# The Neural Basis of
# Sodium Appetite

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# ABSTRACT

Fluid homeostasis, which maintains a stable internal environment, is critical for survival. Body fluid is tightly monitored and regulated through its main components, water and salt. Here, I focus on the aspect of sodium regulation when sodium is the main cation in the extracellular fluid and is also required for primary metabolism. The depletion of sodium induces the retention of sodium but also a central mechanism to obtain sodium from the external sources. This need for sodium specifically drives animals towards sodium consumption, called sodium appetite. Even though sodium appetite is specific for only sodium ion, sodium appetite observed as an innate behavior across the animal kingdom.

Sodium appetite is strictly regulated by both peripheral sensory signals and central appetite signals. Due to the development of genetic tools, I was able to investigate the neural basis of sodium appetite from searching sodium appetite dedicated neurons. Here, I identify two genetically defined neural circuits in mice that control sodium intake. The activation of these neurons drives robust sodium intake in sated animals. Particularly, prodynorphin expressing neurons in the pre-locus coeruleus shown specific consumption to sodium compounds, including rock salt. In terms of loss-of-function, inhibition of these neurons selectively reduced sodium consumption. It was further shown that these neurons receive sodium depleted signals by aldosterone-sensitive neurons.

Previously, it was suggested that taste signals have a central role in sodium satiation. I demonstrate that the oral detection of sodium rapidly suppresses sodium appetite neurons. The blockage of the sodium taste or gastric infusion of sodium abolished the sodium suppression in the sodium appetite neurons. Consistently, gastric infusion of sodium did not cause sodium satiation. Moreover, retrograde-viral methods showed that specific inhibitory neurons partially mediate sensory modulation in the bed nucleus of the stria terminalis.

Together, I identified a specific neural population as a functional unit for sodium appetite. By knowing the dedicated circuits for sodium appetite, I demonstrated chemosensory and physiological signals regulate the neural circuits. The genetically defined neural population

can be handle as an entry point of further investigation of the neural basis of sodium appetite.

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  S.L. conceived the research program, designed and carried out experiments, analyzed data and wrote the paper

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# Equal contribution

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# NOMENCLATURE

**ECF extracellular fluid**

**BBB blood-brain barrier**

**CNS central neural system**

**PNS peripheral neural system**

**TRP transient receptor potential**

**ENaC epithelial Na+ channel**

**PKD2L1 polycystic-kidney-disease-like channel**

**MR mineralocorticoid receptor**

**ANG angiotensin**

**AT ANG II receptors**

**RAS renin-angiotensin system**

**ACE angiotensin coverting enzyme**

**ChR2 channelrhodopsin-2**

**CVOs circumventricular organs**

**LT lamina terminalis**

**SFO subfornical organ**

**OVLT organum vasculosum laminae terminalis**

**Pre-LC pre-locus coeruleus**

**BNST bed nucleus of the stria terminalis**

**NTS nucleus of the tractus solitarius**

**CGRP calcitonin gene-related peptide**

**nNOS neural nitric oxide synthase**

**PDYN prodynorphin**

**HSD2 11-β-hydroxysteroid dehydrogenase 2**

*Chapter 1*

FLUID HOMEOSTASIS

Maintaining a stable internal environment is essential for function and survival. The body continuously protects and adjusts from various physical and chemical challenges. For example, the chemical property of obtained food or temperature change can threaten the metabolism by affecting the enzymes and cells. To preserve a stable internal environment, the body must detect the off-range from a set point and take action to the imbalance stimulus. This physiological process is called homeostasis. The concept of homeostasis was widely used and adapted after Cannon in the 1930s[1]. The basic theme of homeostasis starts from the imbalance, which is outside the set point. Then, the body detects the imbalance through receptors and conveys such information to the control center. Lastly, the control center tries to balance the internal body environment using effectors. After the adjustment, the body receives both feedforward and feedback signals during or after the action of recovery. In this chapter, I will focus on body fluid homeostasis in the aspects of water and sodium regulation, which maintain a particular internal environment from the challenge of osmolarity and volumetric stimulus.

## 1.1. Fluid imbalance

In humans, our total body fluid is about 60% of our whole body weight. The body fluid is strictly regulated within 0.2% to 0.5% of body weight[2]. The body fluid is conceptually divided into the intracellular and extracellular fluid (ECF) by the cell membrane. When the intracellular fluid is defined as the fluid located interior of the membrane, the ECF is the fluid that bathes the cells. The transfer of water and the ionic molecule between the membrane is restricted because of the

semipermeable property of the cellular membrane. This restriction determines the composition of the solute, thus determining the osmotic pressure between the intracellular and ECF. Both intracellular fluid and ECF have its homeostasis to maintain a precise balance. Yet, they both share a goal to maintain a stable amount of solute and the volume of the cell.

The fluid imbalance critically affects the cell. It is believed that the body maintains a certain range of osmolality and volume of the ECF[3]. The osmotic imbalance will drive the fluid in or out of the cell, and the intracellular property will be affected. When the cell is given high (hyper) osmotic stimulus, the cell gets dehydrated. On the other hand, low (hypo) osmotic stimulus can expand the cell by increasing the intracellular volume[3]. Although the cell itself has a mechanism to adjust such a stimulus by changing the ion composition[4], a larger scale of fluid regulation will be required for the ECF.

The ECF frequently encounters loss or excess of body fluid. For instance, body fluid is lost with urine or sweat[5]. Considering that a solution is divided into solute and solvent, two different types of dehydration can occur: intracellular (osmotic) and extracellular (hypovolemic). When the solution, mainly water, is relatively depleted in the ECF, it increases the osmolality, which develops an osmotic force that drives the solvent of the intracellular fluid to the ECF. One example of such deprivation is called hypernatremia, defined as increased serum sodium concentration[6]. Meanwhile, when water is sufficient or easily accessible, but sodium or another solute is depleted, the total volume of ECF is reduced to maintain a constant osmolality. This reduction of ECF is called hypovolemia. Sodium deficiency is one of the examples of hypovolemia[7]. In a multicellular organism, intracellular fluid regulation has a limit to

counterbalance fluid imbalance due to capacity. Thus, the requirement of ECF regulation is essential.

When an imbalance of fluid continues, the imbalance leads to chronic changes to the cells. This stress will affect the cell's metabolism and induce neurovascular and cognitive dysfunction. One of the examples is hypertension. Although it is controversial whether dietary sodium has a linear correlation with hypertension, multiple rodent studies demonstrated that a high sodium diet induces hypertension[8]. High sodium dietary rodents were demonstrated to cause a change of genetic expressions, including cognitive dysfunction[9,10]. Recent studies show that a high sodium diet can cause accumulation of hyperphosphorylated tau, a hallmark protein of Alzheimer's diseases[9]. Understanding the fluid homeostasis will also provide us insights into the development of medical treatment. I will review the component of fluid homeostasis to understand further how the change of ECF detects and achieves the fluid balance.

## 1.2. Sensors of fluid homeostasis

In the homeostasis model, the imbalance stimulus is detected, then the effectors take action to compensate for the imbalance, begging the question how is body fluid detected? The body fluid can be mainly monitored by the solute and the volume of the fluid. The change of the solute can be a parameter as osmolality change. Besides the perception of osmolality, the size of the body fluid can be detected as pressure change, including mechanosensory. Here, I will review the organs and potential cellular receptors that detect the imbalance of the body fluid.

*Sensory organs of fluid homeostasis*

Multiple organs seem to be involved in the detection of fluid imbalance, including the liver and spleen. Among these organs, the primary sensory and effector of the fluid homeostasis are the kidney[11]. In humans, the kidney filters total blood volume every 4-5 minutes, approximately 180 liters per day[12]. The kidney detects fluid imbalance with various mechanisms. For example, the sensation of the body volume flow is mediated by the change of the renal perfusion pressure, and pressure perception of the sympathetic nerve[13,14]. In parallel, the macula densa cells in the distal nephron can detect salt concertation. Salt is detected through apical NaCl transport mechanisms, including the furosemide-sensitive Na+:2Cl−:K+ cotransporter (NKCC2) and apical Na+:H+ exchanger (NHE)[13,15]. Based on this information, the kidney releases signals to the effectors.

The baroreceptors within the circulation also monitor the body fluid. Arterial baroreceptors are located in the carotid sinus and the aortic arch. Baroreceptors increase firing depending on the degree of tension due to fluid volume change[16,17]. Activation from the baroreceptors are known to be relayed to the brain regions, nucleus of the tractus solitarius (NTS), and ventrolateral medulla[18]. Baroreceptors can facilitate the secretion of hormones related to fluid regulation[19]. As a result, decreased blood pressure increases the renal plasma activity, and both sodium and water excretion[20]. In addition, balloon inflation, which induces stretch of the atria, is able to regulate sodium and water excretion[21].

The brain itself was suggested to directly sense chemical and physical changes in the body fluid [3,22-24]. The hypertonic sodium of the intracerebral fluid induces immediate fluid intake and antidiuresis[22,25]. It was proposed that brain region outside of the blood-brain barrier (BBB), circumventricular organs (CVOs) has a critical role as a osmosensor[26-30]. It was demonstrated

that these neurons respond to direct osmolality change[31,32]. These CVOs also express various receptors or channels related to fluid homeostasis[33]. I will futher discuss the functional studies related to the CVOs in the next chapter.

*Receptors of fluid homeostasis*

I have reviewed the sensory organs which are involved in fluid homeostasis. However, the receptor of this osmosensation and mechanosensation in the organs remains to be explored. Various channels were proposed to be the detectors of the fluid imbalance. Here, I overview the potential channels involved in osmosensation or mechanosensation, which contribute to fluid homeostasis in the aspects of knockout studies.

One of the proposed channels as a fluid sensor is transient receptor potential (TRP) channels. Although the endogenous ligand of TRP is not apparent, a different type of TRP channel was found to be expressed in the fluid-related organs, including the kidney and brain[3,34]. Among TRP channels, TRPV was proposed as an osmosensor from the comparison of homologs of OSM-9, an ion channel involved in mechanosensation[35]. Among TRP, TRP vanilloid 1 and 4 (TRPV1 and TRPV4) were discovered to have a role in osmosensory channels. TRPV4 is a nonselective ion channel which is suggested to be activated by osmotic pressure[36]. TRPV4 was suggested to be involved in the osmosensing of hepatic vessels. A study determined water ingestion evoked a response in the hepatic afferent which was not observed in TRPV4 knockout[37]. In another study, the role of TRPV4 was highlighted as a central osmosensor. Lower vasopressin levels were observed more often after osmotic load in the TRPV4 knockout mice than in wild-type animals. Also, it was reported that knockout of TRPV4 mice has higher plasma osmolality than wild-type. On a behavioral level, knockout of TRPV4 showed reduced daily water intake and

slight impairment to acute hypertonic stimulus[38]. However, other researchers reported no significant water intake behavior from the knockout of TRPV4[39]. Therefore, the role of TRPV4 in the CNS and its involvement in osmoregulation remains controversial.

TRPV1 was also proposed as osmosensory in the CNS. TRPV1 was shown to be involved in triggering vasopressin in response to hyperosmolality. TRPV1 knockout mice did not show response to hyperosmotic stimulus response in supraoptic nucleus vasopressin-releasing neurons[40]. It was also reported that TRPV1 knockout mice show decreased water intake after osmotically-stimulated thirst[41]. Similar to TRPV4, it was also declared a controversial behavioral water intake result of TRPV1 knockout[39,42]. Together, the role of TRP channels in fluid homeostasis remains a mystery. Although the interpretation was based on global knockouts instead of selective knockouts, it will require further studies to pinpoint the role of TRP channels along in vivo manipulation in selective matter. For instance, the central mechanism was investigated by electrophysiological recording, but the behavior consequence the specific channel remain unclear. It will require further investigation into whether TRP expressing neural populations are able to induce central mechanisms like thirst or sodium intake along with transcriptomic analysis.

As sodium is the main cation of the body fluid, the detection of sodium is critical for fluid imbalance detection. Distinct sodium channels were proposed as the receptors of fluid homeostasis. Nobly, the epithelial Na+ channel (ENaC) family, which selectively permeates sodium ions, was also introduced to have a role in the detection of sodium. It is well established that in the taste system, ENaC is an important channel for sodium detection[43,44]. The blockage of the ENaC in taste was sufficient to inhibit the detection of sodium[45,46]. In the kidney, ENaC

played the role of an effector involving the absorption of sodium, while NKCC2 and NHE detected salt[13,15]. ENaC was also proposed as a central sodium sensor by the result that the ENaC alpha subunit was expressed in the circumventricular organs. ENaC alpha subunit expressing neurons were activated when hypotonic saline was acutely injected[47].

Another sodium channel, the Na(x) channels, which are a weak voltage-sensitive sodium ion channel, was suggested for the central sensory mechanism. These channels were found to be expressed in the circumventricular organs, which are known to govern thirst[48]. The knockout of the Na(x) channel has shown increased sodium preference, but did not increase the total intake amount of fluid in thirsty animals. Furthermore, the effect was observed when the Na(x) was specifically a knockout in the circumventricular organs[32]. However, whether there is involvement of Na(x) channel in the hypovolemic stimulus, or if the osmolality and sodium concentration is not changed or low, is not clear.

Recently, a mechanosensitive ion channel Piezo was identified and was largely investigated for its physiological role[49-55]. Piezo channels were found to be expressed in the kidney and baroreceptors. The knockout of the Piezo channel was demonstrated to abolish the reflex of aterial baroreceptors such as phenylephrine or sodium nitroprusside. Meanwhile, a single knockout of piezo 1 or 2 did not affect the baroreceptor reflex. Furthermore, this study has done gain-of-function using optogenetics on the neural population that expressed piezo 2. They were able to reproduce blood pressure regulation by the activation of piezo 2 expressing neurons[49]. The piezo channel has been nicely demonstrated from molecular receptors to the neural mechanism of the baroreflex. Whether the Piezo channel has a role in the brain is not yet known.

Outside of the highlighted channels, various receptors were proposed and demonstrated for the detection of fluid imbalance. The proposed receptors were also found in different organs and play different roles in the physiological system. The difficulty of selective manipulation and different expression level may have led to a controversial result. Global ablation, for instance, in other organs, can indirectly evolve secondary effects. Viral targeting[56] and genetic profiling[57] have become a solution to understand the role of the receptors and explain of the conflicting physiological results.

## 1.3. Receptor to Effector: Endocrine system and PNS

After receptors capture the fluid imbalance, how is the information carried to the proper effectors? Multiple aspects of fluid regulation must be controlled simultaneously, such as retention and intake behaviors. To orchestrate the effectors, the sensory information is transmitted and amplified using the endocrine system or peripheral nervous systems (PNS). Endocrine is explicitly detected by the receptors of the effectors and has a different cellular dynamic but mostly shares the same goal. For instance, angiotensin II (ANG II) is a hormone regulates the reabsorption of water and sodium but also regulates blood pressure and sympathetic activity[7,58]. Also, the detection from nerves can govern the peripheral nervous system or convey to the effector on the CNS. Here, we will review the endocrine and the PNS, which carry and amplify the imbalance to the effectors.

*Endocrine system and effectors*

*A) Angiotensin II from renin-angiotensin system*

One of the endocrine systems studied for fluid homeostasis is the renin-angiotensin system (RAS)[7,59]. Although renin-angiotensin system has various roles in the body, including cell proliferation, inflammation, and fibrosis[60], here I will mainly focus on the aspect of fluid regulation. In fluid regulation, the renin-angiotensin system is mostly highlighted in the view of secretion of ANGII and aldosterone. ANG is well-conserved signal for fluid imbalance accross the animal kingdom[61]. Although renin-like activity is observed in other organs, the kidney mainly controls the secretion of renin into the circulation response to the fluid simulation. The RAS is initiated with renin, a protease protein secreted by the kidneys. Renin then coverts to ANG I. This step is considered the rate-limiting stage. Then, ANG I is converted into ANG II by angiotensin converting enzyme (ACE). The body has a distinct mechanism to regulate each step. Finally, ANG II will control various effectors including the kidney itself, the vascular system, the adrenal gland, and the brain to regulate the body fluid[7].

ANG II is normally found to be around 2 to 10 pg/ml of blood in humans and 4 to 20 mg/ml of blood in rats[62]. Increased ANG II level was observed in hypovolemia and water deprivation but not in hyperosmotic stimuli were applied[63-66]. On the other hand, both systemic and intracranial administration of ANG II was able to trigger homeostatic response such as secretion of vasopressin, baroreceptor reflex, and fluid intake[65-70]. If ANG II is a messenger which encodes hypovolemia, then the blockage of ANG II will abolish homeostasis action. Deletion of angiotensinogen, which is the precursor of RAS, causes death before weaning, and has been shown to impair in the kidney, while, increased copy of angiotensin increased blood pressure as well as hypertension[71]. Other studies showed reported low blood pressure in another type of angiotensinogen deficient mice[72]. Along with blood pressure regulation, the defect in urine dilution mechanisms was also characterized by the angiotensinogen mutant mice[73].

Then, what will happen when the interaction between ANG II and effectors are blocked? Due to the fact that ANG II induces homeostatic response mediated by the activation of ANG II receptors, studies of the antagonist or knockout of the ANG II receptor were conducted to prove the role of ANG II as a physiological messenger. Studies have identified at least two distinct types of ANG II receptors: AT1 and AT2[7,74,75]. An antagonist of AT1, losartan, was able to abolish the effect of ANG II-induced homeostasis response[76-81]. The knockout of a subtype of AT1, AT1a, had the phenotype of hypertension, low plasma vasopressin, and high excretion of water[82]. Knockout of AT1a mice were not able to respond to water deprivation and excreted water, although elevated vasopressin level was similar[83]. A knockout of another subtype of AT1, AT1b, showed minor effects in fluid regulation, but double knockout of both subtypes of AT1a and b showed the severe phenotype of fluid regulation failure[84].

On the other hand, AT2 receptors are also demonstrated using antagonists to be involved in the fluid homeostasis[81,85,86]. Although knockout of AT2 has not shown significantly in daily intake of water as in AT1 knockout, it was observed that water-restricted animals show reduced water intake than wild-type[87]. For blood pressure, the deletion of AT2 had high basal blood pressure and was not able to control blood pressure after acute ANG II infusion[88]. Together, it was concluded that ANG II mediates various effectors to regulate body fluid.

Later, I will review the modulation of ANG II in the CNS. It is well established that ANG II is a critical molecule inducing fluid intake behavior. Intracranial injection of ANG II was able to trigger central mechanisms including vasopressin secretion and fluid intake[7,67-69,81]. However, the source of ANG II for the central mechanisms remains debatable. The generation of ANG II is suggested by two pathways: systemic and central. First, ANG II is generated by the peripheral

and transmits the fluid imbalance signals to the CNS by circulation. The expression of ANG II receptors in the CVOs makes sense in this view. Although it is a supraphysiological amount of ANG II, systemic administration of ANG II was able to induce fluid intake[66,70,89]. However, ANG II receptors are also found in brain regions with blood brain barrier[90-93]. Also, the local generation of renin and the angiotensin-converting enzyme is variously found in different tissues[94,95]. These results raise another possibility that ANG II is generated within the neuron. This local generation of ANG II is more like a neurotransmitter rather than a neurohormone[96]. A study also reports the production of ANG II within the intracellular fluid, which makes ANG II as an intracrine, not endocrine system[97].

*B) Aldosterone*

One of the other major impacts of ANG II is the production of a mineral corticoid hormone, aldosterone. Aldosterone is a mineral corticoid hormone, which is synthesized in the glomerular zone of the adrenal cortex[98]. Aldosterone secretion is increased mostly because of the evaluate ANG II in hypovolemia[7,99]. Interestingly, it was also shown that water-deprived animals maintain a low aldosterone level until water was provided. Probably, this is because the repair from the hyperosmolality led to hypovolemia. Otherwise, adrenocorticotropin and potassium regulated the aldosterone level[100].

Aldosterone was largely studied to induce genomic changes to increase sodium retention. Aldosterone upregulates the channels, including luminal ENaC and potassium channels, and serosal Na+/K+-ATPase for sodium reabsorption[101]. Another example of aldosterone effectors effect is the increased transcription of the Na+/H+-exchanger, which is the luminal thiazide-sensitive Na+/Cl− cotransporter (NCC) of the colon and kidney[100,102,103]. These genomic

changes from aldosterone conclusively drive sodium reabsorption and potassium excretion in the kidney to conserve sodium.

On the other hand, aldosterone was demonstrated to show rapid non-genomic actions. Aldosterone significantly changed systemic vascular resistance and cardiac output within 3 min while genomic action is expected to occur after at least 30 mins[100,104]. The effector of the non-genomic pathway of aldosterone for the fluid balance is the luminal Na+/H+-exchanger and potassium channels[105]. However, the receptors of the non-genomic action of aldosterone and the role of the non-genomic in systemic level remain to be investigated.

The deficiency of aldosterone causes impairments in the body fluid. In human clinical studies, diseases such as Addison's disease implied that the lack of aldosterone impairs urine concentration, and in some cases, increases sodium intake[7]. Furthermore, experimental demonstration of the ablation of the adrenal gland by Richter in rodents was able to reproduce symptoms, including increased sodium intake, which was shown in aldosterone deficiency patents. The adrenalectomy animals have shown high sodium loss and were not able to survive without supplementary sodium[106]. The knockout of the aldosterone synthase mice demonstrated low plasma sodium and high plasma potassium to sodium restriction. These animals also had impaired sodium reabsorption along with low volume and osmolality of urine[107]. Although the animals did not survive after 2 weeks, the deficiency of mineralocorticoid receptors (MR) also demonstrated similar phenotypes of adrenalectomy, like hyperkalemia, hyponatremia, and the renal excretion of sodium ion[108]. The antagonist studies also reconciled the sodium diuresis in the blockage of MR[109,110].

