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CNS sites activated by renal pelvic epithelial sodium channels (ENaCs) in response to hypertonic saline in awake rats

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Abstract

In some patients, renal nerve denervation has been reported to be an effective treatment for essential hypertension. Considerable evidence suggests that afferent renal nerves (ARN) and sodium balance play important roles in the development and maintenance of high blood pressure. ARN are sensitive to sodium concentrations in the renal pelvis. To better understand the role of ARN, we infused isotonic or hypertonic NaCl (308 or 500 mOsm) into the left renal pelvis of conscious rats for two hours while recording arterial pressure and heart rate. Subsequently, brain tissue was analyzed for immunohistochemical detection of the protein Fos, a marker for neuronal activation. Fos-immunoreactive neurons were identified in numerous sites in the forebrain and brainstem. These areas included the nucleus tractus solitarius (NTS), the lateral parabrachial nucleus, the paraventricular nucleus of the hypothalamus (PVH) and the supraoptic nucleus (SON). The most effective stimulus was 500 mOsmNaCl. Activation of these sites was attenuated or prevented by administration of benzamil (1 μ M) or amiloride (10 μ M) into the renal pelvis concomitantly with hypertonic saline. In anesthetized rats, infusion of hypertonic saline but not isotonic saline into the renal pelvis elevated ARN activity and this increase was attenuated by simultaneous infusion of benzamil or amiloride. We propose that renal pelvic epithelial sodium channels (ENaCs) play a role in activation of ARN and, via central visceral afferent circuits, this system modulates fluid volume and peripheral blood pressure. These pathways may contribute to the development of hypertension.

Keywords

renal afferent nerves; epithelial sodium channels (ENaCs); arterial pressure regulation; fluid volume regulation; hypertonic saline; c-fos activation; CNS projections

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1. Introduction

Renal denervation has been shown to attenuate or delay the development of experimental hypertension in several animal models (for reviews, see Katholi, 1985; Wyss et al., 1992). Based on these and other observations, clinical trials were undertaken to treat resistant primary hypertension in humans by catheter-based radiofrequency ablation of renal nerves (Krum et al., 2009; Esler et al., 2010). Results from the first two clinical studies suggested that renal denervation was successful in reducing arterial pressure for at least three years (Esler et al., 2014; Krum et al., 2014) although a recent study failed to confirm these findings (Bhatt et al., 2014). It has long been posited that elevated central sympathetic drive is a common contributing factor in essential hypertension (Esler and Kaye, 1998; Schlaich et al., 2004; Joyner et al., 2008; Malpas, 2009). Increased muscle sympathetic nerve activity in hypertensive patients has been reported to be reduced by catheter-based renal denervation (Schlaich et al., 2013; Hering et al., 2014). The mechanism by which renal denervation interrupts the development of hypertension or might interfere with central sympathetic outflow has not been elucidated.

Several investigators have suggested that ARN may trigger excess sympathetic activity contributing to the development of hypertension (Katholi, 1985; Ciriello and de Oliveira, 2002; Veelken and Schmieder, 2014). Renal afferent nerves are located predominantly in the renal pelvis with the majority containing CGRP and/or Substance P (Ferguson and Bell, 1988; Marfurt and Echtenkamp, 1991; Kopp, 2015). Renal afferent fibers carry both mechano- and chemoreceptor-sensitive nerves (Dietz and Gilmore, 1991; DiBona and Kopp, 1997; Kopp, 2011). Thus, these fibers are sensitive to various stimuli, including ischemia, as well as changes in ionic composition of urine and hydrostatic pressure within the renal pelvis (Recordati et al., 1980; Kopp et al., 1998). In the rat, chemosensitive neurons have been identified that respond to changes in NaCl concentration, as well as other chemoreceptor stimuli (Recordati et al., 1980; Kopp, 2011). The signaling of these renal afferents has been shown to play an important role in body fluid homeostasis and cardiovascular regulation, as stimulation of these fibers leads to release of vasopressin as well as changes in arterial pressure (Simon et al., 1989).

Renal afferent fibers arise from sensory neurons in the dorsal root ganglia (DRG) of caudal thoracic and rostral lumbar spinal segments (Ciriello and Calaresu, 1983; Knuepfer et al., 1988; Weiss and Chowdhury, 1998). Electrical stimulation of the renal nerve activated *c-fos* in several sites in the brain including the nucleus of the solitary tract (NTS), the subfornical organ (SFO), the paraventricular nucleus of the hypothalamus (PVH), and the supraoptic nucleus (SON) (Day and Ciriello, 1985; Solano-Flores et al., 1997). Multisynaptic ARN projections to the NTS, PVH, SON and circumventricular organs have also been mapped using the transneuronal tracer pseudorabies virus (Weiss and Chowdhury, 1998; Huang and Weiss 1999; Huang et al., 2002) but this technique does not discriminate between afferent and efferent projections. These data demonstrate the existence of central renal projections but do not discriminate between specific mechano- or chemoreceptor inputs from the kidney.

Epithelial sodium channels (ENaCs) exist in principal cells in the renal collecting duct and mediate the apical entry of sodium (Rossier, 2014). ENaCs have been identified

immunohistochemically in urothelium covering the pelvis and ureters (Hager et al., 2001). ENaCs are important in ion transport and in maintaining osmotic gradients between the tubular fluid and the blood. ENaCs are regulated by aldosterone and also sensitive to a host of other paracrine and autocrine factors (Pearce et al., 2014). Mechanoreceptor transduction in the heart and kidney have been reported to be dependent on activation of ENaCs since afferent nerve activity is prevented by low concentrations of amiloride (Kopp et al., 1998; Ditting et al., 2003). Frelin et al. (1988) determined that ENaCs can be inhibited by lower doses of amiloride or benzamil without interfering with other Na dependent channels. Therefore, ENaCs play a role both in regulating sodium excretion and reabsorption and in pressure sensitive renal afferent pelvic nerves.

