CNS Neuroplasticity and Salt Sensitive Hypertension Induced by Prior Treatment with Sub-Pressor Doses of Angiotensin II or Aldosterone

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Running Title: CNS Neuroplasticity and Induced Salt-Sensitivity

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ABSTRACT

Although sensitivity to high dietary NaCl is regarded to be a risk factor for cardiovascular disease, the causes of salt-sensitive hypertension remain elusive. Previously we have shown that rats pretreated with sub-pressor doses of either angiotensin II (ANG II) or aldosterone (Aldo) show sensitized hypertensive responses to a mildly pressor dose of ANG II when tested after an intervening delay. The current studies investigated whether such treatments will induce salt-sensitivity. In studies employing an Induction-Delay-Expression experimental design, male rats were instrumented for chronic blood pressure (BP) recording. In separate experiments, ANG II, Aldo or vehicle were delivered either subcutaneously or intracerebroventricularly during Induction. There were no sustained differences in BP during Delay prior to being given 2% saline. While consuming 2% saline during Expression, both ANG II- and Aldo-pretreated rats showed significantly greater hypertension. When hexamethonium was used to assess autonomic control of BP, no differences in the decrease of BP in response ganglionic blockade were detected during Induction. However, during Expression the fall was greater in sensitized rats. In separate experiments, brain tissue collected at the end of Delay showed increases in message or activation of putative markers of neuroplasticity (i.e., brain-derived neurotrophic factor, p38 mitogen-activated protein kinase, cAMP response element-binding protein). These experiments demonstrate that prior administration of non-pressor doses of either ANG II or Aldo will induce salt-sensitivity. Collectively our findings indicate that treatment with sub-pressor doses of ANG II and Aldo initiate central neuroplastic changes that are involved in hypertension of different etiologies.

Key words: angiotensin II, aldosterone, sensitization, neuroplasticity, salt-sensitivity
INTRODUCTION

Many epidemiological studies indicate that high sodium chloride (i.e., salt) intake is associated with an increased risk of cardiovascular disease (5, 42). In the United States, about one half of all hypertensive patients are salt sensitive (45), and heritability analyses consistently indicate that there is a significant contribution of genetic factors to blood pressure (BP) related salt-sensitivity (21). The vasculature (19), kidney (35) and central nervous system [CNS; (18)] have all been implicated in salt-sensitivity, but the mechanisms associated with sodium chloride as an inducing or exacerbating factor of hypertension are far from clear.

Recent studies from our laboratory (48, 49) have shown that the hypertensive response to systemically administered angiotensin II (ANG II) can be markedly amplified by small non-pressor doses of ANG II or aldosterone (Aldo) administered several days earlier. The enhancing effects of earlier treatment with these factors are examples of response sensitization. Both of these models of hypertensive response sensitization are dependent upon the functional integrity of the brain renin-angiotensin-aldosterone system (RAAS) and can be reproduced by directly delivering ANG II or Aldo to the brain (48, 49). In addition, both models produce persistent neurochemical changes in the subfornical organ, median preoptic nucleus and the organum vasculosum which collectively are referred to as the structures of the lamina terminalis (LT). These changes outlast the presence of the exogenous ANG II or Aldo, and mRNA for many of the components of the brain RAAS remain elevated for at least one week after the termination of the sensitizing pretreatments (48, 49). The sustained changes in components of the brain RAAS suggest that neuroepigenetic mechanisms mediate the process of sensitization of the hypertensive response.
Neuroplasticity involving long term molecular and structural changes in the CNS has been demonstrated to be associated with sensitization of many physiological and behavioral responses. Examples of response sensitization mediated by CNS neuroplasticity include functional modification of neural pathways implicated in pain (4), pleasure [i.e., drugs of abuse (47)], baroreceptor and chemoreceptor reflexes (23), intermittent hypoxia (32), and exercise (24), as well as long-term potentiation and depression in the hippocampus and in many other brain regions (2). Neuroplasticity in the neural networks controlling many functions is essential during early development and for memory formation and maintenance throughout life. However, in some cases, CNS neuroplasticity-mediated sensitization will result in pathological conditions such as pain-related allodynia/hyperalgesia (36) and the craving for abused drugs (11).