On the other hand, it has been demonstrated that excessive aldosterone also triggers abnormal sodium intake in rodents[111]. Human clinical studies also reported excessive mineralocorticoid patients to show syndromes of sodium intake and high blood pressure[7]. Further studies suggested one of the causes of this excessive aldosterone secretion is due to the failure control of the affinities of aldosterone-MR, which is mediated by HSD2 11-β-hydroxysteroid dehydrogenase 2 (HSD2)[112]. Additional studies have demonstrated colocalization of HSD2 with MR in target tissues[113]. The specific knockout of HSD2 in the brain was able to show an increase in sodium intake[113]. It was proposed that HSD2 increases the affinity of MR-aldosterone by inactivating the glucocorticoid[114,115]. Together, aldosterone is a critical endocrine involved in fluid balance.

*C) Vasopressin*

Vasopressin is an antidiuretic nonapeptide hormone, particularly synthesized from the central nervous system[116]. The secretion of vasopressin is mediated by the neural circuit unlike other endocrines. Vasopressin-expressing neurons were proposed to detect osmolality changes from the circumventricular organs[3]. When vasopressin has a critical role in fluid homeostasis, it is also involved in social and sexual behavior[117,118]. It was shown vasopressin release is increased when the osmolarity is decreased or the extracellular volume is decreased[20,24,116]. At least a 40% reduction of blood volume showed significant secretion of vasopressin[119].

The role of vasopressin was implied in a disease called diabetes inspidius, which is characterized by high water intake due to the excessive excretion of water[120-122]. Vasopressin was able to reverse high water intake symptoms, blocking the water excretion[123]. It was demonstrated that Brattleboro rats had hereditary diabetes insipidus, which is caused by a single base deletion in

the vasopressin precursor[124]. In humans, it was found the patient with nephrogenic diabetes insipiudus was shown to carry a mutation in the vasopressin receptor 2 gene[122].

As vasopressin was found to be involved in fluid regulation, vasopressin activate the water retention via the effectors from the kidney and circulatory systems. When the knockout of vasopressin has low durability to be characterized[125], further investigations were held on the receptors of the vasopressin effectors. There are three subtypes of vasopressin receptors; V1a, V1b, and V2. The receptors are categorized based on the cellular cascade; V1a and V1b is mediated by Gq proteins which activate phospholipase C activity; V2 mediated by Gs proteins, and acts through the cyclic AMP system. Each receptor has a role in fluid regulation[126]. Knockout studies of V1a demonstrated low basal blood pressure. It was also observed that these knockout animals had impaired baroreceptor reflex-like basal levels of atrial natriuretic peptide[127]. On the other hand, V1b knockout mice were shown to have high baseline daily water intake and urine volume, but no change in water intake and urine volume was found upon water deprivation[128]. The role of V1b is known to regulate pituitary adrenocorticotropin and corticotrophin-releasing hormone. Consistently, The knockout of V1b has low basal circulating adrenocorticotropin and corticotrophin-releasing hormone[129]. In addition, V1b knockout also reported decreased aggression[130].

Lastly, the V2 receptor distinctly expressed in the kidneys. The defect of V2 is one of the causes of diabetes insipidus, which results in excessive loss of water[131]. The vasopressin increase reabsorption of water via the activation of the V2 receptor in collecting duct cells of the kidney. The activation of V2 receptors translocates vasopressin-regulated water channel aquaporin2 in the collecting duct[132]. This increase in permeability allows water reabsorption.

*Peripheral system*

Peripheral detection of fluid imbalance can also be transmitted via neural pathways. The information is a relay in nerves such as vagus and glossopharyngeal nerve, and reaches to the CNS[133]. The denervation of the nerves which respond to osmotic change have shown impairments in intake behavior after fluid imbalance[133-140]. However, it is not clear whether intake behavior is a reduction of detection of fluid imbalance or the lack of feedback from ingestion. Numerous studies have demonstrated a visceral pathway's role, especially the hepatic osmosensory and gastrointestinal tract, for the detection of the ingested load, while the baroreceptors were proposed to monitor the body fluid. I will discuss the gastrointestinal tract and hepatic portal vein later as feedback for ingestion.

In terms of fluid imbalance detection, the baroreceptors detect the fluid balance and convey via nerves. Baroreceptors are located in the carotid sinus nerve, and aortic depressor nerve. Baroreceptors are expressed on the afferent of the glossopharyngeal (carotid) and vagus (aortic arch) nerves, in which the cell bodies exist in the petrosal and nodose ganglia[17]. Denervation studies were demonstrated to increase the variability of arterial pressure and impair baroreflex[139,140]. A recent denervation study identified that the double knockout of piezo1 and piezo 2 channels in the petrosal and nodose ganglia blocks the detect of the afferent and facilitates reproduction of increased variability and blunt baroreflex[49]. Meanwhile, the activation of the piezo2-expressing neurons using ChR2 immediately reduced blood pressure[49,52].

## 1.4. Fluid ingestion: Thirst and sodium appetite

Fluid homeostasis regulates the income and outcome of the solvent and solute. Mainly, the kidney controls the retention of water and sodium. However, there is a limit to maintain water and sodium, especially when the animal encounters depletion. In this case, when water or sodium is required, such nutrients must be obtained from the external source. A central neural mechanism is involved in inducing specific and correct intake behavior. The animal must know what exact nutrient is lacking in the body, which makes the intake behavior a goal-orientated behavior. In fluid homeostasis, animals develop a specific appetite toward water or sodium. When thirst is a desire to drinking water or liquid, sodium appetite is a desire for sodium[132]. Each appetite has a distinct mechanism to be modulated. Here, I will review how fluid intake behavior can be induced and modulated by physiological signals.

*Thirst*

The appetite that drives water consumption, thirst, is one of the main behaviors to obtain the solvent of the body fluid. Physiological signals that trigger thirst were largely researched with in the emerging studies of both endocrinology and neuroscience[7,33,141-145]. Furthermore, studies have suggested that thirst is not only regulated by homeostatic signals from depletion but also by anticipatory signals that can potentially induce water deprivation, such as circadian rhythm and post-ingestion signals[33,141,145,146].

Thirst is generally viewed as a homeostasis response toward fluid challenges by osmolality and volumetric stimuli, known as osmotic thirst and volumetric thirst[147]. Injection of high osmolality, which induces increased solute concentration, including hypernatremia to animals, was able to induce immediate and robust water drinking. On the other hand, volumetric stimuli such as bleeding and sodium deficiency also trigger water intake along with sodium intake[148]. Then, how

does the stimulus trigger the activation of the CNS? One mechanism is the osmolality change

can be directly detected by the central osmosensory. Water deprivation or osmotic stimuli

increases the plasma osmolality[149-151]. It was proposed that the central osmosensors are located

outside the BBB. Hence, the CVOs were suggested as a site of osmosensory. Further research

found osmotic response in the circumventricular organs, which were critical in thirst[48,152,153]. On

the other hand, osmolality changes can be detected in the peripheral osmosensors. These sensory

mechanisms will be more focused on this review as a feedback mechanism of the ingestion.

In additional to the central osmosensory mechanism, it is expected that the endocrine signals

trigger thirst. For the hypovolemia, physiological signals such as ANG II are the critical regulator

driving the thirst[63-66]. The ANG II receptor was found in the circumventricular organ and also

found in the brain region within the BBB[154,155]. The intracerebroventricular injection of ANG II

was sufficient to drive immediate thirst[67]. Meanwhile, the blockage of the central ANG II

receptor largely attenuated thirst[156,157]. Furthermore, direct ANG II injection into the CVOs was

able to produce drinking[158,159], while lesion of the CVOs was able to abolish intravenous

angiotensin drinking[160,161]. Therefore, ANG II has a critical role in water-drinking behaviors.

Thirst can be triggered by an anticipatory factor that is triggered ahead of the consequence of

other types of homeostatic regulation. These feedforward response trigger thirst without actual

fluid imbalance[33]. For instance, prandial thirst, is a mechanism to prevent the solute load before

it impacts the body fluid[150]. Another remarkable example is that circadian factors can also induce

thirst [33]. Circadian-induced thirst was independent of serum osmolality. Furthermore, it

demonstrated regulation of the central neural circuit of the circadian clock. Particularly, the

suprachiasmatic nucleus can drive thirst neurons to induce thirst[162].

*Sodium appetite and hypovolemic thirst*

Sodium is an essential and a main cation for various function of the body. When the body sodium, but not necessary serum sodium level, is depleted the animal developed a specific appetite toward sodium, knowns as sodium appetite[5]. In the view of fluid homeostasis, sodium is the principal cation in the ECF. Therefore, sodium is one of the main targets for fluid homeostasis. The lack of sodium can drive the body fluid's extracellular dehydration, and to compensate the dehydration, it induces the decrease in body fluid, hypovolemia. Sodium appetite is one of the outcomes of the hypovolemia-induced intake in fluid homeostasis.

Some hypovolemic stimuli such as polyethylene glycol and formalin were able to induce delayed sodium intake occupied with water intake[163-165]. This is could be explained by slow procedure of sodium loss. On the other hand, pure sodium appetite can be facilitated using loop diuretic, furosemide, with a sodium deficient diet[166,167]. Although, sodium appetite expressed animals did not necessarily have low plasma sodium concentration[5,168]. It seems that sodium appetite does not have a direct osmosensor like thirst. Though it was reported that infusion of mannitol cerebrospinal fluid increases sodium intake, it is still not as rapid as hypotonic sodium triggered thirst[169]. It seems that sodium appetite is activated by a secondary messenger to activate the central neural circuits instead of the direct detection of low osmolality or sodium level.

Sodium appetite was first experimentally characterized by Richter. Robust sodium intake was demonstrated by the ablation of the adrenal gland in rodents[106]. Further studies identified that aldosterone from the adrenal gland was a key element for sodium appetite. Adrenalectomized rat's high sodium intake phenotype were rescued by the injection of deoxycorticosterone, a

precursor of aldosterone[111]. It was explained that the absence of aldosterone causes the excretion of sodium, then the loss of sodium motivates a drive toward sodium intake.

Ironically, further studies found that high dose injection of deoxycorticosterone was able to induce sodium intake in rodents[111,170,171]. The explanation for the high dosage of aldosterone induced sodium intake was proposed as a result of the central action of aldosterone. It is expected that aldosterone penetrates the BBB because aldosterone is a steroid hormone. However, experimental demonstrations showed that aldosterone actually has a poor penetration rate with the BBB[115,172]. Even worse, corticosterone, which competes with aldosterone, had a higher penetration rate than aldosterone[173]. Although a potential mechanism might exist to increase the chance that aldosterone can interact with the CNS[115], it remains as a question whether the aldosterone interacts with the CNS. Unfortunately, unlike ANG II, mineral corticoid receptors had a low expression in the circumventricular organs[92,93,174]. However, research was able to identify mineral corticoid receptors in the central amygdala, and the nucleus of the solitary tract was activated after chronic infusion of aldosterone[175-177]. I will discuss in the next chapter the central neural circuits related to this discovery.

On the other hand, sodium intake in adrenalectomy mice which lacks aldosterone could not be explained by aldosterone. An alternative explanation for this paradox was ANG II[178,179]. The paradox was able to be explained by ANG II-inducing sodium appetite. It was found that ANG II is increased in sodium-depleted animals[180]. Consistently, studies have shown that a high dose of intracranial ANG II was able to induce not only water intake but also sodium intake[68,69]. There is a conflicting view of whether ANG II contributes to sodium appetite origin from the peripheral or the central system. A number of studies have demonstrated that the intravenous

injection, not central, of captopril, an antagonist of the ACE enzyme, was able to reduce sodium appetite[181-183]. Conversely, other studies have shown the opposite result, that the central administration of captopril, not peripheral, was not able to abolish sodium intake[184-186]. Both results can be interpreted differently based on incomplete blockage of ACE enzyme, but it seems both components of the RAS can contribute to the generation of sodium appetite[187].

Sudies on the observation of the involvement of ANG II and aldosterone, suggested that sodium appetite is formed by a synergy of ANG II and aldosterone. It was demonstrated that the blockage of the aldosterone was not sufficient to reduce sodium appetite completely, but that concurrent inhibition of ANG II abolishes sodium appetite[188], whereas intracerebroventricular infusion of ANG II exhibits sodium intake with chronic deoxycorticosterone injection[189]. Another explanation for sodium appetite is the disinhibition theory[190]. The experimental demonstration that intracranial injection of ANG II can elicit both water and sodium intake, given an idea that sodium intake tonically suppressed and can be disinhibited to induce sodium appetite. Central oxytocin[190] and serotonin[191] were proposed to account for the inhibition of sodium intake. These complicated interactions between physiological signals may explain the slow induction of sodium appetite compared to immediate thirst mediated by intracranial ANG II and hypertonic saline[7].

The anticipatory factor of sodium appetite is not well defined. Studies have reported need-free (non-homeostasis) sodium intake. These need-free sodium intakes were developed after experiencing repeated sodium depletion. ANG II and aldosterone levels were not increased during the need-free status[192]. More research reported increase in sodium intake during pregnancy and lactation, which requires extra ECF[193].

Sodium appetite and thirst are not exhibited unless the body recognizes the need for sodium or water. For example, animals do not drink high concentrations of sodium because it is aversive[44]. However, when animals are sodium depleted, this aversive response is switched to attractive toward sodium. So far, I have overviewed the physiological aspects of sodium appetite and thirst. Physiological signals were able to activate the central mechanism of appetite. Then, how does the internal state form an appetite for a specific nutrient? It is expected that a central mechanism is involved to form these goal-oriented intake behaviors. To investigate these neural principles underlying appetite, pinpointing the neural circuit of appetite is required. I will further discuss this in the next chapter related to the central mechanism of appetite.

## 1.5. Feedback and feedforward mechanism of ingestive behavior

Although the animal can generate appetite and satisfy its need, the animal encounters another challenge: how much to consume. An excessive amount of consumption will again result in fluid imbalance. Then, the body must proceed another round to recover from the ingestive consequence. However, the consumed nutrient cannot impact the body fluid on a real-time basis, especially in a multicellular organism level due to the multi barriers and procedures. It is known that intake behaviors are finished ahead of the ingested solution, reflecting fluid homeostasis[194-196]. Therefore, animals evolved to monitor ingestion instead of awaiting the net effect of the body fluid to estimate the post-ingestive consequences[33,145]. This information led to the termination of a meal, called satiation[142,143,146].

How can the ingested fluid be monitored to get an estimate of consumption? It was demonstrated that various mechanosensory and chemosensory systems from the oral cavity like oropharyngeal and gastrointestinal tracts send signals and reduce intake[33,141,142,145,146,150]. The

ingestion sensory information is integrated into the nervous system and provides feedback to the CNS[197]. In the perspective of behavior, these ingestive sensory are a feedback to the brain, but they is also a feedforward regulation in terms of prediction of the post-ingestion consequence[141]. Here, I will review the peripheral detection of the ingested fluid, which regulates the fluid intake.

*Oral cavity: Taste*

The oral cavity is the first contact-based detection occuring in ingestion. Mainly in the oral cavity, the taste system is the primary gate to distinguish and trigger specific behavioral outputs, such as drinking and eating. Distinct tastes are specifically detected by taste receptors: sweet, bitter, sour, salty, and umami[198]. The taste receptor cells carry the chemosensory information to the chorda tympani and greater superficial petrosal nerve, which projects to the NTS[43].

In addition to the basic five tastes, a recent study suggested that the removal of bicarbonate triggers a water response[199]. A polycystic-kidney-disease-like channel (PKD2L1), which is described as taste receptor cells for sour[200], was identified as the main receptor for water response. Chorda tympani nerve recording showed that the genetic knockout of PKD2L1 abolished water-evoked responses in taste nerves. It was also characterized using a behavior assay when ChR2 was expressed in the PKD2L1 cells. Only water-deprived animals continuously licked light without water. However, the activation of PKD2L1 cells in the oral cavity did not show a satiated effect on thirst. The animal was continuously licking the light even though it was an empty spout[199]. It seems that PKD2L1 cells are more involved in the detection of water rather than the feedback mechanism for thirst satiation.

The feedback mechanism was demonstrated in sodium taste upon sodium appetite. The salt pathway was demonstrated to be mediated by different pathways. One pathway is mediated by ENaC. It was demonstrated by blocking the ENaC channel using amiloride or knockout of ENaC, abolishing the sodium response in the chorda tympani nerve[46]. Meanwhile, a high concentration salt triggers the aversive pathways of bitter and sour[44]. It was further demonstrated that the ablation or blockage of ENaC definitively abolishes sodium intake in sodium-depleted animals[46]. Sodium taste was not only important for sodium detection but also for sodium satiation. Sodium-depleted animals consumed the same amount of sodium regardless of quality of sodium[201,202]. Meanwhile, the direct infusion of sodium into the stomach bypassing the oral cavity did not mediate strong suppression of sodium appetite unless a long-duration was applied, whereas normally, ingestion of sodium shows quick quenching of sodium appetite[202-204].

*Oropharyngeal and gastrointestinal tract*

Outside of the oral cavity, the oropharyngeal and the gastrointestinal tract provides the chemical or mechanical sensory of the ingestion to the CNS[205]. Early studies have suggested that oropharyngeal signals might be involved in satiation[194,206]. It was demonstrated in dogs that the oropharyngeal motor activity and swallowing was a critical factor in reducing the secretion of vasopressin drastically. Drinking and vasopressin level reduced before the water left the stomach. Meanwhile, eating food that contains water did not reduce the vasopressin secretion, although it immediately satiated thirsty animals[194]. The same phenomena were observed in humans[206]. The intragastric load of water, bypassing the oropharynx, also had no effect on vasopressin secretion[207], although a recent study has shown reduced neural activity in vasopressin neurons in the supraoptic nucleus after intragastric loading and water cue in mice[208,209]. The

oropharynx signals are transmitted to the glossopharyngeal and vagus nerve[210], whether the denervation eliminates the rapid inhibition of vasopressin secretion from water ingestion will require further investigation.

Furthermore, stimulation of the gastrointestinal tract was able to reduce appetite in deficient conditions[202,204,209,211-214]. Chemosensory inputs from the gastrointestinal tract provide the information of the osmolality and nutrients. The intragastric infusion of water in a water deprivation animal was able to satiate thirst within few minutes, whereas saline did not affect the water intake[209,214]. Meanwhile, the gastrointestinal tract seems to have a minor role in sodium satiation. The intragastric infusion of sodium for sodium appetite did not affect sodium satiation. After a certain amount of time, the intragastric sodium was able to suppress sodium appetite, however, whether this is due to the gastrointestinal tract or absorbed sodium is unclear[202].

The gastrointestinal tract is predicted to innervate this information to the vagus nerve. Vagotomy results show some impairment in food and fluid intake[135,215,216]. Recently, it was shown the vagotomy was able to reduce the satiation effect of intragastric infusion in thirst[209]. Previous studies in the vagus nerve conducted various responses upon the different qualities of ingestion load[217]. However, the effect of denervation requires further investigation because the vagotomy could also affect the efferents[205]. Whether the gastrionitestinal tract sends distinct signals upon specific type of nutrients[218] will require additional investigation.

On the other hand, mechanosensation such as distention also provides a feedback for satiation[55]. The distension of the stomach by inflation of stomach balloon was able to partially reduce water intake but still less than water infusion, but this effect was abolished after vagotomy[219]. A recent study has identified the vagus nerve, response to the stomach or intestinal stretch. Furthermore,

the activation of the genetically-identified neurons was able to change gut motility[220]. Other studies have identified this distension information conveyed from vagus to brainstem and hindbrain[220,221].

*Hepatic portal vein*

The hepatic portal vein was proposed as an osmosensory site due to its connection to the spleen and gastrointestinal tract to the liver. The hepatic portal vein is one of the windows to monitor the ingested fluid before the body. It was demonstrated that water infusion into the hepatic portal vein induces diuretic response[222]. When a neural pathway from the hepatic to the lateral hypothalamus was suggested[223], it was identified that vagal and spinal afferent innervates to the hepatic portal vein[224]. The vagal afferent was demonstrated in response to osmolality change. In consist, the hepatic vagotomy shown impaired regulation of urine concentration against acute osmotic change[138].

Other studies also suggested that the splanchnic afferent pathway is also involved in osmosensory of the hepatic portal vein[225]. Further research has identified that the splanchnic afferent mediates osmosensing via TRPV4 channels. Isolated thoracic dorsal root ganglia neurons, calcium influx was found in response to hypoosmotic stimulation. The hypoosmotic response was abolished when TRPV4 was blocked or genetically deleted[37]. With other nerves, the hepatic portal vein also requires precise dissection and in vivo experiments to demonstrate the correlation of osmosensory and feedback regulation of ingestion.

How the feedback information from the vagus afferent conveys to the CNS is also largely studied due to the recent genetic approaches. It is now possible to approach dissection of the

vagus nerve genetically. Already, studies of selective recording and manipulation in the vagus nerve has been published[49,51,52,220,226-228]. Understanding the feedback mechanism will further increase the understanding of what signals can conduct satiation for appetite.