In the present study, we identified specific central sites activated in response to renal afferent stimulation by intrarenal pelvic infusion of isotonic or hypertonic sodium chloride solutions. Sites of activation were identified using immunohistochemical staining for the protein Fos, a product of the immediate early proto-oncogene *c-fos*. We verified activation of renal afferent nerves by recording sensory nerves in isoflurane-anesthetized rats using a similar pelvic infusion procedure. We also determined that ENaCs were responsible for both physiological and immunohistochemical responses to hypertonic saline using benzamil and amiloride. By identifying these central sodium-sensitive projections, we hoped to gain a better understanding of the role of renal afferents in the control of central sympathetic drive and the development of hypertension.

2. Materials and Methods

2.1 Studies in Conscious Rats

2.1.1 Surgical Preparation—Male Sprague-Dawley rats (n=56, 220–300g BW, Harlan Laboratories, Indianapolis, IN) were housed in individual cages on a 12-12 hour light-dark cycle. All recovery surgical procedures were performed using aseptic technique. All wounds were sutured and treated with Bactroban cream (GlaxoSmithKline, Research Triangle Park, NC) and with antibiotic therapy using enrofloxacin (Baytril, 10 mg/Kg, sc, Bayer Corp, Pittsburgh, PA) and analgesic/anti-inflammatory agent carprofen (Rimadyl, 20 mg/Kg, ip, Zoetis, Inc., Kalamazoo, MI). All surgical and experimental procedures were approved by the St. Louis University Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines as described in the Guide for the Care and Use of Laboratory Animals (2011).

Arterial and venous cannulas were implanted under isoflurane anesthesia. The femoral artery and vein were exposed and cannulated. The cannulas were tunneled subcutaneously and exited through a cutaneous incision between the scapulae. The cannulas were attached to the underlying muscle and plugged for later use.

Dual-lumen (n=42) or triple-lumen (n=14) cannulas (CR2L32 Cth or CR3L32 Cth, ReCathCo, Allison Park, PA) were implanted into the renal pelvis of the animals to allow for infusion of various concentrations of saline during the experiment. A left flank incision was made in the abdomen of the animals to expose the ureter for retrograde insertion of a sterile multi-lumen cannula (32 awg each) into the pelvis which was then sutured in place. The

cannula was tunneled subcutaneously and exteriorized between the scapulae and all cutaneous wounds were sutured. The renal pelvic cannulae were left open to allow for the continual flow of urine from the renal pelvis. The animals were allowed at least five days for recovery before being tested.

2.1.2 Experimental Protocol in Conscious Rats—Rats were moved to a quiet room in their home cage and allowed at least 12 hours to acclimate before infusions while maintaining the scheduled light:dark cycle. On the day of the experiment, one cannula was connected to a peristaltic infusion pump (Gilson Minipuls 2, Middleton, WI) while the other cannula was connected to the same pump for withdrawal in a push-pull fashion. Pumps were calibrated to deliver 50 μ l/min (range of 40–75 μ l/min) and to remove a similar volume. Those rats with a third pelvic cannula were connected to a strain gauge for measuring pelvic pressure. Intrarenal pelvic perfusions of hypertonic (500 mOsm, n=8) or normal NaCl (308 mOsm, n=8) were performed in conscious freely moving rats for 2 hours. A group of instrumented rats was used as control (n=10), undergoing the same experimental protocol without receiving an infusion. Two other control groups were given hypertonic saline (500 mOsm) and benzamil (1 μ M, n=7) or amiloride (10 μ M, n= 5) to determine whether pelvic ENaC mediated activation of sensory afferent nerves. Arterial pressure and heart rate were monitored continuously through the femoral artery using a strain gauge. All animals undergoing the NaCl infusion were monitored for signs of irritation and, in the case of those with triple lumen cannulas, for increases of pelvic pressure (>10 mmHg). Excessive ambulation or alerting responses were interpreted as possible increased intrarenal pressure leading to irritation. Rats exhibiting these signs (n=6) or measured elevated pelvic pressure (n=2) were excluded from the results.

2.1.3. Immunohistochemistry—After 120 min of intrarenal NaCl infusion (see above) or sham treatment in awake rats the animals were anesthetized with 4–5% isoflurane, and then, given pentobarbital (65 mg/kg, i.v.). They were perfused through the heart with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Their brains were removed immediately, and stored in fixative for one to four days. In several rats (n=4), the perfusion was deemed incomplete since significant artifact (including erythrocytes) were noted in the brain. These rats were excluded from the analysis.

Brains from the rats were cut in the transverse plane at 50 μ m on a freezing microtome and the sections were stored in 0.1 M sodium phosphate buffer (pH = 7.4) containing 0.1% sodium azide. Single-label immunohistochemistry for Fos was performed on every fifth section, using the ABC-DAB histochemical technique. Free-floating sections were incubated overnight at room temperature in an antiserum raised in rabbit diluted at 1:8,000 (Fos “Ab-5” from Oncogene; now PC38, Calbiochem, San Diego, CA). The antiserum was raised against a synthetic peptide (SGFNADYEASSSRC) corresponding to amino acids 4–17 of human Fos (Calbiochem data sheet). On the following day, after a 5-minute KPBS wash and a 2.5-hour incubation in biotinylated secondary antibody solution (1:250 biotinylated donkey anti-rabbit; Jackson Immunoresearch, West Grove, PA), sections were again washed in KPBS, then incubated in an avidin-biotin-horseradish peroxidase complex (ABC; Vector,

Burlingame, CA), washed again, then reacted for 10–15 minutes with diaminobenzidine with cobalt chloride (DAB; Sigma-Aldrich, Inc.), to produce a black reaction product.

2.2. Afferent Renal Nerve Activation in Anesthetized Rats

2.2.1. Surgical Preparation—We also studied the effects of the same stimuli on ARN activity in anesthetized rats. Male Sprague-Dawley rats (n=16, 230–310g BW) were anesthetized with isoflurane (1.8%) and prepared with arterial, venous and renal pelvic cannulae. The femoral artery and vein were cannulated for arterial pressure determination and drug administration, respectively. After laparotomy, the left ureter was cannulated with a dual or triple lumen catheter (ReCathCo, Inc., Allison Park, PA). Subsequently, the left renal nerve was gently dissected and placed on bipolar stainless steel electrodes under mineral oil for recording renal nerve activity. Nerve activity was amplified (50,000× with Grass P55 Preamp and Dagan Quad Differential Amplifier), processed with a moving averager (100 ms time constant, MA-821/RSP, CWE, Inc.) and recorded using WINDAQ Pro+ Data Acquisition (DATAQ Instruments, Dayton, OH) at 4000 samples/sec. Recordings were made under isoflurane (1.5%) after a stabilization period of 10–20 min.

