Oxytocin-receptor-expressing neurons in the parabrachial nucleus regulate fluid intake

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Brain regions that regulate fluid satiation are not well characterized, yet are essential for understanding fluid homeostasis. We found that oxytocin-receptor-expressing neurons in the parabrachial nucleus of mice (OxtrPBN neurons) are key regulators of fluid satiation. Chemogenetic activation of OxtrPBN neurons robustly suppressed noncaloric fluid intake, but did not decrease food intake after fasting or salt intake following salt depletion; inactivation increased saline intake after dehydration and hypertonic saline injection. Under physiological conditions, OxtrPBN neurons were activated by fluid satiation and hypertonic saline injection. OxtrPBN neurons were directly innervated by oxytocin neurons in the paraventricular hypothalamus (OxtrPVH neurons), which mildly attenuated fluid intake. Activation of neurons in the nucleus of the solitary tract substantially suppressed fluid intake and activated OxtrPVH neurons. Our results suggest that OxtrPVH neurons act as a key node in the fluid satiation neurocircuitry, which acts to decrease water and/or saline intake to prevent or attenuate hypervolemia and hyponatremia.
Fig. 1 | Oxtr<sup>Cre</sup> activation suppresses fluid but not food intake. a, b, TdTomato expression in PBN of Oxtr<sup>Cre</sup>/::Ai14 reporter mice (n = 3; a) and adult expression of mCherry fluorescence in Oxtr<sup>Cre</sup> neurons (b) following injection of AAV-DIO-mCherry in a 9-week-old Oxtr<sup>Cre</sup> male mouse (n = 7). dl, dorsolateral; el, external lateral; scp, superior cerebellar peduncle. Scale bar represents 100 μm. c, Representative example of electrophysiological activity in an Oxtr<sup>Cre</sup> neuron using cell-attached configuration. We observed increased spiking after application of the Oxtr agonist TGOT (0.2 μM), which was inhibited by coadministration of the Oxtr antagonist atosiban (1 μM; the same neuron) (n = 4 of 4 Oxtr<sup>Cre</sup> neurons). d, Injection of AAV-DIO-hM3Dq:mCherry in Oxtr<sup>Cre</sup> neurons. Gray and black triangles denote foxP and fox2722 sites, respectively. e-g, Acute Oxtr<sup>Cre</sup> activation with CNO revealed no significant change in food intake at baseline or after 24 h of fasting (n = 7 per group; two-way repeated measures (RM) ANOVA; food: interaction F(8,96) = 0.3424; water: interaction F(8,96) = 0.2901, P = 0.9282; water: interaction F(8,96) = 0.210; water: interaction F(8,96) = 0.3809, P = 0.9678; 24-h fast: interaction F(8,96) = 1.143, P = 0.3381) (e); decreased water and NaCl consumption following 24-h dehydration in the presence of food (n = 7 per group; two-way RM ANOVA; NaCl: interaction F(8,96) = 12.63, P < 0.0001; water: interaction F(8,96) = 39.75, P < 0.0001) (f) and in the absence of food (n = 6 per group; two-way RM ANOVA; NaCl: interaction F(8,80) = 8.173, P < 0.0001; water: interaction F(8,80) = 22.31, P < 0.0001) (g); decreased water consumption at baseline (n = 7 per group; three-way mixed design ANOVA; NaCl: interaction (2,875,34,501) = 1.593, P = 0.210; water: interaction (2,099,25,183) = 4.464, P = 0.021) (h); and no significant change in Ensure or water intake following 24 h of caloric deprivation (n = 6 hM3Dq, 7 mCherry; two-way RM ANOVA; Ensure: interaction F(8,88) = 0.3809, P = 0.9282; water: interaction F(8,88) = 0.5037, P = 0.8505). Data are expressed as mean ± s.e.m. mCh, mCherry; veh, vehicle. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05. See Supplementary Table 2 for statistical analyses.

them food. The mice expressing hM3Dq decreased both water and NaCl intake, similarly to when food was present; however, control (mCherry-expressing) mice drank less water but drank more NaCl when food was absent (Fig. 1g and Supplementary Fig. 3b). We also investigated the effects of Oxtr<sup>PBN</sup> stimulation at the start of the dark cycle without prior fluid deprivation. We observed a significant...
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that OxtrPBN neurons may be involved in mediating dehydration-induced anorexia.

Apparently, the highly palatable nature of Ensure overrode the ability of Oxtr PBN stimulation to suppress fluid intake. Because mice drank only minimal water in this experiment, we repeated it, but returned fixed amounts of Ensure (1.6 or 0.7 ml); however, both hM3Dq- and mCherry-expressing mice still drank limited amounts of water (Supplementary Fig. 3d,e), suggesting that the presence of even small amounts of Ensure decreases concurrent water intake. We assessed the effects of activating OxtrPBN neurons on food intake during mild dehydration and observed decreased food intake (control diet) in hM3Dq-injected mice (Fig. 2a), suggesting that OxtrPBN neurons may be involved in mediating dehydration-induced anorexia.

We also examined the effect of OxtrPBN activation after salt depletion by injecting mice on a sodium-depleted diet with the diuretic furosemide (40 mg kg⁻¹ ip, twice, 1 d apart) and removing access to saline (0.3 M NaCl) for 48 h. OxtrPBN stimulation suppressed water, but not NaCl, intake (Fig. 2b), suggesting that it does not suppress salt appetite. We also assessed the effect of OxtrPBN stimulation after generating thirst by two different mechanisms: increasing osmolality and inducing volume loss. To increase osmolality, we injected hypertonic 0.5 M NaCl or equiosmolar 1 M mannitol ip; whereas we induced volume loss by injecting 30% polyethylene glycol (PEG) subcutaneously, which progressively draws out ECF without affecting osmolarity. We observed that OxtrPBN stimulation suppressed water intake after hypertonic saline injection in hM3Dq- versus mCherry-expressing mice (Fig. 2c); however, there was no significant difference in overnight water intake (P = 0.208), suggesting a rebound effect in fluid intake (Supplementary Fig. 3f). OxtrPBN stimulation also decreased fluid intake after mannitol and PEG injections (Fig. 2d,e). Throughout the experiments, we observed that mice retained similar daily baseline NaCl and water intake (Supplementary Fig. 3g). Following experimentation, we confirmed the targeting and activation of OxtrPBN neurons by injecting CNO 2 h before perfusion, and we observed Fos expression in 72 ± 4% of hM3Dq-expressing neurons by injecting CNO 2 h before perfusion, and we observed Fos expression in 72 ± 4% of hM3Dq-expressing neurons.

Fig. 2 | OxtrPBN activation decreases food intake in dehydrated mice and suppresses water intake following 48-h salt depletion and thirst-inducing conditions. a, Acute OxtrPBN stimulation decreases food intake when mice are dehydrated (n = 6 hM3Dq, 7 mCherry; two-way RM ANOVA; interaction F(4,44) = 7.143, P = 0.0002). b–e, Acute OxtrPBN stimulation also decreases water; but not NaCl, consumption following 48-h salt depletion (n = 7/group; two-way RM ANOVA; NaCl: interaction F(8,96) = 1.408, P = 0.2028; water: interaction F(8,96) = 35.57, P < 0.0001). b, 0.5 M saline ip injection (n = 7/group; two-way RM ANOVA; NaCl: interaction F(4,48) = 0.08955, P = 0.9853; water: interaction F(4,48) = 11.37, P < 0.0001) (e), 1 M mannitol ip injection (n = 7/group; two-way RM ANOVA; NaCl: interaction F(4,48) = 3.638, P = 0.0114; water: interaction F(4,48) = 24.56, P < 0.0001) (d) and 30% PEG sc injection (n = 6 hM3Dq, 7 mCherry; two-way RM ANOVA; NaCl: interaction F(4,44) = 0.4042, P = 0.8046; water: interaction F(4,44) = 11.54, P < 0.0001) (e). f, Following CNO administration, Fos was robustly expressed in OxtrPBN and adjacent neurons in hM3Dq-injected mice. Scale bar represents 100 μm. Data are expressed as mean ± s.e.m. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05. See Supplementary Table 2 for statistical analyses.
neurons (and some adjacent neurons) in the PBN (Fig. 2f and Supplementary Fig. 3h).

