Salt-Induced Hypertension in a Mouse Model of Liddle Syndrome Is Mediated by Epithelial Sodium Channels in the Brain

James W. Van Huysse, Md Shahrier Amin, Baoli Yang, Frans H.H. Leenen

Abstract—Neural precursor cell expressed and developmentally downregulated 4-2 protein (Nedd4-2) facilitates the endocytosis of epithelial Na channels (ENaCs). Both mice and humans with a loss of regulation of ENaC by Nedd4-2 have salt-induced hypertension. ENaC is also expressed in the brain, where it is critical for hypertension on a high-salt diet in salt-sensitive rats. In the present studies we assessed whether Nedd4-2 knockout (−/−) mice have the following: (1) increased brain ENaC; (2) elevated cerebrospinal fluid (CSF) sodium on a high-salt diet; and (3) enhanced pressor responses to CSF sodium and hypertension on a high-salt diet, both mediated by brain ENaC. Prominent choroid plexus and neuronal ENaC staining was present in −/− but not in wild-type mice. In chronically instrumented mice, ICV infusion of Na-rich artificial CSF increased mean arterial pressure 3-fold higher in −/− than in wild-type mice. ICV infusion of the ENaC blocker benzamil abolished this enhancement. In telemetered −/− mice on a high-salt diet (8% NaCl), CSF [Na⁺], mean arterial pressure, and heart rate increased significantly, mean arterial pressure by 30 to 35 mmHg. These mean arterial pressure and heart rate responses were largely prevented by ICV benzamil but only to a minor extent by SC benzamil at the ICV rate. We conclude that increased ENaC expression in the brain of Nedd4-2 −/− mice mediates their hypertensive response to a high-salt diet by causing increased sodium levels in the CSF, as well as hyperresponsiveness to CSF sodium. These findings highlight the possible causative contribution of central nervous system ENaC in the etiology of salt-induced hypertension. (Hypertension. 2012;60:691-696.)

Key Words: salt-dependent hypertension ■ brain epithelial sodium channels ■ central nervous system ■ telemetry ■ benzamil

Neural precursor cell expressed and developmentally downregulated 4-2 protein (Nedd4-2) is a ubiquitin ligase that polyubiquitylates the epithelial Na channel (ENaC). The ubiquitylation facilitates the endocytosis of ENaC from the plasma membrane, leading to its degradation in proteosomes. Patients with mutations in the ENaC genes that cause a gain of function of ENaC have salt-sensitive hypertension (Liddle syndrome). These mutations occur in regions of the ENaC genes encoding PY motifs that interact with WW domains of Nedd4-2. The gain of function of ENaC in this case is attributed to disruption of the ENaC-Nedd4-2 interaction, resulting in decreased removal of ENaC from the plasma membrane. The gene knockout of Nedd4-2 in mice produces overexpression of all 3 ENaC subunits in the kidney and results in salt-dependent hypertension. The Nedd4-2−/− mouse thus serves as a model of Liddle syndrome.

ENaC is also expressed in the brain, both in the choroid plexus (CP) and in neurons. Chronic ICV infusion of the ENaC blocker benzamil abolishes the sympathetic hyperactivity and hypertension caused by chronic ICV infusion of Na⁺-rich artificial cerebrospinal fluid (CSF; aCSF) or by a high-salt diet in salt-sensitive rats, such as Dahl salt-sensitive (S) rats. Dahl S rats also exhibit an increase in CSF [Na⁺] on high salt, as well as enhanced sympathoexcitatory and pressor responses to CSF [Na⁺].

Because Nedd4-2 is also expressed in the brain, we hypothesized that ENaC expression is increased in the brains of Nedd4-2−/− mice, which contributes to the salt-induced hypertension in this model. Accordingly, we first assessed brain ENaC expression and evaluated whether Nedd4-2−/− mice also have increased CSF [Na⁺] on a high-salt diet and enhanced pressor responses to sodium in the CSF. We then assessed whether central ENaC blockade by ICV infusion of benzamil can prevent both the enhanced pressor responses to sodium and the hypertension on a high-salt diet.