There are many neurochemical changes associated with neuroplasticity. Some of these are unique for a particular functional system such as dopamine in natural and drug reward systems (43) or substance P in pain pathways (40). On the other hand, there are some molecules that seem to be ubiquitous in their involvement in neuroplasticity. One extracellular signal that has been implicated in many models of neuroplasticity is brain derived neurotrophic factor [BDNF; (50, 12)]. Consequently, finding evidence of a sustained increase in BDNF mRNA and protein and related molecular changes after treatments that enhance the hypertensive response would provide further evidence that the sensitization of hypertension produced by prior delivery of non-pressor doses of ANG II or Aldo involves cellular and molecular processes similar to those found in other cases or types of neuroplasticity and functional sensitization.
The initial goals of the following experiments were to determine if salt-sensitivity could be induced following the protocols we have used to sensitize ANG II-induced hypertension (48, 49), and to test if such sensitization was due to alterations of the sensitizing factors on the CNS. A second objective was to investigate the role of the sympathetic nervous system in the induction and expression of salt-sensitivity. The final aim was to determine if there are sustained molecular changes in putative markers of neuroplasticity produced by the sensitizing ANG II and Aldo treatments.

The design of the experiments followed the *Induction-Delay-Expression* paradigm used in previous work (48, 49). This involves systemic or central treatments that are delivered for a period of induction to sensitize the hypertensive response (*Induction*). This is followed by a period of rest (*Delay*), to be reasonably certain that the exogenous sensitizing agents are metabolized, and to demonstrate the persistence of the sensitized state. *Delay* is followed by the stimulus conditions that produce hypertension, and the response is studied throughout a period of expression (*Expression*). In the present work, this design was also followed in an experiment where the same pretreatments of low, non-pressor doses of either ANG II or Aldo were administered subcutaneously during *Induction*, but at the conclusion of *Delay*, brain tissue was collected to determine if canonical indices of neuroplasticity were present in structures of the LT.
METHODS

Animals/Animal Care

Male Sprague-Dawley rats (10 wk old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed in temperature- (22±0.2 C) and light- (12/12 h dark/light) controlled animal quarters. The rats were adapted to the laboratory for at least 7 days before experimental procedures were initiated. They had free access to rat chow (7013 NIH-31 modified rat diet, 0.25% NaCl) and drinking water, except where otherwise indicated in the experimental protocol. All experiments were conducted in accordance with the National Institutes of Health recommendation to follow the Guide for the Care and Use of Laboratory Animals of the National Research Council of the (U.S.) National Academies and were approved by The University of Iowa Animal Care and Use Committee.

Surgical Procedures

Telemetry probe implantation: Implantable rat transmitters (TA11PA-C40; Data Sciences International, St. Paul, MN) were used for chronic recording of BP and heart rate (HR). For implantation, rats were anesthetized with a ketamine-xylazine mixture (100 and 10mg/kg). The femoral artery of the rat was accessed with a ventral incision, the right femoral artery was isolated, and the catheter of a telemetry probe was inserted into the vessel. Through the same ventral incision, a pocket along the right flank was formed, and the body of the transmitter was slipped into the pocket. The incision was then closed with surgical staples.

Osmotic pump implantation: In the course of the experiments, rats were implanted with osmotic minipumps to deliver vehicle, ANG II, or Aldo either subcutaneously (sc) or
intracerebroventricularly (icv). For systemic delivery, osmotic pumps (Model 2001; Alzet, Cupertino, CA) were implanted sc in the back under isofluorane anesthesia. For central administration, an icv cannula was implanted into the right lateral ventricle (the coordinates: 0.9 mm caudal; 1.5 mm lateral to bregma; 4.5 mm below the skull surface). An osmotic minipump (Model 2001, Alzet,) attached to the icv cannula was implanted sc in the back.

Experimental Rationale and General Protocol: The present set of experiments employed an Induction-Delay-Expression experimental design, as previously described (48, 49). During Induction, a sub-pressor dose of Aldo, ANG II, or vehicle was delivered subcutaneously (sc, protocol I) or centrally (icv, protocol II) by osmotic minipump for 1 wk. This week was followed by a 1-wk Delay. After Delay, the rats’ drinking water was changed to 2% saline for 2 weeks of Expression. Cardiovascular parameters were measured throughout the experiments. Body weight was measured weekly. Drinking behavior was assessed as a daily amount averaged over 7 days; since Expression consisted of a two-week period, there is an average of the first 7 days as well as the final 7 days presented in Tables 2 and 3.

Experimental Protocol I: Sensitization of 2% saline-induced hypertension by induction with either systemic ANG II or Aldo: The objective of this experiment was to determine if the procedures employing very low doses of systemic ANG II or Aldo that induced the sensitization of slow pressor ANG II-induced hypertension (48, 49) could be used to sensitize the hypertensive response to another hypertension-generating stimulus, i.e., 2% saline as the sole drinking fluid (38, 10, 27). Rats were randomly assigned to one of three groups (n=10/group): 1) Induction with sc vehicle (isotonic saline; CONTROL), 2) Induction with sc ANG II (10
ng/kg/min, Sigma, St. Louis, MO), or 3) **Induction** with sc Aldo (750 ng/h; Sigma). Rats were implanted with radiotelemetry devices, and after 7 days of recovery, BP and HR were recorded for 7 days (baseline). Rats were then briefly anesthetized to implant osmotic minipumps sc to deliver vehicle, ANG II, or Aldo during **Induction**. After **Induction** and following **Delay**, 2% saline was given during **Expression**. The change in BP during each phase of the experiment was assessed and compared to baseline BP as described in more detail below.