Oxtr<sup>PBN</sup> neurons are necessary for protection against hypernatremia. To ascertain whether Oxtr<sup>PBN</sup> neurons are necessary for fluid regulation, we chronically inactivated them by bilaterally injecting AAV carrying a Cre-dependent tetanus toxin light chain (AAV1-DIO-GFP:TetTox)<sup>14</sup> or control (AAV1-DIO-YFP) into the PBN of Oxtr<sup>Cre</sup> mice (Fig. 3a and Supplementary Fig. 4a,b). We found no significant difference in baseline NaCl preference at 0.075 M (P = 0.6292), 0.3 M (P = 0.3364) or 0.5 M (P = 0.9224) (Supplementary Fig. 4c), and no substantial difference in baseline fluid intake after vehicle injection, either acutely (2 h) or overnight (Supplementary Fig. 4d,e). Following 24-h fluid deprivation, however, we observed increased NaCl, but not water, intake in TetTox-injected mice (Fig. 3b), which remained increased overnight, suggesting a longer term effect on fluid intake than that for hM<sub>4</sub>D<sub>q</sub>-injected mice (Supplementary Fig. 4f). We also observed increased 0.3 M NaCl intake after hypertonic (0.5 M) saline ip injection, but not after salt depletion or other tests of fluid intake (Fig. 3c,d and Supplementary Fig. 4g,h). Following experimentation, we observed no significant correlation between TetTox-GFP expression and fluid intake (for NaCl, P = 0.5408; for water, P = 0.3905; Supplementary Fig. 4i). Overall, these results suggest that Oxtr<sup>PBN</sup> neuron activity prevents excessive NaCl ingestion following dehydration and hypertonic saline injection.

We also investigated acute inhibition of Oxtr<sup>PBN</sup> neurons by injecting AAV carrying a Cre-dependent hM<sub>4</sub>Di:mCherry transgene (AAV1-DIO-hM<sub>4</sub>Di:mCherry)<sup>30</sup> bilaterally into the PBN (Fig. 3e and Supplementary Fig. 5a,b), and observed increased NaCl intake after CNO injection in hM<sub>4</sub>Di-injected mice relative to controls at the start of the dark cycle (Fig. 3f). There was increased fluid intake (NaCl and water) after 24-h fluid deprivation, but the effect was less robust than that observed for TetTox-injected mice (Supplementary Fig. 5c). There was no difference in fluid intake during the light cycle, suggesting that acute Oxtr<sup>PBN</sup> inhibition does not spontaneously induce drinking, and there was no difference in feeding (Supplementary Fig. 5d,e). Following experimentation, we examined the extent of hM<sub>4</sub>Di expression, but observed no significant correlation of expression with fluid intake (for NaCl, P = 0.2208; for water, P = 0.2013; Supplementary Fig. 5f).

Oxtr<sup>PBN</sup> neurons are activated under physiological conditions following fluid satiation and hypertonic saline. To assess whether
Oxtr<sup>Cre</sup> mice are activated physiologically by fluid and salt satiation and by hypertonic saline. 

Fig. 4 Oxtr<sup>Cre</sup> neurons are activated physiologically by fluid and salt satiation and by hypertonic saline. **a**, Representative histological sections of Fos expression in Oxtr<sup>Cre</sup>−Ai14 mice in caudal PBN from control, salt-returned, salt-depleted, fluid-returned, fluid-deprived, 1M saline-injected and normal-saline-injected mice. Scale bar represents 100 µm. 

**b,g** Quantification of caudal Oxtr<sup>PBN</sup> coexpression of Fos and Oxtr in salt-depletion experiments (n = 4 salt returned, 4 salt depleted, 3 control; one-way ANOVA; Fos/Oxtr: interaction F(2,7) = 11.41, P = 0.0063; Oxtr/Fos: interaction F(2,7) = 26.02, P = 0.0006) (b,c); fluid-deprivation experiments (n = 4 fluid returned, 3 fluid deprived; unpaired two-tailed Student’s t test; Fos/Oxtr: t(5) = 4.825, P = 0.0078; Oxtr/Fos: t(5) = 3.430, P = 0.0186) (d,e); and hypertonic saline injected experiments (n = 4 for 1M saline, 3 normal saline; unpaired two-tailed Student’s t test; Fos/Oxtr: t(5) = 4.033, P = 0.0100; Oxtr/Fos: t(5) = 1.353, P = 0.2341) (f,g). 

Injection of AAV-DIO-GCaMP6 in Oxtr<sup>PBN</sup> neurons. Gray and black triangles denote lhx1 and lhx222 sites, respectively. 

**h,i** Raster plot of normalized fluorescent Ca<sup>2+</sup> activity during water bouts for each Oxtr<sup>PBN</sup> neuron (n = 94 neurons in 3 mice). 

**j** Raster plot of normalized fluorescent Ca<sup>2+</sup> activity during Ensure bouts for each Oxtr<sup>PBN</sup> neuron (n = 85 neurons in 3 mice). 

**l** Average Oxtr<sup>PBN</sup> fluorescent Ca<sup>2+</sup> activity for water, Ensure and empty bottle. 

**m** Average Oxtr<sup>PBN</sup> fluorescent Ca<sup>2+</sup> activity comparing before bout versus after bout (two-way RM ANOVA: interaction F(2, 257) = 17.01, P < 0.0001). 

**n** Oxtr<sup>PBN</sup> fluorescent Ca<sup>2+</sup> activity following injection of CNO ip; ΔF/F = F - F<sub>0</sub> (n = 83 neurons in 2 mice; one-way RM ANOVA: interaction F(8,656) = 134.7, P < 0.0001; different letters denote significant difference from other columns). Data are expressed as mean ± s.e.m. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05. See Supplementary Table 2 for statistical analyses.
OxtrPBN neurons are involved in fluid and salt intake under physiological conditions, we examined Fos expression in OxtrPBN neurons of OxtrCre/AAV1-DIO-GCaMP6m mice following 48-h salt deprivation or 24-h fluid deprivation. We observed significantly increased Fos expression in caudal OxtrPBN neurons when NaCl and/or water were returned compared with control mice or mice that remained salt or water deprived, suggesting that OxtrPBN neurons are activated following fluid satiation (Fig. 4a–c). There were no substantial differences in Fos expression in mid- and rostral-PBN populations, revealing differential activation throughout the PBN (Supplementary Fig. 6a–d). We also examined Fos expression under hypernatremic conditions by injecting 1 M NaCl ip into OxtrCre/AAV1-DIO-GCaMP6m mice. This treatment increased Fos expression in caudal OxtrPBN neurons compared with 0.15 M NaCl (normal saline) injection (Fig. 4a,f,g and Supplementary Fig. 6e,f).

We assessed real-time OxtrPBN neuron activity by injecting AAV1-DIO-GCaMP6m into the PBN of OxtrCre/AAV1-DIO-GCaMP6m mice and measured calcium fluorescence after returning water to water-deprived mice (Fig. 4h and Supplementary Videos 1 and 2). We observed low fluorescence in OxtrPBN neurons during dehydration, which remained low when the water spout was returned; however, we observed a rapid rise in Ca<sup>2+</sup> fluorescence when mice began drinking, which decreased between bouts (Fig. 4i,l,m). This pattern was observed in ~52% of OxtrPBN neurons during water intake, but not during Ensure intake or when given an empty bottle, suggesting that OxtrPBN neurons respond to noncaloric fluid ingestion, but not liquid diet or motor movements associated with licking (Fig. 4i–m).

We co-injected AAV-DIO-hM<sub>3</sub>Dq:mCherry into two mice and observed increased calcium fluorescence within 5 min that peaked at ~15 min and lasted at least 2 h, suggesting that OxtrPBN neurons have minimal overlap with CGRP<sup>PBN</sup> neurons (6 ± 2%; Fig. 5a).

