Possible role of brain salt-inducible kinase 1 in responses to central sodium in Dahl rats

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In Dahl salt sensitive (S) rats, Na⁺ entry into the cerebrospinal fluid (CSF) and sympatho-excitatory and pressor responses to CSF Na⁺ are enhanced. Salt-inducible kinase 1 (SIK1) increases Na⁺/K⁺-ATPase activity in kidney cells. We tested the possible role of SIK1 in regulation of CSF [Na⁺] and responses to Na⁺ in the brain. SIK1 protein and activity were lower in hypothalamic tissue of Dahl S (SS/Mcw) compared to salt-resistant SS.BN13 rats. Intra-cerebroventricular (icv) infusion of the protein kinase inhibitor staurosporine at 25 ng/d, to inhibit SIK1 further increased BP and HR, but did not affect the increase in CSF [Na⁺] or hypothalamic aldosterone in Dahl S on high salt. Icv infusion of Na⁺-rich artificial CSF caused significantly larger increases in renal sympathetic nerve activity, BP and HR in Dahl S versus SS.BN13 or Wistar rats on regular salt. Icv injection of 5 ng staurosporine enhanced these responses, but the enhancement in Dahl S rats was only 1/3 that in SS.BN13 and Wistar rats. Staurosporine had no effect on BP and HR responses to icv Ang II or carbachol, whereas the specific protein kinase C inhibitor GF109203X inhibited pressor responses to icv Na⁺-rich artificial CSF or Ang II. These results suggest that the SIK1 -Na⁺/K⁺-ATPase network in neurons acts to attenuate sympatho-excitatory and pressor responses to increases in brain [Na⁺]. The lower hypothalamic SIK1 activity and smaller effect of staurosporine in Dahl S rats suggests that impaired activation of neuronal SIK1 by Na⁺ may contribute to their enhanced central responses to sodium.

**Key words:** salt-inducible kinase, salt-induced hypertension, renal sympathetic nerve activity, CSF [Na⁺].
In Dahl salt-sensitive (S) rats, both enhanced Na\(^+\) entry from the blood into the cerebrospinal fluid (CSF) and enhanced neuronal excitatory responses to Na\(^+\) contribute to salt-induced sympathetic hyperactivity and hypertension (17, 19). In Dahl S on high salt, enhanced Na\(^+\) transport, in part via Na\(^+\)/K\(^+\)-ATPase in the choroid plexus (1, 17), increases Na\(^+\) entry into the CSF and CSF [Na\(^+\)]. High salt intake in Dahl S rats or chronic intra-cerebroventricular (icv) infusion of Na\(^+\)-rich artificial CSF (aCSF) in Wistar rats appears to increase local production of aldosterone in the hypothalamus (14, 20) and thereby increases release of endogenous ouabain-like factor (EO) (14, 20, 40). EO may inhibit Na\(^+\)/K\(^+\)-ATPase in the choroid plexus (23) and thereby attenuate Na\(^+\) entry into the CSF (17). All 3 α-isoforms of Na\(^+\)/K\(^+\)-ATPase are also expressed in hypothalamic nuclei such as the paraventricular (PVN) and supraoptic (SON) nuclei (26). Microinjection of ouabain into the PVN causes dose-related increases in BP and HR, which can be prevented by an AT\(_1\) receptor blocker (7). Thus, EO may inhibit Na\(^+\)/K\(^+\)-ATPase in the membrane of neurons, increasing intracellular Ca\(^{2+}\) (30), and thereby enhance activity of angiotensinergic sympatho-excitatory pathways leading to hypertension (7).

Salt-inducible kinase 1 (SIK1) has been shown to regulate aldosterone synthesis as well as intracellular [Na\(^+\)] (38). SIK1 represses gene expression of CYP11A, CYP11B1 and CYP11B2, and attenuates ACTH-induced steroidogenesis in the adrenal cortex (36, 38). SIK1 also is part of a cell sodium-sensing network that regulates active sodium transport by Na\(^+\)/K\(^+\)-ATPase through a calcium-dependent process in kidney cells (32). The SIK1 gene is located within a BP quantitative trait locus (QTL) on chromosome 20p12, which may contribute ~25 mmHg increase in BP in congenic spontaneously hypertensive-Lewis
rats on high salt (24, 31). In Milan hypertensive rats, Na⁺/K⁺-ATPase activity in proximal tubule cells is increased via activation of the SIK1-Na⁺/K⁺-ATPase network (28, 33), Basal SIK1 phosphorylation and sodium transport were increased in a cell line of proximal tubule origin by a hypertension-linked mutation of human α-adducin (33).

SIK1 has also been identified in rat brain, and SIK1 mRNA increases up to 8 fold in the hippocampus and cortex following depolarization (6). We hypothesized that the SIK1-Na⁺/K⁺-ATPase network in the choroid plexus or neuronal membrane contributes to regulation of CSF [Na⁺], hypothalamic aldosterone and neuronal excitability, and dysregulation of this SIK1-Na⁺/K⁺-ATPase network in the brain contributes to salt-induced hypertension.

