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Vagal Afferent Input Alters the Discharge of Osmotic and ANG II-Responsive Median Preoptic Neurons Projecting to the Hypothalamic Paraventricular Nucleus

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Abstract

The goal of the present study was to determine the effect of activating vagal afferent fibers on the discharge of median preoptic (MnPO) neurons responsive to peripheral angiotensin II (ANG II) and osmotic inputs. Vagal afferents were activated by electrical stimulation of the proximal end of the transected cervical vagus nerve (3 pulses, 100 Hz, 1 ms, 100–500 μ A). Of 21 MnPO neurons, 19 were antidromically activated from the hypothalamic paraventricular nucleus (PVH) (latency: 10.3 ± 1.3 ms, threshold: 278 ± 25 μ A). MnPO-PVH cells had an average spontaneous discharge of 2.1 ± 0.4 Hz. Injection of ANG II (150 ng) and/or hypertonic NaCl (1.5 Osm/L, 100 μ l) through the internal carotid artery significantly ($P < 0.01$) increased the firing rate of most MnPO-PVH neurons (16/19, 84%). Vagus nerve stimulation significantly ($P < 0.01$) decreased discharge ($-73 \pm 9\%$) in 10 of 16 (63%) neurons with an average onset latency of 108 ± 19 ms. Among the remaining 6 MnPO-PVH neurons vagal activation either increased discharge ($177 \pm 100\%$) with a latency of 115 ± 15 ms ($n = 2$) or had no effect ($n = 4$). Pharmacological activation of chemosensitive vagal afferents with phenyl biguanide produced an increase ($n = 3$), decrease ($n = 2$), or no change ($n = 6$) in discharge. These observations indicate that a significant proportion of ANG II- and/or osmo-sensitive MnPO neurons receive convergent vagal input. Although the sensory modalities transmitted by the vagal afferents to MnPO-PVH neurons are not presently known, the presence of inhibitory and excitatory vagal-evoked responses indicates that synaptic processing by these cells integrates humoral and visceral information to subserve potentially important cardiovascular and body fluid homeostatic functions.

Keywords

blood pressure; thirst; vasopressin; hyperosmolality; sympathetic

Abbreviations

Median preoptic nucleus, MnPO; Hypothalamic paraventricular nucleus, PVH; Angiotensin II, ANG II; Arterial blood pressure, ABP; Mean arterial blood pressure, MABP; Osmole, Osm; Phenylephrine, PE; Sodium nitroprusside, SNP

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1. Introduction

The median preoptic (MnPO) nucleus serves as a forebrain integration site for both humoral and visceral signals related to body fluid homeostasis and cardiovascular regulation (Johnson and Loewy, 1990; Johnson *et al.*, 1996). Lesions of the MnPO disrupt the drinking behavior in response to hyperosmolality, ANG II and hypovolemia (Mangiapane *et al.*, 1983; Gardiner and Stricker, 1985; Cunningham *et al.*, 1991; Cunningham *et al.*, 1992), attenuate vasopressin secretion during increase plasma osmolality and ANG II levels (Mangiapane *et al.*, 1983; Gardiner *et al.*, 1985), and blunt centrally mediated pressor responses to hypertonic saline and ANG II (O'Neill and Brody, 1987; Yasuda *et al.*, 2000). Each of the aforementioned stimuli increase Fos immunoreactivity, a marker of synaptic activation, in MnPO neurons (Oldfield *et al.*, 1994; Larsen and Mikkelsen, 1995; Potts *et al.*, 1999). In some cases, Fos immunoreactivity was reported in MnPO neurons that project to the hypothalamic paraventricular nucleus (PVH) and/or supraoptic nucleus (Oldfield *et al.*, 1994; Larsen and Mikkelsen, 1995). Consistent with the notion that osmolality and ANG II increase the excitability of MnPO neurons, electrophysiological studies in vivo have demonstrated that hyperosmolality and circulating ANG II significantly increased the neuronal firing rates of MnPO neurons (McAllen *et al.*, 1990; Tanaka *et al.*, 1993; Tanaka *et al.*, 1995; Aradachi *et al.*, 1996; Stocker and Toney, 2005).

