Gαi<sub>2</sub>-Protein–Mediated Signal Transduction
Central Nervous System Molecular Mechanism Countering the Development of Sodium-Dependent Hypertension

Richard D. Wainford, Casey Y. Carmichael, Crissey L. Pascale, Jill T. Kuwabara

Abstract—Excess dietary salt intake is an established cause of hypertension. At present, our understanding of the neuropathophysiology of salt-sensitive hypertension is limited by a lack of identification of the central nervous system mechanisms that modulate sympathetic outflow and blood pressure in response to dietary salt intake. We hypothesized that impairment of brain Gα<sub>i2</sub>-protein–gated signal transduction pathways would result in increased sympathetically mediated renal sodium retention, thus promoting the development of salt-sensitive hypertension. To test this hypothesis, naïve or renally denervated Dahl salt-resistant and Dahl salt-sensitive (DSS) rats were assigned to receive a continuous intracerebroventricular control scrambled or a targeted Gα<sub>i2</sub>-oligodeoxynucleotide infusion, and naïve Brown Norway and 8-congenic DSS rats were fed a 21-day normal or high-salt diet. High salt intake did not alter blood pressure, suppressed plasma norepinephrine, and evoked a site-specific increase in hypothalamic paraventricular nucleus Gα<sub>i2</sub>-protein levels in naïve Brown Norway, Dahl salt-resistant, and scrambled oligodeoxynucleotide-infused Dahl salt-resistant but not DSS rats. In Dahl salt-resistant rats, Gα<sub>i2</sub> downregulation evoked rapid renal nerve-dependent hypertension, sodium retention, and sympathoexcitation. In DSS rats, Gα<sub>i2</sub> downregulation exacerbated salt-sensitive hypertension via a renal nerve-dependent mechanism. Congenic-8 DSS rats exhibited sodium-evoked paraventricular nucleus-specific Gα<sub>i2</sub>-protein upregulation and attenuated hypertension, sodium retention, and global sympathoexcitation compared with DSS rats. These data demonstrate that paraventricular nucleus Gα<sub>i2</sub>-protein–gated pathways represent a conserved central molecular pathway mediating sympathoinhibitory renal nerve-dependent responses evoked to maintain sodium homeostasis and a salt-resistant phenotype. Impairment of this mechanism contributes to the development of salt-sensitive hypertension. (Hypertension. 2014;65:00-00.) • Online Data Supplement

Key Words: Blood pressure regulation • central G-protein–coupled receptors • renal sympathetic nerves • salt-sensitive hypertension • sympathetic nervous system

Excess dietary salt intake is a well-established cause of human hypertension, and salt-sensitive patients with hypertension have a 3-fold increased risk of adverse cardiovascular events. Despite the adverse impact of salt sensitivity, the mechanism(s) by which dietary salt evokes increased blood pressure remain to be fully elucidated. It has been hypothesized that among many proposed mechanisms, impaired sympathetic nervous system activity plays a critical role in the development of salt-sensitive hypertension, a theory supported by increased activation of the sympathetic nervous system in both animal models of salt-sensitive hypertension and salt-sensitive humans. In the setting of high salt intake, the regulation of central sympathetic outflow to the kidneys, via the renal sympathetic nerves, is of critical importance because of the direct impact of the renal nerves on renal sodium reabsorption, blood volume, total peripheral resistance, and long-term blood pressure. A key role for the renal sympathetic nerves in blood pressure regulation is highlighted by reports that renal nerve ablation attenuates Dahl salt-sensitive (DSS) hypertension and hypertension in human subjects.

At present, the central nervous system (CNS) mechanisms that mediate reduced central sympathetic outflow to the kidney to facilitate sodium homeostasis and normotension require further investigation. In response to increased sodium intake, sodium-sensitive receptors, present in the circumventricular organs and hypothalamic paraventricular nucleus (PVN), activate multiple brain G-protein–coupled receptor (G<sub>PCR</sub>) systems (eg, the α<sub>1</sub>-adrenoceptor) to inhibit renal sympathetic nerve activity to facilitate natriuresis, thus maintaining fluid and electrolyte homeostasis and normotension. Despite our understanding of the role of multiple G<sub>PCR</sub>’s in the regulation of renal sympathoinhibition and subsequent natriuresis, there remains a paucity of knowledge regarding potential common down-stream signal transduction pathways that are...
activated by GPCR’s to evoke these regulatory responses in vivo. Our laboratory has recently reported that brain G\(\alpha_i\)-subunit protein pathways, which mediate GPCR-stimulated signal transduction postligand binding, are required to mediate the natriuresis produced by acute central \(\alpha_2\)-adrenoceptor stimulation, an acute isotonic volume expansion, and acute isovolumetric sodium loading in conscious Sprague-Dawley rats.

In chronic studies, we demonstrated in the Sprague-Dawley rat that (1) high salt intake evoked upregulation of hypothalamic PVN G\(\alpha_i\)-subunit proteins, and (2) downregulation of brain G\(\alpha_i\)-protein expression evoked the development of renal nerve-dependent salt-sensitive hypertension. Collectively, these findings suggest brain G\(\alpha_i\)-subunit protein signal transduction as a potential common pathway mediating GPCR-evoked natriuresis.

Based on our prior findings, we hypothesized that high dietary sodium intake stimulates upregulation of PVN G\(\alpha_i\)-subunit proteins to suppress central sympathetic outflow, particularly to the kidneys, to maintain fluid and electrolyte balance and normotension in multiple salt-resistant phenotypes. Furthermore, we predict that failure to upregulate PVN G\(\alpha_i\)-subunit proteins in response to salt contributes to the pathophysiology of salt-sensitive hypertension. We investigated this hypothesis in the salt-resistant Brown Norway (BN) and Dahl salt-resistant (DSR) rat phenotypes and in the DSS rat model. After our observation of no increase in brain G\(\alpha_i\)-protein expression in response to salt intake in the DSS rat, additional studies were conducted in transgenic 8-congenic DSS rats to assess the impact of upregulation of PVN G\(\alpha_i\)-protein levels on the development of DSS hypertension.