In the present study we examined whether SIK1 in the brain contributes to: 1) increases in Na⁺ entry into CSF and in hypothalamic aldosterone in Dahl S rats on high salt; and 2) excitatory BP, HR and sympatho-excitatory responses to CSF [Na⁺]. To assess the role of SIK1, we employed central administration of staurosporine. Staurosporine inhibits SIK1 activity at 5-10 nM (2.4-4.7 ng/ml, with MW=467) in vitro (22, 32), and at higher concentrations inhibits other kinases, eg. protein kinase C (PKC) with IC₅₀ of ~50 nM (2). We assessed: 1) SIK protein and activity in hypothalamic tissue of Dahl S and salt-resistant consomic SS.BN13 rats, and the concentrations of staurosporine needed to inhibit SIK1 activity; 2) effects of icv staurosporine on excitatory BP, HR and renal sympathetic nerve activity (RSNA) responses to icv infusion of Na⁺-rich aCSF in Dahl S, SS.BN13 and Wistar rats; 3) effects of chronic icv infusion of staurosporine on CSF [Na⁺], resting BP and HR, and hypothalamic aldosterone and corticosterone content in Dahl S and SS.BN13 rats on high salt for 2 weeks; and 4) as “control” studies, effects of
icv staurosporine on responses to icv Ang II and carbachol, and of the specific PKC inhibitor GF109203X on pressor responses to icv Na⁺-rich aCSF and icv Ang II.
Methods

Five to six week old male Dahl S rats (SS/Mcw) and salt-resistant consomic (SS.BN13) rats, and Wistar rats were purchased from Charles River, Montreal, Canada. SS.BN13 rats show functional and histological responses to high salt, which are similar to normotensive strains (4), and are used as salt-resistant control for Dahl S rats (4, 11). The animals were housed on a 12-hour light/dark cycle and fed a standard commercial rat chow with regular salt (101 µmol Na⁺/g, Harlan Laboratories, WI, USA, cat. # TD.2019) and tap water ad libitum. All experiments were approved by the University of Ottawa Animal Care Committee, and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication NO. 85-23, revised 1996).

Effects of icv injection of staurosporine on responses to acute increases in CSF [Na⁺]

Effects on responses of BP, HR and renal sympathetic nerve activity (RSNA): Dahl S and SS.BN13 and Wistar rats (2 groups/strain, n=5-8/group) remained on regular salt intake. Under isoflurane anesthesia a 23 gauge stainless steel cannula was placed just above the right lateral cerebro-ventricle as a guide cannula and fixed on the skull with dental cement (19). At least 5 days following the head surgery, the rats were anesthetized with isoflurane and the right femoral artery and vein were cannulated with PE10/PE50 tubings, which were tunneled to the back of the neck. Through a flank incision, a pair of silver electrodes was then placed around and fixed to the left renal nerve with silicone rubber (Wacker Sil-Gel, 604 AB) and exteriorized as described previously (19).

At least 4 hours after recovery from the anesthesia in the original cage, the intra-arterial catheter was connected to a transducer to record BP and HR with a Grass polygraph (19). The electrodes were connected to a high-impedance probe, and the electrical signals were
amplified 20,000-50,000 times by a band-pass amplifier (Grass P511, Grass technologies/Astro-Med Inc.) with a high- and low-frequency cutoff of 1000 and 30 Hz, respectively. The filtered signals were channeled to a rectifying voltage integrator (Grass 7P10) and recorded through the Grass polygraph. The RSNA signal (in mV), together with BP and HR, were also fed into an on-line computer with a Grass data acquisition and analysis program (Polyview 2.0). The signal noise for RSNA was determined after the rat had been killed at the end of the study, and subtracted from the total activity (19).

After a 30 min rest, baseline MAP, HR and RSNA were recorded in unrestrained animals for 10 min. A 26-gauge stainless steel needle was then inserted into the guide cannula so that its tip protruded into the lateral cerebro-ventricle. Via a PE tubing, the other end of the needle was connected to a 500 μl Hamilton micro-syringe, which was mounted on a Sage 355 infusion pump for icv infusion. Following a 20 min rest, Na⁺-rich aCSF (300 mmol/l Na⁺) was infused at 3.8 μl/min for 8 min. About 20 min after responses had subsided, staurosporine (5 ng/2μl ) or vehicle (2 μl aCSF with 0.5% ethanol) was injected icv in 2 groups of rats for each strain. After 3 min, icv infusion of Na⁺-rich aCSF was repeated at the same rate for 8 min. Five min before each icv infusion, the vasopressin V₁ receptor antagonist D-(CH₂)₅-Tyr-(Me)AVP (30 μg/kg in 0.1-0.2 ml saline, Sigma Chemical Co.) was injected iv, to exclude the effects of vasopressin release by the Na⁺-rich aCSF. Iv injection of the vasopressin antagonist did not affect resting MAP, RSNA and HR.

*Effects on CSF [Na⁺]:* In a second set of 12 Wistar rats, an icv cannula was inserted as described above. In addition, a hole was drilled in the skull on the sagittal midline immediately rostral to the interparietal-occipital bone suture for CSF withdrawal from the
cisterna magna. The rats were divided into 3 groups: 1) icv injection of vehicle then icv infusion of aCSF (n=4); 2) icv injection of vehicle then icv infusion of Na⁺-rich aCSF (n=4); 3) icv injection of staurosporine then icv infusion of Na⁺-rich aCSF (n=4). Via a micro-syringe, staurosporine (5 ng/2 μl) or vehicle (2 μl aCSF with 0.5% ethanol) was injected icv under anaesthesia. After 3 min, aCSF or Na⁺-rich aCSF (300mmol/l Na⁺) was infused icv at 3.8μl/min for 20 min. About 30 sec before the end of the icv infusion, a 25-gauge, Pencan® pencil point spinal needle (B. Braun Medical Inc.) was inserted into the hole above the cisterna magna at a 70-75° angle to the skull surface, and advanced about 7.5 mm. With an 1 ml syringe, 150-200μl of CSF was withdrawn at < 10μl/sec, for measurement of [Na⁺] with a Na⁺-sensing electrode (model MI-425, Microelectrodes).

