Glucocorticoids Induce Nondipping Blood Pressure by Activating the Thiazide-Sensitive Cotransporter


Abstract—Blood pressure (BP) normally dips during sleep, and nondipping increases cardiovascular risk. Hydrochlorothiazide restores the dipping BP profile in nondipping patients, suggesting that the NaCl cotransporter, NCC, is an important determinant of daily BP variation. NCC activity in cells is regulated by the circadian transcription factor per1. In vivo, circadian genes are entrained via the hypothalamic-pituitary-adrenal axis. Here, we test whether abnormalities in the daily variation of circulating glucocorticoid influence NCC activity and BP control. C57BL6/J mice were cycled at the peak (1:00 AM) and trough (1:00 PM) of BP. We found no day:night variation in NCC mRNA or protein but NCC phosphorylation on threonine53 (pNCC), required for NCC activation, was higher when mice were awake, as was excretion of NCC in urinary exosomes. Peak NCC activity correlated with peak expression of per2 and bmal1 (clock genes) and sgk1 and tsc22d3 (glucocorticoid-responsive kinases). Adrenalectomy reduced NCC abundance and blunted the daily variation in pNCC levels without affecting variation in clock gene transcription. Chronic corticosterone infusion increased bmal1, per1, sgk1, and tsc22d3 expression during the inactive phase. Inactive phase pNCC was also elevated by corticosterone, and a nondipping BP profile was induced. Hydrochlorothiazide restored rhythmicity of BP in corticosterone-treated mice without affecting BP in controls. Glucocorticoids influence the day:night variation in NCC activity via kinases that control phosphorylation. Abnormal glucocorticoid rhythms impair NCC and induce nondipping. Night-time dosing of thiazides may be particularly beneficial in patients with modest glucocorticoid excess. (Hypertension. 2016;67:00-00.)

DOI: 10.1161/HYPERTENSIONAHA.115.06977

Key Words: cardiovascular disease ■ circadian rhythm ■ diabetes mellitus ■ hypertension ■ risk factors

Blood pressure (BP) displays daily variation in healthy people, peaking midmorning and falling during night-time sleep.1 Loss or attenuation of the nocturnal BP dip (referred to as nondipping) incurs significant health risk by promoting cardiac, renal, and vascular injury.2 Nondipping is an early characteristic of diabetes mellitus preceding and predicting the development of microalbuminuria.3 In patients with chronic kidney disease, nocturnal hypertension is associated with a faster rate of decline in renal function.4,5 Even if clinic or mean 24-hour ambulatory BP is normal, loss of dipping significantly increases the risk of dialysis or incident cardiovascular mortality.6,7 Lowering sleep BP alone reduces risk of cardiovascular disease,8 suggesting that restoration of normal day:night BP variation is an important therapeutic goal.

Clinical studies show that the day:night variation in BP is strongly correlated with renal sodium excretion.9 The kidney is an important long-term regulator of BP and impaired pressure natriuresis may contribute to nocturnal hypertension10:10:10. BP remains elevated during sleep to facilitate sodium excretion and maintain 24-hour sodium balance.11,12 Certainly, hypertensive and prehypertensive patients have a blunted day:night pattern of sodium excretion and excrete a greater fraction of their daily output at night.13 Moreover, dietary sodium restriction can restore a dipping BP profile in salt-sensitive hypertensive patients,14 presumably by reducing sodium load below the day-time excretory threshold. Mechanistically, hydrochlorothiazide, either alone15 or in combination with amlodipine16 or valsartan,17 reduces night-time BP and restores dipping BP in nondipping hypertensive patients. This suggests that inappropriate activation of the thiazide-sensitive NaCl cotransporter (NCC) is a strong determinant of nocturnal BP.

NCC activity is modulated by multiple hormonal systems acting through a regulatory cascade of serine/threonine kinases, including WNKs, SPAK, and OSR1.18 These control
phosphorylation at conserved residues in the N terminus, and thereby influence NCC activity and trafficking into the apical membrane. The distal tubule also expresses the canonical circadian transcription factors per1/2, bmal1, clock, and cry1/2, and a recent cell-line study demonstrated transcriptional regulation of NCC and WNK kinases by per1. It is increasingly evident that circadian transcription factors influence renal function. Glucocorticoids play an important role in the entrainment of renal clocks to the day:night cycle. In the aldosterone-sensitive distal nephron, glucocorticoid actions are normally limited because of metabolism by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). However, modest glucocorticoid excess promotes sodium retention by activating transport in the aldosterone-sensitive distal nephron. Such perturbations, whether iatrogenic or because of conditions such as Cushing syndrome, also impair BP rhythmicity and induce a nondipping BP profile.

Because 11βHSD2 is only expressed in some cells of the distal convoluted tubule (DCT), we hypothesized that glucocorticoids set the day:night variation of NCC activity. Using the complementary approaches of adrenalectomy and chronic corticosterone infusion, we found that flattening the day:night variation in hypothalamic–pituitary–adrenal axis activity also flattened the variation in NCC activity, inducing a nondipping BP, which could be restored by thiazide therapy.

