Organum Vasculosum of the Lamina Terminalis Detects NaCl to Elevate Sympathetic Nerve Activity and Blood Pressure

Brian J. Kinsman, Sarah S. Simmonds, Kirsteen N. Browning, Sean D. Stocker

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Abstract—High-salt diet elevates NaCl concentrations in the cerebrospinal fluid to increase sympathetic nerve activity (SNA) in salt-sensitive hypertension. The organum vasculosum of the lamina terminalis (OVLT) resides along the rostral wall of the third ventricle, lacks a complete blood–brain barrier, and plays a pivotal role in body fluid homeostasis. Therefore, the present study used a multifaceted approach to examine whether OVLT neurons of Sprague–Dawley rats are intrinsically sensitive to changes in extracellular NaCl concentrations and mediate the sympathoexcitatory responses to central NaCl loading. Using in vitro whole-cell recordings, step-wise increases in extracellular NaCl concentrations (2.5–10 mmol/L) produced concentration-dependent excitation of OVLT neurons. Additionally, these excitatory responses were intrinsic to OVLT neurons because hypertonic NaCl evoked inward currents, despite pharmacological synaptic blockade. In vivo single-unit recordings demonstrate that the majority of OVLT neurons (72%, 13/19) display concentration-dependent increases in neuronal discharge to intracarotid (50 μL/15 s) or intracerebroventricular infusion (5 μL/10 minutes) of hypertonic NaCl. Microinjection of hypertonic NaCl (30 nL/60 s) into the OVLT, but not adjacent areas, increased lumbar SNA, adrenal SNA, and arterial blood pressure in a concentration-dependent manner. Renal SNA decreased and splanchnic SNA remained unaffected. Finally, local inhibition of OVLT neurons with the GABA_A receptor agonist muscimol (24 nL/10 s) significantly attenuated the sympathoexcitatory and pressor responses to intracerebroventricular infusion of 0.5 mol/L or 1.0 mol/L NaCl. Collectively, these findings indicate that OVLT neurons detect changes in extracellular NaCl concentrations to selectively alter SNA and raise arterial blood pressure. (Hypertension. 2017;69:163-170. DOI: 10.1161/HYPERTENSIONAHA.116.08372.)

Key Words: blood pressure ■ hypothalamus ■ osmoreceptor ■ sodium ■ sympathetic

Time-controlled studies in both humans and rodents suggest that a high-salt diet elevates cerebrospinal fluid (CSF) NaCl concentrations to subsequently increase sympathetic nerve activity (SNA) and arterial blood pressure (ABP).1,2 For example, a high-salt diet elevates plasma or CSF NaCl concentrations by 3 to 6 mmol/L in salt-sensitive subjects.3–6 Similarly, elevated CSF Na+ concentrations have been reported in Dahl salt-sensitive, spontaneously hypertensive, and Grollman Renal Wrap rats.7–9 Prior studies suggest that the putative NaCl-sensing neurons reside within the anteroventral third ventricular region (AV3V).10–13 Lesion of AV3V prevents/attenuates the sympathoexcitatory and pressor response to central hypernatremia14–16 and neurogenic forms of salt-sensitive hypertension.17–19 AV3V comprises several hypothalamic nuclei bordering the rostral third ventricle, including the periventricular preoptic, medial preoptic, ventral median preoptic, organum vasculosum of the lamina terminalis (OVLT), and fibers of passage from the subfornical organ (SFO).20 The OVLT and SFO have incomplete blood–brain and CSF–brain barriers and are uniquely exposed to circulating neurohormonal factors.

Some evidence suggests that OVLT may contain neurons that detect elevations in NaCl to increase SNA and ABP in response to central hypernatremia. For instance, hypernatremia increases the number of Fos-positive neurons within OVLT.19,20 Second, electric stimulation of OVLT neurons increases SNA.21,22 However, contemporary knowledge regarding OVLT neuronal responses to hyperosmotic stimuli are premised on electrophysiological responses to hypertonic mannitol, but not NaCl.23 The distinction between hypertonic NaCl versus mannitol is important because central infusion of hypertonic NaCl elicits sympathoexcitation, but hypertonic mannitol does not.24–26

This study used a multifaceted approach to establish whether OVLT neurons are excited by physiological elevations...
in NaCl concentrations and mediate downstream NaCl-dependent sympathoexcitatory increases in ABP. Because these experiments represent the first step to identify a role for OVLT in NaCl-dependent responses, experiments were conducted in Sprague–Dawley rats. In vitro studies reveal that OVLT neurons display intrinsic, concentration-dependent excitation during 2% to 5% increases in NaCl concentrations. Additional in vivo neurophysiological experiments demonstrate that central hypernatremia excites OVLT neurons to increase SNA and ABP.

Methods

All the experimental procedures conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State College of Medicine and University of Pittsburgh School of Medicine. Male Sprague–Dawley rats (250–400 g; Charles River Laboratories) were housed in a temperature-controlled room (22±1°C) with a 12-hour dark-light cycle, fed standard chow (Harlan Teklad Global Diet 2018), and given access to deionized water. A detailed methods section is available in the online-only Data Supplement.

In Vitro Electrophysiological Recording of OVLT Neurons

Whole-cell patch clamp recordings of spontaneously active OVLT neurons were obtained from coronal hypothalamic sections in adult Sprague–Dawley rats. Slices were continuously perfused by oxygenated and heated (31°C) Krebs Buffer (composition in mmol/L): 126 NaCl, 25 NaHCO3, 2.5 KCl, 2.4 CaCl2, 1.2 NaHPO4, and 11 glucose (pH 7.4 and 295 mOs/m/L). Hyperosmotic solutions (+2.5, +5, or +10 mmol/L NaCl) were prepared by the addition of NaCl to the extracellular control solution (+0 mmol/L NaCl). OVLT neurons were classified as NaCl sensitive (NaCl-S) by evidence of a >25% increase in peak action potential (AP) firing rate in response to a +5 mmol/L NaCl stimulus (3-minute duration). This firing rate was calculated as the number of spikes averaged over a 60-s interval. Only 1 OVLT slice was obtained per rat, and 1 to 2 OVLT neurons were recorded per slice. Different sets of slices were used for each experimental set.