**Experimental Protocol II**: Sensitization of 2% saline-induced hypertension by induction with either ANG II or Aldo administered icv: Because both systemically delivered ANG II and Aldo induced sensitization of 2% saline-induced hypertension, the next experiment was designed to determine if administration of ANG II and Aldo directly to the CNS would sensitize the response to 2% saline, as seen in our previous studies involving sensitization of ANG II-hypertension (44,45). Rats were randomly assigned to one of three groups (n=9/group): 1) **Induction** with icv vehicle (isotonic aCSF; “control”), 2) **Induction** with icv ANG II (1 ng/kg/min), and 3) **Induction** with icv Aldo (10ng/hr). The **Induction-Delay-Expression** paradigm used in Experimental Protocol I was followed except that during **Induction** vehicle, ANG II or Aldo were delivered by the icv route rather than sc. Additionally, in this protocol, the contribution of the autonomic nervous system to maintaining baseline BP was tested by ganglionic blockade administered on day 7 of baseline, on day 6 of **Induction**, and on day 12 of **Expression**. To do this, in a subset of animals, hexamethonium (30 mg/kg; Sigma) was injected intraperitoneally (ip) after recording resting BP for approximately 5 min. BP was then recorded for 10 minutes following the injection. The resting period was averaged to obtain a single resting BP; the response value was calculated as an average of 40-60 s during the maximum fall in BP.
Experimental Protocol III: Induction of changes of mRNA and protein expression in the lamina terminalis: Three additional groups of rats without telemetry probes were treated with sc vehicle, ANG II or Aldo during Induction using the same treatment parameters that were used in Protocol I except that at the end of a 1-wk Delay, the animals were euthanized by decapitation. The brains were rapidly collected and the ensemble of LT-related structures dissected from surrounding tissue and prepared for determination of mRNA and protein analysis.

Measurement of mRNA Expression in the LT: Total RNA was isolated immediately from fresh LT tissue samples using the Trizol method (Invitrogen, Grand Island, NY). Total RNA was reverse transcribed using random hexamers following the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA). cDNA was amplified and analyzed using a C1000 Thermocycler System (Bio-Rad, Hercules, CA). Changes in mRNA expression levels were normalized to expression of GAPDH and calculated using the ΔΔCt method. Results are expressed as relative fold change from control, mean fold change ± SE. The sequences of the primers for rt-PCR for BDNF, vascular endothelial growth factor (VEGF); and the BDNF receptor, tropomyosin-related kinase B (TrkB); are provided in Table 1. The primers were purchased from Integrated DNA Technologies (Coralville, IA).

Measurement of Protein Expression in the LT: Fresh LT tissue was harvested and immediately sonicated in Tris buffer with added protease inhibitors to isolate total cellular protein. Protein samples were mixed with equal volumes SDS-PAGE buffer and loaded on a 10% SDS-PAGE gel for electrophoresis and then transferred to a PVDF membrane (Bio-Rad). Membranes were
probed with anti-rabbit antibody for anti-BDNF (1:200, ANT-010, Alomone Labs Ltd, Israel), anti-rabbit antibody for anti-phosphorylated-p38 mitogen activated protein kinase (p-p38 MAPK; 1:1000, Cell Signaling, Danvers, MA), anti-rabbit antibody for anti-p38 mitogen activated protein kinase (p38 MAPK; 1:1000, Cell Signaling), anti-rabbit antibody for anti-phosphorylated-cyclic AMP response element binding protein (p-CREB) (1:1000, Cell Signaling), and anti-rabbit antibody for anti-CREB (1:1000, Cell Signaling). This was followed by horseradish peroxidase-labeled mouse anti-rabbit antibody (Cell Signaling) and then subjected to enhanced chemiluminescence reagents (Supersignal Substrate Western Blotting, Thermo Scientific, Rockford, IL). Band intensities were quantified with the Imager software (Bio-Rad) and were normalized to β-actin (1:5000, Sigma).

Cardiovascular Data Analysis: Baseline BP and HR data were collected for 7 days of baseline and for 28 days throughout Induction, Delay and Expression. BP and HR are presented as mean daily values averaged over 24 hours. A single mean value for each phase of the experiment was calculated for each animal by averaging over 5 days of baseline, 5 days of Induction, 5 days of Delay, and finally, the last 5 days of Expression. Difference scores for BP and HR were calculated for each animal and a repeated measure ANOVA was conducted. Post-hoc analysis was performed using the Bonferroni test. Statistical significance was set at $P \leq 0.05$.

