DREADD-induced Activation of Subfornical Organ Neurons Stimulates Thirst and Salt Appetite

Haley Nation¹*, Marvin Nicoleau²*, Brian J. Kinsman¹, Kirsteen N. Browning¹, and Sean D. Stocker¹

*Co-first author

Department Neural & Behavioral Sciences¹
Penn State College of Medicine, Hershey PA 17033
Department of Biochemistry and Molecular Biology²
Franklin & Marshall College, Lancaster PA 17603

Abbreviated Title: DREADD activation of SFO neurons stimulates thirst

Corresponding/Submission Author

Sean D. Stocker, PhD
500 University Drive H166
Room C4723
Penn State College of Medicine
Hershey, PA 17033
Ph: 717-531-0003 ext 285573
Fax: 717-531-7667
Email: sstocker@hmc.psu.edu

Total Number of Words: 
Number of Figures: 4
Number of Tables: 0
ABSTRACT

The subfornical organ (SFO) plays a pivotal role in body fluid homeostasis through its ability to integrate neurohumoral signals and subsequently alter behavior, neuroendocrine function and autonomic outflow. The purpose of the present study was to evaluate whether selective activation of SFO neurons using virally-mediated expression of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) stimulated thirst and salt appetite. Male C57Bl/6 mice (12-15 weeks) received an injection of rAAV2-CaMKII-HA-hM3D(Gq)-IRES-mCitrine targeted at the SFO. At 2 wks later, acute injection of clozapine N-oxide (CNO) produced dose-dependent increases in water intake of mice with DREADD expression in the SFO. CNO also stimulated the ingestion of 0.3M NaCl. Acute injection of CNO significantly increased the number of Fos-positive nuclei in the SFO of mice with robust DREADD expression. Furthermore, in vivo single-unit recordings demonstrate that CNO significantly increases the discharge frequency of both AngII- and NaCl-responsive neurons. In vitro current-clamp recordings confirm bath application of CNO produces a significant membrane depolarization and increase in action potential frequency. In a final set of experiments, chronic administration of CNO approximately doubled 24-h water intake without an effect on salt appetite. These findings demonstrate DREADD-induced activation of SFO neurons stimulates thirst, and DREADDs are a useful tool to acutely or chronically manipulate neuronal circuits influencing body fluid homeostasis.

Keywords: Hypothalamus, angiotensin II, sodium, Fos, electrophysiology
NEWS & NOTEWORTHY

SFO neurons play a pivotal role in body fluid homeostasis and autonomic function including neurogenic forms of hypertension and cardiovascular disease. In the present study, we provide direct evidence that DREADD technology is a useful tool to manipulate SFO neuronal activity. The experiments reported herein indicate that acute or chronic activation of SFO neurons stimulates excessive fluid intake. Therefore, SFO neurons may be a potential therapeutic target for the treatment of body fluid homeostatic disorders. Future research is needed to identify the exact neuronal populations and signaling mechanisms that may underlie these responses during varying physiological challenges and diseases.
INTRODUCTION

The central nervous system plays a pivotal role in body fluid homeostasis through the ability of specialized neurons in the forebrain lamina terminalis to detect and integrate neurohumoral signals and subsequently alter behavior, neuroendocrine function, and autonomic outflow (Coble et al. 2015; McKinley et al. 2004; Toney and Stocker 2010). The lamina terminalis spans the rostral wall of the 3rd ventricle and includes two circumventricular organs: the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT). Neurons in these regions lack a complete blood brain barrier and therefore can readily detect changes in circulating substances that other brain regions cannot (McKinley et al. 2003). For example, studies using in vivo single-unit recordings (Gutman et al. 1988; Tanaka et al. 1985) or immunocytochemical localization of Fos (Kinsman et al. 2014; Larsen and Mikkelsen 1995; Oldfield et al. 1994; Taylor et al. 2008) indicate SFO neurons are responsive to either NaCl or the peptide hormone angiotensin II (AngII). Activation of SFO neurons increases water intake (Simpson et al. 1978; Simpson and Routtenberg 1978; 1973; Smith et al. 1995), plasma vasopressin levels (Ferguson and Kasting 1986), and arterial blood pressure (Gutman et al. 1985). Furthermore, lesion or interruption of neurotransmission in the SFO attenuates thirst, vasopressin secretion, and/or changes in sympathetic nerve activity and blood pressure in response to multiple stimuli (Hosutt et al. 1981; Mangiapane et al. 1984; Osborn et al. 2012; Simpson et al. 1978; Simpson and Routtenberg 1978; 1973; Sunn et al. 2002; Thrasher et al. 1982; Thunhorst et al. 1999; Tiruneh et al. 2013). In addition to water homeostasis, SFO neurons have also been implicated in sodium balance. Experimental paradigms employed in rodents to produce a salt appetite increase Fos expression in
SFO neurons (Thunhorst et al. 1998), and lesion of the SFO attenuates the ingestion of salt solutions (Thunhorst et al. 1999). Despite the evidence to support a role for SFO neurons in body fluid homeostasis, the neuronal populations or signaling mechanisms within SFO that contribute thirst and salt appetite are not well understood.

