the following segments: The proximal tubule, the thin descending limb, the thin ascending limb (only in the long looped nephron) and the thick ascending limb of the loop of Henle, the distal convoluted tubule and the connecting tubule which empties into the collecting duct together with approximately eleven other nephrons ([31], [33]) (Figure 1.2).
The gradual buildup of urine concentration is facilitated by the medullary architecture: Descending and ascending tubules with varying water permeability are surrounded by

straight arterioles. Substance specific transport across the single-layered epithelia of the tubule system is realized by channels, transporters and pumps located at the luminal or basolateral site of the polarized cells ([29]).

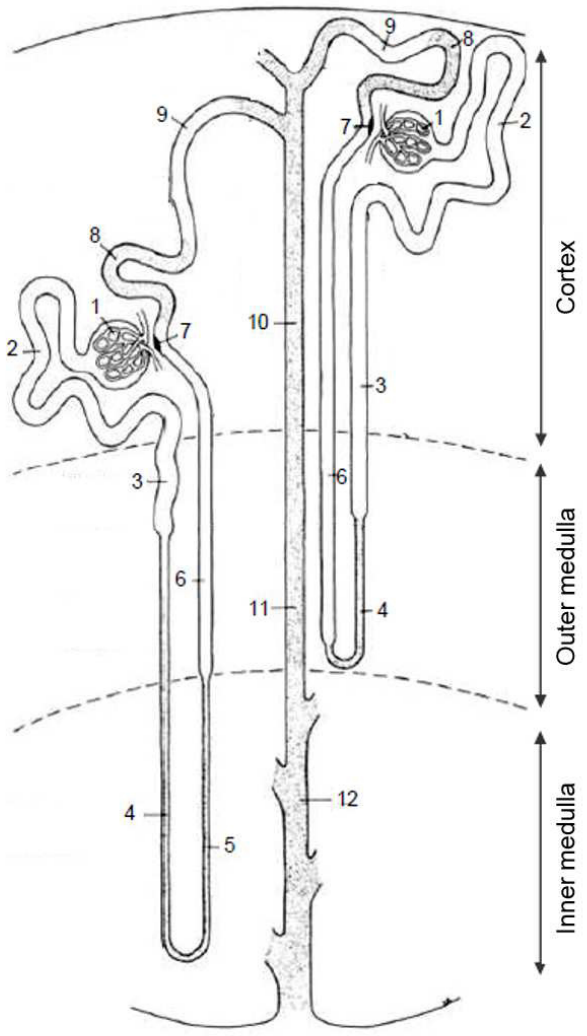

Figure 1.2. Structure of the Nephron Shown is the schematic of a juxtamedullary and a cortical nephron. Juxtamedullary nephrons have a longer loop of Henle and the hairpin reaches to the inner zone of the medulla. They are responsible for the development of the osmotic gradient that concentrates the urine. 1: glomerulus, 2: proximal convoluted tubule, 3: proximal straight tubule, 4: thin descending limb, 5: thin ascending limb, 6: the thick ascending limb of the loop of Henle, 7: macula densa, 8: distal convoluted tubule, 9: connecting tubule, 10-12: collecting duct. Schematic from Kriz and Bankir ([29]).

The renal corpuscule consists of the glomerulus and the Bowman's capsule. At the glomerulus, blood enters via an afferent arteriole and leaves through an efferent arteriole ((Figure 1.2). Through the glomerular blood pressure in the capillaries within the

glomerular network, water and solutes are filtered out of the blood. The renal filtration barrier is composed of the capillary endothelium, a basement membrane, and adjacent podocytes, which build a slit diaphragm that has the ability to discriminate among molecules with different size and electrical charge ([25], [27], [29], [28]).

Solutes up to an effective radius of $1.8\,nm$ can freely pass the barrier while passage for larger molecules becomes progressively difficult. Blood cells or the majority of proteins will not enter the Bowman's space but remain in the plasma. Filtration of anionic compounds is more difficult due to the anionic components within the filtration barrier. Water and small solutes such as glucose, amino acids, small proteins and electrolytes are filtered and transferred into the renal tubule system ([25], [27]).

Around $180\,l$ primary urine per day are produced through glomerular filtration. 99 % of the water and a large amount of the solutes are reabsorbed along the renal tubule, creating a highly concentrated secondary urine. A final volume of approximately $1.5\,l$ are excreted per day ([34], [35]).

1.2.1 The Distal Tubule – Key Role in Volume Regulation

To ensure renal excretion, glomerular filtration rate (GFR) has to be kept constant even at fluctuations of systemic blood pressure. For this, intra- and extrarenal mechanisms exist to effectively regulate fluid and electrolyte homeostasis. The distal tubule which consists of the thick ascending limb (TAL) and the distal convoluted tubule (DCT) herein plays a substantial role. Distal tubule cells possess a high density of Na^+-K^+-ATPase as well as specific salt transporters which are target of widely used diuretic drugs ([36], [34]). Another characteristic of TAL and DCT is their impermeability for water. Together, this allows for effective separation of salt and water in the distal tubule ([37]).

The Thick Ascending Limb

The TAL reabsorbs around 25 % of the filtered sodium chloride (NaCl). The active NaCl reabsorption in this water-impermeable segment results in a hypoosmolar urine. For this reason, the TAL is also termed the "diluting segment" ([37], [36]). The key component for

sodium reabsorption in the TAL is the apical Na^+-K^+-$2Cl^-$ cotransporter (NKCC2). Following the electrochemical gradient that is generated by the basolateral Na^+-K^+-ATPase, NKCC2 transports Na^+, K^+ and $2Cl^-$ electroneutrally into the cytoplasm ([36], [35]).

NaCl uptake via NKCC2 is particularly important in specialized macula densa cells which are located in the late portion of the TAL. The macula densa is an area of closely packed cells lying in direct contact with the vascular pole of the renal corpuscule ([38]). They act as a NaCl sensor and are responsible for two mechanisms: the tubuloglomerular feedback (TGF) and the activation of the renin-angiotensin-aldosterone system (RAAS). As a consequence of these mechanisms, the tone of afferent and efferent arterioles can be adjusted to keep renal filtration at a constant level ([39], [40]). In case of increased renal filtration through increased systemic blood pressure, NaCl delivery to the macula densa is enhanced. This stimulates macula densa cells to secrete adenosine, the mediator of the TGF. Adenosine causes vasoconstriction of the afferent arteriole of the glomerulus which finally reduces the GFR ([41], [42]).

In case of reduced GFR induced by low systemic blood pressure, reduced chloride delivery to the macula densa stimulates the secretion of renin by specialized smooth muscle cells in the wall of afferent arterioles, so called juxtaglomerular cells ([43], [44]). Renin secretion initiates a cascade that ultimately forms angiotensin II. This hormone has strong vasoconstrictive effects, systemically as well as intrinsically with a potential preference on the efferent arterioles in the kidney, which consequently increases the GFR ([45]). Besides, angiotensin II stimulates the secretion of aldosterone and AVP. Release of these two hormones results in elevated sodium uptake with water following through the tubular epithelium. This in turn increases blood volume and subsequently raises the systemic blood pressure ([46], [47]). In contrast, when renal filtration is too high, the macula densa downregulates the GFR by restricting the RAAS in order to avoid glomerular damage.

Together, the tubuloglomerular feedback and the RAAS are pivotal mechanisms regulated directly and indirectly by the macula densa to maintain blood pressure and renal filtration via intrinsic and extrinsic mechanisms ([48]).

The Distal Convoluted Tubule

Approximately 5 - 10 % of the filtered sodium is reabsorbed in the DCT ([34]). The DCT is functionally divided into an early (DCT1) and a late (DCT2) segment. Electroneutral transport of sodium and chloride across the apical membrane of the DCT is driven by the gradient created by the Na^+-K^+-ATPase. DCT cells are characterized by the Na^+-Cl^- cotransporter (NCC) ([49], [50]). NCC is expressed in the entire DCT, but in the late DCT2 its expression overlaps with the epithelial sodium channel ENaC. However, NCC is chiefly responsible for the sodium uptake in the DCT ([51], [52]). The DCT2 builds a portion of the aldosterone-sensitive distal nephron which includes the connecting tubule and the collecting duct. Different studies have shown that angiotensin II and aldosterone stimulate NCC activity ([53], [54]), and AVP also plays a role in stimulation of NCC activity ([55]). Due to its ability to adapt to changes in hormonal stimuli and precisely adjust sodium balance, the DCT is a critical nephron segment for the salt and water homeostasis and volume regulation.

Since the distal tubule plays a key role in blood pressure regulation understanding the mechanisms of how CNIs activate NKCC2 and NCC will help improve the treatment of CNI-induced hypertension.

1.3 Expression and Regulation of NKCC2 and NCC

1.3.1 Gene and Protein Expression

NKCC2 and NCC belong to the group of solute carriers (SLC) and are members of the SLC12 family of electroneutral cation-chloride-coupled cotransporters. The SLC12A1 gene encodes NKCC2 and the SLC12A3 gene encodes NCC ([56], [50]). The basic protein structure of NKCC2 and NCC is of high similarity, comprising 12 transmembrane-spanning domains, flanked by a short cytoplasmic N-terminal and a large cytoplasmic C-terminal domain, and segment 7 and 8 are connected by a long hydrophilic extracellular loop ([57]) (Figure 1.3).

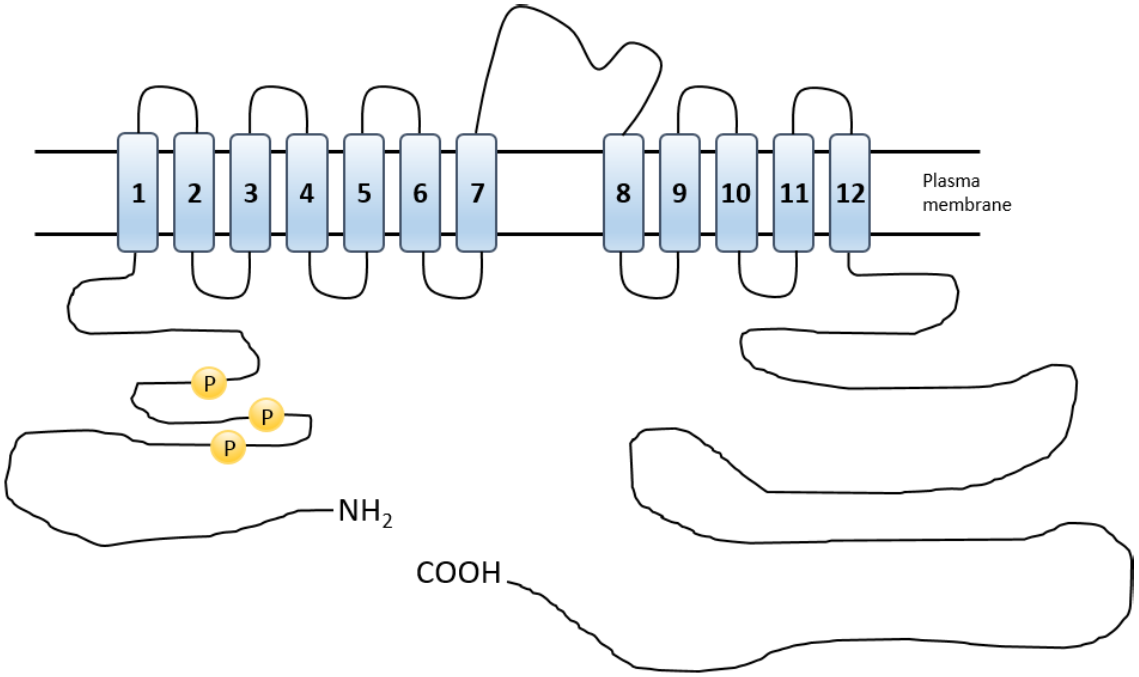

Figure 1.3. Schematic Structure of the Highly Homologous Distal Salt Transporters NKCC2 and NCC Basic structure of the NKCC2 and NCC consisting of 12 transmembrane-spanning domains, a short cytoplasmic N-terminal (NH_2) and a large cytoplasmic C-terminal (COOH) domain. Segment 7 and 8 are connected by a long hydrophilic extracellular loop. Most of the known conserved regulatory phosphorylation (P) sites are located within the N-terminal domain. Schematic modified from Ares et al. ([57])

Both SLC12A1 and SLC12A3 give rise to different isoforms of NKCC2 and NCC, respectively. Alternative splicing of exon 4 of the SLC12A1 gene yields three full length transcripts of 1,099 amino acids (SLC12A1a, b and f) that differ in their distribution along the TAL and transport affinity for Na^+, K^+ and Cl^- ([58], [59]). In rodent kidney, additional alternative splicing leads to the generation of three NKCC2 isoforms with truncated C-terminal ends. Some studies suggest that the various NKCC2 isoforms cooperate in salt reabsorption in the TAL, since different salt concentrations can be detected in this part of the nephron ([60], [61]). The predicted molecular mass of NKCC2 is 121 kDa; however, immunoblotting of NKCC2 shows an apparent molecular mass of 160 kDa, conferred by two N-glycosylations at positions between transmembrane domain 7 and 8. This glycosylation is likely involved in maturation and membrane targeting of NKCC2 ([58], [62]).

The NCC gene gives rise to three alternative splice products, all of a size around $1,020$ - $1,030$ amino acids, of which isoform 3 is the dominant form in the human kidney and

isoforms 1 and 2 are lacking in rodents ([63], [64]). Like NKCC2, NCC possesses two N-glycosylations, located in the 4th extracellular loop, and they have been shown to be essential for efficient function and surface expression. The non-glycosylated protein has a molecular mass of 113 kDa, but the complexly glycosylated protein shows a broad band between 125 and 160 kDa when detected by immunoblotting ([65], [66], [67]).

1.3.2 Trafficking and Phosphorylation

Function of most transmembrane proteins is regulated by trafficking. It is still unclear how trafficking and phosphorylation increase function of NKCC2 and NCC. While increased trafficking and phosphorylation usually correlate with activation, the underlying mechanisms remain to be clarified. However, different studies have shown potential mechanistic links and suggest that adequate regulation of NKCC2 phosphorylation and trafficking facilitates its affinity to chloride and, thus, transport function ([68], [69], [70]).
In a dynamic process of endocytosis and exocytosis the transporters are balanced between residing in the apical membrane and in subapical vesicles. Upon certain stimuli they undergo exocytosis to increase surface expression which ultimately enhances Na^+ resorption ([71], [72]). The rate of exocytosis and the increase in apical abundance are most likely mediated via the cAMP/PKA pathway ([73], [57], [74]). Endocytosis of NKCC2 and NCC is reportedly regulated by the clathrin-mediated pathway ([75], [76]). Recycling of the salt transporters via exocytosis of the intracellular pool presumably facilitates a faster and more efficient NaCl reabsorption ([68]).

Like many proteins, NKCC2 and NCC are regulated by phosphorylation, being activated or inhibited by this type of post-translational modification. Conserved serine and threonine phosphorylation sites have been described for NKCC2 and NCC in both their N- and C-terminal domains. Most of the regulatory phosphorylation sites are located on the N-terminal domain ([57]) (Figure 1.3).
Regulation of NKCC2 by phosphorylation has first been described in 2003 ([77]). In NKCC2, the three threonine sites T96, T101 and T114 (mouse and rat sequence, equivalent to T100, T105 and T118 in human NKCC2) play a critical role. Individual mutations of these threonine residues reduce NKCC2 activity; however, at least two of the sites have to be mutated to completely abolish transport function ([70]). The serine residue S126

(mouse, rat) is another important stimulatory site. With immunoblots, phosphorylation at this site is hardly detectable at baseline conditions but found phosphorylated when NKCC2 activity is increased, and mutants lacking this site have strongly decreased NKCC2 function ([69], [78]). It has been demonstrated that T101 and S126 play the most important role for NKCC2 activity since combined mutation of the two residues abolishes NKCC2 function ([69]). For NCC regulation, the critical phosphorylation sites are T53, T58 and S71, localized within the N-terminal region of the protein. Activity of the transporter has been demonstrated to correlate with phosphorylation of the threonine sites, particularly with T58 ([79], [80], [81]). In contrast to NKCC2, an individual mutation of T58 is sufficient to severely inhibit NCC function. However, mutations in S71 also markedly reduce NCC function making it an important phospho-site for NCC regulation ([82], [83]).

Whether phosphorylation of NCC takes place at the apical membrane or within subapical vesicles is still under debate. For NKCC2, increased phosphorylation was shown to coincide with its translocation to the apical membrane ([77]). For NCC, some studies provide evidence that increased levels of the phosphorylated cotransporter are accompanied by trafficking towards the plasma membrane; other studies report no correlation of phosphorylation and trafficking processes ([84], [85]).

Since modulation of surface expression and malfunction of phosphorylation are associated with major defects in salt reabsorption, these mechanisms play a major role in the regulation of NKCC2 and NCC activation.

1.3.3 Activation by the WNK-SPAK/OSR1 Kinase Cascade

NKCC2 and NCC are regulated by similar pathways that involve a kinase cascade of WNK (with-no-lysine [K] kinase), SPAK (Ste20-related proline/alanine-rich kinase) and OSR1 (oxidative stress-response kinase 1) (Figure 1.4).

The homologous SPAK and OSR1 are serine/threonine kinases with overlapping renal expression along the distal nephron. SPAK (encoded by STK39) and OSR1 (encoded by OXSR1) were found to interact with NKCC2 and NCC via binding directly to a docking motif (RFXV for NKCC2 and RFXI for NCC) within the N-terminus of the transporters.

A conserved docking site within the non-catalytical C-terminal of SPAK/OSR1 called CCT (conserved C-terminal) was identified to be critical for this interaction ([86], [69], [82], [23]). Studies on transgenic mouse models with modified SPAK expression revealed that the T96 and T101 residues of NKCC2 are in fact the SPAK- and OSR1-dependent phosphorylation sites and that these sites are associated with the regulation of blood pressure ([23], [87]). For NCC, several studies illustrate the relevance of all three phosphorylation sites (T53, T58, S71) for transport activity, sodium reabsorption and blood pressure regulation ([82], [80], [87]).

As members of the Ste20 kinase family, SPAK/OSR1 are able to coordinate downstream as well as upstream molecules. In the kidney, known upstream regulators of SPAK/OSR1 are the WNK kinases. During the last decade, WNK kinases have been subject of intense research since they were found to play a role in blood pressure regulation and activate SPAK/OSR1 kinases by phosphorylation ([88], [89]). WNK (with-no-lysine [K]) kinases are a unique subfamily of serine/threonine kinases that have their catalytic lysine residue located in an unusual subdomain, distinguishing it from other kinase families. Four homologs of WNK kinases have been identified in human, of which at least WNK1 (encoded by WNK1) and WNK4 (encoded by WNK4) are expressed in rats and mice ([90], [89], [91]). WNK kinases are activated by osmotic stress and changes in chloride concentration ([92], [82]). They comprise a phosphorylation site at serine 382 (in rodents) which can directly interact with chloride ions. Low chloride levels reportedly activate WNK1, apparently by enabling autophosphorylation within the kinase domain ([93], [94]). The S382 residue is conserved in all WNK isoforms, suggesting that the autocatalytic mechanism and its inhibition by chloride binding happens accordingly in all four WNK transcripts ([95]).

For the interaction with SPAK/OSR1, WNK kinases possess the same RFX[I/V]-motif that is present on NKCC2 and NCC ([96], [97]). SPAK and OSR1 are regulated by WNK1 and WNK4 through phosphorylation at threonine residues within their catalytic domain of the T-loop (in rodents: T243 in SPAK, T185 in OSR1) and at serine residues within a regulatory domain of the S-motif (in rodents: S383 in SPAK, S325 in OSR1) ([88], [98], [99]).

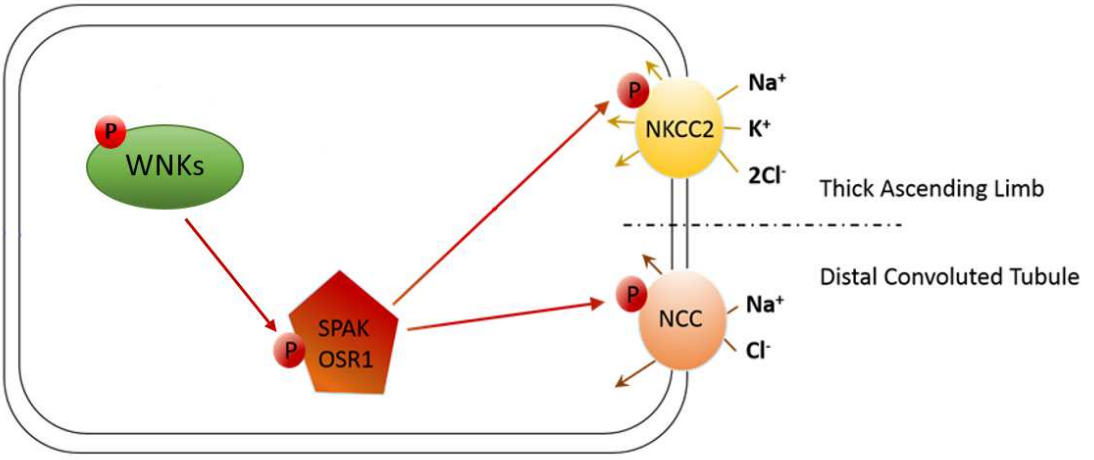

Figure 1.4. Activation of NKCC2 and NCC by the WNK-SPAK/OSR1 Kinase Cascade NKCC2 and NCC, the salt transporters of the thick ascending limb and the distal convoluted tubule, respectively, are activated by phosphorylation processes through a kinase cascade involving SPAK/OSR1 and WNK kinases.

1.3.4 Endocrine Control of NKCC2 and NCC

Both NKCC2 and NCC are regulated by hormonal signaling pathways which affect sodium reabsorption and arterial blood pressure. One of the most studied and most potent hormones stimulating NKCC2 activity is arginine vasopressin (AVP) ([100], [101], [102]). AVP can induce an increase of phosphorylation levels at the important regulatory sites T96 and T101 and thereby activate NKCC2 transport ([77]). Hormones like AVP exert their effects via an increase of intracellular cyclic adenosine monophosphate (cAMP) levels which in turn activates the protein kinase A (PKA). Experiments with cAMP and PKA stimulation have shown to control NKCC2 function but only phosphorylate the S126 site within the N-terminal domain of NKCC2, leaving T96 and T101 sites unaffected ([103], [104]). This suggested that phosphorylation at site S126 is PKA-dependent whereas AVP-induced T96 and T101 phosphorylation must be regulated by other kinases activities, e.g. SPAK or OSR1. It has further been shown that the vasopressin receptor 2(V2R)-specific agonist desmopressin increases the abundance of phosphorylated SPAK/OSR1 suggesting that SPAK mediates the effects of AVP on distal sodium reabsorption ([105]).

In the DCT, a stimulatory effect of AVP on NCC function and salt reabsorption has early been demonstrated by Elalouf et al. ([55]). The crucial phosphorylation sites of NCC, T53, T58 and S71, are regulated by AVP and there is compelling evidence that this effect

is mediated via SPAK/OSR1 ([20], [106], [105], [23]).

As mentioned in chapter 1.1, an activated renin-angiotensin-aldosterone-system (RAAS) has stimulatory effects on sodium reabsorption in the nephron. In the DCT, both aldosterone and angiotensin II are able to stimulate NCC expression and modulate NCC phosphorylation, either independently or as a result of combined effects. The activation of NCC by aldosterone and angiotensin II is likely mediated by SPAK ([81], [54], [107], [108]).

The discovery of the WNK-SPAK/OSR1 signaling cascade has helped understanding the mechanisms of blood pressure regulation and the development of hypertension. The kinase cascade provided a new link between hormones and salt transport in the distal nephron ([105], [109], [110], [108]). However, the impact of endocrine pathways in the pathomechanism of hypertension and especially CNI-induced hypertension still remain to be clarified.

1.3.5 Pathophysiology of NKCC2 and NCC

Dysfunction of the distal salt transporters perturbs sodium reabsorption and may deteriorate blood pressure. Loss of function mutations within the genes encoding NKCC2 and NCC cause Bartter's and Gitelman's syndrome, respectively. While symptoms differ among patients, both diseases are characterized by pronounced salt wasting, hypokalemia, and potentially low blood pressure despite high plasma renin activity and high aldosterone concentrations ([111], [64]).

Mutations in the genes encoding members of the WNK-SPAK/OSR1 cascade are associated with the development of hypertension. Mutations in WNK1 and WNK4 have been identified to be responsible for the development of the rare monogenic disease FHHt (familial hyperkalemic hypertension) or otherwise named Gordon syndrome or pseudohypoaldosteronism type 2, characterized by hyperkalemia and hypertension ([112], [24]). Mutations in the WNK1 gene are large deletions which increase WNK1 expression, and mutations in WNK4 lead to accumulation of WNK4. The overabundance of WNK protein stimulates the downstream signaling cascade by phosphorylating SPAK/OSR1 which in turn phosphorylate NCC. Activation of NCC, the major pathogenic factor of FHHt, leads

to an excess in sodium reabsorption in the DCT which subsequently causes hypertension ([83], [109], [10]).

Since adverse effects associated with calcineurin inhibitors used in immunosuppressive therapy resemble FHHt symptoms, it is tempting to assume that CNIs affect the same signaling pathway. In fact, several studies have shown that CNIs stimulate NKCC2 and NCC function ([16], [113]). The fact that FHHt-related hypertension is a sole result of NCC activation while for this syndrome increased levels of phosphorylated NKCC2 have not been reported indicates that NCC is sufficient to induce hypertension in certain conditions. It remains unclear why in FHHt the accumulation of WNK protein is not associated with a stimulation of NKCC2 function, although multiple studies have demonstrated that WNK kinases are signaling via SPAK/OSR1 to NKCC2/NCC, providing compelling evidence that WNKs can activate NKCC2.

Only recently, two upstream regulators of WNK kinases, cullin-3 (CUL3, 89 kDa) and kelch-like protein 3 (KLHL3, 65 kDa), have been discovered and FHHt-causing mutations were identified within their encoding genes. KLHL3 (encoded by KLHL3) is an adaptor protein that brings WNK kinases in close proximity with the E3 ubiquitin-protein ligase CUL3 (encoded by CUL3) which is able to ubiquitylate WNK and thereby tag it for degradation ([114], [115]). Mutations found in the KLHL3-CUL3 complex all lead to impaired degradation of WNK, either due to impairing the interaction of KLHL3 with CUL3 or WNK, or due to ubiquitylation of KLHL3 itself ([116], [117], [118]).
Different expression levels of CUL3 and KLHL3 may play a role in the regulation of NKCC2 and NCC in FHHt or CNI-induced renal side effects. However, it is still unclear whether CUL3 and KLHL3 are differentially expressed along the distal nephron and, thus, might have differential effects on NKCC2 and NCC function.

1.4 Effects of Cyclosporine and Tacrolimus on NKCC2 and NCC Function

Besides their systemic action described in chapter 1.1 the two calcineurin inhibitors cyclosporine and tacrolimus exert local effects on the kidney. Especially in the renal distal

nephron, these effects play a crucial role in the development of hypertension ([16], [18], [17]). Interestingly, NKCC2 and NCC are differentially affected by the two drugs. While CsA has been shown to increase both NKCC2 and NCC activity, tacrolimus is suggested to merely stimulate NCC function. The fact that calcineurin inhibition locally affects the distal nephron suggests that the calcineurin phosphatase is involved in the regulation of NKCC2 and NCC. The two transporters are under control of phosphorylation and dephosphorylation processes and studies have indeed provided compelling evidence that they are a target of calcineurin ([68], [19]).

1.4.1 Calcineurin

Calcineurin or protein phosphatase 3 (PP3, formerly called PP2B) is a Ca^{2+}-calmodulin-dependent serine/threonine phosphatase that is activated by increased intracellular Ca^{2+} concentrations. The full protein consists of a catalytic subunit of approximately 60 kDa (PP3-A/CnA) encoded by the PPP3C gene and of a regulatory subunit of 19 kDa (PP3-B/CnB) encoded by PPP3R. Three isoforms with a sequence identity of roughly 80 % exist of the catalytic subunit: Isoform α, β and γ. The latter is only expressed in testis and brain and is absent at least in the healthy kidney ([119], [120]). Two isoforms exist of the regulatory calcineurin B subunit: CnB1 which is ubiquitously expressed and CnB2 which is only found in testis ([121]). The catalytic calcineurin A subunit possesses a phosphatase domain that is necessary for interaction with phosphorylated substrates. The regulatory CnB subunit binds calcium and calmodulin and, upon increased cytosolic Ca^{2+} levels, facilitates the conformational change that is needed for activation of the CnA phosphatase domain ([122], [123]).