The excitability of MnPO neurons is also affected by visceral signals that arise from the hindbrain. For example, in vivo single-unit recordings of MnPO neurons have demonstrated that these cells are barosensitive (Knuepfer *et al.*, 1985; Tanaka *et al.*, 1993; Aradachi *et al.*, 1996; Stocker and Toney, 2005). The MnPO is innervated by catecholaminergic neurons in hindbrain regions such as the nucleus tractus solitarius and A1 cell population (Saper and Levisohn, 1983; Saper *et al.*, 1983), and previous studies have demonstrated that neurons in these regions are responsive to changes in baroreceptor input (Stornetta *et al.*, 1999; Verberne *et al.*, 1999). Interestingly, stimulation of the A1 region has been reported to increase cell discharge of MnPO neurons (Tanaka *et al.*, 1992). However, neurons in the A1 region also receive input from cardiopulmonary receptors and vagal afferents (Day *et al.*, 1992; Gieroba and Blessing, 1993; Verberne *et al.*, 1999). Interestingly, changes in circulating blood volume increases Fos immunoreactivity in MnPO neurons (Potts *et al.*, 2000). This raises the possibility that MnPO neurons may receive input arising from vagal afferent fibers. Therefore, the present study sought to determine whether activation of vagal afferent fibers alter the excitability of MnPO neurons that are responsive to circulating ANG II and osmotic inputs. We focused on the population of MnPO neurons with axonal projections to the PVH (MnPO-PVH) since the PVH appears to coordinate neuroendocrine, autonomic, and behavioral responses to stress.

2. Results

2.1 Basal Discharge and Antidromic Response Characteristics

We performed extracellular single-unit recordings from 21 MnPO neurons with a basal discharge of 1.7 ± 0.4 Hz (range: 0.0 – 6.9 Hz). Of these units, 19 were antidromically activated from the PVH with an average onset latency of 10.3 ± 1.3 ms (range: 6–25 ms) and antidromic threshold of 278 ± 25 μ A (range: 130–500 μ A, 0.5 ms pulse duration). These MnPO-PVH neurons displayed an average basal discharge of 2.1 ± 0.4 Hz. The average conduction velocity was 0.19 ± 0.04 m/s (range: 0.08–0.30 m/s) thereby suggesting the axons of these neurons were unmyelinated. Only 5% (1 of 19) of MnPO-PVH units were antidromically activated from both sides of the PVH thereby confirming previous observations in our laboratory that the vast majority of MnPO-PVH neurons project to the PVH unilaterally (Stocker and Toney, 2005).

2.2 Effect of Vagal Afferent Fiber Activation on the Activity of MnPO-PVH Neurons that Increased Neuronal Discharge to ANG II and/or Osmotic Stimulation

In order to determine whether activation of vagal afferent fibers altered the discharge of ANG II- or osmotic-responsive MnPO-PVH neuron, we initially identified a population of MnPO-PVH neurons that displayed changes in neuronal firing rates to ICA injection of ANG II (150 ng) or hypertonic NaCl (1.5 Osm/L, 100 μ l). The vast majority of MnPO-PVH neurons (16/19, 84%) increased cell discharge to either ANG II and osmotic stimulation (n=9), ANG II stimulation only (n=3), or osmotic stimulation only (n=4). In these neurons, ICA injection of ANG II increased cell discharge by $280 \pm 59\%$ ($P < 0.01$; baseline discharge: 2.0 ± 0.7 Hz) and was associated with a significant increase in MABP of 49 ± 9 mmHg ($P < 0.01$; baseline MABP: 107 ± 4 mmHg). ICA injection of 1.5 Osm/L NaCl increased cell discharge by $384 \pm 93\%$ ($P < 0.01$; baseline discharge: 2.4 ± 0.6 Hz) and produced a small but significant increase in MABP of 10 ± 3 mmHg ($P < 0.05$; baseline MABP: 102 ± 4 mmHg). As previously reported, the increase in cell discharge preceded any change in MABP (Stocker and Toney, 2005). There were no differences in baseline firing rates among MnPO-PVH neurons that increased cell discharge to ANG II versus osmotic stimulation. Moreover, a neuron displayed a similar increase in cell discharge during ANG II stimulation regardless of whether it increased cell discharge to hypertonic NaCl (data not shown). The converse was also true. These findings confirm previous observations from our laboratory with a larger population of MnPO-PVH neurons (Stocker and Toney, 2005).

Of the 16 MnPO-PVH neurons that increased cell discharge to ANG II and/or osmotic stimulation, electrical stimulation of vagal afferent fibers (3 pulses, 1 ms, 100 Hz) significantly decreased cell discharge in 10 of 16 (63%) of these units by $-73 \pm 9\%$ ($P < 0.01$) with an average latency of 108 ± 19 ms. Of the remaining units, electrical stimulation of vagal afferent fibers increased cell discharge in 2 of 16 (12%) of MnPO-PVH neurons by $177 \pm 100\%$ with an average latency of 115 ± 15 ms. Activation of the vagus nerve failed to alter the neuronal firing rates in 4 of 16 (25%) of MnPO-PVH neurons. These responses are summarized in Table 1.

In a subset of these neurons (n=11), vagal afferent fibers were also activated by intravenous injection of the serotonergic receptor₃ agonist PBG (100 μ g/kg, iv). In unilateral vagotomized rats, PBG significantly decreased MABP by -27 ± 3 mmHg ($P < 0.001$) and produced variable changes in cell discharge. Of those MnPO-PVH neurons tested, cell discharge either increased (n=3) by $214 \pm 60\%$ (baseline: 1.8 ± 0.7 Hz, $P < 0.05$), decreased (n=2) by $-53 \pm 15\%$ (baseline: 2.5 ± 1.3 Hz, $P < 0.05$), or did not change (n=6, baseline: 2.7 ± 1.3 Hz). The responses to PBG did not correlate with the directional effect of electrical stimulation of the vagal afferent nerves (Table 1).