2.2.2. Experimental Protocol—Responses to acute administration of phenylephrine (2 µg/Kg, iv) and nitroprusside (6 µg/Kg, iv) on renal nerve activity were recorded initially to verify sympathetic nerve recording. Subsequently, the central end of the renal nerve was cut to record only afferent activity. We obtained control data for one min before beginning a push-pull infusion of the renal pelvis with a Gilson peristaltic pump in the same manner as performed in conscious rats. The renal pelvis was perfused for 10 min with saline (308 mOsm or 500 mOsm, n=8 and 15, respectively) while recording arterial pressure, heart rate and renal nerve activity. Rats were allowed to recover for at least 10 min between infusions since several received more than one infusion. Finally, we infused hypertonic (500 mOsm) saline with 1 µM benzamil or 10 µM amiloride for 10 min (n=6 each group) while recording hemodynamics and nerve activity. This was the final experiment in each rat due to the administration of a sodium channel inhibitor.

At the completion of the experiment, the local anesthetic bupivacaine (0.25%) was administered to the renal nerve for one minute. The anesthetic solution was removed and replaced with mineral oil to record background activity (noise). This value was subtracted from all recordings to remove background activity.

2.3. Statistical Analysis

Arterial pressure and heart rate were measured at 20 min intervals during the 2 hour pelvic infusion of fluids and compared by analysis of variance. In anesthetized rats, arterial pressure, heart rate, and renal nerve activity were recorded at 30 sec intervals for the first two min then at 60 sec intervals for the remaining 8 min infusion. We calculated the area under the curve using an arithmetic analysis for each segment (change multiplied by 30 or 60 s) and analyzed the resulting data by analysis of variance (ANOVA).

We used ImageJ software to count activated cells in specific nuclei. We analyzed cell counts in CNS sites after different interventions using a Kruskal-Wallis one-way ANOVA. Differences $p < 0.05$ were considered significant in all analyses.

2.4. Drugs and chemical reagents

Sodium chloride (pharmaceutical grade), paraformaldehyde, benzamil, and amiloride, were obtained from Sigma Chemical, Inc. (St. Louis, MO). NaCl concentrations are expressed as mOsm/L. Bupivacaine HCl (Sensorcaine, 0.25%) was purchased from Astra Pharmaceutical Products, Inc. (Westborough, MA).

3. Results

3.1 Anatomical Mapping

Arterial pressure and heart rate were recorded during infusion of various concentrations of sodium chloride into the renal pelvis. There was no significant change in arterial pressure or heart rate in response to any of the stimuli (Table 1). We excluded eight rats with behavioral responses suggesting that intrapelvic pressure was elevated or with measured increases of intrapelvic pressure > 10 mm Hg and four rats due to inadequate perfusion. Results from the remaining 44 rats are described below.

Fos immunoreactivity was observed in several central structures within the brainstem, forebrain and telencephalon after renal pelvic stimulation with hypertonic saline (500 mOsm), whereas considerably fewer neurons were activated in response to normal saline (308 mOsm) or hypertonic saline plus benzamil or amiloride. These groups will be discussed together in comparison to the results obtained from the control animals. The location and identification of specific cell sites was determined using the atlas of Swanson (2004).

3.1.1. Brain Stem—Although little Fos-immunoreactivity was observed in control rats or those with isotonic saline infusion in the renal pelvis, a large number of Fos-immunoreactive neurons were consistently seen in the NTS of animals after infusion of hypertonic saline (Fig. 1A–C, Table 2). The bulk of the labeling was present in the medial NTS. Considerably less labeling was observed in rats with concurrent amiloride or benzamil administration (Fig. 1D). Nonparametric analysis of the differences between the groups did not demonstrate significant differences ($p = 0.71$, Fig. 2). Likewise, the area postrema (AP) had intense Fos labeling after hypertonic saline with occasional sparse labeling after normal saline (Fig. 3A–C, Table 2). After hypertonic saline with amiloride or benzamil (Fig. 3D, Table 2), there appeared to be fewer Fos-labeled neurons but this was not significant ($p = 0.55$, data not shown).

Other medullary nuclei had occasional labeling with hypertonic saline. The rostral ventrolateral medulla (RVLM) had several labeled neurons after hypertonic saline in 5 rats. While some labeling was also noted in RVLM after normal saline, there was no labeling observed in control animals. Most animals that received normal or hypertonic NaCl infusion also had Fos-labeling within the A1/C1 region of the medulla. Fos-staining was rarely observed in the A1/C1 region of the control animals or after benzamil or amiloride plus

hypertonic saline. One rat had labeling in the nucleus ambiguus (NAmb) after hypertonic saline.

In the midbrain, labeling was observed most consistently in the lateral parabrachial nucleus (PB). While few neurons were labeled in control or isotonic saline-infused rats, hypertonic saline resulted in dense Fos-labeling in the inner external lateral (el-inner) and dorsal lateral (dl) regions (Fig. 4). This labeling was considerably more prevalent after hypertonic saline but occasionally observed in isotonic saline treated rats in the PBdl. In the PB of two control animals, weak Fos-staining was seen in the dorsolateral region or the el-outer region. There was very little Fos labeling observed in the PBel after treatment with benzamil plus hypertonic saline although there was still some Fos-immunoreactivity after amiloride in some rats (Fig. 4D). Analysis of the differences demonstrated a possible effect ($p=0.087$) due to a reduction in the hypertonic response compared to hypertonic saline with amiloride (Fig. 2).