Although ubiquitously expressed, the effects of activated calcineurin are best described in T cells, where it activates the transcription factor NFAT through dephosphorylation and thus induces gene expression of immunomodulatory genes (see chapter 1.1). In the kidney, CnAα-CnB and CnAβ-CnB compose two functional enzymes; however, their renal expression pattern is still controversial. Furthermore, different studies suggest distinct cellular functions for the two isoforms ([124], [13]). While *in vitro* experiments show that CnAα and CnAβ have similar catalytic activity on various substrates, they seem to differ

in their physiological function as CnAα and CnAβ knockout mice exhibit different phenotypes. Mice lacking the β isoform have an immature immune system, whereas mice lacking the α isoform can still be immunosuppressed but manifest developmental defects and kidney dysfunction ([125], [126], [127]). Thus, particularly in the kidney, the two catalytic isoforms appear to have distinct functions.
Since calcineurin reportedly plays a role in distal nephron function ([128], [68]) and the renal effects of calcineurin inhibition differ depending on the administered CNI ([19], [129]), it has been suggested that the expression pattern of the calcineurin isoforms is the key to this phenomenon.

Another tempting assumption is that calcineurin isoforms differ in substrate specificity. Calcineurin binds its substrates via certain recognition sites in the respective proteins or peptides. The PxIxT motif and the LxVP motif were detected in NFAT and other calcineurin substrates ([130], [131], [132]). While roughly 50 calcineurin substrates have been experimentally confirmed so far, almost 600 potential substrates were identified based on bioinformatics analysis. It has been postulated that the two binding motifs establish the affinity of calcineurin substrates or regulators in cooperation ([133]).
CnAα and CnAβ further have significant sequence variance within their N-terminal region which may cause substrate specific binding of the isoforms. Whether substrate specificity of the catalytic subunits or their differential expression along the nephron is the key to the distinct effects of calcineurin inhibition remains to be clarified.

1.4.2 Calcineurin Inhibition

Inhibition of calcineurin through CNIs occurs via binding of CsA and tacrolimus to their respective intracellular receptors, the cyclophilins and Fkbp12, which collectively are known as immunophilins. The hydrophobic groove at the interface of the CnA and CnB subunits that interacts with the LxVP motif of calcineurin substrates has been identified to be the docking site of the CNI-immunophilin-complex which competes with calcineurin substrates. By binding to calcineurin, the CNI-immunophilin-complex completely inhibits

enzymatic activity of the phosphatase, thus suppressing the phosphatase-controlled activation of NFAT and preventing lymphokine gene expression ([134], [135], [132]).

CsA and tacrolimus have been effectively used for immunosuppression for more than 20 years. The cyclic peptide CsA, extracted from a soil fungi, and the macrolide tacrolimus, isolated from a soil bacterium, are both highly lipophilic and have a similar molecular weight (approximately 1.200 g/mol and 800 g/mol, respectively) ([136], [137]). With regard to immunosuppression, tacrolimus is more potent exhibiting a lower risk of rejection than CsA. Tacrolimus is also less nephrotoxic than CsA but is associated with more neurotoxic and gastrointestinal problems ([138], [139]). However, both drugs can ultimately cause volume expansion and hypertension, but the local effects on the renal distal nephron are dissimilar. CsA is able to stimulate NKCC2 and NCC function but tacrolimus only increases NCC activity ([68], [18], [17], [140], [15]). A potential reason for this phenomenon could be a differential expression of the CNI-binding proteins, the immunophilins, in the distal nephron.

Besides being the endogenous receptors of CNIs, immunophilins have peptidyl-prolyl-cis/trans-isomerase activity facilitating protein folding, and as chaperones they are involved in protein trafficking and molecular assembly. Cyclophilin A (CypA) and cyclophilin B (CypB), encoded by PPIA and PPIB, are the isomerases most sensitive to CsA-inhibition. The 18 kDa protein CypA is considered to be the principal target of CsA, it binds the drug with high affinity. CypB, a 24 kDa protein, contains an ER signal sequence that is absent in CypA and is believed to play a role in the secretory pathway and maturation of proteins destined to the plasma membrane ([141], [142]). The tacrolimus-binding protein Fkbp12 (encoded by FKBP1A) is the smallest protein of the Fkbp family (12 kDa) containing the minimal sequence for a peptidyl-prolyl isomerase. Besides the isomerase function and the immunosuppressive activity of the tacrolimus-Fkbp12 complex, Fkbp12 associates with membrane receptors and seems to play a role in their regulation ([143], [144]).

While cyclophilins and members of the Fkbp family equally inhibit calcineurin activity, they have dissimilar protein structure, and their renal expression remains to be precisely clarified.

That CNI-induced hypertension is mediated not only by local and intraepithelial but also by paracrine and endocrine mechanisms has been suggested in several *in vitro* and *in vivo* studies, and these effects seem to be more pronounced with CsA than with tacrolimus administration ([145]). CsA-induced vascular effects such as AVP-mediated contraction of smooth muscle cells have been described ([146]). Also, renin as well as cyclooxygenase-2 (COX-2) expression has been shown to be affected by CsA and tacrolimus treatment ([145], [21]). COX-2 is constitutively expressed in renal macula densa cells and its secretion stimulates the local prostaglandin pathway ([147]). Different prostaglandins are implicated in the regulation of blood pressure via modulation of the glomerular filtration rate, renin synthesis and NKCC2 function ([148], [149]). Since COX-2 transcription is regulated by the NFAT transcription factor and thus depends on calcineurin signaling ([150], [151]) it has been suggested that suppression of COX-2 by calcineurin inhibition is one major factor that complicates the use of CNIs. Altogether, paracrine and endocrine pathways such as AVP signaling, COX-2 expression and the RAAS may aggravate the well-known CsA-induced adverse effects.

In view of their broad clinical use, unraveling the local and systemic mechanisms of calcineurin inhibition that affect renal homeostasis would be highly beneficial.

Chapter 2

Hypotheses, Aims and Study Design

The calcineurin inhibitors (CNIs) cyclosporine A (CsA) and tacrolimus are effective immunosuppressive agents for the treatment of several autoimmune diseases and the prevention of organ rejection, since they block the calcineurin-NFAT signaling pathway and stop the expression of inflammatory response genes. When chronically administered, however, CNI treatment may be accompanied by severe side effects particularly affecting the kidney which manifests in hypertension and electrolyte disorders. Previous studies have indicated that calcineurin inhibition is associated with activation of the crucial cation coupled cotransporters NKCC2 and NCC in the distal nephron, suggesting the involvement of the calcineurin phosphatase in the regulation of the transporters. Interestingly, tacrolimus has been shown to only stimulate NCC function, whereas CsA affects transport activity in the entire distal nephron. Additionally, various studies suggest that tacrolimus-induced activation of NCC and concomitant increase of arterial blood pressure primarily rely on local calcineurin inhibition. In contrast, CsA-induced hypertension may partially be caused by systemic effects of calcineurin inhibition, e.g. stimulated vasopressin signaling or activation of the renin angiotensin aldosterone system. In order to improve the benefit/risk ratio of CNI treatment, we aimed to uncover the mechanism behind the distinct effects of CsA and tacrolimus in the distal nephron.

Aim 1: The first part of this study focused on the characterization of the key factors regulating the inhibition of calcineurin and the activation of the distal salt transporters. With this, we sought to gain further insight into the molecular mechanisms underlying the local signaling of calcineurin inhibition. We hypothesized that the distinct effects of CsA and tacrolimus are due to differential expression of crucial proteins involved in calcineurin inhibition.

Aim 2: The main objective was to differentiate between local and systemic effects of CsA in the regulation of the distal salt transporters NKCC2 and NCC. Thus, we sought to describe acute and chronic effects of CsA administration *in vivo* and *in vitro*, evaluate physiological changes and study the role of endocrine factors in this context. We speculated that additional systemic effects of calcineurin inhibition might be key to the different CNI-sensitivities of the distal nephron.

Aim 3: In a third approach we sought to investigate the *in vivo* effects of CsA treatment in the absence of NCC. In a previous study, tacrolimus treatment did not induce hypertension in NCC knockout mice. We speculated that the impact of CsA treatment on blood pressure is more diverse and that potential systemic mechanisms affect renal homeostasis.

The present study aimed to contribute to a better understanding of the mechanisms of CNI-induced hypertension and may help improve the clinical use of CNIs and alleviate their negative side effects.

Chapter 3

Material and Methods

3.1 Animals, Tissues, Treatments

All animals, except for the NCC knockout mouse model, were bred and held at the research facilities for experimental medicine (Forschungseinrichtungen für experimentelle Medizin, FEM) and later in the facilities of the CharitéCrossOver (CCO) at the Charité University Medicine of Berlin. All animals used in this study were adult male rats and mice, respectively, which were kept at a standard diet and tap water ad libitum.

Wistar rats, outbred albino rats developed in the Wistar Institute ([152]), were used as wildtype (WT) model. For evaluation of short-term CsA effects, WT rats were divided into groups (n=5 for biochemical evaluation and at least n=4 for morphology) and injected intraperitoneally (i.p.) with CsA (30 mg/kg body weight, Sandimmune, Novartis, Nürnberg, Germany) or vehicle (Chremophor, Sigma-Aldrich, München, Germany) for 1 h and 4 h, respectively. At end point, rats were sacrificed and the kidneys removed and decapsulated for biochemical analysis. For morphologic evaluation, anesthetized rats (ketamine/xylazine, 0.016 mg/g BW and 0.12 mg/g BW, Sigma-Aldrich) were perfusion-fixed retrogradely via the aorta abdominalis ([153]).

For long-term CsA studies, WT rats received a daily dose of CsA (30 mg/kg body weight) or vehicle (Chremophor) for 14 days by subcutaneous injection and animals were divided into groups (n=10 for physiological analysis and n=4 for morphology). For physiological analysis, animals were individually placed in metabolic cages and received normal food and tap water ad libitum. 24 h urine was collected to determine urine sodium and

creatinine concentrations as well as plasma renin activity. Mean arterial blood pressure measurement was performed applying the non-invasive tail-cuff method on anesthetized rats. For evaluation of NKCC2 activity, furosemide tests were performed by injecting one dose of furosemide into the peritoneum (40 mg/kg body weight, Sigma-Aldrich) and collecting urine 4 h following injection. Blood was collected via the tail vein during sacrifice prior to organ collection. Perfusion fixation was performed for morphologic evaluation.
To evaluate the effects of calcineurin inhibition in the absence of AVP, Brattleboro rats (n=6 per group) were injected with CsA (30 mg/kg body weight, i.p.) or vehicle (Chremophor), sacrificed 4 h after injection, and kidneys were removed. Brattleboro rats lack endogenous AVP due to a mutation in the AVP gene precursor which disturbs the ability to concentrate urine and causes central diabetes insipidus ([154]).
For characterization of protein and mRNA expression patterns along the nephron, wild-type (WT) mice with a Balb/c background were utilized. For morphologic analysis, mice were perfusion fixed and *in situ* hybridization and immunofluorescent labeling were performed on paraffin embedded kidney sections. For biochemical analysis, microdissected tubule segments from mouse kidneys were supplied by the group of Markus Bleich (Institute of Physiology, Kiel, Germany) and qPCR and immunoblotting was performed on lysates from tubule sections.
These experiments were approved by the Regional Office for Health and Social Affairs Berlin (LAGESO permission G0220/12).

For studies of the effects of CsA treatment in mice lacking the distal salt transporter NCC, the NCC knockout (KO) mouse model was applied. The NCC KO animals were generated by the group of David Ellison at the Oregon Health and Science University, Oregon, US. The knockout of the NCC gene is achieved by the disruption of exon 12 through insertion of a neo gene. NCC KO and WT mice can be distinguished by genotyping using a standard RT-PCR protocol ([155]). Experiments on this mouse model were performed at the facilities of David Ellison's group and were approved by the OHSU Institutional Animal Care and Usage Committee (Protocol IS03286).
For evaluation of long-term CsA effects in NCC KO mice, animals were divided into groups and injected daily with CsA (30 mg/kg body weight, i.p.) or vehicle (Chremophor) for 12 days. Blood pressure measurement (n=6 mice per group) was performed using the radio telemetry method. For morphologic evaluation, anesthetized mice were perfusion fixed (at least n=2) ([153]) and immunofluorescent labeling was performed on paraffin

embedded kidney sections. For biochemical analysis using immunoblotting, mice (n=5) were sacrificed at end point and the kidneys removed and decapsulated.

3.2 Perfusion Fixation and Tissue Embedding

The animals were killed by *in vivo* perfusion fixation under ketamine/xylazine anesthesia. The kidneys were perfused retrograde via the aorta abdominalis using PBS (phosphate buffered saline) with sucrose (330 mosmol, pH 7.4) for 30 s followed by infusion of 3 % paraformaldehyde in PBS (pH 7.4) for 5 min ([153]). Kidneys were removed and cut into halfs. Kidney pieces were processed for embedding in paraffin, Tissue Freezing Medium (Leica Biosystems, Nussloch, Germany)) or LR White medium (Electron Microscopy Sciences, München, Germany). For paraffin embedding, additional fixation was achieved by incubating kidney pieces in 4 % formalin (Thermotex, Berin, Germany) at 4°C overnight. The tissues were then stored in 330 mosmol sucrose + 0.02 % sodium azide and paraffin embedded at the Institute for Pathology (Charité University Medicine Berlin, CCM) or at the Histopathology Shared Resource of the Oregon Health and Science University, respectively. For cryo embedding, tissues were subsequently incubated in 800 mosmol sucrose and then shock-frozen in liquid nitrogen-cooled isopentane and Tissue Freezing Medium for subsequent cryostat sectioning. For electron microscopy, tissues were stored in 3°C paraformaldehyde plus 0.05 % glutaraldehyde at 4°C followed by embedding in LR white resin for subsequent preparation of ultrathin sections.

3.3 Cell Culture

All cells were cultivated in 75 cm^2 cell culture flasks at 95 % humidity, and 5 % CO_2. Rat medullary thick ascending limb cells (raTAL) ([156]) were cultured in renal epithelial growth medium (Promo Cell, Heidelberg, Germany) with 1 % penicillin/streptomycin. Mouse distal convoluted tubule cells (mDCT) ([157]) were cultured in RPMI (Roswell Park Memorial Institute) medium (Biochrom, Berlin, Germany) with 10 % fetal calf serum and 1 % penicillin/streptavidin at 37°C. Cells were grown to confluent monolayers, stimulated with 1 μM CsA (Santa Cruz Biotechnology, Heidelberg, Germany), 10 μM desmopressin (DDAVP, Sigma-Aldrich), or both agents simultaneously in culture medium for 4 h and

then harvested in Igepal lysis buffer (Sigma-Aldrich). Whole cell lysates were prepared for immunoblotting.

3.4 Antibodies

All antibodies used in this work were validated in previous studies by either preabsorption tests or by using respective knockout control samples. Specific primary and secondary antibodies used for immunoblotting, immunofluorescence and *in situ* hybridization are listed in table A.1 and A.2.

3.5 Immunofluorescence

For immunofluorescence analysis, paraffin sections (4 μm) were dewaxed with xylene and rehydrated through an ethanol series (100 % - 70 %) followed by boiling for 6 min in citrate buffer (0.02 M citric acid, 0.09 M sodium citrate; pH 6) for antigen retrieval. Cryo- (7 μm) sections were incubated in 0.5 % (v/v) Triton X-100 (Sigma-Aldrich) in PBS for antigen retrieval. All tissues sections were washed in PBS prior to incubation with primary antibodies diluted in blocking medium (1 h, overnight). Multiple stainings were separated by washing steps and fluorescent Cy2-, Cy3- or Cy5-conjugated antibodies were applied for detection. Evaluation of sections was performed using a Zeiss confocal microscope (LSM 5 Exciter) and signals were analysed with respect to localization and intensity. Kidney sections were double-labeled with antibodies against NKCC2 or NCC, respectively, in order to identify TAL and DCT. For evaluation of signal intensities of pNKCC2, pNCC and pSPAK/OSR1, double-labeling was performed applying antibodies against the total protein. Micrographs were obtained using ZEN2008 software (Zeiss, Jena, Germany) and ImageJ software ([158]) was applied to analyze signal intensities in individual tubular profiles. Mean signal values of phospho-signals within 2 μm distance to the apical membrane were normalized to respective non-phospho signals. In cases where the respective non-phospho antibody was not available, signals were normalized to background signals. For evaluation of pWNK1, signal intensities were normalized by colocalized NCC signal in the DCT. Analysis of two to six animals per group with at least 20 similar tubular profiles per individual were performed in a blind fashion. For COX-2

signal quantification at the macula densa and adjacent TAL portions, cells histochemically positive for COX-2 were counted.

3.6 Immunoblotting

Whole kidneys and microdissected nephron segments were homogenized in buffer containing 250 mM sucrose, 10 mM triethanolamine and protease inhibitors (Complete, Roche Diagnostics, Berlin, Germany), sonicated and centrifuged (1000 xg for 10 min) to remove nuclei. The same protocol was used for protein extraction of cell lysates. Proteins were electrophoretically separated using 10 % polyacrylamide minigels and then transferred onto nitrocellulose membranes (Macherey-Nagel, Düren, Germany). Membranes were blocked with respective blocking buffer for 30 min and subsequently incubated with primary antibody (see A.1) for 1 h at room temprature or overnight at 4°C. HRP-conjugated secondary antibodies (see A.2) were applied for detection following three washing steps between antibodies. Membranes were then incubated with chemiluminescent reagent (ECL Western Blotting Detection Reagents, Amersham, UK) and signals were detected using the ChemoCam Imager ECL (Intas, Göttingen, Germany). Densitometric evaluation was performed using ImageJ software ([158]).

3.7 Quantitative PCR

RNA from cell and tissue samples was extracted using the RNA extraction kit (Stratec biomedical, Birkenfeld, Germany). Reverse transcription into cDNA was performed in a two-step reaction. For denaturation of RNA and hybridisation of Oligo(dT)18 primers (Bioline GmbH, Luckenwalde, Germany), samples were first incubated 10 min at 70°C. Next, Tetro Reverse Transcriptase (Promega, Mannheim, Germany), 10 mM dNTPs (Bioline GmbH) and 10 U Ribolock RNase Inhibitor were added and incubated 2 h at 37°C for cDNA synthesis. Quantitative PCR was performed using HOT FIREPol EvaGreen qPCR Mix (Solis BioDyne, Tallinn, Estonia) using primers specific for target genes (see table A.3). Experiments were run for 40 cycles and subsequent melting curve generation in a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany).

The $\Delta\Delta$Ct method was used for gene expression analysis and expression was normalized against β-actin or against whole kidney control samples for tubule segments.

3.8 In Situ Hybridization

For evaluation of renin mRNA as well as for localization of CypA and CypB, *in situ* hybridzation was performed on perfusion-fixed paraffin embedded mouse and rat kidney sections. Digoxygenin (DIG)-labeled (Sigma-Aldrich) antisense RNA probes were generated via transcription from full length cDNA clones. Sections were dewaxed in xylene and ethanol, digested with proteinase K (Roche) for membrane permeabilization and hybridized with the respective probes at room temperature overnight. Anti-DIG-alkaline phosphatase-conjugated antibody (Dako) was applied for detection of hybridized probes and visualization was performed by incubating the sections with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche). Sections were analyzed with a Leica DMRB microscope (Leitz).

3.9 Ultrastructural Analysis

For analysis of NKCC2 and NCC distribution, transmission electron microscopy was performed on perfusion-fixed ultrathin LR white medium kidney sections. Primary antibodies against NKCC2 and NCC and 10 nm nanogold-labeled secondary antibodies (Amersham) were applied for detection. Visualization was performed using transmission electron microscopy (TECNAI G-2, Thermo Fisher Scientific, Berlin, Germany). Immunogold signals of NKCC2 in TAL and NCC in DCT, respectively, were quantified on at least 10 profiles and four cells per profile per individual animal. Signals within a 20 nm distance to the plasma membrane were defined as membrane-bound, whereas signals detected above 20 nm until the nuclear envelope were defined as cytoplasmic.

3.10 Genotyping

Identification of the genotype of wildtype and NCC knockout mice was achieved applying a standard genotyping protocol using tissue from tail biopsies. DNA was amplified in

a PCR reaction with one forward (5-AGGGTCAAGGGCACGGTTGGC-3) and two reverse primers (1: 5-GGTAAAGGGAGCGGGTCCGAGG-3; 2: 5-GCATGCTCCAGACTGCCTTG-3) creating PCR products of different size depending on the genotype. The forward primer and the reverse primer 1 correspond to the intron sequences flanking exon 12 which is disrupted in NCC KO mice through insertion of the neo gene. In wildtype mice, these primers will amplify a 265 base pair product. The reverse primer 2 is complementary to sequences within the neo gene. Thus, in NCC KO, the forward primer and the reverse primer 2 amplify a 188 base pair product.

3.11 Blood Pressure Measurements

Evaluation of mean arterial blood pressure in rats receiving long-term CsA treatment was performed at the Max-Delbrück-Center for Molecular Medicine (MDC Berlin, Germany) applying the non-invasive tail-cuff method on anesthetized rats.
Evaluation of blood pressure in NCC KO mice was performed using the radio telemetry method, the gold standard for the monitoring of arterial pressure in conscious mice. The technique involves a surgical procedure under anesthesia where a thin, flexible catheter is inserted from the left carotid artery into the aortic arch and the telemetry probe is implanted under the skin ([159]). Data were recorded by the implanted telemetry device at 10 min intervals and 24 h mean values were plotted.

3.12 Statistics

Samples from microdissected tubule sections were considered as independent and unrelated groups. Quantitative PCR data from these samples were determined applying one-way ANOVA with Tukey's HSD post hoc test in order to identify the sample-to-sample differences. For statistical evaluation of the effects of different treatments in mice, rats and cell culture, parametric Student's t-test or non-parametric Mann-Whitney-test was applied to test for significant differences between groups. Two-way ANOVA with Bonferroni correction was used for physiological data received from metabolic cage experiments and for evaluation of different treatments in wildtype and NCC KO mice. P-values of $P < 0.05$ were considered significant. All data are expressed as means ± SEM.

Chapter 4

Results – Part I: Key Factors Involved in Local Calcineurin Inhibition

CNI therapy is accompanied by hypertension and salt retention mediated, among other factors, by activation of the renal sodium chloride cotransporters. CsA and tacrolimus herein display disparate effects with respect to the activation of NKCC2 and NCC. We hypothesized that this is due to the differential expression of key factors involved in calcineurin inhibition. Thus, in this part of the study, localization of the two pivotal calcineurin isoforms CnAα and CnAβ, and the immunophilins CypA, CypB and Fkbp12 was analyzed along the nephron applying qPCR, immunoblotting and *in situ* hybridization. Since the recently identified adapter and ubiquitylation proteins KLHL3 and CUL3 might play a role in hypertension, we further hypothesized that their expression pattern may have differential effects on NKCC2 and NCC function in calcineurin inhibition conditions. Therefore, analysis of KLHL3 and CUL3 localization was performed applying the same protocols. Characterization of the expression pattern of these factors provides valuable insights into the molecular mechanisms underlying calcineurin inhibition on a local level.

4.1 Localization of Calcineurin Isoforms

Limited information is available about the renal expression of calcineurin. There is evidence that CnAα, the predominant isoform in the kidney, is the major isoform in the cortex while CnAβ is primarily expressed in the medullary TAL ([160], [120], [15]). Our group was recently able to confirm CnAβ expression in the TAL ([68]). To provide a clear picture of calcineurin expression in the nephron, quantitative PCR and immunoblotting were performed on microdissected mouse nephron segments. PCR analysis revealed ubiquitous mRNA expression of both CnAα and CnAβ isoforms with highest expression levels in the two distal tubule segments TAL and DCT, but no significant differences between the segments (Figure 4.1 A). Immunoblotting confirmed the presence of both isoforms in all nephron segments including the glomerulus (Figure 4.1 B).

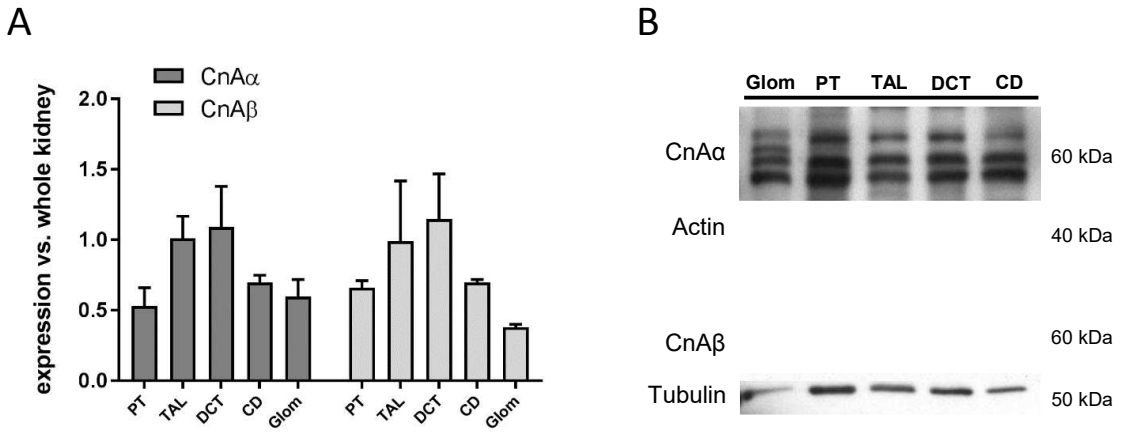

Figure 4.1. Calcineurin Isoforms Are Expressed Ubiquitously along the Nephron. (**A**) Quantitative PCR analysis of calcineurin isoforms CnAα and CnAβ on lysates of microdissected tubule sections. Expression was normalized to whole kidney. Data are the means ± SEM. (**B**) Representative immunoblots of lysates from microdissected tubule sections showing immunoreactive signals for CnAα and CnAβ, both at approximately 60 kDa; β-actin (approximately 40 kDa) and tubulin (approximately 50 kDa) served as loading control. PT: proximal tubule, TAL: thick ascending limb, DCT: distal convoluted tubule, CD: collecting duct, Glom: glomerulus.

These results disprove the hypothesis that the distinct effects of local calcineurin inhibition are based on differential expression of the calcineurin isoforms in the distal nephron. Nevertheless, differences in post-translational regulation cannot be excluded and substrate specificity of the two isoforms may play a pivotal role. However, differences in the regulation of the sodium chloride cotransporters in the TAL and DCT by the calcineurin phosphatase do not seem to be based on the expression pattern of CnAα and CnAβ.

4.2 Localization of Immunophilins

Next, we hypothesized that the immunophilins, the cytosolic receptors of CsA and tacrolimus, are differentially expressed along the nephron since the TAL and the DCT differ in their sensitivity to CsA and tacrolimus. To shed light on this question, our group has recently investigated the renal expression of CypA, CypB and Fkbp12 by performing immunoblots on lysates from microdissected nephron segments, and detected each of the three immunophilins in all segments ([68]). To confirm this observation on the mRNA level, additional quantitative PCR on lysates from the isolated nephron segments was performed and confirmed the ubiquitous expression along the entire nephron (Figure 4.2). The CsA binding proteins CypA and CypB appeared with highest expression in the distal tubule segments TAL and DCT; however, differences were not statistically significant. Overall expression of the tacrolimus binding protein Fkbp12 was quite low showing similar levels within the different segments and no significant changes in TAL and DCT (Figure 4.2).