## 1.6. Conclusion

Appetite is not a reflex to increase the amount of intake, but is more a complicated mechanism. We have viewed appetite as an action of homeostasis, which involves detection, signaling, feedforward, or feedback mechanisms. Appetite is expected to be managed by the CNS because of its goal-oriented aspect. Further understanding of how intake is controlled will require identifying central appetite circuits. Understanding the central appetite circuits will not only provide us an insight into the physiological input regulation but also into the neural principle of how central circuits form behavior. In the next chapter, I will review and conduct the neural circuit for sodium appetite.

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*C h a p t e r   2*

# CENTRAL NEURAL CIRCUIT OF SODIUM APPETITE

Lee, Sangjun, et al. "Chemosensory modulation of neural circuits for sodium appetite." Nature 568.7750 (2019): 93-97. https://doi.org/10.1038/s41586-019-1053-2

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After the experimental demonstration of sodium intake by Richter, further studies identified the physiological signals underlying sodium appetite[1-3]. This raises a general question of whether a dedicated neural circuit for sodium appetite exists. Series of histology, lesion, and pharmacology approaches have shown multiple brain regions, including the lamina terminalis, hindbrain, and extended amygdala are involved in sodium appetite[3-5]. It was not a surprise that multiple brain regions are involved since sodium appetite is a complex behavior that includes neural principles such as motivation and satiation. However, to pinpoint the central mechanism, it is necessary to find the fundamental unit of sodium appetite, which can replicate the sodium appetite by the neural activation. With the development of neural tools, it is possible to directly label and stimulate a genetically defined neural populations[6]. This paradigm allows investigating the causal relationship between the neural activity of the defined neural population and sodium appetite. Taking advantage of previous enriched studies, I was able to investigate neural populations for sodium appetite.

## 2.1. Introduction: Search for sodium appetite dedicated neurons

*Relative region based on the lesion, electrical stimulation, and histological studies*

Enriched physiological studies highlighted the possibility that physiological signals, like endocrine, can activate the central neural system (CNS) to trigger intake behavior. The circumventricular organs (CVOs) which lack the blood-brain barrier (BBB) were primarily studied as a window to interact with physiological signals[7]. Also, the CVOs expressed angiotensin (ANG) II receptors, when ANG II is a major hormone related to fluid balance[5]. Numerous studies showed that the lamina terminalis demonstrates increased neural activity after sodium depletion by expression of the immediate early gene, including c-Fos [8-10].

Furthermore, the ablation of the CVOs in lamina terminalis (LT), including the subfornical organ (SFO), vascular organ of lamina terminalis (OVLT) shown impair in sodium intake with various hypovolemic stimulus[11-15]. Conversely, other studies were not able to observe a similar impair in sodium intake[14,16]. Although the different stimuli and various effects of the lesion were shown, the lesion studies were able to show the involvement of LT.

In addition to the LT, the area posterma, which also lacks BBB in the hindbrain, was also implied to be involved in sodium intake. Histological and lesion studies have suggested the potential role of the area posterma in sodium intake[17,18]. Some studies reported increased c-Fos expression after sodium depletion and increased activation of aldosterone related receptors near the area posterma[19]. The lesion in the area posterma was demonstrated to increase sodium intake in sated animals[20]. Another region in the hindbrain, the parabrachial nucleus, was suggested to be a relay for sodium appetite[21]. Studies have shown that the lesion of the parabrachial nucleus enhanced

sodium appetite[22]. Furthermore, studies were able to define neural population in the surrounding area of the locus coeruleus (pre-LC) and lateral parabrachial nucleus that expresses forkhead box protein P2 (FOXP2) overlapped with c-Fos expression induced by sodium depletion[23,24].

Besides the hindbrain and the CVOs, diverse brain region were also suggested to be involved in sodium appetite, including the bed nucleus of the stria terminalis (BNST), medial and central amygdala, thalamus, lateral hypothalamus, and zona incetra[25-37]. However, the heterogeneity of the brain remained a caveat. Most brain regions contain both excitatory neurons and inhibitory neurons and mediate diverse functions. Studies had controversial results depending on the preciseness and degree of the lesion. Therefore, to further investigate the neural principle of sodium appetite, it is required to selectively target and manipulate the neural population even within the same brain region.

*Pharmacological approaches of the neural circuits for sodium appetite*

Instead of stimulation or lesion of a brain region, studies used a pharmacological approach such as infusion of hormones or neural transmitters to dissect the circuit within a brain region. Because of the known physiological signals underlying sodium appetite, the neural circuitry of sodium appetite was invesigated from the assumption that physiological signals ANG II and aldosterone directly interact with the CNS[2]. Fortunately, researchers identified ANG II and aldosterone receptors in various brain regions, both with and without BBB[38-41]. ANG II receptors were expressed in different regions, including brain regions lacking the BBB, the LT, and area posterma[38,39]. Regardless of the origin of ANG II, the high dose of intracranial injection of ANG II drives a robust water and sodium intake[42,43]. It seems to be debatable that the high

dosage of angiotensin can trigger secondary natriuresis[42]. However, when the lower concentration of ANG II were intracranially injected, the animal favor water than sodium[44].

In parallel, several brain regions, including the central amygdala, hippocampus, and septum were found to express aldosterone-receptors or related proteins[40,41,45]. Previously, the injections of antisense oligodeoxynucledotide, antagonist to the genomic action of mineralocorticoid receptor, into the amygdala suppress desoxycorticosterone-induced sodium appetite[28,46]. Surprisingly, central implantation of aldosterone into the amygdala induced a rapid nongenomic activation of sodium intake, whereas exogenous aldosterone could induce sodium intake when it was chronically administered[28]. Another study has identified a neural population surrounding the region of the area posterma, the nucleus of the solitary tract (NTS) interact with aldosterone[19]. These neural populations were found activated in sodium-depleted animals and co-expressed corticosteroid-11-beta-dehydrogenase isozyme 2 (HSD2). The expression of HSD2 indicated that direct aldosterone interaction might occur with this population[47]. Consistently, it has been shown that the chronic infusion of aldosterone into 4th ventricular can activate these populations and further increase sodium intake without changing water intake[48-50]. Also, mineralocorticoid antagonist RU 28318 infusion into 4th ventricular attenuates sodium intake, but not the into the lateral ventricular[51].

Another approach to understanding the neural basis of sodium appetite is identifying the dedicated neurotransmitter. Neural peptides such as opioids were implied to be involved in central sodium regulation[52]. Further pharmacological studies pinpointed that mu-opioid signaling in the central amygdala is involved in sodium appetite. Research showed that direct administration of mu-opioid receptor antagonists but no other types of opioids abolished

sodium intake[53]. Meanwhile, the agonist was able to increase sodium intake along with water[54].

Another brain region, the parabrachial nucleus, was widely studied to research the neural transmitter dedicated to sodium appetite. Consistent with lesion studies[22], a study demonstrated that the infusion of antagonist or agonist of neural transmitters such as oxytocin, GABA, and serotonin into the PBN accurately could manipulate the sodium intake [55-60]. When methysergide, an antagonist for serotonin receptors 1 and 2, was bilaterally applied into the parabrachial nucleus, and the ANG II-induced drinking amplified both saline intake and water intake[58]. The parabrachial nucleus seems to have a general role in controlling fluid intake. It was further proposed that the parabrachial nucleus tonically suppresses sodium appetite. Therefore, sodium appetite was explained by the disinhibition of the parabrachial nucleus. This disinhibitory-induced sodium appetite was first suggested from an experiment that showed pre-administration of oxytocin receptor antagonist in lateral vesicular was able to increase the saline intake of intracerebroventricular angiotensin injection[61]. It was suggested angiotensin originally encoded both water and sodium drinking, but sodium drinking was suppressed unless the inhibitory inputs were released.

The pharmacological approaches have significantly provided insight into the central action within the brain region along with the cell-type profile and a further implication of a molecular mechanism. However, whether the ligand-receptor interaction drives the neural activity remains as a caveat during the manipulation, especially because the temporal resolution is not clear. The application of ligand does not guarantee the immediate neural firing of the selective neurons due to the cellular pathways. Also, neurotransmitters or neuroendocrines can induce secondary effect during infusion. For example, aldosterone and ANG II could result in secondary effects like urination, which could also influence saline intake. While searching for the functional unit of

sodium appetite, it remains a challenge to search for a neural population that has a direct correlation between the neural activity of the defined neural population and sodium appetite.

*System neurobiological approaches of the neural circuits for sodium appetite*

Due to the recent development of neural tools, now it is possible to target specific neurons based on the genetic profile. Such genetic tool became a powerful tool to investigate the functional role of neural populations. If the genetic profile is identified, Cre recombinase drivers and viral methods can be used to access a specific neural population. Furthermore, the neural activity of genetically defined neurons can be monitored or manipulated using genetically engineered proteins. For manipulation, Channelrhodopsin-2 (ChR2), a cation channel can be opened by a specific wavelength light or ligand. These tools can trigger time-locked excitation or inhibition by the control of light. In this way, instead of the consideration of the ligand-receptor and intracellular cascade dynamic activation, it is possible to directly activate the neural population and investigate behavioral or other functional outputs. While, for neural activity observation, calcium sensors, green fluorescent calmodulin M13 fusion protein (GCaMP), are used to visualize the change of calcium flux during neural activation. The GCaMP recording facilitates population recording on a real-time basis using fiber photometry[62]. Together, the system neuroscience approach simplifies (sometimes oversimplifies) the neural activity with functional outputs.

Recent studies have shown the existence of dedicated neurons for thirst in the LT[63-65]. Along with the previous studies that ANG II receptors are expressed in the CVOs, researchers investigated the loss-of and gain-of-function of defined neural populations in sodium intake. A study has performed selective ablation of ANG II receptor in the SFO, and demonstrated

impairment in sodium appetite[25]. Further dissection was held using the projector patterns. The role of individual trajectory neurons was able to be investigated by the photostimulation of the terminal instead of the cell-body[66]. It was identified that subpopulation of ANG II receptor (Agtr1a)-expressing neurons (mostly overlap with nNOS) in the SFO, projects to the ventral BNST (vBNST). In this research, they show the inhibition of this population (SFO$^{Agtr1a}$→vBNST) has reduced sodium intake in the sodium-depleted animal. The activation of this population showed an increased preference toward sodium intake in the water-deprived animal, although sodium intake in sated animals was not demonstrated[25]. Together, the subpopulation of SFO excitatory neurons, which specifically projects to ventral BNST, was necessary but not sufficient to drive sodium appetite.

In parallel, the neuronal population which expressed HSD2 in the nucleus tractus solitarius was investigated. Previous studies heavily supported that this neural population in the nucleus tractus solitarius will be involved in sodium intake and be activated by administration of aldosterone [48,51]. Further study identified that sodium depletion involved genomic change of ion channel composition of the NTS$^{HSD2}$ neurons, HCN, and Nav1.5 channels, which creates spontaneous pacemaker-like activity[67].

The functional role of NTS$^{HSD2}$ neurons was investigated. The selective ablation of NTS$^{HSD2}$ neurons using caspase3 significantly reduced sodium appetite[67]. Although another study showed that the chronic inactivation of NTS$^{HSD2}$ neurons were lethal, the acute inhibition of NTS$^{HSD2}$ neurons using chemogenetics lowered the sodium intake[68]. Together, NTS$^{HSD2}$ neurons were suggested to be necessary for sodium appetite, which matched with the previous antagonist result[51].

However, the sufficiency of NTS[HSD2] neurons in sodium intake required additional interpretation. One study demonstrated that the activation of NTS[HSD2] neurons increased sodium intake when the animal was in the home-cage environment during the dark cycle[68]. Another study did not observe sodium intake unless under the same condition from the other study[68] or concurrent signals of ANG II, such as injection of ANG II or water deprivation[67]. This requirement of concurrent conditions was explained by the synergy of ANG II and aldosterone in sodium appetite, meaning that the activation NTS[HSD2] additionally requires the activation of ANG II to induce sodium intake[2]. However, increased ANG II has not been observed under those condition[68], which showed robust sodium intake in home-cage environment[67]. Meanwhile, slice recording in HSD2 neurons demonstrated that HSD2 neurons increase firing upon acute ANG II but not aldosterone. The increased firing was observed when aldosterone was chronically applied via minipump[67]. It made sense that chronic aldosterone administration was required to induce sodium intake, however, with the behavior result, it remains unclear whether the HSD2 neurons play a central node of the central ANG II and aldosterone.

A few other candidates of a neural circuit of sodium appetite, more focused on the suppression of sodium intake, were proposed in the lateral parabrachial nucleus. Oxytocin receptor-expressing neurons that are distinct from calcitonin gene-related peptide (CGRP) neurons, which are known to suppress appetite, were identified to induce the satiation of thirst and sodium appetite[59,69]. Similarly, serotonin 2c receptor-expressing neurons were proposed play a role in tonic suppression of sodium intake. These populations overlapped with CGRP neurons but were distinct from Oxytocin receptor expressing-neurons. The inhibition of serotonin 2c receptor-expressing neurons was able to induce sodium appetite, but not water intake. However,

for the activation of serotonin 2c receptor-expressing neurons suppress fluid intake in general, and also food intake, which could be explained by the overlap of CGRP neurons[70]. Another recent study also identified that prodynorphin neurons in the parabrachial nucleus, which is distinct from oxytocin and CGRP neurons, can suppress fluid intake. This study further demonstrated the role of the feedback mechanism in fluid ingestion, including digestive tract distension[71]. Together, these populations seem to have a general role in suppression of fluid intake rather than the acute inhibition of sodium appetite.

The introduction of neural tools, including optogenetics, has taken us one step further in understanding appetite neural circuits. Previous studies have identified few candidates for sodium appetite dedicated neural population. However, the sufficiency of these neural populations in sodium intake requires additional conditions to induce sodium intake. These prerequisite conditions remain as a caveat to investigating the neural basis of sodium appetite. It is expected that the functional unit should replay sodium appetite without any additional conditions. In other appetites, such as thirst and hunger, dedicated neurons were identified to drive sufficient appetite without other motivational factors[63-65,72-77]. Therefore, further studies were necessary to identify sodium appetite-inducing neurons.

## 2.2. Characterization of sodium appetite dedicated neurons

To identify sodium appetite neurons, I have started from revisiting the previous histology studies. I first screened immediate early genes expression, c-Fos, in the sodium-deprived rodent brain with furosemide diuresis. As previous studies indicated, I was able to find distinct c-Fos expression in two brain regions: OVLT in the hypothalamus and pre-LC in the hindbrain. I

further investigated genetic markers that overlap with c-Fos positive neurons and characterize

the causal relationship between the neural activity of the identified neurons and intake behavior.

*Characterization of OVLT^PDYN and OVLT^nNOS neurons*

OVLT is one of the CVOs which lacks the BBB[7]. Recent studies were able to induce drinking

by the activation of OVLT neurons[74,78]. OVLT neurons strongly express c-Fos when the animal

was water-deprived or sodium-depleted, while no activation was observed in sated and rescued

animals (Fig. 1a)[79]. Interestingly, a distinct c-Fos expression pattern was observed between water-

deprived and sodium-depleted animals. The c-Fos expression was more spread from the center

in water-deprived animals than sodium depleted animals (Extended Fig. 1a). To further search

for a genetic marker for the sodium depletion induced c-Fos positive neurons, I screened several

genetic markers using immunohistochemistry and Cre driver crossed with fluorescence reporter

lines. I have identified two potential markers that overlap with the c-Fos positive neurons. One

population was neural nitric oxide synthase (nNOS), which previously showed that nNOS-

expressing neurons in the SFO can be activated upon water-deprivation[63,64]. Consistently, nNOS

expressing neurons in OVLT (OVLT^nNOS) were also activated upon sodium depletion (Fig. 1b).

Furthermore, I found that prodynorphin (PDYN)-expressing neurons in OVLT (OVLT^PDYN)

overlapped with the sodium depletion induced c-Fos expression (Fig. 1c). It was further

characterized that PDYN neurons were a subpopulation of nNos-expressing neurons (Extended

Fig. 1b).

Next, to investigate the causal relationship between the neural activity of the identified neuronal

population and sodium intake, adeno-associated virus (AAV) encoding Cre-dependent ChR2

(AAV-DIO-ChR2) was infected in nNOS-Cre and PDYN-Cre animals (Fig. 1d and e, left

panel). Animals were given two bottles, NaCl and water, upon photostimulation. Photostimuation of $OVLT^{nNOS}$ neurons induced water intake (Fig. 1d, right panel) but not sodium intake (Fig 1d, middle panel). However, the activation of $OVLT^{PDYN}$ neurons induced sodium intake (Fig. 1e, middle panel) as well as water (Fig. 1e, right panel). It was surprising that a subpopulation was able to induce both water and sodium intake, while the activation of $OVLT^{nNOS}$ neurons specifically drink water intake. When considering both water and sodium intake, it is hard to conclude that these neurons induce sodium appetite, but maybe could include hypovolemic thirst. Although, the photostimulation of $OVLT^{PDYN}$ neurons prefers sodium instead of potassium or sodium with amiloride (Extended Fig. 1c).

*Characterization of pre-$LC^{PDYN}$ neurons*

Another distinct neural activation upon sodium depletion was found in the pre-LC. Pre-LC was also one of the downstream regions of $NTS^{HSD2}$ neurons[67,68,80]. Compare to OVLT, c-Fos expression was distinctly found only in sodium-depleted condition, not in sated, rescued, and water-deprived animals (Fig. 2a). Almost all c-Fos positive neurons were excitatory, whereas approximately 60% of pre-LC excitatory neurons expressed c-Fos (Fig. 2b). In other words, not all excitatory neurons in pre-LC were involved in sodium depletion. After screening several genetic lines that potentially overlapped with the c-Fos positive neurons, I found that PDYN expression faithfully (>90%) overlaps with sodium-depletion-activated neurons (Fig. 2c and Extended Data Fig. 2d). Notably, pre-$LC^{PDYN}$ neurons co-expressed Foxp2, a previous genetic marker for sodium depletion-sensitive neurons in the pre-LC[24] (Extended Data Fig. 2).

Next, I investigated the functional significance of these neurons for sodium appetite by gain- and loss-of-function approaches. For optogenetic activation of pre-$LC^{PDYN}$ neurons, we infected

AAV-DIO-ChR2 into the pre-LC of PDYN-Cre animals. This neural manipulation triggered robust sodium ingestion from a high concentration of NaCl solution (0.5 M) that is usually aversive[81] under sated conditions, but not water or empty bottle (Fig. 2d, e) or even from rock salt (Fig. 2f). The photostimulation-induced appetite was able to produce sodium intake even in low-concentrate sodium solutions (Extended Fig. 3a). In addition, appetite was observed regardless of sex or time of the day (Extended Data Fig. 3b). The photostimulation of pre-LC[PDYN] neurons induced a specific appetite toward sodium but not potassium (Extended Data Fig. 3c, left panel). Previously, it was reported that sodium detection is mediated by ENaC in the taste system and can be blocked with an ENaC blocker, amiloride[82,83]. The animal prefers sodium without amiloride more than with amiloride (Extended Data Fig. 3c, right panel). Amiloride did not trigger additional aversion in preference tests (Extended Data Fig. 3d). Together, it was shown that pre-LC[PDYN] neurons specifically induce a robust sodium appetite in sated animals.

Moreover, loss-of-function studies revealed the functional necessity of pre-LC[PDYN] neurons for sodium appetite. Photoinhibition of pre-LC[PDYN] neurons specifically suppressed sodium ingestion under sodium-depleted conditions (Fig. 2h and Extended Data Fig. 4a-d). Chemogenetic inhibition also attenuates sodium intake of sodium-depleted animals (Extended Data Fig. 4e-h). Both gain-of-function and loss-of-function experiments demonstrate that pre-LC[PDYN] neurons have a critical role in sodium appetite.

Whether pre-LC[PDYN] neurons are elicited a sustained sodium signal was additionally investigated. In other appetites, activation of appetite neurons have a distinct mechanism of driving consumption. In hunger, the photostimulation of agouti-related protein neurons within the

arcuate nucleus induced a sustained activation. Once hunger neurons were given a certain duration of stimulation, the animal showed persisted feeding even after the stimulation was terminated. However, in thirst, as soon the activation was terminated, the animal did not consume any water[84]. I investigated whether the activation of pre-LC$^{PDYN}$ also induce a sustained signal. As a result, sodium consumption required concurrent stimulation of pre-LC$^{PDYN}$ neurons with sodium presentation (Fig. 2g). These data demonstrate that the ongoing activity of pre-LC$^{PDYN}$ neurons is required for driving sodium appetite.

## 2.3. Valence underlying sodium appetite

One of the major motivations to study appetite neural mechanism is to explain how the internal state can drive a specific goal-orientated behavior. Classical behavioral studies suggest a model that nutrient deficiency evokes negative internal states, which operates animals toward consumption to alleviate such discomfort[85]. Previously, it was proposed that neural circuit of appetite induces motivation through a negative valence[63,86]. By knowing which neurons are activated during sodium depletion, it was possible to investigate the valence encoded in the activation sodium appetite neurons.