**Methods**

Specific protocols and detailed methods are provided in the expanded Methods in the online-only Data Supplement.

**Results**

Elevated Dietary Sodium Intake Differentially Impacts CNS G\(\alpha_i\)-Subunit Expression in Naive DSR and DSS Rats

DSR rats exhibited a classical normotensive phenotype, featuring suppression of neural (plasma norepinephrine) and humoral (PRA) sodium retaining mechanisms (Figure S1 in the online-only Data Supplement) after high salt intake. In these normotensive DSR rats, high salt intake produced a site-specific

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**Animals and Surgery**

Male DSR, DSS, BN, and 8-congenic DSS rats aged 10 to 14 weeks were used in this study. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Chronic intracerebroventricular infusion of a control scrambled or targeted G\(\alpha_i\)-oligodeoxynucleotide was performed as previously described before random assignment of animals to a standard (total NaCl content 0.4%) or high-salt diet (total NaCl content 8%) for a 21-day experimental period. In certain studies after 21 days of high salt intake, animals were acutely instrumented for blood pressure measurement as previously described. Separate cohorts underwent radiotelemetry probe implantation before sham or bilateral renal denervation (RDNX) as described previously. Sham or bilateral RDNX animals were then assigned to chronic blood pressure monitoring via radiotelemetry or chronic metabolic balance studies. Plasma renin activity (PRA) and plasma norepinephrine were analyzed by ELISA. Brain G\(\alpha_i\)-subunit protein levels were assessed by immunoblotting as previously described.

**Statistics**

Data are expressed as mean±SEM. Differences occurring between treatment groups (eg, scrambled versus G\(\alpha_i\)-oligodeoxynucleotide) were assessed by a 2-way repeated measures ANOVA, with treatment group being one fixed effect and time the other, with the interaction included. The time (min) was then the repeated factor. Post hoc analysis was performed by a Newman–Keuls test to compare variations among the groups. Statistical significance was defined as probability (P) <0.05.
increase in PVN \( \text{G}_{i2} \)-protein expression (Figure 1A and 1B). In contrast, DSS rats exhibited hypertension, sodium and water retention, and a failure to suppress neurally mediated sodium reabsorption (Figure S1) that was accompanied by no change in PVN \( \text{G}_{i2} \)-protein expression levels (Figure 1A and 1C) when maintained on a high salt intake.

**Central \( \text{G}_{i2} \)-Subunit Protein-Gated Pathways Counter the Development of Salt-Sensitive Hypertension**

The salt-resistant and salt-sensitive phenotypes exhibited by Dahl rats were not altered by the central infusion of a control scrambled oligodeoxynucleotide during high salt intake (Figure 2A and 2B). High salt treatment produced significant upregulation of hypothalamic PVN \( \text{G}_{i2} \)-proteins in scrambled oligodeoxynucleotide-infused DSR, but not DSS rats (Figure 2C and 2D), without altering brain \( \text{G}_{i1}, \text{G}_{i3} \), or \( \text{G}_{o} \) protein levels (Figure S2). In Dahl rats fed a high but not a normal sodium content diet, central \( \text{G}_{i2} \)-oligodeoxynucleotide infusion resulted in the development of salt-sensitive hypertension in the DSR rat and exacerbated the magnitude of hypertension in the DSS rat (Figure 2A). In both Dahl phenotypes maintained on a high-salt diet and receiving a scrambled or \( \text{G}_{i2} \)-oligodeoxynucleotide infusion, PRA was suppressed, as seen in naive rats (Figure 2A). In contrast to the suppression of plasma norepinephrine in scrambled oligodeoxynucleotide-infused DSR rats, plasma norepinephrine levels were significantly elevated in \( \text{G}_{i2} \)-oligodeoxynucleotide–infused DSR rats challenged with a high-salt diet (Figure 2A). In response to increased dietary salt intake, DSS rats receiving a \( \text{G}_{i2} \)-oligodeoxynucleotide infusion exhibited significantly increased levels of plasma norepinephrine versus animals receiving a control scrambled oligodeoxynucleotide treatment. In both Dahl rat phenotypes, we observed no difference in plasma sodium concentration or osmolality between experimental treatment groups (Tables S1 and S2). Illustrated as an index of the salt sensitivity of blood pressure, scrambled oligodeoxynucleotide-infused DSR rats exhibited a classical salt-resistant phenotype. \( \text{G}_{i2} \)-oligodeoxynucleotide infusion caused a significant reduction in the slope of the pressure-natriuresis relationship in DSR rats (Figure 2B), reflecting increased salt sensitivity of blood pressure. In the DSS phenotype, as in the DSR rat, intracerebroventricular \( \text{G}_{i2} \)-oligodeoxynucleotide infusion increased salt sensitivity by reducing the slope of the blood pressure, sodium excretion relationship (Figure 2B). In these animals for which physiological data are presented in Figure 2A and 2B, targeted intracerebroventricular oligodeoxynucleotide infusion produced selective and efficacious downregulation of global CNS \( \text{G}_{i2} \)-proteins (Figure 2C and Figure S2) as we have previously reported in the Sprague-Dawley rat.22 In these animals, central \( \text{G}_{i2} \)-oligodeoxynucleotide infusion selectively blocked the

