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Role of superior laryngeal nerve and Fos staining following dehydration and rehydration in the rat

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ABSTRACT

Immunohistochemistry for Fos was used to determine the role of the superior laryngeal nerve in conscious rats following water deprivation and rehydration. Adult male rats were subjected to either unilateral superior laryngeal nerve section (SLNX) or sham surgery. Two weeks later rats from each surgical group were water deprived for 48 h or water deprived for 46 h and given access to water for 2 h prior to perfusion. Controls were allowed *ad libitum* access to water. Brains were processed for Fos using a commercially available antibody. Changes in plasma osmolality and hematocrit were not significantly different between SLNX and sham following any of the treatments. Water intake in rats was not significantly affected by SLNX. In the supraoptic nucleus (SON) of sham rats, water deprivation significantly increased Fos staining while water intake following dehydration prevented this increase. Water deprivation significantly increased Fos staining in the SON of SLNX rats. Following water intake after 46 h water deprivation in SLNX rats, Fos staining in the ipsilateral SON was significantly greater than the contralateral SON and significantly lower than 48 h water deprivation. In the nucleus of the solitary tract (NTS) of sham rats, both water deprivation and water intake produced significant increases in Fos staining bilaterally compared to euhydrated controls. In SLNX rats, water deprivation significantly increased Fos in both ipsilateral and contralateral NTS that was not different from sham rats. SLNX significantly decreased Fos staining in the ipsilateral NTS of rats given access to water after dehydration compared to the corresponding sham treated rats. Fos staining was not affected in the contralateral NTS of SLNX rats given access to water after dehydration. This suggests that the superior laryngeal nerve contributes to changes in Fos staining in the NTS and SON following water intake in dehydrated rats.

1. Introduction

The supraoptic (SON) and paraventricular nuclei of the hypothalamus contain magnocellular neurosecretory cells that release the hormones vasopressin and oxytocin into the systemic circulation from nerve terminals in the posterior pituitary [1–3]. The regulation of the electrical activity of these neurons is critical for determining the circulating levels of both peptide hormones [4]. The physiological control of vasopressin release from the posterior pituitary is vital for the maintenance of body fluid homeostasis, and inappropriate vasopressin release contributes to the pathophysiology of several chronic disease states including congestive heart failure and hepatic cirrhosis [5–8]. Neurohypophyseal oxytocin is involved in lactation and parturition and may also play a role in sodium homeostasis [9–11]. In some species, both vasopressin and oxytocin release are regulated by plasma osmolality [4]. A number of other non-osmotic factors also influence vasopressin and oxytocin release but these mechanisms are not as well characterized.

A number of studies have demonstrated that water intake can rapidly reduce plasma vasopressin in several species including humans [12–20]. The central nervous system mechanisms mediating these effects have not been defined. Oropharyngeal cues appear to play an important role in the inhibition of vasopressin associated with water intake in many of these species [12–14,17–19]. In contrast, the inhibition of vasopressin release after water intake is reportedly mediated by the post-absorptive signals in the rat [20,21]. However, there have been a few observations that suggest oropharyngeal afferents participate in the regulation of magnocellular neurons in the rat. For example, a study by Shingai et al. [22] demonstrated that infusions of water into the oral cavity of anesthetized rats produced an increase in urine flow and that these results were dependent on the integrity of the superior laryngeal nerves. Other investigators have shown that the application of hypertonic or hypotonic solutions into the oral cavity of anesthetized rats will increase or decrease the activity of putative magnocellular neurosecretory cells [23,24]. We

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have recently observed that sham rehydration with water significantly attenuates the reduction in Fos staining in the SON that is produced when dehydrated rats drink water [25]. This suggests that pre-absorptive signals from the gastrointestinal tract or the oral cavity related to water intake do influence the activity of neurons in the SON in the rat.

The major afferents of the oral cavity include the chorda tympani branch of cranial nerve VII, the laryngeal and pharyngeal branches of cranial nerve IX in addition to the superior laryngeal branch of the vagus nerve [26]. The nucleus of the solitary tract (NTS) is the primary site of termination for these afferents [26–30] and previous studies have shown that Fos staining in this region is increased by water deprivation and further increased by fluid intake [31,32]. Pre-absorptive signals related to fluid intake from the superior laryngeal nerve or other oropharyngeal and gustatory afferents would be relayed to the NTS and could contribute to the increase in Fos staining that has been observed in the NTS following water or saline ingestion.

In the present study, we tested the hypothesis that oropharyngeal afferents associated with the superior laryngeal nerve contribute to the regulation of SON magnocellular neurons and the NTS by testing the effects of chronic unilateral superior laryngeal nerve section (SLNX) on Fos staining in unanesthetized rats given access to water for 2 h after 46 h water deprivation.

2. Material and methods

All experiments were conducted on adult male Sprague–Dawley rats (200–300 g bw, Charles Rivers). Prior to surgery, the rats were individually housed in a temperature controlled room on a 12:12 light/dark cycle with light onset at 700 h. Food and water were available *ad libitum* except during the experiments. All experiments using unanesthetized rats were conducted during the light phase of the LD cycle in the rat's home cage. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio according to NIH guidelines.