To investigate whether sodium appetite neurons encode a specific valence, I used a two-chamber real-time place preference assay and operant assay. I found that photostimulation of pre-LC$^{PDYN}$ neurons significantly reduced occupancy time in the compartment paired with light (Fig. 3a). Thus, the activation of pre-LC$^{PDYN}$ neurons transmits an aversive stimulus. We next tested if animals would actively attempt to reduce the aversive state mediated by pre-LC$^{PDYN}$ neurons. I used an operant assay where the animal has to press a lever to pause continuous photostimulation of the pre-LC (Fig. 3b, left panel). I was able to observe a robust lever-press

behavior to stop stimulation (Fig. 3b, right panel and Extended Data Fig. 5). Thus, pre-LC[PDYN] neurons transmit a negative valence signal upon activation.

## 2.4. Conclusion

Here, I was able to identify two neural populations that induced sufficient sodium appetite. While OVLT[PDYN] neurons induced both water and sodium consumption, I was able to observe the activation of pre-LC[PDYN] neurons which generated a goal-oriented behavior specifically toward to sodium. Photostimulation of the PDYN neurons consumed a high concentration of sodium, which is generally aversive. Furthermore, both photostimulation-induced sodium intake was rapidly exhibited without a prerequisite condition. It is interesting that distinct neural populations, OVLT[PDYN] and pre-LC[PDYN] neurons, can encode different modules such as sodium appetite and hypovolemic thirst. In the aspect of sodium appetite, Pre-LC[PDYN] neurons were able to reconcile specific sodium appetite. Pre-LC[PDYN] neurons will be further investigated as an entry point to the neural basis of sodium appetite. In the next chapter, I will also tackle several aspects, including sodium satiation, by manipulation and observation of pre-LC[PDYN] neurons.

## 2.5. Methods and acknowledgements

*Animals*

All procedures followed animal care guidelines from NIH for the care and use of laboratory animals and California Institute of Technology Institutional Animal Care and Use Committee (1694–14). Animals at least six weeks old were used for experiments. The following mice were purchased from the Jackson Laboratory: C57BL/6J, stock number 00064. Slc17a6-Cre, stock number 016963. Ai75D, stock number 025106, Ai3, stock number 007903. nNOS-Cre, stock

number 017526. PDYN-Cre mice were provided by B. Lowell (Harvard Medical School) and M. Krashes (NIH). Ai110 line was provided by D. Anderson (Caltech). Mice were housed on a 13 h: 11h light: dark cycle with ad llbitum access to food and water except for specific depletion experiments (water, sodium). Male and female mice were used for experiments.

*Viral constructs*

The following AAV viruses were purchased from the UNC Vector Core AAV2-EF1a-DIO-eYFP (4.6 x 1012 genome copies per ml), AAV1-EF1a-DIO-ChR2-mCherry (5.1 x 1012 genome copies per ml), AAV5-Ef1a-DIO iC++-eYFP (4.5 x 1012 genome copies per ml). The following AAV viruses were purchased from the UPenn virus core, AAV5-EF1a-DIO-ChR2-eYFP (3.3 x 1013 genome copies per ml), AAV1-EF1a-DIO-ChR2-mCherry (2.0 x 1013 genome copies per ml). The following AAV viruses were purchased from Addgene, AAV8-hSyn-DIO-hM4D(Gi)-mCherry (1.9 x 1013 genome copies per ml), AAV5-hSyn-DIO-mCherry (1 x 1013 genome copies per ml), AAV8-Ef1a-DIO-iC++-eYFP (8.5 x 1013 genome copies per ml) was purchased from the Stanford Virus vector core.

*Surgery*

Mice were anaesthetized with a mixture of ketamine (1 mg/mL) and xylazine (10 mg/mL) in isotonic saline, intraperitoneally injected at 10 μl /g body weight. Ketoprofen was subcutaneously administered at 5 μl /g body weight. The animal was placed in a stereotaxic apparatus (Narishige Apparatus) with a heating pad. The three-dimensional MRI coordinate system was used as a reference for the injection site coordinates. Viral constructs were injected using a microprocessor-controlled injection system (Nanoliter 2000, WPI) at 100 nl /min. The

coordinates for OVLT are AP: -2400, ML: 0 DV: -4800 and pre-LC are AP: -9000, ML: ±1000, DV: -3900 (60-100 nl injection).

For optogenetic experiments, implants were made with a 200 μm fiber bundle (FT200EMT, Thorlabs) glued to a ceramic ferrule (CF230 or CFLC230, Thorlabs). For photometry, customized implants (400 μm diameter, Doric Lenses) were used. A fiber implant was placed 200-300 μm (for optogenetic) or 0-50 μm (for photometry) above the virus injection site. Histology position of fiber implant was confirmed after data collection. Data from implant disposition was not included. After surgery, all animals were placed in a clean cage placed on a heating pad overnight and then were housed in the animal facility. Behavioral and histological assays were performed after at least 10 days of recovery. At the end of experiments, all animals were sacrificed for histological examination.

*Optogenetic and chemogenetic manipulations*

For ChR2 photostimulation. 473 nm laser pulses (20ms, 20Hz) were delivered via an optic cable (MFP-FC-ZF, Doric Lenses) using a pulse generator (Sapphire 9200 from Quantum composers or SYS-A310 from WPI). The laser intensity was maintained at 5-10 mW at the tip of the fiber. Unless otherwise noted, photostimulation was delivered for 1 s at 3 s intervals throughout the behavior session. For iC++ photoinhibition[33], 473 nm laser was continuously turned on throughout the session at 3 mW at the tip of the fiber. For chemogenetic manipulation[34] (Extended Data Fig. 3e-h), CNO (Sigma, 10 mg/kg) or vehicle (water) was administered intraperitoneal 20 min before the sodium consumption experiment.

*Preference assay*

To induce sodium appetite, animals were injected with furosemide (Sigma) at a dose of 50mg/kg body weight. Low sodium diet (TD. 90228, ENVIGO) was provided for 2 days after the injection of furosemide. For water-restriction experiments, animals were kept in their home cage without water, and were provided with 1 mL of water daily. For food restriction experiments, animals were deprived of food up to 24 hrs with normal water provided. All assays were performed in a custom gustometer (Dialog Instruments)[77]. All sodium-depleted animals were trained in a gustometer before experiments. Animals which licked at least 150 licks during the 30-min session were used for further behavioral assays. After every sodium-depletion round, animals were recovered for at least 4 days with the normal diet.

For appetite specificity assay (Extended Data Fig. 1c and Fig. 3c), three different solutions were presented to animals during the same session, and their preference was measured as a lick number. For each trial, 20 sec of photostimulation was delivered to the animal with an inter-trial-interval of 60 secs. I used water, 0.5 M KCl, 0.5 M NaCl, or 0.5 M NaCl + 30 μM amiloride for preference assay. For sodium consumption assay (Fig. 2e, g, and h), animals were given ad libitum access to 0.5 M NaCl, water or an empty spout for either 7.5 or 30 min. For short term sodium consumption assay (Fig. 1d and e), animals were given access for 5 sec after first lick to 0.5 M NaCl or water.

*Rock salt intake behavioral assay*

Sodium-depleted animals were acclimatized for 1 hour in an acrylic box (50 cm X 25 cm X 25 cm) with a rock salt (Halite Himalayan Crystal Salt). Then the lick events of rock salt were monitored for 30 min using a webcam under sated, sodium depleted, or photostimulated conditions. The start and end of bouts were manually annotated and quantified.

*Histology*

Mice were anaesthetized and were perfused with PBS followed by 4% PFA in PBS (pH 7.4). The brain was dissected and fixed in 4% PFA at 4 °C for overnight. Fixed samples were sectioned into 100 μm coronal sections using a vibratome (Leica, VT-1000 s). For immunohistochemistry (IHC), brain sections were incubated in a blocking buffer (10% Donkey serum, 0.2% Triton-X) for 1-2 hrs. Then sections were incubated with primary antibodies diluted in blocking buffer: rabbit anti-NOS1 (1:500, Santa Cruz, sc-648), rabbit anti-NOS1 (1:500, Therm Fisher, 61-7000), goat anti-c-Fos (1:500, Santa Cruz, SC-52G), rabbit anti-c-Fos (1:1000, Millipore ABE457), chicken anti-GFP (1:1000, Abcam, ab13970), rat anti-mCherry (1:500, Thermo Fisher, M11217), and sheep anti-Foxp2 (1:2000, R&D systems, AF5647). Samples were incubated with primary antibodies overnight. After washing three times with PBS, the sections were incubated with secondary antibodies (1:500 dilutions, Jackson laboratory) in blocking buffer for 4 h. Fluorescence in situ hybridization (FISH) was carried out in frozen brain sections using the RNAscope fluorescent multiplex kit (Advanced Cell Diagnostics) following the manufacturer's instructions. IHC staining was applied for eYFP after FISH.

*Slice electrophysiology*

250-μm coronal slices were obtained using a vibratome (VT-1000s, Leica) in ice-cold sucrose-aCSF (artificial cerebrospinal fluid) solution (Sucrose 213, KCl 2.5, NaH2PO4 1.2, NaHCO3 25, glucose 10, MgSO4 7, CaCl2 1, in mM at pH 7.3), and then incubated in HEPES-holding aCSF (NaCl 92, KCl 2.5, NaH2PO4 1.2, NaHCO3 30, HEPES 20, glucose 25, Na-ascorbate 5, thiourea 2, Na-pyruvate 3, MgSO4 2, CaCl2 2 , in mM at pH 7.3-7.4). Slices were recovered at 34.5 °C for 45 min and then held at room temperature until use.

For patch-clamp recording, slices were placed in a recording chamber perfused with aCSF (NaCl 124, KCl 2.5, NaH2PO4 1.2, NaHCO3 25, glucose 10, MgSO4 1, CaCl2 2, in mM, at pH 7.3) on an upright microscope (Examiner.D1, Zeiss). Whole-cell recordings were achieved using glass pipettes (5–8 MΩ) filled with intracellular solution (for DREADD and iC++ experiments, K-gluconate 145, NaCl 2, KCl 4, HEPES 10, EGTA 0.2, Mg-ATP 4, Na-GTP 0.3). Electrical signals were filtered at 3k Hz with Axon MultiClamp 700B (Molecular Devices) and collected at 20 kHz with Axon Digidata 1550A (Molecular Devices).

To obtain light-evoked responses, the light beam from an LED light source (X-Cite 120LED, Excelitas Technologies) was delivered through an optical filter (475/30) and then 40x water-immersion objective (Zeiss) onto neurons. For iC++ experiments, the light was turned on continuously for 10s, while for DREADD experiments, 10 µM CNO was puffed (10 sec) using a glass pipette.

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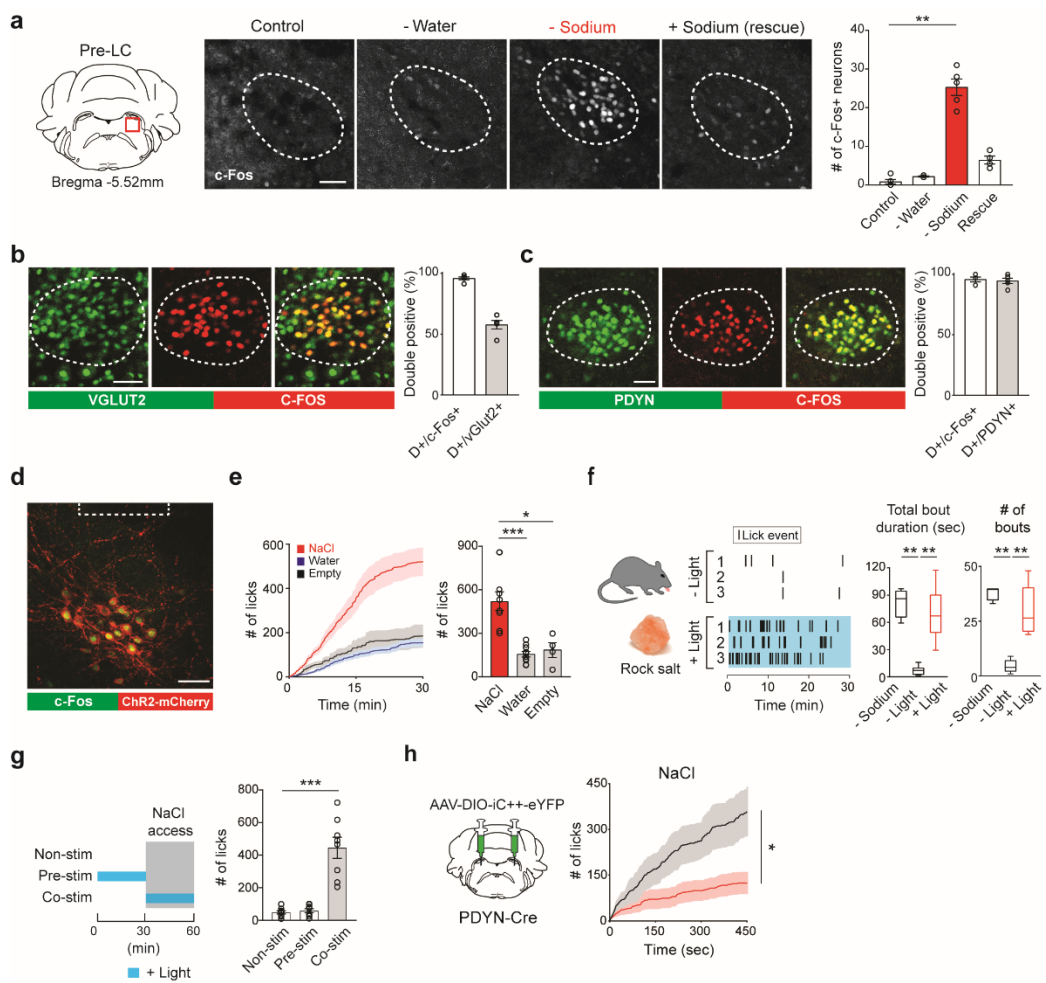48     Gasparini, S. *et al.* Aldosterone infusion into the 4th ventricle produces sodium appetite with baroreflex attenuation independent of renal or blood pressure changes. *Brain research* **1698**, 70-80, doi:10.1016/j.brainres.2018.06.023 (2018).

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**a** Bregma - 0.46 mm | Control | - Water | - Sodium | + Sodium (rescue) | c-FOS | # of c-Fos+ neurons

**b** nNOS | c-Fos | Double positive (%) | D+/c-Fos+ | D+/nNOS+

**c** PDYN | c-Fos | Double positive (%) | D+/c-Fos+ | D+/PDYN+

**d** nNOS | c-Fos | NaCl | Water | # of licks per 5 sec | eYFP | ChR2

**e** PDYN | c-Fos | NaCl | Water | # of licks per 5 sec | eYFP | ChR2
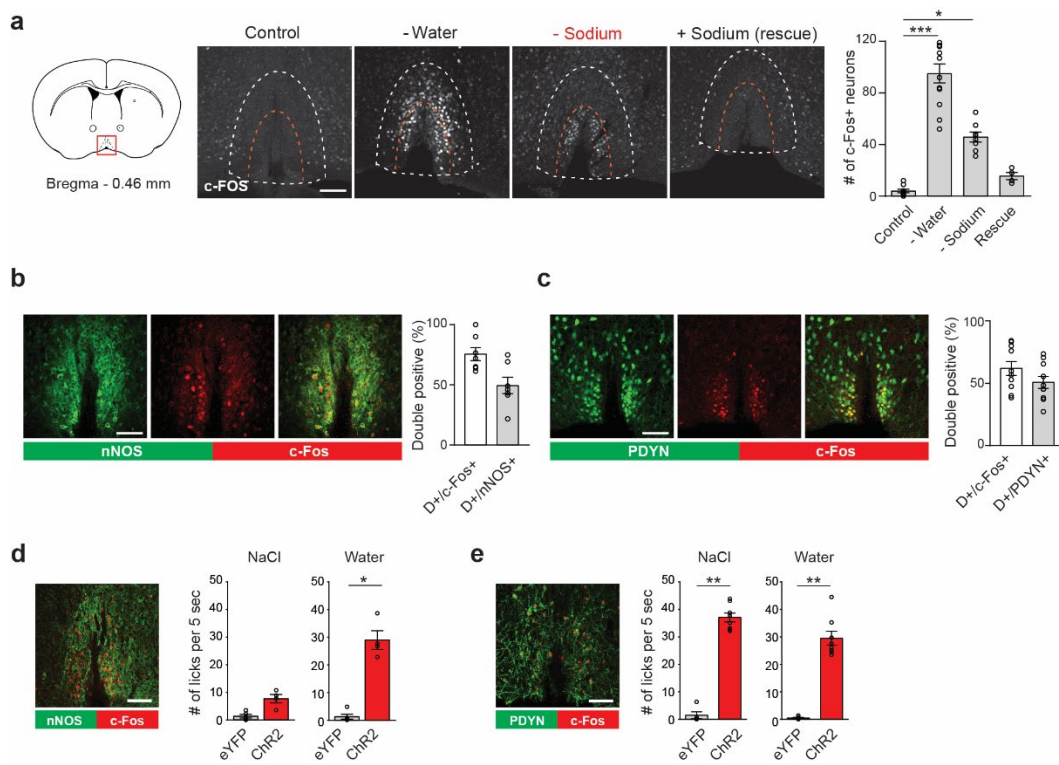
**Figure 1. Genetic and functional identification of sodium appetite neurons in the OVLT. a,** c-Fos expression in sated (control), water-deprived, sodium-depleted, or sodium-repleted (rescue) animals. Quantified data are shown (9 sections from 7 mice for control, 12 mice for water-deprived, 8 mice for sodium-depleted, 4 mice from 2 for rescue). Scale bar, 75 μm. **b,** Sodium-depletion activates OVLT nNOS-positive neurons (7 sections from 6 mice). Scale bar, 50 μm. D+ denotes double-positive. **c,** Sodium-depletion induced c-FOS expression overlaps with PDYN-positive neurons visualized in PDYN/Ai3 transgenic mice (10 sections from 9 mice). Scale bar, 50 μm. **d,** Representative image of optic fiber placement in the OVLT (middle panel). Photostimulation of OVLT$^{nNOS}$ neurons triggered ingestion of water (right panel) solution not sodium (0.5 M, left panel) (n = 5 for eYFP, n = 4 mice for ChR2). **e,** Photostimulation of OVLT$^{PDYN}$ neurons triggered ingestion of both sodium (0.5 M, middle panel) and water (right panel) solution (n = 5 for eYFP, n = 8 mice for ChR2) not sodium (0.5 M). Scale bar, 100 μm. .*$P<0.05$, **$P<0.01$ and ***$P<0.001$ by Kruskal–Wallis (Dunn's multiple comparison), or two-tailed Wilcoxon test, two-tailed Mann-Whitney test. Data presented as mean ± s.e.m. The mouse brain in this figure has been reproduced from the mouse brain atlas[79].

**a** Pre-LC | Bregma -5.52mm | Control | - Water | - Sodium | + Sodium (rescue) | c-Fos | # of c-Fos+ neurons — Control, - Water, - Sodium, Rescue

**b** VGLUT2 | C-FOS | Double positive (%) — D+/c-Fos+, D+/vGlut2+

**c** PDYN | C-FOS | Double positive (%) — D+/c-Fos+, D+/PDYN+

**d** c-Fos | ChR2-mCherry

**e** # of licks vs Time (min) — NaCl, Water, Empty; # of licks — NaCl, Water, Empty

**f** Lick event; - Light; Rock salt; + Light; Total bout duration (sec); # of bouts — - Sodium, - Light, + Light

**g** Non-stim, Pre-stim, Co-stim; NaCl access; (min); + Light; # of licks — Non-stim, Pre-stim, Co-stim

**h** AAV-DIO-iC++-eYFP; PDYN-Cre; NaCl; # of licks vs Time (sec)

**Figure 2. Genetic and functional identification of sodium appetite neurons in the pre-LC. a,** c-Fos expression in sated (control), water-deprived, sodium-depleted, or sodium-repleted (rescue) animals. Quantified data are shown (n = 4 mice for rescue, n = 5 mice for other conditions). **b,** Sodium-depletion activates pre-LC excitatory neurons (n = 5 mice). D+ denotes double-positive. **c,** c-Fos expression fully overlaps with PDYN-positive neurons visualized in PDYN/Ai110 transgenic mice (n = 5 mice). **d,** Representative image of optic fiber placement in the pre-LC. **e,** Photostimulation of pre-LC$^{PDYN}$ neurons triggered ingestion of NaCl solution (0.5 M, n = 10 mice) compared to water (n = 10 mice) or empty spout (n = 4 mice). **f,** Photostimulated mice showed robust licking behavior toward rock salt (n = 4 for – Sodium and n = 8 for the rest). Raster plots of 3 out of 8 mice are shown. **g,** A scheme of photostimulation and sodium presentation (left panel). The number of licks for 30 min was quantified (right panel, n = 8 mice). **h,** Photoinhibition of pre-LC$^{PDYN}$ neurons by iC++ significantly reduced sodium intake (n = 7 mice). Scale bar, 50 μm. *P<0.05, **P<0.01 and ***P<0.001 by Kruskal–Wallis, Friedman test (Dunn's multiple comparison), or two-tailed Wilcoxon test. Data presented as mean ± s.e.m. Box plots show median, quartiles (boxes), and range (whiskers).