We investigated the effect of CGRP<sup>PBN</sup> activation on fluid intake by injecting AAV1-DIO-hM<sub>3</sub>Dq:mCherry into the PBN of CalcaCre/AAV1-DIO-GCaMP6m mice (Fig. 5b). CGRP<sup>PBN</sup> neuron stimulation is known to decrease feeding and mediate many aversive responses<sup>15,24</sup>. We observed minimal coexpression of Oxtr and CGRP in PBN neurons (6 ± 2%; Fig. 5a). We found that injection of CGRP<sup>PBN</sup> neurons decreased both food and fluid intake. We compared the expression pattern of neurons expressing Oxtr or calcitonin-gene-related peptide (CGRP, which is encoded by Calca gene) in the PBN. CGRP<sup>PBN</sup> neuron stimulation is known to decrease feeding and mediate many aversive responses<sup>15,24</sup>. We observed minimal coexpression of Oxtr and CGRP in PBN neurons (6 ± 2%; Fig. 5a). We investigated the effect of CGRP<sup>PBN</sup> activation on fluid intake by injecting AAV1-DIO-hM<sub>3</sub>Dq:mCherry into the PBN of CalcaCre/AAV1-DIO-GCaMP6m mice (Fig. 5b). CGRP<sup>PBN</sup> neuron stimulation is known to decrease feeding and mediate many aversive responses<sup>15,24</sup>. We observed minimal coexpression of Oxtr and CGRP in PBN neurons (6 ± 2%; Fig. 5a). We investigated the effect of CGRP<sup>PBN</sup> activation on fluid intake by injecting AAV1-DIO-hM<sub>3</sub>Dq:mCherry into the PBN of CalcaCre/AAV1-DIO-GCaMP6m mice (Fig. 5b). CGRP<sup>PBN</sup> neuron stimulation is known to decrease feeding and mediate many aversive responses<sup>15,24</sup>. We observed minimal coexpression of Oxtr and CGRP in PBN neurons (6 ± 2%; Fig. 5a). We investigated the effect of CGRP<sup>PBN</sup> activation on fluid intake by injecting AAV1-DIO-hM<sub>3</sub>Dq:mCherry into the PBN of CalcaCre/AAV1-DIO-GCaMP6m mice (Fig. 5b). CGRP<sup>PBN</sup> neuron stimulation is known to decrease feeding and mediate many aversive responses<sup>15,24</sup>. We observed minimal coexpression of Oxtr and CGRP in PBN neurons (6 ± 2%; Fig. 5a).
mice, whereas Oxtr
stimulation resulted in a total fluid intake of ~29% of that of control mice (Fig. 1f and Supplementary Table 1). After 48-h salt depletion, CGRP stimulation decreased both NaCl and water intake (Fig. 5e). Overall, these results suggest that CGRP stimulation inhibits all ingestive behaviors (food, fluid and salt), but is less effective than Oxtr stimulation in decreasing fluid intake. We injected CNO 2 h before perfusion and observed Fos expression in 67 ± 7% of the hM3Dq-expressing neurons, as well as in non-hM3Dq expressing neurons in the dorsolateral PBN, suggesting that CGRP neurons might activate dorsolateral PBN neurons (Fig. 5f).

**Oxtr neurons project to brain regions involved in fluid regulation.** To investigate downstream projections of Oxtr neurons, we injected AAV-DIO-synaptophysin:mCherry into the PBN of OxtrCre mice, which revealed prominent projections to the central nucleus of amygdala (CeA), bed nucleus of stria terminalis (BNST), organum vasculosum of lamina terminalis (OVLT), anteroventral periventricular (AVPV) and median preoptic nuclei (MnPO) (n=2) (b). ac, anterior commissure. Scale bars represent 200 µm. c, Injection of AAV-DIO-hM3Dq:mCherry in Oxtr neurons and AAV-DIO-YFP into Oxtr neurons. d, Projections of Oxtr neurons to Oxtr neurons. Scale bar represents 100 µm. e, f, Chemogenetic activation of Oxtr neurons increases Fos in Oxtr neurons (e) and Oxtr neurons (f). 3V, third ventricle. Scale bar represents 100 µm (n=3). g, Injection of AAV-DIO-ChR2:YFP in Oxtr neurons and AAV-DIO-mCherry into Oxtr neurons. h, Action potentials evoked by 5-ms blue LED pulses at 20 Hz in an Oxtr neuron in cell-attached configuration (three sweeps) (n=5/23 Oxtr neurons). i, Synaptic currents evoked by 2-ms LED pulses in voltage-clamped Oxtr neuron at ~70 mV. 12 consecutive sweeps are shown, and they reveal a brief synaptic delay and sub-millisecond jitter (control). These EPSCs were inhibited in the presence of 20 µM CNQX and 50 µM d-AP5, antagonists of AMPA and NMDA glutamate receptors (red, five sweeps). The currents were recorded in the presence of 100 µM picrotoxin (n=4 of 13 Oxtr neurons).
central nucleus of the amygdala, bed nucleus of the stria terminalis, organum vasculosum of the lamina terminalis (OVLT), anteroventral periventricular nucleus (AVPV) and median preoptic nucleus (MnPo) (Fig. 6a,b), and less prominent projections to other brain regions (Supplementary Fig. 7a,b). Many of these regions are activated following excessive fluid intake2,25. By comparison, CGRP-PBN neurons do not project to the OVLT, AVPV or MnPo25, which are more selectively involved in regulating fluid intake26,27.

**Oxtr**PBN neurons receive projections from OxtPVH neurons. Because OxtrPBN activation suppressed fluid intake, we investigated the effect of PVH oxytocin-expressing neurons (OxtPVH) on fluid intake2,28. We confirmed projections from OxtPVH to OxtrPBN neurons by injecting AAV1-DIO-hM3Dq:mCherry into the PVH and AAV-DIO-YFP into the PBN of OxtPVH+/−:OxtrPBN+/− mice, which revealed mCherry-positive axon fibers in the PBN (Fig. 6c,d). After CNO injection, Fos expression increased in both OxtPVH and OxtrPBN neurons, primarily in the caudal OxtrPBN neurons (20 ± 4%; Fig. 6e,f and Supplementary Fig. 7c). To ensure that the mCherry-positive axons were arising from OxtPVH and not OxtrPVH neurons, we injected AAV1-DIO-synaptophysin:mCherry into the PVH of OxtPVH+/− mice and observed no visible fibers in the PVH and PBN (Fig. 6e,f and Supplementary Fig. 7c).

**Fig. 7 | OxtPVH neuron activation attenuates water consumption.** a. Injection of AAV-DIO-hM3Dq:mCherry in OxtPVH neurons. b–d. Acute OxtPVH activation revealed no significant change in NaCl or water consumption at baseline (n = 8 hM3Dq, 7 mCherry; three-way mixed design ANOVA; NaCl: interaction F(1.749,22.735) = 0.748, P = 0.468; water: interaction F(2.480,32.236) = 1.250, P = 0.305) (b), but water consumption was decreased after 24-h dehydration (n = 8 hM3Dq, 7 mCherry; two-way RM ANOVA; NaCl: interaction F(8,104) = 0.1871, P = 0.9922; water: interaction F(8,104) = 3.456, P = 0.0014) (c) and after 0.5 M saline ip (n = 8 hM3Dq, 7 mCherry; two-way RM ANOVA; NaCl: interaction F(4,52) = 2.052, P = 0.1007; water: interaction F(4,52) = 5.763, P = 0.0006) (d). e. Injection of AAV-DIO-GFP:TetTox in OxtPVH neurons. f. Chronic inactivation of OxtPVH neurons increased NaCl and water consumption after vehicle injection (n = 6 TetTox, 8 YFP; two-way RM ANOVA; NaCl: interaction F(8,96) = 2.046, P = 0.0489; water: interaction F(8,96) = 5.036, P < 0.0001). g. Injection of AAV-DIO-hM3Dq:mCherry in OxtrPVH neurons and bilateral cannula implantation into PBN. h. Infusion of CNO into PBN decreased water consumption after 24-h dehydration (n = 6 per group; two-way RM ANOVA; NaCl: interaction F(8,80) = 0.1334, P = 0.9975; water: interaction F(8,80) = 2.108, P = 0.0444). Data are expressed as mean ± s.e.m. ***P < 0.001; **P < 0.01; *P < 0.05. See Supplementary Table 2 for statistical analyses.
**Fig. 8 | Activation of NTS or CCKNTS neurons decreases fluid intake and activates OxtrPBN neurons.**

- **a.** Injection of AAV-hM₃Dq:mCherry in NTS and AAV-DIO-YFP in OxtrPVH neurons.
- **b,c.** After 24-h dehydration, NTS activation suppressed NaCl and water consumption \((n = 6 \text{ hM}_3\text{Dq}, 5 \text{ DsRed}; \text{two-way RM ANOVA}; \text{NaCl: interaction } F(8,56) = 11.88, P < 0.0001; \text{water: interaction } F(8,72) = 17.94, P < 0.0001)\) (b), as well as food intake \((n = 6 \text{ hM}_3\text{Dq}, 5 \text{ DsRed}; \text{two-way RM ANOVA}; \text{interaction: } F(4,36) = 91.69, P < 0.0001)\) (c).
- **d.** Following CNO administration, Fos was robustly expressed in external lateral and dorsolateral PBN in hM₃Dq-injected mice in caudal and rostral PBN. Scale bar represents 100 μm.
- **e.** Injection of AAV-hM₃Dq:mCherry in CCKNTS neurons. After 24-h dehydration, CCKNTS activation decreased NaCl and water consumption \((n = 5 \text{ hM}_3\text{Dq}, 4 \text{ mCherry}; \text{two-way RM ANOVA}; \text{NaCl: interaction } F(8,56) = 3.047, P = 0.0065; \text{water: interaction } F(8,56) = 5.693, P < 0.0001)\).
- **f.** RNAseq in situ hybridization image from CCK⁺/⁻ mice demonstrating coexpression of Oxtr mRNA and Fos mRNA in PBN \((n = 3)\). Scale bar represents 200 μm.