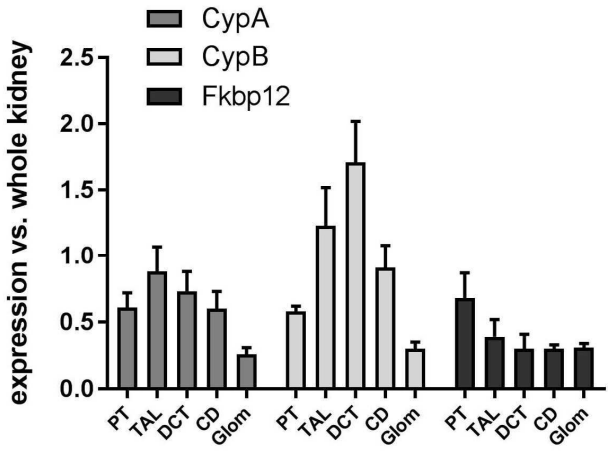

Figure 4.2. Immunophilins Are Ubiquitously Expressed along the Nephron. Quantitative PCR analysis of immunophilins CypA, CypB and Fkbp12 on lysates of microdissected tubule sections. Expression was normalized to whole kidney. Data are the means ± SEM. PT: proximal tubule, TAL: thick ascending limb, DCT: distal convoluted tubule, CD: collecting duct, Glom: glomerulus.

In situ hybridization on perfusion-fixed mouse and rat kidney sections was further performed to verify mRNA expression of the immunophilins in the distal nephron. Strong

signal intensities were detected for both CypA and CypB in the TAL and DCT, confirming the high expression of cyclophilins in the distal tubule segments (Figure 4.3 A and B). Fkbp12 expression could not be evaluated by *in situ* hybridization for technical reasons.

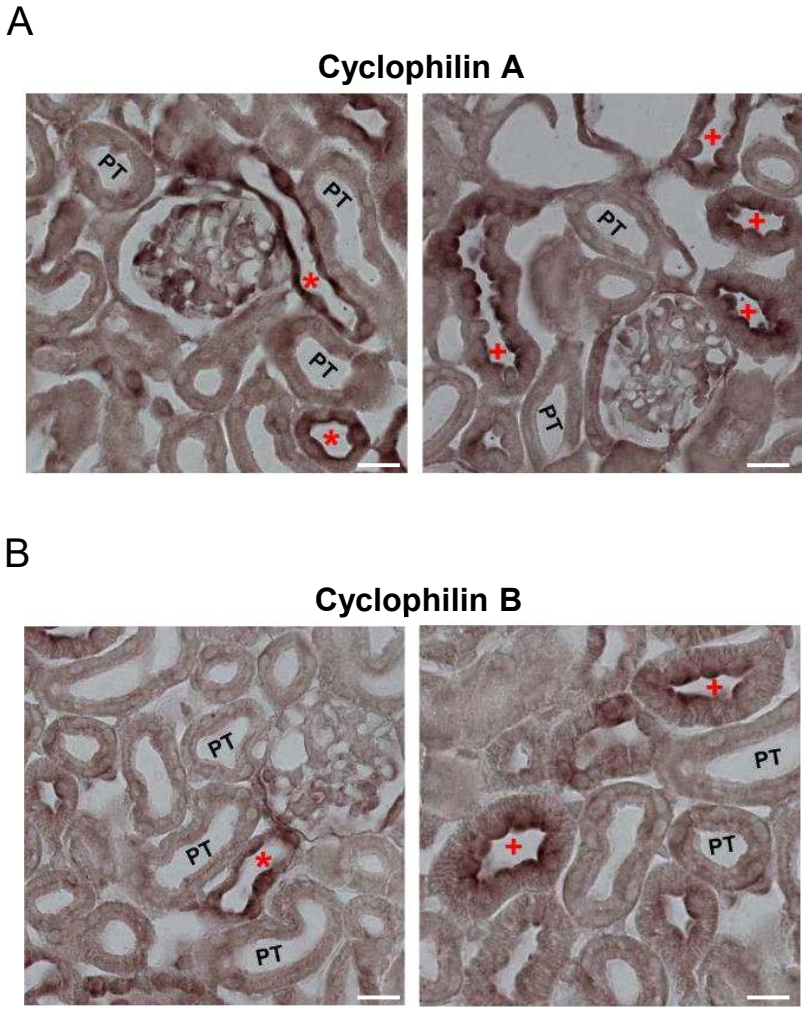

Figure 4.3. CsA Binding Proteins Cyclophilin A and Cyclophilin B Are Highly Expressed in the Distal Nephron. (**B**) Representative images of rat kidney sections showing mRNA signal of (**A**) cyclophilin A and (**B**) cyclophilin B detected by *in situ* hybridization. Strong signal intensities were found in the thick ascending limb (TAL, *) and the distal convoluted tubule (DCT, +), weaker expression was detected in the proximal tubule (PT) and other segments confirming qPCR analysis. Bars indicate 20 μm.

The detection of the three immunophilins CypA, CypB and Fkbp12 in all nephron segments does not explain the different sensitivity to calcineurin inhibitors in the distal tubule. Thus, the distinct effects of CsA and tacrolimus have to be mediated by other mechanisms.

4.3 Localization of KLHL3 and CUL3

The next step was to clarify the renal expression of the two recently identified WNK regulators, CUL3 and KLHL3, which are key factors in the ubiquitylation process of WNKs. Mutations in the genes encoding these proteins cause familial hyperkalemic hypertension (FHHt) and lead to accumulation of WNK protein which in turn upregulates NCC activity. Since only in the DCT signaling pathways seem to be affected by this, we asked whether CUL3 and KLHL3 were differentially expressed along the nephron. Quantitative PCR and immunofluorescent staining was performed on isolated nephron segments to characterize the expression pattern on the mRNA and the protein level, respectively. qPCR revealed KLHL3 mRNA expression in all nephron segments with highest expression levels in the DCT. CUL3 mRNA was detected in all nephron segments with no statistical significance between segments (Figure 4.4).

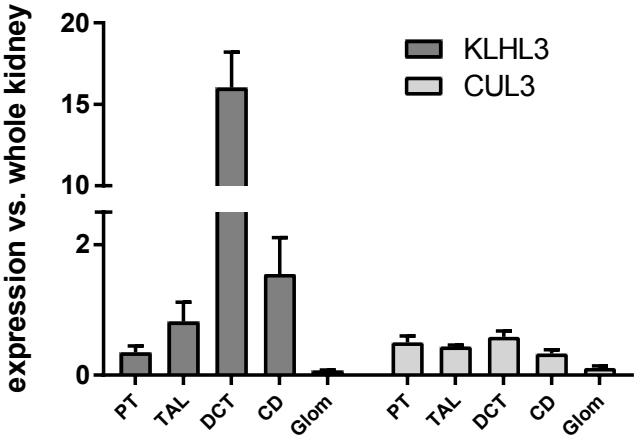

Figure 4.4. WNK Regulators KLHL3 and CUL3 Are Ubiquitously Expressed along the Nephron. Quantitative PCR analysis of WNK regulators KLHL3 and CUL3 on lysates of microdissected tubule sections. Expression was normalized to whole kidney. Strongest KLHL3 expression was detected in the distal nephron and collecting duct with highest levels in the DCT. CUL3 mRNA was relatively low in all segments. Data are the means ± SEM. PT: proximal tubule, TAL: thick ascending limb, DCT: distal convoluted tubule, CD: collecting duct, Glom: glomerulus.

Immunofluorescence staining revealed that medullary TAL and DCT are enriched with KLHL3 protein. Low to intermediate expression was found in the other tubule sections with the thin descending limb lacking any expression. Immunofluorescence further confirmed ubiquitous expression of CUL3, however, with lower signal intensities in the distal nephron. Additionally, intercalated cells of the collecting duct displayed high expression

levels of KLHL3 (Figure 4.5). These results illustrate the vital role of the WNK regulators KLHL3 and CUL3 in the distal nephron; however, they do not explain the differential actions of CNI in this area.

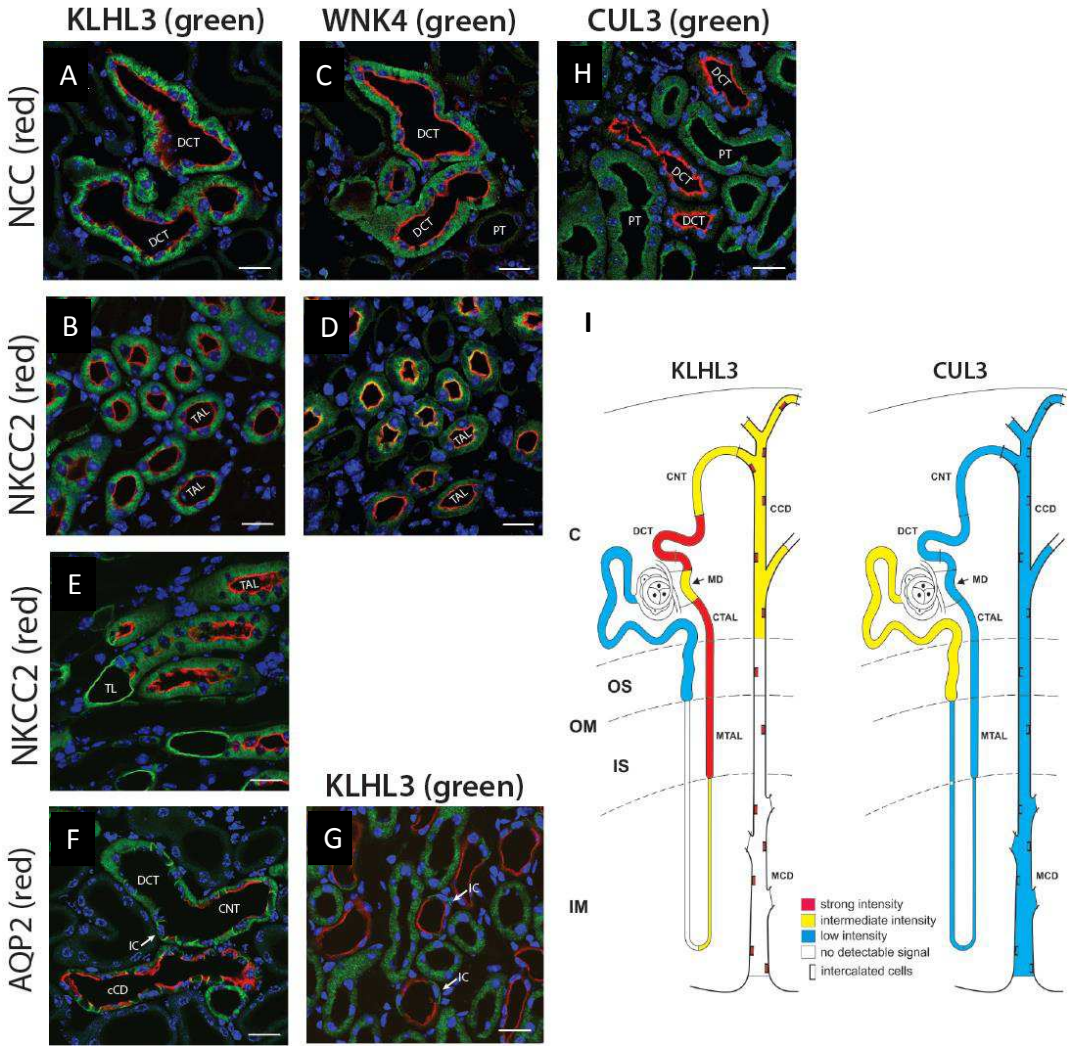

Figure 4.5. Protein Expression of KLHL3 and CUL3 along the Nephron. Immunofluorescence of KLHL3 and CUL3 on paraffin embedded kidney sections as evaluated by confocal microscopy. (**A, B**) KLHL3 in DCT segments (identified by NCC) and TAL (identified by NKCC2). (**C, D**) Colocalization with WNK4 in DCT and TAL (butterfly sections of **A** and **B**). (**E**) KLHL3 in the medulla with robust expression along the thin limb (TL) and TAL. (**F**) KLHL3 in the cortex at the junction of a DCT, CNT, and cortical collecting duct (cCD) with enriched expression in intercalated cells (IC). CNT and cCD were identified by aquaporin 2 (AQP2). (**G**) KLHL3 in the medullary collecting duct (mCD) where it is expressed in intercalated, but not principal cells. (**H**) CUL3 was detected most highly in proximal tubules (PT) and expression in DCT was present but low. (**I**) Schematic expression sites of KLHL3 and CUL3. OM: outer medulla; OS: outer stripe, IS: inner stripe; IM: inner medulla; C: cortex; MD: macula densa; intercalated cells indicated by boxes. Bars indicate 20 μm ([161]).

Chapter 5

Results – Part II: Local and Systemic Effects of Calcineurin Inhibition

Since we showed that some major regulators of NKCC2/NCC activation display a similar expression pattern in the TAL and the DCT, we supposed that other mechanisms must be the key to the different CNI-sensitivities of the distal nephron segments. Various studies suggest that, besides the effects of local calcineurin inhibition, CsA-induced hypertension relies on additional systemic effects. To differentiate between local and systemic effects of calcineurin inhibition in the regulation of the distal salt transporters and blood pressure, in this part of the study acute and chronic effects of CsA treatment were investigated *in vivo* and *in vitro*. Additionally, the role of vasopressin was examined in this context. The characterization of the different effects of CsA treatment significantly contributes to a better understanding of the mechanisms of action of CNI.

5.1 CsA Treatment Activates NKCC2 and NCC on the Post-Translational Level

To assess acute and chronic effects of CsA treatment, Wistar rats were treated for three time periods. 1 h and 4 h were selected as short-term periods whereas 14 d was defined

as long-term treatment. 1 h treatment served to evaluate fast changes on the post-translational level such as phosphorylation and trafficking. 4 h treatment was sought to assess potential early changes in mRNA expression and protein abundance. The 14 d treatment period served for the evaluation of changes in electrolyte handling, blood pressure and endocrine mechanisms.

5.1.1 Acute CsA Treatment Induces NKCC2 and NCC Activation *in vivo*

First, Wistar rats were treated with CsA or vehicle (Veh) for 1 h and 4 h, respectively. Protein abundance and phosphorylation levels of NKCC2 and NCC were evaluated by immunoblotting. In order to analyze activation of the transporters via the SPAK cascade, the T96/T101 phosphorylation site of NKCC2 and the S71 phosphorylation site of NCC were detected, respectively, using specific phospho-antibodies (see section 1.3.3). Phosphorylation levels of NKCC2 and NCC were increased after both 1 h (+131 % for pNKCC2 and +190 % for pNCC, $P < 0.05$, Figure 5.1 A, C) and 4 h (+197 % for pNKCC2 and +410 % for pNCC, $P < 0.05$, Figure 5.1 B, D) post CsA administration compared to the vehicle control groups. Total protein abundance was unchanged after both time points.

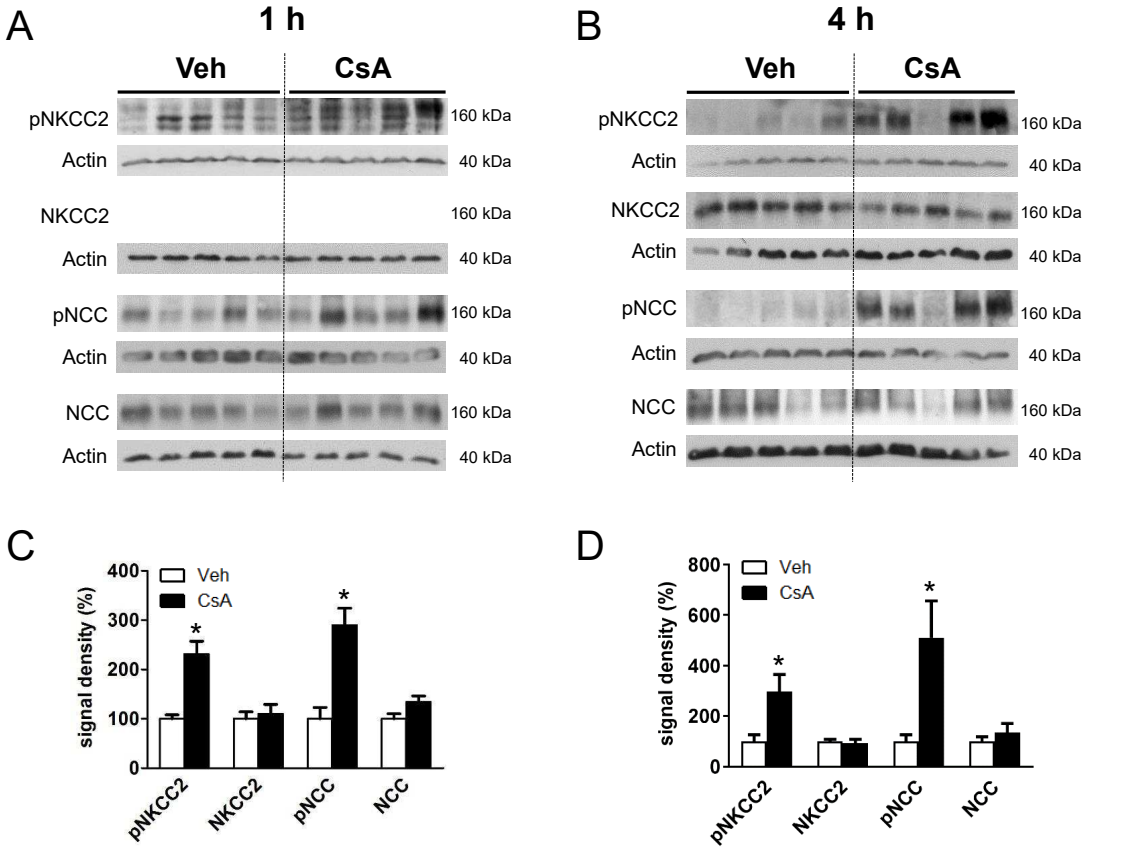

Figure 5.1. Short-Term CsA Treatment Stimulates Phosphorylation of NKCC2 and NCC. Representative immunoblots of kidney lysates from rats treated with cyclosporine A (CsA) for (**A**) 1 h and (**B**) 4 h, showing immunoreactive signals for total NKCC2, phosphorylated NKCC2 (pNKCC2), total NCC, and phosphorylated NCC (pNCC), all at approximately 160 kDa; β-actin served as loading control (approximately 40 kDa). (**C, D**) Graphs showing respective densitometric evaluation of immunoreactive signals normalized to loading controls. Levels of pNKCC2 and pNCC were increased after short-term cyclosporine A (CsA) treatment compared to the vehicle (Veh) control group, but no change in their total protein abundance was detected. Data are the means ± SEM; $^*P < 0.05$ ([162]).

Trafficking of NKCC2 and NCC was assessed by applying immunogold electron microscopy on ultrathin kidney sections. After 1 h of CsA treatment surface expression of the transporters was unchanged in both TAL and DCT. After 4 h of CsA treatment a moderate increase of NKCC2 and NCC signal in the plasma membrane was detected within the respective nephron segment compared to the control group (+20 % for NKCC2 and +13 % for NCC, $P < 0.05$, Figure 5.2). These data indicate that acutely administered CsA activates NKCC2 and NCC function by increasing their phosphorylation levels and stimulating their trafficking.

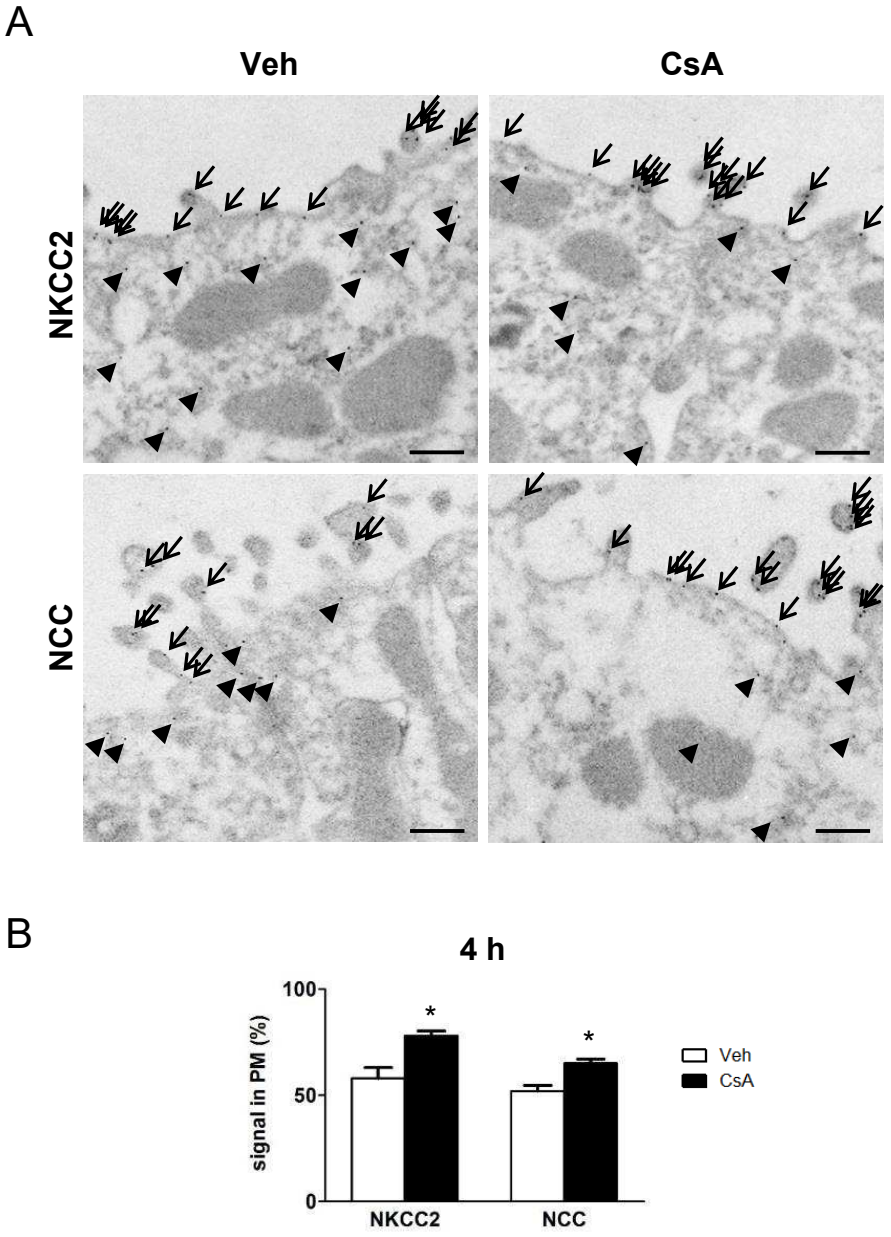

Figure 5.2. Short-term CsA Treatment Stimulates Trafficking of NKCC2 and NCC. (**A**) Representative immunoelectron microscopic images showing cellular distribution of NKCC2 and NCC in the plasma membrane (arrows) and the cytoplasm (arrowheads) in kidneys from vehicle-treated (Veh) and cyclosporine A (CsA)-treated rats; 5 nm gold grain labeling. (**B**) Numerical quantification of NKCC2 and NCC signals in plasma membrane (PM) per respective total cellular signals. 4 h CsA increased the surface expression of NKCC2 and NCC compared to the Veh control group. Plots showing the means ± SEM; $^*P < 0.05$. Bars indicate 1 μm ([162]).

5.1.2 Acute CsA Treatment Activates the WNK-SPAK/OSR1 Cascade *in vivo*

To assess the phosphorylation status of SPAK and OSR1, the kinases responsible for NKCC2 and NCC function, we next used confocal microscopy on paraffin embedded kidney sections from rats treated with CsA or vehicle. Activation of SPAK/OSR1 after short-term (1 h and 4 h) CsA treatment was analyzed by evaluating apical abundance of their phosphorylated species in the TAL and the DCT. Due to the high homology of the two kinases, phospho-antibodies detect the phospho-sites of both SPAK and OSR1. Signals of their phosphorylated regulatory domain (pS-SPAK/OSR1) were normalized to colocalized SPAK signals.

Interestingly, in the TAL short-term CsA treatment did not induce significant changes of pSPAK/OSR1 levels, albeit a trend could be detected (Figure 5.3 A, C). In contrast, in the DCT substantially increased apical signals were detected upon short-term CsA administration (Figure 5.3 B, C). This was true for both time points (+49 % after 1 h CsA and +94 % after 4 h CsA, $P < 0.05$; (Figure 5.3 C, D)).

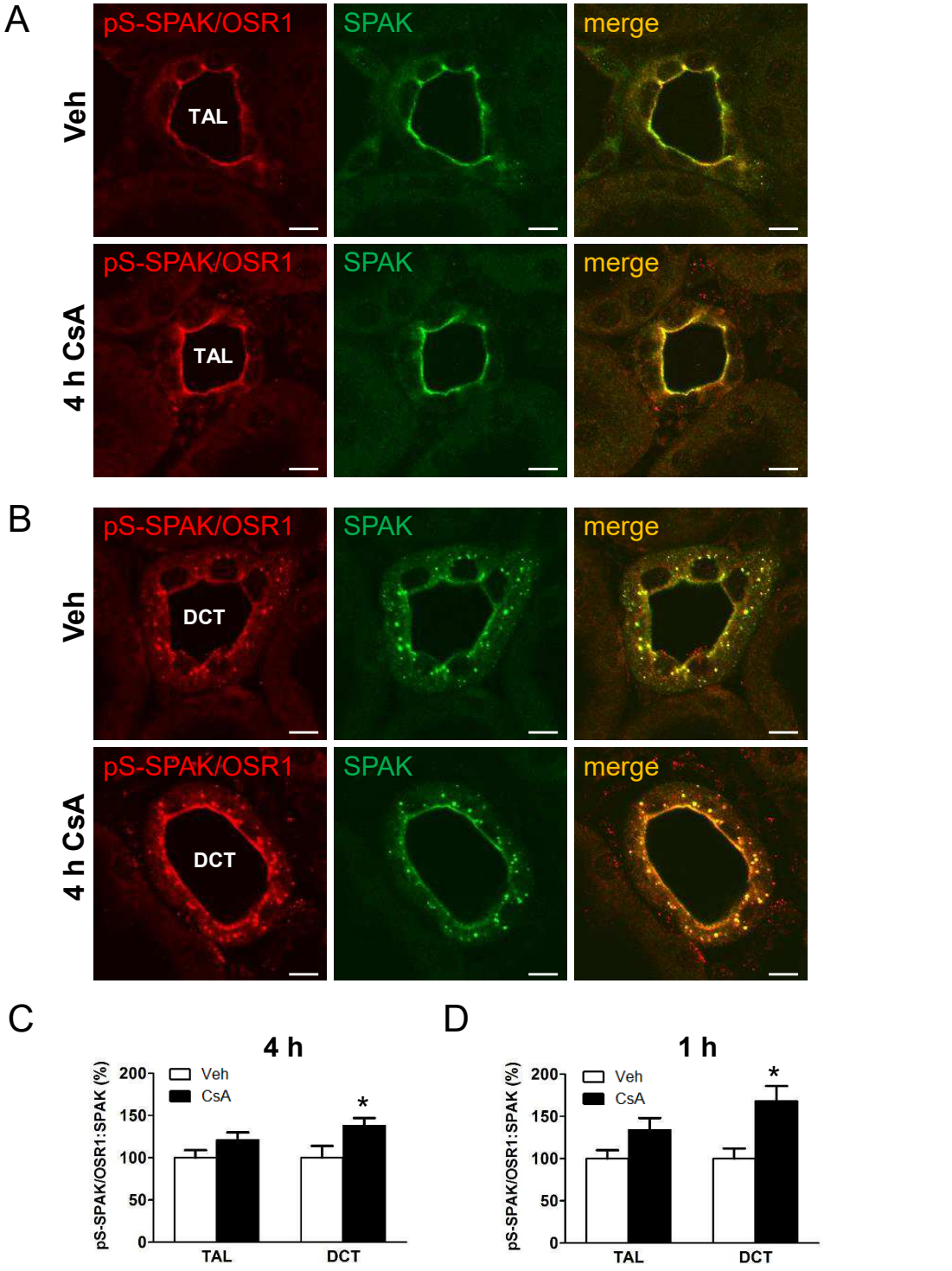

Figure 5.3. Increased Phosphorylation Levels of pS-SPAK/OSR1 after Short-Term CsA Treatment in the Distal Convoluted Tubule (**A, B**) Representative images of kidney sections from vehicle- (Veh) or cyclosporine A (CsA)-treated rats (4 h) showing immunofluorescent labeling of phosphorylated regulatory SPAK/OSR1 domain (pS-SPAK/OSR1) and double-labeling for SPAK in (**A**) the thick ascending limb (TAL) and in (**B**) the distal convoluted tubule (DCT). Identification of TAL and DCT was accomplished according to morphological criteria and specific SPAK signal patterns (predominant apical signal in TAL vs. apical and punctate cytoplasmic signal in DCT). (**C, D**) Graphs showing pS-SPAK/OSR1:SPAK signal ratio in TAL and DCT of rats treated with Veh or CsA for 4 h (**C**) and 1 h (**D**) as evaluated using ZEN and ImageJ software; respective immunofluorescent images for 1 h are not shown. Short-term CsA treatment increased the abundance of pS-SPAK/OSR1 in the DCT, but not in the TAL. Data are the means ± SEM; $^*P < 0.05$. Bars indicate 10 μm ([162]).