**a**

eYFP    ChR2

- Light

+ Light

Photostimulation

0    1 (Time %)

Time in preferred side (%)

eYFP
ChR2

Pre   1    2    3

Session

**b**

Light ON

Light pause

# of lever press

eYFP
ChR2

Time (min)

ChR2

# of lever press

+ Light   - Light

eYFP

+ Light   - Light
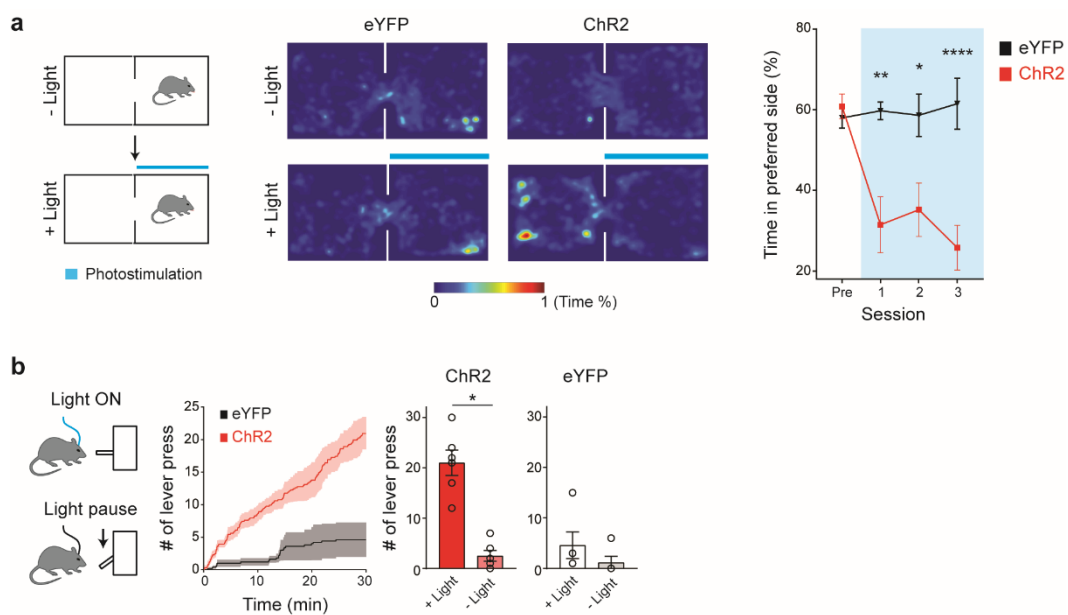
**Figure 3. Activation of pre-LC<sup>PDYN</sup> neurons drives an aversive motivational signal. a,** Two-chamber real-time place preference assay (left panel). Place preference of a representative animal with or without photostimulation (middle panel). Blue bars indicate the side with light. Quantified data are shown (n = 8 mice for eYFP, n = 10 mice for ChR2). **b,** Negative reinforcement assay. Animals were continuously photostimulated (20 Hz) in the chamber, which was paused for 20 sec by each lever press. Cumulative and a total number of lever press were quantified (n = 5 and 6 mice for eYFP and ChR2). *P<0.05, **P<0.01, ****P<0.0001 by two-way repeated measures ANOVA (Sidak's multiple comparisons test) or two-tailed Wilcoxon test. Data presented as mean ± s.e.m.

**a**

c-Fos spread (um)

- Water
- Sodium

**

**b**

PDYN    nNOS

Double positive (%)

D+/PDYN+    D+/nNOS+

**c**

KCl        Ami

# of licks per 5 sec

NaCl    KCl        - Ami    + Ami

*        *
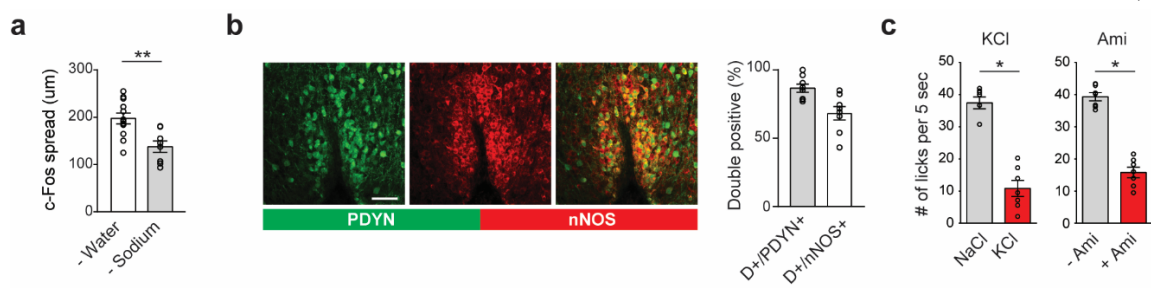
**Extended Data Figure 1. Additional histological and behavior analysis of the OVLT. a,** Distant c-Fos expression pattern in the OVLT between water-deprived and sodium depleted animals (n = 12 mice for water-deprivation, n = 8 mice for sodium-depletion). **b,** PDYN-expressing neurons are subpopulation of nNOS-expressing neurons (n = 8 mice). **c,** OVLT$^{PDYN}$-stimulated animals preferred NaCl over KCl (left panel, n = 7 mice). 0.5 M solutions were used for NaCl and KCl. NaCl consumption was reduced in the presence of amiloride (30 μM, right panel, n = 7 mice). Scale bar, 50 μm. *P<0.05, **P<0.01 by two-tailed Mann-Whitney test or two-tailed Wilcoxon test. Data presented as mean ± s.e.m.

**a**

Day: 0    1

-Water    +Normal diet / -Water

-Sodium    FURO    +Low sodium diet / +Water    2

+Sodium (Rescue)    FURO    +Normal diet / +Water

**b**

# of licks per 5 sec

■ 0.5 M NaCl
□ Water

Control   - Water   - Sodium   + Sodium (Rescue)

**c**

Subfornical organ    Pre-LC

c-Fos

- Water (48 hrs)

**d**

eYFP    *PDYN mRNA*

**e**

PDYN    Foxp2
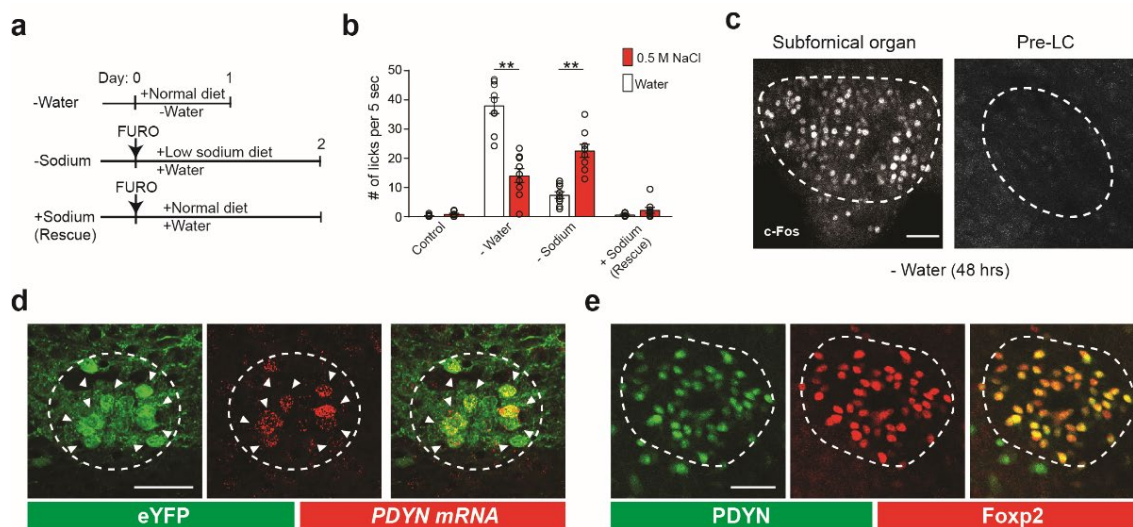
**Extended Data Figure 2. Behavioral paradigms for sodium appetite induction and histological analysis of the pre-LC. a,** Experimental protocols for inducing thirst and sodium appetite. Intraperitoneal injection of furosemide (50 mg/kg body weight) was used to induce sodium appetite. **b,** Sodium-depleted animals showed a strong preference for sodium while water-deprived animals preferred water over sodium (n = 9 mice). **c,** Water-deprivation for 48 hrs induced robust c-Fos expression in the subfornical organ. However, it did not activate the pre-LC (one out of 4 mice). **d,** Fluorescence in situ hybridization (FISH) showing that PDYN-Cre expression (visualized in Ai3 transgenic line, green) overlaps with endogenous PDYN transcripts in the pre-LC (red, one out of 2 mice). **e,** Pre-LC[PDYN] neurons also overlap with Foxp2 expression, a known marker in the pre-LC[24,64] (93.8 ± 1.1%, n = 3 mice). Scale bar, 50 μm. **P<0.01 by two-tailed Wilcoxon test. Data presented as mean ± s.e.m.
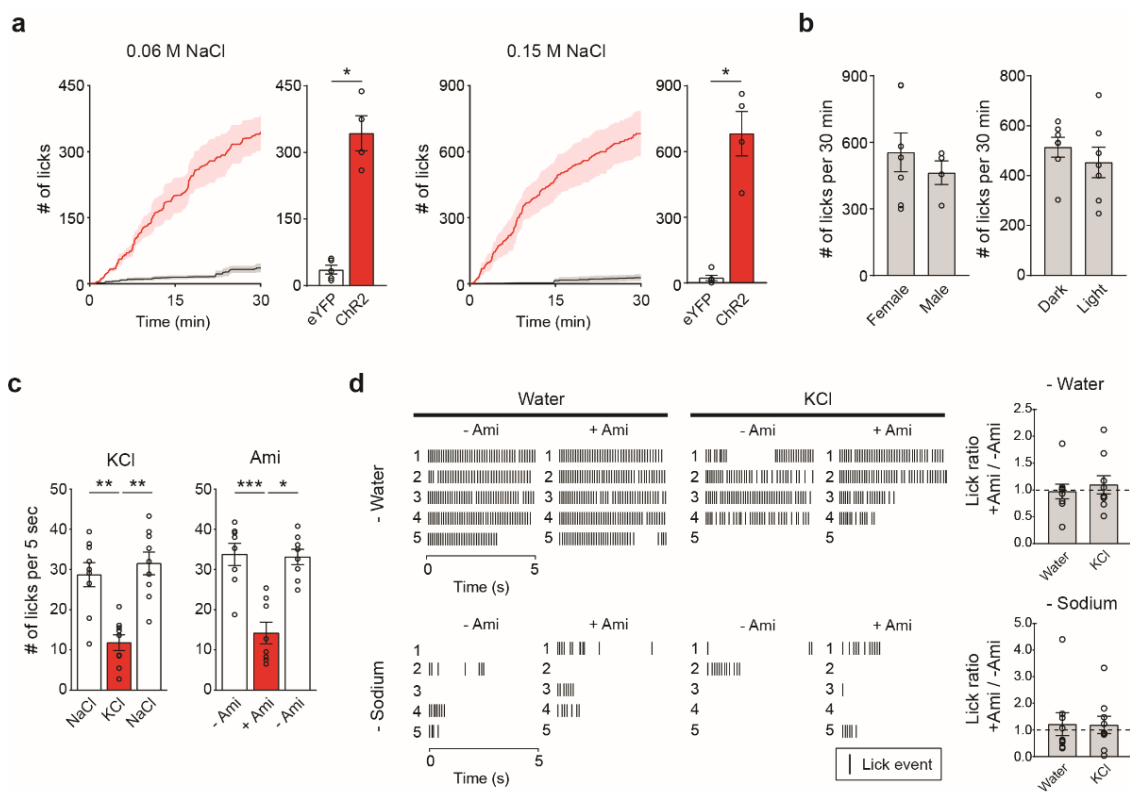
**Extended Data Figure 3. Sodium appetite induced by the photostimulation of pre-LC$^{PDYN}$ neurons. a,** Photostimulation of pre-LCPDYN neurons increased intake of a lower concentration of NaCl (0.06 M and 0.15 M, n = 5 mice for eYFP, n = 4 mice for ChR2). **b,** Photostimulation triggered sodium appetite in both sexes (left panel, n = 7 for female, n = 4 mice for male), at any time of the day (right panel, n = 7 mice). Data were partially reanalyzed from Fig. 1e and g. **c,** Pre-LC$^{PDYN}$-stimulated animals preferred NaCl over KCl (left panel, n = 9 mice). NaCl consumption was reduced in the presence of amiloride (right panel, n = 8 mice). 0.5 M solutions were used for NaCl and KCl. **d,** Representative plots showing lick events during the 5-sec of water or KCl access (left panels). The effect of amiloride on water and KCl intake was quantified under water-deprivation and sodium-depletion (right panels). The total number of licks from 5 trials with amiloride was averaged and divided by that without amiloride (n = 9 mice). *P<0.05, **P<0.01, ***P<0.001 by two-tailed Mann-Whitney test or Friedman test (Dunn's multiple comparison). Data presented as mean ± s.e.m.

**a**

Light ON for 10s

20mV

5 s

**b**

iC++

**c**

- Water

# of licks

iC++

-Light  +Light

**d**

- Sodium        - Water

# of licks

eYFP

-Light  +Light    -Light  +Light

**e**

AAV-DIO-hM4Di(Gi)-mCherry

PreLC

CNO

20mV

10s

**f**

hM4Di

**g**

- Sodium        - Water

# of licks

**

hM4Di

Vehicle  CNO    Vehicle  CNO

**h**

- Sodium        - Water

# of licks

mCherry

Vehicle  CNO    Vehicle  CNO
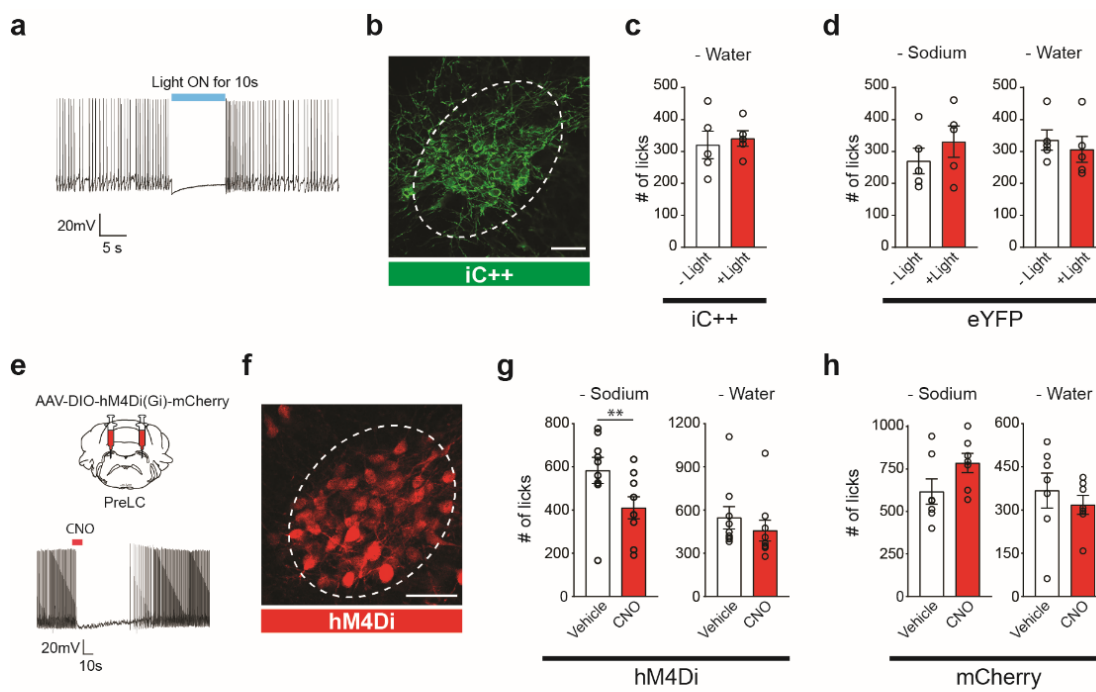
**Extended Data Figure 4. Optogenetic and chemogenetic inhibition of pre-LC<sup>PDYN</sup> neurons. a,** Electrophysiological recording in fresh brain slices. Illumination of 473 nm light strongly suppressed firing of pre-LC$^{PDYN}$ neurons expressing iC++ (10 out of 10 neurons from 2 mice). **b,** A representative image of AAV-DIO-iC++-eYFP expression in the pre-LC of a PDYN-Cre animal (one out of 7 mice). **c,** Suppression of pre-LC$^{PDYN}$ did not affect water intake in water-deprived animals (n = 5 mice). **d,** AAV-DIO-eYFP controls for optogenetic inhibition (n = 5 mice). **e,** AAV-DIO-hM4Di-mCherry was bilateral injected into the pre-LC. A representative recording demonstrates chemogenetic inhibition of pre-LC$^{PDYN}$ neuron by CNO (13 out of 14 neurons from 2 mice). **f,** A representative image of AAV-DIO-hM4Di-mCherry expression in the pre-LC (one out of 9 mice). **g,** Chemogenetic inhibition of pre-LC$^{PDYN}$ neurons reduced sodium intake in sodium-depleted animals. The same manipulation did not affect thirst (n = 9 mice). **h,** CNO administration did not affect thirst or sodium appetite in AAV-DIO-mCherry injected animals (n = 7 mice). Scale bar, 50 μm. **P<0.01 by two-tailed Wilcoxon test. Data presented as mean ± s.e.m.

**a**

```
┌──────────────────┐        Foot shock
│   Habituation    │
└──────────────────┘
         ↓
┌──────────────────┐
│ Footshock training │
└──────────────────┘
         ↓                 Foot shock OFF
┌──────────────────┐
│ PreLC stimulation │
└──────────────────┘
```

**b**

Foot shock avoidance

# of level press

80

60

40

20

0

+FS (ChR2)  - FS (ChR2)  + FS (eYFP)  - FS (eYFP)

**c**

Without foot shock

# of level press

15

10

5

0

1    2    3    4    - Light

\*

+ Light Session
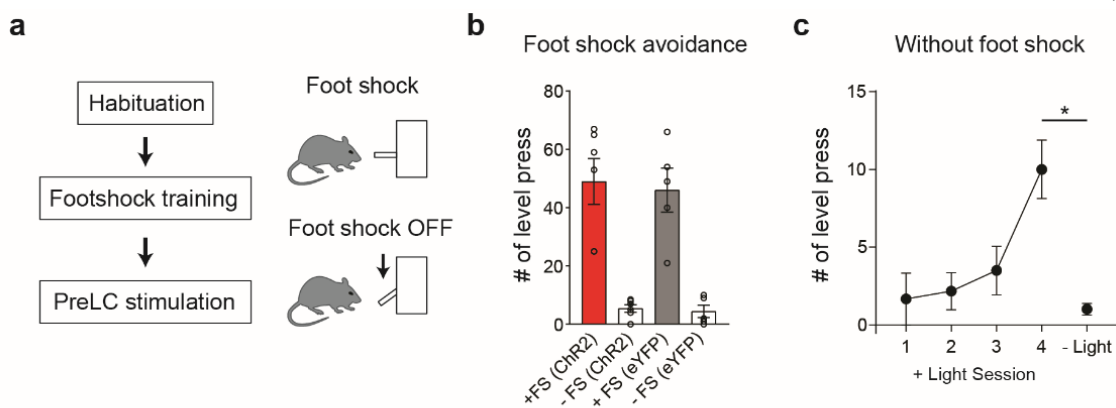
**Extended Data Figure 5. Training paradigm for negative reinforcement assay. a,** A diagram of training paradigm using foot shock. Each lever press pauses continuous foot shock for 20 sec. **b,** A total number of lever press in each condition during the 30-min session (n = 5 for eYFP and n = 6 mice for ChR2). **c,** Animals were conditioned to perform lever press without foot shock pre-training sessions (n = 6 mice). *P<0.05 by two-tailed Wilcoxon test. Data presented as mean ± s.e.m.

*Chapter 3*

# MODULATION OF SODIUM APPETITE

Lee, Sangjun, et al. "Chemosensory modulation of neural circuits for sodium appetite." Nature 568.7750 (2019): 93-97. https://doi.org/10.1038/s41586-019-1053-2

Appetite can be simply characterized as an increase in intake. However, appetite encodes an integrated neural basis more than just increased intake. Central appetite circuits actively receive various sensory and behavioral modulations on a real-time basis and regulate appetite. For instance, central appetite circuit can be modulated by sensory informations. A recent recording of hunger neurons in the arcuate nucleus has demonstrated in mice that sensory information, such as vision and even smell can sufficiently suppress the neural activity of appetite neurons[1,2]. Furthermore, sensory information provides a prediction of the physiological impact on the body[3]. This is critical for an animal to estimate the amount of consumption to prevent overconsumption. In addition, the appetite circuit forms a goal to recognize it needs and changes the response toward its need. The activation of the appetite circuit is developed from body physiological signals as I reviewed in the first chapter. Together, central appetite circuits are expected to integrate these signals and regulate intake behaviors. Along with the genetic tools to manipulate, tools to monitor the neural activity on a real-time basis were largely applied to neuroscience. With the identified specific neural population for sodium appetite, I was able to proceed with the investigation of the neural basis of sodium appetite.

### 3.1. Sensory modulation of pre-LC$^{PDYN}$ neurons

In the previous chapter, I showed that stimulation of the pre-LC$^{PDYN}$ neurons did not induce any prolonged effect on sodium intake. Only when the presentation of sodium was given with concurrent stimulation, the animal exhibited sodium intake. This implies that the sodium appetite is driven by the ongoing activity of the pre-LC $^{PDYN}$ neuron. To further investigate the neural activity of pre-LC$^{PDYN}$ neurons, fiber photometry recording[4] from pre-LC$^{PDYN}$ neurons was utilized during sodium consumption (Fig. 1a and Extended Data Fig. 1a). I found that the neural activity of pre-LC$^{PDYN}$ neurons rapidly and persistently suppressed upon sodium ingestion (Fig. 1a, c). The reduction was observed upon low concentration (Extended Data Fig. 1b). This robust inhibition was not observed when animals licked water or an empty bottle (Fig. 1b, c, and Extended Data Fig. 1c, d).