Effects of icv injection of staurosporine on responses to icv injection of Ang II and carbachol

In 12 Wistar rats, effects of icv injection of staurosporine on responses to icv injection of Ang II or carbachol were tested. Rats were instrumented with an icv guide cannula and an intra-arterial catheter in the femoral artery. Four hours after recovery from the anesthesia for arterial cannulation, BP and HR responses to icv injection of Ang II (n=6) or cholinergic mimetic carbachol (n=6), each at 2 doses (15), were examined. Following a 20 min rest, 30 ng/2μl Ang II or 25 ng/2μl carbachol was injected icv. After the BP and HR responses had reached a plateau, 90 ng/2μl Ang II or 75 ng/2μl carbachol was injected. About 20 min after responses had subsided, staurosporine (5 ng /2μl) was injected icv. Three min later, the two doses of Ang II or carbachol were repeated. Ang II and carbachol were purchased from Sigma Co., and dissolved in aCSF.
Effects of icv injection of PKC inhibitor GF109203X on responses to icv Na⁺-rich aCSF and Ang II.

GF109203X is a highly selective, cell-permeable PKC inhibitor with a similar structure to staurosporine. In vitro, the effective concentration of GF109203X is 1 μM (34) or 0.4 μg/ml. In rats, micro-injection of GF109203X into the NTS at 0.08 μg/side inhibits Ang II- induced inhibition of baroreflex gain (34). Considering the production rate and total volume of CSF in rats, we estimated that the dose for acute icv administration needs to be at least 0.2 μg to inhibit PKC in the brain. Two groups (n=8/group) of Wistar rats on regular salt were instrumented with an icv guide cannula, and an intra-arterial catheter in the femoral artery 5 days later. About 18 h after arterial cannulation, BP and HR were recorded for 10 min in resting rats, and via a stainless steel needle Na⁺-rich aCSF was infused icv at 3.8 μl/min for 8 min. About 20 min after responses had subsided, Ang II (25 and 75 ng/2 μl) was injected icv. Twenty min after the responses had subsided, the selective PKC inhibitor GF109203X (0.5μg/4μl, Cayman Chemical) or vehicle (4 μl aCSF with 2% DMSO) was injected. After 3 min, icv infusion of Na⁺-rich aCSF and injection of Ang II were repeated.

Effects of chronic icv infusion of staurosporine in Dahl rats.

In 2 groups of Dahl S rats and 2 groups of SS.BN13 rats (n=7-8/ group) under isoflurane anesthesia, an L-shaped, 23 gauge stainless steel cannula was placed into the right lateral cerebro-ventricle (14). Via polyethylene tubing the other end of the cannula was connected to an osmotic minipump (Alzet, Model 2002, rate: 0.5μl/h) placed subcutaneously (sc) for icv infusion of either staurosporine (Sigma Chemical Co.) at 25 ng/day or vehicle [artificial CSF (aCSF) with 0.5% ethanol]. In our previous studies, icv
infusion of aCSF with 1-2% ethanol at the same rate had no effects on resting hemodynamics or behaviour (37). The rate for icv infusion of staurosporine was estimated from in vitro studies and the total volume and production rate of CSF. Considering that the CSF production rate is about ~6 ml/d and total CSF volume is ~0.5 ml in rats of ~300 g body weight. (5, 10), icv infusion of staurosporine at 25 ng/d may result in a CSF concentration of ~4 ng/ml (25 ng/6ml) or 8.5 nM. Intravenous or subcutaneous rates are in the 30 mg/kg/day range (41), and it is therefore unlikely that the icv infusion at 25 ng/day would cause relevant peripheral effects. High salt diet (1370 μmol Na⁺/g, Harlan Laboratories, WI, USA, cat. # TD.79119) was started in all 4 groups following the surgery and lasted for 2 weeks.

At the end of the 2-week icv infusion and high salt intake, under isoflurane anesthesia a PE catheter (PE10 fused to PE50) was placed into the right femoral artery. The catheter was filled with heparinized saline and exteriorized on the back of each rat. The next morning about 18 hour after the arterial cannulation, the arterial line was connected to a pressure transducer, Grass polygraph and tachograph. After a rest of about 30 min, BP and HR were recorded for 5 minutes. The average BP and HR recorded over these 5 min was used as the resting BP and HR. The rat was then re-anesthetized with isoflurane and 100-200μl CSF was collected from the cisterna magna at <10μl/sec (14). After decapitation, the whole brain was removed, frozen quickly in dry ice and stored at -80°C. The whole hypothalamus and hippocampus were dissected according to Glowinski and Iversen (8).

The accuracy of the placement of the icv cannulae were verified by either injection of blue dye after the short-term study or by visual examination during tissue collection in
the chronic study. A few rats (3 out of 61) with unsuccessful icv cannulation in the acute experiments were excluded.

**SIK1 protein and activity in the hypothalamus.**

SIK1 activity was measured in immuno-purified hypothalamic tissue, using $[^\gamma-32P]-$ATP (Perkin Elmer, 10Ci/mmol 2mCi/ml), the synthetic peptide substrate syntide-2 (6). (Santa Cruz product #sc-201151), and the phosphocellulose binding technique (3). Briefly, the hypothalami from 4 Dahl R and 4 Dahl S rats were first homogenized in lysis buffer, centrifuged at 12,000g for 20 min at 4°C, then replicate aliquots of the supernatants (containing 250µg total protein) were prepared. After immunoprecipitation with anti-SIK1 and Protein A/G + Agarose (Santa Cruz product #s sc-83754 and sc-2003 respectively). the purified SIK1 was resuspended in 55 µl 2x kinase buffer (100 mM Tris-HCl pH 7.2, 50mM MgCl$_2$, 4mM EDTA, 10mM EGTA, 25mM β-glycerophosphate, with 0.5mM DTT added freshly) and 17.5µl was added to an equal volume of reagent mixture containing 1.2µCi $[^\gamma-32P]$ATP, 200µg BSA, and 10µl substrate (1 mg/ml) in ddH$_2$O, on ice. After 20 min incubation at 30°C, an aliquot of each reaction mixture was spotted onto a labeled P81 cellulose phosphate paper disc which was immediately washed 5x 5 min in 75mM H$_3$PO$_4$, then counted (3 min) in CytoScint-ES cocktail (MP Biomedicals #882453) using a Packard Tri-Carb 2100TR LS Analyzer. SIK1 activity was directly proportional to the cpm, a measure of the $[^\gamma-32P]$ incorporated into the synthetic substrate. A known amount of SIK1 (25 ng) was added to one of the replicates for each sample to calculate the actual SIK1 activity in that sample.