Methods
Detailed Methods are available in the online-only Data Supplement.

Animals
C57BL/6J/Ola mice (Harlan, United Kingdom) were used between 2 and 4 months of age. Mice were acclimatized to a 12-hour light:dark cycle for at least 2 weeks before experiments and given free access to water and standard chow (maintenance diet 1; Special Diet Services, Essex, United Kingdom). The lights were turned on at 7:00 AM local time, and this was defined as Zeitgeber time 0 (ZT0); lights were turned off at 7 PM (ZT12). Experiments were performed under university license from the UK Home Office and after approval by the University veterinary services.

Kidney Collection
Mice were culled by cervical dislocation <1-minute after removal from the holding room. Culls were performed at both 1:00 PM (ZT6) and 1:00 AM (ZT18) local time. The kidneys were rapidly excised and decapsulated, snap-frozen on dry ice and stored at −80°C.

Bilateral Adrenalectomy
This was performed under isoflurane anesthesia and buprenorphine (Vetergesic 0.1 mg/kg sc) analgesia. After recovery, adrenalectomized and control mice were individually housed and given ad libitum access to 0.9% saline and tap water.

Chronic Corticosterone Infusion
Elastomer pellets were used to encapsulate corticosterone for slow release. In vitro, the release rate was ≈3.7 mg/kg per day, and this was maintained during a 4-week period. Pellets were implanted subcutaneously under isoflurane anesthesia and buprenorphine analgesia (Vetergesic 0.1 mg/kg sc).

Plasma Corticosterone Measurements
Approximately 20-μL blood was sampled from conscious mice via the tail vein at local time 7:00 AM and 7:00 PM, unless otherwise stated. Plasma was stored at −80°C before measurement of corticosterone concentration by commercial EIA (Enzo Life Sciences, Exeter, United Kingdom).

Daily Variation in Renal Excretion
Control mice were single housed in metabolic cages, and after 5 days of acclimatization, food intake, water intake, urine output, and body weight were measured at 12-hour intervals. The concentration of sodium in urine was measured by flame photometry and used to calculate excretion rate in μmol/h. Urine concentration of both aldosterone and corticosterone was measured by ELISA.

Measurement of NCC in Urinary Exosomes
The number of exosomes in urine samples was measured by Nanoparticle Tracking Analysis (NanoSight Ltd, Amesbury, United Kingdom), as described.

Immunoblotting
Western blots were performed on homogenized whole-kidney samples as we have described, using primary antibodies against NCC, pThr53-NCC, anti-pThr58-NCC, anti-pSer71-NCC, and anti-NKCC2. Images were developed by electrochemiluminescence and quantified by densitometry using ImageJ, as described. The cross-reactivity of NCC phospho-antibodies was determined by immunoprecipitation experiments. Anti-pThr53-NCC and anti-pSer71-NCC were selective for NCC but anti-pThr58-NCC showed significant cross-reactivity with NKCC2 (Figure S1).

Quantitative Polymerase Chain Reaction
RNA was extracted from whole kidney and used for quantitative polymerase chain reactions with the Roche Universal ProbeLibrary (Data Supplement; Table). TB, HPRT, and 18S rRNAs were used as endogenous control genes.

Immunofluorescence
Kidneys were fixed by aortic perfusion of 4% paraformaldehyde and then embedded in paraffin, sectioned at 5 μm and mounted onto glass slides. Sections were double-immunostained to colocalize NCC expression with glucocorticoid receptor, mineralocorticoid receptor (MR), or 11βHSD2.

BP Measurement
Radiotelemetry devices were inserted into male C57BL/6 mice under isoflurane anesthesia. Data were collected during a 1-minute period every 30 minutes at an acquisition rate of 1 KHz. Basal measurements were obtained >7 days before corticosterone (n=5) or blank (n=6) elastomer pellets were implanted subcutaneously. After 11 days, hydrochlorothiazide (80 mg/kg per day) was administered in drinking water. Hydrochlorothiazide concentration in plasma samples taken on the final experimental day was measured by liquid chromatography–mass spectrometry.

Statistical Analysis
Data are presented as mean±95% confidence interval. The number of biological replicates (n) for each experimental group is given in the figure legends along with the test used for statistical analysis. The arithmetic mean BP was calculated for periods of subjective day, when mice were asleep, and subjective night, when mice were awake. Cosinor analysis was also used to calculate mesor (rhythm-adjusted mean) as the central tendency, period, amplitude, and acrophase. Data were smoothed using a moving average during 5 hours. For each experimental phase (baseline, corticosterone, and thiazide), the smoothed data for each individual mouse was collected in bins of 5 consecutive days and fitted by the least squares methods to a cosinor curve using software available at www.circadian.org, as described. Robustness/prominence of the rhythm was calculated as the percentage of the variance accounted for by the cosinor model. Statistical significance of the goodness of fit was P<0.000001 for each analysis and was corrected for multiple tests.
We measured the excretion of NCC in urinary exosomes as a noninvasive surrogate of transporter abundance in the DCT apical membrane. As for pNCC abundance, exosomal NCC excretion had a marked day:night variation and was higher during the active phase (Figure 1D).