The periaqueductal gray (PAG) at the level of the superior colliculus (SC) also had Fos-immunoreactivity within the dorsomedial (dm) and dorsolateral (dl) regions after hypertonic saline infusion (Table 2). Labeling was also consistently found in the ventrolateral PAGvl of pelvic infusion of hypertonic saline. Some of the isotonic saline-infused animals also displayed inconsistent labeling within the lateral PAG, although this did not appear to be related to hypertonic saline infusion. A few control animals contained Fos-labeled neurons within the PAGdm and PAGdl, but lacked labeling in the PAGvl. All animals, including controls, had limited Fos-staining present in the dorsal raphe of the PAG (PAGdr) at the level of the SC. At the level of the inferior colliculus (IC), Fos was observed in the PAGdm in at least half of the rats treated with hypertonic saline but only seen in one control animal and not observed in after treatment with amiloride or benzamil plus hypertonic saline. At the level of the IC, sparse Fos-labeling was noted within the PAGvl of all animals treated with hypertonic saline whereas control rats had very few labeled neurons in the PAG. At the level of the IC, all animals, including controls, lacked Fos-staining within the PAGdr. Weak Fos-labeling was present within the locus coeruleus (LC) of two of the eight rats infused with hypertonic saline. Control animals did not have Fos-labeling present within the LC.

3.1.2. Diencephalon—A number of sites in the hypothalamus were labeled after hypertonic saline infusion including the PVH, the supraoptic nucleus (SON), the lateral hypothalamic area (LHA) and the periventricular nucleus of the hypothalamus (PV). We observed Fos-immunoreactivity in the PVH after renal pelvic infusion (Fig. 5). Considerably fewer neurons were apparent in the PVH in control animals, or in isotonic saline infusion compared to hypertonic saline. Fos-labeled cells were most prevalent in the medial and dorsal parvocellular regions of the PVH after hypertonic saline infusion and were attenuated by either amiloride or benzamil treatment (Fig. 5D, Table 2). While there was labeling of neurons in several rats in the magnocellular region, the labeled cells appeared to be smaller, parvocellular neurons. Indeed, the parvocellular cell counts in the lateral and ventrolateral PVH were significantly increased by hypertonic saline ($p=0.003$ and $.016$, respectively) as determined by ANOVA (Fig. 2).

The SON had fewer or no Fos-labeled neurons in control or isotonic saline-infused rats (Fig. 6A,B, Table 2). In contrast, hypertonic saline activated many neurons in the SON and this activation appeared to be reduced by treatment with benzamil or amiloride (Fig. 6C,D) although this difference was not observed by ANOVA ($p=0.45$, data not shown). Hypertonic saline also increased Fos-staining within the dorsomedial nucleus of the hypothalamus (DMH), although this staining was fairly weak. All hypertonic saline-infused animals showed Fos-labeling within the periventricular hypothalamus (PV). This labeling was absent or reduced in isotonic saline infused or control animals. Fos-labeling was observed in the lateral hypothalamic area (LHA) of all saline infused animals. The intensity of this staining appeared greater in those rats with hypertonic saline infusion but this was not obvious. The LHA of control animals lacked Fos-labeled neurons. Fos-staining was present in both the lateral and medial preoptic areas (LPO and MPO) of several rats with pelvic infusion of hypertonic saline (Table 2). The intensity of this staining was more consistent within the MPO. Animals with isotonic saline infusions showed weak Fos-labeling within the LPO. Control animals rarely exhibited Fos-labeling within the LPO or MPO. The Fos-immunoreactivity in the MPO was not always reduced by amiloride or benzamil co-administration. In a similar manner, the median preoptic nucleus (MnPO) and the anteroventral preoptic nucleus (AVP) appeared to have many activated neurons with hypertonic saline but still had significant labeling after amiloride and, to a lesser extent, benzamil co-administration. The anteroventral periventricular nucleus of the hypothalamus (AVPV) also had some Fos-immunoreactivity after hypertonic saline infusion that was prevented by amiloride or benzamil and not observed in control rats (Table 2). There was limited Fos-labeling in the suprachiasmatic nucleus (SCN) although this did not appear to be related to pelvic infusions since it was observed occasionally in control rats. Likewise, Fos-labeling was observed in the arcuate nucleus of the hypothalamus (ARH) of some rats after hypertonic saline but the labeling was sparse and greater than in control rats.

We observed occasional labeling in rostral circumventricular organs. Fos-labeling was present in the organum vasculosum laminae terminalis (OVLT) of several animals that received hypertonic NaCl infusions. Fos in these rats was concentrated within the dorsal cap of the OVLT whereas fewer Fos-labeled neurons were present in the OVLT of control or saline infused rats (Table 2). Fos labeling in the OVLT was prevented by benzamil but not by amiloride coadministration (Table 2). Fos-labeling within the subfornical organ (SFO) was present in only one animal that received hypertonic saline and in two control rats.

Consistent labeling in the thalamus was observed in the paraventricular nucleus of the thalamus (PVT) of rats after pelvic perfusion of isotonic or hypertonic saline. Fos-staining was also present in the PVT of two control animals, although one was quite weak. Fos-labeling was consistently present in the paraventricular nucleus (PSTh) of all saline infused animals. No Fos-labeled neurons were present in the PSTh of either control animal or in benzamil or amiloride-treated rats.

3.1.3. Telencephalon—We noted Fos-immunoreactivity in the central nucleus of the amygdala (CeA) of most rats after hypertonic saline infusion (Fig. 7). Fos-labeled neurons were less commonly observed after isotonic saline perfusion of the renal pelvis and rare in control rats (Table 2). Co-administration of benzamil or amiloride with hypertonic saline

appeared to attenuate the Fos-labeling in the CeA (Fig. 7D, Table 2) but this was not significant ($p=0.186$, Fig. 2).

As shown in Table 2, Fos-staining was observed within many subnuclei of the bed nucleus of the stria terminalis (BST). Staining was most significant after hypertonic saline, specifically within the region of the dorsomedial nucleus (BSTdm), fusiform nucleus (BSTfu), and parastrial nucleus (PS). There was a significant effect of hypertonic saline on Fos-labeling in the BSTfu ($p=0.044$, Fig. 2). Fos-labeling was also present in the dorsal BST, most consistently in the region of the oval nucleus (BSTov) and juxtacapsular nucleus (BSTju). Fos-staining was rarely present in either the dorsal or ventral BST of saline-infused rats. In a similar manner, Fos-labeled neurons within the insular cortex (Icx) were found but they were not substantially different between groups.