Immunoblots revealed that total protein abundance of SPAK and OSR1 were not affected by acute CsA administration as compared to the vehicle treated control group (Figure 5.4).

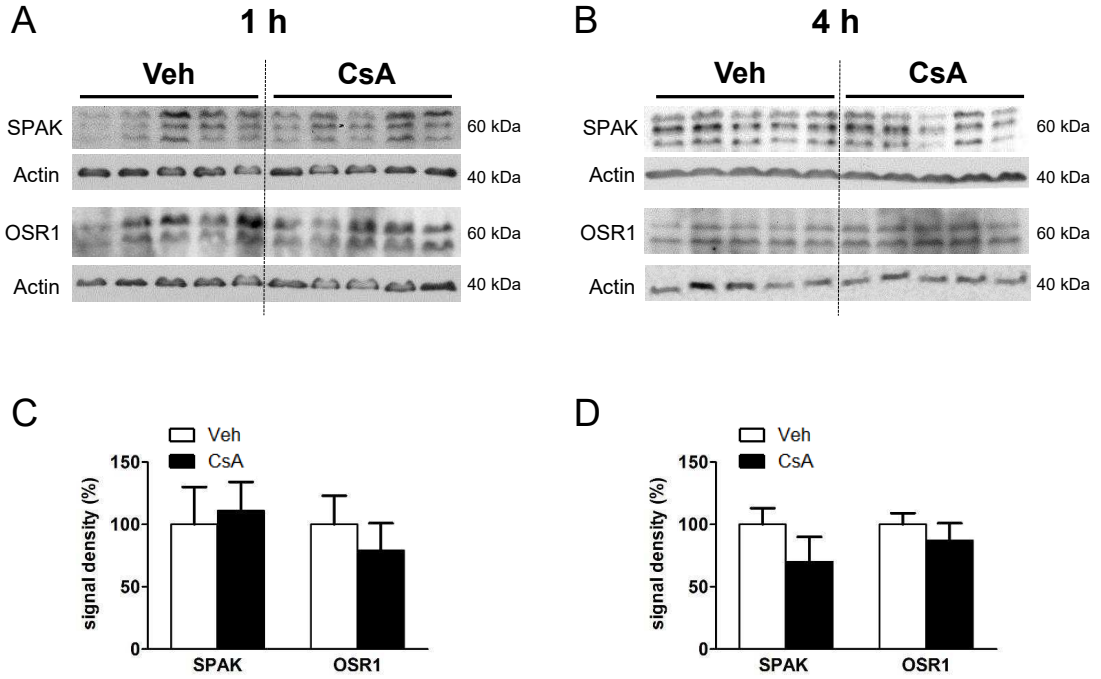

Figure 5.4. Short-Term CsA Treatment Has no Effect on Total SPAK and OSR1 Protein Abundance. Representative immunoblots of kidney lysates from rats treated with cyclosporine A (CsA) for (**A**) 1 h and (**B**) 4 h, showing immunoreactive signals for SPAK and OSR1, both at approximately 60 kDa; β-actin served as loading control (approximately 40 kDa). (**C, D**) Graphs showing respective densitometric evaluation of immunoreactive signals normalized to loading controls. Levels of SPAK and OSR1 were unchanged after short-term CsA treatment compared to the Veh control group. Data are the means ± SEM; $^*P < 0.05$ ([162]).

Since WNK kinases are the known upstream regulators of SPAK/OSR1, we next assessed the phosphorylation level of WNK1, the predominant isoform expressed in the DCT ([163]). The S382 phosphorylation site of WNK1 is reported to be required for activation of the SPAK/OSR1 kinases (see section 1.3.3). The antibody used in this study detects the S382 phospho-site of both pWNK1 and pWNK4. Therefore, individual changes of WNK1 and WNK4 phosphorylation may not be detectable due to overlapping signals. pWNK1 signals were normalized to overlapping NCC signals. We could not detect any change in the phosphorylation level of WNK1 upon acute CsA administration as evaluated by confocal microscopy (Figure 5.5).

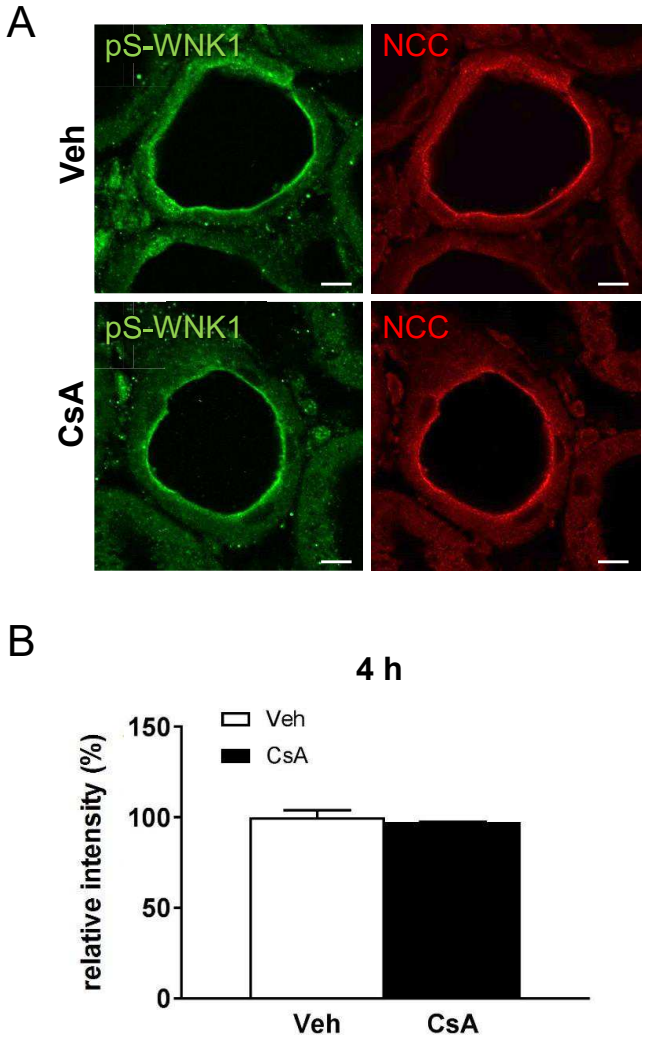

Figure 5.5. Phosphorylation Levels of pS-WNK1 after Short-Term CsA Treatment in the Distal Convoluted Tubule (**A**) Representative images of kidney sections from vehicle- (Veh) or cyclosporine A (CsA)-treated rats (4 h) showing immunofluorescent labeling of phosphorylated WNK1 domain (pS-WNK1) and double-labeling for NCC in the distal convoluted tubule. Lacking change of phosphorylation levels upon acute CsA treatment might be due to individual changes of pWNK1 and pWNK4 which cannot be detected with the antibody used here. (**B**) Graphs showing relative signal intensities of phosphorylated WNK signals normalized to colocalized NCC signals as evaluated using ZEN and ImageJ software. Data are the means Data are the means ± SEM; $^*P < 0.05$. Bars indicate 10 μm.

5.1.3 Acute CsA Treatment Does not Affect mRNA of the WNK-SPAK/OSR1 Cascade

Next, mRNA expression profiles were investigated applying qPCR. After 4 h of CsA treatment no changes were detected for WNKs, SPAK, OSR1, NKCC2, and NCC (Figure 5.6). These data provide further evidence that short-term calcineurin inhibition using CsA activates the WNK-SPAK/OSR1 cascade and their renal substrates, NKCC2 and NCC,

on the post-translational level by increasing their apical abundance and phosphorylation but not mRNA levels.

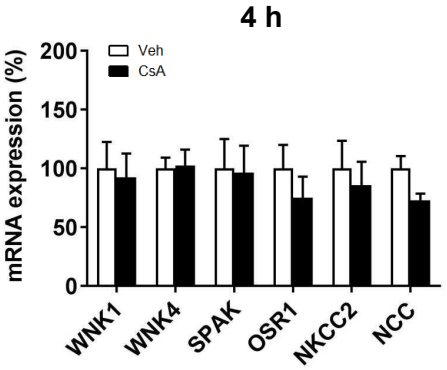

Figure 5.6. Short-Term CsA Treatment Has No Effect on mRNA Expression of the WNK-SPAK/OSR1-NKCC2/NCC Cascade Quantitative PCR analysis of WNK1, WNK4, SPAK, OSR1, NKCC2, NCC mRNA in kidney lysates from rats treated with vehicle (Veh) or cyclosporine A (CsA) for 4 h. All results were normalized to GAPDH expression. Data are the means ± SEM; $^*P < 0.05$ ([162]).

5.1.4 Chronic CsA Treatment Induces NKCC2 and NCC Activation *in vivo*

To evaluate long-term effects, Wistar rats were treated with CsA or vehicle for 14 days. Phosphorylation levels of NKCC2 and NCC were analyzed using confocal microscopy. NKCC2 and NCC labeling was used to identify TAL and DCT, respectively, and for normalization of phosphorylation signals to colocalized signals. This revealed a CsA-induced increase in the abundance of their phosphorylated species without concomitant changes in the total protein levels (pNKCC2: +46 %, pNCC: +19 %, $P < 0.05$, Figure 5.7).

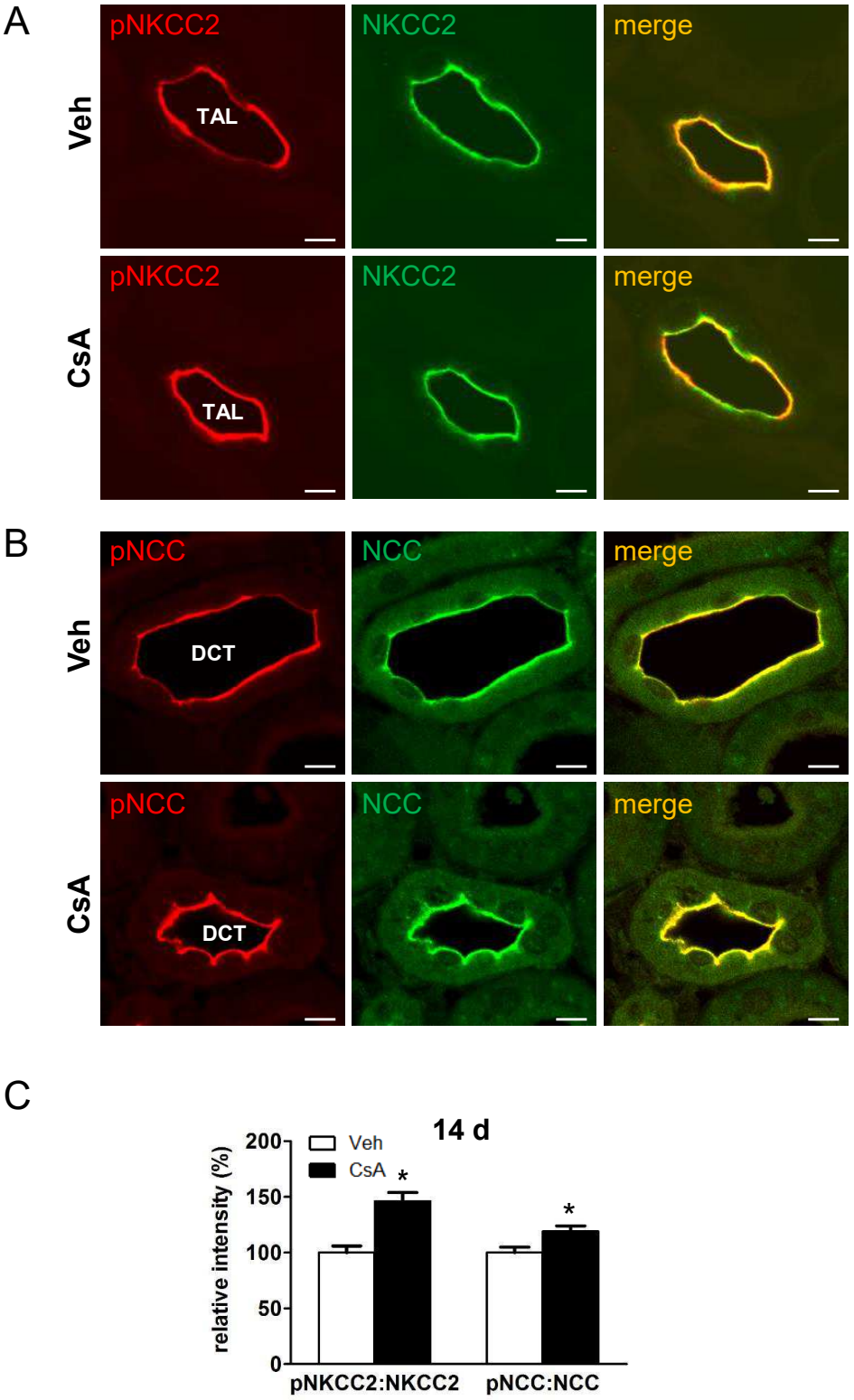

Figure 5.7. Increased Phosphorylation Levels of pNKCC2 and pNCC after Long-Term CsA Treatment (**A, B**) Representative images of kidney sections from vehicle- (Veh) or cyclosporine A (CsA)-treated rats (14 d) showing immunofluorescent labeling of (**A**) pNKCC2 and double-labeling for NKCC2 in the thick ascending limb TAL or of (**B**) pNCC and double-labeling for NCC in the distal convoluted tubule (DCT). (**C**) Graphs showing relative signal intensities of phosphorylated NKCC2 and phosphorylated NCC signals normalized to colocalized total NKCC2 or NCC signals as evaluated using ZEN and ImageJ software. Data are the means ± SEM; $^*P < 0.05$. Bars indicate 10 μm ([162]).

5.1.5 Chronic CsA Treatment Activates SPAK/OSR1 *in vivo*

Activation of SPAK/OSR1 kinases was analyzed by evaluating apical abundance of their phosphorylated species using confocal microscopy. Signals were analyzed for the regulatory (pS-SPAK/OSR1) and the catalytic form (pT-SPAK/OSR1) and normalized to colocalized SPAK/OSR1 signals. Relative signal intensities displayed upregulation of the phosphorylated catalytic as well as regulatory SPAK phospho species; however, only in the DCT (pT-SPAK/OSR1: +123 %, pS-SPAK/OSR1: +36 %; $P < 0.05$, Figure 5.8). Total protein abundance remained unchanged.

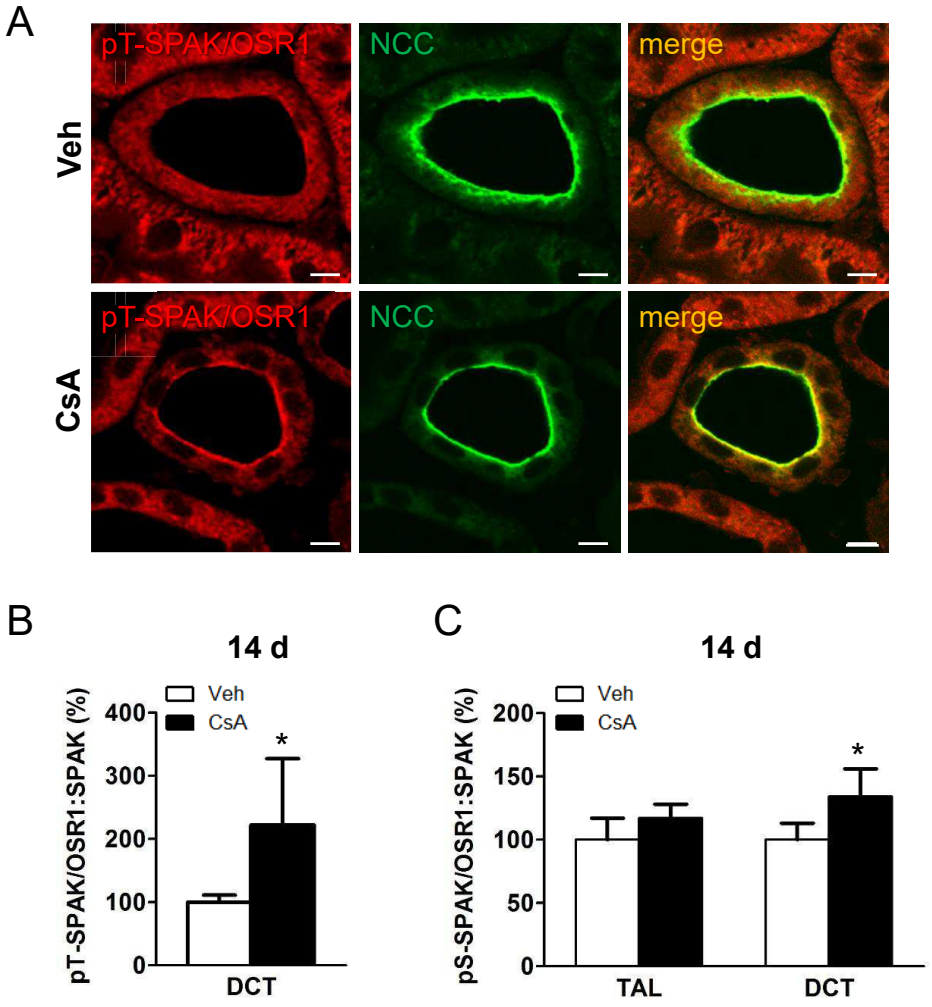

Figure 5.8. Increased Phosphorylation Levels of pSPAK/OSR1 after Long-Term CsA Treatment in the Distal Nephron (**A**) Representative images of kidney sections from vehicle- (Veh) or cyclosporine A (CsA)-treated rats (14 d) showing immunofluorescent labeling of phosphorylated catalytic SPAK/OSR1 domain (pT-SPAK/OSR1) and double-labeling for NCC in the distal convoluted tubule (DCT). (**B, C**) Graphs showing (**B**) pT-SPAK/OSR1:SPAK signal ratio in DCT of rats treated with Veh or CsA for 14 d and (**C**) pS-SPAK/OSR1:SPAK signal ratio in TAL and DCT as evaluated using ZEN and ImageJ software; respective immunofluorescent images for pS-SPAK/OSR1:SPAK are not shown. Long-term CsA treatment increased the abundance of catalytic pT-SPAK/OSR1 and regulatory pS-SPAK/OSR1 in the DCT, but not in the TAL. Data are the means ± SEM; $^*P < 0.05$. Bars indicate 10 μm ([162]).

5.1.6 Chronic CsA Treatment Does not Affect mRNA of the WNK-SPAK/OSR1 Cascade

Subsequently, qPCR was performed to assess whether CsA-induced activation of the transporters and their regulating kinases is a complete result of post-translational modification. Chronic CsA administration did not alter mRNA expression of WNKs, SPAK, NKCC2

and NCC; however, OSR1 expression was moderately increased (+24 %, $P < 0.05$; Figure 5.9). These data suggest that long-term CsA treatment, just like the short-term treatment, activates the distal salt transporters and their activating kinases chiefly by post-translational mechanisms.

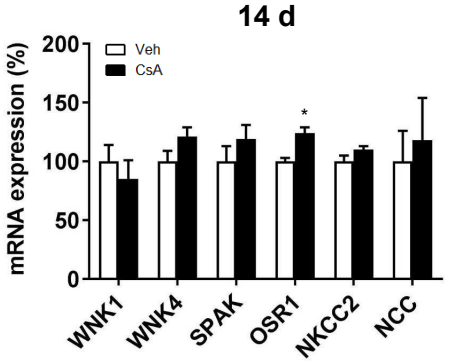

Figure 5.9. Long-Term CsA Treatment Has no Effect on mRNA Expression of WNK, SPAK, NKCC2 and NCC Quantitative PCR analysis of WNK1, WNK4, SPAK, OSR1, NKCC2, NCC mRNA in kidney lysates from rats treated with vehicle (Veh) or cyclosporine A (CsA) for 4 h. All results were normalized to GAPDH expression. Only OSR1 shows moderately increased expression upon CsA treatment. Data are the means ± SEM; $^*P < 0.05$ ([162]).

5.2 Effects of CsA on Endocrine and Paracrine Regulation of NKCC2 and NCC

Since calcineurin inhibition, particularly using CsA, reportedly modulates endocrine and paracrine pathways with effects on distal salt handling, we next analyzed the function of the juxtaglomerular apparatus upon CsA treatment. Local COX-2 and renin synthesis was evaluated by qPCR, immunofluorescence and *in situ* hybridization. On the mRNA level, COX-2, which is regulated by the calcineurin-NFAT pathway, ([145]) was expectedly suppressed by acute and chronic CsA treatment (4 h: −41 %, 14 d: −54 %, $P < 0.05$; Figure 5.10).

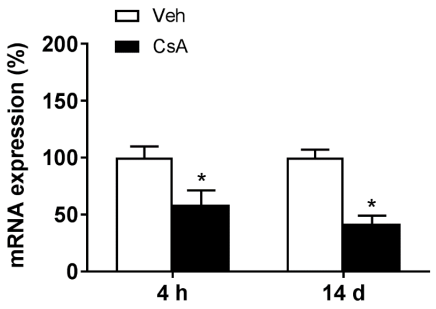

Figure 5.10. Short- and Long-Term CsA Treatment Downregulates COX-2 mRNA Expression Quantitative PCR analysis of COX-2 mRNA in kidney lysates from rats treated with vehicle (Veh) or cyclosporine A (CsA) for 4 h and 14 d. All results were normalized to GAPDH expression. Data are the means ± SEM; $^*P < 0.05$.

Immunofluorescence confirmed the suppressed juxtaglomerular COX-2 expression after chronic CsA administration (−93 %, $P < 0.05$; Figure 5.11 A, C). Furthermore, renin mRNA expression was upregulated upon long-term CsA treatment as shown by *in situ* hybridization on kidney sections (Figure 5.11 B, D).

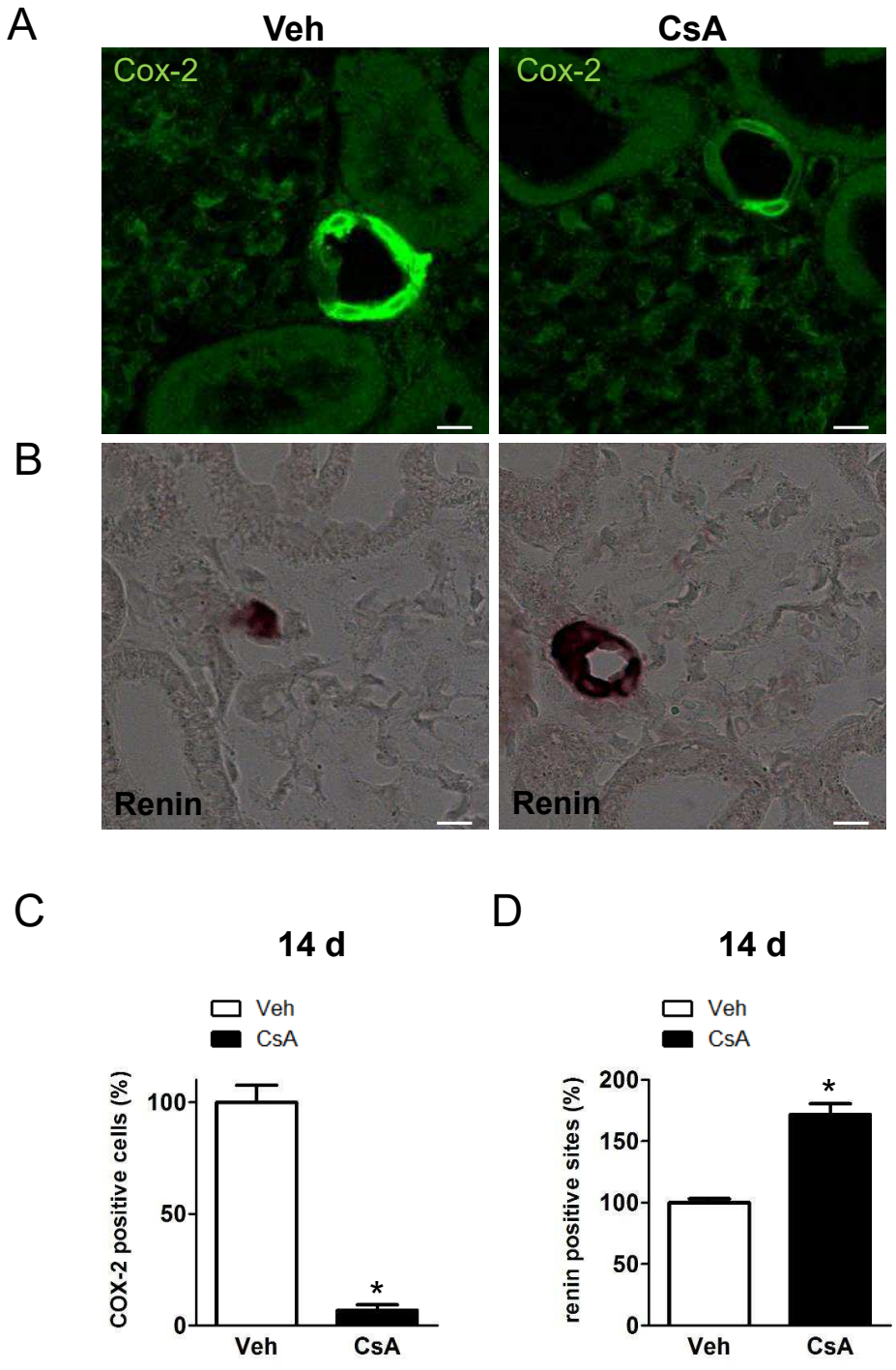

Figure 5.11. Regulation of Endocrine and Paracrine Factors by Long-Term CsA Treatment (**A**) Representative images of macula densa regions in kidney sections from vehicle- (Veh) or cyclosporine A (CsA)-treated rats (14 d) showing immunofluorescent labeling of juxtaglomerular COX-2 expression. (**B**) Representative images of afferent arterioles in kidney sections from Veh- and CsA-treated rats showing renin mRNA signal detected by *in situ* hybridization. (**C, D**) Graphs showing numerical quantification of (**C**) COX-2 positive cells and (**D**) renin positive sites normalized for respective glomeruli numbers. Data are the means ± SEM; $^*P < 0.05$. Bars indicate 10 μm ([162]).

Additionally, plasma renin activity was determined in chronically treated rats and was strongly increased after 14 d CsA (+966 %, $P < 0.05$; Figure 5.12).

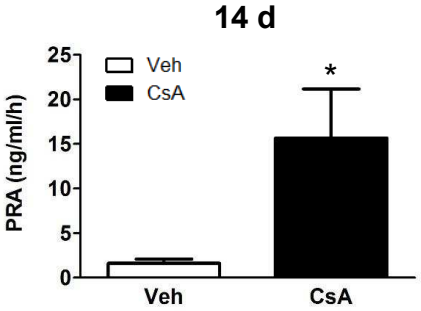

Figure 5.12. Long-Term CsA Stimualtes Plasma Renin Activity Plasma renin activity in vehicle- (Veh) and cyclosporine A- (CsA; 14 d) treated rats. Data are the means ± SEM; $^*P < 0.05$ ([162]).

These results suggest that CsA stimulates renal salt reabsorption via additional systemic mechanisms.

5.3 CsA-Induced Salt Retention and Hypertension

For evaluation of physiological changes induced by CsA treatment, rats were kept in metabolic cages and blood pressure was measured. Urinary salt excretion was markedly decreased on day 1 (−31 %, $P < 0.05$; Figure 5.13 A) and day 10 (−51 %, $P < 0.05$; Figure 5.13 A) in CsA treated animals compared with the vehicle-treated control group. Mean arterial blood pressure displayed a moderate but significant increase on day 7 of treatment compared to the control group (113 (Veh) vs. 121 (CsA) mmHg; $P < 0.05$; Figure 5.13 B).