To ascertain whether OxtrPVH neurons can directly activate OxtrPBN neurons, we injected AAV1-DIO-ChR2:YFP into the PVH and AAV-DIO-mCherry into the PBN of OxtrPVH mice and observed minimal Fos expression in OxtrPBN neurons after CNO (Supplementary Fig. 7d). We also injected AAV1-DIO-hM₃Dq:mCherry into the PVH and AAV-DIO-YFP into the PBN of OxtrPVH mice and observed minimal Fos expression in OxtrPBN neurons after CNO (Supplementary Fig. 7e–g).

**Activation of OxtrPVH neurons attenuates fluid intake.** To assess the effect of OxtrPVH activation on fluid intake, we injected AAV1-DIO-hM₃Dq:mCherry bilaterally into the PVH of OxtrPVH mice (Fig. 7a). OxtrPVH stimulation did not significantly attenuate baseline NaCl or water intake following CNO or vehicle injection in hM₃Dq-injected mice.
or control mice (Fig. 7b); however, OxtPvh stimulation did attenuate water intake following 24-h fluid deprivation and osmolality-induced thirst (hypertonic saline and mannitol) (Fig. 7c,d and Supplementary Fig. 7i), but not following salt depletion or volume depletion (PEG) (Supplementary Fig. 7j,k). These results suggest that OxtPvh stimulation attenuates fluid intake, but does so less robustly than OxtrPbn stimulation. As previously reported, there was no significant difference in feeding at baseline (P = 0.9325) or after fasting (P = 0.9670)2 (Supplementary Fig. 7l). Following CNO, we observed Fos expression in 64 ± 5% of hM, Dq-expressing OxtPvh neurons (Supplementary Fig. 7m).

We also inactivated OxtPvh neurons by injecting AAV1-DIO-GFP: TetTox bilaterally to test whether these neurons have a physiological role in fluid intake similar to that of OxtrhbR neurons (Fig. 7e and Supplementary Fig. 8a,b). OxtPvh inactivation revealed no substantial difference in 24-h baseline saline preference (Supplementary Fig. 8c); however, TetTox-injected mice increased fluid intake, particularly water intake, following vehicle injection at the start of the dark cycle and during several other tests of fluid intake (Fig. 7f and Supplementary Fig. 8d–h), suggesting that OxtPvh neurons have a role in reducing water intake under physiological conditions.

To assess the role of PBN projections from OxtPvh neurons, we injected AAV1-DIO-hMdq:mCherry or AAV-DIO-mCherry bilaterally into the PVH of OxtPvh mice and implanted a bilateral cannula over the PBN (Fig. 7g and Supplementary Fig. 9a). Intra-PBN infusion of CNO (1 mM; 500 nl bilaterally) during the light cycle following 24-h fluid restriction decreased water intake in the hM, Dq-injected mice compared with controls (Fig. 7h). For comparison, intraperitoneal CNO also decreased water intake following 24-h dehydration in the light cycle (Supplementary Fig. 9b). As with previous cohorts, there was no significant difference in baseline NaCl intake (P = 0.3627) or water intake (P = 0.1763) following CNO or vehicle ip injections at the start of the dark cycle (Supplementary Fig. 9c).

Following experimentation, we confirmed targeting and cannula placement (Supplementary Fig. 9d). To validate that delivery of CNO to the PBN activates OxtrhbR neurons, we injected OxtPvh−/−:OxtrhbR mice with AAV1-DIO-hMdq:mCherry in the PVH, AAV1-DIO-YFP in the PBN and a bilateral cannula over the PBN (Supplementary Fig. 9e). When mice were infused with CNO 2 h before perfusion, Fos was induced in 22 ± 2% OxtPvh neurons (Supplementary Fig. 9f), suggesting that the effect may be mediated either by projections of OxtrhbR to OxtrPbn neurons or by antidiromic activation to OxtrPvh cell bodies, which then activate axon collaterals projecting to other brain regions10. We observed Fos expression in 26 ± 8% of OxtPvh neurons (Supplementary Fig. 9f), but only scattered, low-level Fos expression in the nucleus of the solitary tract (NTS) region, which receives projections from the PVH (Supplementary Fig. 9g). These data suggest that activation of OxtPvh projections to OxtrPbn neurons provides a small attenuation of fluid intake, indicating that other neuronal inputs to OxtrPbn neurons are required for more robust fluid suppression.

NTS neurons also suppress fluid intake and activate OxtrPbn neurons. Previous research has suggested that the NTS and the adjacent area postrema modulate fluid intake and provide substantial input to the PBN14,15. To investigate the effect of the medial NTS on fluid intake, we injected nonselective AAV-hM,Dq:mCherry or control nonselective AAV-DsRed into the NTS of OxtPvh−/− mice and AAV-DIO-YFP into the PBN to fluorescently label OxtrPbn neurons (Fig. 8a and Supplementary Fig. 9i). Following 24-h dehydration, we activated NTS neurons with CNO and observed a large decrease in fluid (water and NaCl) and food intake in hM, Dq-injected mice relative to controls (Fig. 8b,c). Mice did not display overt signs of distress or impaired movement. We injected CNO 2 h before perfusion and observed robust Fos expression in hM,Dq-expressing NTS neurons (Supplementary Fig. 9j,k) and in 33 ± 6% of OxtrPbn neurons and other neurons in the dorsolateral and external lateral rostral PBN (Fig. 8d).

Previous studies have identified cholecystokinin (CCK) as a neuuropeptide that decreases fluid intake following infusion into the PBN14, CCK is expressed in NTS neurons (CCKNTS) that project to the PBN and decrease food intake by directly activating CGRP PBN neurons15. We investigated whether CCKNTS neurons also decrease fluid intake by bilaterally injecting AAV1-DIO-hMdq:mCherry into the NTS of Ccktm/c mouse (Fig. 8e and Supplementary Fig. 9f). Following 24-h dehydration, hM,Dq-injected mice significantly decreased both NaCl and water intake (Fig. 8f). This decrease was not as robust as that evoked by OxtrPbn stimulation, but was larger than that evoked by CGRP PBN stimulation (Supplementary Table 1), suggesting that it is not merely mediated via CCKNTS projections to CGRP PBN neurons. We observed Fos expression in the NTS and in both the dorsolateral and external lateral PBN regions (Supplementary Fig. 9m,n). To investigate whether OxtrPbn neurons were specifically activated, we performed in situ hybridization for Fos and Oxtr mRNA in hM, Dq-injected mice and found that 19 ± 2% of OxtrPbn neurons expressed Fos (Fig. 8g). We also observed that both application of a CCK agonist, CCK-8 (30 nM), increased the firing rate in both OxtrPbn and CGRP PBN neurons (Fig. 8h), which is indicative of functional CCK receptors in both PBN populations. A comparison of 2-h and 4-h fluid intake after stimulating each of the neuronal populations following 24-h dehydration revealed a range of effects on fluid consumption. From largest to smallest, the effects on total fluid intake were nonspecific NTS > OxtrPbn > CCKNTS > CGRP PBN > OxtrPvh (Supplementary Table 1). Of these, only OxtrPbn and OxtrPvh stimulation selectively decreased noncaloric fluid intake.

Discussion
Our results identify a population of Oxtr-expressing neurons in the PBN that regulate noncaloric fluid intake. These neurons receive direct inputs from hypothalamic OxtPvh neurons, are activated by NTS neurons and project to several forebrain regions (Supplementary Fig. 10a). OxtrPbn neurons are predominantly separate from CGRP PBN neurons, which decrease both food15 and fluid intake (see Fig. 5), suggesting that distinct neuronal populations mediate different aspects of ingestive behaviors.