Recent technological advances such as optogenetics permit a more detailed evaluation and dissection of various neuronal populations and circuits (Deisseroth 2015; Roth 2016). In regard to SFO neurons and body fluid homeostasis, Oka and colleagues (Oka et al. 2015) reported that optogenetic activation of SFO neurons by expressing Channelrhodopsin under control of the CamKII1α-promoter produced an immediate increase in water, but not salt, intake in water-replete mice. On the other hand, activation of neurons expressing the vesicular GABA transporter inhibited thirst (Oka et al. 2015).

An alternative approach to manipulate SFO neurons are chemogenetic tools such as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) (Urban and Roth 2015). DREADDs may represent an advantage over optogenetic approaches as neuronal activity can be easily manipulated acutely or chronically. Therefore, the purpose of the present study was to employ a DREADD-based approach to activate SFO neurons and examine the impact on thirst and salt appetite. We report that expression of hM3D(Gq) under the CamKII promoter in SFO neurons acutely stimulated water intake and salt appetite after injection of CNO. Chronic activation of SFO neurons over several days nearly doubled 24-h water intakes without an effect on salt appetite. Additionally, we provide the first in vivo single-unit recordings of neuronal excitation using DREADDs.
MATERIAL and 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. Male C57BL/6 mice (12-15 weeks of age, The Jackson Laboratory, Bar Harbor, ME; https://www.jax.org/strain/000664) were anesthetized with 2-2.5% isoflurane (in 100% O₂) and placed into a stereotaxic frame. After a small craniotomy, rAAV2-CaMKII-HA-hM3D(Gq)-IRES-mCitrine (1x10¹² molecules/mL, 50nL over 5 min, UNC Gene Therapy Core) was injected into the SFO using glass micropipettes angled 87 degrees to avoid the midsagittal sinus and coordinates in reference to Bregma: -0.4mm caudal, 0.15mm lateral, -2.8mm ventral. Mice were treated with ampicillin (100mg/kg, sc), buprenex (0.03 mg/kg, sc), and carprofen (5mg/kg, sc). Animals were singly-housed in a temperature controlled room with a 12-h light-dark cycle, given access to distilled water and normal laboratory chow (Harlan Teklad #2018), and acclimated to the testing procedures daily.

Acute Thirst and Salt Appetite Studies. Cumulative water intakes (±0.05mL) were measured over 180 min using 10mL graduated drinking tubes after injection of CNO (Tocris; 0.03, 0.06, 0.1, 0.3, 1.0, and 3.0 mg/kg, sc) or vehicle (10 µL per g body weight). A CNO stock solution (20-40 mg/mL in DMSO) was made daily and subsequently diluted with isotonic saline. The various concentrations were tested in a randomized order separated by >3 days. After thirst studies were completed, mice were
fed a low-sodium diet (0.01%, Research Diets D17010; New Brunswick, NJ) and given access to drinking tubes containing distilled water and 0.3M NaCl for 1 week. Then, cumulative water and 0.3M NaCl intakes (±0.05mL) were measured after injection of CNO (3mg/kg, sc) or saline as described above. In every experiment, the drinking tubes were weighed before and at the end of the experiment to verify the volumetric measurement. We did not observe any differences (±0.1g) between volume versus mass.