Molecular Data Analysis: Comparisons between groups were made on the average values obtained in the above molecular methods (RT-PCR and Western blot) using an unpaired t-test. Statistical significance was considered at $P=0.05$ or higher.
RESULTS

The sensitization of 2% saline-elicited hypertension induced by systemic administration of low doses of either ANG II or Aldo: Mean daily BP and HR responses over the course of the baseline and induction, delay and expression in three groups of rats treated with sc administered vehicle, ANG II or Aldo during induction are presented in Figure 1. In comparison to the baseline period, treatment with low doses of ANG II and of Aldo had no effect on BP (Figure 1A, C) or HR (Figure 1B, D) during either induction or delay. Providing 2% saline as the sole fluid significantly increased BP during expression in all groups (F(4,52)=2.467, P<0.05); however, this increase was greatest in animals pre-treated with either ANG II or Aldo (P<0.05). HR fell throughout the experimental protocol (F(4,52)=3.038, P<0.05); however no differences could be detected between groups at any time point.

Presented in Table 2 are the body weights and fluid intakes for the three groups during baseline, induction, delay and early and late expression. There were no differences in body weight or water intake among the groups during induction or delay, and most importantly, there were no differences in the intake of 2% saline during expression.

The sensitization of 2% saline-elicited hypertension induced by icv administration of low doses of either ANG II or of Aldo: Mean daily BP and HR responses over the course of baseline, induction, delay and expression in the three groups of rats treated with icv vehicle, ANG II or Aldo are presented in Figure 2 A, B. In comparison to the baseline period, treatment with the low dose of Aldo had no effect on BP (Figure 2A, C) or HR (Figure 2B, D) during either induction or delay. In the case of icv administration of ANG II there was a significant increase in BP.
The body weights and fluid intakes for the three groups during baseline, Induction, Delay, and early and late Expression are presented in Table 3. Not unexpectedly, icv ANG II during Induction resulted in an increase in water intake that returned to baseline after this period. No differences in body weight or fluid intake were detected among the three groups during any other phase of the experiment.

Shown in Figure 3 are the effects of ganglionic blockade with hexamethonium during baseline, Induction, and Expression. When tested during Induction, ganglionic blockade had no greater effect on the fall in BP in the groups given icv sub-pressor doses of ANG II and Aldo than hexamethonium had in animals receiving icv vehicle (Figure 3). However, the fall in BP produced by ganglionic blockade at the end of Expression was greater in all animals (F(4,20)=2.90, P<0.05) but was significantly greater (P<0.05) in animals pre-treated icv with either ANG II or Aldo, indicating that the BP of the sensitized rats was more dependent on sympathetic tone during this phase.
The effect of treatment with sc ANG II or Aldo given during Induction on mRNA and protein expression in the lamina terminalis at the end of Delay: In LT-associated tissues harvested at the end of Delay, sc ANG II and Aldo infusions given during Induction resulted in increased mRNA expression of BDNF but not VEGF or TrkB (Figure 4). Increased protein expression of BDNF was also detected (Figure 5). The ANG II and Aldo sensitizing treatments given during Induction were also associated with significant increases in the phosphorylated form of the second messenger, p38 MAPK, and of the transcription factor, CREB, at the end of Delay (Figures 6 and 7). There was no change in the total p38 MAPK or CREB (Figures 6 and 7).

DISCUSSION

There are four important new findings reported in this paper. First, the hypertensive response to orally consumed 2% NaCl is sensitized by pre-exposure to non-pressor doses of systemically administered ANG II and Aldo. Second, sensitization of the hypertensive response to 2% NaCl can be produced by directly administering ANG II and Aldo to the brain. Third, although icv ANG II and Aldo administered during Induction enhanced the hypertensive response to 2% NaCl presented during Expression, there was no apparent change in the contribution of the sympathetic nervous system to maintaining BP at the end of Induction. However, the contribution of sympathetic tone was significantly enhanced by the end of Expression in sensitized animals. Fourth, systemic treatments used for inducing sensitization produced changes in BDNF and increased amounts of p-p38 MAPK and p-CREB in LT-associated tissues. It is notable that these changes in BDNF and the phosphorylation states of p38 MAPK and CREB persisted well after the cessation of ANG II or Aldo administration, and this was at a
time when BP was at control levels. The significance of these four findings will be elaborated upon in the following discussion.