Figure 1 provides an example of a MnPO-PVH neuron that increased cell discharge to both osmotic (Fig. 1A) and ANG II (Fig. 1B) stimulation by 160% and 256%, respectively. Chemical stimulation of vagal afferents with PBG did not alter cell discharge; however, electrical stimulation of the vagus nerve significantly decreased cell discharge by -98% with a latency of 75 ms (Fig. 1C). Figure 2 provides an example of a barosensitive MnPO-PVH neuron that increased cell discharge in response to ICA injection of ANG II. ICA injection of hypertonic NaCl produced a delayed decrease in cell discharge that occurred after the small increase in MABP rather than the injection itself (Fig. 2A); however, an increase in MAP produced by inflation of an aortic cuff or iv injection of PE significantly decreased firing rate (Fig. 2A). Therefore, it is likely that the decrease in cell discharge after ICA injection of hypertonic NaCl was due to the associated-increase in MABP. ICA injection of ANG II only did not alter cell discharge but did increase MABP by 54 mmHg (Fig. 2B). When the ANG II-induced increase in ABP was attenuated (27 mmHg), ANG II significantly increased cell discharge by 368% (Fig. 2B, b). This indicates that the ANG II-evoked increase in ABP masked the ANG II-induced increase in cell discharge. Therefore, such neurons were deemed ANG II-

responsive. Interestingly, both pharmacological and electrical activation of vagal afferents significantly decreased cell discharge (Figs. 2A, C), indicating that inputs from chemically-sensitive visceral endings comprise at least a portion of the inhibitory vagal drive to such ANG II responsive cells.

Figure 3 provides an example of a MnPO-PVH neuron that increased cell discharge to ICA injection of hypertonic NaCl but not isotonic saline, i.v. PE (Fig. 3A) or ICA ANG II (Fig. 3B). Electrical stimulation of the vagus nerve with 3 pulses (100 Hz) produced a biphasic response with an initial decrease in cell discharge of -94% followed by an increase of 95% (Fig. 3C, a). This effect was observed when the vagus nerve was stimulated with 1 pulse at the same stimulus intensity (Fig. 3C, b) but not with 1 pulse at one-fifth the stimulus intensity (Fig. 3C, c). The lack of either an initial decrease or longer latency increase in cell discharge by the latter stimulus suggests that activation of C-fibers, and not A-fibers, is likely to mediate the overall cellular response to vagal stimulation. This example was the only neuron that displayed a biphasic response to electrical stimulation of vagus afferent fibers characterized by an initial decrease in cell discharge.

A small number of MnPO-PVH neurons that increased cell discharge to ANG II and/or osmotic stimulation also increased cell discharge to vagal afferent fiber activation. Figure 4 provides an example of a MnPO-PVH neuron that increased neuronal discharge during ICA injection of hypertonic NaCl whereas isotonic saline, baroreceptor stimulation, and ANG II had no effect (Figs. 4A, B). Pharmacological activation of vagal afferents with PBG significantly increased cell discharge (Fig. 4A). Electrical stimulation of vagal afferent fibers (3 pulses) significantly increased cell discharge by 470% with an onset latency of 85 ms followed by a significant decrease in unit discharge by -83% with a latency of 140 ms (Fig. 4C, a). When the stimulation frequency was reduced to 1 pulse, the magnitude of this effect was reduced but still increased cell discharge by 91% with a latency of 85 ms (Fig. 4D b).

2.3 Effect of Vagal Afferent Fiber Activation on the Activity of MnPO-PVH Neurons that Decreased Neuronal Discharge to ANG II and/or Osmotic Stimulation

A small number of MnPO-PVH neurons displayed a decrease in neuronal discharge to ANG II (n=1), osmotic (n=1), and both ANG II and osmotic stimulation (n=1). In these units, ICA injection of ANG II decreased neuronal discharge by $-82 \pm 17\%$ ($P < 0.01$; baseline discharge: 2.0 ± 1.0 Hz, n = 2) and this was associated with a significant increase in MABP of 56 ± 8 mmHg ($P < 0.05$; baseline MABP: 121 ± 2 mmHg). ICA injection of 1.5 Osm/L NaCl decreased neuronal discharge in 2 of these MnPO-PVH neurons by $-79 \pm 17\%$ ($P < 0.05$; baseline discharge: 2.5 ± 1.3 Hz) and produced a small increase in MABP of 11 ± 4 (baseline MABP: 116 ± 5 mmHg). The decrease in cell discharge preceded any change in MABP. Electrical stimulation of the vagal afferent fibers significantly decreased neuronal discharge in 2 of these MnPO-PVH units by $-43 \pm 4\%$ with an average latency of 78 ± 38 ms (Table 1). Activation of vagal afferent fibers did not alter firing rate of the MnPO-PVH neuron that decreased cell discharge to ANG II. The basal discharge, latency to vagal stimulation, and conduction velocity among MnPO-PVH neurons did not differ with respect to the effect of vagal stimulation (Table 1).