Experiment 1: OVLT Neuron Excitation by Step-Wise Hypertonic NaCl

In current clamp, mean membrane potential and firing rates were measured throughout step-wise increases in bath NaCl concentration during baseline (+0 mmol/L NaCl; 3–5 minutes), +2.5 mmol/L NaCl (3 minutes), +5 mmol/L NaCl (3 minutes), +10 mmol/L NaCl (10 minutes) hypertonic NaCl stimuli. These variables (final 30 s for each stimulus) were compared across baseline, +2.5 mmol/L NaCl, and +5 mmol/L NaCl stimuli.

Experiment 2: OVLT Neuron Excitation by Sustained Hypertonic NaCl

In current clamp, mean firing rates were measured throughout a sustained increase in bath NaCl concentration during baseline (+0 mmol/L NaCl; 3–5 minutes) and +5 mmol/L NaCl (10 minutes) hypertonic NaCl stimuli. Mean firing rates (60 s) were compared across baseline, the 5- to 6-minute midpoint of +5 mmol/L NaCl, and the final 9 to 10 minutes of +5 mmol/L NaCl.

Experiment 3: Intrinsic NaCl Sensitivity of OVLT Neurons

These experiments were performed in the presence of 30 μmol/L bicuculline, 1 μmol/L kynurenic acid, and 1 μmol/L tetrodotoxin. In voltage clamp, mean current responses were measured throughout a sustained increase in bath NaCl concentration during baseline (+0 mmol/L NaCl; 3 minutes) and in response to either +5 mmol/L or +10 mmol/L NaCl (10 minutes). Mean current responses (60 s) were compared across stimuli and time points.

In Vivo Electrophysiology Experiments

Rats were anesthetized with Inactin (120 mg/kg IV) and prepared for simultaneous recordings of ABP (brachial artery and vein) and SNA (lumbar, renal, splanchnic, and adrenal) as described previously.12,17,24 A brain cannula was implanted into the lateral ventricle for intracerebroventricular (ICV) infusion of 0.15, 0.5, or 1.0 mol/L NaCl in artificial CSF (aCSF, 5 μL/10 minutes). The OVLT was targeted through a dorsal or ventral approach (please see online-only Data Supplement). Surgical preparation required ≈2 hours and was followed by 5 to 10 hours of experimental procedures. Different sets of rats were used per in vivo experimental set. Responses to each NaCl stimulus were measured in every rat within an experimental set.

Experiment 4: OVLT Single Unit Responses to Central Hypernatremia

To facilitate the identification of NaCl-responsive OVLT neurons, the tip of a nonocclusive intracarotid catheter was placed into the internal carotid artery at 1.5 mm rostral to the carotid bifurcation by insertion through the ascending pharyngeal artery. Through a ventral surgical approach, extracellular recordings of OVLT neurons were performed during intracarotid (0.15 or 0.5 mol/L NaCl, 50 μL/15 s) or ICV (0.15, 0.5, or 1.0 mol/L NaCl, 5 μL/10 minutes) infusion of isotonic or hypertonic NaCl. Intracarotid injection of 0.5 mol/L NaCl should increase carotid NaCl concentrations ≈6% to 7% (assuming carotid blood flow is 5.5 mL/min), whereas ICV infusion of 0.5 or 1.0 mol/L NaCl increases CSF [Na+] by ≈3 mmol/L (2%) and ≈7 mmol/L (5%).13

Experiment 5: OVLT Activation by Direct Injection of Hypertonic NaCl

Hypertonic NaCl (0.5, 1.0, or 1.5 mol/L) or aCSF (30 nL/60 s) was microinjected in a randomized sequence into the OVLT or at sites 500 μm adjacent. Variables were recorded for an additional 45 minutes.

Experiment 6: OVLT Activation by Mucimol During ICV Infusion of Hypertonic NaCl

The GABA-A agonist mucimol (5 mmol/L/24 mL) or aCSF (24 mL/10 s) was injected into the OVLT or at sites 500 μm adjacent. Ten minutes later, ICV infusion of 0.15, 0.5, or 1.0 mol/L NaCl (5 μL/10 minutes) was performed in a randomized manner, and variables were recorded for an additional 60 minutes.

Statistics

Analyses and graphs were prepared with SigmaPlot 11 or Systat 10.2. All data are presented as mean±SEM. Step-wise paradigm AP firing rate and membrane potential data were analyzed by comparing firing rates at baseline and in response to hypertonic NaCl stimuli by 2-way repeated measures analysis of variance and post hoc Holm–Sidak tests to evaluate difference between groups. Sustained paradigm AP firing rates were analyzed with Friedman repeated measures analysis of variance on ranks and post hoc Tukey tests. Inward current data were analyzed by 1-way repeated measures analysis of variance for +5 and +10 mmol/L hypertonic NaCl stimuli and post hoc Bonferroni comparisons. Differences between inward current responses to +5 and +10 mmol/L NaCl stimuli were compared via inward current averages from the final 1 minute of NaCl stimulation followed by Student’s t test. Passive and active membrane properties between NaCl-S and NaCl nonsensitive (NaCl-NS) OVLT neurons were compared with either Mann–Whitney U tests or Student’s t tests depending on whether the data satisfied normality. Data from in vivo experiments were averaged into 1 minute bins. Peak changes in all variables were compared with a 5-minute baseline segment and analyzed by 1- or 2-way analysis of variance. When significant F values were obtained, layered Bonferroni paired or independent t tests were performed to identify differences. Data for intracarotid infusions were averaged in 1-s bins, and peak changes (2 s) were compared with a 30-s baseline segment using a t test. P<0.05 was statistically significant for all comparisons.
Results

Experiment 1: OVLT Neuron Excitation by Step-Wise Hypertonic NaCl

An initial goal was to establish whether OVLT neurons detect physiological changes in NaCl concentrations. Using whole-cell recordings, we identified 9/16 (56%) OVLT neurons as NaCl-S defined by >25% increase in AP firing rate in response to +5 mmol/L NaCl. The remainder were NaCl-NS. Next, AP firing rates and membrane potentials were measured at baseline (+0 mmol/L) and in response to small, step-wise hypertonic NaCl stimuli (+2.5 and +5 mmol/L; 3 minutes each). Hypertonic NaCl induced concentration-dependent increases in NaCl-S, but not NaCl-NS, neuron AP firing rate (Figure 1A and 1B) and membrane depolarization (Figure 1A and 1C).