Then, a question rises as to what exact sensory information suppresses pre-LC$^{PDYN}$ neurons. The sensory information that can represent the sodium level of the ingestion can be the osmolality of the solution or the direct sodium detection, sodium taste. I examined if this persistent inhibition is selectively driven by chemosensory detection of sodium. One hypothesis is that the pre-LC$^{PDYN}$ neurons can be suppressed by osmolality. In contrast to NaCl, no suppression was observed by KCl (0.5 M, Fig. 1d and Extended Data Fig. 1e), excluding the possibility of osmolality detection. Importantly, I tested whether sodium taste mediates the inhibition of pre-LC$^{PDYN}$ neurons during sodium consumption. When the sodium taste receptor was blocked by amiloride[5,6], the neural activity of pre-LC$^{PDYN}$ neurons was fully abolished (Fig. 1e and Extended Data Fig. 1e).

Moreover, simple contact with NaCl solution was sufficient to induce robust suppression for several minutes (Fig. 1f). Together, these results suggest that oral chemosensory signals are critical for the acute modulation of sodium appetite neurons.

## 3.2. Chemosensory modulation of sodium satiation

Both thirst and sodium appetite are quenched within a short time before the systemic homeostatic recovery[7-9]. The termination of a meal, satiation, is the key mechanism of the estimation of the amount of needed nutrients ahead of the actual extracellular recovery. Without such prediction, the animal requires additional energy and time to recover from the overconsumption[3,10-13]. Because of the rapid response, it was expected that sensory-mediated inhibition would modulate the intake behavior. In thirst, it was demonstrated that the gulping action was able to temporarily suppress the thirst neurons in the lamina terminalis[14]. For sodium appetite, sodium taste was proposed in previous studies as the sensory input for sodium satiation[8]. Animals consume the same amount of sodium solution regardless of the sodium contents such as NaCl vs. $Na_2CO_3$[15]. Also, intragastric infusion of sodium that bypasses the orosensory systems was not able to satiate the sodium-depleted animal. Although, the infusion of sodium did reduce sodium intake in a longer timescale than satiation, which could be explained by the actual body fluid recovery[15,16].

Given the significant role of the pre-LC[PDYN] population for sodium intake, I hypothesized that the taste-mediated inhibition of these neurons contribute to the satiation of sodium appetite. I examined this possibility using photometry recording combined with intragastric infusion (Fig. 2a)[16,17]. Surprisingly, I found that gastric

preloading of sodium chloride in sodium-depleted mice did not affect subsequent sodium ingestion, whereas oral NaCl contact quickly quenched the appetite (Fig. 2b, left panel). Sodium appetite was reduced only after a significant time of intragastric infusion (41.3 ± 6.0 % after 2 hrs, n = 6 mice). Previously, it was reported that the intragastric infusion was sufficient to satiate thirsty and hungry animals[18-21]. As a control experiment, I confirm that intragastric water and glucose infusion in thirsty and hungry animals suppressed water/food consumption shortly after the infusion (Fig. 2b middle and right panels). Although it remains unclear how the post-ingestive factors regulate sodium appetite, I was able to identify that sodium taste signals are critical for sodium satiation.

If pre-LC[PDYN] neurons are involved in taste-mediated satiation, it is predicted that the neural activity also should be suppressed by sodium taste. When sodium was consumed orally, the neural activity of pre-LC[PDYN] neurons drastically reduced (Fig. 2c, e), while the intragastric infusion of sodium in the same animals did not trigger any response (Fig. 2d, e). These results suggest that sodium taste signals facilitate satiation of sodium appetite via persistent suppression of pre-LC[PDYN] neurons.

## 3.3. Physiological inputs of pre-LC[PDYN] neurons

Proven that the activation of pre-LC[PDYN] neurons drives sodium appetite and receives sensory information, my results indicate a model that the pre-LC integrates the internal physiological signals and sensory information to regulate sodium intake. I further approach the neural architecture of the pre-LC[PDYN] circuit, where and how these inputs can modulate pre-LC[PDYN] neurons. Unlike the other appetite neurons, pre-LC[PDYN] neurons are not exposed from the BBB, thus, the neurons have difficulty

communicating with the physiological signals. Then, how are the preLC[PDYN] neurons activated by physiological signals? Previous studies showed that the pre-LC receive afferents from the HSD2 neurons in the NTS (NTS[HSD2]neurons)[22,23]. NTS[HSD2] neurons were proposed to interact with aldosterone[23,24]. Aldosterone is one of the main endocrines to generate sodium appetite[25]. I tested if NTS[HSD2]neurons carry the physiological signals to pre-LC[PDYN] neurons. Firstly, I have to confirm the connectivity between NTS[HSD2] and pre-LC[PDYN] neurons. Using ChR2-assisted circuit mapping[26], I found that a majority of recorded pre-LC[PDYN] neurons received monosynaptic excitatory inputs from NTS[HSD2] neurons (Fig. 3a and Extended Data Fig. 2a). Additionally, I confirmed the optogenetic stimulation of NTS[HSD2] neurons increased c-Fos expression in partial pre-LC[PDYN] neurons in sated animals (Extended Data Fig. 2b).

Next, I examined the functional significance of this connection. If the pre-LC[PDYN] neurons receive interoceptive information from the NTS[HSD2], it was expected that the ablation of NTS[HSD2] will reduce the activation of pre-LC[PDYN] neurons. As expected, the ablation of NTS[HSD2] neurons by caspase greatly attenuated c-Fos expression in pre-LC[PDYN] neurons after sodium-depletion (Fig. 3b and Extended Data Fig. 2c, d). In consistent with previous studies, the ablation of NTS[HSD2] neurons significantly abolish sodium intake in sodium depleted animals[23]. Together, the data showed the potential physiological signals are driven from excitatory NTS[HSD2] neurons to pre-LC[PDYN] neurons.

## 3.4. Sensory inputs of pre-LC[PDYN] neurons

I further searched for neural circuits that mediate taste-dependent inhibitory component by monosynaptic rabies tracing (SAD-$\Delta$G-BFP)[27] from pre-LC[PDYN] neurons. The tracing experiments identified several brain regions with most prominent inputs from the dorsal area of the bed nucleus of the stria terminalis (dBNST), and the central amygdala (Fig. 3c and Extended Data Fig. 3a). Because the BNST was previously shown to contribute to sodium consumption[28-30], I focused our functional analysis on the dBNST neurons projecting to pre-LC circuit. In dBNST, a majority of rabies-positive neurons were the inhibitory population that also expressed PDYN (dBNST[PDYN], Fig. 3d). I also confirm that these populations express GAD as an inhibitory marker (Extended Data Fig. 3b). It was confirmed in slice recording that dBNST[PDYN] neurons send monosynaptic inhibitory inputs to pre-LC[PDYN] neurons (Fig. 3e). When it is assumed that dBNST neurons projecting to pre-LC mediates rapid satiation signals, dBNST neurons should be activated upon sodium ingestion. To verify this idea, retrograde canine adenovirus (CAV2)-Cre was injected in the pre-LC and AAV-flex-GCaMP6s was injected in the dBNST. I was able to label dBNST neurons projecting to pre-LC (Extended Data Fig. 3c). The neural recording from dBNST to pre-LC neurons demonstrated that they responded upon sodium intake under sodium-depleted conditions, which were strongly inhibited by amiloride (Fig. 3f).

### 3.5. Conclusion

I explored the architecture and the functional significance of the sodium appetite circuit. The neural circuitry controls sodium appetite and consequent ingestive behavior. Multiple hindbrains and forebrain structures, including the NTS, BNST, and amygdala,

were suggested to be involved in sodium intake regulation[31]. Interestingly, pre-LC$^{PDYN}$ neurons have anatomical relations with the past-studied brain regions (Fig. 3c and Extended Data Fig. 4), suggesting their integral roles in sodium appetite. Together, I was able to replay sodium appetite, with photostimulation of a distinct population. Further investigation will be required to explore other behavioral aspects, including the value change of sodium which can be started as using the pre-LC$^{PDYN}$ as an entry point.

## 3.6. Methods and adknowledgements

*Animals*

All procedures followed animal care guidelines from NIH for the care and use of laboratory animals and California Institute of Technology Institutional Animal Care and Use Committee (1694–14). Animals at least six weeks old were used for experiments. C57BL/6J, stock number 00064 from the Jackson Laboratory. HSD2-Cre mice were provided by A. and G. Fejes-Tóth (Dartmouth Medical School). PDYN-GFP mice were provided by D. Kong (Tufts University School of Medicine). PDYN-Cre mice were provided by B. Lowell (Harvard Medical School) and M. Krashes (NIH). Mice were housed on a 13 h: 11h light: dark cycle with ad llbitum access to food and water except for specific depletion experiments (water, food, sodium). Male and female mice were used for experiments.

*Viral constructs*

The following AAV viruses were purchased from the UNC Vector Core AAV1-CAG-flex-RG (3.0 x 1012 genome copies per ml), AAV1-EF1a-flex-TVA-mCherry (6.0 x

1012 genome copies per ml), AAV2-EF1a-DIO-eYFP (4.6 x 1012 genome copies per ml), AAV5-flex-taCasp3-TEVp (4.5 x 1012 genome copies per ml). The following AAV viruses were purchased from the UPenn virus core, AAV1-hSyn1-flex-GCaMP6s-WPRE-SV40 (2.28 × 1013 genome copies per ml), AAV5-EF1a-DIO-ChR2-eYFP (3.3 x 1013 genome copies per ml). SAD- ΔG-BFP (1.7 x 109 genome copies per ml) was purchased from Salk. CAV-Cre (1.5 x 1013 genome copies per ml) was purchased from Plateforme de Vectorologie de Montpellier.

*Surgery*

Mice were anaesthetized with a mixture of ketamine (1 mg/mL) and xylazine (10 mg/mL) in isotonic saline, intraperitoneally injected at 10 μl /g body weight. Ketoprofen was subcutaneously administered at 5 μl /g body weight. The animal was placed in a stereotaxic apparatus (Narishige Apparatus) with a heating pad. The three-dimensional MRI coordinate system was used as a reference for the injection site coordinates. Viral constructs were injected using a microprocessor-controlled injection system (Nanoliter 2000, WPI) at 100 nl /min. The coordinates for pre-LC are AP: -9000, ML: ±1000, DV: -3900 (60-100 nl injection), for dBNST are AP: -3100 ML: 1100 DV: -3600 (100 nl injection), for NTS are AP: -10800 ML: ±150 DV: -5100, -5300 (100-300 nl injection each).

For optogenetic experiments, implants were made with a 200 μm fiber bundle (FT200EMT, Thorlabs) glued to a ceramic ferrule (CF230 or CFLC230, Thorlabs). For photometry, customized implants (400 μm diameter, Doric Lenses) were used. A fiber implant was placed 200-300 μm (for optogenetic) or 0-50 μm (for photometry) above

the virus injection site. Histology position of fiber implant was confirmed after data collection. Data from implant disposition was not included. For IG infusion, catheter construction and implantation closely followed as described previously[16,17]. IG catheters were custom made using silastic tubing (Dow Corning, 508-002), tygon tubing (Instech, BTPE-25) and pinport (Instech, PNP3F25-50). For photometry recording, IG surgery was performed after animals recovered from the initial implantation of an optic fiber. After surgery, all animals were placed in a clean cage placed on a heating pad overnight and then were housed in the animal facility. Behavioral and histological assays were performed after at least 10 days of recovery. For ablation experiments, AAV-flex-taCasp3-TEVp or AAV-hSyn-DIO-mCherry (control) was injected. These animals were sodium-depleted after 2-3 weeks of recovery. At the end of experiments, all animals were sacrificed for histological examination. For fiber implantation experiments, we occasionally observed that the position of an implanted fiber shifted in the hindbrain due to cranial deformation.

*Optogenetic and chemogenetic manipulations*

For ChR2 photostimulation. 473 nm laser pulses (20ms, 20Hz) were delivered via an optic cable (MFP-FC-ZF, Doric Lenses) using a pulse generator (Sapphire 9200 from Quantum composers or SYS-A310 from WPI). The laser intensity was maintained at 5-10 mW at the tip of the fiber. Unless otherwise noted, photostimulation was delivered for 1 s at 3 s intervals throughout the behavior session.

*Preference assay*

To induce sodium appetite, animals were injected with furosemide (Sigma) at a dose of 50mg/kg body weight. Low sodium diet (TD. 90228, ENVIGO) was provided for 2 days after the injection of furosemide. For water-restriction experiments, animals were kept in their home cage without water, and were provided with 1 mL of water daily. For food restriction experiments, animals were deprived of food up to 24 hrs with normal water provided. All assays were performed in a custom gustometer (Dialog Instruments) or Biodaq monitoring system (Research Diets Inc)[14]. All sodium-depleted animals were trained in a gustometer before experiments. Animals which licked at least 150 licks during the 30-min session were used for further behavioral assays. After every sodium-depletion round, animals were recovered for at least 4 days with the normal diet.

For photometry recording, animals were given either 5 or 10 min access to stimuli. To examine sodium specific responses of pre-LC$^{PDYN}$ neurons (Fig. 1d, e), animals were presented with three solutions during the session. First, animals were given 5 min access to 0.5 M KCl (Fig. 3d) or 0.5 M NaCl + 0.1 mM amiloride (Fig. 1e). Then animals had 5 min access to water. Finally, animals were given 5 min access to 0.5 M NaCl. The interval between trials was 5 min.

*Intragastric infusion*

In Fig. 2b, 0.5 M NaCl, deionized water, or glucose solution (5 M) was infused via an intragastric catheter[16,17]. Solutions were delivered at 0.1 ml/ min for 5 min (water and sodium) or 10 min (food) using an infusion syringe pump (NE-300, New Era Pump Systems Inc). 10 min after gastric infusion, animals were given access to nutrients and their consumption was quantified for 10 min (for water and 0.5 M NaCl), or 30 min (for

normal chow). Either air infusion (for water), or water infusion (for sodium and food) was used as a control stimulus. In Fig. 2d, either 0.15 M NaCl, water, or air was infused at a rate of 0.1 ml/ min while recording the neural activity by photometry. For control, oral ingestion (Fig. 2c), the same set of animals were given access to 0.15 M NaCl.

*Fibre photometry*

For all photometry assays, animals were acclimatized for 10-15 min in the chamber before stimuli were presented. Bulk fluorescence signals were collected using fibre photometry as previously described[14]. Briefly, data were extracted and subjected to a low-pass filter at 1.8 Hz. A linear function was used to scale up the 405-nm channel signal to the 490-nm channel signal to obtain the fitted 405-nm signal. The resultant $\Delta F/F$ was calculated as (raw 490 nm signal – fitted 405 nm signal)/ (fitted 405 nm signal). $\Delta F/F$ was then time-binned by a factor of 2.5 times the sampling frequency and down-sampled to 10 Hz. For all bouts, the mean fluorescence for 5 min before the first lick was calculated and subtracted from the entire session. The licks from the lickometer were simultaneously recorded. The area under the curve (AUC) was quantified by integrating the baseline-subtracted fluorescence signals for 30 sec after the start of the bout. For Extended Data Fig. 1d, the data were quantified as $\Delta F/F$ change between 1 sec prior to, and at the first lick (0 sec). For IG infusion experiments (Fig. 2e), AUC was quantified during the 5-min of infusion.

*Retrograde Viral tracing*

For monosynaptic rabies tracing 40 of pre-LC[PDYN], 100 nl of a mixture of AAV1-CAG-flex-RG and AAV1-EF1a-flex-TVA-mCherry (4:1 ratio) was injected to the pre-LC. Two weeks later, 200 nl of SAD-ΔG-BFP was injected into the pre-LC. The mice were euthanized a week later.

To label the dBNST→pre-LC circuit, 100nl of CAV-Cre was injected into the pre-LC followed by the injection of AAV5-DIO-mCherry or AAV1-flex-GCaMP6S into the dBNST. These animals were used for experiments at least two weeks after the injection.

*Histology*

Mice were anaesthetized and were perfused with PBS followed by 4% PFA in PBS (pH 7.4). The brain was dissected and fixed in 4% PFA at 4 °C for overnight. Fixed samples were sectioned into 100 μm coronal sections using a vibratome (Leica, VT-1000 s). For immunohistochemistry (IHC), brain sections were incubated in a blocking buffer (10% Donkey serum, 0.2% Triton-X) for 1-2 hrs. Then sections were incubated with primary antibodies diluted in blocking buffer: goat anti-c-Fos (1:500, Santa Cruz, SC-52G), rabbit anti-c-Fos (1:1000, Millipore ABE457), rabbit anti-GAD65+GAD67 (1:500, Abcam, ab183999), chicken anti-GFP (1:1000, Abcam, ab13970), rat anti-mCherry (1:500, Thermo Fisher, M11217), sheep anti-Foxp2 (1:2000, R&D systems, AF5647), and rabbit anti-HSD211β2 (1:300, proteintech, 14192-1-AP). Samples were incubated with primary antibodies overnight. After washing three times with PBS, the sections were incubated with secondary antibodies (1:500 dilutions, Jackson laboratory) in blocking buffer for 4 hr. For an exception, GAD65+GAD67 staining was performed without Triton-X.

250-μm coronal slices were obtained using a vibratome (VT-1000s, Leica) in ice-cold sucrose-aCSF (artificial cerebrospinal fluid) solution (Sucrose 213, KCl 2.5, NaH2PO4 1.2, NaHCO3 25, glucose 10, MgSO4 7, CaCl2 1, in mM at pH 7.3), and then incubated in HEPES-holding aCSF (NaCl 92, KCl 2.5, NaH2PO4 1.2, NaHCO3 30, HEPES 20, glucose 25, Na-ascorbate 5, thiourea 2, Na-pyruvate 3, MgSO4 2, CaCl2 2 , in mM at pH 7.3-7.4). Slices were recovered at 34.5 °C for 45 min and then held at room temperature until use.

For patch-clamp recording, slices were placed in a recording chamber perfused with aCSF (NaCl 124, KCl 2.5, NaH2PO4 1.2, NaHCO3 25, glucose 10, MgSO4 1, CaCl2 2, in mM, at pH 7.3) on an upright microscope (Examiner.D1, Zeiss). Whole-cell recordings were achieved using glass pipettes (5–8 MΩ) filled with intracellular solution (for glutamatergic postsynaptic currents, Cs(CH3)SO3 145, NaCl 2, HEPES 10, EGTA 0.2, QX-314 bromide 5, Mg-ATP 4, Na-GTP 0.3, in mM, at pH 7.25). Electrical signals were filtered at 3k Hz with Axon MultiClamp 700B (Molecular Devices) and collected at 20 kHz with Axon Digidata 1550A (Molecular Devices).

To obtain light-evoked responses, the light beam from an LED light source (X-Cite 120LED, Excelitas Technologies) was delivered through an optical filter (475/30) and then 40x water-immersion objective (Zeiss) onto neurons. For CRACM experiments, 2-msec light pulses were given either 5 times at 1 Hz for 4 cycles or 1 time for 20 cycles. To verify GABAergic connections, picrotoxin (PTX, 100 μM) was applied through perfusion, whereas for glutamatergic connections, 6-Cyano-7-nitroquinoxaline-2,3-

dione (CNQX, 10 µM) and DL-2-Amino-5-phosphonopentanoic acid (APV, 25 µM) were used.