Under physiological conditions, we observed increased Fos expression in a subset of OxtrPbn neurons (~20%) after rehydration, suggesting that these neurons are engaged in fluid satiation. Using calcium fluorescence, we observed real-time activity in OxtrPbn neurons, which was low during dehydration and remained low before consumption, suggesting that OxtrPbn neurons do not respond to anticipatory cues, unlike neurons that express agouti-related protein or vasopressin4,15. Drinking water precipitated a rapid rise in OxtrPbn calcium activity, which decreased between bouts, suggesting that these neurons interact with thirst-related neural circuits to adjust the overall level of fluid intake. We observed no difference in calcium activity for Ensure or for an empty bottle. One limitation of our study was that photobleaching of OxtrPbn neurons occurred when the recording was extended, leaving us unable to quantitatively assess overall calcium activity changes over a prolonged period; however, qualitatively, calcium activity appeared to increase over the course of rehydration. We observed that ~52% of OxtrPbn neurons responded to water, as measured by GCaMP fluorescence, although this is likely to be an underestimate because some neurons with low levels of GCaMP6 expression became photobleached. These results also suggest that Fos reveals only the subset of OxtrPbn neurons with the greatest activity.

Chemosensitive activation of ~72% of OxtrPbn neurons suppressed both NaCl and water intake, but activation of ~20% of caudal
Oxtr<sup>PBN</sup> neurons following Oxt<sup>PVH</sup> activation only mildly decreased water intake. Inactivating Oxt<sup>PVH</sup> neurons predominately increased NaCl intake. Taken together, these results suggest that Oxtr<sup>PBN</sup> neurons provide an overall inhibitory effect on total fluid intake, with the amount of fluid intake and the type of fluid intake (NaCl and/or water) being dependent on the percentage of Oxtr<sup>PBN</sup> neurons that are activated.

Results from inactivation studies suggest that Oxtr<sup>PBN</sup> neurons are necessary for preventing excessive NaCl intake. In the case of dehydration, the body engages physiological mechanisms to reduce NaCl and retain water to prevent hypernatremia—for example, by inducing a dehydration-induced natriuresis<sup>36,37</sup>. Our results suggest that Oxtr<sup>PBN</sup> neurons are activated following dehydration to prevent excessive NaCl ingestion. In addition, Oxtr<sup>PBN</sup> activation decreased food intake during dehydration, but not after fasting, suggesting that the decrease may be a result of a (0.25%) in the food rather than its caloric content<sup>38</sup>. In the case of hypertonic saline injection, hypernatremia is known to increase thirst and water intake<sup>44-48</sup>, as well as to stimulate renal NaCl excretion<sup>46</sup>. Our results suggest that Oxtr<sup>PBN</sup> activation also decreases NaCl ingestion. Activation and inactivation studies revealed that Oxtr<sup>PBN</sup> neurons did not alter NaCl intake after salt depletion and did not alter food intake except during dehydration, suggesting they are not involved in salt appetite or caloric-containing food consumption. Overall, our results suggest that activation of Oxtr<sup>PBN</sup> neurons is essential for maintaining fluid homeostasis, as they decrease NaCl and/or water intake to prevent or attenuate hypervolemia and/or hypernatremia (Supplementary Fig. 10b).

The hypothalamus is an integrative center of the brain that coordinates responses to maintain homeostatic setpoints<sup>11</sup>. Whole-cell patch-clamp recordings have revealed increased firing of oxytocin-expressing neurons under hypertonic conditions and decreased firing in response to angiotensin II, which signals hypovolemic conditions<sup>44</sup>, suggesting that hypothalamic oxytocin-related neurons may be activated predominantly by hypervolemic hypertonic conditions. We expected that activating Oxtr<sup>PVH</sup> neurons would decrease NaCl intake; instead, we observed only a mild attenuation of water intake. A possible explanation is that Oxtr<sup>PBN</sup> neurons are already engaged in preventing excessive NaCl intake, and further activation via Oxtr<sup>PVH</sup> stimulation provides only a small extra attenuation of total fluid intake. In addition, activating Oxtr<sup>PVH</sup> may release peripheral oxytocin and induce renal sodium excretion<sup>29</sup>, which, in conjunction with a mild attenuation of water intake, may decrease ECF volume.

The NTS receives peripheral signals, including baroreceptor input, via inputs from cranial nerves IX and X, and is known to suppress fluid intake<sup>21,41</sup>. We observed that activating NTS neurons, including CCK<sup>NTS</sup> neurons, substantially reduced fluid intake, suggesting that NTS neurons may provide a large functional input to the PBN to reduce fluid intake. Although we did not demonstrate direct projections to Oxtr<sup>PBN</sup> neurons, we observed Fos expression in ~33% of Oxtr<sup>PBN</sup> neurons after nonspecific activation of the NTS and in ~19% of Oxtr<sup>PBN</sup> neurons after activation of CCK neurons in the NTS, suggesting that Oxtr<sup>PBN</sup> neurons are regulated by several distinct inputs from the NTS.

Overall, our results identify Oxtr as a marker for PBN neurons that are involved in regulating fluid intake. Studying the neural circuitry of fluid satiation may help to increase our understanding of body fluid homeostasis.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-017-0014-z.

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Author contributions

P.J.R. and R.D.P. conceived and designed the study. P.J.R. performed and analyzed the experiments. S.I.R and P.J.R. performed the immunohistochemistry and counting of cells. V.A.D. performed electrophysiological experiments. C.A.C. performed GCaMP6 studies. R.D.P. generated *OxtrCre* mice and provided equipment and reagents. P.J.R. wrote the manuscript with input from R.D.P. and other authors.

Competing interests

The authors declare no competing financial interests.

Additional information

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Methods

Mice. Experiments were approved by the University of Washington Animal Care and Use Committee and performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mouse lines were bred on a C57Bl/6J background and backcrossed ≥6 generations. The following mouse lines were used in these experiments: heterozygous Oxtr<sup>Cre</sup>-GFP and Calca<sup>Cre</sup>-GFP (developed in our laboratory); heterozygous Cck<sup>Cre</sup>-GFP and Oxtr<sup>Cre</sup> mice (Jackson Laboratory), and Oxtr<sup>Cre</sup>:Oxtr<sup>Cre</sup> mice. The Oxtr<sup>Cre</sup> and Oxtr<sup>Cre</sup>-GFP mice lines were crossed to Cre-dependent Gr(ROSA)26Sortm<sup>14;Cre</sup> germ line mosaic mice (Allen Institute, Aii14) to reveal expression patterns.

Most behavioral experiments were performed on male mice (7–14 weeks old at start of experimentation). Combined cohorts of male and female mice were used in the following experiments: Oxtr<sup>Cre</sup> mice following fluid deprivation; Oxtr<sup>Cre</sup>-GFP mice injected with non-specific hM<sub>D</sub>4 or DsRed in the NTS; behavioral studies on Oxtr<sup>Cre</sup> mice, including Oxtr<sup>Cre</sup> mice implanted with cannulae; and Calca<sup>Cre</sup>-GFP mice. In these experiments, female mice were distributed evenly between experimental and control groups and data were combined, as we found no significant difference in water or saline intake, as previously reported in the literature for rats<sup>43</sup>.

Animals in each litter were randomly assigned to either experimental or control groups. Before stereotaxic surgery, mice were group housed and maintained on a rodent diet (Picolah, number 5053) with water available ad libitum in a 12-h light:dark cycle at 22°C. Mice were at least 7 weeks old before surgery. Following surgery, mice were single housed and allowed to recover for at least 1 week before experimentation. All fluid and food experiments were performed with at least two cohorts of mice and data were combined, unless otherwise stated.

During salt-related experiments, mice were fed on a sodium-deficient diet (0.2% NaCl) and also tested on a liquid diet, Ensure (Abbott Laboratories). Mice were at least 7 weeks old before surgery. All fluid and food experiments were performed with at least two cohorts of mice and data were combined, unless otherwise stated.