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**Figure 2.** A, Mean arterial pressure (MAP) (mm Hg), plasma norepinephrine (NE; nmol/L) and plasma renin activity (ng/mL per hour), (B) index of salt sensitivity, and (C) \( \text{G}_{i2} \)-subunit protein expression in the brain cortex, paraventricular nucleus (PVN), and ventrolateral medulla (VLM) of male Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats receiving an intracerebroventricular (i.c.v.) infusion of a scrambled (SCR) or \( \text{G}_{i2} \)-oligodeoxynucleotide (ODN; 25\( \mu \)g/6\( \mu \)l per day) maintained for 21 days on a normal or high salt intake (mean±SEM, N=6/group). *P<0.05 vs respective normal salt intake group value. τP<0.05 vs DSR high salt intake group value. D, Representative immunoblots illustrating ODN-mediated downregulation of brain \( \text{G}_{i2} \)-subunit protein levels in DSR and DSS rats.
hypotensive, but not bradycardic, response to acute intracerebroventricular injection of the \(\alpha_1\)-agonist guanabenz (Figure S3), a finding consistent with our prior observations after acute\(^{20}\) and chronic\(^{22}\) central G\(\alpha_i\)-protein downregulation.

**Development of Salt-Sensitive Hypertension in Central G\(\alpha_i\)-Oligodeoxynucleotide–Infused Rats Is Renal Nerve Dependent**

Bilateral RDNX abrogated the development of rapid-onset dietary salt-induced hypertension produced by continuous central G\(\alpha_i\)-oligodeoxynucleotide infusion in DSR rats (Figure 3A–3C). As observed in intact DSR G\(\alpha_i\)-oligodeoxynucleotide–infused rats, we observed a significant decrease in the slope of the pressure-natriuresis relationship in sham RDNX rats, a change abolished by RDNX (Figure 3C). Mechanically, in response to 21-day high-salt intake, G\(\alpha_i\)-oligodeoxynucleotide–infused bilateral RDNX DSR rats did not exhibit elevated global sympathetic activity or an increased depressor response to ganglionic blockade as was observed in hypertensive sham-operated animals (Figure 3D and 3E). Furthermore, in sham-operated G\(\alpha_i\)-oligodeoxynucleotide–infused DSR rats, we observed a significant elevation in both estimated plasma volume and estimated blood volume after high-salt intake, a change that was abolished in RDNX rats (Figure 3F and 3G).

In sham RDNX central G\(\alpha_i\)-oligodeoxynucleotide–infused DSS rats, we observed an increase in speed and magnitude of salt-induced hypertension compared with control scrambled oligodeoxynucleotide–infused rats—an effect prevented by bilateral RDNX (Figure 4A). DSS rats that underwent a sham RDNX procedure exhibited a significant elevation in 24-hour sodium balance during the first 5 days of high-salt intake compared with bilateral RDNX animals (Figure 4B). As in intact DSS G\(\alpha_i\)-oligodeoxynucleotide–infused rats, we observed a decrease in the slope of the pressure-natriuresis relationship in sham RDNX rats that was accompanied by a small (=8 mm Hg) parallel shift in the pressure-natriuresis relationship, a change abolished by RDNX (Figure 4C). Sham RDNX DSS rats also exhibited enhanced increases in plasma norepinephrine content (Figure 4D) and an increased peak depressor response to chlorisondamine-mediated ganglionic blockade (Figure 4E) compared with high-salt intake RDNX or intact animals. In sham RDNX DSS G\(\alpha_i\)-oligodeoxynucleotide–infused rats, we observed significant elevations in estimated plasma volume and estimated blood volume after 21 days of high-salt intake, the magnitude of which was attenuated in RDNX rats (Figure 4F and 4G). There were no differences in plasma sodium or plasma osmolality between DSR and DSS G\(\alpha_i\)-oligodeoxynucleotide–infused experimental treatment groups in sham or RDNX animals (Tables S3 and S4). RDNX was verified by observation of a significant reduction in kidney norepinephrine content in denervated versus sham-operated animals (Figure 4S).

**Chromosome 8 Substitution Restores Sodium-Evoked CNS G\(\alpha_i\)-Subunit Protein Upregulation and Attenuates the Development of DSS Hypertension**

A high-salt challenge increased PVN G\(\alpha_i\)-protein expression in the BN (Figure 5A and 5B) and 8-congenic DSS rat...
During high-salt intake, BN rats exhibited stable blood pressure (Figure 6A), sodium homeostasis (Figure 6B), suppression of plasma norepinephrine content (Figure 6C), and no change in blood or plasma volume (Figure 6E and 6F). Compared with naive DSS rats, 8-congenic DSS rats display a slower initial rise in mean arterial pressure over the first several days of high-salt intake and a significant reduction in development of sympathetically mediated salt-sensitive hypertension (Figure 6A–6E).

Discussion

High dietary sodium intake evoked a site-specific upregulation of PVN G<sub>αi2</sub>-protein levels in DSR rats. These findings significantly extend our prior report that PVN G<sub>αi2</sub>-proteins are upregulated in Sprague-Dawley rats in response to increased salt intake<sup>22</sup> and demonstrate that this endogenous mechanism is conserved across salt-resistant rat phenotypes. After high salt intake in the DSS rat, which developed salt-sensitive hypertension, we detected no change in the expression of PVN G<sub>αi2</sub>-proteins. The lack of PVN G<sub>αi2</sub>-protein upregulation in response to increased salt intake, in contrast to that observed in Sprague-Dawley<sup>22</sup> and DSR rats, suggests this reflects an underlying central mechanism driving, in part, the development of salt-sensitive hypertension in the DSS phenotype. To determine the significance of a sodium-evoked increase in PVN G<sub>αi2</sub>-proteins in DSR rats, and the potential role(s) of CNS G<sub>αi2</sub>-proteins in the DSS rat, we examined the impact of chronic downregulation of brain G<sub>αi2</sub>-protein levels. Downregulation of brain G<sub>αi2</sub>-proteins was achieved via intracerebroventricular infusion of a G<sub>αi2</sub>-targeted oligodeoxynucleotide sequence that we have demonstrated produces significant and specific downregulation of the target protein in vivo.<sup>19–22</sup> In these studies after oligodeoxynucleotide infusion,
we observed significant downregulation of G\(_{\alpha}\)\(_{i2}\)-proteins in all brain areas studied in DSR and DSS rats that was of comparable magnitude during both normal and high salt intake.