To assess the in vitro inhibition of SIK1 by staurosporine, SIK1 activity was compared in replicate aliquots of the immunopurified tissue preparations to which staurosporine was
added in final concentrations of 0, 5, 25 or 50 nM (determined from initial experiments with active SIK1, Signal Chem #S14-11H-05). The activity in the samples with added staurosporine was expressed as a % of that in the same sample without the inhibitor. In a separate experiment, the identity of the proteins in the immunoprecipitate were confirmed by SDS-PAGE using a 10% Bis-Acrylamide gel. Proteins were transblotted onto a PVDF membrane which was subsequently blocked with 5% milk, incubated at 4°C overnight in rabbit anti-SIK1 antibody (Santa Cruz product #s sc-83754, 1:500 dilution), washed, then incubated at room temperature for 1 hr in goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz #sc-2004, 1:5000). Proteins were visualized with ECL Western Lightning Plus reagents (Perkin Elmer #NEL104001) and an AlphaEase imaging system. For immunoblotting of the total lysates from the same samples, the membrane was reprobed with β-actin antibody (Sigma, #A2228, 1:10,000), for normalization of the SIK1 bands. Specific bands, which were completely eliminated by pre-incubation of the primary antibody with an excess of the immunizing peptide were observed at ~85 and 35 KDa (6, 22, 32).

Aldosterone and corticosterone in hypothalamus and hippocampus

Aldosterone was measured by radioimmunoassay (RIA) after tissue preparation and Sep-Pak extraction as described previously (25) using a rabbit antiserum and [125I]-labeled aldosterone (MP Biomedicals, #07-108216 and #07-108226). The sensitivity for aldosterone was 35 pg/g in hypothalamic tissue (12), and 17 pg/g in the hippocampus. All samples from the experiment were done in one assay, with an intra-assay variation of 7%. Cross re-activities were 0.03 and 0.14 % for corticosterone and DOC, respectively.
For measurement of corticosterone, the brain tissues were prepared as for aldosterone, then the Sep-Pak eluates were further diluted with steroid diluent (MP Biomedicals, #07-166197) before assay using a corticosterone $^{125}$I RIA kit (MP Biomedicals, NY, #07-120103) according to the manufacturer’s instructions. The sensitivity for hypothalamic corticosterone was 0.71 ng/g, and for the hippocampus, 0.33 ng/g. The corticosterone antibody had 0.34% cross reactivity with desoxycorticosterone, and 0.1% with testosterone.

Recovery for the Sep-Pak extraction, measured by spiking with known concentrations, was similar for both aldosterone and corticosterone at $\geq 88\%$. Reagent blanks for the Sep-Pak extraction and subsequent manipulations showed not significant readings.

**Data Analysis:**

Values are expressed as means ± SE. Responses of RSNA were expressed as percentages of resting values. For the chronic study and maximal responses in acute studies, two-way ANOVA was performed. For RSNA, MAP and HR responses to acute icv infusion of Na$^+$-rich aCSF, one way or two way repeated-measures ANOVA was performed. For CSF [Na$^+$] after acute infusion of Na$^+$-rich aCSF, one way ANOVA was performed. In vitro % inhibition of SIK1 activity was analysed by two way ANOVA. For all these test, when the F values were significant for main effect, Bonferroni t-test was used for multiple comparisons. Comparisons for acute responses before versus after staurosporine (both absolute and percentage changes) were analyzed with paired-t test. Statistical significance was defined as $p< 0.05$. 
Results

Hypothalamic SIK1 protein and activity, and inhibition by staurosporine.

For SIK1 protein, both bands were significantly lower by 20-30% in the hypothalamus of the Dahl S rats compared to the SS.BN13 controls (Fig 1). SIK1 activity in the hypothalamus followed the same pattern (Fig 1). Staurosporine at 5, 25 and 50 nM inhibited hypothalamic SIK1 activity by 50-80% in a dose-related manner in both SS.BN13 and S rats (Fig 2). The in vitro % inhibition by staurosporine at corresponding concentrations was somewhat more pronounced in the S rats (p=0.04).

Icv staurosporine and responses to acute increases in CSF [Na⁺].

Among Wistar, SS.BN13 and S rats on regular salt, there were no significant differences in resting MAP (109±4, 107±3, and 114±4 mmHg), HR (398±14, 387±12, and 409±10 bpm) and RSNA (6.7±2.2, 5.6±2.0 and 7.7±2.1 mV). MAP, HR and RSNA started to increase within 1-2 min after the start of icv infusion of Na⁺-rich aCSF, reached a plateau within 3-4 min, and returned to basal levels within 2-3 min after the termination of icv infusion (Fig 3 and 4). This pattern was similar in Wistar and SS.BN13 and S rats, except that the maximal responses were significantly larger in Dahl S rats (Table 1, Fig 3).

No changes in resting MAP, HR and RSNA were observed during the 3 min after icv injection of vehicle or staurosporine (data not shown). Na⁺-rich aCSF –induced increases in MAP, HR and RSNA were not affected by icv vehicle in all 3 strains (Fig 2, 3). In contrast, after icv staurosporine the maximal increases in MAP, HR and RSNA induced by icv infusion of Na⁺-rich aCSF were significantly larger compared to those before staurosporine (Table 1, Fig 3 and 4). Whereas the absolute differences between maximal BP responses after versus before staurosporine were similar in the 3 strains, the
differences of RSNA and HR responses were significantly smaller in Dahl S versus SS.BN13 or Wistar rats (Table 1). The % increases in the maximal responses of MAP, RSNA and HR to staurosporine were similar in SS.BN13 and Wistar rats, and for all 3 parameters significantly larger than in Dahl S rats (Table 1). However, the maximal responses to icv infusion of Na⁺-rich aCSF remained significantly larger after staurosporine in Dahl S rats versus SS.BN13 or Wistar rats (Table 1).