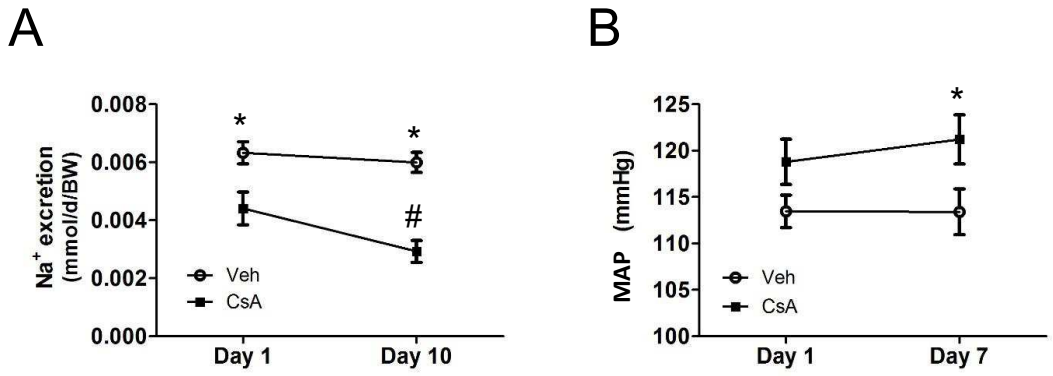

Figure 5.13. Regulation of Sodium Excretion and Mean Arterial Blood Pressure by Long-Term CsA Treatment (**A**) 24 h urine sodium excretion in vehicle- (Veh) and cyclosporine A- (CsA) treated rats on treatment days 1 and 10. Long-term CsA treatment induced marked decreases in urinary salt excretion. (**B**) Mean arterial blood pressure (MAP) in anesthetized Veh- and CsA-treated rats measured by non-invasive tail cuff measurement. Long-term CsA treatment induced a moderate but significant increase in MAP on day 7 of treatment compared to Veh group. Data are the means ± SEM; $^*P < 0.05$ for Veh vs. CsA, $\#P < 0.05$ for day 1 vs. day 10 ([162]).

Previous studies on CNI have reported that tacrolimus-induced salt retention is the chief result of NCC activation ([51], [15]). To demonstrate the respective contribution of NKCC2 in a CsA-induced salt retention and hypertension, we performed a furosemide test. CsA-receiving animals displayed a stronger furosemide-induced salt loss than the vehicle treated group, indicating that NKCC2 function is indeed increased upon CsA treatment (+104 %, $P < 0.05$; Figure 5.14).

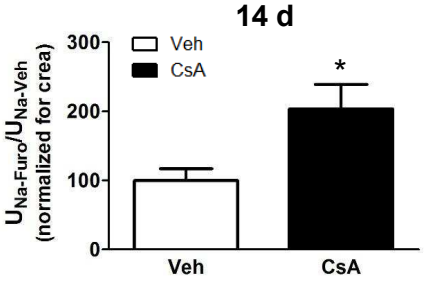

Figure 5.14. Furosemide Test Revealed CsA-Induced Activation of NKCC2 Furosemide test in vehicle- (Veh) and cyclosporine A- (CsA) treated rats, as evaluated by the ratio of urinary sodium excretion after (U_{Na}-Furo) and before furosemide application (U_{Na}-Veh); data were normalized to creatinine excretion. Data are the means ± SEM; $^*P < 0.05$ ([162]).

5.4 CsA-Induced Activation of NKCC2 Depends on AVP

5.4.1 CsA Stimulates NCC but not NKCC2 in AVP-Deficient Brattleboro Rats

To study whether AVP plays a role in CNI-induced activation of NKCC2 and NCC, AVP-deficient Brattleboro rats were treated with CsA or vehicle for 4 h and immunoblot profiles of the distal transporters were compared between the groups. CsA treatment stimulated NCC but not NKCC2 function, as demonstrated by significantly increased levels of pNCC but no change in pNKCC2 or the unphosphorylated species of the two transporters (+68 % for pNCC, $P < 0.05$; Figure 5.15). These data suggest that CsA-induced activation of NKCC2 requires AVP signaling whereas activation of NCC occurs independently of AVP levels.

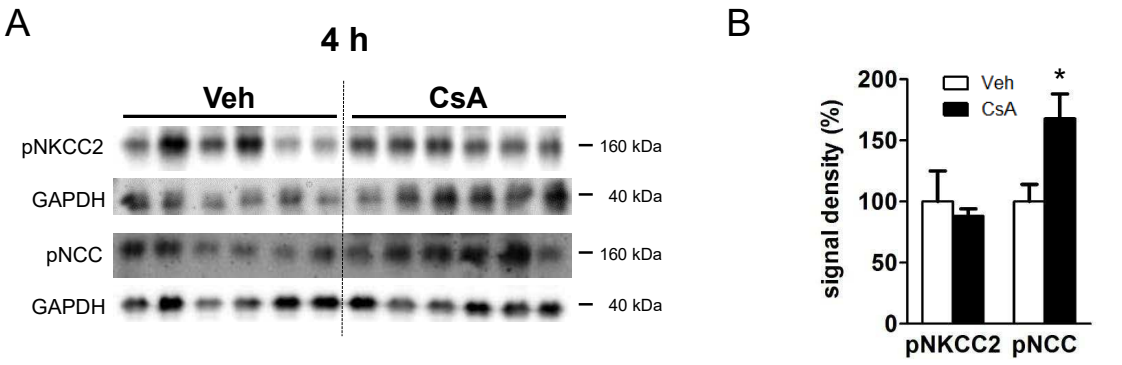

Figure 5.15. Short-term Effects of CsA on NKCC2 and NCC Phosphorylation in Vasopressin Deficient Brattleboro Rats (**A**) Representative immunoblots of kidney lysates from vehicle- (Veh) and cyclosporine A- (CsA, 4 h) treated Brattleboro rats showing immunoreactive signals for pNKCC2 and pNCC (both approximately 160 kDa); GAPDH served as loading control (approximately 40 kDa). (**B**) Densitometric evaluation of immunoreactive signals normalized to loading controls. Short-term CsA treatment increased levels of pNCC but not pNKCC2. Data are the means ± SEM; $^*P < 0.05$ ([162]).

5.4.2 AVP is Required for CsA-Induced Activation of NKCC2 *in vitro*

To prove the hypothesis that CsA-induced activation of NKCC2 and NCC differs in their dependence on AVP, we next studied the local effects of calcineurin inhibition in cultured epithelial cells of the distal tubule. For this, mDCT and raTAL cells were each stimulated with CsA or vehicle for 4 h and immunoblots were performed to analyze NKCC2, NCC and SPAK/OSR1. In mDCT cells, 4 h of CsA strongly increased levels of phosphorylated NCC (+375 % for pNCC, $P < 0.05$) and SPAK/OSR1 (+68 % for pS-SPAK/OSR1,

$P < 0.05$; Figure 5.16 A, C). In contrast, CsA did not induce any significant changes in NKCC2 or SPAK/OSR1 phosphorylation in raTAL cells (Figure 5.16 B, C).

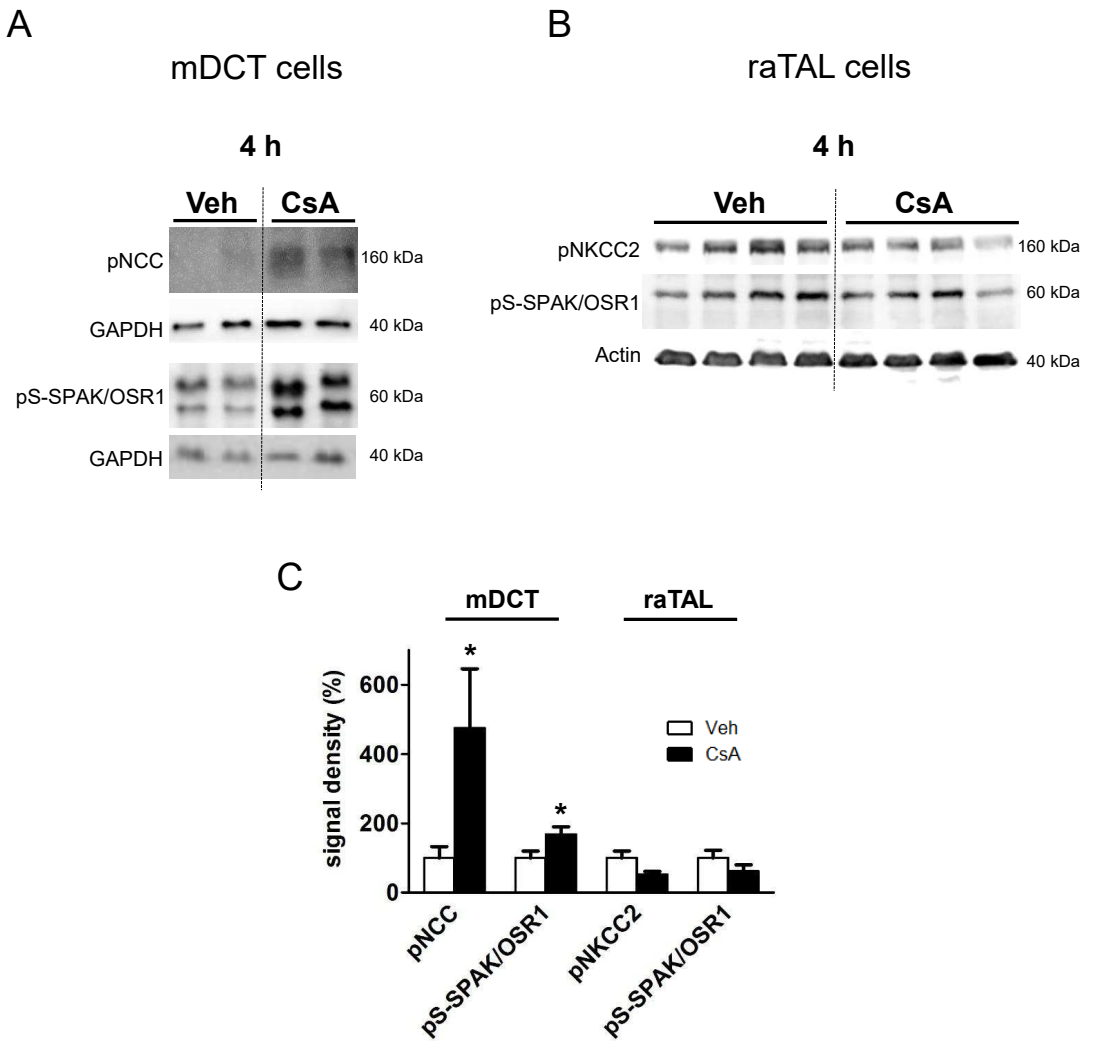

Figure 5.16. CsA Activates NCC but not NKCC2 *in vitro* (**A**) Representative immunoblots of cell lysates from vehicle- (Veh) and cyclosporine A- (CsA, 4 h) treated mDCT cells showing immunoreactive signals for pNCC (approximately 160 kDa) and pS-SPAK/OSR1 (approximately 60 kDa); GAPDH served as loading control (approximately 40 kDa). (**B**) Representative immunoblots of cell lysates from Veh- and CsA-treated raTAL cells showing immunoreactive signals for pNKCC2 (approximately 160 kDa) and pS-SPAK/OSR1 (approximately 60 kDa); β-actin served as loading control (approximately 40 kDa). (**C**) Densitometric evaluation of immunoreactive signals normalized to loading controls. mDCT: mouse distal convoluted tubule cells, raTAL: rat thick ascending limb cells. Data are the means ± SEM; $^*P < 0.05$ ([162]).

To provide further support for the idea that CsA-induced activation of NKCC2 requires AVP signaling, we added the AVP receptor 2 agonist DDAVP to the treatment protocol. DDAVP-stimulated raTAL cells indeed displayed markedly increased phosphorylation levels of NKCC2 (+46 % for DDAVP and +97 % for DDAVP+CsA, $P < 0.05$) and SPAK/OSR1 (+65 % for DDAVP, not significant; +92 % for DDAVP+CsA, $P < 0.05$;

Figure 5.17 A, B). In sum, these results clearly illustrate that local calcineurin inhibition plays a dominant role in the DCT, regulating NCC function. However, compelling evidence is provided for a permissive role of AVP in the TAL, regulating NKCC2 function.

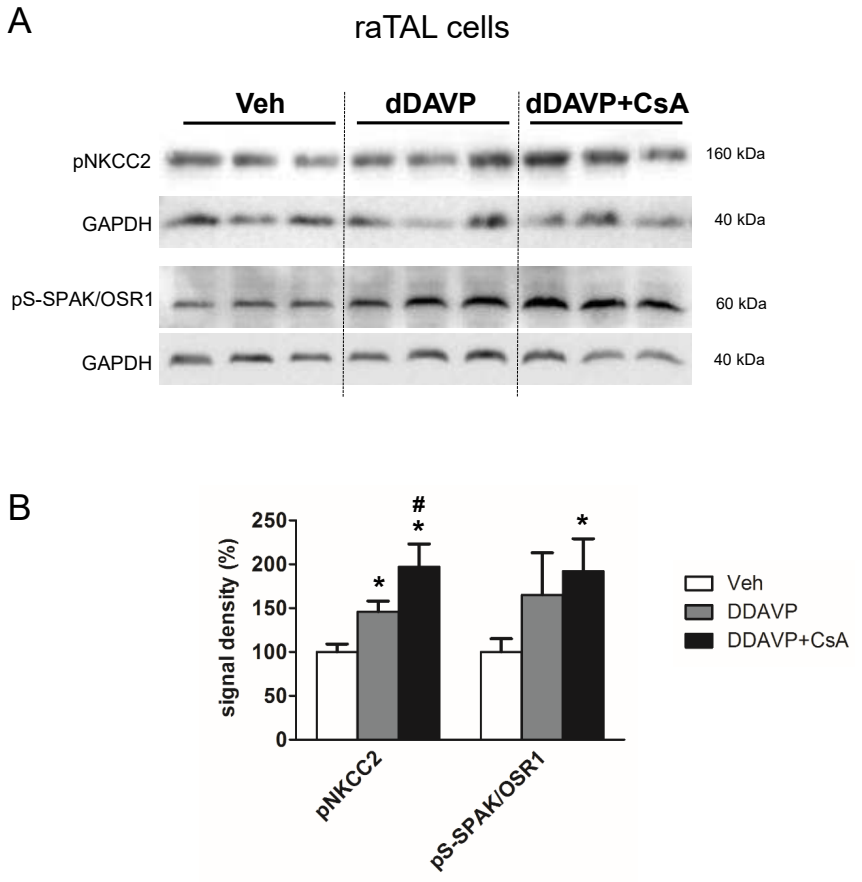

Figure 5.17. DDAVP Stimulates NKCC2 and SPAK/OSR1 Phosphorylation in Cultured TAL Cells (**A**) Representative immunoblots of cell lysates from vehicle- (Veh), desmopressin- (DDAVP), and DDAVP+CsA-treated TAL cells showing immunoreactive signals for pNKCC2 (approximately 160 kDa) and pS-SPAK/OSR1 (approximately 60 kDa); GAPDH served as loading control (approximately 40 kDa). (**B**) Densitometric evaluation of immunoreactive signals normalized to loading controls. Data are the means ± SEM; $^*P < 0.05$ for DDAVP vs. Veh or DDAVP+CsA vs. Veh, $\#P < 0.05$ for DDAVP vs. DDAVP+CsA ([162]).

Chapter 6

Results – Part III: Key Role of NKCC2 in Blood Pressure Regulation

In a collaborative work our group has previously shown that NCC knockout (NCC KO) mice are protected from developing hypertension when treated with the calcineurin inhibitor tacrolimus. That work demonstrated that tacrolimus only stimulates NCC activity and that it has no effect on NKCC2 function. In the present study, we provide further evidence that CsA treatment, in contrast, activates both, NKCC2 and NCC, and we demonstrate that these effects are partially mediated via systemic signaling. Based on these findings we speculated that NKCC2 plays a major role in CsA-induced hypertension. Thus, we hypothesized that CsA treatment, unlike tacrolimus, will induce hypertension regardless of NCC activity. Here, we used the NCC KO mouse model in order to further define the role of NKCC2 and evaluate the effects of CsA in the absence of NCC activity. Mice were treated with CsA and mean arterial blood pressure was monitored over a period of 12 days. Protein expression was evaluated at end point. With this we aimed to gain further insight into the differential effects of the two CNIs tacrolimus and CsA on salt homeostasis and blood pressure.

6.1 Effects of CsA Treatment in NCC Knockout Mice - A Pilot Study

To assess the effects of CsA treatment on blood pressure in NCC KO mice, animals were injected with CsA for 12 days. The well-established radio telemetry method was used to measure blood pressure in unanesthetized mice (see section 3.11). Three days of baseline levels were recorded while a vehicle solution was applied to the mice. Unfortunately, due to technical problems the experiment was finished with only $n = 2$ mice per group and, thus, the results may only give an idea of the actual effects.

By day 5 of CsA treatment, wildtype mice had developed a marked increase in blood pressure (109 mmHG: mean baseline level; 133 mmHg: CsA day 5) and levels remained high throughout the rest of the experiment. NCC knockout mice, despite high variations within the group, also displayed a marked albeit slower increase of blood pressure. By day 6, NCC KO mice had developed high blood pressure (105 mmHG: mean baseline level; 133 mmHG: CsA day 6) and levels remained at a fairly high level throughout the rest of the experiment (Figure 6.1).

These data, although without statistical significance, indicate that CsA might induce high blood pressure regardless of NCC activity, albeit with a certain delay. These results corroborate the hypothesis that NKCC2 plays a key role in CsA-induced hypertension.

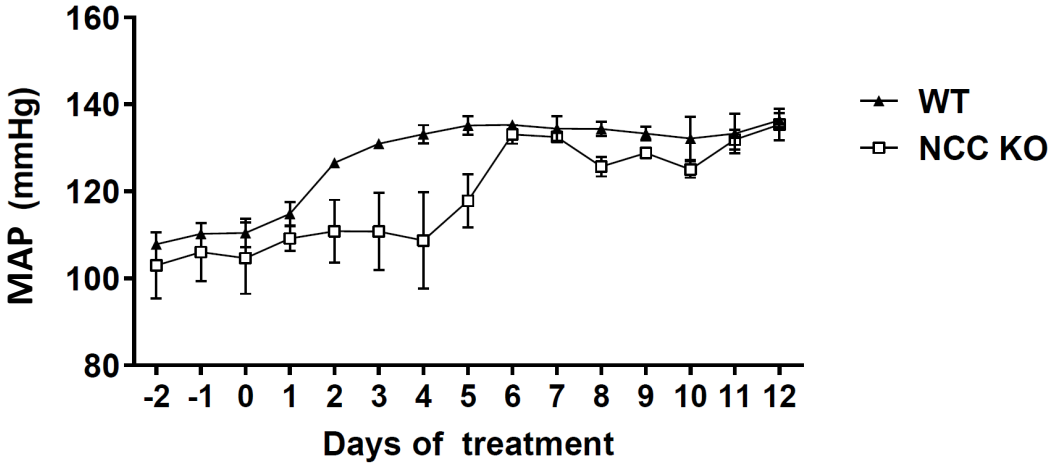

Figure 6.1. CsA Induces Hypertension in NCC KO Mice Mean arterial blood pressure (MAP) in CsA-treated wildtype (WT) and NCC knockout (NCC KO) mice measured by radio telemetry. CsA treatment induced hypertension in WT mice shortly after the beginning of treatment. NCC KO mice also developed hypertension, albeit with a certain delay. Data are the means ± SEM.

In order to provide evidence for the NKCC2 activation, we sought to assess NKCC2 phosphorylation levels in CsA-treated NCC KO mice. To this aim, we analysed immunofluorescent signals in 20 representative TAL motifs in kidneys of WT and NCC KO mice. In both, WT and NCC KO mice, a marked CsA-induced increase of NKCC2 phosphorylation was detected. The increase was slightly stronger in NCC KO mice compared to their WT littermates (Figure 6.2). Since this part of the project was performed during a three months research stay in David Ellison's laboratory at the Oregon Health and Science University, US, the number of available animals was limited. Due to the initial problems with the telemetry method described above, we could only proceed with a small number of animals per group throughout the rest of the study. Proper statistical evaluation was not possible for the immunofluorescent data. However, it is tempting to conclude that the CsA-induced rise in blood pressure observed in NCC KO mice is a result of the strong upregulation of NKCC2 activity, possibly via the induction of AVP signaling.

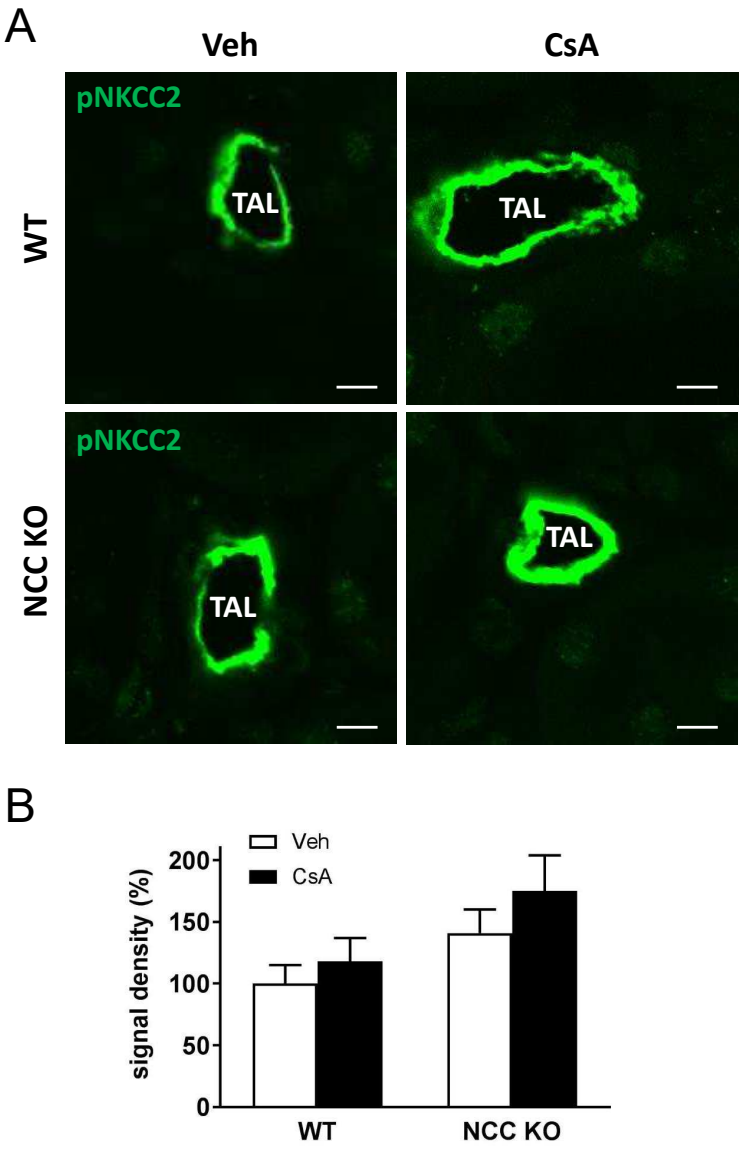

Figure 6.2. Effects of CsA Treatment on NKCC2 Phosphorylation in WT and NCC KO Mice (**A**) Representative images of kidney sections from vehicle- (Veh) or cyclosporine A (CsA)-treated wildtype (WT) and NCC knockout (NCC KO) mice showing immunofluorescent labeling of phosphorylated NKCC2 in the thick ascending limb (TAL). (**B**) Graphs showing pNKCC2 signal in TAL of WT and NCC KO mice treated with Veh or CsA as evaluated using ZEN and ImageJ software. pNKCC2 signal was normalized to background signal. CsA treatment increased the abundance of pNKCC2 in both groups, but to a stronger extent in NCC KO mice. Data are the means ± SEM. Bars indicate 10 μm.

6.2 Stimulated Salt Reabsorption Cascade in the TAL of NCC Knockout Mice

Based on these findings we hypothesized that NCC KO mice possess a compensatory mechanism that upregulates NKCC2 function. To gain further insight into the role of

NKCC2, in a new experiment we assessed baseline protein expression and phosphorylation levels of NKCC2 in untreated wildtype and NCC knockout mice. Immunoblots revealed similar levels of NKCC2 in both groups. Interestingly, phosphorylation levels of NKCC2 were found to be significantly higher in NCC knockout mice compared to wildtype animals (Figure 6.3). Since NCC function is minimized in NCC KO mice, the high levels of phosphorylated NKCC2 might indeed reflect a compensatory mechanism in order to upregulate NKCC2 activity for adjustment of salt homeostasis. Such mechanism would explain that NCC knockout mice display a steady state blood pressure only slightly lower than wildtype mice (Figure 6.1) and develop hypertension on the CsA protocol.

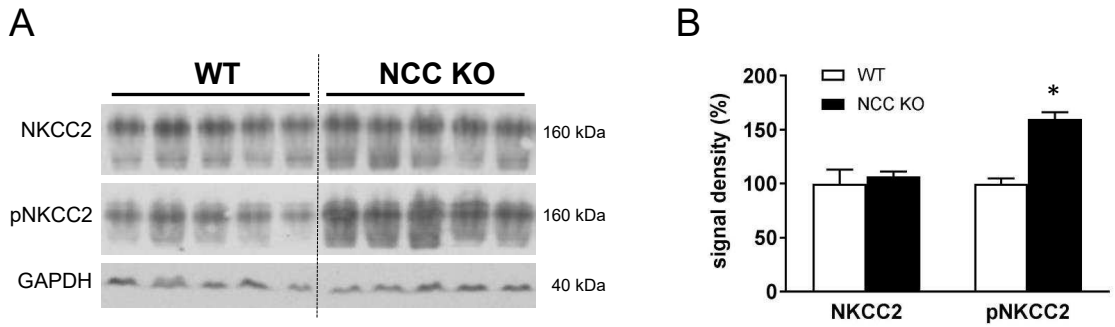

Figure 6.3. Baseline Activity of NKCC2 Is Higher in NCC KO Mice Representative immunoblots of kidney lysates from wildtype (WT) and NCC knockout (NCC KO) mice showing (**A**) immunoreactive signals for total NKCC2 and phosphorylated NKCC2 (pNKCC2), both at approximately 160 kDa; GAPDH served as loading control (approximately 40 kDa). (**B**) Graphs showing respective densitometric evaluation of immunoreactive signals normalized to loading controls. NCC KO mice have higher baseline pNKCC2 levels than their WT littermates, but total protein abundance is not different in the two groups. Data are the means ± SEM; $^*P < 0.05$.

In order to investigate whether the SPAK kinase is responsible for NKCC2 activation, we next analyzed SPAK expression and phosphorylation in WT and NCC KO mice. Immunoblot analysis revealed that SPAK protein expression is similar in both groups. Surprisingly, NCC KO mice displayed slightly lower pS-SPAK levels, albeit not significant (Figure 6.4).

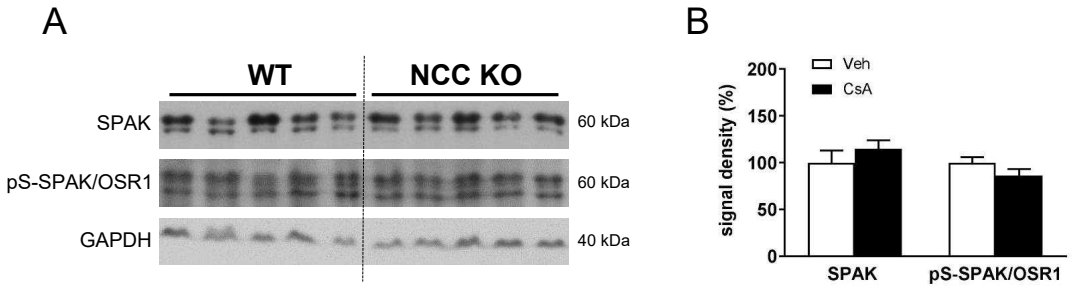

Figure 6.4. Similar Baseline Activity of SPAK in WT and NCC KO Mice Representative immunoblots of kidney lysates from wildtype (WT) and NCC knockout (NCC KO) mice showing (**A**) immunoreactive signals for total SPAK and phosphorylated regulatory pS-SPAK/OSR1, both at approximately 60 kDa; GAPDH served as loading control (approximately 40 kDa). (**B**) Graphs showing respective densitometric evaluation of immunoreactive signals normalized to loading controls. WT and NCC KO mice have similar baseline SPAK levels, pS-SPAK/OSR1 trended to be lower in NCC KO mice than in WT. Data are the means ± SEM.