In Dahl rat phenotypes fed a normal sodium diet, downregulation of brain G\(_{\alpha}\)\(_{i2}\)-proteins did not alter any physiological parameter studied. These data indicate that under baseline sodium intake, downregulation of G\(_{\alpha}\)\(_{i2}\)-proteins does not evoke hypertension and that the remaining minimal expression of CNS G\(_{\alpha}\)\(_{i2}\)-proteins (10%–15% of control) is sufficient to maintain sodium homeostasis. During high salt intake, central G\(_{\alpha}\)\(_{i2}\)-protein downregulation evoked sympathetically mediated salt-sensitive hypertension in the DSR rat and exacerbated the magnitude of salt-sensitive hypertension observed in the DSS phenotype. These data indicate that despite a failure to upregulate PVN G\(_{\alpha}\)\(_{i2}\)-proteins during high salt intake, endogenous brain G\(_{\alpha}\)\(_{i2}\)-protein–gated signal transduction pathways, likely located within the PVN, are acting to attenuate the magnitude of DSS hypertension. This is evidenced by the fact that removal of the influence of the endogenous expression of brain G\(_{\alpha}\)\(_{i2}\)-proteins exacerbated salt sensitivity in the DSS phenotype. In naive DSR and DSS rats, we observed the suppression of PRA after high salt intake as has been previously reported.\(^{24,25}\) After downregulation of CNS G\(_{\alpha}\)\(_{i2}\)-proteins and either development (DSR) or exacerbation (DSS) of salt-sensitive hypertension, we again observed suppression of PRA. The mechanism driving the suppression of PRA in this setting, in which enhanced sympathetic outflow would be assumed to stimulate renin release, remains to be established. However, our prior studies in which downregulation of CNS G\(_{\alpha}\)\(_{i2}\)-proteins did not prevent sodium-stimulated suppression of renin secretion\(^{21,22}\) support the present findings and suggest that PRA is regulated by a mechanism that does not involve brain G\(_{\alpha}\)\(_{i2}\)-proteins.

Radiotelemetry data reveal that downregulation of CNS G\(_{\alpha}\)\(_{i2}\)-proteins evoked an immediate elevation in blood pressure in the DSR rat on high-salt challenge, followed by a slower gradual increase in blood pressure over time. In the DSS rat, an enhanced pressor response to salt intake was also observed after CNS G\(_{\alpha}\)\(_{i2}\)-protein downregulation, after which the rate of development of hypertension was comparable between scrambled and G\(_{\alpha}\)\(_{i2}\)-treated animals. In both Dahl phenotypes, CNS G\(_{\alpha}\)\(_{i2}\)-downregulation evoked a rapid ≈20 mm Hg increase in mean arterial pressure within 2 to 3 days, indicating the dramatic impact of this pathway on blood pressure regulation in response to salt across phenotypes. We hypothesize that the overall blood pressure in the DSS phenotype remained significantly higher than that observed in the DSR rat, despite G\(_{\alpha}\)\(_{i2}\) downregulation, because of the additive nature of G\(_{\alpha}\)\(_{i2}\) downregulation to the existing complex multifactorial nature of DSS. Our data strongly support the hypothesis of distinct phases of salt sensitivity\(^{26}\) and suggest that endogenous CNS G\(_{\alpha}\)\(_{i2}\)-protein pathways are activated and likely undergo upregulation immediately on increased sodium intake to facilitate sodium homeostasis.

Chronic removal of the influence of both the afferent and efferent renal sympathetic nerves, via bilateral RDNX, prevented central G\(_{\alpha}\)\(_{i2}\)-oligodeoxynucleotide–induced sympathetically mediated salt-sensitive hypertension in DSR rats and attenuated the development of salt-sensitive hypertension.
in DSS rats. Recent studies have reported that renal efferent nerve reinnervation has begun 4 weeks after RDNX, as assessed via immunohistochemistry. Therefore, we think, owing to dramatically suppressed renal norepinephrine content at the end of our 21-day sodium challenge, there was negligible functional impact of efferent renal nerve innervation during our studies. Confirming our findings in intact Dahl rats, sham RDNX DSR and DSS animals receiving an intracranial perfusion of Gαi2-oligodeoxynucleotide infusion exhibited a rapid hypertensive response to elevated salt intake with blood pressure remaining elevated during high salt intake. There is a clear association between renal nerve activity and the regulation of sodium excretion, and recent studies have suggested that increased renal nerve release of norepinephrine initiates renal sodium transporter dysregulation to drive the pathophysiology of salt-sensitive hypertension. Our current studies provide clear evidence of a direct role of renal sympathetic innervation of the kidney in the development of salt-sensitive hypertension. Based on our prior studies demonstrating a failure to suppress renal sympathetic nerve traffic and sympathetic outflow in response to pharmacological and physiological stimuli after Gαi2 downregulation, we speculate in these studies that there was a failure to suppress sympathetic outflow to the kidneys. This failure to suppress nerve traffic to the kidney would enhance renal norepinephrine release and increase norepinephrine-mediated sodium reabsorption to evoke the observed sodium retention. In these studies, bilateral RDNX abolished the development of salt sensitivity. We speculate that this reflects the removal of excess efferent stimulation of the kidney and subsequent norepinephrine-mediated renal sodium reabsorption. Owing to the use of RDNX, we are unable to delineate the individual impact of the afferent versus efferent nerves, which interact structurally and functionally, on the observed responses. Supporting our finding of a critical role of the renal sympathetic nerves in countering the development of salt sensitivity is the fact the removal of the influence of the afferent renal nerves, which inhibit efferent nerve traffic during high salt intake, via dorsal rhizotomy evoked the development of salt-sensitive hypertension in the Sprague-Dawley rat. Collectively, these studies highlight the critical, yet minimally explored, importance of the interactions between the afferent and efferent renal nerves in the regulation of blood pressure during high salt intake.