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Tissue processing. Following experimentation, mice were anesthetized with Beuthanasia 0.2 ml ip (effective content 320 mg/kg pentobarbital; Schering, Ploug) and transcardially perfused with a saline buffer solution followed by 4% paraformaldehyde (Electrolysis Microscopy Sciences) in PBS. Brains were removed and placed in parafomaldehyde at 4°C overnight, and then cryoprotected in 30% sucrose in PBS overnight, then OCT (Fisher HealthCare) and kept at ~8°C before processing. Coronal sections of 30 μm thickness were cut from the brain on a cryostat. For all fos studies, every third section was collected (90 μm apart) for quantification, for confirmation of viral targeting, every 10th brain slice was mounted onto glass slides (Fisherbrand Superfrost Plus microscope slides, Fisher Scientific) and coverslipped with Dapi Fluoromount G (Southern Biotech), and remaining sections were collected in PBS for further processing as required.

Although Oxtr<sup>Cre</sup>-GFP mice have GFP fused to Cre, we were unable to visualize GFP even after anti-GFP antibody staining; hence Oxtr<sup>Cre</sup>-GFP neurons were visualized by genetic crosses with Ai14 mice, which allows expression of tdTomato in Oxtr neurons.

Spike optogenetics and electrophysiology. Acute 250-μm coronal slices that included the PBN were prepared from 2–6-month-old mice, which were deeply anesthetized in their housing cages before decapitation. All recordings were performed essentially as previously described<sup>15,24,44</sup>. Neurons of interest were identified by GFP or mCherry expression (CGRPPBN and Oxtr<sup>Cre</sup>PBN, respectively). To observe neuronal activity under minimally invasive conditions, endogenous spike activity and optogenetically evoked spikes were recorded using loose-patch cell-attached configuration with patch pipettes filled with the same artificial cerebrospinal fluid used for slice perfusion (ACSF; in mM: NaCl 115, KCl 3, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 1, NaH<sub>PO</sub> 1, NaH<sub>CO</sub> 25, and d-glucose 11; mOsM 295, pH 7.4 when aerated with carbon, 33°C) and 0 holding current. Synaptic currents were recorded under voltage-clamp conditions and patch pipettes filled with intracellular solution (in mM: 100 cesium methanesulfonate, 25 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPS, 0.4 EGTA, 4 ATP, 0.4 GTP, 10 phosphocreatine, mOsM 295, pH 7.3). Light-evoked action potentials and synaptic currents were initiated by optical activation of ChR2 with 2–5 ms pulses of blue light (up to 3 mW optical power) either from a 473-nm laser (LaserGrow) via an optic fiber positioned over the slice, or from a high power 460 nm LED (UHP-LED, Prinzmatix) via a microscope objective. Synaptic latency was measured as the time between the onset of the blue-light pulse and the onset of synaptic current. Synaptic jitter was calculated as the s.d. in variation of synaptic latency over multiple trials (25–50) in a particular neuron. Currents were elicited at 10-s interval between successive trials, recorded at 2–5 kHz bandwidth, digitized at 20 kHz and acquired by pCLAMP analysis software (Molecular Devices). Drugs were dissolved in ACSF and delivered to slices by local perfusion via a three-barrel system positioned over the slice. The drug application was gravity driven and on-off timing was manually controlled. Where indicated, AMPA and NMDA glutamate receptors were inhibited by a cocktail of CNQX (20 μM, Tocris) and d-AP5 (30 μM, Tocris) added to ACSF, and GABA<sub>A</sub> receptors were inhibited by bicuculline (100 μM, Tocris). Other drugs used included the following: Oxtr agonist, TgOCT (200 μM; Bachem; H-7710); specific Oxtr antagonist, atosiban (1 μM; Sigma-Aldrich, A34880<sup>®</sup>); and CCK-8 (30 nM; Bachem; H-2080<sup>®</sup>). Overall, recordings were made from 45 Oxtr<sup>Cre</sup> neurons from 47 PBN slices derived from 26 Oxtr<sup>Cre</sup>-GFP mice (some crossed with Oxtr<sup>Cre</sup>-GFP mice), of which 11 were male and 15 were female; and from 7 CGRP<sup>Cre</sup> neurons from 7 PBN slices derived from 7 Calca<sup>Cre</sup>-GFP mice (all male).

Immunohistochemistry. Fos studies. Immunohistochemistry was performed on free-floating sections. Sections underwent three 5-min washes in PBST (phosphate-buffered solution with 0.1% Triton X-114 (Sigma-Aldrich)) and were placed in a blocking solution (PBST with 3% normal donkey serum (NDS; Jackson ImmunoResearch)) for 1 h at room temperature (20–22°C) to prevent nonspecific binding. For the primary antibody, sections were incubated with rabbit anti-Fos (1:2,000; Cell Signaling Technology, number PC38) in PBS for 48 h at 4°C, then washed three times (5 min each) in PBST, before being mounted onto glass slides in PBS and coverslipped using Dapi Fluoromount-G, hM<sub>D</sub>, hM<sub>D</sub>4, and synaptophysin studies. Oxtr<sup>Cre</sup>+, Oxtr<sup>Cre</sup>+/+, Oxtr<sup>Cre</sup>+/−, Calca<sup>Cre</sup>-GFP, and Cck<sup>Cre</sup>-GFP mice injected with AAV1-DIO-hM<sub>D</sub>4,Chmerry, AAV1-DIO-hM<sub>D</sub>4,DmCherry, control AAV1-DIO-mCherry and AAV-DIO-synaptophysin mice were injected with anti-DiI (100 nM) to stain to allow visualization of the fluorescent protein signal. To examine neuronal activation, we examined Fos expression in hM<sub>D</sub>4- and control mCherry-injected mice after
Injection with CNO 2 h before perfusion. The primary antibodies were rabbit anti-Ds-Red (1:1,000; Clontech) Takara Bio USA, number 632496) and goat anti-1F5 (1:300, Santa Cruz Biotechnology); the secondary antibodies were Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500; Jackson Immunoresearch, number 711-585-152) and Cy5-conjugated donkey anti-goat IgG (1:500; Jackson Immunoresearch, number 705-175-147).

**TetTox studies.** OxtrCre+/+, OxtrCre+/− and OxstCre+/− mice injected with AAV1-DIO-GFP-TetTox and AAV1-DIO-YFP underwent anti-GFP immunohistochemistry staining using a similar histological procedure to amplify the fluorescent protein signal. The primary antibody was chicken anti-GFP (1:10,000; Abcam, number 13970); the secondary antibody was Alexa Fluor 488-conjugated donkey anti-chicken IgG (1:500; Jackson Immunoresearch, number 703-545-155).

**GFP antibody studies.** To investigate coexpression of Oxtr and CGRP in PBN, we prepared PBN sections from OxtrCre+/−:Ai14 mice. The primary antibody was mouse anti-CGRP (1:5,000; Abcam, ab81887); the secondary antibody was Cy5-conjugated anti-mouse IgG (1:400, Jackson Immunoresearch, number 715-175-150).

**RNAseq.** We performed in situ hybridization (ISH) using the RNAseq assay on brain tissue collected from OxtrCre+/−:Ai14 and CckCre+/− mice. Microscopy was performed as described above then embedded gradually through a series of 10%, 20% and 30% sucrose followed by OCT (Fisher HealthCare). Brains were cut coronally at 10 µm thickness, mounted and stored at −80 °C before processing. On the day of processing, sections were thawed at 20–22 °C before performing ISH according to the manufacturer’s protocol (Advanced Cell Diagnostics). We used probes for Oxtr (Mm-Oxtr-C3 probe, 1:50 dilution) and Fos (Mm-Fos-C2 probe, 1:50 dilution). Following ISH, slides were coverslipped and imaged as described.

**For OxtrCre+/−:Ai14 mice, we imaged TidTomato, fluorescence, then matched sections after ISH (n=3). For CckCre+/− mice, we imaged staining for Oxtr and Fos mRNA after performing ISH (n=3)**.

**Microscopy.** For Fos, in situ and coexpression studies, brain sections were imaged on an Olympus Fluoview FV1200 confocal microscope (Shijuku). To confirm targeted injections, brain sections were imaged using the Nikon upright epifluorescent Eclipse E600 microscope (Minato) or Keyence Fluorescence Microscope EZ-100 (Keyence Europe). A program for calculating brightness or contrast, was used to color images—for example, OxtrCre+/−:Ai14 cells red and AAV1-DIO-YFP-infected neurons green. Images were minimally processed to enhance brightness and contrast for optimal representation. Following imaging, any mouse whose targeted injection site was missed or demonstrated very sparse expression (<5 fluorescent neurons/section), suggesting inadequate injection, was excluded from experimental analysis. In addition, any mouse that was unilaterally injected was included in experimental analyses for stimulatory (one unilateral injection in each group of hM4Dq injected OxtrOslo and OxstCre mice) and control groups, but excluded from inhibitory (TetTox- and hM4Dq-injected) groups. For infusion experiments, mice whose targeted injection was missed were excluded (two mice).