Experiment 2: OVLT Neuron Excitation by Sustained Hypertonic NaCl

The next experiment assessed whether OVLT neuronal excitatory responses to hypertonic NaCl adapted with a more sustained stimulus exposure. Hypertonic NaCl (+5 mmol/L) induced sustained increases in AP firing rate of NaCl-S, but not NaCl-NS, OVLT neurons from baseline versus the 5- to 6-minute midpoint and the final 9 to 10 minutes of stimulation (Figure 1D and 1E). Notably, there was no difference in AP firing rate between 5 to 6 minutes and 9 to 10 minutes. One OVLT neuron hyperpolarized and reduced AP firing rate in response to hypertonic NaCl—that neuron was excluded from this analysis.

Experiment 3: Intrinsic NaCl Sensitivity of OVLT Neurons

We then interrogated whether these excitatory responses to hypertonic NaCl are intrinsic to OVLT neurons or are mediated by synaptic neurotransmission onto OVLT. NaCl-S neurons were first identified in current clamp in the presence of an ionotropic glutamate receptor antagonist kynurenic acid (1 mmol/L) and a GABA\(\text{A}\) receptor antagonist bicuculline (30 \(\mu\)mol/L). Subsequently, in voltage clamp \((V_h=-50 \text{ mV})\) with 1 \(\mu\)mol/L tetrodotoxin to block AP-dependent synaptic inputs, these NaCl-S neurons displayed concentration-dependent inward currents in response to either +5 or +10 mmol/L NaCl (Figure 2A). Both +5 and +10 mmol/L NaCl elicited significant inward currents compared with an isotonic NaCl baseline (Figure 2C). This inward current persisted for the remainder of the hypertonic NaCl stimulation. The average inward current during the final 1 minute of NaCl stimulation was significantly greater in response to +10 mmol/L versus +5 mmol/L NaCl (Figure 2C). Likewise, the magnitude of the integrated inward current (area above the curve) was significantly greater during +10 mmol/L NaCl (-75.25±16.98 pA minutes) versus +5 mmol/L NaCl (-36.69±5.09 pA minutes; \(P=0.023\)).

Post hoc immunofluorescent identification of neurobiotin-filled OVLT neurons revealed that NaCl-S and NaCl-NS cells were distributed throughout all rostrocaudal levels of the OVLT (Figure 3). In addition, there were no significant differences between NaCl-S and NaCl-NS populations based on active and passive membrane properties derived from current pulse-stimulated APs and current–voltage relationships (Table S1 and Figure S1 in the online-only Data Supplement).

Experiment 4: OVLT Single Unit Responses to Central Hypernatremia

To determine the extent by which central hypernatremia alters the activity of OVLT neurons in vivo, we used a ventral approach to perform single-unit recordings of OVLT neurons during intracarotid or ICV infusion of isotonic or hypertonic NaCl.
NaCl. NaCl-S or NaCl-NS OVLT neurons were initially distinguished by the discharge response to intracarotid injection of 0.5 mol/L NaCl (50 μL/15 s). Baseline mean ABP and heart rate were 92±4 mm Hg and 387±12 bpm, respectively (n=12). The majority of OVLT neurons were NaCl-S (72%, 13/18) and increased discharge frequency to intracarotid injection of 0.5 mol/L NaCl (1.2±0.3 to 9.9±1.9 Hz; *P<0.01). Intracarotid injection of 0.5 mol/L NaCl also produced a small increase in mean ABP (9±2 mm Hg; *P<0.05). Intracarotid injection of 0.15 mol/L NaCl did not alter cell discharge (1.1±0.2 to 1.3±0.3 Hz; *P<0.5) or mean ABP (1±2 mm Hg; *P>0.6). These same NaCl-S neurons displayed concentration-dependent increases in cell discharge during ICV infusion of 0.5 or 1.0 mol/L NaCl (Figure 4A and 4B). The increased firing rate occurred within 2 minutes after the onset and remained elevated despite an increased mean ABP. In fact, both intracarotid and ICV infusion of hypertonic NaCl increased cell discharge before any significant changes in mean ABP. On the contrary, a subset of OVLT neurons (5/18, 28%) were NaCl-NS because intracarotid injection of 0.5 mol/L NaCl did not alter cell discharge (1.9±0.5 to 2.1±0.6 Hz; *P<0.6). Similarly, ICV infusion of 0.5 or 1.0 mol/L NaCl did not alter cell discharge in NaCl-NS neurons (Figure 4). Juxtaglomerular labeling revealed that NaCl-S neurons had processes that typically coursed caudally along the third ventricle. NaCl-S or NaCl-NS OVLT neurons were anatomically distributed throughout the OVLT (Figure S2).

**Experiment 5: OVLT Activation by Direct Injection of Hypertonic NaCl**

The next experiment tested the extent by which local changes in extracellular NaCl concentrations within the OVLT elevated SNA and ABP. Again, half of the animals per group were performed using a dorsal (n=3) versus ventral (n=3) approach to target OVLT. Because there were no differences in baseline mean ABP, heart rate, or the responses to local injection of NaCl between ventral versus dorsal approaches, the data were combined. Baseline mean ABP and heart rate were 90±4 mm Hg and 403±11 bpm, respectively. ICV injection of NaCl produced a concentration-dependent increase in lumbar SNA, adrenal SNA, and mean ABP (Figure 5). Typically, these variables began to change as the injection was performed. Renal or splanchnic SNA did not change from baseline values. Importantly, injection of 1.0 mol/L NaCl adjacent to the OVLT (rostral, lateral, and dorsal; n=4 per site) did not produce significant changes in any SNA or mean ABP. Injection sites are illustrated in the online-only Data Supplement (Figure S3).