The rapid development of persistent hypertension in both sham-denervated Dahl phenotypes correlated with dramatically enhanced sodium retention over the first several days of high salt intake. In these animals, sodium balance returned to levels observed in RDNX animals within 7 days while blood pressure remained elevated throughout the 21-day protocol. We think that the observed rapid elevation in blood pressure, after Gαi2 downregulation, is a result of a reduction in the slope of the blood pressure–sodium excretion relationship, indicative of increased short-term salt sensitivity of blood pressure. This has the effect of resetting the pressure-natriuresis set-point to a higher level—a level reached within several days, as hypothesized in current models of the development of salt-sensitive hypertension. These data demonstrate that CNS Gαi2 downregulation decreases the slope of the pressure-natriuresis relationship to drive early onset phase 1 hypertension to maintain sodium homeostasis. During prolonged exposure to high-salt intake (ie, ≥21 days), it is probable that we would see a significant rightward parallel shift in the pressure-natriuresis relationship reflecting the irreversible resetting of blood pressure to a higher level after renal damage, as has been documented in the DSS rat. Furthermore, in these sham-operated animals, we observed increased plasma and blood volume in both DSR and DSS rats after Gαi2 downregulation. Despite the controversial role of an expansion in blood volume in the pathophysiology of DSS, our studies provide evidence for blood and plasma volume expansion, without changes in plasma sodium or osmolality, in the development of salt-sensitive hypertension evoked by central Gαi2 downregulation.

To strengthen our hypothesis that upregulation of PVN Gαi2-subunit proteins represents a conserved central mechanism required for salt-resistance, we conducted studies that demonstrated the BN rat exhibits PVN Gαi2-subunit upregulation, normotension, and sympathoinhibition during high salt intake. Using the 8-congeneric DSS rat, a congenic DSS rat that contains chromosome 8, which encodes the guanine nucleotide binding protein [G protein], alpha inhibiting activity polypeptide 2 (GNAI2) gene from the BN rat, we investigated the impact of restoring the upregulation of PVN Gαi2-proteins on the development of salt sensitivity in the DSS rat. When challenged with a high salt intake, the 8-congeneric DSS rat exhibited upregulation of PVN Gαi2-proteins, attenuation of sodium-evoked hypertension, sodium retention, sympathoexcitation, and stable plasma and blood volume.

Owing to the multiple mechanisms underlying the pathophysiology of DSS, the fact that restoration of the PVN Gαi2-protein upregulation attenuated, not abolished, the magnitude of DSS is expected. In the 8-congeneric DSS rat, we observed a significantly slower increase in blood pressure during the first several days of salt intake versus DSS rats, further supporting a role of Gαi2-proteins in preventing the initiation/development of salt sensitivity.

A key question that remains unanswered, and is beyond the current studies, is the mechanism driving the increased expression of Gαi2 in response to altered sodium intake. Despite no detectable change in either plasma sodium or osmolality in the current studies, we speculate minute changes in plasma sodium/osmolality after sodium ingestion sensed by osmo/sodium-sensitive receptors located in the hypothalamic PVN, the circumventricular organs or the renal afferent nerve terminals, trigger the observed alterations in protein expression. Human studies have reported the presence of a single nucleotide polymorphism in the human GNAI2 gene is associated with increased hypertension risk by the Millennium Genome Project for Hypertension in Japan and in white Italians. Menzagli et al identified a C>G single nucleotide polymorphism in the promoter region of the GNAI2 gene which prevents binding of the specificity protein 1 transcription factor. The DSS rat phenotype contains several C>G single nucleotide polymorphisms in the GNAI2 gene compared with both the Brown Norway and DSR phenotypes, one of which is located in the specificity protein 1–binding domain. We hypothesize that in response to sodium intake, PVN Gαi2-protein upregulation is driven by sodium-evoked stimulation...
of specificity protein 1 binding that is likely impaired in the DSS rat, resulting in deficient specificity protein 1–mediated gene transcription after high salt intake and the observed failure to upregulate PVN G_{\alpha_{i}}-proteins.

**Perspectives**

At present, the central mechanisms acting to facilitate sodium homeostasis and normotension remain to be fully established.42 The observed genetically conserved endogenous increase in PVN G_{\alpha_{i}}-protein expression in multiple salt-resistant phenotypes, and a failure of this response in the DSS rat, is of high physiological significance because of the pivotal role that the PVN plays in the neural network that influences sympathetic outflow and blood pressure regulation.14 Our studies (1) implicate sympathetic innervation of the kidney as a critical component in the pathogenesis of salt-sensitive hypertension and, (2) suggest the underlying pathogenesis of salt-sensitive hypertension involves integration of both the CNS and the kidneys with communication being regulated by the renal sympathetic nerves via a CNS G_{\alpha_{i}}-protein–gated signal transduction pathway. Brain G_{\alpha_{i}} pathways, therefore, represent a central molecular pathway that acts to regulate central sympathetic outflow to the kidneys in response to alterations in salt intake. Significantly, our data support the theoretical dynamic modeling of DSS hypertension, which proposes several phases over the pathophysiology of salt sensitivity of varying duration.35,32 Collectively, these findings demonstrate a critical role for G_{\alpha_{i}}-protein–gated signal transduction in the early response to high salt intake, at which point there are dramatic increases in the set-point of the pressure-natriuresis relationship to maintain sodium homeostasis if the endogenous sympathoinhibitory responses to sodium intake (which we demonstrate require brain G_{\alpha_{i}}-proteins) are impaired.