Compared to icv infusion of aCSF, icv infusion of Na⁺-rich aCSF for 20 min significantly increased CSF [Na⁺] (173±5 versus 154±1 mmol/L, p<0.05). Icv injection of staurosporine did not affect this increase in CSF [Na⁺] induced by icv infusion of Na⁺-rich aCSF (170±6 versus 173±5 mmol/L in rats with icv injection of vehicle).

**Icv staurosporine and responses to icv Ang II and carbachol**

As shown in Table 2, icv injection of Ang II increased BP and HR in a dose-related manner, and icv injection of staurosporine did not affect these responses. Icv injection of carbachol increased BP and decreased HR in a dose-related fashion, and these responses were also not affected by staurosporine.

**Effects of GF109203X on responses to icv Na⁺-rich aCSF and Ang II.**

No changes in resting MAP and HR were observed during the 3 min after icv injection of vehicle or GF109203X. Na⁺-rich aCSF or Ang II –induced increases in MAP were similar before and after icv vehicle (Fig 5). In contrast, after icv GF109203X the increases in MAP by Na⁺-rich aCSF or Ang II were significantly attenuated by 50-70% compared to those after injection of vehicle or before icv GF109203X.

**Chronic icv infusion of staurosporine in Dahl rats on high salt.**
There were no significant differences in initial body weight or weight gain among the 4 groups of rats on high salt, treated with either icv vehicle or staurosporine for 2 weeks (228±12 and 210±9 g or +58±3 and +61±4 g for Dahl S and SS.BN13 rats with vehicle, and 225±13 and 208±13 or +59±4 and +63±3 g for Dahl S and SS.BN13 rats with staurosporine).

After 2 weeks of high salt intake, resting MAP was significantly higher in Dahl S versus SS.BN13 rats treated with icv infusion of vehicle (Fig 6). In SS.BN13 rats, MAP was not affected by icv infusion of staurosporine. In contrast, in Dahl S rats icv infusion of staurosporine further increased MAP significantly by ~15 mmHg. Resting HR tended (p=0.07) to be higher in Dahl S versus SS.BN13 rats treated with icv infusion of vehicle, and was significantly higher in Dahl S rats with icv infusion of staurosporine.

After 2 weeks of high salt diet, CSF [Na⁺] was significantly higher by 5 mmol/L in Dahl S versus SS.BN13 rats treated with vehicle (Fig 6). Staurosporine did not affect the high salt induced increase in CSF [Na⁺] observed in Dahl S rats.

Compared with SS.BN13 rats, Dahl S rats on high salt showed significantly higher aldosterone levels in both the hypothalamus and hippocampus. Corticosterone levels were only increased in the hypothalamus (Fig 7). These increases persisted in Dahl S rats treated with icv infusion of staurosporine (Fig 7).
Discussion

The present study shows that: 1) SIK1 protein and activity are significantly lower in the hypothalamus of Dahl S versus SS.BN13 rats; 2) icv injection of staurosporine significantly enhances excitatory RSNA, BP, and HR responses to icv infusion of Na⁺-rich aCSF; the extent of this enhancement is significantly smaller for RSNA and HR in Dahl S rats compared with SS.BN13 and Wistar rats; 3) icv infusion of staurosporine at a rate of 25 ng/day significantly enhances salt-induced increases in resting BP and HR without affecting the increases in CSF [Na⁺] and hypothalamic aldosterone in Dahl S rats on high salt intake; and 4) icv injection of staurosporine does not affect BP and HR responses induced by icv Ang II or carbachol, whereas icv injection of a specific PKC markedly inhibitor inhibits pressor responses to icv Na⁺-rich aCSF or Ang II.

An increase in intracellular [Na⁺] leads to activation of Na⁺/K⁺-ATPase to increase outward Na⁺ transport. SIK1 may serve as part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process (32). An increase in intracellular [Na⁺] may increase intracellular [Ca²⁺] through the Na⁺/Ca²⁺ exchanger, leading to SIK1 activation. The latter results in de-phosphorylation of the Na⁺/K⁺-ATPase α-subunit and an increase in its activity (32). Considering its peripheral actions, in the CNS SIK1 may potentially affect several components of the pathways leading from increased CSF [Na⁺] to sympatho-excitation and hypertension. To assess the role of SIK1 in the CNS, we used the kinase inhibitor staurosporine. First, icv staurosporine did not affect the increase in CSF [Na⁺] in Dahl S rats on high salt intake. The choroid plexus is the major site for production of CSF (29) and Na⁺/K⁺-ATPase plays a major role in transporting sodium into the CSF (1, 17). The absence of changes in CSF [Na⁺] with
chronic icv infusion of staurosporine in Dahl S and SS.BN13 rats on high salt suggests that in contrast to the renal tubules (33), SIK1 per se may play at the most a minor functional role in Na⁺ transport across the choroid plexus. In addition, this finding suggests that the increase in CSF [Na⁺] in Dahl S rats on high salt diet is not due to increased SIK1 activity in the choroid plexus.

Secondly, chronic icv infusion of staurosporine did not affect the increase in hypothalamic aldosterone and corticosterone in Dahl S rats on high salt. Since central infusion of an aldosterone synthase inhibitor prevents the increase in aldosterone (20), it appears that an increase in CSF [Na⁺] by icv infusion of Na⁺-rich aCSF (14) or by high salt intake in Dahl S rats (20) increases local production of aldosterone and corticosterone in the brain. The absence of an effect of icv infusion of staurosporine on these increases suggests that it is unlikely that SIK1 in the brain plays a functional role in regulation of hypothalamic aldosterone and corticosterone synthesis in response to an increase in CSF [Na⁺]. However, possible effects of staurosporine on aldosterone and corticosterone levels in specific hypothalamic nuclei can not be excluded.