2.4 Location of MnPO-PVH Neurons

As previously reported in our laboratory (Stocker and Toney, 2005), MnPO-PVH neurons responsive to ANG II and/or osmotic stimulation were located throughout the rostral-caudal distribution of the nucleus (not shown). There was no observable difference between the location of MnPO-PVH neurons and the neuronal responses to ANG II, osmotic, and/or vagal stimulation (Figure 5A). Figure 5B and 5C are examples of juxtacellular labeled neurons in the precommissural (-0.2 mm from bregma) and dorsal MnPO (-0.3 mm from bregma). We

were able to successfully entrain and label 11 of 14 (79%) MnPO-PVH cells. In many cases, the axon was observed to course caudally alongside the wall of the third ventricle.

3. Discussion

Anatomical and functional studies suggest the MnPO serves as a forebrain integration site for both humoral and visceral afferent information (Johnson and Loewy, 1990; Johnson *et al.*, 1996). In this regard, our laboratory (Stocker and Toney, 2005) and others (Knuepfer *et al.*, 1985; McAllen *et al.*, 1990; Tanaka *et al.*, 1993; Tanaka *et al.*, 1995; Aradachi *et al.*, 1996) have shown that MnPO neurons are responsive to changes in plasma osmolality, circulating ANG II, and/or changes in ABP. Since the central nervous system integrates these stimuli along with the prevailing level of blood volume to result in appropriate behavioral, neuroendocrine, and autonomic responses to body fluid homeostatic challenges, we examined whether the ongoing activity of MnPO neurons is altered by activation of vagal afferent fibers and whether vagal afferent input specifically targeted MnPO-PVH neurons that were responsive to forebrain ANG II and osmotic stimulation. The present findings provide new insight into the integration of neural and humoral information by the forebrain lamina terminalis as the majority of MnPO-PVH neurons responsive to ANG II and/or osmotic stimulation received input arising from vagal afferent fibers.

A number of studies have demonstrated that hyperosmolality and elevated plasma ANG II levels increase Fos immunoreactivity, a marker of neuronal activation, in MnPO neurons (Oldfield *et al.*, 1994; Larsen and Mikkelsen, 1995; Potts *et al.*, 1999). These observations are consistent with the ability of these stimuli to increase neuronal discharge of MnPO neurons in vivo (McAllen *et al.*, 1990; Tanaka *et al.*, 1995; Aradachi *et al.*, 1996; Stocker and Toney, 2005). In addition, changes in circulating blood volume have been reported to increase Fos immunoreactivity in MnPO neurons (Potts *et al.*, 2000). While these studies provide support for the MnPO as a forebrain integration site, the interpretation of Fos immunoreactivity can be limited as this technique cannot distinguish whether a single neuron responds to multiple stimuli. Thus, it is difficult to determine whether changes in circulating blood volume or changes in vagal afferent nerve activity alter the excitability of MnPO neurons that integrate humoral information related to osmolality and plasma ANG II levels.

The present findings demonstrate that MnPO-PVH neurons responsive to ANG II and/or osmotic stimulation also receive vagal afferent input. In the majority of cases in which ANG II and/or osmotic stimulation increased cell discharge, electrical activation of vagal afferent fibers decreased cell discharge. However, we did observe a small number of MnPO-PVH neurons (n=2) that displayed a decrease in cell discharge to ANG II, osmotic, and/or vagal stimulation. The possibility that such response characteristics represent two classes of MnPO-PVH neurons with distinct functions needs further investigation. Alternatively, the different responses of these two neurons may reflect the complexity of vagal afferent activation by electrical stimuli. Since electrical stimulation of the vagus nerve in the present study was likely to activate both A- and C- type vagal afferents (Fan and Andresen, 1998; Horn and Friedman, 2005) and because the cervical vagus nerve contains afferents originating from a variety of sources including the cardiopulmonary circulation and gastrointestinal tract (Berthoud and Neuhuber, 2000), additional studies will be needed to identify the specific type and origin of vagal afferent input that alters the discharge of MnPO-PVH neurons. This notwithstanding, Potts and colleagues (Potts *et al.*, 2000) have reported that both volume expansion and hypovolemia increase Fos immunoreactivity in MnPO neurons. Moreover, we also observed that activation of cardiopulmonary chemosensitive afferents with PBG altered the discharge in a subset of MnPO-PVH neurons. Although these responses consisted of both an increase and decrease in neuronal firing rate, these observations provide further support that vagal afferent fibers from the cardiopulmonary circulation influence the excitability of MnPO-PVH

neurons. Since these studies were performed in unilateral vagotomized rats to eliminate activation of vagal efferents, the present observations likely underrepresent the number of MnPO-PVH neurons that receive chemosensitive cardiopulmonary input. Altogether, these observations suggest that activation of cardiopulmonary vagal afferents alter the excitability of MnPO neurons. The present findings highlight the presence of neural pathways and synaptic mechanisms that permit changes in vagal afferent activity to alter the discharge of MnPO-PVH neurons.