**Experiment 6: OVLT Inhibition by Muscimol During ICV Infusion of Hypertonic NaCl**

To evaluate whether OVLT neurons mediate the sympatoexcitatory response to changes in CSF NaCl concentrations, OVLT neurons were inhibited by injection of the GABA<sub>A</sub> agonist muscimol before ICV infusion of 0.5 or 1.0 mol/L NaCl. Half of the animals per group were performed using a dorsal (n=4) versus ventral (n=4) approach to target OVLT. Because there were no differences in baseline mean ABP, heart rate, or the responses to ICV infusion of NaCl between ventral versus dorsal approaches, the data were combined. Baseline mean ABP and heart rate were 95±4 mm Hg and 399±13 bpm, respectively. Injection of aCSF into the OVLT did not alter SNA or mean ABP. As previously reported,<sup>11</sup> ICV infusion of NaCl produced concentration-dependent increases in lumbar SNA, adrenal SNA, and ABP (Figure 6). Renal SNA decreased (Figure 6), and splanchnic SNA did not change (data not shown). Inhibition of OVLT neurons with local injection of the GABA<sub>A</sub> agonist did not alter any SNA or mean ABP. However, muscimol pretreatment significantly attenuated the sympatoexcitatory and pressor response to ICV infusion of 0.5 and 1.0 mol/L NaCl (Figure 6). In a separate
set of animals, injection of muscimol rostral, lateral, or dorsal to OVLT did not alter the responses to ICV infusion of 0.5 or 1.0 mol/L NaCl (data not shown). Injection sites are illustrated in the online-only Data Supplement (Figure S4).

Discussion

This study provides the first evidence that physiological elevations in NaCl concentrations excite OVLT neurons to increase SNA and ABP. We have made multiple novel observations that support this conclusion: (1) in vitro whole-cell recordings demonstrate that OVLT neurons display concentration-dependent excitation to hypertonic NaCl (+2.5–10 mmol/L), (2) OVLT neurons are intrinsically NaCl-S because hypertonic NaCl stimulates an inward current in the presence of synaptic blockade, (3) intracarotid or ICV infusion of hypertonic NaCl produced concentration-dependent increases in OVLT neuronal discharge in vivo, (4) local OVLT microinjection of hypertonic NaCl produced concentration-dependent increases in lumbar SNA, adrenal SNA, and ABP, and (5) inhibition of OVLT neurons with local injection of the GABAA receptor agonist muscimol prevented the sympathoexcitatory response to ICV hypertonic NaCl.

CSF NaCl concentrations are elevated by +3 to 8 mmol/L in salt-sensitive hypertension.4–9 Moreover, the elevation in CSF Na+ concentration may precede the development of salt-sensitive hypertension in Dahl salt-sensitive and spontaneously hypertensive rats fed a high-salt diet.4–9 Prior studies using electrolytic lesions or Fos immunocytochemistry suggest that the AV3V region, and more specifically OVLT, contain putative NaCl-responsive cells14,29–31; yet, evidence to indicate that such neurons directly sensed physiological changes in NaCl concentrations was absent. The present study provides the first evidence on a cellular level that hypertonic NaCl (+2.5–10 mmol/L) causes concentration-dependent increases in OVLT neuron AP discharge frequency in vitro. These responses are likely intrinsic to OVLT neurons because hypertonic NaCl produced an inward current during pharmacological blockade of synaptic neurotransmission. The response onsets to hypertonic NaCl were stimulus locked, which suggests that metabotropic receptor activation is less likely to initiate these responses. Still, this does not exclude possible contributions from atypical signaling molecules (eg, ATP, lactate, hydrogen sulfide) originating from ependymal, astrocytic, or vascular elements within OVLT. These in vitro cellular responses were...
confirmed by in vivo single-unit recordings to demonstrate that OVLT neurons were sensitive to physiological changes in plasma or CSF NaCl concentrations (2%–5%). Although the response magnitude to hypertonic NaCl was greater in vivo versus in vitro, these differences could be attributed to experimental preparation (ie, slice temperature, intact synaptic inputs). Nevertheless, both approaches provide clear evidence that OVLT neurons can sense and respond to discrete changes in extracellular NaCl concentrations.

Lesion of the AV3V region attenuates sympathoexcitatory responses to acute hypernatremia and also attenuates/prevents every neurogenic experimental model of salt-sensitive hypertension. The current findings provide novel evidence to suggest OVLT neurons mediate these effects. First, local injection of hypertonic NaCl into the OVLT, but not adjacent to the OVLT, produced concentration-dependent increases in SNA and ABP. Second, inhibition of OVLT neurons by local injection of the GABA<sub>A</sub> agonist muscimol attenuated the sympathoexcitatory responses to ICV NaCl. Inhibition of neurons immediately adjacent to OVLT did not affect the sympathoexcitatory effect of central NaCl loading. Altogether, these site-specific effects indicate that the OVLT is a key neural substrate for NaCl-dependent regulation of ABP. The contribution of OVLT neurons to salt-sensitive hypertension is limited, with the exception of 1 investigation in which OVLT lesion attenuated angiotensin II plus high-salt hypertension.

While local injection of muscimol into OVLT largely attenuated the sympathoexcitatory responses to ICV NaCl, the response was not completely eliminated. A second potential NaCl-sensing site is the SFO. Prior studies have reported that the SFO contains NaCl-responsive neurons, and interruption of SFO neurotransmission attenuated pressor responses to ICV infusion of hypertonic NaCl. Although SFO lesions in rats only attenuate salt-sensitive hypertension in angiotensin II plus high-salt model, manipulation of SFO signaling in mice attenuates deoxycorticosterone acetate–salt hypertension. Hence, the SFO may also contribute to NaCl-sensing and salt-sensitive hypertension.

Central or peripheral infusion of hypertonic NaCl increases lumbar or muscle SNA in rodents and humans, respectively, but either has no effect or decreases renal SNA. Although a few studies report that intracarotid infusion of 0.75 to 1.50 mol/L NaCl (300 μL) increases renal SNA, these renal sympathoexcitatory may be attributed to the extremely large increases in forebrain NaCl concentration (≈15%–100%). Indeed, our preliminary data indicate that intracarotid infusion of 1.0 mol/L NaCl (50 μL/15 s) raises the firing rate of OVLT neurons to >40 Hz (unpublished observation). Thus, these high-magnitude intracarotid NaCl stimuli may activate OVLT and other sympathoregulatory centers. In the present study, ICV infusion of hypertonic NaCl decreased renal SNA. As reported and discussed previously, this renal sympathoinhibitory response is mediated by a specific subset of neurons in the rostral ventrolateral medulla and attributed to a baroreflex-mediated inhibition or a centrally mediated natriuretic response.