**Adrenalectomy Reduces NCC Expression and Blunts Day:Night pNCC Variation**

Adrenalectomy significantly reduced circulating corticosterone levels and abolished day:night variation (Figure 2A). Although slc12a3 mRNA levels were not changed by adrenalectomy (Figure S8), total NCC protein was reduced by \(\approx 50\%\) (Figure 2B). There was no significant day:night variation in pNCC levels in adrenalectomized mice (Figure 2C), and the variation in tsc22d3 expression was blunted (Figure S8). The day:night variation in the circadian genes per2, cry1, and bmal1 was maintained after adrenalectomy (Figure S8).

**Clamping Plasma Corticosterone Abolishes Daily pNCC Variation**

Next, we implanted slow-release corticosterone pellets, which eliminated the day:night variation and clamped corticosterone in the midphysiological range at \(\approx 200\) nmol/L (Figure 3A). The day:night variation in aldosterone levels was not affected by this maneuver (Figure 3B). Slc12a3 mRNA (Figure S9) and total NCC abundance (Figure 3C; Figure S10) were not changed by chronic corticosterone infusion but pNCC variation was abolished (Figure 3D; Figure S10). This reflected increased pNCC levels during the inactive phase in corticosterone-treated mice. Glucocorticoid clamping also increased expression of tsc22d3 and sgk1 during the inactive period, as well as levels of cry1, bmal1 and per1 (Figure S9). A small but significant reduction in active phase urinary sodium excretion was recorded in corticosterone-treated mice (Figure 3E), which was not attributable to reduced food intake (Figure 3F). Sodium excretion during the inactive phase was not different from controls.

**Clamping Plasma Corticosterone Induces a Nondipping BP Profile**

SBP (Figure 4A) and diastolic blood pressure (DBP; Figure 4B) were measured in mice by radiotelemetry before and after implantation of slow-release corticosterone (n=5).
or control (n=6) pellets and then during thiazide therapy. Before implantation, daily rhythms of BP, heart rate, and locomotor activity were robust; acrophase, amplitude, and mesor were not different between groups (Table). Pellets were then implanted and, after recovery from anesthetic, the vehicle group immediately regained the daily rhythm of SBP (Figures 4A, 5A, and 5C) and DBP (Figures 4B and 5D), which were not different from preimplant values. The corticosterone-treated group took longer to regain 24-hour periodicity and when they did, SBP was raised during sleep compared with baseline values in all mice (Figure 5A and 5C). Corticosterone treatment increased DBP both in the active and in the inactive phases (Figure 5D).

Changes to the day:night variation in SBP did not reflect changes in either locomotor activity or heart rate, as assessed by telemetry (Figure S1).

Cosinor analysis was performed on 5-day bins of steady-state SBP (indicated by the blue boxes on Figure 4A). Corticosterone treatment significantly (P<0.001) increased mesor and significantly decreased both the amplitude and robustness (P<0.001) of the underlying rhythm (Table).

### Chronic Thiazide Therapy Restores a Dipping BP Profile

In the next phase of the experiment, the mice were given hydrochlorothiazide in drinking water, achieving a plasma concentration of ≈15 μmol/L in both the groups. This did not alter BP in the control group but had a significant antihypertensive effect in the corticosterone-treated mice (Figure 5B). Thiazide reduced SBP during the inactive phase without affecting SBP during the active phase (Figure 5C). A similar profile was observed for DBP, being reduced by hydrochlorothiazide treatment when mice were asleep but remaining elevated during the active phase (Figure 5D). Cosinor analysis showed that hydrochlorothiazide restored a robust day:night rhythm for SBP in corticosterone-treated mice, reducing mesor and increasing amplitude to levels not significantly different from controls (Table).

---

**Figure 1.** Day:night variation of gene expression (A), total NaCl cotransporter (NCC) protein (B), phosphorylated NCC (pT53-NCC; C), and exosomal NCC (D) in C57BL6 mice. Kidneys were harvested at Zeitgeber time (ZT) 6 (day) and ZT18 (night) for immunoblots and quantitative polymerase chain reaction using whole kidney homogenate. A–C, Exosomal NCC was measured by Nano-tracking gated for 20- to 100-nm particles labeled with Q-dot conjugated NCC antibody in 12-hour urine samples collected between ZT0–12 (day/sleep) and ZT12–0 (night/active). Two-way ANOVA with post hoc Sidak correction was carried out on gene expression data (n=15). Day:night total (n=6) and phosphorylated NCC (n=6) normalized densities in arbitrary units were compared by unpaired t tests. Exosomal NCC was corrected for urinary volume and analyzed by paired t tests. *P<0.0001, †P<0.001, ‡P<0.01, nonsignificant (ns) P>0.05. All data are mean±95% confidence interval.
Discussion

We find that NCC has a day:night variation of activity determined by phosphorylation of existing protein rather than by gene transcription. This variation is influenced by the rhythm of circulating glucocorticoid. If this hormonal profile is flattened, NCC is inappropriately activated during the inactive phase, inducing a nondipping BP profile. Hydrochlorothiazide, which inhibits NCC, reduced BP during the inactive phase only in corticosterone-treated mice, restoring a robust day:night variation in BP.