In contrast to the above “negative” results, icv staurosporine markedly enhanced sympatho-excitatory and pressor responses to an increase in CSF [Na⁺]. Icv infusion of Na⁺-rich aCSF caused parallel increases in RSNA, BP and HR, and these responses were significantly enhanced by 75% with icv injection of staurosporine in Wistar or SS.BN13 rats. One may speculate that a sodium -induced activation of neuronal SIK1 and thereby Na⁺/K⁺-ATPase attenuates sodium -induced sympatho-excitatory and pressor responses. Removal of this compensatory mechanism by inhibiting SIK1 with staurosporine unmasks the “full” sympatho-excitatory and pressor responses to CSF Na⁺. Icv
staurosporine did not affect the increase in CSF [Na\(^+\)] caused by the icv infusion of Na\(^+\)-rich aCSF, making it unlikely that the enhanced responses are due to larger increases in CSF [Na\(^+\)]. Alternatively staurosporine affects several kinases in a concentration-dependent manner (2, 22) and may cause a generalized enhancement of sympathoexcitatory responses. This is unlikely. First, estimated CSF concentrations for staurosporine in the present study are ~4 ng/ml, or 8.5 nM. As shown in Fig 2, at this concentration staurosporine inhibits SIK1 activity by 50-60%. This IC\(_{50}\) is consistent with previous studies (22, 32). Higher concentrations are needed to inhibit other kinases, with eg an IC\(_{50}\) of ~50 nM for PKC (2). Secondly, inhibition of these kinases would be expected to attenuate responses to icv Na\(^+\) rather than enhance responses. Sympathoexcitatory and pressor responses to icv Na\(^+\)-rich aCSF or high salt diet in Dahl S rats are mediated by angiotensinergic sympatho-excitatory pathways and can be blocked by icv infusion of an AT\(_1\) -receptor blocker (16, 18, 35). Activation of several kinases such as PKC is involved in Ang II signaling in the central nervous system (9, 39). Staurosporine did not affect the pressor responses induced by central AT\(_1\) receptor stimulation with icv Ang II. Responses to central muscarinic receptor stimulation with carbachol, which also involve activation of PKC, were also not affected by staurosporine. In contrast, the selective PKC inhibitor GF109203X inhibited by 60-70% the pressor responses to both icv Na\(^+\)-rich aCSF and icv Ang II. Similarly, icv infusion of a selective inhibitor of p44/42 mitogen-activated protein kinase also markedly attenuates increases in BP, RSNA and neuronal activity in the PVN induced by icv injection of Ang II (39).

Excitatory RSNA, HR and BP responses to icv Na\(^+\)-rich aCSF are significantly larger in Dahl S versus SS.BN13 or Wistar rats. After staurosporine, all these responses were
increased in the 3 strains, but both absolute and percent increases in RSNA and HR and percent increase in BP responses were significantly less in Dahl S versus SS.BN13 and Wistar rats. The absolute increases in BP responses by staurosporine were similar in the Dahl S rats compared to the 2 controls, possibly reflecting enhanced arterial responses in Dahl S rats. These findings suggest that the neuronal SIK1-network is less effective in Dahl S rats and as a result its inhibition by staurosporine leads to smaller increases in the RSNA, BP and HR responses to CSF Na⁺. These functional studies do not clarify which components of the SIK1 -Na⁺/K⁺-ATPase network are different or differently regulated in Dahl S versus SS.BN13 and Wistar rats. In the whole hypothalamus, SIK1 protein and enzymatic activity were significantly lower in Dahl S versus SS.BN13 rats. On the other hand, inhibition of enzymatic activity by staurosporine in vitro was somewhat larger in Dahl S rats. These findings may indicate that expression of SIK1 per se is decreased in Dahl S rats, and that in vivo regulators of SIK1 protein expression may be less active in Dahl S rats compared to the control strains.

Chronic icv infusion of staurosporine significantly increased BP and HR only in Dahl S and not SS.BN13 rats on high salt. Since the increase in CSF [Na⁺] in Dahl S rats on high salt was not affected by icv infusion of staurosporine, it appears that the further increase in neuronal responsiveness to CSF Na⁺ by staurosporine contributes to this larger increase in BP and HR in Dahl S rats on high salt. This finding also suggests that the neuronal SIK1-network – although less effective – still plays a buffering role in Dahl S rats in attenuating the hypertension. Chronic icv infusion of staurosporine had no effect on the BP of SS.BN13 rats on high salt. CSF [Na⁺] does not increase in Dahl salt-resistant (R) rats on high salt intake (17), and an increase in CSF/interstitial [Na⁺] appears
to be needed to activate the neuronal SIK1- Na\(^+\)/K\(^+\)-ATPase network, and for inhibition by staurosporine to have an effect. Supporting this conclusion, acute injection of staurosporine also did not affect resting RSNA, BP and HR in all 3 strains.

After icv staurosporine, the absolute BP, RSNA and HR responses to icv Na\(^+\)-rich aCSF were still larger in Dahl S rats versus the other 2 strains, suggesting that other mechanisms also contribute to increased neuronal responsiveness to CSF Na\(^+\) in Dahl S rats. We previously demonstrated (13) that in Dahl S rats, variants within C10 QTL2 on chromosome 10 contribute to gain-of-function of central mechanisms determining neuronal responses to CSF [Na\(^+\)]\(^↑\). Whether these mechanisms interact with the SIK1-Na\(^+\)/K\(^+\)-ATPase network will require further study.