With respect to the increased pNKCC2 levels in NCC KO mice we had expected up-regulation of SPAK phoshphorylation. To gain better understanding of the mechanism responsible for NKCC2 activation, we analyzed TAL and DCT motifs in WT and NCC KO kidneys by immunofluorescent labeling of the two SPAK/OSR1 phosphorylation sites. Interestingly, we found that kidneys of the NCC KO mice lack the apical signal of the regulatory pS-SPAK/OSR1 form in their DCTs. Instead, in the DCT only cytosolic pS-SPAK/OSR1 was detected (Figure 6.5) while WT mice displayed normal pS-SPAK/OSR1 distribution (apical in TAL, apical and cytosolic in DCT). The missing apical expression of regulatory SPAK/OSR1 might reflect a deficiency of SPAK function in NCC KO mice due to NCC depletion.

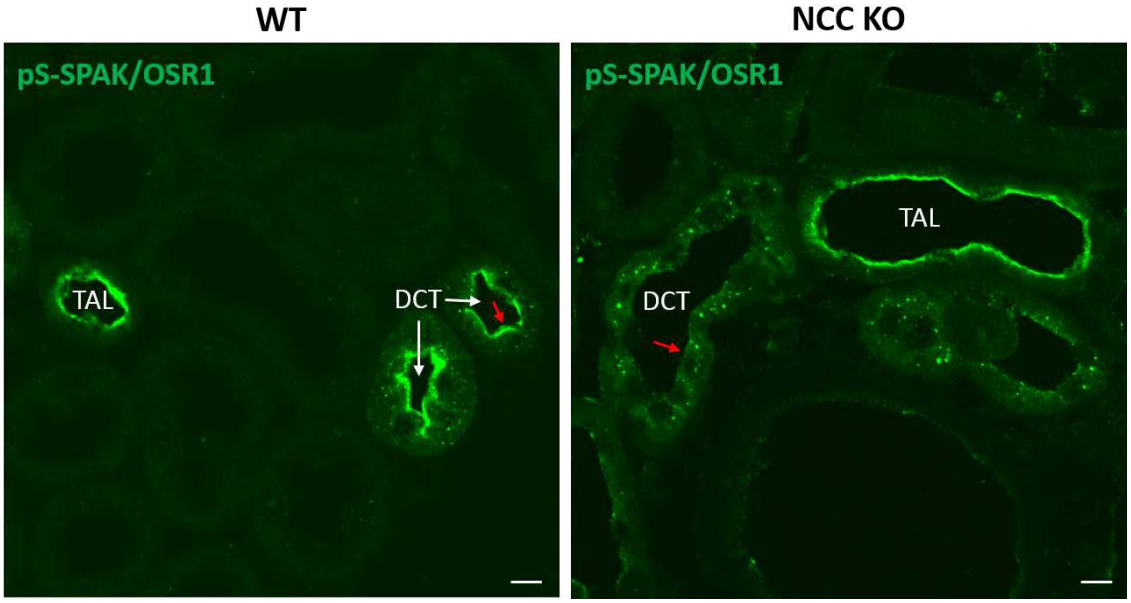

Figure 6.5. Expression Profile of Regulatory pS-SPAK/OSR1 in WT and NCC KO Mice Representative images of kidney sections from wildtype (WT) and NCC knockout (NCC KO) mice showing immunofluorescent labeling of phosphorylated regulatory SPAK/OSR1 domain (pS-SPAK/OSR1) in the thick ascending limb (TAL) and the distal convoluted tubule (DCT). In WT mice, pS-SPAK/OSR1 shows apical expression in the TAL, and apical and cytosolic expression in the DCT. NCC KO mice lack the apical expression in the DCT. Bars indicate 10 μm.

For the catalytic pT-SPAK/OSR1 form, we saw a reversed picture in NCC KO mice compared to WT mice. pT-SPAK/OSR1, which in wildtype mice shows strong apical expression in the DCT and mild apical expression in the TAL, could not be detected in the DCT of NCC KO mice at all. However, in NCC KO mice, pT-SPAK/OSR1 was found to be expressed in the apical membrane of the TAL (Figure 6.6). These data indicate that the SPAK kinase is not fully functional in NCC KO mice but compensates this lack of function through enhanced activity in the TAL, thereby balancing NKCC2 function and salt transport.

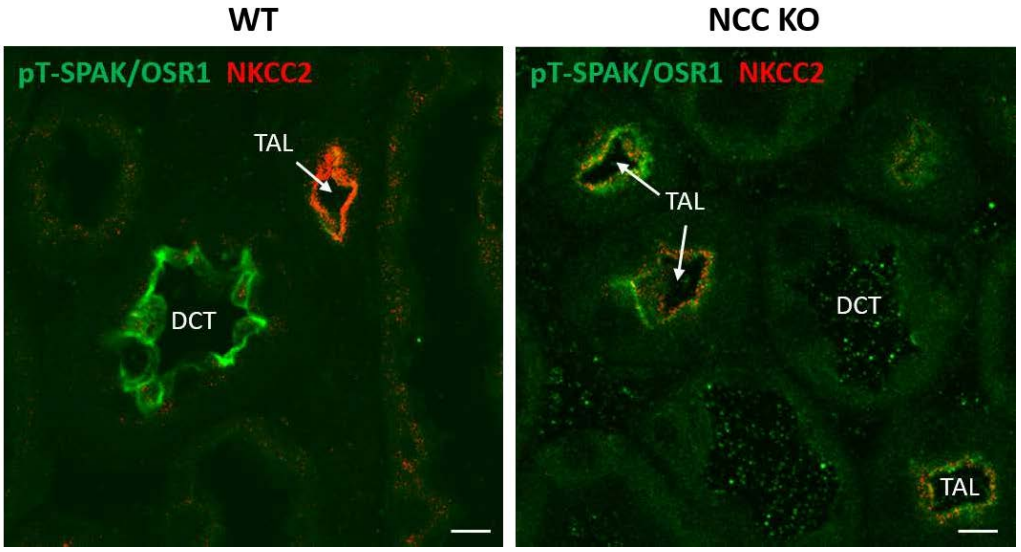

Figure 6.6. Expression Profile of Catalytic pT-SPAK/OSR1 in WT and NCC KO Mice Representative images of kidney sections from wildtype (WT) and NCC knockout (NCC KO) mice showing immunofluorescent labeling of phosphorylated catalytic SPAK/OSR1 domain (pT-SPAK/OSR1) in the thick ascending limb (TAL) and the distal convoluted tubule (DCT). In WT mice, pT-SPAK/OSR1 shows strong apical expression in the DCT and lower apical expression in the TAL. NCC KO mice lack the apical expression in the DCT, but display apical expression in the TAL. Bars indicate 10 μm.

Taken together, these data underline the significance of NKCC2 in the regulation of blood pressure and its involvement in the development of hypertension. They further corroborate the hypothesis of a potentiating effect of CsA-induced AVP signaling on NKCC2 activation. Additionally, SPAK expression and function seem to adapt to distal salt transport activity and to be able to compensate for deteriorated resorption in the DCT.

Chapter 7

Discussion

Since their market launch over 20 years ago, the calcineurin inhibitors (CNIs) cyclosporine A and tacrolimus have been increasingly used for immunosuppression after organ transplantation and in the treatment of several autoimmune diseases. Due to the great treatment success and significant prolongation of patient survival, long-term treatment with calcineurin inhibitors (CNIs) became a standard treatment protocol ([1], [2]). However, severe side effects associated with chronic CNI administration complicate their use. The kidney, the essential organ for blood pressure regulation, is most affected by CNI treatment. One of the early symptoms occurring in a majority of patients is elevated blood pressure that often manifests as hypertension. Besides, the most prevalent adverse effect of chronic CNI treatment is renal damage ([7], [8]).

The kidney controls blood pressure via regulation of water and electrolyte homeostasis. While filtering the whole body blood volume, the kidney precisely coordinates tubular reabsorption and secretion processes to permanently ensure a constant blood filtration rate ([38],[36], [35]). Since Na^+ ions are the predominant ions in the interstitium, reabsorption of sodium is essential for blood pressure regulation. Increased sodium uptake stimulates water uptake which increases extracellular fluid volume and finally enhances blood pressure. The distal tubule herein plays a key role as a substantial amount (up to 35 %) of sodium is reabsorbed in the TAL and the DCT ([34], [37], [36], [51]).

Regarding CNI administration, the distal tubule becomes particularly interesting as CNIs differentially affect the two major salt transporters, NKCC2 and NCC, expressed in this

part of the nephron. Acute and chronic CNI treatment is associated with activation of NKCC2 and NCC, and activation of the transporters can lead to hypertension at long-term. Previous studies suggested that tacrolimus treatment only stimulates NCC, whereas CsA treatment affects both NKCC2 and NCC function ([68],[18], [17], [140], [19], [129]). CsA and tacrolimus also differ in their systemic effects and, thus, have distinct side effects. Therefore, both drugs are indispensable for individual patient care and various disease patterns ([138], [139]).

Unraveling the mechanism of how NKCC2 and NCC are regulated will thus be of high clinical value for the improvement of blood pressure control upon CNI treatment. Since not only CNIs but certain other drugs as well as hereditary diseases and life-style factors can induce hypertension, anti-hypertensive treatment has become increasingly important ([22], [23], [24]). Therefore, elucidating the regulation of the distal salt transporters can further contribute to a better understanding of the development of high blood pressure in general.

In this study we provide new insights into the effects of CsA on salt handling in the distal nephron. While activation of NKCC2 and NCC is regulated by a kinase cascade comprising members of the WNK family and the two homologous SPAK and OSR1 kinases ([68], [23]), it has been suggested that the transporters and the kinases are also regulated by the calcineurin phosphatase since calcineurin inhibition affects NKCC2 and NCC function. Thus, we hypothesized that differential expression of the key factors regulating calcineurin inhibition might be the key for the distinct effects of CsA and tacrolimus in the distal nephron. To investigate this, we first localized the renal isoforms of the catalytic subunit of calcineurin, CnAα and CnAβ, and showed that both isoforms are ubiquitously expressed along the nephron. Other groups suggested that CnAα is primarily expressed in the renal cortex while CnAβ is the major isoform of the medullary TAL ([160], [123], [15]). In this study, we could not detect any significant differences in the expression of the two isoforms along the nephron on either the mRNA or the protein level. These results indicate that the distinct effects of CsA and tacrolimus are not mediated by differential expression of calcineurin isoforms. Instead, differences in post-translational regulation or substrate specificity might be responsible for different effects of calcineurin inhibition. In fact, it has been postulated by Sheftic et al. ([133]) that two recognition sites detected in calcineurin substrates, the PxIxT motif and the LxVP motif, regulate the binding

affinity of calcineurin substrates ([130], [131], [132]). But neither the two transporters NKCC2 and NCC nor their upstream regulating kinases possess these binding motifs. However, when co-immunoprecipitating, they show multiple interactions suggesting scaffolding mechanisms between calcineurin and its substrates ([68], [105]). For example, a recent study of our group revealed that SORLA (sorting-related receptor with A-type repeats), an intracellular receptor involved in sorting and trafficking of various proteins, interacts with CnAβ and modulates dephosphorylation and activation of NKCC2 ([132], [68]). Thus, substrate specificity of calcineurin isoforms as a reason for the differential effects of CNIs cannot be ruled out.

We next hypothesized that the CNI-binding proteins, the immunophilins, which are essential for calcineurin inhibition, are differentially expressed along the nephron. Since tacrolimus treatment only affects NCC function, it was very tempting to suggest that the tacrolimus-binding protein Fkbp12 is expressed primarily in the DCT. Analogically, we presumed to detect the CsA-binding proteins CypA and CypB in both, TAL and DCT, since CsA treatment is associated with activation of NKCC2 and NCC. Moreover, a previous study revealed that renal deletion of Fkbp12 in mice abolishes the effects of tacrolimus on NCC phosphorylation ([164]). However, we found no significant differences in CypA, CypB and Fkbp12 expression on the mRNA or protein level. Thus, we concluded that other mechanisms must be the key to the different CNI sensitivities of the TAL and the DCT. Finally, we analyzed expression patterns of two proteins involved in WNK ubiquitylation, KLHL3 and CUL3. Since mutations in KLHL3 and CUL3 cause hypertension via activation of NCC, a phenotype that resembles the renal effects of tacrolimus treatment, we suggested that these proteins are majorly expressed in the DCT. However, we found strong expression of KLHL3 in both the TAL and the DCT and lower but ubiquitous expression of CUL3 along the entire nephron. In sum, these data provide compelling evidence that the distinct effects of CsA and tacrolimus are not mediated by differential expression of key factors within the signaling pathways regulating NKCC2 and NCC.

Thus, we pursued a different approach to unravel the mechanisms behind the distinct effects of CNIs. Various studies suggest that tacrolimus-induced hypertension and NCC activation primarily rely on local effects of calcineurin inhibition while CsA-induced elevation of blood pressure may partially be caused by systemic effects. To assess local and

systemic effects of CsA treatment we analyzed the effects of acute and chronic treatment protocols in rat TAL and DCT. Acute treatment served for evaluation of fast changes on the post-translational level such as phosphorylation and trafficking as well as potential early changes in mRNA expression and protein abundance. Chronic treatment served to evaluate the impact of endocrine/paracrine mechanims on electrolyte handling and blood pressure. With regard to the local effects of calcineurin inhibition in distal nephron cells, our findings in the short- and long-term treated rats corroborate and extend previous reports on the role of post-translational modification of NKCC2 and NCC by phosphorylation ([68], [77]). Conflicting data exist regarding the effects of CNI treatment on protein content of the kinases and the salt transporters in animals and cultured cells ([17], [18], [140], [165], [166], [19]). However, with the treatment protocols used in this study, no substantial changes in total protein abundance or mRNA levels of the transporters or WNK and SPAK/OSR1 were detected. Additional evidence for the activated state of the two transporters is provided by the parallel increase in surface expression upon CsA treatment. Previous studies hypothesize that phosphorylation stabilizes the transporters within the plasma membrane ([77], [84], [85]). Our data support this idea of a correlation of phosphorylation and trafficking processes. Since we observed that the initial effects of CsA treatment were increased phosphorylation levels of the transporters followed by increased surface expression after 4 h of CsA, we conclude that phosphorylation of NKCC2 and NCC might stimulate trafficking in order to augment transport function.

Taken together, these results highlight the role of post-translational regulation of NKCC2 and NCC by phosphorylation/dephosphorylation reactions. Our data indicate that increased levels of phosphorylated NKCC2 and NCC are, at least in part, a result of the activation of the WNK-SPAK/OSR1 kinase cascade. Additionally, local inhibition of the calcineurin phosphatase might play a role in the regulation of NKCC2 and NCC activation ([167], [168]). However, it remains to be clarified how calcineurin binds to the distal salt transporters. It is known that substrates of calcineurin possess recognition sites for calcineurin binding, the PxIxT and the LxVP motif, but these sites have not been detected in NKCC2 and NCC nor in the WNK-SPAK/OSR1 kinases. Thus, they might interact with the calcineurin phosphatase via scaffolding proteins ([68], [105]). Additionally, other phosphatases like the protein phosphatase 1 may be involved in the regulation of NKCC2 and NCC and mediate the effects of calcineurin ([168]).

Apart from the local effects in the renal distal tubule cells, CNIs reportedly stimulate

endocrine and paracrine mechanisms as well as renal sympathetic innervation and may thereby facilitate renal salt reabsorption ([145], [21], [169]). In our rat model, CsA treatment clearly increased renin expression and activity, likely reflecting enhanced sympathetic activity and stimulated renin synthesis in juxtaglomerular cells ([21]). Activation of the renin-angiotensin-aldosterone-system can be induced by COX-2 secretion which stimulates the local prostaglandins pathway and subsequently activates renin expression. This leads to upregulation of the glomerular filtration rate and NKCC2 function ([148], [149]). However, COX-2 expression was suppressed in CsA-treated animals, indicating that stimulation of renin activity was unrelated to paracrine mechanisms within the juxtaglomerular apparatus. Since transcription of COX-2 is controlled by the nuclear factor of activated T cells (NFAT), which remains inactive under calcineurin inhibition conditions, downregulation of COX-2 upon CsA treatment was an expected observation ([145], [150]). While the effects of renal COX-2 suppression on salt homesostasis are complex ([5]), global inhibition of COX-2 can result in salt-sensitive hypertension ([170]). Thus, synergistic effects of a stimulated RAAS and inhibition of COX-2 may enhance salt reabsorption along the distal nephron and thereby contribute to the development of CNI-induced hypertension ([16], [170]).

Another principal component of the endocrine control of NKCC2 and NCC is arginine vasopressin (AVP) ([100], [101], [55]). While the vasopressin V2 receptor (V2R) is reportedly expressed along the entire distal nephron ([171], [172], [84]), the impact of AVP signaling on salt reabsorption processes might be different in the TAL and the DCT ([173]). In fact, kidneys of AVP-deficient Brattleboro rats display almost complete absence of phosphorylated NKCC2, whereas levels of phosphorylated NCC are less affected in this model ([84], [20]). In our study, short-term CsA treatment induced an increase of NCC phosphorylation in kidneys of Brattleboro rats but did not stimulate NKCC2, suggesting that activation of NKCC2 depends on AVP signaling. Our in vitro studies on cultured distal nephron cells, where systemic effects can be excluded, support these results. Herein, CsA induced activation of NCC and SPAK in cultured mDCT cells but did not change NKCC2 and SPAK function in raTAL cells. These findings support previous suggestions that particularly the TAL strongly depends on the presence of AVP ([102]). This is further substantiated by one of our recent studies showing that rats with a segment-specific overexpression of a dominant-negative V2R mutant exhibit substantial deficits in NKCC2

phosphorylation and function ([172]), underlining the major role of the TAL in the urinary concentration process. Overall, these data provide compelling evidence that AVP is permissive for CsA-induced activation of NKCC2, while local calcineurin inhibition is sufficient for the regulation of NCC.

The molecular mechanism underlying the stimulatory effects of AVP on NKCC2 and NCC function are still unclear, but several studies suggest facilitating effects of AVP on relevant kinases such as WNK-SPAK/OSR1 kinases, protein kinase A (PKA), or AMP-activated protein kinase ([77], [55], [105]). Stimulation of basal SPAK/OSR1 activity may be achieved via activation of PKA through induction of intracellular cAMP release ([103], [104], [105]). Furthermore, it has been increasingly recognized that sensing of intracellular chloride concentration is a major function of WNK kinases. For instance, it has been demonstrated that low intracellular chloride concentration stimulates WNK to activate NCC ([174]). Besides, in a previous study we have shown that uromodulin, a protein expressed in the TAL and potentially involved in urinary concentrating mechanism, contributes to AVP-induced NKCC2 phosphorylation via effects on intracellular chloride concentration ([175], [176]). It is therefore tempting to speculate that AVP either affects chloride concentration or modulates chloride sensitivity of WNK-SPAK/OSR1 kinases in TAL and DCT cells. Therefore, decreased function of NKCC2-activating kinases in the absence of AVP may explain that CsA treatment fails to enhance NKCC2 phosphorylation in Brattleboro rats or cultured raTAL cells lacking AVP.

To confirm the essential role of AVP in NKCC2 activation, we added DDAVP to the CsA-treated raTAL and mDCT cells and found clear increases of phosphorylation levels of SPAK/OSR1 and NKCC2 in raTAL cells. Thus, in the presence of AVP, CsA treatment induces activation of NKCC2 and might thereby contribute to salt retention occuring at the long-term.

Taken together, these data confirm that in DCT cells local calcineurin inhibition is sufficient to induce NCC activation, whereas in the TAL, stimulation by AVP appears to be indispensable for activation of NKCC2. While stimulatory effects of AVP signaling have been described for both, TAL and DCT, the different sensitivities of the two segments are still unclear, but the distal nephron seems to adapt to AVP depending on the

treatment protocol ([55], [105], [20]). Direct and indirect effects of AVP on the distal salt transporters cannot be ruled out but remain to be elucidated.

Our analysis highlights that NKCC2 function depends on systemic signaling. Since the systemic effects associated with calcineurin inhibitors have been reported to be more pronounced with CsA treatment than with tacrolimus and tacrolimus being less nephrotoxic than CsA ([145], [138], [139]), we suggested that activation of NKCC2 is a key mechanism in CsA-induced hypertension. CsA-induced activation of systemic factors may explain the differential effects of CsA and tacrolimus in the distal nephron. Thus, we assumed that CsA treatment, unlike tacrolimus, enhances blood pressure in NCC KO mice. In a collaborative study we have previously applied the NCC KO model and therein we were able to show that tacrolimus-induced hypertension is chiefly mediated by NCC activation ([19]). In that study, NCC KO mice were protected from developing high blood pressure upon tacrolimus treatment. In contrast, in a pilot study we detected an increase in blood pressure in CsA-treated NCC KO mice by day 6 of treatment. These results suggest that CsA induces hypertension regardless of NCC activity, supporting the pivotal role of NKCC2 in CsA-induced hypertension. In line with this, NCC KO mice displayed higher baseline levels of phosphorylated NKCC2 and the CsA-induced increase of pNKCC2 was stronger than in the wildtype group. The upregulation of NKCC2 activity in NCC KO mice may likely reflect a compensatory mechanism counterbalancing the lack of NCC function. However, due to limited animal numbers these data were not statistically significant.

Compensatory reactions of the SPAK kinase have been reported previously ([177], [105]). The findings of the present study further point to the ability of SPAK to compensate for imbalanced salt transport in the distal nephron. While total SPAK protein levels were similar in WT and NCC KO mice, apical expression of regulatory pS-SPAK/OSR1 and catalytic pT-SPAK/OSR1 in the DCT of NCC KO mice was below detection levels, likely reflecting a diminished demand for NCC activating kinases. Instead, enhanced apical expression of phosphorylated SPAK kinase was detected in the TAL of NCC KO mice suggesting that SPAK might balance NKCC2 function as well as salt transport in the TAL of NCC KO mice. Since in NCC KO mice baseline blood pressure was slightly lower and CsA-induced increase of blood pressure was delayed compared to their wildtype littermates, SPAK in the TAL seemed not to be able to fully compensate for the lack of NCC.

Notably, NCC KO mice resemble many features of SPAK KO mice where a tendency toward salt wasting is attenuated by activation of NKCC2 ([177]). In line with our findings on AVP signaling and the fact that compensatory effects in the kidney are commonly stimulated by endocrine factors ([147], [148], [149], [106]), the upregulation of NKCC2 might be triggered by AVP. Alternative kinase pathways such as PKA might mediate the effects of AVP ([69], [57])

In sum, the data of the present study underline the key role of NKCC2 in CsA-induced hypertension and expand the understanding of the involvement of AVP signaling in this context.

Chapter 8

Conclusion

In this study, we present a novel mechanism that might be key to the more diverse effects of CsA treatment compared to tacrolimus. We succeeded in localizing all major components within the signaling pathways regulating the function of the renal distal salt transporters. Our analysis indicates that the distinct effects of the two CNIs CsA and tacrolimus are not mediated via differential expression of those components. We further show that CsA-induced activation of NKCC2 and NCC chiefly occurs at the post-translational level via enhanced phosphorylation of the transporters and their activating kinases. With the use of an AVP-deficient rat model and *in vitro* experiments on cultured distal tubule cells we were able to show that CsA-induced activation of NKCC2, unlike NCC, depends on AVP signaling. These results provide compelling evidence that NKCC2 plays a pivotal role in the development of hypertension upon CsA treatment. In fact, though in a small group of animals, we demonstrate that CsA induces high blood pressure regardless of NCC activity, thus underlining the strong impact of NKCC2 function in blood pressure regulation. Based on these findings, we conclude that differential effects of CsA and tacrolimus may be mediated via AVP signaling in the TAL.

In sum, this study provides a better understanding of the mechanisms of CNI-induced hypertension and may help clinical therapeutic strategy.

Chapter 9

Perspectives

The present study provides new insights into the mechanism of CsA-induced renal side effects. Based on previous findings on the effects of CNIs, our data suggest to favour the use of tacrolimus in certain situations. Even though CsA is more frequently used in the post-transplantational therapy, the use of tacrolimus gradually increases and alternative immunosuppressant drugs are also increasingly prescribed ([4]). Regarding safety and efficacy, tacrolimus is superior to CsA exhibiting less renal complications with less need for antihypertensive treatment ([139]). However, CsA treatment is associated with less post-transplant diabetes and reduced severity of liver fibrosis ([178]). Thus, a conversion to CsA or other immunosuppressive drugs can be appropriate in some cases and might be advantageous especially for lower risk patients ([138]). Our data further support the use of furosemide diuretics and suggest AVP blocker, V2R antagonists or other substances that specifically inhibit NKCC2 function to control blood pressure in CsA-treated patients.

A previous study which focused on the effects of tacrolimus suggested that the toxic side effects in the kidney arise from local calcineurin inhibition ([164]). Our findings also point to the involvement of the calcineurin phosphatase in the regulation of salt transport and thus blood pressure control, at least in the DCT. To reduce calcineurin inhibitor toxicity, we envision novel agents that directly target immune cells or specific calcineurin isoforms. Future experiments should grant a better insight as to whether different effects of CNIs additionally arise from substrate specific binding of calcineurin.

Altogether, this study can help improve blood pressure control and may contribute to a

better quality of life for patients who depend on successive immunosuppressive therapy.