**Fos Studies and Immunocytochemistry.** After the completion of behavioral experiments, mice were given access to normal laboratory chow and distilled water for >1 week. Then, animals were injected with CNO (3.0 mg/kg, sc) or vehicle as described above but denied access to food or water. At 90 min later, mice were deeply anesthetized with isoflurane (3% in 100% O₂) and perfused transcardially with isotonic saline and 4% paraformaldehyde. Brains were post-fixed overnight at 4°C, sectioned at 30µm using a vibratome, and processed immunocytochemically for Fos protein (Kinsman et al. 2014; Taylor et al. 2008) and/or HA-tag to assess DREADD expression. Sections were incubated in a rabbit anti-cFos antibody (1:4000, EMD Biosciences Ab5-PC38) at 4°C for 48 hrs and subsequently visualized with an AlexaFluor594 goat-anti-rabbit antibody (1:250, Molecular Probes). Then, sections were incubated with a mouse anti-HA antibody (1:750, Cell Signaling #2367) at 4°C for 48hrs and subsequently visualized using a biotin-XX goat anti-mouse IgG (1:250, Molecular Probes), avidin-biotin amplification (ABC Vectastain Kit, Vector Laboratories), and streptavidin AlexaFluor 488 (1:250, Molecular Probes). Sections were mounted onto slides and
coverslipped with VectaShield. Fos-positive nuclei and HA-immunofluorescence were quantified in one representative section for each structure by two blinded individuals using a Nikon 90i microscope and NIS elements software.

In Vivo and In Vitro Electrophysiology. Mice received an SFO injection of the rAAV2-CaMKII-HA-hM3D(Gq)-IRES-mCitrine at least 3 weeks before experiments. Then, in vivo single-unit recordings of SFO neurons were performed in mice anesthetized with isoflurane using glass electrodes (10-30MΩ) angled 86° from the midsagittal sinus and filled with 4% Neurobiotin dissolved in 0.5M sodium acetate (pH 7.4) (Stocker et al. 2015; Stocker and Toney 2005). A multi-barrel glass pipette was lowered into the lateral ventricle to test SFO neuronal responsiveness to 0.5M NaCl (100nL over 5s) or Angiotensin II (20ng in 100nL over 5s). Then, CNO (0.3 mg/kg, IV) was injected through a femoral venous catheter (100 uL). CNO was tested once per animal. At the end of recordings, cells were juxtacellulary labelled (Pinault 1996), and animals perfused transcardially with 4% paraformaldehyde. Neurobiotin-filled cells and HA-tag expression were visualized by overnight incubations with AlexaFluor594 or mouse anti-HA antibody and Goat Anti-Mouse AlexaFluor 488, respectively.

For in vitro patch-clamp experiments, mice were injected with Fluorogold (0.2mL of 3.75 mg/mL, ip) to facilitate identification of SFO neurons as fluorogold will also label areas lacking a complete blood brain barrier. Approximately 3 days later, mice were deeply anesthetized with isoflurane, decapitated, and the brain harvested into ice-cold Krebs’ solution (in mM: 126 NaCl; 25 NaHCO3; 2.5 KCL; 1.2 MgCl2; 2.4 CaCl2; 1.2
NaH$_2$PO$_4$; and 11 dextrose, maintained at pH 7.4 by bubbling with 95/5% O$_2$/CO$_2$).

Coronal slices (250um) containing the SFO were cut and slices incubated in Krebs' solution at 30±1°C for at least 90min prior to use. A single slice was placed in a custom-made perfusion chamber (500ul volume) mounted onto the stage of a Nikon E600FN microscope equipped with UV epifluorescent filters. Slices were maintained at 32±1°C by continuous perfusion with warmed Krebs' solution. Fluorogold-containing SFO neurons were identified under UV epifluorescence and electrophysiological recordings were made under brightfield illumination using DIC (Nomarski) optics. Whole cell patch clamp recordings were made using pipettes (4-5MΩ tip resistance) filled with a potassium gluconate solution (in mM: 28 K-gluconate; 10KCl, 0.3 CaCl$_2$; 1 MgCl$_2$; 10 HEPES, 1 EGTA, 2 Na$_2$ATP; 0.25 NaGTP, adjusted to pH 7.36 with KOH) and a single electrode voltage clamp amplifier (Axoclamp 200B, Molecular Devices, Union City, CA).