3.2. Electrophysiological Responses of Afferent Renal Nerves in Anesthetized Rats

Using isoflurane-anesthetized rats, we recorded renal afferent nerve responses to push-pull perfusion of the left renal pelvis with normal or hypertonic saline (with or without benzamil or amiloride) in order to determine the extent of activation of sensory nerves using these stimuli. There were no differences in resting arterial pressure, heart rate or basal renal nerve activity between these groups before pelvic perfusion. Whereas normal saline had little effect on afferent nerve activity, hypertonic NaCl (500 mOsm) elicited an increase in ARN activity that was attenuated by simultaneous administration of 1 μ M benzamil or 10 μ M amiloride as shown in the example in Fig. 8. Arterial pressure and heart rate were not substantially affected by these infusions (Fig. 9). When the area under the curve was analyzed for the 10 min perfusion, there was a significant attenuation of the increase in renal nerve activity with hypertonic saline when benzamil or amiloride was added to the infusate (Fig. 9).

4. Discussion

We provided evidence that specific central sites are activated by ARN stimulation in response to NaCl infusion, and thus identified structures that integrate sensory information from the kidneys. Two concentrations of NaCl (500 and 308 mEq/L) were used under the assumption that only hypertonic NaCl would activate renal sodium sensitive receptors and thus cause increased ARN activity. However, there were gradations of activation within some sites activated that had some Fos after normal saline. These stimuli were likely selective for renal pelvic sodium receptors as they evoked an increase in ARN activity but did not alter arterial pressure or heart rate. Overall, we observed consistent activation of supraspinal sites that have been implicated in blood pressure and fluid volume regulation.

Numerous studies have identified the CNS projections of renal afferent fibers. Pseudorabies virus has been used to map renal sympathetic nerves (Schramm et al., 1993; Sly et al., 1999) and renal sensory nerves (Weiss and Choudhury 1998; Huang and Weiss 1999; Huang et al., 2002) although this approach is not specific for afferents or efferents. The putative afferent projections to the CNS identified several nuclei including the AP, the NTS, the PBel, the PVH and the SON. Electrical stimulation of the renal nerve activates neurons in the PVH and SON (Calaresu and Ciriello, 1981; Day and Ciriello, 1985). Solano-Flores et al. (1997)

used electrical stimulation to activate ARN and identify central projections using Fos-immunoreactivity within both the forebrain and brainstem. Although this approach activated ARN in a non-specific, supraphysiological manner, many CNS sites were identified. The results from the present study are consistent with these findings, for the most part, despite the fact that we used a selective stimulus (hypertonic sodium) delivered to the renal pelvis. Interestingly, several additional central sites were activated in response to ARN stimulation with hypertonic saline, yet we did not note the activation of some previously identified CNS structures. The sites that exhibited consistent Fos-immunolabeling after intrarenal pelvic hypertonic NaCl infusion include the NTS, AP, PBel and PAG, as well as several regions of the hypothalamus, including the PVH, SON, MPO and telencephalic sites such as the CeA.

4.1. Brain Stem

NTS neurons were consistently activated by infusion of hypertonic saline in the renal pelvis (Fig. 1). Within the brain stem, Solano-Flores et al. (1997) identified Fos-immunolabeling within the NTS, which is consistent with several other tract tracing studies of ARN (Weiss and Chowdhury, 1998; Huang and Weiss, 1999; Sly et al., 1999). The NTS is known to respond to administration of a saline load by numerous non-renal routes (Kobashi et al., 1993; Carlson et al., 1997; Morita et al., 1997; Rinaman et al., 1997; Geerling and Loewy, 2007). Similarly, activation of the area postrema (AP) was also reported after electrical stimulation of ARN (Solano-Flores et al., 1997) or salt loading (Kobashi et al., 1993; Rinaman et al., 1997; Carlson et al., 1997; Geerling and Loewy, 2007). Furthermore, activation within the region of the rostral ventrolateral medulla (RVLM) and rostromedial medulla (RMM) including the areas known to contain A1 noradrenergic and C1 adrenergic cells (Solano-Flores et al., 1997) was observed, although this was not consistently seen in our studies with hypertonic saline.

We also observed Fos-immunoreactivity in the parabrachial nucleus particularly in the external lateral region in response to hypertonic saline. This particular subnucleus of the PB has been shown by others to respond to salt loading (Kobashi et al., 1993; Carlson et al., 1997; Rinaman et al., 1997). The lateral external parabrachial nucleus likely plays a key role in regulation of fluid volume and sodium balance and is dependent on projections from the NTS for these responses (Geerling and Loewy, 2007).

4.2. Diencephalon

In the diencephalon, hypertonic NaCl infused in the renal pelvis induced Fos-activation in the PVH (Fig. 4). Several investigators have reported that renal afferents are linked multisynaptically to parvocellular PVH neurons (Solano-Flores et al., 1997; Huang and Weiss 1999; Fitch et al., 2000). Fitch and Weiss (2000) proposed that ARN activation of suprabulbar sites was mediated by the renin angiotensin system (RAS), not from central afferent systems originating from the kidney, in response to furosemide administration since renal denervation did not prevent the Fos activation of diencephalic sites. We noted numerous Fos-immunoreactive neurons in the PVH, particularly in response to renal pelvic administration of hypertonic saline. These cells in the PVH appeared to be parvocellular neurons but they were both in the medial and lateral subdivisions of the PVH. Our data suggest that hypertonic saline activated specific pelvic ARN since benzamil or amiloride

attenuated the activation of these CNS sites. This stimulus would be expected to reduce renin-angiotensin system (RAS) activity if the perfusate accessed the collecting ducts so this is unlikely are response to humoral activation of the RAS.