Methods

Mouse Source and Husbandry

Nedd4-2−/− and wild-type (WT) mice were obtained in-house from a breeding colony established from founders that were transferred from the University of Iowa. Mice were housed in group cages before surgery but

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From the Hypertension Unit (J.W.V.H., M.S.A., F.H.H.L.), University of Ottawa Heart Institute, Ottawa, Ontario, Canada; Department of Medicine (J.W.V.H., F.H.H.L.), University of Ottawa, Ottawa, Ontario, Canada; Departments of Biochemistry, Microbiology, and Immunology (J.W.V.H.), Pathology and Laboratory Medicine (M.S.A.), and Cellular and Molecular Medicine (F.H.H.L.), University of Ottawa, Ottawa, Ontario, Canada; Department of Obstetrics and Gynecology (B.Y.), University of Iowa, Iowa City, IA.
Correspondence to Frans H.H. Leenen, University of Ottawa Heart Institute, 40 Ruskin St, H-3238, Ottawa, Ontario K1Y 4W7, Canada (E-mail fleenen@ottawaheart.ca); or James W. Van Huysse, University of Ottawa Heart Institute, 40 Ruskin St, H-3213, Ottawa, Ontario K1Y 4W7, Canada (E-mail jvanhuysse@ottawaheart.ca).
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were housed individually postoperatively. Housing was provided in a temperature-controlled environment with a 12:12-hour light-dark cycle. Water and standard chow (0.3% NaCl) were provided ad libitum, except when a high-salt diet (8% NaCl, Harlan Laboratories, Madison, WI) was substituted for standard chow. The present studies were carried out in accordance with guidelines established by the National Institutes of Health and the Canadian Council on Animal Care and were approved by the University of Ottawa Animal Care Committee. Studies were performed predominantly in males because of their larger size. When females were used in an experimental group, an equivalent number was used in most control groups. Moreover, blood pressure (BP) and heart rate (HR) responses to ICV Na\textsuperscript{+}-rich aCSF are similar in male and female mice.\textsuperscript{13}

**Genotyping**

The Nedd4-2 gene knockout involves a deletion of exons 6 to 8.\textsuperscript{8} Previously published methods were used for genotyping,\textsuperscript{9} using DNA extracted from the tail. Dual PCR reactions were run in parallel for each sample, each using a separate set of primers in which the reverse primer was specific for the WT or \(\sim\) allele. A forward primer (P1, 5\textsuperscript{\prime}TGAACCTATT-GCTTACCTC\textsuperscript{3\prime}) was common to both reactions. The reverse primer P2 (5\textsuperscript{\prime}GTTCATGCTGAAGCCTTAGCCATCTCATGAA\textsuperscript{3\prime}) was the reverse complement of a sequence located within the region deleted by the knockout. Thus, the 150-bp amplicon for the WT and \(\sim\) allele was identified in each sample. For both WT or a separate set of primers in which the reverse primer was specific for the tail. Dual PCR reactions were run in parallel for each sample, each using 35 PCR cycles were run, at 94°C, 62°C, and 72°C (30 seconds each) in succession for denaturing, annealing, and extension, respectively. These cycles were preceded by a 94°C denaturation period (5 minutes) and were followed by extension at 72°C (7 minutes).

**Immunohistochemistry for ENaC Expression in the Brain**

ENaC expression was studied in the CP and neurons of the pyramidal cortex, subfornical organ, supraoptic nucleus, and paraventricular nucleus of the hypothalamus. Mice were perfused with ice-cold PBS containing 4% paraformaldehyde (pH 7.4) after a lethal dose of pentobarbital and the brains were immediately removed and stored in the same solution at 4°C until the time of immunohistochemistry for ENaC. Tissue microarrays were prepared to include areas of the brain containing the lateral ventricles and hypothalamus. Immunohistochemistry with anti-\(\alpha\), -\(\beta\) and -\(\gamma\)ENaC antibodies was performed on 3 tissue microarray slides to include the major areas of interest from all of the samples and evaluated at multiple levels.\textsuperscript{13,14} Details regarding the quality and specificity of the ENaC antibodies were published previously.\textsuperscript{56,14} Distribution and intensity of staining in the cytoplasm and membrane were scored blindly in each of these areas. The score for distribution was represented as the percentage of cells that stained positive from 0 to 3 (0, no staining; 1, <30%; 2, 30% to 70%; and 3, >70% of cells stained). Staining intensity was scored as 0 to 3 (0, no staining; 1, mild; 2, moderate; and 3, strong). The 2 scores for each area were multiplied to yield a composite value (range, 0 [0, 0] to 9 [3, 3]).