Data were filtered at 2kHz, digitized via a Digidata 1400 interface, stored on a computer, and analyzed using pClamp10 software (Molecular Devices). Recordings with series resistance >20MΩ were eliminated from the study. Neurons were current clamped at approximately -60mV. Electrotonic potentials sufficient to hyperpolarize the membrane approximately 10mV, were applied to the neuron every 5s. CNO (10uM) was applied via superfusion, for a period of time sufficient for the response to reach plateau, or for 2min if no response was noted. The CNO-induced response was measured as the peak change in membrane potential relative to baseline. At the conclusion of the recording, Neurobiotin (2.5%) included in the recording pipette was injected into the neuron by passing subthreshold depolarizing current pulses (400ms duration, 0.8Hz for 20min). After removal of the pipette, the membrane was allowed to re-seal for 5-10min before
the slice was fixed in Zamboni’s fixative at 4°C for at least 24hrs. Neurobiotin-filled cells and HA-tag were visualized as described above.

Chronic Thirst and Salt Appetite Studies. A final set of experiments was performed to determine whether chronic activation of SFO neurons using DREADDs altered thirst and salt appetite. Mice were injected with rAAV2-CaMKII-HA-hM3D(Gq)-IRES-mCitrine and given access to water, 0.3M NaCl and Na+ deficient chow as described above for at least 2 weeks. 24-h intakes were recorded for several days. Then, CNO was administered through both drinking tubes for 3 successive days. The initial CNO concentration was 0.025 mg/mL and adjusted daily based on the 24-h intake of the previous day to yield ~3mg/kg.

Statistical Analysis. All data were analyzed by an ANOVA with repeated measures (when appropriate) followed by a layered Bonferroni with correction (Systat Software V10.2). Cell discharge was averaged in 1 min bins; baseline discharge was calculated by a 3-min average. In vitro experiments calculate baseline and 30s peak responses in $V_m$ or discharge. A P<0.05 was statistically significant.
RESULTS

CNO Increases Water and 0.3M NaCl Intake. Post-hoc analysis of HA-immunofluorescence identified two primary groups of mice based on the absence or presence of DREADD expression in the SFO (Figure 1A). These mice are referred to as SFO-x or SFO mice, respectively. SFO mice displayed strong HA-immunofluorescence localized to the SFO without expression dorsal or rostral in the hippocampal commissure, caudal in the hippocampus, or ventral in the thalamus. In these animals, the immunofluorescence was present throughout the rostral-caudal, dorsal-ventral, and medial-lateral extent of SFO (Figure 1, iii-iv). In contrast, the majority of SFO-x mice had no detectable expression in any brain structure, or a few SFO-x mice had a small amount of HA-immunofluorescence present in the surrounding structures such as the hippocampus or ventral in the thalamus. Finally, there were 2 mice that displayed HA-immunofluorescence in SFO but the expression was limited to 10-15 SFO neurons. These animals did not respond to CNO (data not shown) and were not included in the analysis due to the clear delineation in expression between SFO versus SFO-x mice.

For acute experiments, body weight did not differ between SFO and SFO-x mice (26.8±1.4 vs 27.4±0.3g; n=13 and 12 per group, respectively). Injection of 3.0mg/kg CNO significantly increased water intake of SFO mice within 15 min (Figure 1B). In fact, the dipsogenic response in SFO mice was observed over a range of CNO doses (0.1-3.0mg/kg, sc; Figure 1C). In marked contrast, CNO did not stimulate water intake in SFO-x mice (Figure 1C). To assess whether DREADD-induced activation of SFO neurons stimulated salt-appetite, SFO mice were given access to both water and 0.3M NaCl. Injection of CNO significantly increased the ingestion of water and 0.3M NaCl.
(Figure 1D); however, CNO stimulated a significantly greater increase in water versus 0.3M NaCl intake.