Figure 2. Day:night variation of plasma corticosterone (A and B), total NaCl cotransporter (NCC; C), and phosphorylated NCC (pNCC) protein (D) in adrenalectomized (ADX) and adrenal intact C57BL6 mice. A pooled profile of 24-hour plasma corticosterone was generated from composite terminal and spot plasma collections taken from ADX and control mice (A, n=8–20). Matched 2-way ANOVA was only performed on Zeitgeber time (ZT) 0 and ZT12 spot plasma samples collected concomitantly (B). The bar at the top of A and B indicates periods of light and dark. Kidneys were harvested at during the day when mice were asleep (ZT6) or at night when mice were awake (ZT18). Signal densities for immunoblots were normalized to Coomassie signal densities and compared with 2-way ANOVAs followed by post hoc Sidak correction. *P<0.0001, †P<0.001, ‡P<0.01, §P<0.05, nonsignificant (ns) P>0.05. All data are mean±95% confidence interval.

Daily NCC Activity Is Determined by Phosphorylation

Around 4000 genes in the DCT exhibit day:night variation at the transcriptional level, of which ≈10% fit a circadian profile with a period of 24 hours. Genes found with circadian rhythmicity included clock transcription factors, such as per1/2, cry1/2, and bmal1. Glucocorticoid-induced genes, such as the kinase sflk1 and the transcriptional regulator tsc22d3, also displayed a circadian profile of transcription at whole-kidney level. Our data are consistent with this literature (day:night
transcriptional changes were found in per2, cry1, bmal1, sgk1, and tsc22d3); however, a limitation of our work is that it lacked the temporal resolution to detect rhythmic changes that have been reported for per1,19,32 cry2,19,32 mo25α,32 cul3,32 and WNK132 expression. It is also important to note that we assessed transcription at the whole-kidney level and cannot, therefore, discriminate between glucocorticoid-sensitive regions of the kidney and those with minimal glucocorticoid sensitivity (ie, the aldosterone-sensitive distal nephron) because of expression of 11βHSD2. In such regions sgk1 and tsc22d3 might be regulated by aldosterone.

Slc12a3, which encodes NCC, had no day:night rhythm of transcription,19,32 as we have confirmed here. Our data at the whole-kidney level underscore the concept that day:night variation in NCC activity is regulated by post-transcriptional modification of existing protein.19 Of the 3 important residues thus far defined, our data show day:night control of phosphorylation of Thr53, but not Ser71. We do not present data for Thr58 phosphorylation, as we found significant cross-reactivity of this antibody with NKCC2, which is present in our samples.

pNCC abundance is often used as a surrogate marker for NCC activity as it correlates with thiazide-sensitive transport in cells34,32 and in the intact kidney.39 We chose not to assess thiazide-sensitive sodium transport directly because the requisite approaches either increase corticosterone35 or abolish day:night rhythms of renal function.36 We instead used the excretion of NCC in urine exosomes to provide a noninvasive snap-shot of transporter abundance in the apical membrane of the DCT.37,38 Exosomal NCC excretion was higher when mice were active, as recently described in healthy humans.39 In combination, these complementary experimental strategies indicate that thiazide-sensitive sodium transport is normally reduced during the inactive phase, correlating with the fall in BP and the lower ambient glucocorticoid level.

How Do Glucocorticoids Influence Daily Variation in NCC Activity?
The circadian rhythm of circulating glucocorticoids can entrain renal clock gene transcription.22 One hypothesis is that circadian transcription factors are the link between circulating corticosterone and phosphorylation/activation of NCC. Per1 does regulate NCC activity, binding the promotor of slc12a3 and wnk4 to positively regulate transcription in a DCT cell line.20 In our study, adrenalectomy abolished the daily variation of pNCC abundance but did not uniformly affect the variation in clock gene expression. A clearer relationship was observed in mice chronically infused with glucocorticoid. In this scenario, increased pNCC levels during the inactive phase were associated with increased transcription of per1, cry1, and bmal1. A clearer relationship to pNCC abundance across all experimental paradigms was with sgk1 and tsc22d3 expression. An alternative hypothesis is that the circadian variation in glucocorticoids directly controls NCC activity by engaging a network of regulatory kinases downstream of a corticosteroid...
receptor and upstream of the WNK/SPAK/OSR1 kinase cascade. Indeed, synthetic glucocorticoids increase the expression/activity of NCC, and recent studies show that sgk1 enhances NCC activity indirectly by suppressing degradation of WNK1 kinase. Because we found that most DCT cells do not express 11βHSD2, corticosterone could induce sgk1 expression through either MR or glucocorticoid receptor activation: MR is the most plausible route because MR antagonists abolish the endogenous rhythm of the WNK1/SPAK/OSR1 phosphorylation cascade. Nevertheless, an important limitation to our study is that gene expression and protein phosphorylation were assessed at only 2 time points. This lack of temporal resolution precludes cosinor analysis of these data sets and limits our ability to assess the relationship between glucocorticoid-activated transcriptional pathways and NCC activation.