If the OVLT is the putative NaCl-S site to regulate SNA and ABP, then what is the underlying mechanism by which these neurons detect changes in extracellular NaCl concentrations? Because ICV and intracarotid administration of hypertonic NaCl stimulates SNA and raises ABP, but infusion of equiosmolar mannitol does not, there are likely distinct cellular processes that differentiate between hyperosmolality versus hypertonic NaCl. For example, supraoptic nucleus neurons respond to hyperosmotic mannitol stimuli via a mixed cationic inward current, whereas hypertonic NaCl evokes an inward current without clear ionic reversal potential. There are several potential mechanisms that may underlie cellular NaCl sensing. First, within the supraoptic nucleus, chronic NaCl loading collapses the chloride gradient, converts synaptic GABAergic inputs from inhibitory to excitatory, and causes a partially vasopressin-dependent elevation in ABP. Second, the Na<sub>x</sub> channel has also been implicated in Na<sup>+</sup> sensing along the lamina terminalis. Within the rat median preoptic nucleus, Na<sub>x</sub> mediates a sodium leak current that is regulated by the Na<sup>+</sup>/K<sup>+</sup> ATPase α1 isoform to facilitate neuronal

![Figure 6](http://hyper.ahajournals.org/)

A, Arterial blood pressure (ABP), mean ABP (gray line), and integrated lumbar, adrenal, and renal sympathetic nerve activity (SNA) during intracerebroventricular (ICV) infusion of 0.5 mol/L NaCl (6 μL/10 min) after microinjection of aCSF or the GABA<sub>A</sub> agonist muscimol (2.5 mmol/L/24 nL). Splanchnic SNA did not change during any injection or infusion (data not shown). B, Means±SEM of peak changes, *P<0.05 vs 0.15 mol/L, #P<0.05 vs muscimol.
sodium sensing. 46 Within the mouse SFO, hypertonic NaCl was found to augment an Na\textsubscript{\text{A}}-mediated sodium leak current in ependyocytes to stimulate lactate release and excite proximal GABAergic interneurons. 37 Third, ICV administration of benzamil, a nonvoltage gated sodium channel blocker, attenuates vasopressin secretion, sympathoexcitatory responses to CSF hypernatremia, and salt-sensitive hypertension. 48–50 This data suggests that one of several benzamil-sensitive channels (ie, sodium–calcium antiporters, sodium–proton pumps, acid-sensing ion channels, or epithelial sodium channels) represents the putative NaCl sensor. Nevertheless, the above mechanisms have not yet been identified to function within OVLT neurons.

**Limitations**

In the current study, OVLT neurons were classified as NaCl-S based on a ≥25% increase in peak firing rate during +5 mM\textsubscript{NaCl}/L NaCl. This classification method may underestimate the number of NaCl-S OVLT neurons. Interneuronal differences in the timing and magnitude of the response may reflect differences in expression or function of the unidentified NaCl-sensing protein(s). Second, multiple in vivo methods were used to raise central NaCl concentrations (ICV, intracarotid, OVLT microinjection). Although it is difficult to directly measure local NaCl concentrations in the OVLT, NaCl concentrations were estimated or measured in the CSF to change 3% to 5%. 13 Finally, in vivo experiments were conducted in anesthetized animal preparations. Although anesthetics will alter the magnitude of the responses, the distinct advantage of the preparation was the novel insight gained from simultaneous recordings of multiple sympathetic nerves and single-unit recordings. However, future experiments are needed to extend these observations into awake and chronic models of salt-sensitive hypertension.

**Perspectives**

Herein, we have provided the first electrophysiological evidence that a majority of OVLT neurons are intrinsically excited by 2% to 5% elevations in NaCl concentrations. These responses are functionally significant because hypernatremia-induced activation of OVLT neurons elevates lumbar SNA, adrenal SNA, and ABP. The implication of the current findings is that ingestion of a high-salt diet elevates CSF NaCl concentrations to activate OVLT neurons, which elevate blood pressure. Surprisingly, little data exist regarding the function of OVLT neurons in salt-sensitive hypertension. Consequently, follow-up studies are needed to evaluate alterations in OVLT neuron function in salt-sensitive models and the chronic contribution of OVLT to salt-sensitive hypertension. For now, the undiscovered mechanism(s) of NaCl-sensing by OVLT neurons remains an enticing prospect of future investigation.

**References**


Organum vasculosum of the lamina terminalis (OVLT) neurons are intrinsically excited by physiologically relevant elevations in extracellular NaCl concentrations.

In vivo, site-specific activation of OVLT neurons by hypertonic NaCl solutions differentially modulates sympathetic nerve activity. In vivo, central hypernatremia increased the discharge frequency of OVLT neurons to subsequently elevate lumbar sympathetic nerve activity.

What Is Relevant?

Rodents and humans affected by salt-sensitive hypertension demonstrate similar (3–8 mmol/L) elevations in blood and cerebrospinal fluid NaCl concentrations in response to a high-salt diet. This suggests that pathological activation of OVLT neurons could contribute to salt-sensitive hypertension.

Summary

OVLT neurons are excited by physiological increases in NaCl concentrations to selectively regulate sympathetic nerve activity and elevate arterial blood pressure.
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ONLINE SUPPLEMENT

The Organum Vasculosum of the Lamina Terminalis Detects NaCl to Elevate Sympathetic Nerve Activity and Blood Pressure

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SUPPLEMENTARY METHODS

Animals. All of the experimental procedures conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State College of Medicine and University of Pittsburgh School of Medicine. Experiments were conducted in male Sprague-Dawley rats (250-400g, Charles River Laboratories) housed in a temperature-controlled room (22±1°C) with a 12-hour dark:light cycle. Rats were fed standard chow (Harlan Teklad Global Diet 2018) and given access to deionized water.