Appendix A

Supplementary Tables

A.1 Primary Antibodies

Antigen	Species	Source of Supply
β-actin	mouse	Sigma Aldrich
CnAα	rabbit	Millipore
CnAβ	rabbit	Millipore
CUL3	rabbit	provided by D. H. Ellison, US
CypA	rabbit	Abcam
CypB	rabbit	Abcam
COX-2	goat	Santa Cruz Biotechnology
Fkbp12	rabbit	Abcam
GAPDH	rabbit	Santa Cruz Biotechnology
KLHL3	rabbit	provided by D. H. Ellison, US
NCC	rabbit	provided by D. H. Ellison, US
NKCC2	guinea pig	[153]
OSR1	sheep	University of Dundee, UK
phospho-S71-NCC	rabbit	Pineda Antikörper-Service
phospho-T96/T101-NKCC2	rabbit	[77]
phospho-S383-SPAK/pS325-OSR1	sheep	University of Dundee, UK
phospho-T243-SPAK/pT185-OSR1	sheep	University of Dundee, UK
phospho-S382-WNK1	sheep	University of Dundee, UK
SPAK	rabbit	[177], US
WNK4	rabbit	provided by J. A. McCormick, US

Table A.1. Validated Primary Antibodies Used in this Study Listed are all antibodies used for immunoblotting, immunofluorescence and in situ hybridization

A.2 Secondary Antibodies

Species	Source of Supply
DyLight488 donkey anti rabbit IgG	DIANOVA, Germany
Cy2 donkey anti guinea pig IgG	DIANOVA, Germany
Cy3 donkey anti rabbit IgG	DIANOVA, Germany
Cy3 donkey anti goat IgG	DIANOVA, Germany
Cy3 donkey anti sheep IgG	DIANOVA, Germany
Cy3 donkey anti guinea pig IgG	DIANOVA, Germany
Cy5 donkey anti Polyclonal swine anti rabbit IgG/HRP	Dako, Denmark
Goat anti mouse IgG/HRP	Dako, Denmark
Polyclonal swine anti guinea pig IgG/HRP	Dako, Denmark
Rabbit anti sheep IgG/HRP	Dako, Denmark
Polyclonal rabbit anti goat IgG/HRP	Dako, Denmark
Anti-DIG-alkaline phosphatase-conjugated antibody	Dako, Denmark

Table A.2. Validated Secondary Antibodies Used in this Study Listed are all antibodies used for immunoblotting, immunofluorescence and in situ hybridization

A.3 qPCR Primers

Gene	Forward Primer	Reverse Primer
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
β-actin	ACGGCCAGGTCATCACTATT	GCCACAGGATTCCATACCCA
CnAα	CCAACACTCGCTACCTCTTC	GTGCCTACATTCATGGTTTCC
CnAβ	GCAACCATGAATGCAGACACC	CAAGGGGCAAGCTGTCAAAAG
CypA	CTGGACCAAACACAAACGGT	TGCCTTCTTTCACCTTCCCA
CypB	GCACAGGAGGAAAGAGCATC	TGAGCCATTGGTGTCTTTGC
KLHL3	GCCATGAAGTACCACCTCCT	ACCAACCACAATCATGACCTTG
CUL3	GCACCATGTCGAATCTGAGC	TTCATCCATGGTCATCGGAAA
WNK1	AAGTATGCCTCAGTCCGTGG	ACTTTCGGTGGACAGGTAGG
WNK3	GAGCTACAGGACCGCAAATTA	TCGAACTATATTGGGATGCTGGA
SPAK	TGCCAGACGAGTATGGATGA	CCACAGCTCATCTTTGACCA
OSR1	AAAGACGTTTGTTGGCACCC	GCCCCTGTGGCTAGTTCAAT
NKCC2	TGTGAAGTTTGGATGGGT	CCGCTTCTCCTACAATCC
NCC	TGATCATCCTTACCTTGCCCA	ACGTTCTCCTGGTTACCTCG
COX-2	TGACAGCCCACCAACTTACA	TCCTTATTTCCTTTCACACCCA

Table A.3. qPCR Primers Used in this Study Listed are all primers used for quantitative PCR

A.4 Primers for In Situ Hybridization

Gene	Primer
CypA Forward	AGTTCCAAAGACAGCAGAAAACT
CypA Reverse	TAATACGACTCACTATAGGATGCCAGGACCTGTATGCTT
CypB Forward	ACAGGAGAGAAAGGATTTGGC
CypB Reverse	TAATACGACTCACTATAGGCCAGGCTCTCTACTCCTTGG
Renin Forward	TCTCTGGGCACTCTTGTTGCTCTGGACCTCTTGTAGCTT
Renin Reverse	AGTCTCCCGACAGACACAGCCAGCTTTGGACGAATCTTGCTCAAGAAA

Table A.4. In Situ Hybridization Primers Used in this Study Listed are all primers used for in situ hybridization

References

[1] A. A. Fernandez-Ramos, V. Poindessous, C. Marchetti-Laurent, N. Pallet, and M.-A. Loriot, "The effect of immunosuppressive molecules on t-cell metabolic reprogramming," *Biochimie*, vol. 127, pp. 23–36, 2016.

[2] I. J. Saldanha, O. Akinyede, and K. A. Robinson, "Immunosuppressive drug therapy for preventing rejection following lung transplantation in cystic fibrosis," *The Cochrane database of systematic reviews*, no. 11, p. CD009421, 2015.

[3] D. C. Brennan, "Long-term trends in allograft survival," *Advances in chronic kidney disease*, vol. 13, no. 1, pp. 11–17, 2006.

[4] C.-L. Chou, C.-Y. Chou, Y.-Y. Huang, M.-S. Wu, C.-C. Hsu, and Y.-C. Chou, "Prescription trends of immunosuppressive drugs in post-heart transplant recipients in taiwan, 2000-2009," *Pharmacoepidemiology and drug safety*, 2014.

[5] M. Naesens, D. R. J. Kuypers, and M. Sarwal, "Calcineurin inhibitor nephrotoxicity," *Clinical journal of the American Society of Nephrology : CJASN*, vol. 4, no. 2, pp. 481–508, 2009.

[6] A. J. Olyaei, A. M. de Mattos, and W. M. Bennett, "Immunosuppressant-induced nephropathy: pathophysiology, incidence and management," *Drug safety*, vol. 21, no. 6, pp. 471–488, 1999.

[7] C. R. Williams and J. L. Gooch, "Calcineurin inhibitors and immunosuppression - a tale of two isoforms," *Expert reviews in molecular medicine*, vol. 14, p. e14, 2012.

[8] A. Busauschina, P. Schnuelle, and F. J. van der Woude, "Cyclosporine nephrotoxicity," *Transplantation proceedings*, vol. 36, no. 2 Suppl, pp. 229S–233S, 2004.

[9] L. E. Kassel and L. E. Odum, "Our own worst enemy: pharmacologic mechanisms of hypertension," *Advances in chronic kidney disease*, vol. 22, no. 3, pp. 245–252, 2015.

[10] P. W. Flatman, "Cotransporters, wnks and hypertension: Important leads from the study of monogenetic disorders of blood pressure regulation," *Clinical science (London, England : 1979)*, vol. 112, no. 4, pp. 203–216, 2007.

[11] J. Roy and M. S. Cyert, "Cracking the phosphatase code: docking interactions determine substrate specificity," *Science signaling*, vol. 2, no. 100, p. re9, 2009.

[12] F. Rusnak and P. Mertz, "Calcineurin: Form and function," *Physiological reviews*, vol. 80, no. 4, pp. 1483–1521, 2000.

[13] H. Jiang, F. Xiong, S. Kong, T. Ogawa, M. Kobayashi, and J. O. Liu, "Distinct tissue and cellular distribution of two major isoforms of calcineurin," *Molecular immunology*, vol. 34, no. 8-9, pp. 663–669, 1997.

[14] E. S. Masuda, R. Imamura, Y. Amasaki, K. Arai, and N. Arai, "Signalling into the t-cell nucleus: Nfat regulation," *Cellular signalling*, vol. 10, no. 9, pp. 599–611, 1998.

[15] E. J. Hoorn, S. B. Walsh, J. A. McCormick, A. Fürstenberg, C.-L. Yang, T. Roeschel, A. Paliege, A. J. Howie, J. Conley, S. Bachmann, R. J. Unwin, and D. H. Ellison, "The calcineurin inhibitor tacrolimus activates the renal sodium chloride cotransporter to cause hypertension," *Nature medicine*, vol. 17, no. 10, pp. 1304–1309, 2011.

[16] E. J. Hoorn, S. B. Walsh, J. A. McCormick, R. Zietse, R. J. Unwin, and D. H. Ellison, "Pathogenesis of calcineurin inhibitor-induced hypertension," *Journal of nephrology*, vol. 25, no. 3, pp. 269–275, 2012.

[17] C. Esteva-Font, E. Ars, E. Guillen-Gomez, J. M. Campistol, L. Sanz, W. Jimenez, M. A. Knepper, F. Torres, R. Torra, J. A. Ballarin, and P. Fernandez-Llama, "Ciclosporin-induced hypertension is associated with increased sodium transporter of the loop of henle (nkcc2)," *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*, vol. 22, no. 10, pp. 2810–2816, 2007.

[18] S. Damiano, R. Scanni, R. Ciarcia, S. Florio, and G. Capasso, "Regulation of sodium transporters in the kidney during cyclosporine treatment," *Journal of nephrology*, vol. 23 Suppl 16, pp. S191–8, 2010.

[19] E. J. Hoorn, J. H. Nelson, J. A. McCormick, and D. H. Ellison, "The wnk kinase network regulating sodium, potassium, and blood pressure," *Journal of the American Society of Nephrology : JASN*, vol. 22, no. 4, pp. 605–614, 2011.

[20] K. Mutig, T. Saritas, S. Uchida, T. Kahl, T. Borowski, A. Paliege, A. Böhlick, M. Bleich, Q. Shan, and S. Bachmann, "Short-term stimulation of the thiazide-sensitive na+-cl- cotransporter by vasopressin involves phosphorylation and membrane translocation," *American journal of physiology. Renal physiology*, vol. 298, no. 3, pp. F502–9, 2010.

[21] A. Kurtz, R. Della Bruna, and K. Kuhn, "Cyclosporine a enhances renin secretion and production in isolated juxtaglomerular cells," *Kidney international*, vol. 33, no. 5, pp. 947–953, 1988.

[22] P. A. Heidenreich, J. G. Trogdon, O. A. Khavjou, J. Butler, K. Dracup, M. D. Ezekowitz, E. A. Finkelstein, Y. Hong, S. C. Johnston, A. Khera, D. M. Lloyd-Jones, S. A. Nelson, G. Nichol, D. Orenstein, P. W. F. Wilson, and Y. J. Woo, "Forecasting the future of cardiovascular disease in the united states: A policy statement from the american heart association," *Circulation*, vol. 123, no. 8, pp. 933–944, 2011.

[23] F. H. Rafiqi, A. M. Zuber, M. Glover, C. Richardson, S. Fleming, S. Jovanovic, A. Jovanovic, K. M. O'Shaughnessy, and D. R. Alessi, "Role of the wnk-activated spak kinase in regulating blood pressure," *EMBO molecular medicine*, vol. 2, no. 2, pp. 63–75, 2010.

[24] F. H. Wilson, S. Disse-Nicodème, K. A. Choate, K. Ishikawa, C. Nelson-Williams, I. Desitter, M. Gunel, D. V. Milford, G. W. Lipkin, J. M. Achard, M. P. Feely, B. Dussol, Y. Berland, R. J. Unwin, H. Mayan, D. B. Simon, Z. Farfel, X. Jeunemaitre, and R. P. Lifton, "Human hypertension caused by mutations in wnk kinases," *Science (New York, N.Y.)*, vol. 293, no. 5532, pp. 1107–1112, 2001.

[25] K. Besseghir and F. Roch-Ramel, "Renal excretion of drugs and other xenobiotics," *Renal physiology*, vol. 10, no. 5, pp. 221–241, 1987.

[26] S. GLABMAN, H. S. AYNEDJIAN, and N. BANK, "Micropuncture study of the effect of acute reductions in glomerular filtration rate on sodium and water reabsorption by the proximal tubules of the rat," *The Journal of clinical investigation*, vol. 44, pp. 1410–1416, 1965.

[27] L. WERKO, J. EK, E. VARNAUSKAS, H. BUCHT, B. THOMASSON, and H. ELIASCH, "The relationship between renal blood flow, glomerular filtration rate and sodium excretion, cardiac output and pulmonary and systemic blood pressures in various heart disorders," *American heart journal*, vol. 49, no. 6, pp. 823–837, 1955.

[28] B. J. Potter, "The effect of an intravenous infusion of hypertonic saline on renal mechanisms and on electrolyte changes in sheep," *The Journal of physiology*, vol. 184, no. 3, pp. 605–617, 1966.

[29] W. Kriz and L. Bankir, "A standard nomenclature for structures of the kidney. the renal commission of the international union of physiological sciences (iups)," *Kidney international*, vol. 33, no. 1, pp. 1–7, 1988.

[30] A. B. Donnersberger and D. R. Donnersberger, *A laboratory textbook of anatomy and physiology: Cat version.* Sudbury, Mass.: Jones and Bartlett Publishers, 9th ed. ed., 2010.

[31] A. Benninghoff and D. Drenckhahn, *Anatomie: Makroskopische Anatomie, Embryologie und Histologie des Menschen.* München [u.a.]: Urban & Schwarzenberg, 17., durchges. aufl. ed., 2008.

[32] M. H. Little, *Kidney development, disease, repair and regeneration.* Amsterdam: Elsevier, 2016.

[33] R. J. Alpern, S. C. Herbert, D. W. Seldin, and G. H. Giebisch, *Seldin and Giebisch's the kidney: Physiology & pathophysiology.* Amsterdam and Boston: Elsevier Academic Press, 4th ed. ed., 2008.

[34] K. Hierholzer and M. Wiederholt, "Some aspects of distal tubular solute and water transport," *Kidney international*, vol. 9, no. 2, pp. 198–213, 1976.

[35] R. Greger, "Ion transport mechanisms in thick ascending limb of henle's loop of mammalian nephron," *Physiological reviews*, vol. 65, no. 3, pp. 760–797, 1985.

[36] M. B. Burg, "Tubular chloride transport and the mode of action of some diuretics," *Kidney international*, vol. 9, no. 2, pp. 189–197, 1976.

[37] C. M. Bennett, B. M. Brenner, and R. W. Berliner, "Micropuncture study of nephron function in the rhesus monkey," *The Journal of clinical investigation*, vol. 47, no. 1, pp. 203–216, 1968.

[38] M. Burg and D. Good, "Sodium chloride coupled transport in mammalian nephrons," *Annual review of physiology*, vol. 45, pp. 533–547, 1983.

[39] M. B. Burg, S. PAPPER, and J. D. ROSENBAUM, "Factors influencing the diuretic response to ingested water," *The Journal of laboratory and clinical medicine*, vol. 57, pp. 533–545, 1961.

[40] S. Nielsen, A. B. Maunsbach, C. A. Ecelbarger, and M. A. Knepper, "Ultrastructural localization of na-k-2cl cotransporter in thick ascending limb and macula densa of rat kidney," *The American journal of physiology*, vol. 275, no. 6 Pt 2, pp. F885–93, 1998.

[41] J. Schnermann, H. Osswald, and M. Hermle, "Inhibitory effect of methylxanthines on feedback control of glomerular filtration rate in the rat kidney," *Pflugers Archiv : European journal of physiology*, vol. 369, no. 1, pp. 39–48, 1977.

[42] S. Thomson, D. Bao, A. Deng, and V. Vallon, "Adenosine formed by 5'-nucleotidase mediates tubuloglomerular feedback," *The Journal of clinical investigation*, vol. 106, no. 2, pp. 289–298, 2000.

[43] T. A. Kotchen, W. J. Welch, J. N. Lorenz, and C. E. Ott, "Renal tubular chloride and renin release," *The Journal of laboratory and clinical medicine*, vol. 110, no. 5, pp. 533–540, 1987.

[44] T. K. Keeton and W. B. Campbell, "The pharmacologic alteration of renin release," *Pharmacological reviews*, vol. 32, no. 2, pp. 81–227, 1980.

[45] R. M. Carey, "The intrarenal renin-angiotensin system in hypertension," *Advances in chronic kidney disease*, vol. 22, no. 3, pp. 204–210, 2015.

[46] P. H. Baylis, "Regulation of vasopressin secretion," *Bailliere's clinical endocrinology and metabolism*, vol. 3, no. 2, pp. 313–330, 1989.

[47] E. E. Windhager, *Handbook of physiology.* Bethesda, Md.: American Physiological Soc, 1992.

[48] M. Carlstrom, C. S. Wilcox, and W. J. Arendshorst, "Renal autoregulation in health and disease," *Physiological reviews*, vol. 95, no. 2, pp. 405–511, 2015.

[49] D. H. Ellison, H. Velázquez, and F. S. Wright, "Thiazide-sensitive sodium chloride cotransport in early distal tubule," *The American journal of physiology*, vol. 253, no. 3 Pt 2, pp. F546–54, 1987.

[50] G. Gamba, S. N. Saltzberg, M. Lombardi, A. Miyanoshita, J. Lytton, M. A. Hediger, B. M. Brenner, and S. C. Hebert, "Primary structure and functional expression of a cdna encoding the thiazide-sensitive, electroneutral sodium-chloride cotransporter," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 7, pp. 2749–2753, 1993.

[51] G. Gamba, "Molecular physiology and pathophysiology of electroneutral cation-chloride cotransporters," *Physiological reviews*, vol. 85, no. 2, pp. 423–493, 2005.

[52] R. Schmitt, D. H. Ellison, N. Farman, B. C. Rossier, R. F. Reilly, W. B. Reeves, I. Oberbaumer, R. Tapp, and S. Bachmann, "Developmental expression of sodium entry pathways in rat nephron," *The American journal of physiology*, vol. 276, no. 3 Pt 2, pp. F367–81, 1999.

[53] T. Wang and G. Giebisch, "Effects of angiotensin ii on electrolyte transport in the early and late distal tubule in rat kidney," *The American journal of physiology*, vol. 271, no. 1 Pt 2, pp. F143–9, 1996.

[54] G. H. Kim, S. Masilamani, R. Turner, C. Mitchell, J. B. Wade, and M. A. Knepper, "The thiazide-sensitive na-cl cotransporter is an aldosterone-induced protein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 24, pp. 14552–14557, 1998.

[55] J. M. Elalouf, N. Roinel, and C. de Rouffignac, "Effects of antidiuretic hormone on electrolyte reabsorption and secretion in distal tubules of rat kidney," *Pflugers Archiv : European journal of physiology*, vol. 401, no. 2, pp. 167–173, 1984.

[56] P. Igarashi, G. B. Vanden Heuvel, J. A. Payne, and B. r. Forbush, "Cloning, embryonic expression, and alternative splicing of a murine kidney-specific na-k-cl cotransporter," *The American journal of physiology*, vol. 269, no. 3 Pt 2, pp. F405–18, 1995.

[57] G. R. Ares, P. S. Caceres, and P. A. Ortiz, "Molecular regulation of nkcc2 in the thick ascending limb," *American journal of physiology. Renal physiology*, vol. 301, no. 6, pp. F1143–59, 2011.

[58] D. B. Mount, A. Baekgaard, A. E. Hall, C. Plata, J. Xu, D. R. Beier, G. Gamba, and S. C. Hebert, "Isoforms of the na-k-2cl cotransporter in murine tal i. molecular characterization and intrarenal localization," *The American journal of physiology*, vol. 276, no. 3 Pt 2, pp. F347–58, 1999.

[59] J. A. Payne and B. r. Forbush, "Alternatively spliced isoforms of the putative renal na-k-cl cotransporter are differentially distributed within the rabbit kidney," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 10, pp. 4544–4548, 1994.

[60] I. Carota, F. Theilig, M. Oppermann, P. Kongsuphol, A. Rosenauer, R. Schreiber, B. L. Jensen, S. Walter, K. Kunzelmann, and H. Castrop, "Localization and functional characterization of the human nkcc2 isoforms," *Acta physiologica (Oxford, England)*, vol. 199, no. 3, pp. 327–338, 2010.

[61] M. Oppermann, D. Mizel, G. Huang, C. Li, C. Deng, F. Theilig, S. Bachmann, J. Briggs, J. Schnermann, and H. Castrop, "Macula densa control of renin secretion and preglomerular resistance in mice with selective deletion of the b isoform of the na,k,2cl co-transporter," *Journal of the American Society of Nephrology : JASN*, vol. 17, no. 8, pp. 2143–2152, 2006.

[62] K. Fiedler and K. Simons, "The role of n-glycans in the secretory pathway," *Cell*, vol. 81, no. 3, pp. 309–312, 1995.

[63] N. Mastroianni, A. Bettinelli, M. Bianchetti, G. Colussi, M. de Fusco, F. Sereni, A. Ballabio, and G. Casari, "Novel molecular variants of the na-cl cotransporter gene are responsible for gitelman syndrome," *American journal of human genetics*, vol. 59, no. 5, pp. 1019–1026, 1996.

[64] D. B. Simon, C. Nelson-Williams, M. J. Bia, D. Ellison, F. E. Karet, A. M. Molina, I. Vaara, F. Iwata, H. M. Cushner, M. Koolen, F. J. Gainza, H. J. Gitleman, and R. P. Lifton, "Gitelman's variant of bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive na-cl cotransporter," *Nature genetics*, vol. 12, no. 1, pp. 24–30, 1996.

[65] B. Ko, A. C. Mistry, L. Hanson, R. Mallick, L. L. Cooke, B. K. Hack, P. Cunningham, and R. S. Hoover, "A new model of the distal convoluted tubule," *American journal of physiology. Renal physiology*, vol. 303, no. 5, pp. F700–10, 2012.

[66] R. S. Hoover, E. Poch, A. Monroy, N. Vazquez, T. Nishio, G. Gamba, and S. C. Hebert, "N-glycosylation at two sites critically alters thiazide binding and activity of the rat thiazide-sensitive na(+):cl(-) cotransporter," *Journal of the American Society of Nephrology : JASN*, vol. 14, no. 2, pp. 271–282, 2003.

[67] S. Kunchaparty, M. Palcso, J. Berkman, H. Velazquez, G. V. Desir, P. Bernstein, R. F. Reilly, and D. H. Ellison, "Defective processing and expression of thiazide-sensitive na-cl cotransporter as a cause of gitelman's syndrome," *The American journal of physiology*, vol. 277, no. 4 Pt 2, pp. F643–9, 1999.

[68] A. Borschewski, N. Himmerkus, C. Boldt, K. I. Blankenstein, J. A. McCormick, R. Lazelle, T. E. Willnow, V. Jankowski, A. Plain, M. Bleich, D. H. Ellison, S. Bachmann, and K. Mutig, "Calcineurin and sorting-related receptor with a-type repeats interact to regulate the renal na(+)-k(+)-2cl(-) cotransporter," *Journal of the American Society of Nephrology : JASN*, vol. 27, no. 1, pp. 107–119, 2016.

[69] C. Richardson, K. Sakamoto, P. de los Heros, M. Deak, D. G. Campbell, A. R. Prescott, and D. R. Alessi, "Regulation of the nkcc2 ion cotransporter by spak-osr1-dependent and -independent pathways," *Journal of cell science*, vol. 124, no. Pt 5, pp. 789–800, 2011.

[70] I. Gimenez and B. Forbush, "Regulatory phosphorylation sites in the nh2 terminus of the renal na-k-cl cotransporter (nkcc2)," *American journal of physiology. Renal physiology*, vol. 289, no. 6, pp. F1341–5, 2005.

[71] P. A. Ortiz, "camp increases surface expression of nkcc2 in rat thick ascending limbs: role of vamp," *American journal of physiology. Renal physiology*, vol. 290, no. 3, pp. F608–16, 2006.

[72] M. B. Sandberg, A. B. Maunsbach, and A. A. McDonough, "Redistribution of distal tubule na+-cl- cotransporter (ncc) in response to a high-salt diet," *American journal of physiology. Renal physiology*, vol. 291, no. 2, pp. F503–8, 2006.

[73] P. Meade, R. S. Hoover, C. Plata, N. Vázquez, N. A. Bobadilla, G. Gamba, and S. C. Hebert, "camp-dependent activation of the renal-specific na+-k+-2cl- cotransporter is mediated by regulation of cotransporter trafficking," *American journal of physiology. Renal physiology*, vol. 284, no. 6, pp. F1145–54, 2003.

[74] C. Dathe, A.-L. Daigeler, W. Seifert, V. Jankowski, R. Mrowka, R. Kalis, E. Wanker, K. Mutig, S. Bachmann, and A. Paliege, "Annexin a2 mediates apical trafficking of renal na^+-k^+-$2cl^-$ cotransporter," *The Journal of biological chemistry*, vol. 289, no. 14, pp. 9983–9997, 2014.

[75] G. R. Ares and P. A. Ortiz, "Dynamin2, clathrin, and lipid rafts mediate endocytosis of the apical na/k/2cl cotransporter nkcc2 in thick ascending limbs," *The Journal of biological chemistry*, vol. 287, no. 45, pp. 37824–37834, 2012.

[76] L. L. Rosenbaek, M. L. A. Kortenoeven, T. S. Aroankins, and R. A. Fenton, "Phosphorylation decreases ubiquitylation of the thiazide-sensitive cotransporter ncc and subsequent clathrin-mediated endocytosis," *The Journal of biological chemistry*, vol. 289, no. 19, pp. 13347–13361, 2014.

[77] I. Giménez and B. Forbush, "Short-term stimulation of the renal na-k-cl cotransporter (nkcc2) by vasopressin involves phosphorylation and membrane translocation of the protein," *The Journal of biological chemistry*, vol. 278, no. 29, pp. 26946–26951, 2003.

[78] S. A. Fraser, I. Gimenez, N. Cook, I. Jennings, M. Katerelos, F. Katsis, V. Levidiotis, B. E. Kemp, and D. A. Power, "Regulation of the renal-specific na+-k+-2cl-co-transporter nkcc2 by amp-activated protein kinase (ampk)," *The Biochemical journal*, vol. 405, no. 1, pp. 85–93, 2007.

[79] D. Pacheco-Alvarez, P. S. Cristobal, P. Meade, E. Moreno, N. Vazquez, E. Munoz, A. Diaz, M. E. Juarez, I. Gimenez, and G. Gamba, "The na+:cl- cotransporter is activated and phosphorylated at the amino-terminal domain upon intracellular chloride depletion," *The Journal of biological chemistry*, vol. 281, no. 39, pp. 28755–28763, 2006.

[80] G. Gamba, "Regulation of the renal na+-cl- cotransporter by phosphorylation and ubiquitylation," *American journal of physiology. Renal physiology*, vol. 303, no. 12, pp. F1573–83, 2012.

[81] M. Chiga, T. Rai, S.-S. Yang, A. Ohta, T. Takizawa, S. Sasaki, and S. Uchida, "Dietary salt regulates the phosphorylation of osr1/spak kinases and the sodium chloride cotransporter through aldosterone," *Kidney international*, vol. 74, no. 11, pp. 1403–1409, 2008.

[82] C. Richardson, F. H. Rafiqi, H. K. R. Karlsson, N. Moleleki, A. Vandewalle, D. G. Campbell, N. A. Morrice, and D. R. Alessi, "Activation of the thiazide-sensitive na+-cl- cotransporter by the wnk-regulated kinases spak and osr1," *Journal of cell science*, vol. 121, no. Pt 5, pp. 675–684, 2008.

[83] S.-S. Yang, T. Morimoto, T. Rai, M. Chiga, E. Sohara, M. Ohno, K. Uchida, S.-H. Lin, T. Moriguchi, H. Shibuya, Y. Kondo, S. Sasaki, and S. Uchida, "Molecular pathogenesis of pseudohypoaldosteronism type ii: Generation and analysis of a wnk4(d561a/+) knockin mouse model," *Cell metabolism*, vol. 5, no. 5, pp. 331–344, 2007.

[84] K. Mutig, A. Paliege, T. Kahl, T. Jöns, W. Müller-Esterl, and S. Bachmann, "Vasopressin v2 receptor expression along rat, mouse, and human renal epithelia with focus on tal," *American journal of physiology. Renal physiology*, vol. 293, no. 4, pp. F1166–77, 2007.

[85] L. L. Rosenbaek, M. Assentoft, N. B. Pedersen, N. MacAulay, and R. A. Fenton, "Characterization of a novel phosphorylation site in the sodium-chloride cotransporter, ncc," *The Journal of physiology*, vol. 590, no. 23, pp. 6121–6139, 2012.

[86] K. Piechotta, J. Lu, and E. Delpire, "Cation chloride cotransporters interact with the stress-related kinases ste20-related proline-alanine-rich kinase (spak) and oxidative stress response 1 (osr1)," *The Journal of biological chemistry*, vol. 277, no. 52, pp. 50812–50819, 2002.

[87] S.-S. Yang, Y.-F. Lo, C.-C. Wu, S.-W. Lin, C.-J. Yeh, P. Chu, H.-K. Sytwu, S. Uchida, S. Sasaki, and S.-H. Lin, "Spak-knockout mice manifest gitelman syndrome and impaired vasoconstriction," *Journal of the American Society of Nephrology : JASN*, vol. 21, no. 11, pp. 1868–1877, 2010.

[88] A. N. Anselmo, S. Earnest, W. Chen, Y.-C. Juang, S. C. Kim, Y. Zhao, and M. H. Cobb, "Wnk1 and osr1 regulate the na+, k+, 2cl- cotransporter in hela cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 29, pp. 10883–10888, 2006.

[89] T. Moriguchi, S. Urushiyama, N. Hisamoto, S.-i. Iemura, S. Uchida, T. Natsume, K. Matsumoto, and H. Shibuya, "Wnk1 regulates phosphorylation of cation-chloride-coupled cotransporters via the ste20-related kinases, spak and osr1," *The Journal of biological chemistry*, vol. 280, no. 52, pp. 42685–42693, 2005.

[90] B. Xu, J. M. English, J. L. Wilsbacher, S. Stippec, E. J. Goldsmith, and M. H. Cobb, "Wnk1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain ii," *The Journal of biological chemistry*, vol. 275, no. 22, pp. 16795–16801, 2000.

[91] X. Min, B.-H. Lee, M. H. Cobb, and E. J. Goldsmith, "Crystal structure of the kinase domain of wnk1, a kinase that causes a hereditary form of hypertension," *Structure (London, England : 1993)*, vol. 12, no. 7, pp. 1303–1311, 2004.