The neural pathways and synaptic mechanisms by which vagal and/or baroreceptor information is relayed to MnPO neurons remains largely unexplored. The MnPO is innervated by medullary neurons of the nucleus tractus solitarius and ventrolateral medulla (Saper and Levisohn, 1983; Saper *et al.*, 1983); these neurons contain catecholaminergic enzymes and/or neuropeptide Y (Saper *et al.*, 1983; Kawano and Masuko, 1999). Within the ventrolateral medulla, previous studies have demonstrated that these neurons with ascending projections to the hypothalamus receive baroreceptor, cardiopulmonary, and vagal input (Li *et al.*, 1992; Gieroba and Blessing, 1993; Stornetta *et al.*, 1999; Verberne *et al.*, 1999) and stimulation of the A1 area has been reported to increase cell discharge of MnPO-PVN neurons through the likely activation of α_1 -adrenoceptors (Tanaka *et al.*, 1992). Alternatively, neurons of the nucleus tractus solitarius respond to cardiopulmonary and vagal afferent fiber activation (Raybould *et al.*, 1988; McCann and Rogers, 1992; Hines *et al.*, 1994; Sevoz-Couche *et al.*, 2000), and orthodromic stimulation of this region typically produces an increase in MnPO cell discharge (Aradachi *et al.*, 1996). Therefore, cardiopulmonary and/or vagal inputs may alter the excitability of MnPO neurons through mono- or polysynaptic pathways via the nucleus tractus solitarius or ventrolateral medulla. The ascending projections from these two hindbrain regions are largely catecholaminergic (Saper and Levisohn, 1983; Saper *et al.*, 1983), and hypovolemia has been reported to enhance catecholamine turnover in the MnPO (Wilkin *et al.*, 1987; Tanaka *et al.*, 2003). In vitro patch-clamp recordings of MnPO neurons have revealed two actions of noradrenaline: a α_2 -mediated membrane hyperpolarization or a α_1 -mediated depolarization (Bai and Renaud, 1998b). The interaction of noradrenaline with MnPO neurons responsive to ANG II is quite complex and could reflect intrinsic properties of these neurons as supported by Renaud and co-workers who showed that noradrenaline-sensitive MnPO neurons exhibit hyperpolarization-activated inward rectification and rebound excitation (Bai and Renaud, 1998b, a), the latter appearing to involve activation of a T-type Ca^{++} conductance that is enhanced by ANG II (Spanswick and Renaud, 2005). Altogether, these observations raise the possibility that ascending noradrenergic pathways to the MnPO may transmit information regarding vagal and/or baroreceptor information and thereby alter the excitability or gain of MnPO neuronal responses to osmotic and/or ANG II stimulation. In this regard, blockade of α -adrenoceptors or destruction of catecholaminergic fibers in the MnPO attenuates ANG II-stimulated water intake (Cunningham and Johnson, 1989; Tanaka, 2002). Future studies are needed to definitively establish the synaptic mechanism within the MnPO, and the origin and phenotype of afferent projections to the MnPO that relay vagal and/or baroreceptor information.

Previous studies strongly indicate that the MnPO plays a pivotal role in autonomic, neuroendocrine, and behavioral responses to body fluid homeostatic challenges (Johnson and Loewy, 1990; Johnson *et al.*, 1996). The present findings along with our previous study (Stocker and Toney, 2005) demonstrate that MnPO-PVH neurons integrate multiple signals including circulating ANG II, osmotic, baroreceptor, and visceral information arising from vagal afferent fibers. The possible convergence of these inputs likely has functional significance as visceral signals such as the prevailing level of ABP and blood volume influence responses evoked by elevations in circulating ANG II and plasma osmolality. For example, several investigators have postulated that the pressor action of exogenously administered ANG II masks the ability of ANG II to stimulate thirst, vasopressin secretion, and sympathetic

outflow. In this regard, barodenervation or prevention of the ANG II-evoked increase in ABP does enhance thirst and acutely increases sympathetic outflow (Robinson and Evered, 1987; Schreihöfer *et al.*, 2000; Stocker *et al.*, 2001, 2002; Xu and Sved, 2002; Stocker *et al.*, 2004). To the extent it has been examined, similar observations have been reported during increases in plasma osmolality (Weiss *et al.*, 1996; Chen and Toney, 2001; Stocker *et al.*, 2001, 2002). Due to these functional observations, an attractive hypothesis arises that the ability of ANG II and hyperosmolality to evoke appropriate physiological responses depends upon the prevailing level of ABP and/or blood volume. In essence, these visceral inputs alter the gain or excitability of MnPO neurons to ANG II and/or osmotic inputs. The present findings along with our previous study (Stocker and Toney, 2005) provide substantial insight into the possible neuronal populations that may serve as body fluid homeostatic integrators within the central nervous system.