**Fos experiments.** For salt-depletion and fluid-deprivation experiments, OxtrCre+/−:Ai14 mice were single-housed and placed on a sodium-deficient diet and given access to both 0.5% NaCl and water for 5 d of habituation. For salt depletion, mice were divided into three groups: (1) salt returned, (2) salt depleted and (3) given access to both 0.5 M NaCl and water for 5 d of habituation. For salt depletion, mice were divided into two groups: (1) fluid returned (n=3 or 4 per group). Mice in group 1 were injected with furosemide (5 mg ml−3 or 4 per group). In group 1 were treated with furosemide (5 mg ml−3) daily for 2 d and denied access to saline (but not water), to develop a salt appetite. On day 3, saline was returned and mice were perfused 2 h later (during the light cycle). Mice in group 1 underwent a similar protocol; however, saline was not returned before perfusion. Mice in group 3 had no furosemide challenge and were perfused on day 3. For fluid deprivation, mice were divided into two groups: (1) fluid returned and (2) fluid deprived (n=3 or 4 per group). Mice in group 1 were deprived of saline and water for 24 h, and were perfused 2 h after fluid was returned (during the light cycle). Mice in group 2 were also deprived of saline and water for 24 h, which were not returned before perfusion. For hypertonic saline injections, mice were single-housed for 5 d with ad libitum access to food and water, and divided into two groups: (1) 1 M saline (10 g l−1 body weight) injection and (2) normal saline injection (n=3 or 4 per group). Each mouse was injected with saline and perfused 2 h later (during the light cycle). Quantification of Fos expression was performed on six 30-µm-thick coronal brain sections 90 min after either 3 or 4 per group). Each mouse was injected with saline and perfused 2 h later (during the light cycle). Therefore, the major portion of the PBN where Oxtr is expressed. The investigator who quantified Fos was blinded to the identity of the conditions. Given that there was differential activation throughout the PBN, the sections were evenly divided into caudal, middle and rostral regions. An estimate of total Oxtr-expressing neurons was made by multiplying by 3.

For drinking and salt-related studies, mice had access to both water and saline, and were placed on a sodium-depleted diet (diet #9901603, Research Diets), unless otherwise stated, to allow accurate measurement of both fluid and salt uptake.

**Calcium imaging.** Mice were prepared for calcium imaging as described. Briefly, 3 weeks after AAV1-DIO-GCaMP6m and AAV1-DIO-hDqCherry viral injection, mice were anesthetized (as described above) and implanted with a miniature microscope lens (0.5 mm diameter, 10×/0.17 NA, catalog #100-000588) that allowed visualization of the fluorescent activity during the lens implantation. Because basal fluorescence from Oxtr neurons was low, we treated the mice with CNO before lens implantation to facilitate visualization and lens placement. The lens was targeted to be ~200–300 µm above the neuropil using the following coordinates: −4.80 mm posterior to bregma, −1.40 mm lateral from midline, and −3.00 mm ventral to skull surface. One week after lens implantation, mice were anesthetized and a baseplate (Inscopix, catalog #100-000279) was implanted above the lens. The baseplate provides an interface for attaching the miniature microscope during calcium imaging experiments, but at this point, a baseplate cover glass (Inscopix, catalog #010-000241) was attached to prevent damage to the microendoscope lens. Calcium fluorescence was recorded at five frames per second, 200-ms exposure time, and 50% LED power using a miniature microscope from Inscopix (nVista). The recording parameters were based on pilot studies that demonstrated the least amount of photobleaching while allowing sufficient detection of fluorescent activity. We used Ethovision (XT 10, Noldus Technology) to trigger and synchronize calcium recordings with behavioral video recordings.

**Water deprivation and rehydration.** Mice were water-deprived for 24 h. On the experimental day, mice were briefly anesthetized with isoflurane to attach the microscope, and then allowed to acclimate in their home cage for 1 h. After the acclimation period, baseline fluorescence was recorded for 30 s, a water bottle was placed in the cage, and fluorescence was continuously recorded for 10 min. On separate test days, we placed an empty bottle or a bottle with Ensure in the cage. We adjusted the focus of the microscope between tests, and counted numbers of neurons for slightly differing numbers of neuron with these test days. Calcium recording files were spatially downsampled (factor of 4), motion-corrected (Inscopix, Mosaic v1.2), and fluorescent traces from individual neurons were extracted using constrained non-negative matrix factorization for microendoscopic data (CNMF-E). The key parameters used for CNMF-E were: [1, 7, 14; P; 10, 55; 0.85 (ref. 31)]. We then normalized (0 to 1) individual fluorescence traces taken from the entire 10-min recording period. Photobleaching was apparent during the recording session, so we were unable to adequately measure changes in activity over extended periods of time (for example, 10-min session); instead, we analyzed the change in fluorescence activity for 5 s before and 5 s during each bout. Bouts were defined as uninterrupted mouth contact with the water spout, and the beginning of an inter-bout interval was >10 s of not contacting the spout. The data from every bout were averaged for each neuron across the 10-min recording session. ‘Activated neurons’ were defined as neurons that had an average fluorescence (after time point 0) of >2 s.d. above baseline (baseline = average fluorescence from −5 to 0 s). Following the recording session, we injected mice with CNO, which aided in identifying all the neurons. These rehydration studies were conducted during the first hour of the dark cycle.

**CNO-induced activation.** Mice were briefly anesthetized with isoflurane to attach the microscope, then allowed to acclimate in their home cage for 1 h. After acclimation, we recorded baseline fluorescence activity for 30 s, followed by an injection of CNO (1 mg/kg). We non-continuously recorded (20 s for each time point) fluorescence at 5, 10, 15, 20, 25, 30, 45, 60 and 120 min after CNO injection. The calcium recording files were spatially downsampled (factor of 2), motion-corrected, and fluorescent traces were extracted using individual component analysis and principal component analysis (Inscopix, Mosaic v1.2). The change in fluorescence (ΔF/ΔF0) was calculated as (Ft − F0)/F0, where Ft was the average fluorescence 30 s before injection and F0 was the average fluorescence during a particular time point. These studies were conducted during the light cycle.

**Food, water, and saline intake.** For acute feeding assays, mice were placed in BioDAQ chambers (Research Diets) which measured food intake electronically. Mice were individually housed, had access to water and body weight was measured during the day and measured during the night. Mice were supplied with a rodent diet (D12450B, Research Diets), habituated to cages for 5–7 d and habituated to saline injections for 2–3 d. To measure food intake during the dark cycle, mice were injected with CNO 30 min before lights out and had access to food removed until lights out. To measure response after a fast, food was removed for 24 h, then mice were injected with CNO 30 min before the return of food (in the morning). To measure food intake during the light cycle, mice were injected with CNO in the morning, then denied access to food for 30 min to allow time for the CNO to take effect. The experimenter was not blinded to the identity of the experimental versus control groups.

For drinking and salt-related studies, mice had access to both water and saline, and were placed on a sodium-depleted diet (diet #9901603, Research Diets), unless otherwise stated, to allow accurate measurement of both fluid and salt uptake.
intake. Tubes were placed into angled ports at the front of a cage and weighed manually (in grams; converted to milliliters), taking care to prevent dripping.

For TetTox-injected mice, baseline salt intake was monitored at three different concentrations (0.075, 0.3 and 0.5 M) for 3 d each (after 1 d of habituation); for subsequent experiments, a saline concentration of 0.3 M was used, unless otherwise stated. The order of experiments was baseline food and/or saline studies, 48-h salt depletion, 24-h fluid deprivation, hypertonic (0.5 M) saline ip, mannitol (1 M) ip and PEG 30% subcutaneous injections (these last three were randomly ordered). All these experiments were performed at the start of the dark cycle, unless otherwise noted. Recovery time was at least 7 d following salt depletion, and at least 3 d following other experiments.