CNO Increases Fos Immunoreactivity in SFO Neurons. To determine whether CNO activates SFO neurons, Fos immunoreactivity was assessed in SFO and SFO-x mice. Injection of CNO versus vehicle in SFO mice significantly increased the number of Fos-positive nuclei in the SFO (Figure 2A). Fos positive nuclei were observed throughout the rostral-caudal, dorsoventral, and mediolateral extent of the SFO. In SFO mice, the Fos-positive nuclei were associated with strong HA-immunofluorescence. Interestingly, injection of CNO also increased the number of Fos-positive nuclei in several efferent targets including the median preoptic nucleus, organum vasculosum of the lamina terminalis, supraoptic nucleus, and hypothalamic paraventricular nucleus (Figure 2B). It is noteworthy that injection of CNO in SFO-x mice did not statistically increase Fos expression in any of the above structures.

CNO Excites SFO Neurons. In vivo single-unit recordings demonstrate that systemic injection of CNO increases the discharge of SFO neurons (n=6) responsive to either ICV AngII (n=3) or 0.5M NaCl (n=3) (Figure 3A). CNO significantly increased the discharge of all 6 SFO neurons from $6.2\pm2.2 \text{ Hz}$ to $22.0\pm7.2 \text{ Hz}$ ($P<0.05$). The response occurred within 1-2 min and discharge remained elevated for >45 min. It is noteworthy that CNO did not increase the firing rate of 3 HA-negative neurons located ~400µm dorsal to the SFO in SFO mice (data not shown). Moreover, CNO did not alter neuronal
discharge in SFO-x mice (baseline: 9.4±1.8 Hz vs peak: 10.7±2.4 Hz; n=5). A final set of
in vitro current clamp recordings reveal that bath application of 10µM CNO in SFO vs
SFO-x mice produced a significant membrane depolarization (SFO: 6.2±0.8 mV vs
SFO-x: 0.2±0.6mV, n=4 per group; P<0.01) and increase in action potential frequency
(Figure 3B).

Chronic Activation of SFO Neurons Stimulates Thirst. Chronic administration of
CNO in the water and 0.3M NaCl drinking tubes significantly increased 24-h water
intake but did not affect the ingestion of 0.3M NaCl in SFO mice (Figure 4). In fact, CNO
doubled 24-h water intakes on Day 2 (112±34%) and Day 3 (117±26%). The excessive
water intake persisted for 2 days after CNO administration was stopped (Day 4:
107±28%, Day 5: 51±12% versus baseline intakes). Interestingly, the body weight of
SFO mice increased during chronic CNO administration (Day 0: 26.6±1.0g vs Day 4:
27.0±1.0g, P<0.05; Δbody weight: 0.4±0.1g). In contrast, chronic CNO administration to
SFO-x mice did not alter water and 0.3M NaCl intake (Figure 4) or body weight (Day 0:
26.2±0.9g vs Day 4: 26.1±1.0g, P>0.6; Δbody weight: -0.1±0.1g). The average daily
dose of CNO was 3.8±0.4 and 3.1±0.2 mg/kg in SFO versus SFO-x mice, respectively.
DISCUSSION

The present study employed a chemogenetic approach using DREADDs to acutely and chronically activate SFO neurons and assess its impact on thirst and salt appetite. There are several novel findings including: 1) acute CNO injection dose-dependently stimulated water intake in SFO mice, 2) acute CNO produce a small but significant increase in salt appetite, 3) acute CNO injection significantly increased Fos expression in SFO neurons (and downstream efferent targets), 4) \textit{in vivo} single-unit recordings demonstrate CNO produced an immediate and sustained increase in cell discharge that persisted for >45 min, 5) \textit{in vitro} patch-clamp recordings demonstrate that CNO depolarized neurons and increased action potential frequency, and 6) chronic administration of CNO doubled 24-h water intake without an effect on salt appetite. Collectively, these findings indicate that DREADDs can be used to acutely and chronically manipulate SFO neuronal activity and alter body fluid homeostasis.