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**a** NaCl

AAV-flex-GCaMP6s

PDYN-Cre

eYFP
GCaMP6s

**b** Water

eYFP
GCaMP6s

**c** GCaMP6s    eYFP

**d**

NaCl
KCl

**e**

- Ami
+Ami

**f** 1 sec NaCl access
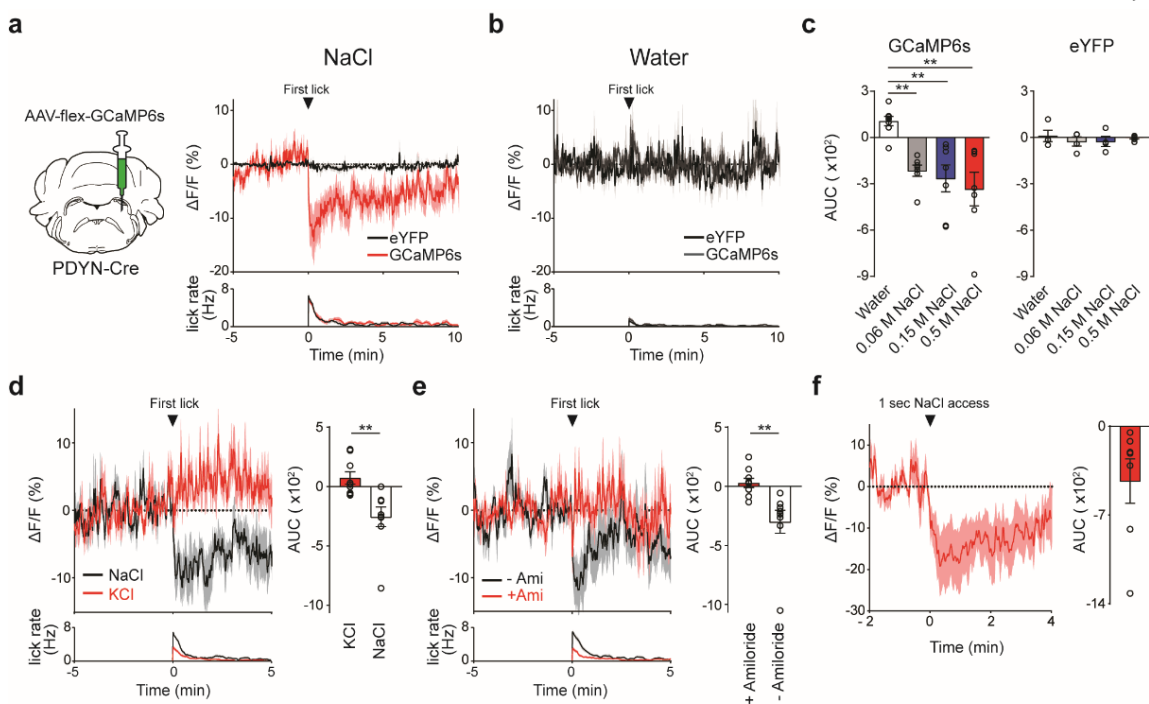
**Figure 1. Sodium appetite neurons are rapidly modulated by sodium taste signals. a,** Photometry recording of GCaMP6s signals from pre-LC$^{PDYN}$ neurons (n = 7 mice for eYFP and GCaMP6s). **b,** No suppression was observed when the animals licked water (n = 4 and 8 mice for eYFP and GCaMP6s). **c,** Fluorescent change (AUC) was calculated upon consumption of water and NaCl solutions (n = 4, 7, and 7 mice for eYFP, 0.06 M, and 0.15 M NaCl, respectively). **d,** Ingestion of KCl did not affect pre-LCPDYN neuron activity (n = 9 mice). **e,** Blocking the ENaC by amiloride eliminated inhibition (n = 9 mice). **f,** A brief NaCl intake for 1 sec induced persistent suppression (n = 7 mice). **P<0.01 by Kruskal–Wallis test (Dunn's multiple comparison) or two-tailed Wilcoxon test. Data presented as mean ± s.e.m.

**a** Optical recording / Intragastric (IG) infusion

**b**

- Sodium

NaCl intake (# of licks)

Control | NaCl IG | NaCl Oral

*

- Water

Water intake (# of licks)

Control | Water IG | Water Oral

*

***

- Food

Food intake (g)

Control | Glucose IG

**

**c** Oral / NaCl

ΔF/F (%)

Time (min)

**d** IG infusion

NaCl | Water | Air

Time (min)

eYFP
GCaMP6s

**e**

GCaMP6s

AUC (×10³)

NaCl IG | Water IG | Air IG | NaCl Oral

* | * | **

eYFP

AUC (×10³)

NaCl IG | Water IG | Air IG | NaCl Oral
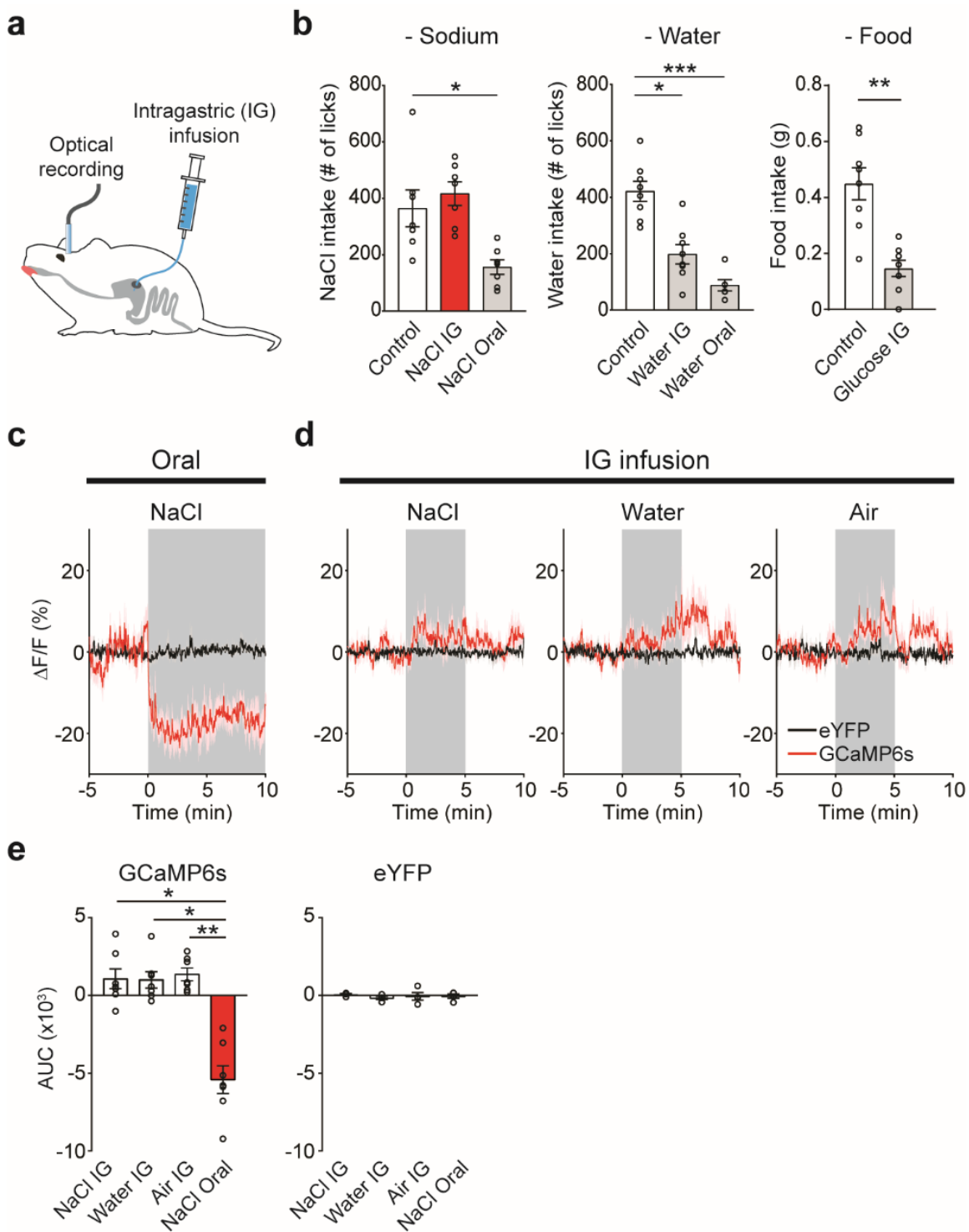
**Figure 2. Oral sodium detection promotes satiation of sodium appetite by suppressing pre-LC$^{\text{PDYN}}$ neurons. a,** Simultaneous optical recording of pre-LC$^{\text{PDYN}}$ neurons and intragastric (IG) infusion. **b,** The effects of gastric infusion of sodium, water, and glucose (5M) on subsequent ingestive behaviors for 10 min (n = 7 mice for NaCl, n = 6 mice for water oral, n = 8 mice for controls, water IG and glucose IG). **c,** Oral sodium consumption suppressed pre-LC$^{\text{PDYN}}$ neuron s activity (n = 4 and 7 mice for eYFP and GCaMP6s). **d,** However, fluorescence signals were not affected by IG infusion of NaCl, water, or air. **e,** Quantification of c and d. *P<0.05, **P<0.01 and ***P<0.001 by Friedman, Kruskal–Wallis test (Dunn's multiple comparison), or two-tailed Wilcoxon test. Data presented as mean ± s.e.m.

**a** AAV-DIO-ChR2 / NTS^HSD2 / Pre-LC^PDYN / HSD2-Cre/PDYN-GFP / CNQX/APV / 20 pA / 10 ms

**b** AAV-flex-Casp3 / NTS^HSD2 / Pre-LC / HSD2-Cre

NTS / + Casp3 / Control / HSD2

# of HSD2 neurons / + Casp3 / - Casp3 / ****

Pre-LC (- sodium) / + Casp3 / Control / c-Fos / Foxp2

# of c-Fos+ neurons / + Casp3 / - Casp3 / ****

**c** AAV-flex-TVA + RG / SAD-ΔG-BFP (EnvA) / PDYN-Cre / PreLC / CeA / dBNST / PVN / NTS

# of RV+ cells / CeA / dBNST / PVN / NTS

**d** dBNST / +RG / -RG / SAD-BFP / PDYN

**e** AAV-DIO-ChR2 / dBNST^PDYN / Pre-LC^PDYN / PDYN-Cre/Ai75D / PTX / 100 pA / 0.5 s

**f** dBNST → pre-LC neurons / ΔF/F (%) / - Ami / + Ami / Time (s) / AUC / + Ami / - Ami / *

**Figure 3. Pre-LC$^{PDYN}$ neurons receive both homeostatic and sensory inputs. a,** NTS$^{HSD2}$ neurons send monosynaptic inputs to pr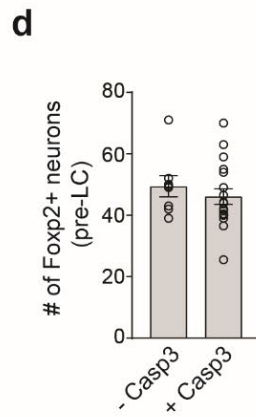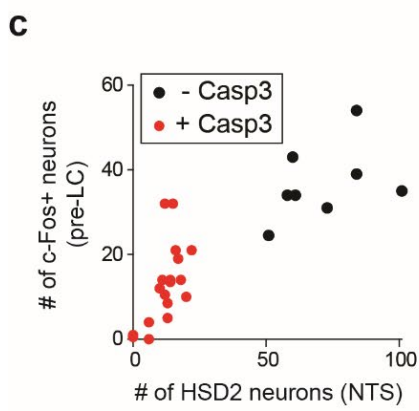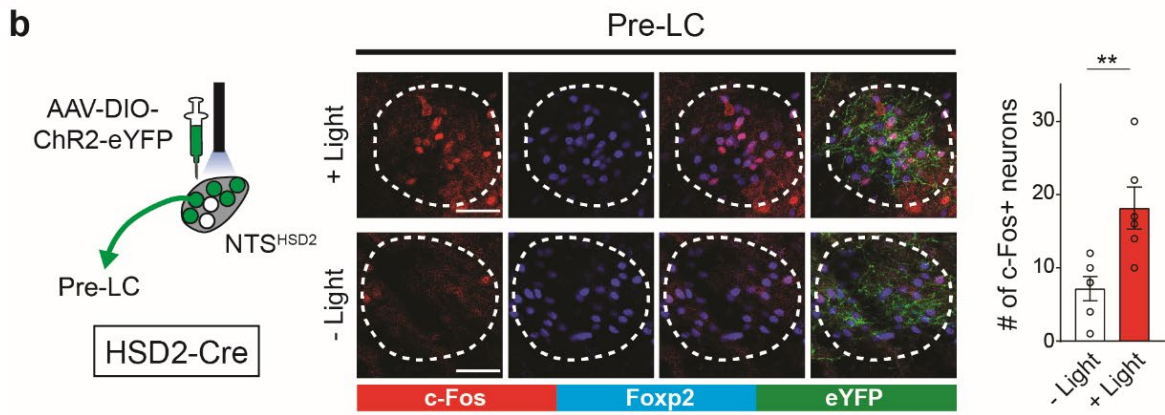e-LC$^{PDYN}$ neurons (23/38 neurons) with an EPSC latency 6.3 msec. **b,** Ablation of NTS$^{HSD2}$ by AAV-flex-Casp3 (left panels) drastically reduced the pre-LC activity under sodium depletion (right panels, n = 18 from 9 mice for + Casp3, and n = 8 from 4 mice for – Casp3). Pre-LC$^{PDYN}$ neurons were visualized by Foxp2 immunostaining. **c,** Monosynaptic rabies tracing from pre-LC$^{PDYN}$ neurons (left panel). Representative images of the pre-LC, CeA, dBNST, PVN, and NTS (middle panels), and the number of SAD-positive neurons (right panel) was quantified (n = 5 mice). **d,** Monosynaptic dBNST$^{PDYN}$ → pre-LC$^{PDYN}$ projections. A magnified image from c showing that SAD-ΔG-BFP overlaps with PDYN expression in the dBNST (upper panels, 71.7 ± 6.8%, n = 5). Control tracing experiments without RG are shown (bottom panels). **e,** Monosynaptic inhibitory connections of dBNST$^{PDYN}$ → pre-LCPDYN (28/44 neurons) with an IPSC latency of 7 msec. f, GCaMP6s was retrogradely delivered to dBNST → pre-LC neurons by infecting CAV-Cre in the pre-LC and AAV-flex-GCaMP6s in the dBNST. Shown are calcium responses of dBNST → pre-LC neurons toward sodium with or without amiloride (n = 7 mice). Scale bar, 50 μm. *P<0.05, ****P<0.0001 by two-tailed Wilcoxon or two-tailed Mann-Whitney test. Data presented as mean ± s.e.m.
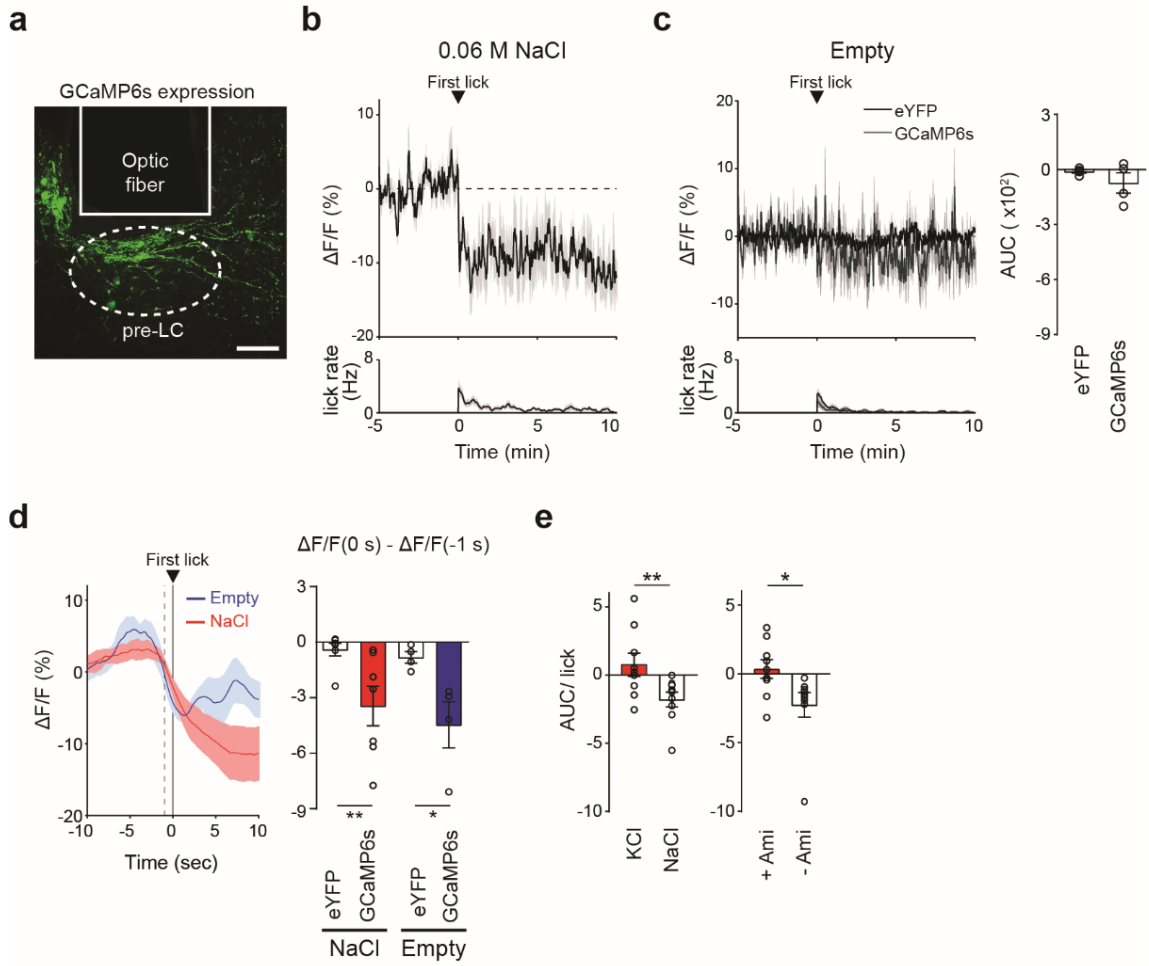
**Extended Data Figure 1. In vivo activity of pre-LC$^{\text{PDYN}}$ neurons upon ingestive behaviors. a,** Placement of an implanted optic fiber and GCaMP6s expression in the pre-LC. Scale bar, 50 μm. **b,** A low concentration of NaCl exhibited inhibitory effects on pre-LC$^{\text{PDYN}}$ neurons (0.06 M, n = 7 mice). **c,** Licking empty spout had no inhibitory effect on pre-LC$^{\text{PDYN}}$ neurons (n = 4 mice for eYFP, n = 4 mice for GCaMP6s). **d,** Peristimulus time histogram of GCaMP signals around the start of sodium ingestion. Data were magnified from c and Fig. 1a. Fluorescence changes (ΔF/F) from -1 to 0 sec was calculated. **e,** Activity change per lick was quantified for Fig. 3d and 3e. *P<0.05, **P<0.01 by two-tailed Wilcoxon or two-tailed Mann-Whitney test. Data presented as mean ± s.e.m.

**a**

PDYN-GFP

PDYN | c-Fos

**b**

AAV-DIO-ChR2-eYFP

NTS^HSD2

Pre-LC

HSD2-Cre

Pre-LC

+ Light

- Light

c-Fos | Foxp2 | eYFP

# of c-Fos+ neurons

\*\*

- Light    + Light

**c**

# of c-Fos+ neurons (pre-LC)

- Casp3
+ Casp3

# of HSD2 neurons (NTS)

**d**

# of Foxp2+ neurons (pre-LC)

- Casp3    + Casp3
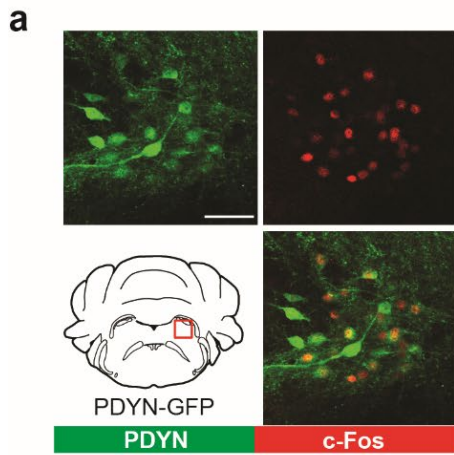
**Extended Data Figure 2. Functional analysis of the NTS$^{HSD2}$→ pre-LC$^{PDYN}$ connections. a,** Functional validation of PDYN-GFP transgenic animals. Similar to PDYN-Cre line, GFP-positive neurons in the pre-LC mice were activated by sodium-depletion in PDYN-GFP mice (One out of 2 mice). **b,** A diagram of optogenetic stimulation of HSD2 neurons. Foxp2-positive pre-LC neurons express c-Fos after HSD2 stimulation (n = 6 hemispheres from 3 mice). **c,** Relationship between the number of HSD2 neurons in the NTS and c-Fos-positive neurons in the pre-LC. >95% of c-Fos-positive neurons expressed Foxp2. **d,** Number of Foxp2-positive neurons was not affected by the ablation of HSD2 neurons (n = 18 hemispheres from 9 mice for + Casp3, and n = 8 hemispheres from 4 mice for – Casp3). Scale bar, 50 μm. **P<0.01 by two-tailed Wilcoxon test. Data presented as mean ± s.e.m.