4. Experimental Procedure

4.1 Animals and General Methods

Experiments were performed in 18 male Sprague-Dawley rats (300–375 g, Charles River Laboratories) using methodology described previously (Stocker and Toney, 2005). Briefly, rats were anesthetized with 3% isoflurane and instrumented with femoral arterial and venous catheters. Then, isoflurane anesthesia was replaced with a mixture of α -chloralose (75 mg/kg, iv) and urethane (750 mg/kg, iv). Rats were tracheotomized, paralyzed with a continuous infusion of gallamine triethiodide (25 mg/kg/hr at 0.25 ml/hr, iv), and artificially ventilated with oxygen-enriched room air. End-tidal PCO₂ was maintained between 4.0–5.5% by adjusting ventilation rate (60–90 breaths/min) or tidal volume (2–3 ml). Additional catheters were placed into the brachial artery and right jugular vein for recording ABP and administration of drugs, respectively. Then, the rat was placed into a stereotaxic head frame with the skull level between bregma and lambda. A craniotomy was performed to gain access to the MnPO and PVH. Throughout the experiment, body temperature was maintained at 37±1 °C with a heated water-circulation pad. An adequate depth of anesthesia was assessed by the absence of a withdrawal reflex (before neuromuscular blockade) or a pressor response to foot pinch. Supplemental doses of anesthetic (10% of initial dose) were given as necessary. All experimental and surgical procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky College of Medicine and University of Texas Health Science Center at San Antonio.

4.2 Electrophysiological Recordings

Extracellular recordings of MnPO neurons were performed with an AxoClamp 2B amplifier in bridge mode (Axon Instruments) and glass microelectrodes filled with 5% Neurobiotin dissolved in 0.9% NaCl and tip resistance of 10–30 M Ω in vivo. The electrode was angled 6–8° from the midsagittal plane to gain access to the midline without impinging on the midsagittal sinus. To identify both spontaneously-active and quiescent MnPO-PVH neurons, antidromic stimulation was performed as described previously (Stocker and Toney, 2005). Briefly, separate concentric bipolar stimulating electrodes were placed into the left and right PVH using the following coordinates: 1.7–2.0 mm caudal to bregma, 0.5–0.7 mm lateral to the midline, and 7.6–7.8 mm ventral to the brain surface. Initially, the placement of the electrodes was verified in vivo by an increase in MABP (>15 mmHg) in response to a brief train of electrical pulses (100 Hz, 1 ms pulse duration, 200 μ A). Antidromic stimulation was performed by applying square-wave current pulses (0.5 ms, 1 Hz) with varying amplitude (<1.0 mA) to determine threshold intensity. The stimulation pulses alternated between electrodes positioned in the left and right PVH. Units were considered to be antidromic according to criteria used previously (Lipski, 1981; Stocker and Toney, 2005): 1) constant onset latency, 2) ability to follow high frequency stimulation (>250 Hz), and 3) collision of the stimulus-evoked spike by

a spontaneous action potential. The latter two criteria were not always met due to interference with the stimulus artifact or a lack of spontaneous activity. All neurons in the present study satisfied at least two criteria.

4.3 Experimental Protocol

After a MnPO-PVH neuron was identified, baseline activity was recorded for 5 min. Then, neuronal responses to hyperosmotic NaCl (1.5 Osm/L), ANG II (150 ng), isotonic NaCl vehicle (0.3 Osm/L) delivered through an internal carotid artery catheter (ICA) advanced 1.5–2.0 mm rostral to the carotid bifurcation via the occipital artery were examined. All solutions were delivered over 10 s in a volume of 100 μ l. Since ANG II stimulation can increase ABP and our previous study (Stocker and Toney, 2005) indicates that this increase in ABP may mask the direct effect of ANG II to increase cell discharge in a subset of MnPO-PVH neurons, we also determined whether these neurons were barosensitive by measuring changes in neuronal discharge to phenylephrine (4–20 μ g/kg, iv), sodium nitroprusside (4–20 μ g/kg, iv), or inflation of a cuff placed around the descending aorta distal to the renal vessels. If a barosensitive MnPO-PVH neuron was unresponsive to ANG II stimulation, the trial was repeated >5 min later but sodium nitroprusside (4–12 μ g/kg, iv) was coadministered with ANG II. We did not perform parallel experiments with osmotic stimulation since we have previously reported that changes in cell discharge during osmotic stimulation occur much sooner than those associated with baroreceptor activation (Stocker and Toney, 2005). To determine whether vagal afferent inputs alter the discharge of MnPO-PVH neurons, the left cervical vagus was isolated at the level of the carotid bifurcation and the aortic depressor nerve was carefully isolated to prevent activation of arterial baroreceptor afferents. The vagus nerve was mounted on a stainless steel bipolar electrode, cut distally, and covered with a mixture of mineral oil and vaseline. The vagus nerve was stimulated electrically with application of 1–3 square-wave current pulses (1 ms, 100 Hz) at varying amplitude (100–500 μ A). In a subset of neurons, vagal afferent fibers were activated with the serotonergic receptor₃ agonist phenyl biguanide (100 μ g/kg, iv). After each test, discharge was allowed to return to baseline values and all tests were separated by a minimum of 5 min.