**Dosages and Formulations**

Benzamil for ICV or SC infusion was dissolved into a mixture of 85% aCSF-15% polypropylene glycol (aCSF; 148 mmol/L of Na) or 85% Na-rich aCSF-15% polypropylene glycol (255 mmol/L of Na final concentration). The dose of benzamil for acute studies (7.4 ng/min) was established empirically, because to our knowledge a dose for acute ICV benzamil in mice has not been published previously. The benzamil dose (2.64 \(\mu\)g/d) used for chronic ICV infusion markedly decreases sympathetic activity in aortic banded mice on high salt.\textsuperscript{23} aCSF contained (in millimoles): NaCl (117.00), KCl (2.50), NaH\textsubscript{2}PO\textsubscript{4}·2H\textsubscript{2}O (0.65), Na\textsubscript{2}HPO\textsubscript{4}·2H\textsubscript{2}O (2.27), Na\textsubscript{2}SO\textsubscript{4} (0.50), Mg\textsubscript{2}Cl\textsubscript{2}·6H\textsubscript{2}O (2.14), CaCl\textsubscript{2} (1.00), and NaHCO\textsubscript{3} (27.00) and was adjusted to pH 7.0.

**Acute Responses to ICV Na-Rich aCSF**

WT and \(\sim\) mice received a guide cannula that was implanted into a lateral brain ventricle 6 to 8 days before study. One day before study, under isoflurane anesthesia each mouse received a carotid arterial catheter. The next day, in conscious WT and \(\sim\) mice baseline BP and HR were recorded for 10 minutes before the start of an ICV infusion of aCSF (148 mmol/L of Na) for 30 minutes at 0.4 \(\mu\)L/min. The aCSF infusion was immediately followed by an ICV infusion of Na-rich aCSF (235 mmol/L of Na) for 90 minutes, also at 0.4 \(\mu\)L/min. This rate of infusion causes modest increases in BP and HR in WT mice.\textsuperscript{13} To assess possible osmolar effects, a separate group of \(\sim\) mice was infused with mannitol in aCSF, at equivalent osmolality to the Na-rich aCSF (ie, 491 versus 337 osmol/L for aCSF).

To determine whether the enhanced BP and HR responses to ICV Na-rich aCSF in \(\sim\) mice can be blocked by ICV benzamil, a similar protocol was used, except that during the first ICV infusion period, benzamil (7.4 ng/min) was infused for 30 minutes. This was followed by an ICV infusion for 90 minutes of the same rate of benzamil combined with Na-rich aCSF. A control group of \(\sim\) mice received the same 2 consecutive ICV infusions, combined with vehicle. The effects of benzamil were also evaluated in WT mice using the same protocol.

**Effects of Chronic ICV Benzamil on Hypertension Induced by High-Salt Diet**

A telemetry transducer/transmitter (model TA11PA-C10, Data Sciences International, St Paul, MN) was implanted into 9- to 12-week-old mice 16 to 18 days before the start of a high-salt diet, with the tubing inserted into the abdominal aorta and glued into place with Vetbond. The transmitter/battery was placed in the peritoneal cavity. To conserve the telemetry battery, the transmitter was turned off for part of days 2, 4, and 6 and the entire nights 2, 4, and 6. A high-salt diet started on day 0 and continued for 10 days. BP recordings began the morning of day 3. After 24 hours of baseline BP monitoring, an ICV infusion of benzamil (2.64 \(\mu\)g/d) was given from day 2 to the end of the study on day 10 via a lateral ventricular guide cannula that was connected to an SC implanted ALZET model 1002 osmotic minipump (flow rate, 0.25 \(\mu\)L/h). Control groups received ICV vehicle or SC benzamil at the same rate as ICV.