**How Do Glucocorticoids Induce a Nondipping BP Profile?**

We chronically infused corticosterone such that plasma levels were held midway between the physiological peak and trough. This rapidly induced nondipping BP, reflecting a significant rise in BP during the inactive phase. Our approach models the loss of nocturnal BP observed in patients receiving chronic glucocorticoid therapy. We also discount a major role for reduced arterial compliance because DBP increased (throughout the 24-hour cycle), rather than fell, during corticosterone treatment. The most plausible explanation of our data are that corticosterone activates the WNK/SPAK/OSR1 cascade and clamps NCC on throughout the 24-hour cycle. This impairs the normal modulation of BP by pressure natriuresis, and in consequence BP does not fall during sleep to facilitate sodium excretion and maintain balance. Indeed, corticosterone infusion did not affect overall sodium balance but reduced sodium excretion during the active phase. This is consistent with data from normotensive humans: those excreting a greater proportion of the daily sodium load during sleep have a reduced dip during sleep. This concept is reinforced by data in adrenalectomized mice. These animals do not activate NCC during periods of activity and cannot maintain sodium balance by modulating renal excretion: BP lacks day:night variation because peak BP is significantly reduced.

**Perspectives**

Hydrochlorothiazide monotherapy was previously shown to reduce night-time BP in essential hypertensive patients, but only those with a nondipping BP profile. Night-time dosing of hydrochlorothiazide (in combination with valsartan)
is more effective than daytime dosing at reducing BP during sleep.\textsuperscript{17} Our study provides mechanistic insight into these outcomes and suggests that thiazides, or thiazide-like diuretics with a longer half-life could improve BP control over the 24-hour cycle in certain patients. Measuring urinary exosomal NCC in morning urine samples would be an effective and noninvasive means of patient stratification. We predict benefit in nondipping patients on glucocorticoid therapy or those with abnormal peripheral glucocorticoid metabolism. Restoring dipping BP profile through thiazide diuretics would be a cost-effective way of reducing cardiovascular risk in these patients.

Acknowledgments

T.S. Peltz was awarded a scholarship by The Physiological Society. We thank the British Heart Foundation Centre of Research Excellence Award for supporting the telemetry studies.

Sources of Funding

J.R. Ivy and A.R. Howarth were funded by PhD studentships from the British Heart Foundation (FS/11/78/29328 and FS/13/52/30637).

Disclosures

None.

References


Figure 5. A, Corticosterone (gray lines) increased systolic blood pressure (SBP) when mice were asleep but not during the active period. B, Chronic hydrochlorothiazide (HCTZ) treatment lowered sleep-time SBP in corticosterone-treated mice (gray line) but not in controls (black line). The bar at the top of A and B indicates periods of subjective day (when mice were asleep) and night (when mice were awake). The analysis was performed on 5-day bins of raw SBP data (blue box in Figure 4) and a group average calculated every 30 minutes in the 24-hour cycle. The average SBP (C) and diastolic blood pressure (DBP; D) were also calculated during subjective day (Zeitgeber time [ZT] 3–8) when mice were asleep and night (ZT15–20), when mice were awake. Data were analyzed by matched 2-way ANOVA with post hoc Sidak correction. *P<0.05, nonsignificant (ns) P>0.05, n=6 (vehicle), n=5 (corticosterone). Data are means±95% confidence interval (CI), where thick lines are mean and thin-dotted lines are 95% CI.


Glucocorticoids Induce Nondipping Blood Pressure by Activating the Thiazide-Sensitive Cotransporter


Hypertension. published online March 7, 2016;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2016/03/07/HYPERTENSIONAHA.115.06977

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2016/03/07/HYPERTENSIONAHA.115.06977.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
SUPPLEMENTARY MATERIAL

Glucocorticoids induce non-dipping blood pressure by activating the thiazide-sensitive co-transporter

Jessica R. Ivy1, Wilna Oosthuyzen1, Theresa Peltz1, Amelia R. Howarth1, Robert W. Hunter1, Neeraj Dhaun1, Emad A.S. Al-Dujaili2, David J. Webb1, James, W. Dear1, Peter W. Flatman3, Matthew A. Bailey1

1The British Heart Foundation Centre for Cardiovascular Science The University of Edinburgh; 2Dietetics, Nutrition and Biological Sciences Department, Queen Margaret University and 3The Centre for Integrative Physiology, The University of Edinburgh, United Kingdom

Correspondence to: Matthew A. Bailey, PhD
University/BHF Centre for Cardiovascular Science,
The Queen’s Medical Research Centre,
The University of Edinburgh,
47 Little France Crescent,
Edinburgh, EH16 4TJ United Kingdom

Email Matthew.Bailey@ed.ac.uk
Supplementary Methods:

**Measurement of NCC in urinary exosomes**: The number of exosomes in urine samples was measured by Nanoparticle Tracking Analysis (NTA, NanoSight Ltd, Amesbury, UK), as described. NCC-containing exosomes were identified by fluorescence after conjugation of a rabbit anti-NCC antibody (AB3553; EMD Millipore) to a quantum dot (Qdot 605 Anti-body Conjugation Kit; Invitrogen, UK). For fluorescent NTA analysis, a 532nm (green) laser diode excited the Qdots with a long-pass filter (430nm) so that only fluorescent particles were tracked and labeled-particle concentration determined by NTA software.