4.4 Histology

At the end of experiments, the cell was juxtacellularly labeled with neurobiotin (Molecular Probes, Eugene, OR, USA) as described previously in our laboratory (Stocker and Toney, 2005) and by others (Pinault, 1996; Schreihöfer and Guyenet, 1997). A cell was entrained for 15–120 s, and only 1 entrainment was attempted per experiment. If a cell was not entrained, the site was marked by applying DC current through the recording electrode (5 μ A, 5 min). In a few experiments in which a cell was lost before juxtacellular labeling was attempted, we searched for additional MnPO-PVH neurons and the location of the first cell was reconstructed based upon histology. PVH stimulation sites were verified by applying DC current (50 μ A, 15 s). Then, animals were perfused transcardially with 4% paraformaldehyde. Brain were removed, post-fixed in 4% paraformaldehyde at 4°C for 3–7 days and sectioned at 50 μ m. Cells (or recordings sites) and antidromic stimulation sites were visualized as described previously (Stocker and Toney, 2005). All antidromic stimulation sites were located in the middle to caudal third of the PVH and consistently encroached on the posterior magnocellular, dorsal and ventrolateral parvocellular, and/or lateral parvocellular subnuclei.

4.5 Statistical Analysis

Basal discharge of MnPO neurons was determined from a representative 3 min rate-meter record (1 s bins) when ABP was stable. Differences in basal firing rate, antidromic latency, or antidromic threshold were analyzed using independent t test (Systat 10.2, Systat Software, Inc. Richmond, CA, USA). Changes in unit discharge in response to hyperosmotic, ANG II, and

baroreceptor stimulation were analyzed by comparing the average 30-s baseline discharge to the average discharge of a 5-s segment after the onset of the stimulus. Values were log-transformed and then statistically analyzed using a paired t test. For the effects of vagal stimulation on unit discharge, peristimulus time histograms were constructed using 5 ms bins from a minimum of 300 sweeps. Baseline discharge (250 ms before stimulus onset) was compared to the peak discharge (minimum 50 ms duration) by a paired t-test. The latency to the response was also noted. For all comparisons, a P value < 0.05 was considered statistically significant.

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Fig. 1. Activation of vagal afferent fibers decreased the discharge of MnPO-PVH neurons responsive to osmotic and ANG II stimulation

(A) ICA injection of hypertonic saline significantly increased cell discharge whereas isotonic saline had no effect. This cell was not barosensitive as a PE-evoked increase in ABP did not change neuronal firing rate. Activation of vagal afferents by intravenous injection of PBG did not alter cell discharge. (B) ICA injection of ANG II increased cell discharge. (C) A peristimulus time histogram (5 ms bins) shows that electrical stimulation of the vagus nerve with 3 pulses (100 Hz) at 500 μA significantly decreased cell discharge (* $P < 0.05$) with a latency of 75 ms. (D) This neuron was antidromically activated from the left PVH with a constant latency of 9 ms (a) and stimulus intensity of 150 μA. Note that the antidromic spike was collided with a spontaneous action potential (b). ▼, spontaneous spike; ▽, antidromic spike; s, antidromic stimulus artifact

Fig. 2. Activation of vagal afferent fibers decreased the cell discharge of a MnPO-PVH neuron responsive to ANG II stimulation

(A) ICA injection of hypertonic saline decreased cell discharge and increased MAP. However, this neuron was barosensitive as an increase in ABP produced by inflation of an aortic cuff or injection of PE produced a robust decrease in cell discharge. Chemical activation of vagal afferents by PBG rapidly silenced cell discharge. (B) ICA injection of ANG II did not alter cell discharge (*a*), but when the ANG II-evoked increase in ABP was attenuated with SNP, ICA injection of ANG II produced a clear increase in cell discharge (*b*). (C) A peristimulus time histogram (5 ms bins, 327 sweeps) shows that electrical stimulation of the vagus nerve with 3 pulses (100 Hz) at 500 μA significantly decreased cell discharge (**P*<0.05) with a latency of 80 ms. (D) This neuron was antidromically activated from the left PVH with a constant latency of 14 ms (*a*) and stimulus threshold of 300 μA. Note that the antidromic spike was collided with a spontaneous spike (*b*). ▼, spontaneous spike; ▽, antidromic spike; s, antidromic stimulus artifact