**Measurement of CSF [Na]**

Groups of WT and Nedd4-2 \(\sim\) mice remained on a regular diet or were placed on a high-salt diet for 8 to 10 days and some \(\sim\) mice for 4 days. On the last day, under isoflurane anesthesia 2 to 5 \(\mu\)L of CSF was collected from the cisterna magna of each animal, and in most cases pairs of CSF samples from the same genotype were pooled. The combined samples were diluted 1:8 with distilled water before assay. A sodium-sensitive microelectrode (Microelectrodes Inc, Bedford, NH) was used to measure [Na] in the diluted sample. A set of standards ranging from 128 to 174 mmol/L [Na] in the same genotype were pooled. The combined samples were diluted 1:8 with distilled water before assay. A sodium-sensitive microelectrode (Microelectrodes Inc, Bedford, NH) was used to measure [Na] in the diluted sample. A set of standards ranging from 128 to 174 mmol/L [Na] in the same genotype were pooled. The combined samples were diluted 1:8 with distilled water before assay. A sodium-sensitive microelectrode (Microelectrodes Inc, Bedford, NH) was used to measure [Na] in the diluted sample. A set of standards ranging from 128 to 174 mmol/L [Na] in the same genotype were pooled.
Baseline BP and HR before ICV infusions of aCSF and Na-rich aCSF were similar in the 2 genotypes (legend for Figure 3). Infusion of aCSF (148 mmol/L of Na) for 30 minutes did not change BP or HR (Figure 3). Na-rich aCSF (235 mmol/L of Na) increased BP significantly in both genotypes, but the responses of the −/− mice were ≈3-fold greater than those of WT mice (P < 0.05, −/− versus WT; Figure 3). Na-rich aCSF increased HR significantly in both −/− and WT genotypes, but the HR response was ≈2-fold greater in the −/− versus WT mice (P < 0.05 versus WT; Figure 3). ICV infusion of mannitol for 45 minutes at the same rate or twice the rate did not change BP or HR in −/− mice (for mean arterial pressure, +2.0 ± 0.4 [n = 5] and +3.0 ± 0.7 mmHg [n = 3], respectively).

To determine whether the exaggerated BP and HR responses to acute increases in CSF [Na] in −/− mice are mediated by ENaC in the brain, benzamil was included in the protocol. There was no significant change in BP or HR in −/− or WT mice treated with aCSF, whether benzamil was present or not (Figure 4). In −/− mice treated with ICV Na-rich aCSF-containing vehicle, BP increased to a similar extent as that seen for −/− mice given Na-rich aCSF in Figure 3 (≈20–25 mmHg, P < 0.05 versus baseline and versus BP response in WT mice; Figure 4). ICV benzamil markedly (P < 0.05 versus vehicle) reduced the BP response to ICV Na-rich aCSF in −/− mice to levels that were not different from the WT response but did not affect the response in WT mice (Figure 4). ICV benzamil inhibited (P < 0.05) the increase in HR in both strains (Figure 4).

### Table 1. Cellular Distribution of ENaC Subunits in the Choroid Plexus, Cortex, SON, PVN, and SFO of Nedd4-2−/− Mice and WT Controls

<table>
<thead>
<tr>
<th>ENaC Subunits</th>
<th>Cytoplasm</th>
<th>Plasma Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choroid plexus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>3.9 ± 0.4</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>β</td>
<td>1.3 ± 0.4</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>γ</td>
<td>2.1 ± 0.4</td>
<td>4.3 ± 0.7*</td>
</tr>
<tr>
<td>Pyramidal cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>4.0 ± 0.2</td>
<td>6.1 ± 0.2*</td>
</tr>
<tr>
<td>β</td>
<td>2.6 ± 0.4</td>
<td>4.9 ± 0.9*</td>
</tr>
<tr>
<td>γ</td>
<td>4.0 ± 0.6</td>
<td>5.1 ± 0.5*</td>
</tr>
<tr>
<td>SON</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>2.2 ± 0.6</td>
<td>3.0 ± 1.2</td>
</tr>
<tr>
<td>β</td>
<td>2.4 ± 0.4</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>γ</td>
<td>1.2 ± 0.2</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>PVN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>1.2 ± 0.2</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>β</td>
<td>1.2 ± 0.2</td>
<td>2.7 ± 0.5*</td>
</tr>
<tr>
<td>γ</td>
<td>1.0 ± 0.04</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>SFO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>4.9 ± 1.2</td>
<td>4.7 ± 1.1</td>
</tr>
<tr>
<td>β</td>
<td>3.8 ± 0.7</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>γ</td>
<td>2.9 ± 0.6</td>
<td>2.5 ± 0.6</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 7 per group for choroid plexus and cortex; n = 5 per group for the 3 nuclei). Units for cytoplasmic and membranous distribution are arbitrary (see Methods section). SFO indicates, subfornical organ; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; WT, wild-type; ENaC, epithelial Na channel.