**Limitations**

There are some limitations to consider. Firstly, icv infusion and whole hypothalamus assays do not provide insights into the central pathways/nuclei where SIK1 is operative. Local injections of staurosporine and assays in punches of nuclei may be useful in this regard. Secondly, food (and salt) intake were not measured and an increase in BP could result from increased salt and water consumption by staurosporine. However, the gain of body weight, visible food intake on high salt diet and CSF [Na\(^+\)]\(^↑\) were similar in Dahl S rats on high salt with and without icv infusion of staurosporine, and no behavioral changes were observed in staurosporine-treated rats. It appears unlikely that staurosporine itself further increased salt intake and thereby increased BP. Thirdly, BP and HR were measured through an arterial catheter 18 h after cannulation and a 30 min rest. These are not as ideal as data obtained by telemetry, and likely overestimate resting values somewhat.
Perspectives and significances

The present study provides new insights into intra-neuronal mechanisms regulating neuronal responses to CSF Na\(^+\) and the resulting sympatho-excitation and salt-sensitive hypertension. Neuronal SIK1 -Na\(^+\)/K\(^+\)-ATPase network appears to play an important functional role in the responses to increases in brain [Na\(^+\)] so as to attenuate CSF Na\(^+\) - induced sympatho-excitatory and pressor responses. The lower hypothalamic SIK1 protein and activity and lower effectiveness of this buffering mechanism in Dahl S rats suggests that variations in the neuronal SIK1- Na\(^+\)/K\(^+\)-ATPase network, possibly variants in the SIK1 gene (22, 31), may contribute to salt-sensitivity in Dahl S rats.

Acknowledgement

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Legends for the figures

**Fig 1.** SIK1 protein and activity in the hypothalamus of Dahl SS.BN13 and S rats after 2 weeks high salt diet. Specific bands, eliminated by pre-incubation of the antibody with immunizing peptide, were detected at ~85 and 36 KDa. Band densities, in arbitrary units, were normalized to β-actin as loading control. For the activity assay, activity was measured in duplicate aliquots of each purified sample, after addition of 25 ng SIK1 to one aliquot, and is expressed as ng equivalent.

Data are means±SE analyzed with t-test. (n=4/group for immunoblotting; n=7 for SS.BN13 and n=5 for Dahl S for the activity assay).

*p<0.05 between strains.

**Fig 2.** In vitro % inhibition of SIK1 activity by staurosporine at 5-50 nM in hypothalamic tissue of Dahl S and SS.BN13 rats.

Data are mean±SE (n=4/per concentration), expressed as % activity of corresponding control tissue and analyzed with two way ANOVA.

Between strains: F=4.57, p=0.04; between treatments: F=109.50, p<0.0001; between strains x treatments: F=0.555, p=0.64

*p<0.05, vs control; a: p<0.05, vs 5 or 25 nM.

**Fig 3.** Changes in MAP, renal sympathetic nerve activity (RSNA) and HR in response to icv infusion of Na⁺-rich aCSF before and after icv staurosporine (5 ng, black circles) or vehicle (white circles) in Wistar rats.

Data are mean±SE (n=6-8/group) and compared with one way repeated-measures ANOVA.

Between treatments (staurosporine vs vehicle):
for MAP, $F=54.3$ (p=0.0002); for RSNA, $F=43.1$ (p=0.003); for HR, $F=32.8$ (p=0.0004).

* p<0.05, vs.vehicle.

**Fig 4.** Changes in MAP, renal sympathetic nerve activity (RSNA) and HR in response to icv infusion of Na$^+$-rich aCSF before (left panels) and after (right panels) icv staurosporine (5 ng) or vehicle in Dahl S and SS.BN13 rats on regular salt intake. Data are mean±SE (n=6-9/group).

Data after injection of staurosporine or vehicle were compared with two way repeated-measures ANOVA.

Between treatments (staurosporine vs. vehicle):
for BP, $F=22.4$ (p=0.0003); for RSNA, $F=55.1$ (p=0.0001); for HR, $F=26.3$ (p=0.0002).

Between strains (see also Table 1):
for BP, $F=13.1$ (p=0.003); for RSNA $F=11.9$ (p=0.007); for HR, $F=15.2$ (p=0.008).

* p<0.05, vs. vehicle in Dahl S rats; a: p<0.05, vs. vehicle in Dahl R rats.

BP, RSNA and HR in SS.BN13 with staurosporine and Dahl S with vehicle did not differ significantly.

**Fig 5**

A) Changes in MAP in response to icv infusion of Na$^+$-rich aCSF before and after icv GF109203X. (black circles) or vehicle (white circles) in Wistar rats.

Data are mean±SE (n=8/group) and compared with one way repeated-measures ANOVA.

Between treatments (GF109203X vs vehicle): $F=61.3$ (p=0.0001).

* p<0.05, vs. vehicle.

B) Maximal increases in MAP in response to icv injection of Ang II at 25 and 75 ng before and after icv GF109203X. (black bars) or vehicle (white bar) in Wistar rats.
Data are mean±SE (n=8/group) and compared with student t-test.

* p<0.05, vs. after icv vehicle.

**Fig 6:** Resting mean arterial pressure (MAP) and HR, and CSF sodium concentration in Dahl S and SS.BN13 rats on high salt and icv infusion of staurosporine at 25 ng/day or vehicle for 2 weeks.

Data are mean±SE (n=7-8/group) and analyzed with 2-way ANOVA.

For MAP, degree of freedom (DF) = 27, F=43.1 (p=0.0001) between strains; F=11.1 (p=0.003) between treatments; F=1.95 (p=0.175) between strain x treatment.

For HR, DF=27, F=1.62 (p=0.215) between strains; F=4.73 (p=0.01) between treatments; F=4.3 (p=0.06) between strain x treatment.