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**Disclosures**

None.

**References**


### Novelty and Significance

**What Is New?**

- These studies report that sodium evoked upregulation of paraventricular nucleus Gαi2-proteins represents a conserved central molecular mechanism that is required to suppress renal nerve–dependent sympathetic outflow to maintain sodium homeostasis and normotension. We provide the first evidence demonstrating that impairment of this signal transduction pathway contributes to the pathophysiology of Dahl salt-sensitive hypertension.

**What Is Relevant?**

- Increased understanding of the central molecular pathways acting to regulate blood pressure, which are currently poorly understood, is likely to increase significantly the development of new therapeutic modalities to treat hypertension.

**Summary**

**Gαi2-βγ-Mediated Signal Transduction: Central Nervous System Molecular Mechanism Countering the Development of Sodium-Dependent Hypertension**

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Ga\textsubscript{i2}-PROTEIN MEDIATED SIGNAL TRANSDUCTION: A CNS MOLECULAR MECHANISM COUNTERING THE DEVELOPMENT OF SODIUM-DEPENDENT HYPERTENSION

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Short title: CNS Ga\textsubscript{i2} proteins and salt-sensitive hypertension

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Online Methods

Animals
Male Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats were purchased from Harlan Laboratories Inc, IN (Harlan site, Indianapolis #2024), Brown Norway (BN) rats were purchased from Charles Rivers (Charles Rivers site, Kingston #K90) and 8-congenic Dahl salt-sensitive rats (8-congenic DSS) were purchased from the Medical College of Wisconsin, WI. All experiments were conducted on male rats aged 10-14 weeks. Animals were housed individually in a temperature (range 68-79°F) and humidity (range 30-70%) controlled facility under a 12-h light/dark cycle. Following completion of surgical procedures (see below), rats were randomly assigned to receive a standard rodent diet (Teklad Global Diet, Harlan Laboratories, WI, Teklad Global 18% Protein rodent diet #2918, 18% protein, 5% crude fat, 5% fiber, total NaCl content 0.4% [174 mEq Na+/kg]) or high sodium diet (Test Diet, IN, Basal diet #5G01, 22% protein, 5.5% crude fat, 5% fiber, modified to contain total NaCl content 8% [1,378 mEq Na+/kg]) and water ad libitum for a 21-day experimental period. All procedures were approved by the Louisiana State University Health Sciences Center or Boston University School of Medicine Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical Procedures

Intracerebroventricular oligodeoxynucleotide infusion: Chronic down-regulation of brain Gaia2 proteins was achieved by continuous intracerebroventricular (i.c.v.) infusion of a phosphodiesterase oligodeoxynucleotide (ODN) probe that selectively and specifically targets Gaia2 proteins (5′-CTT GTC GAT CAT CTT AGA-3′).1-4 Control animals received an i.c.v. infusion of a scrambled (SCR) ODN (5′-GGG CGA AGT AGG TCT TGG-3′).1-4 In these studies, SCR and Gaia2 ODN’s were dissolved in isotonic saline and infused i.c.v. at 25μg/6μl/day, a technique previously reported by our laboratory to selectively down-regulate Gaia2 subunit proteins throughout the brain of conscious Sprague-Dawley rats.4 To achieve i.c.v. ODN infusion, animals were anesthetized (ketamine, 30 mg/kg intraperitoneally [i.p.] in combination with xylazine, 3 mg/kg i.p.) and stereotactically implanted with a stainless steel cannula into the right lateral cerebral ventricle (Plastics One, VA),1-4 which was connected via silastic tubing to a miniosmotic pump (model 2004; Durect Corporation, CA).4 A National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) search of the Rattus norvegicus Reference Sequence (RefSeq) protein database was conducted to confirm 1) the specificity of the Gaia2 ODN for the Gaia2 rat protein sequence and, 2) the SCR ODN does not match any known rat protein sequence. In addition, our prior studies1-4, and the studies of other laboratories examining the effects of opioid analgesia and opioid induced feeding5-8, have demonstrated the selectivity and specificity of this Gaia2 ODN sequence in the down-regulation of brain Gaia2 proteins in rats.

Acute femoral vein, artery and bladder cannulation: In certain experimental groups, following 21-days normal (0.4% NaCl) or high (8% NaCl) intake, naïve rats (Fig. S1) or animals previously implanted with an i.c.v. cannula connected to a miniosmotic pump delivering a continuous central ODN infusion (Fig. 2) were anaesthetized with sodium methohexital (20mg/kg i.p., supplemented with 10 mg/kg intravenously, [i.v.] as required). Once anaesthetized, rats were instrumented with catheters in the left femoral artery, left femoral vein, and bladder for
the measurement of arterial blood pressure, i.v. administration of saline and/or drugs, and renal function respectively. Rats were then placed in a plexiglass holder and an i.v. infusion of isotonic saline (20µl/min) was maintained for a 2h surgical recovery period prior to experimentation to enable the animal to regain full consciousness and cardiovascular/renal excretory function to stabilize. Mean arterial pressure (MAP) and heart rate (HR) were continuously recorded via the surgically implanted femoral artery cannula using computer-driven BIOPAC data acquisition software (MP150 and AcqKnowledge 3.8.2, CA) connected to an external pressure transducer (P23XL; Viggo Spectramed Inc., CA).  