Sensitization of the hypertensive response and the mechanisms of sensitization

The phenomenon or process of sensitization is operationally defined by administering a stimulus that alters the state of the organism to result in a response of increased magnitude when the stimulus is administered at a later time. Sensitization can also be produced by stimuli other than the one used to test for the expression of a sensitized response. This is referred to as cross-sensitization. The current studies provide additional evidence that sensitization, or more specifically cross-sensitization, of a hypertensive response can be induced by either ANG II or Aldo, two key factors implicated in the regulation of body fluid balance and BP homeostasis. In previous studies we used the same systemic and central treatment parameters to deliver either ANG II or Aldo during Induction and followed these by the same duration of Delay to demonstrate the sensitization and cross-sensitization of slow-pressor ANG II-hypertension (48, 49). Taken together, our previous and the current findings indicate that low levels of ANG II or Aldo can modify the CNS and sensitize different forms of experimental hypertension.

In experimental Protocol II, the contribution of the sympathetic nervous system to the induction of sensitization and to the expression of hypertension was tested by administering a ganglionic blocker during the baseline period, at the end of Induction, and at the end of Expression. Following Induction, the fall in BP produced by hexamethonium was no different than the effect during the baseline period. However, at the end of Expression, ganglionic blockade resulted in a significantly greater decrease in BP in the groups that were sensitized
with either icv ANG II or Aldo. These results indicate that the sustained sensitized state produced by the low doses of ANG II or Aldo probably does not involve a chronic increase in sympathetic tone, but rather that central mechanisms may have become more reactive to pressor stimuli delivered during Expression to generate enhanced sympathetic drive during this later phase.

The current studies along with our previous work employing the same low doses of either ANG II (48) or Aldo (49) to induce sensitization suggest that the CNS can be reprogrammed to generate a greater hypertensive response to subsequently administered pressor challenges. In this respect the sensitization of hypertension resembles other cases of enhanced or sustained responsiveness observed for many physiological and behavioral responses [e.g., pain (4), pleasure (47), chemo- and baro-receptor reflexes (23) and analogues of learning (2)] that are modified and maintained as a result of life experiences (i.e., the presence of antecedent inducing stimuli). Evidence indicates that these types of changes in molar responses involve molecular modifications (i.e., neuroplasticity) in the associated neural networks that control them. In this light it seems reasonable to think that neuroplasticity underlying the sensitization of the hypertensive response involves components of the neural network controlling BP. In part, this network includes structures of the LT (i.e., the subfornical organ, median preoptic nucleus and the organum vasculosum), the paraventricular hypothalamic nucleus (PVN), the rostral ventrolateral medulla, and the interomediolateral cell column of the spinal cord (14).

In earlier work where the BP response to slow pressor ANG II-induced hypertension was sensitized by the same non-pressor doses of ANG II (48) or Aldo (49) that were used in the
present studies, we found that at the end of Delay there were sustained increases in mRNA of several components of the brain renin-angiotensin-aldosterone system (e.g., angiotensin type 1 and mineralocorticoid receptors; angiotensinogen; angiotensin converting enzyme) in LT structures. These increases in expression were not seen at the same time in the PVN. Although these previous experiments provided evidence of sustained neuroplasticity in the LT, it is recognized that this region constitutes only part of the neural network controlling BP, and it is likely that neuroplasticity associated with the process of sensitization of the hypertensive response will occur at multiple central sites. Also plasticity in different components may come into play at different times over the process of induction, maintenance and expression of the sensitized hypertensive response.

Often it is the case that neuroplasticity in a neural network controlling a particular response involves molecular mediators that are unique to that system (i.e., “signature” molecules). For example, substance P and calcitonin gene-related peptide are signature molecules that have been uniquely associated with the neuroplasticity of pain (40). It seems reasonable to propose that one or more of these endogenous brain RAAS factors may be responsible for enhancing neural excitation in response to different pressor stimuli administered during Expression.

Although components of the brain RAAS might be considered as key signature molecules for mediating the sensitization of hypertension, it is likely that there are ubiquitous neuroplasticity-associated factors also involved. Recognizing this, one of the purposes of the present studies was to explore whether there are molecular indicators present in the LT that are commonly seen in other cases or models of neuroplasticity. The current studies investigated whether
there are molecular changes in the LT of 1) two growth factors, BDNF and VEGF, 2) the TrkB receptor, 3) p-38 MAPK, a key intracellular molecule implicated in BDNF signaling, and 4) the transcription factor, CREB, which is important in the control of the synthesis of new proteins. We found that ANG II and Aldo sensitization-inducing treatments produced up-regulation of BDNF mRNA but not for VEGF or TrkB message. Importantly, although the total amounts p38 MAPK and CREB did not change, there was a shift from the unphosphorylated to phosphorylated state which is indicative of their functional activation. Collectively these findings lend further support for a role of the CNS and in particular the LT in the molecular neurobiology of the sensitization of hypertension.