*P < 0.05. **P < 0.01 versus WT.

Next, we determined whether, in −/− mice, ENaC in the brain regulates CSF [Na]. CSF [Na] (in mmol/L) was as follows: 160 ± 2 (n = 7) in WT mice on regular salt, 157 ± 7 (n = 8) in WT mice on high salt, 156 ± 3 (n = 9) in −/− mice on regular Na and increased to 166 ± 1 (n = 3) after 4 days and to 167 ± 4 (n = 10) in −/− after 8 days on high salt (P < 0.05 versus −/− on regular salt). Within 4 days on high salt, Nedd4-2−/− mice treated with ICV vehicle developed increases in daytime and nighttime BP, ≤30 to 35 mmHg after 6 to 8 days (P < 0.01 versus baseline; Figure 5). In contrast, in −/− mice receiving an ICV infusion of benzamil, the salt-induced BP response was <10 mmHg (P < 0.05 versus baseline) during the day and night (P < 0.01 versus vehicle group for both day and night BP responses). SC infusion of benzamil at the same rate did not affect the increase in BP on a high-salt diet during the day but blunted the response by approximately one third (P < 0.05) during the night (Figure 5). The latter effect was significantly less than that of ICV benzamil (30% versus 85% reduction; P < 0.05). After the start of the high-salt diet, HR increased significantly by 30 to 40 bpm in ICV vehicle-treated −/− mice but not in those treated with ICV or SC benzamil (data not shown).
Discussion

The present study demonstrates the major new finding that hypertension induced by a high-salt diet in mice with a homozygous knockout of the Nedd4-2 gene is largely prevented by ICV infusion of benzamil. The −/− mice develop increased CSF [Na] on a high-salt diet and show markedly enhanced BP and HR responses to sodium in the CSF, which can be blocked by ICV benzamil. Altogether, these findings strongly suggest that the salt-induced hypertension in this mouse model of Liddle syndrome depends critically on increased expression of ENaC in the brain.

In rat models of salt-induced hypertension, an increase in CSF [Na] occurs on a high-salt diet and appears to precede the increase in BP.10 In rats, chronic elevations in CSF [Na] by ICV infusion of Na-rich aCSF increase BP via activation of the same systems in the brain (including ENaC) that produce the hypertensive response to a high-salt diet in salt-sensitive strains.7,8,16 Taken together, these
findings suggest a role for increased CSF [Na] in the etiology of the salt-induced hypertension. The mechanisms responsible for the increase in CSF [Na] in Dahl S and spontaneously hypertensive rats on high salt have not yet been elucidated.14 In rats, ENaC is present on both the basolateral and apical membranes of CP cells,5,14 whereas the Na+, K+-ATPase is located only on the apical (CSF facing) side. In normotensive rats, apical ENaC expression predominates and ICV benzamil increases CSF [Na]5. In the present study, the −/− mice show increased expression of all 3 of the ENaC subunits on both the basolateral and apical membranes of CP cells. In −/− mice after 8 to 10 days on a high-salt diet both plasma [Na+]14 and CSF [Na] show clear increases. It appears that, in −/− mice on a high-salt diet the increase in plasma [Na+]14 and the overexpression of ENaC on the basolateral membrane of CP cells lead to increased Na+ entry into the cells and elevated [Na+]i in the CP, which presumably causes apical Na+, K+-ATPase to transport the excess sodium into the CSF. Increased apical ENaC expression may enhance Na+ reabsorption from the CSF, which may contribute to the normal CSF [Na] in −/− mice on regular salt and may attenuate the increase in CSF [Na] on a high-salt diet. Further studies are needed to assess whether increases in both plasma and CSF [Na] precede the increases in BP on a high-salt diet.