Telemetry probe implantation: A radiotelemetry device (PA-C40, DSI, MN) was implanted into the abdominal aorta via the left femoral artery under ketamine anesthesia (30 mg/kg i.p. ketamine; 3 mg/kg i.p. xylazine). In all cases, radiotelemetry probes were implanted at least 5-7 days prior to i.c.v. miniosmotic pump implantation. In animals in which no renal denervation surgery was conducted, blood pressure recording began 3 days post i.c.v. miniosmotic pump implantation. Animals that received a sham or bilateral renal denervation (RDNX) surgical procedure underwent i.c.v. miniosmotic pump implantation followed 3 days later by RDNX. Blood pressure monitoring was initiated 24h post-RDNX. Radiotelemetry data were collected, stored, and analyzed using Dataquest A.R.T. 4.2 software (DSI, MN).

Bilateral Renal Denervation (RDNX): Standard techniques were used to remove the influence of both the afferent and efferent renal sympathetic nerve fibers. In brief, under pentobarbital anesthesia (30 mg/kg i.p.) each kidney was exposed by a dorsal flank incision. Using a dissecting microscope, the renal vein and artery were dissected out of the surrounding fascia and stripped of all visible renal nerve bundles. Following dissection the renal artery was coated with a 10% phenol solution in ethanol to ensure the destruction of any remaining renal nerve fibers. The effectiveness of RDNX was confirmed at the end of the 21-day salt-intake study via ELISA analysis of norepinephrine (NE) content in kidney tissue as per manufacturer’s instructions (ELISA IB89537, IBL America, MN). As shown, (Fig. S4) this surgical procedure resulted in at least 90% suppression of renal NE content at the end of the 21-day sodium intake protocol indicating in these animals there is still clear evidence of functional denervation as previously demonstrated by our group. These data are supported by recent evidence demonstrating significantly reduced efferent nerve innervation of the kidney at the 3-4 week time point following bilateral renal denervation.  

Acute Cardiovascular Studies

Blood pressure measurement: In certain studies (Figs. S1 and 2), after a 21-day normal or high salt-intake acute femoral vein, artery and bladder cannulation was performed. After a 2-h stabilization period in which rats were infused i.v. with isotonic saline (20 µl/min), baseline MAP was recorded continuously over a 30-min period in conscious rats (N=6/treatment group/diet).  

I.c.v. guanabenz administration: In certain studies (Fig. 2), after measurement of baseline MAP, animals received an i.c.v. injection guanabenz (5µg/5µl delivered over a 60-second period) and peak changes in HR and MAP were recorded (N=6/treatment group/diet) to provide pharmacological verification of down-regulation of brain Gai2 proteins. As previously reported,
we observed selective abolishment of the hypotensive but not bradycardic responses to i.c.v. guanabenz administration in animals receiving a central Gα₂, but not SCR, ODN (Fig. S3). 1,4

**Chronic BP Measurement and Autonomic Function Studies**

Baseline blood pressure was recorded by radiotelemetry for five consecutive days immediately following i.c.v. miniosmotic pump implantation. Data was collected via scheduled sampling for 10-s every 10-min in DSR, DSS, BN and 8-congenic DSS cohorts which were either naive, receiving an i.c.v. ODN infusion, or an ODN infusion plus prior RDNX/sham surgery (Figs. 3,4 and 6). Rats were maintained on a normal salt diet (0.4% NaCl) for a 5-day baseline period and were then randomly assigned (N=6 per group) to either a normal (0.4% total NaCl) or high (8% total NaCl) salt diet and blood pressure was recorded for a further 21-days. Autonomic function was assessed before high salt-intake (day 3 of baseline blood pressure measurement period) and on day-21 of high salt-intake by i.p. injection of chlorisondamine (5 mg/kg). At the end of the protocol, whole brains and plasma were collected and stored at −80°C.

**Metabolic Balance Studies**

Metabolic balance studies were conducted in certain treatment groups (Figs. S1 & 6) on a normal salt diet and again on day-21 of the dietary sodium intake period. In certain studies (Figs. 4 & 5), metabolic balance studies were conducted prior to, and on days 1, 2, 3, 5, 7, 14 and 21 of high salt-intake. For these studies, rats were housed in individual metabolic cages (model 18cv, Fenco, MA) with external food containers and water bottles. Metabolic cages were equipped with a double-fine mesh screen that allowed separation of food and feces contamination from urine that was collected in vials that contained a layer of mineral oil to prevent urine evaporation. Rats were randomly assigned to receive a normal or high sodium intake diet. All rats were provided free access to rodent chow and tap water via external trays and bottles, respectively. On the day of study measurements were then made for body weight, food and water intake, and urine output during a 24-h period enabling calculation of daily sodium and water balance.2,4

**Measurement of Brain Gα-subunit Protein Levels**

Following completion of experimental protocols, whole brains were removed and frozen at −80°C. Frontal brain cortex (BC), hypothalamic paraventricular nucleus (PVN) and ventrolateral medulla (VLM) samples were extracted from frozen brains cut on a cryostat using a brain punch tool (Stoelting, IL) as previously described.1-4 Tissue lysates were prepared from brain punch samples and protein levels were quantified using the BCA assay as per manufacturers’ instruction (Thermo Scientific, IL). Lysates were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane (GE Healthcare, NJ). Gαi₁₋₃ and Gαo levels were determined as previously published by our laboratory using commercially available antibodies purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), directed against Gαi₁ (1:100, sc-391), Gαi₂ (1:200, sc-13534), Gαi₃ (1:1000, sc-262) and Gαo (1:200, sc-382); protein levels were normalized to GAPDH (anti-GAPDH 1:1000, ab-9483, Abcam, MA).1-4 Chemiluminescent immunoreactive bands were detected by a horseradish peroxidase-conjugated secondary antibody; data was imaged and semi-quantified using Bio-Rad Quantity One image analysis software. Probing with each antibody was performed sequentially following stripping of the membrane with a commercially available stripping reagent as per manufacturers’ instruction (Bio-Rad Laboratories, CA).