The SON was also consistently labeled particularly in rats receiving hypertonic saline in the renal pelvis (Fig. 5). A potential pathway from the ARN to SON has been suggested in earlier work (Ciriello et al., 2004; Fitch et al., 2000; Huang and Weiss, 1999). Others have proposed input to the SON from sodium loading in the vasculature or CNS (Blanch et al., 2013; Carlson et al., 1997; Moellenhoff et al., 1998; Morita et al., 1997; Rinaman et al., 1997). We suggest that sodium sensitive ENaCs in the renal pelvis increase ARN activity that ultimately reaches neurons in the SON and PVH which regulate vasopressin release (Day and Ciriello, 1985).

Several reports have suggested multisynaptic linkages between the kidney and the lamina terminalis, particularly the OVLT and SFO (Sly et al., 1999; Solano-Flores et al., 1997; Fitch et al., 2000). The SFO was not consistently activated but the OVLT often was activated by hypertonic sodium perfusion of the renal pelvis. This suggests that sodium sensors on renal afferents (presumably ENaCs) may more selectively project to the OVLT or the SFO may be less sensitive and the stimulus (500 mOsmNaCl) was not adequate. Renal chemoreceptors are activated by infusion of hypertonic NaCl (0.5 – 1.0 M) but not stimulated by isotonic NaCl infusion (Recordati et al., 1980). We can only conclude that any previously identified multisynaptic central pathway to diencephalic circumventricular organs from the kidney may be more likely to transduce sodium sensitivity to the OVLT. Alternatively, Ciriello et al. (2004) argued that the renal-induced activation of the SFO may be mediated by angiotensin release after renal artery occlusion. As mentioned, it is unlikely that the RAS was activated by these stimuli.

Other diencephalic structures such as the medial preoptic region may receive renal afferent signals (Solano-Flores et al., 1997). While these sensory systems may be present, our studies did not clearly associate these with activation by hypertonic saline and, more importantly, by inhibition of this response with ENaC inhibitors.

4.3. Telencephalon

Electrical stimulation of the renal nerve activated Fos in the CeA, ventral BST, and the insular cortex (Solano-Flores et al., 1997). We observed activation of these sites at least in some rats with renal pelvic hypertonic NaCl administration. The activation of the CeA was not readily attenuated when ENaC antagonists (benzamil or amiloride) were delivered with hypertonic saline in the renal pelvis. This suggests that this labeling may have not been as selective for specific renal pelvic afferents but could have reflected arousal due to the experimental protocol since there was considerable variability even in control animals in the CeA. Likewise, there was widespread activation of several subnuclei in the BST, particularly in ventral regions, but this activation was also not clearly inhibited by benzamil or amiloride co-administration with hypertonic NaCl in the renal pelvis. Therefore, we cannot conclude that sodium sensitive renal afferents necessarily send sensory information to the BST.

4.4. Limitations of Study

We observed somewhat inconsistent Fos-labeling of hypertonic saline-infused animals in several central sites, including the MPO, MnPO, LC and AVPV. Although rare, these sites were occasionally activated after isotonic saline but Fos-immunolabeling was observed in fewer neurons with this stimulus. Fitch et al., (2000) argued that urine flow, in response to the diuretic furosemide, likely activated Fos in hypothalamic neurons. Although, low pelvic perfusion rates were used to avoid this potential artifact, we cannot rule out an effect of shear stress activating pelvic neurons. In addition, low doses of benzamil and amiloride were used to avoid non-specific (non-ENaC) blockade of sodium sensors. Both benzamil and amiloride can block a number of different sodium channels (e.g., ENaC, ASIC) and exchangers (e.g., $\text{Na}^+\text{-Ca}^{++}$ and Na-H^+ exchange) but are relatively selective for ENaC at concentrations $<10\ \mu\text{M}$ (Frelin et al., 1988; Hirsh et al., 2004).

Several structures within the forebrain and brainstem activated in response to ARN stimulation have also been reported to be also excited by stress or visceral pain (Goebel et al., 2009; Wang et al., 2009). Restraint stress or colonic distension activates neurons in the PVH, SON, NTS, and the ventrolateral medulla, in addition to other sites. In order to control for this, behavioral responses to the push-pull infusions possibly due to stress or pain were exempted from further study. More importantly, we infused isotonic saline and, in some rats, recorded pelvic pressure, to control for these possible non-specific stimuli. In this manner, we reduced the possibility that *c-fos*-activation was induced in response to behavioral stress or renal pain. Nonetheless, this remains a possible alternative explanation for some of the Fos-immunoreactivity.

The CNS sites identified by this study are very similar to studies examining the effects of activation of various osmoreceptors after hypertonic sodium administration as reviewed by others (Bourque et al., 1994; Antunes-Rodrigues et al., 2004). Many authors have reported activation of several sites including circumventricular organs after intravenous (Bisley et al., 1996; Ho et al., 2007), intragastric (Kobashi et al., 1993; Carlson and Osborn, 1998; Carlson et al., 1997; Blanch et al., 2013) intracerebroventricular (Moellenhoff et al., 1998), or intraportal vein administration (Morita et al., 1997). These typically include the NTS, AP, PBel, SON and PVH and have been ascribed to either direct increases in plasma or CSF osmolality or to activation of sodium receptors in the portal vein. The portal vein receptors responsive to gastric sodium loads activate similar CNS sites but appear to be dependent on splanchnic and vagal afferent pathways (Carlson and Osborn, 1998). In our study, activation of similar sites was observed yet the relatively small volume of hypertonic saline infused in the renal pelvis in the present study (50 $\mu\text{l/min}$, 500 mOsm) is not likely to affect plasma osmolality due to leakage. We did not note blood in the pelvic perfusates suggesting that we were only bathing the renal pelvic area, without vascular access. Therefore, these CNS sites likely receive input from numerous sodium receptors in the viscera through various pathways although the SFO may be an exception to this since we did not observe consistent activation of this region that responds to sodium loading (McKinley et al., 1995; Miller et al., 2013).