In Vitro Electrophysiology - General Procedures
Rats were anesthetized deeply with 5% isoflurane and decapitated. The brains were extracted rapidly into oxygenated (95%O₂/5%CO₂), ice-cold Krebs Buffer (KRB) (composition in mM): 126 NaCl, 25 NaHCO₃, 2.5 KCl, 1.2 MgCl₂, 2.4 CaCl₂, 1.2 Na₂HPO₄, and 11 d-glucose (pH 7.4 and 295 mOsm/L). Coronal slices containing OVLT were cut at 250µm thickness on a vibratome with a sapphire blade (Delaware Diamond Knives, Wilmington, DE, USA). OVLT can be identified as the translucent ventral-most midline nucleus in dorsal apposition to the optic chiasm, bound rostrally by the Diagonal Band of Broca, caudally by the 3rd ventricle and located 0-600µm rostral to the anterior commissure. Slices were then incubated at 31±1°C in oxygenated KRB for 60-90min prior to whole-cell recordings. A single slice was then transferred to a perfusion chamber (Volume 500µL) and held in place by a nylon slice anchor (Warner Instruments, LLC, Hamden, CT, USA). Slices were continuously bathed in oxygenated KRB via a gravity-fed perfusion system to the recording chamber at 2-3 mL min⁻¹ and warmed to 31±0.5°C with an SF-28 inline heater and TC-324B temperature controller (Warner Instruments).

Whole-cell recordings were made with borosilicate patch-pipettes pulled to resistance of 5-8MΩ when filled with potassium gluconate intracellular (composition in mM): 128 K-gluconate, 10 KCl, 0.3 CaCl₂, 1 MgCl₂, 10 HEPES, 1 EGTA, 4 NaATP, and 0.5 NaGTP adjusted to pH 7.35 with KOH and osmolarity 280±2 mOsm/L. To immunocytochemically label neurons, 1.25mg/mL of Neurobiotin Tracer (VectorLabs, Burlingame, CA, USA) was added to the intracellular solution. Data were acquired in Clampex 10.3 software with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) at a rate of 10 kHz, filtered at 2 kHz and digitized with a Digidata 1440A interface before being saved on a personal computer and analyzed in pClamp10 (Molecular Devices) and Spike 2.0 software. Only neuronal recordings maintaining a series resistance (ie. pipette + access resistance) <20MΩ were considered of acceptable quality. Prior to any recordings, the theoretical liquid junction potential was corrected. Drugs were applied at concentrations demonstrated previously to be effective and dissolved in KRB. Perfusion solutions were switched with the automatic valve controller ValveLink 8.2 (Automate Scientific, Inc., Berkeley, CA, USA) to apply hypertonic NaCl stimuli and/or pharmacologic antagonists including bicuculline (BIC; 30µM; Alomone Labs Ltd.), tetrodotoxin (TTX; 1µM; Alomone Labs Ltd.), and kynurenic acid (KYN; 1mM; Sigma Aldrich).
OVLT neurons were classified as NaCl-sensitive by evidence of a 25% or greater increase in peak action potential (AP) firing rate in response to a +5mM NaCl stimulus (3 min duration). All current-clamp recordings evaluated OVLT neurons that were spontaneously firing APs when held at -55 mV to -52 mV with current injection.

**Experiment 1: OVLT Neuron Excitation by Step-wise Hypertonic NaCl.** This initial experiment evaluated the lower-limit of OVLT neuron excitation by physiologically-relevant, step-wise hypertonic NaCl stimuli. Current clamp recordings were performed as such: baseline (+0mM NaCl; 3-5 min), +2.5mM NaCl (3 min), +5mM NaCl (3 min), +2.5mM NaCl (1.5 min), and washout (+0mM NaCl; 6-10 min) AP firing rates and membrane potential were measured during the final 60s of baseline and during peak 30s AP firing rate in response to +2.5 and +5mM NaCl stimuli. Membrane potential measurements were made during the same intervals as firing rate measurements. Membrane potential measurements were taken during stable inter-AP intervals for 1 s every 10 s. The change in membrane potential was taken as the difference between the cumulative baseline average and interval of interest average.

**Experiment 2: OVLT Neuron Excitation by Sustained Hypertonic NaCl.** This experiment examined whether OVLT neurons adapt in response to a sustained hypertonic NaCl stimulus. Current clamp recordings were performed as such: baseline (+0mM NaCl; 3-5 min), +5mM NaCl (10 min), and washout (+0mM NaCl; 10-20 min). AP firing rates were measured for the final 1min of baseline, the 5-6min midpoint, and the final 9-10min of +5mM NaCl stimulation.

**Experiment 3: Intrinsic NaCl Sensitivity of OVLT Neurons.** NaCl-sensitivity of OVLT neurons was evaluated in current clamp mode in the presence of 1mM KYN and 30µM BIC during a 3-5min baseline and in response to either a +5mM or +10mM NaCl stimulus (3 min). Following washout, these same NaCl-sensitive OVLT neurons were assessed in voltage-clamp. Neurons were recorded in the presence of 1µM TTX at Vh = -50mV. NaCl-sensitive OVLT neuron whole current responses were recorded during baseline (+0mM NaCl; 3 min), +5mM or +10mM NaCl (10 min), and washout (+0mM NaCl; 20 min).

**OVLT Neuron Passive and Active Membrane Properties:** To measure passive membrane properties, a current-voltage (I-V) relationship was examined for OVLT neurons at holding potentials between -50 mV to -120 mV in 400 ms duration 10mV hyperpolarizing steps (See Figure S1 for I-V curves). Resting membrane potential was derived as the X-intercept of the I-V curve. Input resistance was calculated from the difference in steady state current required to hold a neuron at -50 mV versus -60 mV. To measure active membrane properties, single action potentials (AP) were triggered in OVLT neurons held at -57 mV with 5-30 ms current pulses of magnitude sufficient to trigger an AP at current pulse offset (See Figure S1 for AP traces). AP threshold was measured as the inflection point of the depolarization concavity on a 20 ms timescale. AP 10-90% rise time and rate of rise were measured from the AP threshold to peak depolarization. AP duration was measured as the interval from AP threshold until the equivalent membrane potential was reached upon repolarization. Afterhyperpolarization
(AHP) magnitude was measured as the absolute value of the difference between membrane potential at baseline (-57 mV) and the repolarization nadir. The repolarization rate was evaluated by fitting the interval from the repolarization nadir until the membrane potential returned to baseline to a standard exponential function in Clampfit 10.4. The calculated time constant $\tau$ was taken as an index of repolarization rate. See Table S1 for passive and active membrane properties.