**Immunoblotting**: Western blots were performed as we have described. Briefly, kidneys were homogenized in a sucrose buffer containing phosphatase, kinase, and protease inhibitors, and protein samples were resolved by SDS-PAGE (NuPAGE Novex 3%–8% Tris-acetate gels; Invitrogen, UK) and transferred to PVDF membrane (Amersham Hybond-P GE Healthcare). Primary antibodies used were: rabbit anti-NCC (AB3553; EMD Millipore, 1:1000); sheep anti-pThr53-NCC, anti-pThr58-NCC, anti-pSer71-NCC (each at 1:500), and anti-NKCC2 (1:10,000, all from Division of Signal Transduction Therapy, Dundee University, UK). Secondary antibodies were: goat anti-rabbit IgG-HRP (sc-2030; Santa-Cruz Biotechnology, 1:2000), and donkey anti-sheep IgG-HRP (A3415; Sigma-Aldrich, 1:20,000). Images were developed by electrochemiluminescence and quantified by densitometry using ImageJ. The bottom <50kDa of the gel was coomassie stained and used as a loading control, as described. The cross-reactivity of NCC phospho-antibodies was determined by immunoprecipitation experiments. NCC and NKCC2 were separately pulled-down from whole kidney homogenates and the products probed using pThr53-NCC, pThr58-NCC and pSer71-NCC antibodies. Anti-pThr53-NCC and anti-pSer71-NCC were selective for NCC but anti-pThr58-NCC showed significant cross-reactivity with NKCC2 (See Supplemental Material, Figure S1).

**Quantitative-PCR**: RNA was extracted from frozen kidneys (RNeasy Mini Kit, Qiagen) and treated on-column with DNase I. cDNA was transcribed from 500 ng of total RNA and used for qPCR reactions with the Roche Universal ProbeLibrary (See Supplemental Material, Table 1). TBP, HPRT and 18S rRNAs were used as endogenous control genes. Their expression did not differ across any of the experimental groups. CT values were approximately 25-33, 22-28, 7-15 for TBP, HPRT and 18S, respectively. Test gene CT values ranged from 22 to 37. The level of endogenous control genes was normalized such that day/vehicle/sham was equal to 1.

**Immunofluorescence**: Kidneys were fixed by aortic perfusion of 4% paraformaldehyde and then embedded in paraffin, sectioned at 5μm and mounted onto glass slides. Sections were double-immunostained to co-localise NCC expression with GR, MR or 11βHSD2; all sections were counterstained with DAPI (Immunodetection and Imaging Facility; The University of Edinburgh). Images were acquired using a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope. DAPI, Cy3 and Cy5 were excited using a blue diode 405nm laser and HeNe 546nm and 633nm lasers respectively. Tiled images (~7x7) were captured through a 20X objective lens.

**BP measurement**: Radiotelemetry devices (Model TA-11PAC-10, Data Systems International, UK) were inserted into male C57BL/6 mice under isoflurane anesthesia. After a week of post-operative recovery, data were collected over a 1-minute period every 30 minutes at an acquisition rate of 1kHz. Basal measurements were obtained over 7 days before corticosterone (n=5) or blank (n=6) elastomer pellets were implanted subcutaneously. In order to verify that the plasma corticosterone clamping was sustained throughout the experiment, blood was sampled at ZT0 and ZT12 in vehicle and corticosterone treated mice on the last day of the telemetry experiment i.e. four weeks after implantation. After 11 days, hydrochlorothiazide (HCTZ; 80mg/kg/day) was administered in drinking water. The mice did not change their intake of water following HCTZ dosing. HCTZ concentration in plasma samples taken on the final experimental day was measured by LC/MS.
REFERENCES