ENaCs have been identified in the renal pelvis and collecting ducts (Hager et al., 2001; Pearce et al., 2014; Rossier, 2014). These channels are critical for reabsorption of sodium

and signaling within the kidney. These renal regions are innervated by mechanoreceptors and are affected by substance P release presumably from DRG afferents (Kopp et al., 1998). Our findings are consistent with earlier observations which suggest that ENaCs play an important role in the collecting ducts and renal pelvis and directly affect ARN activity (Ditting et al., 2003; Kopp, et al., 1998).

Fos activation as a marker of neuronal activity has limitations. The relative immunostaining in neurons can lead to false positives (Dampney et al., 1995; Herrera and Robertson, 1996) and Fos protein may not be detectable in all neurons after activation (Dragunow and Faull, 1989; Kovács, 2008). Therefore, the results may not include all neurons activated by the stimulus which could explain some of the variability we observed with specific stimuli. Despite this, congruence with pathway tracing studies (Weiss and Choudhury 1998; Huang and Weiss 1999; Huang et al., 2002) and supraphysiological, non-specific activation of renal afferents (Solano-Flores et al., 1997) suggests numerous sites in the brain that receive renal afferent input. Moreover, the similarity to pathways activated by intragastric or intravenous sodium loading supports the existence of central sodium-sensitive pathways that receive renal input from various sites.

4.5 Conclusions

In summary, we describe a method for selective activation of ARN that was used to determine the CNS cell groups likely to receive information from sodium osmoreceptors in the renal pelvis. Our results demonstrated activation of numerous sites within the forebrain and brainstem known to be involved in autonomic control and fluid balance. Our data suggest that the kidney plays an important role in signaling and likely regulates arterial pressure and fluid volume through a widespread network of sodium receptors in both the kidneys and other visceral sites (e.g., GI tract, hepatic portal vein). Some of the CNS sites activated by renal ENaCs had been reported to be activated by non-specific stimuli or by tract tracing techniques. This approach can be used to characterize other renal afferent projections to better understand their role in blood pressure control. Since the densest afferent innervation of the kidney arises from the renal pelvic wall (Ferguson and Bell, 1988; Marfurt and Echtenkamp, 1991; Kopp, 2011), the sensory nerves innervating this site may be responsible for the therapeutic effects of renal denervation reported in some clinical interventions using catheter-based renal denervation (Esler et al., 2014; Krum et al., 2014). Improved understanding of the role of ARN will determine the role of these nerves in cardiovascular pathology and fluid volume regulation.

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General Abbreviations

ABC	avidin-biotin-horseradish peroxidase complex
ARN	afferent renal nerves

DAB	3,3'-diaminobenzidine
ENaCs	epithelial sodium channels
HR	heart rate
KPBS	potassium phosphate buffer solution
MAP	mean arterial pressure
RAS	renin-angiotensin system
RNA	renal nerve activity

CNS Abbreviations

3V	third ventricle
4V	fourth ventricle
AP	area postrema
ARH	arcuate nucleus hypothalamus
AVP	anteroventral preoptic nucleus
AVPV	anteroventral periventricular nucleus hypothalamus
BST	bed nuclei stria terminalis
BSTdm	bed nuclei stria terminalis, anterior division, dorsomedial nucleus
BSTfu	bed nuclei stria terminalis, anterior division, fusiform nucleus
BSTju	bed nuclei stria terminalis, anterior division, juxtacapsular nucleus
BSTov	bed nuclei stria terminalis, anterior division, oval nucleus
CeA	central nucleus of the amygdala
DMH	dorsomedial nucleus hypothalamus
IC	inferior colliculus
Icx	insular cortex
LC	locus coeruleus
LHA	lateral hypothalamic area
LPO	lateral preoptic area
MnPO	median preoptic nucleus
MPO	medial preoptic nucleus

NAmb	nucleus ambiguous
NTS	nucleus of the solitary tract
OVLT	organum vasculosum laminae terminalis
OX	optic tract
PAG	periaqueductal gray
PAGdl	periaqueductal gray, dorsolateral division
PAGdm	periaqueductal gray, dorsomedial division
PAGdr	periaqueductal gray, dorsal raphe
PAGvl	periaqueductal gray, ventrolateral division
PB	parabrachial nucleus
PBdl	parabrachial nucleus, dorsal lateral part
PBel	parabrachial nucleus external lateral part
PS	parastriatal nucleus
PSTh	parasubthalamic nucleus
PV	periventricular nucleus hypothalamus
PVH	paraventricular nucleus of the hypothalamus
PVHlp	lateral parvocellular PVH
PVHmp	medial parvocellular PVH
PVHvlp	ventrolateral parvocellular PVH
PVT	paraventricular nucleus of the thalamus
RVLM	rostromedial lateral medulla
SC	superior colliculus
SCN	suprachiasmatic nucleus
SCP	superior cerebellar peduncle
SFO	subfornical organ
SON	supraoptic nucleus
TS	solitary tract

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Highlights

- Using a novel technique for selectively activating renal pelvic afferent nerves, we report that infusion of isotonic saline has little effect on most CNS sites previously shown to be activated by renal afferent nerve stimulation.
- Activation of renal pelvic afferents with hypertonic saline increases afferent renal nerve activity and causes *c-fos* activation of specific CNS nuclei involved in blood pressure and fluid volume regulation.
- Co-administration of benamil or amiloride with hypertonic saline in the renal pelvis attenuated both the activation of renal afferent nerves and Fos immunoreactivity in the brain.
- Our findings suggest that renal pelvic afferents sensitive to hypertonic saline may be part of a broad network of sodium sensors that contribute to fluid volume regulation.
- Sodium sensitive afferents arising from the renal pelvis may contribute to the development of hypertension due to its effects on circuits which increase central sympathetic drive.

Figure 1.

Examples of Fos-immunoreactivity in micrographs from an untreated rat (Panel A), a rat treated with isotonic saline (Panel B), a rat treated with hypertonic saline (Panel C), and rat treated with hypertonic saline and amiloride (Panel D) showing the area of the NTS. The bar represents 500 μm . Sections are approximately -14.2 from bregma (Swanson, 2004).

Abbreviations are 4V, fourth ventricle; AP, area postrema, NTS, nucleus of the solitary tract; TS, solitary nucleus.