In rats, ENaC not only plays a role in regulation of CSF [Na] but also mediates the sympathoexcitatory and pressor responses to a chronic increase in CSF [Na].8 The actual cellular, presumably neuronal, location of these channels still needs to be determined. In the −/− genotype, neuronal ENaC staining is significantly increased but, in contrast to the CP and cortex, only for the β subunit in the supraoptic nucleus and paraventricular nucleus of the hypothalamus and not in the subfornical organ. These findings suggest cell-type–specific regulation of ENaC ubiquitination by Nedd4-2. A 90-minute ICV infusion of Na-rich aCSF provokes a ∼3-fold greater pressor response in −/− mice than W/T mice, whereas ICV mannitol does not change BP. The enhancement in the BP response to Na+-rich aCSF is abolished by ICV benzamil, indicating that it is attributed to enhanced ENaC in the brain. Similar to Dahl S rats, the Nedd4-2 −/− mice show both an increase in CSF [Na] on high salt and hypersensitivity to sodium in the CSF. Whether this hypersensitivity persists during a chronic increase in CSF[Na+]11, as is the case in Dahl S rats,11 still needs to be assessed.

The current telemetry data show that, on a high-salt diet, BP of the −/− mice starts to increase after 3 to 4 days and after 7 to 10 days shows marked increases by 30 to 35 mm Hg, similar to the increase reported previously.8 HR increases in parallel, consistent with sympathetic hyperactivity. Central infusion of benzamil prevents most of the increases in BP and HR, indicating that ENaC in the brain is critical for the salt-induced hypertension in this model. Peripheral infusion of benzamil at the ICV rate does not affect the initial rise in BP or the further increase in daytime BP. It does attenuate the further rise in night BP, but this reduction is substantially less than that achieved by ICV benzamil (ie, 30% for SC versus 85% for ICV). This delayed and partial response to SC benzamil may reflect a slowly developing central blockade. Night (ie, active phase) BPs may be more sensitive to such blockade, but peripheral effects cannot be excluded. Although brain ENaC appears to mediate (most of) the salt-induced hypertension, it cannot be deduced from the present data whether the brain effects of high salt in −/− mice are secondary to, enhanced by, or independent of renal sodium retention. In balance studies, no evidence for renal sodium retention was found when −/− mice were placed on a high-salt diet.4 However, even if present, renal effects, per se, appear not sufficient to cause the hypertension, that is, they appear to depend on increased brain ENaC.

Limitation of the Present Study
Two connected limitations should be considered. ENaC is the best characterized target of Nedd4-2. However, as reviewed recently,17 Nedd4-2 may also regulate neuronal voltage-gated sodium channels, and their upregulation in the brain may potentially contribute to the salt-induced hypertension in the −/− mice. Benzamil is able to block these channels as well18 but at several fold higher IC50 than its IC50 for blockade of ENaC. Ito et al18 estimated that the rate of infusion of benzamil used in their and the present studies would result in a CSF concentration of <100 nmol/L and, according to our calculations, 10 to 15 nmol/L. Both concentrations would be in the range required for ENaC blockade, whereas the concentrations required for blockade of voltage-gated sodium channels are several fold higher.

Perspectives
Because the Nedd4-2−/− mouse is a model for Liddle syndrome, the present findings raise the question of whether similar aberrant regulation of CSF [Na] and/or hypersensitivity to sodium in the
CSF contribute to the salt-induced hypertension in Liddle patients or other forms of human salt-dependent hypertension. Sodium transport proteins, such as ENaC and Na\(^{+}\), K\(^{+}\)-ATPase, that are expressed in parallel in the brain, vascular smooth muscle, and kidneys are more the rule than the exception. Therefore, in cases of salt-dependent hypertension where mutations occur in such genes, aberrant neural BP control and/or secretion of sodium into the CSF should also be considered.

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**Disclosures**
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**References**


**Novelty and Significance**

- In salt-dependent hypertension with enhanced activity of these proteins, brain mechanisms should be considered as well.

**Summary**
We demonstrate that Nedd4-2 \(\text{Δ}^{\text{Δ}}\) mice also exhibit increased ENaC in the brain and that brain ENaC is needed for the dietary salt-induced hypertension in this animal model of Liddle syndrome.
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