**Immunohistochemistry and Neuroanatomical Mapping of OVLT Neurons:** At the end of recordings, slices were fixed in 4% paraformaldehyde for 16-20 hrs and rinsed with 10mM PBS. Neurobiotin-filled cells were visualized through successive incubations in an avidin-biotin complex solution (1 hr, room temperature, ABC VECTASTAIN, Vector Laboratories) and streptavidin Alexa Fluor 488 (2h, 1:250; Molecular Probes). Slices were mounted on slides and coverslipped with VECTASHIELD. Neurobiotin-filled OVLT neurons were imaged using a Nikon Eclipse 90i microscope and NIS-Elements software. These neurons were mapped onto neuroanatomical schematics of OVLT on three coronal planes each separated by 250 µm.

**In Vivo Experiments – General Procedures.**

Rats were anesthetized with isoflurane (2-3% in 100% O2) and prepared for simultaneous recordings of ABP (brachial artery and vein) and SNA (lumbar, renal, splanchnic, and adrenal) as described previously 2-4. Animals were artificially ventilated with oxygen-enriched room air. End-tidal CO$_2$ and body temperature were maintained at 3.5-4.5% and 37±0.5ºC, respectively. After rats were placed into a stereotaxic head frame, 26-gauge cannulae were implanted into the left and right lateral ventricle 5. After all surgical procedures were completed, anesthesia was replaced by Inactin (120 mg/kg, IV). Animals also received a continuous infusion of 0.75% NaCl and 0.25% glucose (0.5mL/hr, IV). The level of anesthesia was monitored by the lack of a withdrawal reflex to a foot pinch.

Two experimental approaches were employed to target OVLT. DORSAL APPROACH: After a small craniotomy, a tungsten microelectrode was lowered into the ventral hypothalamus in reference to Bregma (0.0-0.6mm rostral, 0.2-0.5mm lateral, 7.2-7.8mm ventral to dura, 86º from midsagittal plane). Square-wave current pulses (1ms, 1Hz, 100-200µA, 30 sweeps per site) were applied as the microelectrode was moved at 200µm increments in the X-Y-Z plane. Stimulus-triggered averages of SNA were constructed at each site to produce a peak increase in SNA with a latency of 150-200ms as described previously 6. These coordinates were used for subsequent microinjections. VENTRAL APPROACH: To avoid related structures located dorsal to OVLT such as the median preoptic nucleus, a ventral midline approach was used to perform microinjections and single-unit recordings as described elsewhere 7. Briefly, the rat was placed supine into the stereotaxic frame with the incisor bar at +5mm. The lower incisors were split and retracted laterally. The tongue was removed. The hard and soft palate were cauterized and removed with a dental drill. Several (2-4) small holes were drilled into the sphenoid bones. Heated plasticene was injected into the holes to cauterize the underlying venous sinus. Then, the bone and plasticene were removed with a dental drill to visualize the optic nerves and entire optic chiasm/hypothalamus
extending caudal to the rostral edge of the pituitary. Coordinates for OVLT were 0.8-1.2 mm caudal to rostral edge of optic chiasm and 0.8-1.4 mm ventral to surface.

**Experiment 4: OVLT Single Unit Responses to Central Hypernatremia.** Animals were prepared as described above using a ventral approach to expose the hypothalamus. To facilitate the identification of NaCl-responsive OVLT neurons, the tip of a non-occlusive intracarotid catheter (heat-stretched PE-10) was placed into the internal carotid artery at 1.5 mm rostral to the carotid bifurcation by insertion through the ascending pharyngeal artery. Single-unit recordings were performed using glass electrodes (10-25 MΩ) filled with 4% Neurobiotin (dissolved in 0.5% sodium acetate, pH 7.4) and intracellular amplifier in bridge mode (AxoClamp 2B, Molecular Devices). OVLT was probed for spontaneously active units in 2 µm steps. Once a neuron was isolated, neuronal responses to intracarotid injection of 0.15 M or 0.5 M NaCl (50 µL over 15 s). Then, 0.15 M, 0.5 M or 1.0 M NaCl (5 µL over 10 min) were infused intracerebroventricularly. Cells were tested for 0.15 M and either 0.5 M or 1.0 M NaCl. At the end of recordings, cells were juxtacellular-labeled as described previously by applying current pulses (200 ms, 50% duty cycle) of increasing amplitude (1.0-8.0 nA) delivered through the recording electrode for 20-180 s of entrainment. If a cell was not entrained or lost during the recording, the site was marked by applying DC current (5 µA, 5 min). Animals were perfused transcardially with 4% paraformaldehyde. Brains were post-fixed overnight, sectioned at 50 µm on a vibratome, and incubated with Streptavidin AlexaFluor 488 or 594 to visualize filled cells (or recording sites).

**Experiment 5: OVLT Activation by Direct Injection of Hypertonic NaCl.** To test whether local increases in NaCl concentrations produce sympathoexcitation, varying concentrations of hypertonic NaCl were injected into the OVLT. Animals were prepared as described above using either a dorsal or ventral approach. Hypertonic NaCl (0.5, 1.0, or 1.5 M) or aCSF (30 nL) was microinjected in a randomized sequence into the OVLT over 60 s. Variables were recorded for an additional 45 min. Control injections of hypertonic NaCl (1.0 M, 30 nL) were performed 400 µm rostral, lateral, or dorsal to the OVLT.

**Experiment 6: OVLT Inhibition by Muscimol During ICV Infusion of Hypertonic NaCl.** To test the contribution of OVLT neurons to the SNA and ABP response during central hypernatremia, the GABA A agonist muscimol (5 mM, 24 nL) or aCSF (24 nL) was injected into the OVLT over 10 s. At 10 min later, ICV infusion of 0.15 M, 0.6 M, or 1.0 M NaCl (5 µL over 10 min) was performed in a randomized manner, and variables were recorded for an additional 60 min. Each animal received all three ICV infusions but was treated with either muscimol or aCSF only. In a separate set of animals, control injections of muscimol were performed 400 µm rostral, lateral, and dorsal to OVLT. In all experiments, the vasopressin receptor antagonist Manning Compound (10 µg/kg, IV) was administered to eliminate the contribution of vasopressin to the NaCl-induced responses. Experiments were performed in animals using both a dorsal and ventral approach.

All microinjections using either approach were performed using single-barrel glass micropipettes (0.68 mm ID and 6 nL per division) connected to a picopump and
monitored with an eye reticule. Injection sites were marked by the addition of rhodamine or FITC beads (0.2%, Molecular Probes) to all solutions.