A myriad of studies have reported that numerous dipsogenic stimuli (ie, angiotensin II, hypertonic NaCl) increase cell discharge or Fos expression in SFO neurons (Anderson et al. 2000; Gutman et al. 1988; Kinsman et al. 2014; Larsen and Mikkelsen 1995; Oldfield et al. 1994; Sunn et al. 2002; Tanaka et al. 1985; Taylor et al. 2008). In many cases, SFO lesions or interruption of SFO neurotransmission attenuate thirst responses to these stimuli (Hosutt et al. 1981; Mangiapane et al. 1984; Osborn et al. 2012; Simpson et al. 1978; Simpson and Routtenberg 1978; 1973; Sunn et al. 2002; Thrasher et al. 1982; Thunhorst et al. 1999; Tiruneh et al. 2013). In the current study, DREADD-induced excitation of SFO neurons produced a dose-dependent increase in water intake of water-replete mice. The effect was immediate and largely observed in
the first 15-30 min. One remarkable finding in the current study was the minimum dose of CNO to produce a dipsogenic response was \(~0.1\text{mg/kg}\) (Figure 1B). This dose is lower than those doses commonly used in experiments employing DREADDs to manipulate neuronal activity and behavior.

Two other studies have used optogenetic or chemogenetic approaches to manipulate SFO neurons and thirst (Betley et al. 2015; Oka et al. 2015). These studies reported that optogenetic activation of SFO neurons through CamKII promoter or mice expressing Cre under the vesicular glutamate transporter-2 significantly increased the number of licks of water. Furthermore, Oka et al (Oka et al. 2015) reported that mice drank 8% of body weight in 15 min \((\sim 1.7\text{mL})\) during optogenetic activation of SFO neurons at 20Hz. There was no effect on salt appetite. This level of water intake is much higher than those levels reported here or those of mice deprived of water for 48 hrs (Oka et al. 2015) or having substantial plasma hypernatremia (Kinsman et al. 2014; Taylor et al. 2008). On the other hand, our findings suggest that injection of CNO produced an increase in water intake comparable to that evoked by moderate plasma hypernatremia \((\sim 5-10\text{mM})\) (Kinsman et al. 2014). These discrepancies in thirst responses between these studies may be attributed to the manipulated cell population (CaMKII vs glutamatergic neurons) or differences in cellular mechanisms of activation between optogenetics versus DREADDs. For example, optogenetic activation of neurons using channelrhodopsin is mediated by a non-selective cation conductance (Deisseroth 2015), whereas DREADD-induced activation through hM3D(Gq) increases intracellular calcium (Roth 2016). However, there is limited data that directly compares the effect of both approaches on a behavioral outcome while directly measuring
changes in neuronal activity. Therefore, it remains unclear whether these different mechanisms of neuronal depolarization and excitation impact behavior differently.