2.1. Chronic unilateral SLNX

Each rat was anesthetized with isofluorane (drop jar) and maintained with 2% isoflurone delivered by an atomizer with O_2. The left superior laryngeal nerve was accessed using a ventral midline approach. Blunt dissection was used to isolate the trachea and identify the left recurrent superior laryngeal nerve. The dorsal aspect of the superior laryngeal nerve that emerges from the distal end of the trachea was isolated and sectioned [33–35]. Controls were subjected to the same surgical procedure except the left superior laryngeal nerve was not sectioned. Water intake and body weight were monitored for 1 week following surgery. Rats were allowed a 2 week recovery period before being used in the experiments.

2.2. Protocol

Sham and unilateral SLNX rats were randomly divided into three treatment groups: euhydrated controls, 48 h water deprivation, and 46 h water deprivation with 2 h access to water prior to perfusion. The rats given water to drink prior to perfusion did not have access to food. At the end of the protocol, each rat was anesthetized with inactin (100 mg/kg ip) and perfused with 0.1 M phosphate buffered saline (PBS) followed by 300–500 ml of 4% paraformaldehyde in PBS. Prior to the start of the perfusion, a 1–2 ml sample of whole blood was collected via cardiac puncture for measuring plasma osmolality, hematocrit, and plasma protein as previously described [31,36]. Rats were also dissected to verify the nerve sections. The brains were removed and placed in PBS with 30% sucrose for 2–3 days. Each brain was marked on the left side (ipsilateral to the SLNX) and sectioned in a cryostat. Three sets of coronal 40 μm sections were collected in cryoprotectant and store at 20 °C until they were processed for immunohistochemistry.

Serial sections from each forebrain and brainstem were stained for c-Fos (Rabbit anti-c-Fos Ab5, Calbiochem, San Diego, CA) as previously described [31,36,37]. Sections were incubated with the primary antibody (1:30,000) for 72 h at 4 °C. After rinsing, the sections were processed with a biotinylated horse anti-rabbit IgG (Vector Laboratories, Berlingame, CA) diluted 1:200 in PBS for 2 h at room temperature. Sections were reacted with an avidin–peroxidase conjugate (Vectastain ABC Kit; Vector Laboratories) and PBS containing 0.04% 3,3′-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulfate for 10 to 11 min. Separate sets of forebrain sections also were processed for oxytocin immunofluorescence using an anti-mouse primary antibody and a Cy3 label anti-mouse secondary antibody (1:250, Jackson ImmunoResearch, West Grove, PA). Sections were mounted on gelatin coated slides, air dried for 1–2 days and the slides were cover slipped with Permount.

Tissue sections containing regions of interest were inspected using an Olympus microscope (BX41) equipped for epifluorescence. Digital images were acquired using an Olympus DP70 camera connected to a Pentium computer running imaging software (Digital Photo Plus, v.2.2.1.227). Images were adjusted to standardize brightness and contrast. Regions of interest were identified using the rat brain stereotaxic atlas of Paxinos and Watson [38]. Four to six sections from each region were analyzed from each rat. The number of Fos positive cells in the SON or NTS was recorded bilaterally for each section. The number of Fos+ oxytocin labeled cells in the SON were also averaged and analyzed for the water deprived groups. Colocalization was determined by capturing separate images using an IX50 Olympus converted to a DSU confocal microscope with an attached mercury lamp for fluorescence. The bright field Fos image was inverted and adjusted to remove background artifacts, pseudocolored green, and merged with the dark field oxytocin image to observe colocalization as previously described [25]. Artifacts placed in each brain ipsilateral to the SLNX section were used to determine whether the region was from the intact or denervated side. Data from the side of the brain ipsilateral to the nerve section were collected separately from the contralateral, intact side of the brain for later analysis.

2.3. Statistics

Data were analyzed by two-way analysis of variance with Student Newman–Keuls *t*-test for posthoc analysis of significant main effects (SigmaStat, v. 2.03, Systat Software Inc., Point Richmond, CA). Significance was set at $P<0.05$. All values are presented as mean ± one simple SEM.

3. Results

3.1. Water intake and plasma measurements

Chronic, unilateral SLNX did not significantly affect basal plasma osmolality, hematocrit or plasma protein concentration (Table 1). Water deprivation produced comparable increases in plasma osmolality, hematocrit and plasma proteins in both intact and SLNX rats (Table 1). Following water deprivation, intact and SLNX rats drank comparable amounts of water (intact 27 ± 2 ml, SLNX 24 ± 1 ml, $P>0.05$) while plasma osmolality, hematocrit and plasma proteins were not significantly different between the two groups (Table 1).

3.2. Supraoptic nucleus

Unilateral SLNX did not appear to influence Fos staining in the SON of euhydrated controls (Fig. 1A and B) or following 48 h water deprivation (Fig. 1C and D). In both treatment conditions, Fos staining