Fig. 3. Activation of vagal afferent fibers decreased the neuronal discharge of an osmotically-responsive MnPO-PVH neuron

(A) ICA injection of hypertonic NaCl increased cell discharge whereas isotonic saline had no effect. Raising ABP with PE was also without affect. (B) ICA injection of ANG II did not alter cell discharge. (C) Peristimulus time histograms (5 ms bins) were constructed during electrical stimulation of the cervical vagus with 3 pulses (100 Hz) at 500 μA (a, 300 sweeps), 1 pulse at 500 μA (b, 305 sweeps), and 1 pulse at 100 μA (c, 331 sweeps). Note that electrical stimulation of the vagus nerve produced a biphasic response (a, b) with an initial decrease in cell discharge (*, $P < 0.05$; latency: 70 ms) followed by an increase in discharge (†, $P < 0.05$, latency 340 ms). No change in cell discharge was observed with 1 pulse at 100 μA (c). (D) This neuron was antidromically activated from the left PVN with a constant onset latency of 9 ms and activation threshold of 150 μA (a). The antidromic spike collided with a spontaneous action potential (b), and the neuron followed high frequency stimulation (>333 Hz, trace not shown). This neuron could not be antidromically activated from the right PVN (stimulus intensity: 1 mA, traces not shown). ▼, spontaneous spike; ▽, antidromic spike; s, antidromic stimulus artifact

Fig. 4. Activation of vagal afferent fibers increased the discharge of an osmotically- responsive MnPO-PVH neuron

(A) ICA injection of hypertonic NaCl increased cell discharge whereas isotonic saline had no effect. Although stimulating and unloading arterial baroreceptors with PE and SNP, respectively, did not alter cell discharge, activation of vagal afferent fibers with PBG produced a rapid and transient increase in cell discharge. (B) ICA injection of ANG II did not alter the neuronal firing rate of this neuron. (C) Peristimulus time histograms (5 ms bins) triggered by stimulation of the cervical vagus with 3 pulses (100 Hz) at 500 μA (*a*, 460 sweeps) and 1 pulse at 500 μA (*b*, 354 sweeps) reveal a biphasic response with an initial increase in cell discharge (*, $P < 0.05$; latency: 85 ms) followed by an immediate decrease (†, $P < 0.05$; latency: 140 ms). The magnitude of the response was smaller with 1 pulse. The effect was not observed when the stimulus intensity was 100 μA. (D) This neuron was antidromically activated from the left PVH with a constant latency of 6 ms (*a*) and stimulus threshold of 250 μA. The antidromic spike was collided with a spontaneous spike thereby confirming its antidromic nature (*b*). ▼, spontaneous spike; ▽, antidromic spike; s, antidromic stimulus artifact

Fig. 5. Location of osmotic and/or ANG II-responsive MnPO-PVH neurons that displayed an increase or decrease in cell discharge during activation of vagal afferent fibers

(A) As previously reported (Stocker and Toney, 2005), MnPO-PVH neurons responsive to osmotic and ANG II stimulation were located throughout the rostral-caudal distribution of the nucleus (not shown). There was no observable difference between the location of MnPO-PVH neurons that increased (□) or decreased (•) discharge to activation of vagal afferent fibers. (B, C) Examples of juxtacellular-labeled neurons. The neurons are located in the precommisural MnPO (B) and the dorsal MnPO (C) nucleus. Note that the morphology of both neurons is similar – both have a simple bipolar cytoarchitecture with limited dendritic branching. Where sufficiently resolved, presumptive axons appear to emerge from a proximal dendrite, not from

the cell soma. dMnPO, dorsal median preoptic nucleus; vMnPO, ventral median preoptic nucleus; AC, anterior commissure; f, fornix; 3V, 3rd ventricle, OC, optic chiasm.

Table 1
Summary of Discharge Characteristics for MnPO-PVH neurons

n	Electrical Stimulation of Vagal Afferents	Onset Latency (ms)	Basal Discharge (Hz)	Conduction Velocity (m/s)	Effect of PBG (100 µg/kg, iv)
↑ 16	ANG II and/or Osmotic 10/16↓	108 ± 19	2.0 ± 0.7	0.19 ± 0.02	↓ n=2, ↑ n=2, ↔ n=3
	2/16↑ 4/16↔	115 ± 15	2.7 ± 1.9 1.5 ± 0.8	0.24 ± 0.06 0.18 ± 0.03	n=1↑ n=3↔
↓ 3	ANG II and/or Osmotic 2/3↓ 1/3↔	78 ± 38	1.2 ± 0.4 2.2	0.19 ± 0.03 0.07	not tested not tested

Values are mean ± SE.

↓ denotes decrease in cell discharge;

↑ increase in cell discharge;

↔ no change in cell discharge.