Of the molecular factors investigated here, BDNF is probably the most frequently acknowledged to be associated with neuroplasticity and sensitization. This neurotrophic factor has been implicated in almost every type of neuroplasticity (50) including long-term potentiation (41, 31), pain modulation (36, 30), and structural and functional changes in drug addiction (37, 28). BDNF binds to the TrkB receptor (37) to increase the generation of many proteins within cells, including neurotransmitters, neuromodulators, structural proteins and transcription factors. To accomplish this, BDNF/TrkB receptor signaling involves three main intracellular pathways, one of which is the Ras-MAPK-ERK pathway (20, 31). This signaling cascade, along with others, can activate transcription factors such as CREB (31). While the present findings demonstrate persistent molecular changes in the LT structures associated with the process of the induction of salt-sensitivity, they do not directly test the role of BDNF, p38 MAPK, or CREB in the sensitization of hypertension. However, given their identified roles in
mediating the process of sensitization and neuroplasticity generally, they must be considered to be important candidates for future functional experiments.

BDNF and ANG II potentially can interact to influence gene expression (3). It is important to note that that ANG II can mobilize signaling cascades that converge to activate MAPK and CREB (15). This may represent a mechanism for synergism between ANG II and BDNF suggesting the pattern, magnitude and strength of MAPK or CREB activation may influence long-term neuroplasticity associated with the brain RAAS and the sensitization of hypertension. Although implicating the brain RAAS along with BDNF, p38 MAPK, and CREB in the present and previous (48, 49) work provides the basis for future studies, it is important to recognize that there are likely to be many other factors involved in extra- and intra-cellular signaling associated with neuroplasticity triggered by sensitizing stimuli and the hypertensive response.

One of the long standing questions regarding the role of CNS plasticity underlying long-term functional changes induced by experience is how changes in neural function and associated molecular mediators are maintained over the long term? The recent emergence of the field of neuroepigenetics focuses on how life events can alter the probability of gene expression through the modification of chromatin structure or through DNA methylation (29, 44) in neurons. There is an increased appreciation that the majority of cases of human hypertension is probably the result of gene X environmental interactions and that the study of epigenetics will be necessary to fully understand the pathogenesis of high BP (6).

*Salt-Sensitivity and Hypertension*
In unselected samples of humans and experimental animals, there are no clear relationships between salt intake and BP (34). However, there is a subset of salt sensitive individuals whose BP increases when they receive increased NaCl and falls when it is withdrawn. Data from a study following a rigorously specified protocol (45) indicated that of hypertensive patients in the U.S., about 51% are salt sensitive and about 33% are salt resistant and that among normotensive individuals, 26% are salt sensitive and about 58% are salt resistant (19). Similarly, as observed by Dahl from his early studies on salt feeding (7), the BP response to high dietary salt was extremely variable in a common (Sprague-Dawley) strain of laboratory rat. This observation led Dahl and colleagues (8) to selectively breed for salt-sensitivity and salt-resistance.

There are several genetic models of salt sensitive hypertensive rats including the Dahl salt sensitive- [Dahl S; (8)], borderline hypertensive- (26), salt sensitive spontaneously hypertensive-(1) and Lyon genetically hypertensive-rat (10). These strains were initially bred specifically for either a BP increase to high salt intake or first selected for high BP and discovered later to be salt sensitive. To demonstrate salt-sensitivity in rats, various methods have been used to increase body or brain sodium. These include adulteration of laboratory chow with high concentrations of NaCl [e.g., 8% in Dahl’s experiments; (8)], icv infusion of Na⁺ rich artificial cerebrospinal fluid (17) and 1 or 2% saline as the sole fluid source (38,10,27). The current study employed 2% NaCl as the sole source of fluid to elicit hypertension. There is no a priori reason to believe that other methods used to test salt sensitivity would not produce similar results to those obtained in the current experiments. In the present study no consistent effect of salt
loading was seen on HR. The reason for this is unclear, but several other studies conducted on rats and rabbits also found no consistent effects of high salt intake on HR (13, 16, 25, 33, 46).

In addition to the effects of high NaCl intake in genetic models, nearly all forms of experimental hypertension [i.e. those models where high BP is induced by surgical manipulation (e.g., 9) or by drug or hormone delivery (e.g., 39, 22)] are also exacerbated by high salt intake. It is relevant to note that there is an aspect of the induction of salt-sensitivity by sensitization with ANG II or Aldo pretreatments that more closely resembles salt-sensitivity displayed by genetic models of hypertension in comparison to surgical or drug/hormonal experimental models. Both in the genetic and in the ANG II or Aldo induction models, it is not necessary to administer any other treatment at the same time high salt is provided.