### Table S1: Table of qRT-PCR assays.

<table>
<thead>
<tr>
<th>gene</th>
<th>accession number</th>
<th>forward primer</th>
<th>reverse primer</th>
<th>UPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn18s</td>
<td>NR_003278.1</td>
<td>ctcaacagggaaacctcacc</td>
<td>ccgcccaaccctaaagc</td>
<td>77</td>
</tr>
<tr>
<td>Tbp</td>
<td>NM_013684</td>
<td>gggagaatcctgacccaga</td>
<td>gatgggaatctcagggctga</td>
<td>97</td>
</tr>
<tr>
<td>HPRT</td>
<td>NM_013556.2</td>
<td>ctcttcagacctctttt</td>
<td>aaccttggtcatctgctaa</td>
<td>95</td>
</tr>
<tr>
<td>per1</td>
<td>NM_001159367.1</td>
<td>gctctggactgacacct</td>
<td>tgctttagatgcccgttgt</td>
<td>71</td>
</tr>
<tr>
<td>per2</td>
<td>NM_011066</td>
<td>gctctgagactgacaacct</td>
<td>tgctttagatgccccaggtgt</td>
<td>5</td>
</tr>
<tr>
<td>clock-201</td>
<td>NM_007715.5</td>
<td>ccagtcagttgctcattt</td>
<td>tgctttagatgctctgaaa</td>
<td>76</td>
</tr>
<tr>
<td>cry1</td>
<td>NM_007771.3</td>
<td>ggcagagcgctgaactacga</td>
<td>tgactttcccaccaactctca</td>
<td>52</td>
</tr>
<tr>
<td>cry2</td>
<td>NM_009963.4</td>
<td>ggagctcagcaacactgagc</td>
<td>ccgctttgctcactttcacc</td>
<td>11</td>
</tr>
<tr>
<td>amt1 var1</td>
<td>NM_001243048</td>
<td>gaatacattgtctcaaccaacactg</td>
<td>ttagctgccggaaggtggt</td>
<td>97</td>
</tr>
<tr>
<td>amt1 var2</td>
<td>ENSMUST00000106637.1</td>
<td>agtagcgcctccccctgat</td>
<td>tgctttgctcactttcacc</td>
<td>79</td>
</tr>
<tr>
<td>Sgk1</td>
<td>NM_001161845</td>
<td>gattgccagacacacctatg</td>
<td>tgatgctgagagggacttag</td>
<td>49</td>
</tr>
<tr>
<td>TSC22d3 v2</td>
<td>NM_010286.3</td>
<td>tccgtaacctttgatacagtc</td>
<td>tggctttcagacaggggtct</td>
<td>49</td>
</tr>
<tr>
<td>MR</td>
<td>NM_001083906</td>
<td>caaaagagcgctggaagg</td>
<td>ttcttccgaatctttctacataatgc</td>
<td>11</td>
</tr>
<tr>
<td>GR</td>
<td>NM_008173</td>
<td>tgacgtgctggaagctgtaatgt</td>
<td>cattttccagcacaaggt</td>
<td>56</td>
</tr>
<tr>
<td>Hsd11b2</td>
<td>NM_0086289.2</td>
<td>caacttgagggagcagattgt</td>
<td>gctgggtgtggactgcttct</td>
<td>26</td>
</tr>
<tr>
<td>Slc12a3</td>
<td>NM_019415</td>
<td>cctccatcacaatactctcct</td>
<td>cgccccatcttctgtgtaa</td>
<td>12</td>
</tr>
<tr>
<td>Oxsr1</td>
<td>NM_133985</td>
<td>tgctttcaaaagcatccaga</td>
<td>tggaaaaattgtgccctacac</td>
<td>84</td>
</tr>
<tr>
<td>Stk39</td>
<td>ENSMUST00000102715</td>
<td>gtacagctcaggaggttatc</td>
<td>tggctttcggggtctgtct</td>
<td>27</td>
</tr>
</tbody>
</table>
**Table S2:** Parameters obtained from cosinor analysis of C57BL6 mice telemetry data. Data are mean± 95% CI. (n=4)

<table>
<thead>
<tr>
<th>Variable</th>
<th>SBP</th>
<th>DBP</th>
<th>HR</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Period (hours)</strong></td>
<td>23h09±0h22</td>
<td>23h09±0h18</td>
<td>23h48±0h12</td>
<td>23.48±0h12</td>
</tr>
<tr>
<td><strong>Acrophase (ZT)</strong></td>
<td>18h10±0h47</td>
<td>18h09±0h49</td>
<td>17h06±0h05</td>
<td>17h06±0h05</td>
</tr>
<tr>
<td><strong>Amplitude (mmHg/bpm/counts)</strong></td>
<td>10.0±1.8</td>
<td>9.8±1.5</td>
<td>57.0±22</td>
<td>6.24±1.2</td>
</tr>
<tr>
<td><strong>MESOR (mmHg/bpm/counts)</strong></td>
<td>125±2</td>
<td>95±5</td>
<td>507±7</td>
<td>7±2</td>
</tr>
<tr>
<td><strong>Robustness (%)</strong></td>
<td>26.3±4.3</td>
<td>23.9±3.3</td>
<td>12.5±5.0</td>
<td>28.5±1.9</td>
</tr>
</tbody>
</table>
**Immunoprecipitation (IP) for specificity of NCC phospho-antibodies:**

The NCC phospho-motifs are conserved in other related transporters, particularly NKCC2. Threonine 53 and 58 on NCC shares homology with Threonine 96 and 101 on NKCC2. Therefore IP was performed to test the specificity of the NCC phospho-antibodies. Superparamagnetic beads (Dynabeads™ Protein G, Life Technologies) were washed and conjugated to NCC and NKCC2 antibodies according to the manufacturer’s instructions. 2500mg kidney homogenate was added to the bead-antibody mixture and placed on a magnet to “pull-down” proteins bound to the antibodies. The antigens were eluted and resuspended (in 20μL Dynabeads™ elution buffer, Life Technologies, 3μL DTT and 7.5μL LDS) and, along with a standard kidney homogenate, were Western blotted.