DREADD-induced excitation by CNO in SFO mice was demonstrated using multiple approaches including: 1) increase in the number of Fos immunoreactive nuclei, 2) membrane depolarization and increased action potential frequency \textit{in vitro}, and 3) increase neuronal discharge via \textit{in vivo} single-unit recordings. Numerous studies have employed the first two approaches to confirm the actions of CNO on various neuronal systems. However, there are limited data using direct cell recordings \textit{in vivo} to demonstrate the effect and time course of CNO on neuronal activity. The current findings provide such evidence, for the first time, as IV injection of 0.3mg/kg CNO produced an immediate (<2 min) increase in cell discharge that persisted for >45 min. Interestingly, mice did not ingest significant amounts of water after the initial 15 min and may be attributed to the generation of inhibitory signals on thirst such as gastric distension, activation of gut osmoreceptors, or osmotic dilution (Stricker and Sved 2000). The \textit{in vivo} single-unit recordings also indicated that CNO affected both AngII and NaCl-responsive neurons in the SFO. Although there are limited data in mice, prior Fos and in vivo electrophysiological studies in rats suggest AngII and NaCl largely affect different populations of SFO neurons. AngII activates SFO neurons located in the central core whereas hypertonic NaCl activates neurons located in the lateral margins (McKinley et al. 1998; Rosas-Arellano et al. 1996). Unfortunately, there is no evidence for a topographical distribution of functionally distinct neurons in the SFO of mice. Regardless, the present findings demonstrate CNO produced a prolonged excitation of SFO neurons \textit{in vivo}. 
Several studies have reported SFO neurons play an important role in salt appetite (Hosutt et al. 1981; Thunhorst et al. 1999). This idea is supported by studies that have observed a reduction in the ingestion of a salt solution after SFO lesion. Although excitation of SFO neurons by acute injection of CNO did produce a statistically significant increase in both water and 0.3M NaCl intake, the effect on 0.3M NaCl intake (salt appetite) was much less. Indeed, the volume of 0.3M NaCl was very small and unlikely to have a physiological impact. Surprisingly, chronic CNO administration over 3 days did not alter 0.3M NaCl intake. Optogenetic activation of SFO also failed to stimulate salt appetite (Oka et al. 2015). There are two potential explanations for the small or absent effect on salt appetite in these experiments. First, salt appetite is usually observed in volume-depleted animals but the current experiments were performed in volume-replete mice. Second, as noted above, hM3D(Gq) expression affected both AngII and NaCl-responsive SFO neurons. Thus, injection of CNO may activate several populations of SFO neurons that both stimulate thirst but generate opposing signals for salt appetite. That is, AngII stimulates salt appetite but plasma hypernatremia likely opposes the ingestion of 0.3M NaCl. Subsequent studies that selectively target neurochemically or topographically distinct populations of SFO neurons may yield different results.

Chemogenetic and optogenetic tools represent unique approaches to manipulate neuronal activity and elucidate neural circuits and function. However, each approach has distinct advantages and disadvantages. For example, optogenetics permits second to second control of cellular activity, whereas DREADDs activate or inhibit neurons for much longer periods of time without any need for instrumentation. Using this
chemogenetic approach, chronic CNO administration through the drinking tubes to activate SFO neurons approximately doubled water intake and produced a small increase in body weight. Interestingly, the polydipsia observed in SFO mice persisted for 1 day after the CNO administration ceased and may be attributed to the pharmacokinetics of CNO. Although we did not directly assess the underlying mechanisms for the increase in body weight, a plausible explanation is the polydipsia and resultant volume expansion. SFO neurons directly innervate magnocellular, vasopressin neurons of the hypothalamic paraventricular and supraoptic nuclei (McKinley et al. 2003). Therefore, activation of SFO neurons should increase plasma vasopressin levels and stimulate renal water reabsorption. These neuroendocrine effects on kidney function combined with the polydipsia may underlie the increase in body weight. Altogether, these findings highlight the pivotal role of SFO neurons in the acute and chronic regulation of thirst. In addition, the present findings highlight the potential utility of DREADD-based approaches to acutely or chronically manipulate body fluid homeostasis.

A distinct advantage of chemogenetic and optogenetic approaches is the ability to selectively manipulate neurochemically or anatomically distinct populations of cells through cre-lox based approaches. For example, SFO neurons express a number of receptors (angiotensin II type 1A, atrial natuiretic peptide, relaxin, estrogen receptor alpha) and densely innervate several hypothalamic nuclei (McKinley et al. 2003). Although the current study did not investigate the relative contributions of these specific neuronal populations in body fluid homeostasis, future studies can directly address these questions using transgenic animals, anatomical tracers (WGA-Cre), or
constructs with unique promoters. Thus, these approaches will not only permit a selective control of neuronal activity but also provide unique insight into the contribution of specific neuronal populations to these responses on an unprecedented level.
Acknowledgements

The authors thank Dr. Bryan Roth and UNC Viral Core for the rAAV2-CaMKII-HA-hM3D(Gq)-IRES-mCitrine.

Grants

The research was supported by National Heart, Lung, and Blood Institute Grant HL-113270 (S.D.S.) American Heart Association Established Investigator Grant (S.D.S.) and Great Rivers Predoctoral Fellowship 14PRE19530001 (B.J.K).