[92] L. Y. Lenertz, B.-H. Lee, X. Min, B.-e. Xu, K. Wedin, S. Earnest, E. J. Goldsmith, and M. H. Cobb, "Properties of wnk1 and implications for other family members," *The Journal of biological chemistry*, vol. 280, no. 29, pp. 26653–26658, 2005.

[93] A. T. Piala, T. M. Moon, R. Akella, H. He, M. H. Cobb, and E. J. Goldsmith, "Chloride sensing by wnk1 involves inhibition of autophosphorylation," *Science signaling*, vol. 7, no. 324, p. ra41, 2014.

[94] B.-e. Xu, X. Min, S. Stippec, B.-H. Lee, E. J. Goldsmith, and M. H. Cobb, "Regulation of wnk1 by an autoinhibitory domain and autophosphorylation," *The Journal of biological chemistry*, vol. 277, no. 50, pp. 48456–48462, 2002.

[95] J. O. Thastrup, F. H. Rafiqi, A. C. Vitari, E. Pozo-Guisado, M. Deak, Y. Mehellou, and D. R. Alessi, "Spak/osr1 regulate nkcc1 and wnk activity: Analysis of wnk isoform interactions and activation by t-loop trans-autophosphorylation," *The Biochemical journal*, vol. 441, no. 1, pp. 325–337, 2012.

[96] A. C. Vitari, J. Thastrup, F. H. Rafiqi, M. Deak, N. A. Morrice, H. K. R. Karlsson, and D. R. Alessi, "Functional interactions of the spak/osr1 kinases with their upstream activator wnk1 and downstream substrate nkcc1," *The Biochemical journal*, vol. 397, no. 1, pp. 223–231, 2006.

[97] K. B. E. Gagnon, R. England, and E. Delpire, "A single binding motif is required for spak activation of the na-k-2cl cotransporter," *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, vol. 20, no. 1-4, pp. 131–142, 2007.

[98] K. B. Gagnon and E. Delpire, "On the substrate recognition and negative regulation of spak, a kinase modulating na+-k+-2cl- cotransport activity," *American journal of physiology. Cell physiology*, vol. 299, no. 3, pp. C614–20, 2010.

[99] K. B. E. Gagnon, R. England, and E. Delpire, "Characterization of spak and osr1, regulatory kinases of the na-k-2cl cotransporter," *Molecular and cellular biology*, vol. 26, no. 2, pp. 689–698, 2006.

[100] D. A. Molony, W. B. Reeves, S. C. Hebert, and T. E. Andreoli, "Adh increases apical na+, k+, 2cl- entry in mouse medullary thick ascending limbs of henle," *The American journal of physiology*, vol. 252, no. 1 Pt 2, pp. F177–87, 1987.

[101] S. C. Hebert, P. A. Friedman, and T. E. Andreoli, "Effects of antidiuretic hormone on cellular conductive pathways in mouse medullary thick ascending limbs of henle: I. adh increases transcellular conductance pathways," *The Journal of membrane biology*, vol. 80, no. 3, pp. 201–219, 1984.

[102] C. A. Ecelbarger, G. H. Kim, J. B. Wade, and M. A. Knepper, "Regulation of the abundance of renal sodium transporters and channels by vasopressin," *Experimental neurology*, vol. 171, no. 2, pp. 227–234, 2001.

[103] R. Gunaratne, D. W. W. Braucht, M. M. Rinschen, C.-L. Chou, J. D. Hoffert, T. Pisitkun, and M. A. Knepper, "Quantitative phosphoproteomic analysis reveals camp/vasopressin-dependent signaling pathways in native renal thick ascending limb cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 35, pp. 15653–15658, 2010.

[104] H. Amlal, C. Legoff, C. Vernimmen, M. Paillard, and M. Bichara, "Na(+)-k+(nh4+)-2cl- cotransport in medullary thick ascending limb: Control by pka, pkc, and 20-hete," *The American journal of physiology*, vol. 271, no. 2 Pt 1, pp. C455–63, 1996.

[105] T. Saritas, A. Borschewski, J. A. McCormick, A. Paliege, C. Dathe, S. Uchida, A. Terker, N. Himmerkus, M. Bleich, S. Demaretz, K. Laghmani, E. Delpire, D. H. Ellison, S. Bachmann, and K. Mutig, "Spak differentially mediates vasopressin effects on sodium cotransporters," *Journal of the American Society of Nephrology : JASN*, vol. 24, no. 3, pp. 407–418, 2013.

[106] N. B. Pedersen, M. V. Hofmeister, L. L. Rosenbaek, J. Nielsen, and R. A. Fenton, "Vasopressin induces phosphorylation of the thiazide-sensitive sodium chloride cotransporter in the distal convoluted tubule," *Kidney international*, vol. 78, no. 2, pp. 160–169, 2010.

[107] V. Vallon, J. Schroth, F. Lang, D. Kuhl, and S. Uchida, "Expression and phosphorylation of the na+-cl- cotransporter ncc in vivo is regulated by dietary salt, potassium, and sgk1," *American journal of physiology. Renal physiology*, vol. 297, no. 3, pp. F704–12, 2009.

[108] N. van der Lubbe, C. H. Lim, R. A. Fenton, M. E. Meima, A. H. Jan Danser, R. Zietse, and E. J. Hoorn, "Angiotensin ii induces phosphorylation of the thiazide-sensitive sodium chloride cotransporter independent of aldosterone," *Kidney international*, vol. 79, no. 1, pp. 66–76, 2011.

[109] K. T. Kahle, F. H. Wilson, Q. Leng, M. D. Lalioti, A. D. O'Connell, K. Dong, A. K. Rapson, G. G. MacGregor, G. Giebisch, S. C. Hebert, and R. P. Lifton, "Wnk4 regulates the balance between renal nacl reabsorption and k+ secretion," *Nature genetics*, vol. 35, no. 4, pp. 372–376, 2003.

[110] P. San-Cristobal, D. Pacheco-Alvarez, C. Richardson, A. M. Ring, N. Vazquez, F. H. Rafiqi, D. Chari, K. T. Kahle, Q. Leng, N. A. Bobadilla, S. C. Hebert, D. R. Alessi, R. P. Lifton, and G. Gamba, "Angiotensin ii signaling increases activity of the renal na-cl cotransporter through a wnk4-spak-dependent pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 11, pp. 4384–4389, 2009.

[111] D. B. Simon, F. E. Karet, J. M. Hamdan, A. DiPietro, S. A. Sanjad, and R. P. Lifton, "Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the na-k-2cl cotransporter nkcc2," *Nature genetics*, vol. 13, no. 2, pp. 183–188, 1996.

[112] R. D. Gordon, "Syndrome of hypertension and hyperkalemia with normal glomerular filtration rate," *Hypertension (Dallas, Tex. : 1979)*, vol. 8, no. 2, pp. 93–102, 1986.

[113] C. H. Lee and G.-H. Kim, "Electrolyte and acid-base disturbances induced by clacineurin inhibitors," *Electrolyte & blood pressure : E & BP*, vol. 5, no. 2, pp. 126–130, 2007.

[114] L. M. Boyden, M. Choi, K. A. Choate, C. J. Nelson-Williams, A. Farhi, H. R. Toka, I. R. Tikhonova, R. Bjornson, S. M. Mane, G. Colussi, M. Lebel, R. D. Gordon, B. A. Semmekrot, A. Poujol, M. J. Valimaki, M. E. de Ferrari, S. A. Sanjad, M. Gutkin, F. E. Karet, J. R. Tucci, J. R. Stockigt, K. M. Keppler-Noreuil, C. C. Porter, S. K. Anand, M. L. Whiteford, I. D. Davis, S. B. Dewar, A. Bettinelli, J. J. Fadrowski, C. W. Belsha, T. E. Hunley, R. D. Nelson, H. Trachtman, T. R. P. Cole, M. Pinsk, D. Bockenhauer, M. Shenoy, P. Vaidyanathan, J. W. Foreman, M. Rasoulpour, F. Thameem, H. Z. Al-Shahrouri, J. Radhakrishnan, A. G. Gharavi, B. Goilav, and R. P. Lifton, "Mutations in kelch-like 3 and cullin 3 cause hypertension and electrolyte abnormalities," *Nature*, vol. 482, no. 7383, pp. 98–102, 2012.

[115] H. Louis-Dit-Picard, J. Barc, D. Trujillano, S. Miserey-Lenkei, N. Bouatia-Naji, O. Pylypenko, G. Beaurain, A. Bonnefond, O. Sand, C. Simian, E. Vidal-Petiot, C. Soukaseum, C. Mandet, F. Broux, O. Chabre, M. Delahousse, V. Esnault, B. Fiquet, P. Houillier, C. I. Bagnis, J. Koenig, M. Konrad, P. Landais, C. Mourani, P. Niaudet, V. Probst, C. Thauvin, R. J. Unwin, S. D. Soroka, G. Ehret, S. Ossowski, M. Caulfield, P. Bruneval, X. Estivill, P. Froguel, J. Hadchouel, J.-J. Schott, and X. Jeunemaitre, "Klhl3 mutations cause familial hyperkalemic hypertension by impairing ion transport in the distal nephron," *Nature genetics*, vol. 44, no. 4, pp. 456–60, S1–3, 2012.

[116] K. Susa, E. Sohara, T. Rai, M. Zeniya, Y. Mori, T. Mori, M. Chiga, N. Nomura, H. Nishida, D. Takahashi, K. Isobe, Y. Inoue, K. Takeishi, N. Takeda, S. Sasaki, and S. Uchida, "Impaired degradation of wnk1 and wnk4 kinases causes phaii in mutant

klhl3 knock-in mice," *Human molecular genetics*, vol. 23, no. 19, pp. 5052–5060, 2014.

[117] Y. Mori, M. Wakabayashi, T. Mori, Y. Araki, E. Sohara, T. Rai, S. Sasaki, and S. Uchida, "Decrease of wnk4 ubiquitination by disease-causing mutations of klhl3 through different molecular mechanisms," *Biochemical and biophysical research communications*, vol. 439, no. 1, pp. 30–34, 2013.

[118] M. Wakabayashi, T. Mori, K. Isobe, E. Sohara, K. Susa, Y. Araki, M. Chiga, E. Kikuchi, N. Nomura, Y. Mori, H. Matsuo, T. Murata, S. Nomura, T. Asano, H. Kawaguchi, S. Nonoyama, T. Rai, S. Sasaki, and S. Uchida, "Impaired klhl3-mediated ubiquitination of wnk4 causes human hypertension," *Cell reports*, vol. 3, no. 3, pp. 858–868, 2013.

[119] C. B. Klee, H. Ren, and X. Wang, "Regulation of the calmodulin-stimulated protein phosphatase, calcineurin," *The Journal of biological chemistry*, vol. 273, no. 22, pp. 13367–13370, 1998.

[120] J. L. Gooch, J. J. Toro, R. L. Guler, and J. L. Barnes, "Calcineurin a-alpha but not a-beta is required for normal kidney development and function," *The American journal of pathology*, vol. 165, no. 5, pp. 1755–1765, 2004.

[121] K. Ueki, T. Muramatsu, and R. L. Kincaid, "Structure and expression of two isoforms of the murine calmodulin-dependent protein phosphatase regulatory subunit (calcineurin b)," *Biochemical and biophysical research communications*, vol. 187, no. 1, pp. 537–543, 1992.

[122] T. Khatlani, S. Pradhan, Q. Da, F. C. Gushiken, A. L. Bergeron, K. W. Langlois, J. D. Molkentin, R. E. Rumbaut, and K. V. Vijayan, "The b isoform of the catalytic subunit of protein phosphatase 2b restrains platelet function by suppressing outside-in aiibb3 integrin signaling," *Journal of thrombosis and haemostasis : JTH*, 2014.

[123] J. L. Gooch, "An emerging role for calcineurin aalpha in the development and function of the kidney," *American journal of physiology. Renal physiology*, vol. 290, no. 4, pp. F769–76, 2006.

[124] M. Buttini, S. Limonta, M. Luyten, and H. Boddeke, "Distribution of calcineurin a isoenzyme mrnas in rat thymus and kidney," *The Histochemical journal*, vol. 27, no. 4, pp. 291–299, 1995.

[125] B. W. Zhang, G. Zimmer, J. Chen, D. Ladd, E. Li, F. W. Alt, G. Wiederrecht, J. Cryan, E. A. O'Neill, C. E. Seidman, A. K. Abbas, and J. G. Seidman, "T cell responses in calcineurin a alpha-deficient mice," *The Journal of experimental medicine*, vol. 183, no. 2, pp. 413–420, 1996.

[126] O. F. Bueno, E. B. Brandt, M. E. Rothenberg, and J. D. Molkentin, "Defective t cell development and function in calcineurin a beta -deficient mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 14, pp. 9398–9403, 2002.

[127] V. S. F. Chan, C. Wong, and P. S. Ohashi, "Calcineurin aalpha plays an exclusive role in tcr signaling in mature but not in immature t cells," *European journal of immunology*, vol. 32, no. 5, pp. 1223–1229, 2002.

[128] K. Hocherl, F. Kees, B. K. Kramer, and A. Kurtz, "Cyclosporine a attenuates the natriuretic action of loop diuretics by inhibition of renal cox-2 expression," *Kidney international*, vol. 65, no. 6, pp. 2071–2080, 2004.

[129] D. C. Hu, C. Burtner, A. Hong, P. I. Lobo, and M. D. Okusa, "Correction of renal hypertension after kidney transplantation from a donor with gitelman syndrome," *The American journal of the medical sciences*, vol. 331, no. 2, pp. 105–109, 2006.

[130] J. Aramburu, F. Garcia-Cozar, A. Raghavan, H. Okamura, A. Rao, and P. G. Hogan, "Selective inhibition of nfat activation by a peptide spanning the calcineurin targeting site of nfat," *Molecular cell*, vol. 1, no. 5, pp. 627–637, 1998.

[131] J. Roy, H. Li, P. G. Hogan, and M. S. Cyert, "A conserved docking site modulates substrate affinity for calcineurin, signaling output, and in vivo function," *Molecular cell*, vol. 25, no. 6, pp. 889–901, 2007.

[132] A. Rodriguez, J. Roy, S. Martinez-Martinez, M. D. Lopez-Maderuelo, P. Nino-Moreno, L. Orti, D. Pantoja-Uceda, A. Pineda-Lucena, M. S. Cyert, and J. M. Redondo, "A conserved docking surface on calcineurin mediates interaction with

substrates and immunosuppressants," *Molecular cell*, vol. 33, no. 5, pp. 616–626, 2009.

[133] S. R. Sheftic, R. Page, and W. Peti, "Investigating the human calcineurin interaction network using the pilxvp slim," *Scientific reports*, vol. 6, p. 38920, 2016.

[134] J. Liu, M. W. Albers, T. J. Wandless, S. Luan, D. G. Alberg, P. J. Belshaw, P. Cohen, C. MacKintosh, C. B. Klee, and S. L. Schreiber, "Inhibition of t cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity," *Biochemistry*, vol. 31, no. 16, pp. 3896–3901, 1992.

[135] N. A. Clipstone, D. F. Fiorentino, and G. R. Crabtree, "Molecular analysis of the interaction of calcineurin with drug-immunophilin complexes," *The Journal of biological chemistry*, vol. 269, no. 42, pp. 26431–26437, 1994.

[136] A. Fahr, "Cyclosporin clinical pharmacokinetics," *Clinical pharmacokinetics*, vol. 24, no. 6, pp. 472–495, 1993.

[137] K. Iwasaki, "Metabolism of tacrolimus (fk506) and recent topics in clinical pharmacokinetics," *Drug metabolism and pharmacokinetics*, vol. 22, no. 5, pp. 328–335, 2007.

[138] B. F. Leas, S. Uhl, D. L. Sawinski, J. Trofe-Clark, S. Tuteja, J. L. Kaczmarek, and C. A. Umscheid, "Calcineurin inhibitors for renal transplant.," 2016.

[139] B. K. Krämer, G. Montagnino, B. Krüger, R. Margreiter, C. J. Olbricht, R. Marcen, U. Sester, U. Kunzendorf, K.-H. Dietl, P. Rigotti, C. Ronco, S. Hörsch, B. Banas, F. Mühlbacher, and M. Arias, "Efficacy and safety of tacrolimus compared with ciclosporin a in renal transplantation: seven-year observational results," *Transplant international : official journal of the European Society for Organ Transplantation*, 2015.

[140] S. Melnikov, H. Mayan, S. Uchida, E. J. Holtzman, and Z. Farfel, "Cyclosporine metabolic side effects: association with the wnk4 system," *European journal of clinical investigation*, vol. 41, no. 10, pp. 1113–1120, 2011.

[141] G. Sune, E. Sarro, M. Puigmule, J. Lopez-Hellin, M. Zufferey, T. Pertel, J. Luban, and A. Meseguer, "Cyclophilin b interacts with sodium-potassium atpase and is

required for pump activity in proximal tubule cells of the kidney," *PloS one*, vol. 5, no. 11, p. e13930, 2010.

[142] P. Wang and J. Heitman, "The cyclophilins," *Genome biology*, vol. 6, no. 7, p. 226, 2005.

[143] S. Barik, "Immunophilins: For the love of proteins," *Cellular and molecular life sciences : CMLS*, vol. 63, no. 24, pp. 2889–2900, 2006.

[144] M. Tong and Y. Jiang, "Fk506-binding proteins and their diverse functions," *Current molecular pharmacology*, vol. 9, no. 1, pp. 48–65, 2015.

[145] K. Höcherl, F. Dreher, H. Vitzthum, J. Köhler, and A. Kurtz, "Cyclosporine a suppresses cyclooxygenase-2 expression in the rat kidney," *Journal of the American Society of Nephrology : JASN*, vol. 13, no. 10, pp. 2427–2436, 2002.

[146] H. Meyer-Lehnert and R. W. Schrier, "Potential mechanism of cyclosporine a-induced vascular smooth muscle contraction," *Hypertension (Dallas, Tex. : 1979)*, vol. 13, no. 4, pp. 352–360, 1989.

[147] R. C. Harris, J. A. McKanna, Y. Akai, H. R. Jacobson, R. N. Dubois, and M. D. Breyer, "Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction," *The Journal of clinical investigation*, vol. 94, no. 6, pp. 2504–2510, 1994.

[148] G.-H. Kim, "Renal effects of prostaglandins and cyclooxygenase-2 inhibitors," *Electrolyte & blood pressure : E & BP*, vol. 6, no. 1, pp. 35–41, 2008.

[149] A. Paliege, D. Mizel, C. Medina, A. Pasumarthy, Y. G. Huang, S. Bachmann, J. P. Briggs, J. B. Schnermann, and T. Yang, "Inhibition of nnos expression in the macula densa by cox-2-derived prostaglandin e(2)," *American journal of physiology. Renal physiology*, vol. 287, no. 1, pp. F152–9, 2004.

[150] M. A. Iniguez, S. Martinez-Martinez, C. Punzon, J. M. Redondo, and M. Fresno, "An essential role of the nuclear factor of activated t cells in the regulation of the expression of the cyclooxygenase-2 gene in human t lymphocytes," *The Journal of biological chemistry*, vol. 275, no. 31, pp. 23627–23635, 2000.

[151] W. M. Flanagan, B. Corthésy, R. J. Bram, and G. R. Crabtree, "Nuclear association of a t-cell transcription factor blocked by fk-506 and cyclosporin a," *Nature*, vol. 352, no. 6338, pp. 803–807, 1991.

[152] G. Rovera, "The wistar institute," *Molecular medicine (Cambridge, Mass.)*, vol. 3, no. 4, pp. 229–230, 1997.

[153] R. Schmitt, E. Klussmann, T. Kahl, D. H. Ellison, and S. Bachmann, "Renal expression of sodium transporters and aquaporin-2 in hypothyroid rats," *American journal of physiology. Renal physiology*, vol. 284, no. 5, pp. F1097–104, 2003.

[154] H. Schmale and D. Richter, "Single base deletion in the vasopressin gene is the cause of diabetes insipidus in brattleboro rats," *Nature*, vol. 308, no. 5961, pp. 705–709, 1984.

[155] P. J. Schultheis, J. N. Lorenz, P. Meneton, M. L. Nieman, T. M. Riddle, M. Flagella, J. J. Duffy, T. Doetschman, M. L. Miller, and G. E. Shull, "Phenotype resembling gitelman's syndrome in mice lacking the apical na+-cl- cotransporter of the distal convoluted tubule," *The Journal of biological chemistry*, vol. 273, no. 44, pp. 29150–29155, 1998.

[156] B. Eng, S. Mukhopadhyay, C. P. Vio, P. L. Pedraza, S. Hao, S. Battula, P. B. Sehgal, J. C. McGiff, and N. R. Ferreri, "Characterization of a long-term rat mtal cell line," *American journal of physiology. Renal physiology*, vol. 293, no. 4, pp. F1413–22, 2007.

[157] F. A. Gesek and P. A. Friedman, "Mechanism of calcium transport stimulated by chlorothiazide in mouse distal convoluted tubule cells," *The Journal of clinical investigation*, vol. 90, no. 2, pp. 429–438, 1992.

[158] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "Nih image to imagej: 25 years of image analysis," *Nature methods*, vol. 9, no. 7, pp. 671–675, 2012.

[159] D. A. Huetteman and H. Bogie, "Direct blood pressure monitoring in laboratory rodents via implantable radio telemetry," *Methods in molecular biology (Clifton, N.J.)*, vol. 573, pp. 57–73, 2009.

[160] J. M. Foster, P. K. Carmines, and J. S. Pollock, "Pp2b-dependent no production in the medullary thick ascending limb during diabetes," *American journal of physiology. Renal physiology*, vol. 297, no. 2, pp. F471–80, 2009.

[161] J. A. McCormick, C.-L. Yang, C. Zhang, B. Davidge, K. I. Blankenstein, A. S. Terker, B. Yarbrough, N. P. Meermeier, H. J. Park, B. McCully, M. West, A. Borschewski, N. Himmerkus, M. Bleich, S. Bachmann, K. Mutig, E. R. Argaiz, G. Gamba, J. D. Singer, and D. H. Ellison, "Hyperkalemic hypertension-associated cullin 3 promotes wnk signaling by degrading klhl3," *The Journal of clinical investigation*, 2014.

[162] K. I. Blankenstein, A. Borschewski, R. Labes, A. Paliege, C. Boldt, J. A. McCormick, D. H. Ellison, M. Bader, S. Bachmann, and K. Mutig, "Calcineurin inhibitor cyclosporine a activates renal na-k-cl cotransporters via local and systemic mechanisms," *American journal of physiology. Renal physiology*, vol. 312, no. 3, pp. F489–F501, 2017.

[163] A. S. Terker, C. Zhang, K. J. Erspamer, G. Gamba, C.-L. Yang, and D. H. Ellison, "Unique chloride-sensing properties of wnk4 permit the distal nephron to modulate potassium homeostasis," *Kidney international*, 2015.

[164] R. A. Lazelle, B. H. McCully, A. S. Terker, N. Himmerkus, K. Blankenstein, K. Mutig, M. Bleich, S. Bachmann, C.-L. Yang, and D. H. Ellison, "Renal deletion of 12 kda fk506-binding protein attenuates tacrolimus-induced hypertension," *Journal of the American Society of Nephrology : JASN*, 2015.

[165] C. E. Deppe, P. J. Heering, H. Tinel, E. Kinne-Saffran, B. Grabensee, and R. K. Kinne, "Effect of cyclosporine a on na+/k(+)-atpase, na+/k+/2cl- cotransporter, and h+/k(+)-atpase in mdck cells and two subtypes, c7 and c11," *Experimental nephrology*, vol. 5, no. 6, pp. 471–480, 1997.

[166] S. W. Lim, K. O. Ahn, M. R. Sheen, U. S. Jeon, J. Kim, C. W. Yang, and H. M. Kwon, "Downregulation of renal sodium transporters and tonicity-responsive enhancer binding protein by long-term treatment with cyclosporin a," *Journal of the American Society of Nephrology : JASN*, vol. 18, no. 2, pp. 421–429, 2007.

[167] J. A. McCormick and D. H. Ellison, "The wnks: atypical protein kinases with pleiotropic actions," *Physiological reviews*, vol. 91, no. 1, pp. 177–219, 2011.

[168] N. Picard, K. Trompf, C.-L. Yang, R. L. Miller, M. Carrel, D. Loffing-Cueni, R. A. Fenton, D. H. Ellison, and J. Loffing, "Protein phosphatase 1 inhibitor-1 deficiency reduces phosphorylation of renal nacl cotransporter and causes arterial hypotension," *Journal of the American Society of Nephrology : JASN*, vol. 25, no. 3, pp. 511–522, 2014.

[169] N. G. Moss, S. L. Powell, and R. J. Falk, "Intravenous cyclosporine activates afferent and efferent renal nerves and causes sodium retention in innervated kidneys in rats," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 23, pp. 8222–8226, 1985.

[170] M.-Z. Zhang, B. Yao, Y. Wang, S. Yang, S. Wang, X. Fan, and R. C. Harris, "Inhibition of cyclooxygenase-2 in hematopoietic cells results in salt-sensitive hypertension," *The Journal of clinical investigation*, vol. 125, no. 11, pp. 4281–4294, 2015.

[171] R. A. Fenton, L. Brønd, S. Nielsen, and J. Praetorius, "Cellular and subcellular distribution of the type-2 vasopressin receptor in the kidney," *American journal of physiology. Renal physiology*, vol. 293, no. 3, pp. F748–60, 2007.

[172] K. Mutig, T. Borowski, C. Boldt, A. Borschewski, A. Paliege, E. Popova, M. Bader, and S. Bachmann, "Demonstration of the functional impact of vasopressin signalling in thick ascending limb by a targeted transgenic rat approach," *American journal of physiology. Renal physiology*, p. ajprenal.00126.2016, 2016.

[173] M. L. A. Kortenoeven, N. B. Pedersen, L. L. Rosenbaek, and R. A. Fenton, "Vasopressin regulation of sodium transport in the distal nephron and collecting duct," *American journal of physiology. Renal physiology*, vol. 309, no. 4, pp. F280–99, 2015.

[174] A. S. Terker, C. Zhang, J. A. McCormick, R. A. Lazelle, C. Zhang, N. P. Meermeier, D. A. Siler, H. J. Park, Y. Fu, D. M. Cohen, A. M. Weinstein, W.-H. Wang, C.-L. Yang, and D. H. Ellison, "Potassium modulates electrolyte balance and blood pressure through effects on distal cell voltage and chloride," *Cell metabolism*, vol. 21, no. 1, pp. 39–50, 2015.

[175] K. Mutig, T. Kahl, T. Saritas, M. Godes, P. Persson, J. Bates, H. Raffi, L. Rampoldi, S. Uchida, C. Hille, C. Dosche, S. Kumar, M. Castañeda-Bueno, G. Gamba, and

S. Bachmann, "Activation of the bumetanide-sensitive na+,k+,2cl- cotransporter (nkcc2) is facilitated by tamm-horsfall protein in a chloride-sensitive manner," *The Journal of biological chemistry*, vol. 286, no. 34, pp. 30200–30210, 2011.

[176] S. Bachmann, K. Mutig, J. Bates, P. Welker, B. Geist, V. Gross, F. C. Luft, N. Alenina, M. Bader, B. J. Thiele, K. Prasadan, H. S. Raffi, and S. Kumar, "Renal effects of tamm-horsfall protein (uromodulin) deficiency in mice," *American journal of physiology. Renal physiology*, vol. 288, no. 3, pp. F559–67, 2005.

[177] J. A. McCormick, K. Mutig, J. H. Nelson, T. Saritas, E. J. Hoorn, C.-L. Yang, S. Rogers, J. Curry, E. Delpire, S. Bachmann, and D. H. Ellison, "A spak isoform switch modulates renal salt transport and blood pressure," *Cell metabolism*, vol. 14, no. 3, pp. 352–364, 2011.

[178] G. Levy, F. G. Villamil, F. Nevens, H. J. Metselaar, P.-A. Clavien, G. Klintmalm, R. Jones, M. Migliaccio, H. Prestele, and R. Orsenigo, "Refine: a randomized trial comparing cyclosporine a and tacrolimus on fibrosis after liver transplantation for hepatitis c," *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, vol. 14, no. 3, pp. 635–646, 2014.