Figure 2.

Comparisons of cell counts in several CNS sites including the CeA (central nucleus of the amygdala), the BSTfu (bed nucleus of the stria terminalis, anterior division, fusiform nucleus), the NTS (nucleus tractus solitarius) shown above, and the PVHlp and PVHvlp (paraventricular nucleus of the hypothalamus, lateral and ventrolateral parvocellular nuclei) and the PBel (parabrachial nucleus, external layer) shown below. Using a Kruskal-Wallis one-way ANOVA, there were significant differences in the BSTfu ($p=0.044$), the PVHlp

($p=0.0032$), and the PVHvp ($p=0.0164$). Data from groups <4 were not included in the analysis. The other regions studied were not significant often due to small n values.

Figure 3.

Examples of Fos-immunoreactivity in micrographs from an untreated rat (Panel A), a rat treated with isotonic saline (Panel B), a rat treated with hypertonic saline (Panel C), and a rat treated with hypertonic saline and benzamil (Panel D) showing the area of the area postrema. The bar represents 500 μ m. Sections are approximately -14.2 from bregma (Swanson, 2004). Abbreviations are 4V, fourth ventricle; AP, area postrema.

Figure 4.

Examples of Fos-immunoreactivity in micrographs from an untreated rat (Panel A), a rat treated with isotonic saline (Panel B), a rat treated with hypertonic saline (Panel C), and a rat treated with hypertonic saline and amiloride (Panel D) showing the area of the parabrachial nucleus. The bar represents 500 μm . Sections are approximately -9.2 from bregma (Swanson, 2004). Abbreviations are dl, dorsolateral parabrachial nucleus; el-inner, external lateral parabrachial nucleus; SCP, superior cerebellar peduncle; vl, ventrolateral parabrachial nucleus.

Figure 5.

Examples of Fos-immunoreactivity in micrographs from an untreated rat (Panel A), a rat treated with isotonic saline (Panel B), a rat treated with hypertonic saline (Panel C), and a rat treated with hypertonic saline and amiloride (Panel D) showing the area of the paraventricular nucleus of the hypothalamus. The bar represents 500 μm . Sections are approximately -1.8 from bregma (Swanson, 2004). Abbreviations are 3V, third ventricle; lp, lateral parvocellular PVH; mp, medial parvocellular PVH.

Figure 6.

Examples of Fos-immunoreactivity in micrographs from an untreated rat (Panel A), a rat treated with isotonic saline (Panel B), a rat treated with hypertonic saline (Panel C), and a rat treated with hypertonic saline and amiloride (Panel D) showing the area of the supraoptic nucleus of the hypothalamus. The bar represents 500 μm . Sections are approximately -1.0 from bregma (Swanson, 2004). Abbreviations are OX, optic tract; SON, supraoptic nucleus.

Figure 7.

Examples of Fos-immunoreactivity in micrographs from an untreated rat (Panel A), a rat treated with isotonic saline (Panel B), a rat treated with hypertonic saline (Panel C), and a rat treated with hypertonic saline and amiloride (Panel D) showing the central nucleus of the amygdala. The bar represents 500 μm . Sections are approximately -1.5 from bregma (Swanson, 2004). Abbreviations are CeA, central nucleus of the amygdala.

Figure 8.

Examples of arterial pressure (AP), renal afferent nerve activity (RNA) and a moving average of RNA in response to hypertonic saline in panel A and in response to hypertonic saline plus amiloride (10 μ M) in panel B from a single rat. The bars beneath each figure represent 10 sec of infusion following a control period before infusion. Background nerve activity, determined after lidocaine exposure, is shown in the lower right.

Figure 9.

Effects of renal pelvic perfusion with isotonic saline (NaCl 308), hypertonic saline (NaCl 500), hypertonic saline + benzamil (NaCl+Bn), and with hypertonic saline + amiloride (NaCl+Am) for 30 and 60 second segments on the arithmetic area under the curve for mean arterial pressure (MAP), heart rate (HR) and for afferent renal nerve activity (RNA).

Significance was determined by one way analysis of variance using a Newman-Keul's post hoc test. * denotes $p < 0.05$ and ** signifies $p < 0.01$.

Table 1

Average change in arterial pressure and heart rate during 2 hour intrapelvic infusion

Stimulus	Arterial Pressure Change (mmHg)	Heart Rate Change (b/min)
Control (no infusion)	1.1 ± 2.1	-3.1 ± 4.8
Normal Saline	4.8 ± 1.7	-8.9 ± 4.1
Hypertonic Saline	-1.8 ± 4.9	12.1 ± 7.3
Hypertonic Saline + Benzamil	3.2 ± 3.5	0.6 ± 7.2
Hypertonic Saline + Amiloride	-1.1 ± 1.9	-6.2 ± 10.2

No significant differences in changes in arterial pressure or heart rate were observed between groups.

Table 2

Activation of Specific CNS Nuclei by Renal Pelvic Stimuli

Brain site	Control	Normotonic NaCl	Hypertonic NaCl	Hypertonic NaCl + Benzamil	Hypertonic NaCl + Amiloride	Solano-Flores et al.
Brain Stem	NAmb	-	+	-	-	N/A
	NTS	+	++	+	+	+
	AP	-	+++	+	+	+
	LC	-	+	-	-	+
	PBdl	++	+++	+	+	N/A
	PBel inner	+	+++	-	++	-
	PAG at SC	+	++	+	+	N/A
	PAG at IC	+	++	+	+	N/A
Diencephalon	PVH	+	+++	+	+	+
	AVP	++	+++	+	++	N/A
	AVPV	-	+	-	-	N/A
	MPO	+	++	+	++	N/A
	LPO	+	+	-	+	N/A
	MnPO	+	+	+	++	N/A
	SON	+	+++	+	+	w
	OVLt	+	++	-	++	+
Telencephalon	SFO	+	-	-	-	+
	Dorsal BST	-	+	+	+	+
	Ventral BST	+	++	+	+	w
	CeA	+	+++	+	+	+
	Icx	+	++	+	+	+