**Figure S1: Cross-reactivity of anti-NCC antibodies**

Immunoprecipitation was used to “pull down” NCC or NKCC2 from 2500 μg of total kidney homogenate. These immunoprecipitation products (+) were then separated on 3-8% Tris Acetate gel, transferred to Hybond P membrane and immunoblotted for the NCC phospho-antibodies and NKCC2. 12 μg of total kidney homogenate (-) was run alongside the IP products and probed directly as a positive control.
Figure S2: 24-hour systolic blood pressure (A), diastolic blood pressure (B), heart rate (C) and activity (D) in C57BL6 mice. Data were collected using radiotelemetry and are shown as hourly mean over 5-day batches (thick line) ±95% CI (thin dotted line) (n=4). The white bar indicates subjective day, when mice were inactive; the black bar indicates the subjective night, when mice were active. Lights were turned on at 7am local time (ZT0).
Figure S3: Urinary aldosterone (A), corticosterone (B), sodium (C) and potassium (D) excretion and food intake (E). Urine samples were collected over subjective day (black circle; ZT0-12), when mice were inactive and over the night (red square; ZT12-0), when mice were active. These data are the average for each mouse over two consecutive days’ measurements. Data are mean ±95% CI. Statistical comparisons were made using paired Student’s t tests, ****P<0.0001 ***P<0.001. **P<0.01 (n=10).
Figure S4: Representative images of immunofluorescent staining of (A) NCC (green) and MR (red) and (B) NCC (green) and GR (red) in male C57BL6 mice. High-powered views of yellow-boxed regions are displayed. Bar, 100μm in large top/bottom panels; 10μm in high-powered middle panels.
Figure S5: Immunofluorescent staining for NCC (green) and 11HSD2 (red) in 3 (A-C) male C57BL6 mice. High-powered views of yellow-boxed regions are given on the right where blue arrows indicate regions in which 11βHSD2 and NCC staining are within the same cell/tubule. Bar, 100μm (left-hand panels); 10μm (right hand panels).
Figure S6: Day:night variation of NCC phosphorylation at Thr53 in female C57BL6 mice. Kidneys were harvested at ZT6 (day/inactive phase) and ZT18 (night/active phase) for immunoblots. No attempt was made to synchronize the oestrus cycles of these mice. Signal densities for immunoblots were normalised to Coomassie signal density and compared by unpaired *t*-tests, **P<0.01, All data are mean ± 95% CI.

Figure S7: Day:night variation of NCC phosphorylation at Ser71 in male C57BL6 mice. Kidneys were harvested at ZT6 (day/inactive phase) and ZT18 (night/active phase) for immunoblots. Signal densities for immunoblots were normalised to Coomassie signal density and compared by unpaired *t*-tests. ns, P>0.05. All data are mean ± 95% CI.
Figure S8: Gene expression in kidneys of adrenalectomised (ADX) and adrenal intact C57BL6 mice. Control and ADX mice were single housed and culled at day 9 post surgery during the subjective day when inactive (black circle; ZT6) or night (red square; ZT18), when active. The encoding gene is given with name of the resulting protein in brackets. Gene expression of each gene was normalised to the average expression of 18S, TBP and HPRT. Data are mean ± 95% CI. Data were analysed with two-way ANOVA followed by post hoc Sidak correction, where ***P<0.001, **P<0.01 *P<0.05, ns P>0.05 (n=5-6).
Figure S9: Gene expression in kidneys of corticosterone (Cort) or vehicle treated C57BL6 mice. Mice were culled during the subjective day when inactive (black circle; ZT6) or night (red square; ZT18), when active. The encoded protein name is given in brackets. Gene expression of each test gene was normalised to the average expression of 18S, TBP and HPRT. Data are mean ± 95% CI. Data were analysed with two-way ANOVA followed by post hoc Sidak correction, where ****P<0.0001, ***P<0.001, **P<0.01 *P<0.05, ns P>0.05 (n=9)
Figure S10: Immunoblots for pT53 NCC (A) pS71 NCC (B) and total NCC (C) in vehicle and corticosterone treated mice. Mice were culled in groups of 3 control and 3 experimental animals during the subjective day when inactive (ZT6) or night (ZT18), when active. The experiment was performed on three separate occasions and immunoblots were performed in these batches (1-3). “R” is reference load, which was a pooled sample that was used to normalise across the three batches of immunoblots.
Figure S11: Heart rate and activity in C57BL6 mice. After 11 days of baseline recording, mice received either corticosterone (red line) or control (black line) pellets. After a further 11 days, all mice received HCTZ (80 mg/kg) in their drinking water. Data displayed here are the moving average (over 5 hours) of heart rate (A) and activity (D) throughout the duration of the experiment. The blue rectangles indicate 5-day bins that were taken forward for further analysis. Summary mean daytime and nighttime heart rate (B, C) and activity (E, F) were obtained by taking the mean over the 5-day bins for subjective day, when mice were inactive (ZT3-8) and night (ZT15-20), when mice were active. Data are mean ± 95% CI, where thick lines are mean and feint lines are 95% CI. Summary data were analysed by matched two-way ANOVA with post hoc Sidak correction. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 ns, not significant P>0.05, n=6 (vehicle) n=5 (corticosterone).