The demonstrations that an enhanced hypertensive response to oral saline intake is sensitized by earlier treatment with low doses of either ANG II or Aldo represent new models of salt sensitive-hypertension. For studying the mechanisms of neuroplasticity in the sensitization of hypertension, these methods have the advantage over other experimental models where high salt must be co-administered along with another hypertension-generating stimulus (e.g., the simultaneous infusion of pressor doses of ANG II or Aldo). Those requiring co-administration confound the processes involved in of induction with those of expression. The Induction-Delay-Expression experimental paradigm can dissociate the process of induction of a predisposing state from the actions of a later stimulus that actually drives the expression of high BP. In other words, the advantage of the Induction-Delay-Expression model is that it is ideal for permitting an analysis of neuroplasticity that is likely to occur during Induction without
the confounding effects of molecular and physiological changes that accompany expression when animals become frankly hypertensive.

**Perspectives and Significance**

The current experiments demonstrate that antecedent systemic or central (icv) administration of low, non-pressor doses of either ANG II or Aldo induces salt-sensitivity. The systemic ANG II and Aldo treatments used to create this type of salt-sensitivity produce molecular changes in forebrain structures implicated in the control of BP and hypertension. These CNS changes persist after termination of the sensitizing treatments. Specifically, increased BDNF mRNA and protein and elevated phosphorylation of p-38 MAPK and of CREB were present at a time when water would have been replaced with 2% saline.

Increases in BDNF and indications of activation of downstream BDNF-associated intracellular signaling molecules are commonly observed in many models of neuroplasticity and response sensitization. The findings of these studies provide two new models for investigating salt-sensitive hypertension and give insight into the nature of the brain neuroplasticity and the sensitization of hypertension.

Both the present and past (48, 49) studies indicate that the hypertensive response can be sensitized and that there are sustained changes in molecular components that are likely candidates as mediators of neuroplasticity underlying the enhanced response. In light of these findings along with a growing recognition of the likely role of epigenetic mechanisms (6) in the pathogenesis of hypertension there is a need to consider a new paradigm for conceptualizing and investigating the mechanisms responsible for this type of disordered BP regulation. A new
perspective requires a shift from focusing only on age- or disease-related damage to systemic receptors (e.g., baro-/chemo-receptors), simple reflex arcs or effectors (e.g., the kidney or blood vessels) as the cause of high BP. Rather there needs to be increased recognition that the pathogenesis of many, or perhaps even most, cases of high BP are associated with CNS plasticity involving neuroepigenetic-mediated mechanisms. Such neuroplastic changes can initially be triggered by life events (i.e., physiological and environmental stressors) that produce a maintained a predisposition to respond to subsequent challenges with enhanced sympathetic and neuroendocrine drive to the distal effectors to alter the course of the long-term maintenance of BP.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.


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Table 1. Primer sequences for real time rt-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Product size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>AAAGGAGCCCCATCACAATC</td>
<td>AACAGGACGGAAACAGAACG</td>
<td>207</td>
<td>NM_012513</td>
</tr>
<tr>
<td>Rattus norvegicus vascular endothelial growth factor</td>
<td>CACATAGGAGAGATGAGCTTC</td>
<td>GCGAGTCTGTGTTTTTCAG</td>
<td>166</td>
<td>BC168708.1</td>
</tr>
<tr>
<td>Rat BDNF receptor Trk 2</td>
<td>AGCAGCCCTGTTATGCTCA</td>
<td>TCGCCAAGTTCTGAAGGAGT</td>
<td>183</td>
<td>M55291</td>
</tr>
</tbody>
</table>

BDNF: brain derived neurotrophic factor.
Table 2. Body weight and drinking volumes during experimental protocol I. Drinking volumes are for water, unless otherwise noted, and reflect the daily average of all animals on the protocol.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>sc ANG II IND (n=10)</th>
<th>sc Aldo IND (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW (g)</td>
<td>Drinking (ml/d)</td>
<td>BW (g)</td>
</tr>
<tr>
<td>Baseline</td>
<td>347±8</td>
<td>27±1</td>
<td>333±8</td>
</tr>
<tr>
<td>Induction</td>
<td>372±6</td>
<td>28±1</td>
<td>361±9</td>
</tr>
<tr>
<td>Delay</td>
<td>385±6</td>
<td>27±1</td>
<td>378±7</td>
</tr>
<tr>
<td>Expression (2% saline)- first week</td>
<td>386±9</td>
<td>40±5</td>
<td>379±7</td>
</tr>
<tr>
<td>Expression (2% saline)- last week</td>
<td>411±9</td>
<td>62±2</td>
<td>404±5</td>
</tr>
</tbody>
</table>
Table 3. Body weight and drinking volumes during experimental protocol II. Drinking volumes are for water, unless otherwise noted, and reflect the daily average of all animals on the protocol.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=9)</th>
<th>ICV ANG II IND (n=9)</th>
<th>ICV Aldo IND (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW (g)</td>
<td>Drinking (ml/d)</td>
<td>BW (g)</td>
</tr>
<tr>
<td>Baseline</td>
<td>326±6</td>
<td>26±2</td>
<td>327±6</td>
</tr>
<tr>
<td>Induction</td>
<td>357±8</td>
<td>28±3</td>
<td>347±5</td>
</tr>
<tr>
<td>Delay</td>
<td>368±8</td>
<td>27±2</td>
<td>347±7</td>
</tr>
<tr>
<td>Expression (2% saline)- first week</td>
<td>365±7</td>
<td>43±4</td>
<td>360±7</td>
</tr>
<tr>
<td>Expression (2% saline)-last week</td>
<td>367±8</td>
<td>59±6</td>
<td>359±9</td>
</tr>
</tbody>
</table>
Figure 1. Hemodynamics of animals subjected to sc infusion of ANG II or Aldo during Induction or Delay; however, during Expression, pre-treated animals displayed a greater increase in MAP from baseline than control animals. Panel B shows the time course of HR during the experiment; panel D is the difference scores for each phase of the experiment. Animals pre-treated with Aldo showed an increased HR during Induction and Delay; however, no difference between groups was detected during Expression. (n=9-10 in each group; *P<0.05 vs. sham-operated rats.)