For CSF [Na⁺], DF=23, F=5.1 (p=0.001) between strains; F=0.83 (p=0.36) between treatments; F=0.38 (p=0.54) between strain x treatment.

*. p<0.05, vs. SS.BN13.  a: p<0.05, vs. Dahl S treated with icv vehicle.

**Fig 7:** Aldosterone and corticosterone content in hypothalamus and hippocampus in Dahl S and SS.BN13 rats on high salt with icv infusion of staurosporine at 25 ng/day or vehicle for 2 weeks.

Data are mean±SE (n=7-8/group) and analyzed with 2-way ANOVA.

For hypothalamic aldosterone, DF=28; F=53.5 (p=0.0001) between strains; F=1.45 (p=0.24) between treatments; F=0.05 (p=0.83) between strain x treatment.

For hypothalamic corticosterone, DF=28; F=12.5 (p=0.001) between strains; F=0.32 (p=0.58) between treatments; F=0.05 (p=0.82) between strain x treatment.

For hippocampal aldosterone, DF=28; F=22.7 (p=0.0001) between strains; F=0.10 (p=0.75) between treatments; F=0.11 (p=0.75) between strain x treatment.
For hippocampal corticosterone, DF=28; F=0.21 (p=0.62) between strains; F=0.14 (p=0.77) between treatments; F=0.10 (p=0.78) between strain x treatment.

* p<0.05, vs. SS.BN13.
References


Table 1: Maximal increases in MAP, renal sympathetic nerve activity (RSNA) and HR by icv infusion of Na\textsuperscript{+}-rich aCSF before and after icv staurosporine (5 ng) in Wistar, Dahl S and SS.BN13 rats.

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg) before</th>
<th>MAP (mmHg) after</th>
<th>MAP increase (%)</th>
<th>RSNA (%) before</th>
<th>RSNA (%) after</th>
<th>RSNA increase (%)</th>
<th>HR (bpm) before</th>
<th>HR (bpm) after</th>
<th>HR increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wistar</strong></td>
<td>11±1</td>
<td>19±2</td>
<td>+8±1</td>
<td>20±3</td>
<td>35±3</td>
<td>+15±2 (70±2%)</td>
<td>18±3</td>
<td>32±3</td>
<td>+14±2 (77±6%)</td>
</tr>
<tr>
<td><strong>SS.BN13</strong></td>
<td>14±2</td>
<td>23±2</td>
<td>+9±1 (69±3%)</td>
<td>23±3</td>
<td>38±3</td>
<td>+15±2 (68±4%)</td>
<td>20±2</td>
<td>32±2</td>
<td>+12±1 (63±4%)</td>
</tr>
<tr>
<td><strong>Dahl S</strong></td>
<td>29±1*</td>
<td>36±3*</td>
<td>7±2</td>
<td>39±2*</td>
<td>48±2*</td>
<td>+9±1* (20±2%)</td>
<td>39±2*</td>
<td>47±2*</td>
<td>+8±1* (26±3%)</td>
</tr>
</tbody>
</table>

Data are mean±SE (for n, see Fig 3 and 4) analyzed with two way ANOVA.

Percent changes in brackets represent percent increases in responses to Na\textsuperscript{+}-rich aCSF after icv staurosporine within strains.

For comparisons of maximal responses between strains either before or after staurosporine:
- for BP, F=68.1 (p=0.0001); for RSNA, F=25.5 (p=0.0002); for HR, F=30.1 (p=0.0002).

For comparisons of maximal responses before and after staurosporine in all strains:
- for BP, F=40.1 (p=0.0001); for RSNA, F=49.9 (p=0.0001); for HR, F=31.9 (p=0.0001).

For comparisons of absolute increases in responses:
- for BP, F=0.58 (p=0.56); for RSNA, F=16.0 (p=0.0001); for HR, F=22.5 (p=0.0001).

For comparisons of % increases in responses:
- for BP, F=51.7 (p=0.001); for RSNA, F=43.3 (p=0.001); for HR, F=33.6 (p=0.001).
Maximal responses after staurosporine are all significantly (p<0.05) increased compared with those before staurosporine.

* p<0.05, vs. SS.BN13 and Wistar rats either before or after staurosporine.

^ p<0.05. vs. SS.BN13 and Wistar rats.
Table 2: Maximal changes in MAP and HR by icv injection of Ang II (30 and 90 ng) or carbachol (25 and 75 ng) before and after icv injection of staurosporine (5 ng) in Wistar rats.

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after</td>
</tr>
<tr>
<td>Ang II, 30 ng</td>
<td>13±2</td>
<td>14±2</td>
</tr>
<tr>
<td>Ang II, 90 ng</td>
<td>30±4 *</td>
<td>28±4 *</td>
</tr>
<tr>
<td>Carbachol, 25 ng</td>
<td>12±2</td>
<td>11±2</td>
</tr>
<tr>
<td>Carbachol, 75 ng</td>
<td>25±3 *</td>
<td>27±4 *</td>
</tr>
</tbody>
</table>

Data are mean±SE (n=5/group), analyzed by paired-t test.

* p<0.05, vs. Ang II at 30 ng or carbachol at 25 ng.
SIK1 protein in hypothalamic tissue

<table>
<thead>
<tr>
<th></th>
<th>SIK1 Activity</th>
<th>SIK1 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS.BN13</td>
<td>1.2 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Dahl S</td>
<td>0.8 ± 0.1</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

*Significant difference (p < 0.05)

Immunoblotting with 40 µg protein from hypothalamic lysates

Fig 1
Fig 2
Wistar rats

Fig 3
Fig 5
Fig 6
aldosterone

Hypothalamus

alderstone

pg/g

0

100

200

300

Hippocampus

pg/g

0

100

200

300

* *

Corticosterone

ng/g

0

20

40

60

80

SS.BN13           Dahl S      veh.       staur.       veh.     staur.       veh.     staur.

Fig 7