**Plasma Renin Activity and Norepinephrine Assays**
Plasma renin activity (PRA) and plasma NE levels were determined as previously described. In brief, following plasma extraction samples were frozen at -80°C until later analysis. For the PRA assay samples were analyzed using a GammaCoat® Plasma Renin Activity 125I RIA Kit (DiaSorin, MN) for the quantitative determination of PRA by the radioimmunoassay of generated angiotensin I as per manufacturers’ instructions. Plasma NE levels were quantified using an ELISA kit (Immuno-Biological Laboratories, Inc., MN) as per manufacturers’ instructions.

**Analytical Techniques**

Urine volume was determined gravimetrically assuming 1g = 1 ml. Urinary and plasma sodium concentrations were measured by flame photometry (model 943, Instrumentation Laboratories, MA). Plasma osmolality was determined using a VAPRO® vapor pressure osmometer (model 5600, Wescor, UT). Plasma hematocrit (Hct) was determined using a micro-hematocrit centrifuge (Adams Readacrit, Clay Adams, NJ). Hct was used to calculate estimated plasma volume (EPV) and estimated blood volume (EBV) using the following equations; 

$$\text{EPV} = (0.065 \times \text{body weight (kg)}) \times (100 - \text{Hct})$$

$$\text{EBV} = \frac{(\text{EPV} \times 100)}{(100-\text{Hct})}$$

**Statistical Analysis**

Data are expressed as mean ± SEM. Differences occurring between treatment groups (e.g., SCR vs. Gai2 ODN) were assessed by a two-way repeated-measures ANOVA, with treatment group being one fixed effect and time the other, with the interaction included. The time (min) was then the repeated factor. Post hoc analysis was performed by a Newman-Keuls test, to compare variations among the groups. Statistical analysis was carried out using Prism 5 (GraphPad Software, CA). Statistical significance was defined as probability ($P$) < 0.05.
REFERENCES


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<th>Dietary Sodium Intake Group</th>
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<th>8% NaCl</th>
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<td>Gαi2</td>
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<td>pNa [mEqL⁻¹]</td>
<td>139.6±2.1</td>
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<td>pOsm [mOsm/kg]</td>
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**Table S1** Plasma sodium (pNa [mEqL⁻¹]) and plasma osmolality (pOsm [mOsm/kg]) in Dahl Salt-Resistant rats receiving an i.c.v. infusion of a SCR or Gαi2 ODN (25µg/6µl/day) measured on day-21 of a normal or high sodium diet. The values are means ± SEM (N=6/group).
Table S2

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<tr>
<td>pNa [mEqL⁻¹]</td>
<td>140.6±2.4</td>
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Table S2 Plasma sodium (pNa [mEqL⁻¹]) and plasma osmolality (pOsm [mOsm/kg]) in male Dahl Salt-Sensitive rats receiving an i.c.v. infusion of a Gαι2 ODN (25µg/6µl/day) measured on day-21 of a normal or high sodium diet. The values are means ± SEM (N=6/group).
<table>
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<td>RDNX</td>
<td>Sham RDNX</td>
<td>RDNX</td>
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<tr>
<td>pNa [mEqL⁻¹]</td>
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<td>pOsm [mOsm/kg]</td>
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Table S3 Plasma sodium (pNa [mEqL⁻¹]) and plasma osmolality (pOsm [mOsm/kg]) in male Dahl Salt-Resistant rats receiving an i.c.v. infusion of a Gαι₂ ODN (25µg/6µl/day) measured on day-21 of a normal or high sodium diet post sham or bilateral RDNX surgery. The values are means ± SEM (N=6/group).
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<td>pNa [mEqL⁻¹]</td>
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<tr>
<td>pOsm [mOsm/kg]</td>
<td>301.4±4.2</td>
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**Table S4** Plasma sodium (pNa [mEqL⁻¹]) and plasma osmolality (pOsm [mOsm/kg]) in male Dahl Salt-Sensitive rats receiving an i.c.v. infusion of a Gαi2 ODN (25µg/6µl/day) measured on day-21 of a normal or high sodium diet post sham or bilateral RDNX surgery. The values are means ± SEM (N=6/group).
Figure S1 (MAP (mmHg), 24-h sodium balance (meq), 24-h water balance (ml), plasma renin activity (ng/ml/h) and plasma NE content (nmol/L) of naïve male DSR and DSS rats maintained for 21-days on a normal or high salt-intake (values are means ± SEM, N=6/group). *P<0.05 versus respective normal salt-intake group value. τP<0.05 versus DSR high salt-intake group value
Figure S2 Ga-subunit protein expression in the brain cortex, PVN and VLM of male DSR and DSS rats receiving an i.c.v. infusion of a SCR or Ga\(\alpha_2\) ODN (25\(\mu\)g/6\(\mu\)l/day) maintained for 21-days on a normal or high sodium diet for which physiological data is presented in Figs. 2A and 2B. The values are means ± SEM. (N=6/group).
Figure S3 Peak change in heart rate (HR; bpm) and mean arterial blood pressure (MAP; mmHg) in male Dahl Salt-Resistant and Dahl Salt-Sensitive rats receiving an i.c.v. infusion of a SCR or Gai₂ ODN (25μg/6μl/day) maintained for 21-days on a normal or high sodium diet for which physiological data is presented in Figs. 2A and 2B. The values are means ± SEM (N=6/group).
Figure S4 Kidney norepinephrine (NE) content (pg/mg) in male Dahl Salt-Resistant and Salt-Sensitive rats receiving an i.c.v. infusion of a Ga\(\alpha\)2 ODN (25\(\mu\)g/6\(\mu\)l/day) measured on day-21 of a high sodium diet post sham or bilateral RDNX surgery. The values are means ± SEM (N=6/group).