Figure 2. Hemodynamics of animals subjected to icv infusion of ANG II or Aldo during Induction. Panel A shows the time course of mean arterial pressure (MAP) during the experiment; panel C is the difference scores from baseline for each phase of the experiment. Animals pre-treated with ANG II displayed an increase in MAP during Induction; during Expression, both animals pre-treated with ANG II and Aldo displayed a greater increase in MAP. Panel B shows the time course of HR during the experiment; panel D is the difference scores for each phase of the experiment. Animals pre-treated with ANG II showed an increased HR only during Induction. (n=9 in each group; *P<0.05 vs. sham-operated rats.)

Figure 3. Contribution of the sympathetic nervous system to baseline BP. Ganglionic blockade was performed during each phase of the experimental protocol. Response is reported as % change from baseline BP recorded just prior to the hexamethonium injection. No difference in
the magnitude of the BP response between sham-operated and sensitized animals during baseline and **Induction** was detected; however, during **Expression**, sensitized animals display a greater change in BP after the hexamethonium treatment. (*P<0.05 vs. sham-operated rats.)

**Figure 4.** Gene expression of BDNF, TrkB, and VEGF in the LT. The LT of Aldo- and ANG II-infused rats were found to have a higher expression of the mRNA for BDNF than sham-operated controls after **Induction** and **Delay**. However, mRNA expression of VEGF and TrkB, respectively, was not different between groups of animals. (n=5 in each group; *P<0.05 vs. sham-operated controls.)

**Figure 5.** Protein expression of BDNF in the LT. The LT of Aldo- and ANG II-infused rats were found to have higher protein expression of total BDNF, including both the precursor (pro-BDNF, corresponding to 27 kD band) and total BDNF (corresponding to 14 kD band). (n=3 in each group; *P<0.05 vs. sham-operated controls.)

**Figure 6.** MAPK phosphorylation after **Induction** and **Delay** in the LT. While no change was detected in total p38 MAPK expression, Aldo- and ANG II-infused rats were found to have a higher p-p38 MAPK expression. (n=3-4 in each group; *P<0.05 vs. sham-operated controls.)

**Figure 7.** CREB phosphorylation after **Induction** and **Delay** in the LT. While no change was detected in total CREB expression, Aldo- and ANG II-infused rats were found to have a higher p-CREB expression. (n=4 in each group; *P<0.05 vs. sham-operated controls.)
Figure 1.

A

MAP (mmHg)

Baseline | Induction | Delay | Expression

control
sc ANG II IND
sc Aldo IND

day

B

HR (bpm)

Baseline | Induction | Delay | Expression

control
sc ANG II IND
sc Aldo IND

day
Figure 1, continued.

C

![Graph C: Delta MAP From Baseline (mmHg)]

- Induction
- Delay
- Expression

Legend:
- □ control
- ◊ sc ANG II IND
- ■ sc Aldo IND

D

![Graph D: Delta HR From Baseline (bpm)]

- Induction
- Delay
- Expression

Legend:
- □ control
- ◊ sc ANG II IND
- ■ sc Aldo IND
Figure 2.

A

MAP (mmHg)

Baseline  Induction  Delay  Expression

Day

B

HR (bpm)

Baseline  Induction  Delay  Expression

Day
Figure 2, continued.
Figure 3.
Figure 4.
Figure 5.

(A) Western blot analysis of BDNF and β-actin expression in control and treated groups. 27KD and 14KD bands are indicated.

(B) Graph showing the ratio of (pro-BDNF+BDNF)/β-actin in control, sc ANG II IND, and sc Aldo IND groups. Significant differences are indicated by *.

Legend:
- □ control
- ◯ sc ANG II IND
- ■ sc Aldo IND
Figure 6.
Figure 7.