Statistics
Analyses and graphs were prepared with SigmaPlot 11 (Systat Software). All data are presented as mean ± S.E.M. Step-wise paradigm AP firing rate and membrane potential data were analyzed by comparing firing rates at baseline and in response to hypertonic NaCl stimuli levels by applying two-way repeated measures ANOVA and post hoc Holm-Sidak tests to evaluate difference between groups. Sustained paradigm AP firing rates were analyzed with Friedman repeated measures ANOVA on ranks and post hoc Tukey tests. Inward current data were analyzed by one-way repeated measures ANOVA for +5mM and +10mM hypertonic NaCl stimuli and post hoc Bonferroni comparisons. Differences between inward current responses to +5mM and +10mM NaCl stimuli were compared via (A) inward current averages from the final 1min of NaCl stimulation followed by Student’s t test and (B) area above the curve analysis and subsequent Mann-Whitney U test. Passive and active membrane properties between NaCl-sensitive and non-sensitive OVLT neurons were compared with either Mann-Whitney U tests or Student’s t tests depending on whether or not the data satisfied normality. Data from in vivo experiments were averaged into 1 min bins. Peak changes in all variables were compared to a 5 min baseline segment and analyzed by one or two-way ANOVA. When significant F values were obtained, layered Bonferroni paired or independent t-tests were performed to identify differences. Data for intracarotid infusions were averaged in 1 s bins, and peak changes (2s) were compared to a 30s baseline segment using a t-test. P<0.05 was statistically significant for all comparisons.
SUPPLEMENTARY RESULTS

Passive and Active Membrane Properties. Throughout in vitro whole-cell recording experiments, the only method available to identify an OVLT neuron as NaCl-sensitive involved bath application of hypertonic NaCl. Thus, we retrospectively analyzed and compared NaCl-S (n = 40 neurons) and NaCl-NS (n = 15 neurons) OVLT neuron passive and active membrane properties in an effort to facilitate more rapid screening and identification of NaCl-S OVLT neurons, and glean insight as to the mechanistic basis of NaCl-sensitivity. Figure S1A-B reveals no significant differences between NaCl-S and NaCl-NS OVLT neuron steady-state I-V relationships from -50 mV to -120 mV. Resting membrane potentials for NaCl-S and NaCl-NS OVLT neurons were similar and comparable to previous reports for OVLT neurons collectively (Table S1). Furthermore, input resistances were not significantly different between NaCl-S (1763 ± 150 MΩ) and NaCl-NS (1560 ± 195 MΩ, P>0.05) OVLT neurons (Table S1). These high input resistances characteristic of OVLT neurons informed our decision to apply a greater magnitude (+10 mM NaCl) hypertonic stimulus when investigating the intrinsic current responses of these neurons in order to evoke a distinguishable inward current signal above the noise. Active membrane properties were analyzed from current pulse triggered action potential traces of OVLT neurons as illustrated in Figure S1C. No significant differences were found between NaCl-S and NaCl-NS OVLT neuron APs on the basis of AP threshold, 10-90% AP rise time, 10-90% AP rate of rise, AP duration, afterhyperpolarization (AHP) magnitude and repolarization constant τ (Table S1; P>0.05).
REFERENCES


**Table S1.** Passive and active membrane properties of NaCl-S versus NaCl-NS OVLT neurons.\(^a\)

<table>
<thead>
<tr>
<th>Membrane Property</th>
<th>NaC-Sensitive</th>
<th>Non-Sensitive</th>
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<tr>
<td>Input Resistance (MΩ)</td>
<td>1763 ± 150</td>
<td>1560 ± 195</td>
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<tr>
<td>Resting Membrane Potential (mV)</td>
<td>-50.50 ± 2.33</td>
<td>-57.67 ± 2.71</td>
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<tr>
<td>AP Threshold (mV)</td>
<td>-42.17 ± 0.59</td>
<td>-42.73 ± 1.08</td>
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<tr>
<td>AP 10-90% Rise Time (ms)</td>
<td>0.68 ± 0.03</td>
<td>0.73 ± 0.03</td>
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<tr>
<td>AP 10-90% Rate of Rise (mV/ms)</td>
<td>83.91 ± 4.70</td>
<td>75.43 ± 4.73</td>
</tr>
<tr>
<td>AP Duration (ms)</td>
<td>4.12 ± 0.21</td>
<td>4.59 ± 0.47</td>
</tr>
<tr>
<td>Afterhyperpolarization Magnitude (mV)</td>
<td>6.54 ± 0.47</td>
<td>7.30 ± 0.80</td>
</tr>
<tr>
<td>Repolarization Constant τ (ms)</td>
<td>194.11 ± 34.08</td>
<td>161.85 ± 23.65</td>
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\(^a\) There were no significant differences in passive or active membrane properties of NaCl-S (40 neurons/34 rats) versus NaCl-NS (15 neurons/12 rats) OVLT neurons. Properties were calculated from I-V curves and AP traces (Fig. 3; see Methods). Values are mean ±SEM.
Figure S1. Comparison of NaCl-sensitive and non-sensitive OVLT neuron passive and active membrane properties. **A**, Whole-cell current traces in response to 10mV hyperpolarization steps. **B**, I-V curves plotting the current responses of all NaCl-sensitive (black squares; 40 neurons/34 rats) and non-sensitive (white circles; 15 neurons/12 rats) OVLT neurons studied. **C**, Whole-cell voltage traces depicting depolarization-induced action potentials (AP) in NaCl-sensitive and non-sensitive OVLT neurons current clamped at approximately -57mV.
Figure S2. OVLT neurons identified by in vivo single unit recording responses to central hypernatremia. A, OVLT neurons were juxtacellularly labeled by entrainment to current pulses applied through the recording electrode containing Neurobiotin and visualized with streptavidin AlexaFluor488. B, The anatomic location of OVLT neurons did not differ between NaCl-S and NaCl-NS cells.
Figure S3. OVLT microinjection sites for hypertonic NaCl. **A,** The anatomic location of injection sites were within OVLT. **B,** OVLT injection site visualized with rhodamine beads.
Figure S4. OVLT microinjection sites for muscimol versus aCSF. **A**, The anatomic location of injection sites were within OVLT. **B**, OVLT injection site visualized with FITC beads.