For baseline fluid experiments in OtxtCre/+ and OxtCre/+ mice, we compared CNO versus vehicle injections in hM3Dq- and mCherry-injected mice, and found no significant difference in fluid intake following vehicle injection. Given that there were multiple fluid intake experiments, we limited the number of experiments performed on individual mice by injecting only CNO and comparing fluid intake in hM3Dq- versus mCherry-injected mice. We observed a difference in NaCl intake between OxttCre/+ and OxttCre/+ mice, which is likely due to a difference in mouse strains obtained from different sources, so we ensured matched litters were used for controls in all experiments. In OxttCre/+ mice, one hM3Dq-injected mouse appeared sick 2 d before PEG injection, so it was not included in that experiment.

Given that mice underwent a series of fluid intake experiments, we also compared daily water and 0.3 M NaCl intake from the start of experimentation (just before the first study) to the end of experimentation (in final week of fluid intake studies). Salt and fluid deprivation were performed as described in the ‘Fos experiments’ section. For hM3Dq–, hM3Dq– and mCherry-injected mice, CNO (1 mg kg⁻¹) ip was injected 30 min before returning fluid. Fluid was returned ~15 min after the start of the dark cycle. For all deprivation experiments, we observed that mice would typically drink a large proportion in the first 15–30 min of the experiment, so we reported the first 2 h in 15-min intervals, unless otherwise stated. Baseline CNO and vehicle (normal saline) injections were also performed 30 min before returning fluid.

Hypertonic (0.5 M) saline and 1 M mannitol were injected ip –10 min before CNO injection, whereas 30% PEG was injected subcutaneously ~30 min before CNO injection to allow extra time to diffuse. In general, these experiments showed increased differentiation of fluid intake over a longer time period, so we reported 4 h in 1-h intervals, unless otherwise stated. For liquid diet (Ensure) experiments, mice were habituated to Ensure and water for 2 d and then deprived of Ensure for 24 h before experimentation.

A separate cohort of mice was used to test limited Ensure intake and fluid intake in the absence of food. For the limited Ensure intake protocol, we initially returned a small quantity of Ensure (1.6 ml or 0.7 ml) after 24 h deprivation; at the end of experimentation, we returned ad libitum Ensure. For fluid intake in the absence of food, we repeated the protocol for 24-h dehydration, but removed food during the experimental period.

A separate cohort of mice was also used to test dehydration-induced food intake. Mice were habituated to BioDAQ chambers and supplied with a rodent diet (D12450B). On the day of experimentation, water and food were removed 7 h before lights out, then food (but not water) was returned at the start of the dark cycle. CNO was injected 30 min before experiments.

Intra-PBN infusions were performed during the light cycle. Infusions were performed using CMA-100 microinjection pump (Bioanalytical Systems) over 5 min. Fluid was returned 5 min after infusion and intake measured for 2 h.

For CalcKCre/+ mice, we performed baseline fluid, rehydration and salt depletion studies, as described above. Rehydration was performed with 0.3 M NaCl and water; baseline fluid and salt depletion experiments were performed with 0.5 M NaCl and water. For GckCre/+ mice and nonspecific hM3Dq-injected mice, we performed rehydration experiments with 0.3 M NaCl and water in hM3Dq versus control mice.

Pharmacological injections. The following agents were prepared in sterile 0.9% saline: CNO (Sigma-Aldrich; C0832), polyethylene glycol (30% v/v; 20000 MW; Sigma-Aldrich; P2263). The following agents were prepared in sterile water: hypertonic NaCl (0.5 M; Fisher Scientific), mannitol (1 M; Sigma-Aldrich; M9647) and furosemide (5 mg ml⁻¹; Hospira). Polyethylene glycol was injected subcutaneously at 0.5 ml per mouse; all other compounds were injected intraperitoneally, including CNO, hypertonic NaCl and mannitol (10 µg g⁻¹ body weight), and furosemide (8 µg g⁻¹ body weight). CNO (1 mM) was also infused intra-PBN.

Statistics and figure preparation. Data analysis and generation of histograms were performed using GraphPad Prism Version 6.01 for Windows (GraphPad Software). Results are expressed as mean ± s.e.m. Statistical significance was determined by different tests appropriate for each dataset: for comparing the means of two groups, unpaired two-tailed Student’s t test; for comparing the means of three or more groups, a one-way ANOVA with Tukey’s post hoc tests; for correlation studies, a Pearson product-moment correlation; for comparing food or fluid intake over time, a repeated measures two-way ANOVA with Sidak’s post hoc tests, unless otherwise noted. Three-way mixed design ANOVAs were performed using IBM SPSS Statistics for Windows v.20 (IBM). If Mauchly’s test of sphericity was violated, a Greenhouse-Geisser correction was applied; if significant interaction was determined, a simple two-way interaction was performed with statistical significance accepted at a Bonferroni-adjusted α level of 0.025. We also tested for equality of variance (Brown-Forsythe test for one-way ANOVAs; or Levene’s test of equality of variances). Data distribution was assumed to be normal, but this was not formally tested. **P < 0.001; ***P < 0.0001; ****P < 0.0001; **P < 0.01; *P < 0.05. Calcium fluorescence data were analyzed using OriginPro v.2016 (OriginLab).

A power analysis was performed for an effective sample size using http://powerandsamplesize.com. Based on a pilot study for fluid deprivation, we used a mean of 1.8 and s.d. of 0.4. Assuming a significance level of 0.05 and power of 0.8, we calculated a sample size of 7 per group if means were 1.5-fold different with a two-tailed Student’s t test. Data and figures were exported into Adobe Illustrator CS6 (Adobe Systems) to prepare figures. Images of sagittal mouse brain were taken from the Motifolio toolkit.

Supplementary statistical analyses and methods checklist are provided in Supplementary Table 2 and the Life Sciences Reporting Summary.

Life Sciences Reporting Summary. Further information on experimental design and reagents is available in the Life Sciences Reporting Summary.

Code availability. No code was written by authors.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

1. Sample size
Describe how sample size was determined.

See Methods - Statistics and figure preparation section - paragraph 2. A power analysis was performed for an effective sample size using http://powerandsamplesize.com. Based on a pilot study for fluid deprivation, we used a mean of 1.8 and standard deviation of 0.4. Assuming a significance level of 0.05 and power of 0.8, we calculated a sample size of 7 per group if means were 1.5-fold different with a two-tailed Student’s t-test.

2. Data exclusions
Describe any data exclusions.

See Methods - Microscopy section. Following imaging, any mouse whose targeted injection site was missed or demonstrated very sparse expression (≤6 fluorescent neurons/section), suggesting inadequate injection, was excluded from experimental analysis. In addition, any mouse that was unilaterally injected was included in experimental analyses for stimulatory (1 unilateral injection in each group of hM3Dq-injected OxtrPBN and OxtPVH mice) and control groups, but was excluded from inhibitory (TetTox- and hM4Di-injected) groups. For infusion experiments, mice whose targeted injection was missed were excluded (2 mice).

3. Replication
Describe whether the experimental findings were reliably reproduced.

See Methods - Mice section - paragraph 3. All fluid and food experiments were performed with at least two cohorts of mice and data were combined, unless otherwise stated.

4. Randomization
Describe how samples/organisms/participants were allocated into experimental groups.

See Methods - Mice section - paragraph 3. Animals in each litter were randomly assigned to either experimental or control groups.

5. Blinding
Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

See Methods - Fos experiments - paragraph 4; and Food and saline intake - paragraph 1.
1) The investigator who quantified Fos was blinded to the identity of the conditions.
2) The experimenter was not blinded to the identity of the experimental versus control groups.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑  | The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☑  | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑  | A statement indicating how many times each experiment was replicated |
| ☑  | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ☑  | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☑  | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
| ☑  | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☑  | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software
Describe the software used to analyze the data in this study.

See Methods - Statistics and figure preparation - paragraph 1
Data analysis and generation of histograms were performed using GraphPad Prism Version 6.01 for Windows.
Three-way mixed design ANOVA were performed using IBM SPSS Statistics for Windows v.20.
Calcium fluorescence data was analyzed using OriginPro v. 2016.

Materials and reagents

Policy information about availability of materials

8. Materials availability
Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used.

9. Antibodies
Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

See Methods - immunohistochemistry section.
The ordering information for the antibodies used are all referenced. The companies provide validation data for histological staining of fixed tissue.

10. Eukaryotic cell lines
a. State the source of each eukaryotic cell line used.
N/A
b. Describe the method of cell line authentication used.
N/A
c. Report whether the cell lines were tested for mycoplasma contamination.
N/A
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.
N/A
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.
   See Methods - Mice section - paragraphs 1 & 2 for details.

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.
   N/A