Conflicts of Interest

None
REFERENCES


Kinsman B, Cowles J, Lay J, Simmonds SS, Browning KN, and Stocker SD. Osmoregulatory thirst in mice lacking the transient receptor potential vanilloid type 1 (TRPV1) and/or type 4 (TRPV4) receptor. Am J Physiol Regul Integr Comp Physiol 307: R1092-1100, 2014.


Oldfield BJ, Badoer E, Hards DK, and McKinley MJ. Fos production in retrogradely labelled neurons of the lamina terminalis following intravenous infusion of either hypertonic saline or angiotensin II. *Neuroscience* 60: 255-262, 1994.


Rosas-Arellano MP, Solano-Flores LP, and Ciriello J. Arcuate nucleus inputs onto subfornical organ neurons that respond to plasma hypernatremia and angiotensin II. *Brain Res* 707: 308-313, 1996.


FIGURE LEGENDS

Figure 1. **(A)** Low and high power digital images of HA immunofluorescence in SFOx (**i, ii**) and SFO (**iii, iv**) mice. Scale bars are 100µm. **(B)** Cumulative water intake of SFO mice (**n=13**) after injection of CNO (3.0mg/kg, sc) or vehicle (0.25mL, sc). **(C)** 60-min cumulative water intake of SFO (**n=13**) or SFO-x (**n=12**) mice plotted as a function of CNO dose. **(D)** 120 min water and 0.3M NaCl intakes of SFO mice after injection of CNO (3.0mg/kg, sc) or vehicle (0.25mL, sc). All values are mean±SEM. *P<0.01 vs vehicle, #P<0.05 vs 0 mg/kg CNO or SFO-x, †P<0.05 water vs 0.3M NaCl.

Figure 2. **(A, TOP)** Fos immunoreactivity in the SFO after injection of CNO in SFO-x (**i**) or SFO (**ii**) mice. The Fos immunoreactive nuclei overlapped with HA-positive cells in SFO mice (**iii-iv**). **(A, BOTTOM)** Mean±SEM of Fos-positive nuclei in the SFO of mice injected with CNO or vehicle. **(B, TOP)** Digital images of Fos immunoreactivity in median preoptic nucleus (MnPO), organum vasculosum of the lamina terminalis (OVLT), supraoptic nucleus (SON) and hypothalamic paraventricular nucleus (PVH) of SFO and SFO-x mice after injection of CNO. **(B, BOTTOM)** Mean±SEM of Fos-positive nuclei in the SFO of mice injected with CNO or vehicle. *P<0.01 versus vehicle, n=4 for all groups. Scale bars are 100µm for all images except **A iv** is 25µm.

Figure 3. **(A)** Two examples of single-unit recordings in SFO mice. SFO neurons were responsive to either ICV AngII (LEFT) or 0.5M NaCl (RIGHT). Injection of CNO (0.3mg/kg, IV) significantly increased SFO neuronal discharge. **(B)** Whole-cell patch-clamp recording of SFO neuron demonstrates bath application of CNO depolarizes $V_m$.
and increases cell discharge. Note: hyperpolarizing potentials are not readily visible in this example due to higher baseline noise. (C) Confocal image of Neurobiotin-filled SFO neuron and HA-tag immunofluorescence.

**Figure 4.** Chronic administration of CNO through the drinking tubes significantly increased 24-h water intake but not 0.3M NaCl intake of SFO mice. CNO did not affect water or 0.3M NaCl intakes of SFO-x mice. Values are mean±SEM. Δ water or 0.3M intakes were calculated by the difference between the 24h intake and the average baseline intake (Day -3 to 0). *P<0.05 vs baseline or SFO-x mice.
Figure 3

**A, In vivo extracellular**

Discharge Rate (Hz)

Raw Cell

**B, In vitro whole-cell**

10µM CNO

**C**

Neurobiotin  HA-Tag  Merged
Figure 4

- **Time (Days)**: -2 0 2 4 6 8
- **Water Intake (mL)**: 1 3 5
- **0.3M NaCl (mL)**: 0 2 4

- **SFO (n=5)**
- **SFO-x (n=6)**

- CNO

- Statistical significance indicated by asterisks: 
  - **** indicates highly significant differences.