**a**
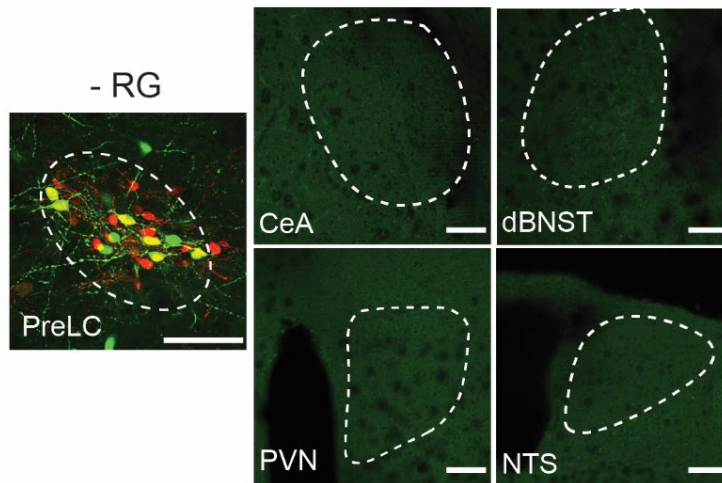


**b**



**c**
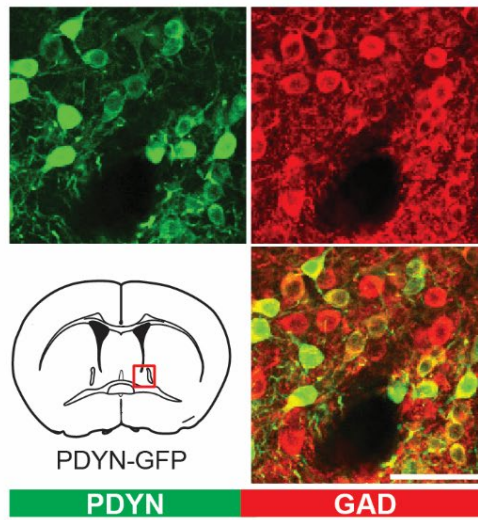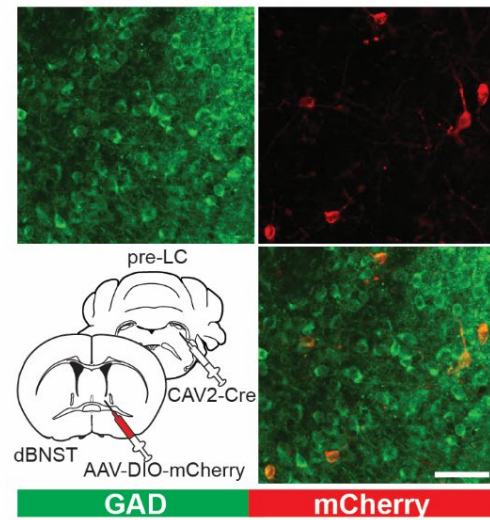
**Extended Data Figure 3. Histological analysis of putative upstream brain structures of pre-LC^PDYN neurons. a,** Control monosynaptic tracing experiments without RG (One out of 3 mice). Scale bar, 100 μm. **b,** A majority of PDYN neurons in the dBNST (green) are inhibitory neurons (red, 77.3 ± 1.7%, n = 3 mice). **c,** CAV-2 positive neurons in the dBNST retrogradely labeled from the pre-LC (red) were inhibitory neurons (One out of 3 mice). Scale bar, 50 μm. The mouse brain in this figure has been reproduced from the mouse brain atlas[32].

**a**



AAV-DIO-ChR2-mCherry

Bregma -5.52 mm

PDYN-Cre

Bregma 0.14 mm    Bregma -0.94 mm    Bregma -0.94 mm    Bregma -1.94 mm

vBNST    PVN    PVT    DMH

Bregma -1.94 mm    Bregma -2.46 mm    Bregma -3.08 mm

pre-LC    ARC    LHA    VTA

**b**



Input

NTS$^{HSD2}$    dBNST    CeA    PVN

PreLC$^{PDYN}$

SFO$^{Agtr1a}$    vBNST    PVT    VTA    LH    DMH/PVN ARC    Output

Preference /valence    Reward    ?
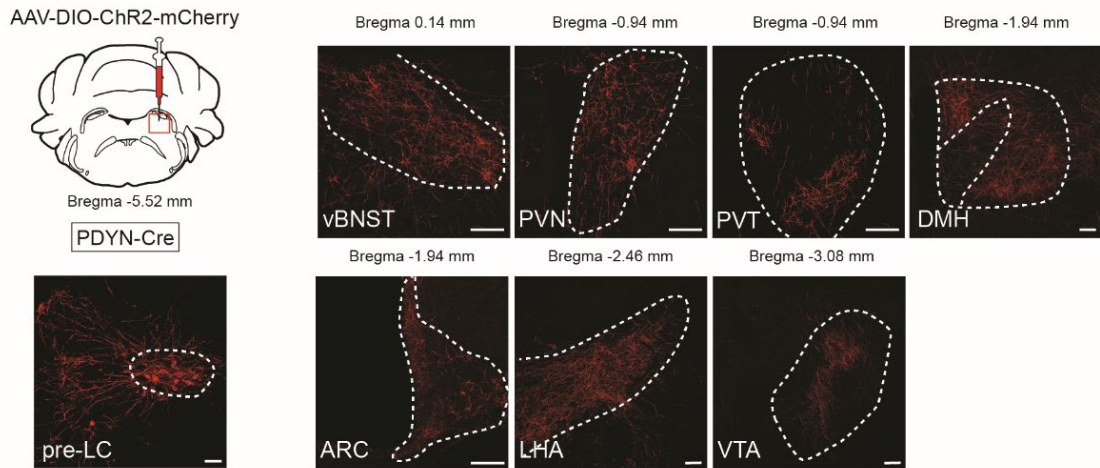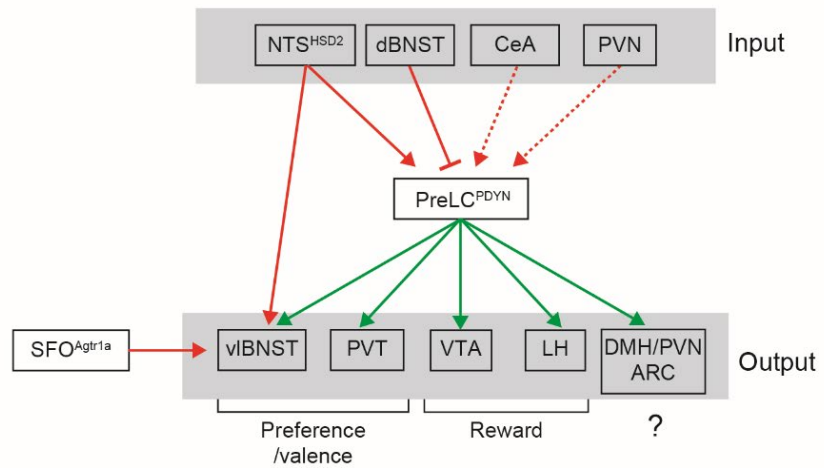
**Extended Data Figure 4. Downstream projections of pre-LC^PDYN neurons. a,** PDYN-Cre mice were injected with AAV-DIO-ChR2-mCherry into the pre-LC. Representative axonal projections are shown (one out of six mice). Arc, arcuate nucleus; vBNST, ventral bed nucleus of the stria terminalis; DMH, dorsomedial hypothalamic nucleus; LHA, lateral hypothalamus area; PVN, paraventricular hypothalamic nucleus; PVT, paraventricular thalamic nucleus; VTA, ventral tegmental area. Scale bar, 50 μm. **b,** A wiring diagram of upstream and downstream neural connections of pre-LC^PDYN neurons.

*Chapter 4*

## FINDINGS AND DISCUSSION

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Recent in vivo analyses of appetite circuits in rodents revealed that ingestive related sensory information rapidly modulates appetite circuits before nutrient absorption in the body. Thirst, hunger, and sodium appetite neurons are all inhibited upon the ingestion of its needs. However, the underlying mechanisms appear to be different for individual appetites. Distinct sensory modulation is involved in the satiation mechanism in each appetite circuit. Hypothalamic hunger neurons are acutely suppressed by food anticipation as well as nutrient detection in the gut. Conversely, thirst neurons are inhibited oropharynx detection like liquid-gulping motion and also from the gut. Meanwhile, here I revealed another aspect of appetite regulation: chemosensory signals. Our circuit analysis revealed that pre-LC$^{PDYN}$ neurons receive multiple inputs from upstream neural populations, including NTS$^{HSD2}$ and BNST$^{PDYN}$ neurons. Defining the genetic identity of these populations would provide further insights for homeostatic and chemosensory regulations of the appetite circuit. Here, I summarized the recent studies of the appetite circuits and further compared the distinct mechanism of the appetite circuits.

## 4.1. Appetite

Ingestion of the appropriate amount of each nutrient factor is crucial for maintaining body homeostasis[1-5]. Improper nutritional balance has been linked to a wide spectrum of disorders[6-8]. For example, too much feeding leads to obesity, whereas high sodium diet poses a risk of cognitive and cardiovascular disorders[6,9,10]. Repeated dehydration in the body is known to damage kidney functions chronically[11]. Thus, optimized ingestive behavior is important for survival and well-being. Modern neurotechnologies have brought us a better understanding of neural circuits underlying body fluid and energy balance at an anatomical and functional level[12-14]. As discussed in this chapter, appetite circuits for different nutrients (energy, water, and sodium) receive unique combinations of sensory modulation arising from different parts of the body[1,15-17]. These signals-chemosensory, hormonal, and gut-to-brain afferent pathways-play key roles in appetite induction and satiation. Why does the brain need such complicated regulations for each nutrient factor? Identification of key neural circuits revealed an important conceptual framework as to how the brain regulates body nutrient homeostasis. This review will focus on three major appetites--hunger, thirst, and sodium craving--and discuss how nutrient-related sensory signals regulate our ingestive behavior. In particular, we will summarize the current understanding of sensory modulation and operating time scale of individual appetite circuits, focusing mainly on mammalian systems. We propose that appetite and satiation signals are specifically tailored for each nutrient factor based on the internal nutritional need. More broadly, the activity of central appetite circuits defines "what" to ingest, while

sensory modulation from the body determines "how much" to consume a given nutrient.

## 4.2. Feedback and feed-forward regulation on appetite

One of the major drives of appetite is homeostatic regulation, a concept adopted by Walter Cannon in the 1930s. In this model, appetite is viewed as a passive process, with nutrient depletion being the major driving factor while nutrient absorption being the quenching factor (post-absorption signals)[18]. Another line of evidence in behavioral and psychophysical experiments suggested that central appetite circuits are actively regulated by sensory signals from the periphery prior to nutrient absorption (pre-absorption signals)[19-21]. From the behavioral perspective, both pre- and post-absorptive regulations are feedback signals as they are consequences of ingestive behaviors. From the homeostatic perspective, post-absorption factors are feedback signals whereas pre-absorptive factors are feed-forward because they precedes changes in homeostatic state[1,14]. For clarity of terminology, we will use the terms from the homeostatic standpoint throughout this review.

## 4.3. Operating time scale of appetite neurons

*Thirst*

There are dedicated neurons and brain circuits underlying major appetites: water, sodium, and energy (food). The common function of these neural circuits is to drive animals toward foraging/consuming specific nutrient factors. However, rapid neural manipulation paradigms (e.g. optogenetics) revealed drastic differences in the operating time of individual appetite neurons (Fig. 1). These differences could have

profound effects on our ingestive behavior. A brain structure called lamina terminalis (LT) in the mammalian forebrain is the primary site that regulates thirst. In the LT, neurons that control water intake are housed in three anatomically linked nuclei, the subfornical organ (SFO), organum vasculosum of LT, and median preoptic nucleus (MnPO). The former two structures lack the blood brain barrier, and directly sense the internal water balance[16,22]. Thirst neurons are mainly excitatory neurons that express genetic markers such as NO synthase (nNos)[23-29]. A series of studies demonstrated that the activity of thirst neurons have a time-locked causal relationship with drinking behavior[23-26,28-33]. For instance, optogenetic stimulation of SFO thirst neurons rapidly drives water intake within a few seconds. Conversely, once the stimulation is turned off, animals rapidly stop water intake [28-30]. The rapid on and off effects were observed in various thirst-related brain nodes including other LT nuclei and their projection site[23-25,27,32]. These findings demonstrate that water-seeking and drinking behavior is precisely time-locked to thirst neuron activity in the brain.

*Sodium appetite*

A similar temporal relationship between neural activity and behavior was observed for sodium appetite circuits. Internal sodium depletion increases a combination of hormonal secretion: aldosterone and ANG II[34,35]. These signals directly stimulate interoceptive neurons located in both the LT and the nucleus

solitary tract (NTS)[2,3,36]. In particular, HSD2- positive neurons in the NTS respond to aldosterone and ANG II[37,38]. Recent studies demonstrated that activation of HSD2 neurons or a subset of SFO neurons increases preference toward sodium through their

downstream sites[32,37,39]. In HSD2 neurons, sodium deficiency was shown to increase spontaneous pacemaker- like activity that correlates with enhanced gene expression of several cation channels[37]. Further studies revealed that interoceptive signals from HSD2 neurons are transmitted to a subset of excitatory neurons in the pre-locus coeruleus (pre-LC)[37,39,40]. These neurons, characterized by the expression of prodynorphin (PDYN) and Foxp2, are causally linked to sodium ingestion in mice[40,41]. Similar to thirst neurons, stimulation of PDYN neurons with optogenetics immediately induces sodium intake in fully sated animals. Termination of light illumination rapidly ceased sodium ingestive behavior[40]. Therefore, the ongoing-activity of thirst and sodium appetite neurons is closely linked to water and sodium intake.

*Hunger*

Another key appetite, hunger, runs on a quite different operating scheme. Need-based feeding is mainly regulated by GABAergic neurons that express agouti-related peptide (AgRP) in the hypothalamic arcuate nucleus (ARC). These interoceptive neurons detect internal energy deficiency through hormonal signals such as ghrelin[42-45]. Similar to LT neurons, AgRP neurons are exposed to circulation due to the lack of the blood brain barrier[46,47]. Numerous studies have demonstrated that artificial stimulation of AgRP neurons triggers voracious feeding behavior in energy-sated animals via downstream neural circuits[30,48-52]. Moreover, cell-type-specific ablation studies showed that AgRP neurons are required for need-based feeding behavior[53-55]. These topics have been covered in exquisite detail by several excellent reviews[1,14,56]. Importantly,

AgRP neurons are known to be "slow" operating neurons for several reasons. First, unlike thirst and sodium appetite circuits, AgRP neuron stimulation does not immediately initiate feeding behavior. Instead, several minutes of delay is reported from the onset of neural stimulation to the ingestive behavior[48]. Second, the termination of AgRP neuron stimulation does not promptly stop eating behavior. Indeed, pre-stimulation of this neural population with ChR2 causes sustained feeding behavior for 30-60 min after stimulus termination[30]. These studies revealed persistent feeding regulation by AgRP neurons. At the physiological level, a single light pulse to AgRP neurons induces asynchronous and continuous (up to 1 sec) postsynaptic currents at downstream projection sites[49]. At the molecular level, neuropeptide Y (NPY) is required for this sustained behavior. Optogenetic stimulation of AgRP neurons in NPY -/- mice induces time-locked feeding without persistent activity[57]. These results suggest that NPY is uniquely required for sustained hunger but how NPY affects the neural circuit activity remains unknown. Either NPY signals at AgRP downstream sites or recurrent activation circuits may contribute to the sustained ingestive phenotype. It should be noted that not all feeding-related neurons are necessarily slow operating: acute feeding could be triggered through GABAergic neurons in a few brain areas including the zona incerta, lateral hypothalamus[58-60]. Optogenetic manipulation of these neurons/projections results in acute feeding regulation within several seconds that resembles binge eating behavior. Although it is unclear whether these fast-operating neurons are related to homeostatic regulation, feeding behavior involves a complex and heterogeneous regulatory processes[61].

Among them, slow behavioral kinetics appears to be a distinct characteristic of AgRP-related neural circuits.

Taken together, appetite for water, sodium, and energy are regulated by specific interoceptive neurons in the LT, NTS-related nuclei, and ARC, respectively. They share a common function to process internal state information and drive appetite. However, the operating time scales are vastly different: time-locked control for thirst and sodium appetite and delayed/sustained control for feeding (Fig. 1). This neural activity behavior relationship has physiological impacts because all appetite neurons described above are rapidly quenched after nutrient intake as discussed in more detail below. One caveat of appetite kinetics is that the observations are largely based on acute optogenetic manipulation. While this is a powerful experimental approach, it is still unclear how precisely such artificial stimulation can recapitulate natural circuit functions in response to endogenous signals[62,63]. Nevertheless, the results from acute stimulation offer an opportunity to investigate the exact neural and molecular mechanisms underlying distinct time scale for individual appetites.

## 4.4. Nutrient-specific satiation signals from peripheral sensory systems

Generally, nutrient absorption by the body is a relatively slow process taking up several minutes to an hour, but much less time is required for consuming necessary amounts of nutrient. Therefore, if animals only rely on homeostatic feedback to terminate ingestive behaviors, they will end up eating/drinking too much. To solve this conundrum, the brain has evolved feed-forward satiation mechanisms [1]. Imagine that you are extremely thirsty after exercising. Drinking a bottle of water should rapidly

alleviate your thirst. This process takes up less than a minute even though the osmotic environment of the body has not changed by then. From behavioral observations like this in the past decades, it became obvious that animals cease ingestive behaviors before the body absorbs nutrients[64-66]. However, only recently did we begin to understand how the brain processes these pre-absorptive alleviation/satiation signals. A series of studies in the past five years demonstrated that central appetite neurons receive rapid feed-forward modulations as summarized below (Fig. 2).

*Oropharyngeal and gastrointestinal modulation of thirst circuits*

Once thirst neurons in the LT are stimulated by ANG II or hyperosmotic signals, it drives water intake behavior[2,16]. Multiple lines of evidence suggest that water intake stimulates rapid satiation signals that quench thirst. The initial checkpoint of drinking exists in the throat[65,66]. In vivo optical recording studies demonstrated that stimulation of oropharyngeal area by liquid gulping inhibits thirst neurons in the LT[25]. Intriguingly, these responses are triggered by rapid ingestion of liquid, but not solid materials. For example, while water intake strongly suppresses thirst neuron activity, eating the same amount of hydrogel (98% water) does not induce this effect [25]. These data suggest that the brain somehow "knows" that animals have drunk liquid and anticipates future water balance. Tactile signals through vagal or other cranial nerves likely mediate gulping-induced initial thirst satiation[25]. The second checkpoint is in the gut that senses osmolality changes. Once liquid passes through the throat, the brain waits for the next satiation signals from the gastrointestinal area to confirm that the ingested liquid will indeed rehydrate the body. Studies in awake behaving animals have shown that SFO

thirst neuron activity is drastically suppressed by hypoosmotic stimuli in the gut[33,67]. Although the precise mechanisms remain unknown, the vagal pathway was suggested to transmit gut osmolality signals to the brain[33,68]. Importantly, oropharyngeal- and gut-mediated sensory signals work in series after water intake[20,66]. Liquid-selective gulping signals initially inhibit thirst neurons. They are followed by osmolality-dependent sustained signals from the gut. In

the brain, these two satiation signals are represented by different GABAergic neural populations in the LT that express glucagon-like peptide 1 receptor (GLP1r). A subpopulation of GLP1r-positive MnPO neurons are activated during drinking behavior, in particular by gulping action[25,33]. Conversely GLP1r-positive SFO neurons respond to gut osmolality change[67].Thus, specific and independent pathways from the body to the brain appear to mediate individual thirst satiation signals.

*Taste modulation of sodium appetite circuits*

Sodium appetite neurons also receive feed-forward inhibition after sodium intake. Similar to water intake, animals rapidly terminate need-based sodium intake before sodium absorption by the body[21,64]. Recent dissection of sodium appetite circuits has shed light on the mechanisms of feed-forward satiation signals. In vivo recording of neural dynamics in the pre-LC showed that taste signals are the major feed-forward signals from the periphery: the activity of sodium appetite neurons (PDYN neurons) were rapidly suppressed upon oral sodium contact in depleted animals[40]. This effect was sodium taste dependent because 1) suppression of PDYN neurons was only observed with sodium ions, 2) blocking sodium taste receptor, ENaC, by amiloride

abolished this suppression[69-71], and 3) gastric sodium infusion (bypassing oral contact) had no inhibitory effect. Thus, for this particular nutrient, oral chemosensory signals play a pivotal role in pre-absorptive satiation. This idea is supported by previous studies showing that oral sodium detection is a critical factor for quenching appetite for sodium. Taste signals are transmitted from the tongue to the brain through facial nerves, but how they modulate sodium appetite neurons is unknown.

*Pre- and post-ingestive modulation of hunger circuits*

Feed-forward regulation also impacts hunger circuits. An elegant set of studies have shown that AgRP neurons are rapidly suppressed by sensory detection of food as well as gut caloric detection. Any food-associated cues including visual, olfactory, and taste are sufficient to suppress AgRP neuron activity[26,72-74]. The association between calorie and sensory cues can be formed even after a single eating episode[52,74]. However, this cue-induced suppression is temporal, because AgRP neuron activity bounces back if no calorie is detected in the gut[26,72-74]. Thus, it is now clear that caloric sensing in the gut is a primary pre-absorptive satiation signal that persistently suppresses AgRP neurons. Importantly, the amplitude of neural suppression is proportional to caloric contents in the gut. How are these satiation signals transmitted to the brain? A piece of evidence suggests that peripheral injection of anorexic hormones inhibit AgRP neurons[72,74]. Some of such hormones (e.g., CCK) stimulate vagus nerves, implying that rapid feed-forward signals are mediated by neurons in the nodose ganglia[75-78]. Alternatively, since the ARC is exposed to circulation, the hormonal effects may be transmitted through the blood circulation. One study investigated the neural basis of

feed-forward AgRP suppression, and identified a genetically defined population that expresses leptin receptor in the dorsal medial hypothalamus[79]. These neurons are 1) GABAergic inhibitory population, 2) rapidly activated upon foraging and feeding, and 3) monosynaptically connected to AgRP neurons. Thus, leptin receptor-positive neurons, at least in part, convey feed-forward modulation to AgRP neurons. Although one neural substrate for rapid satiation is uncovered, many questions remain unanswered. For example, just seeing food does not suppress our craving for food[80,81], but hunger neurons are suppressed by the same stimulus. Thus, the perception of hunger and eating behavior itself may be regulated by overlapping but distinct mechanisms. Another question is the gut-to-brain signal transmission. It was suggested that different hormonal signals are involved in fat-, sugar-, and protein-induced satiation signals from the gut[72,74]. In other species including humans, Drosophila, and C. elegans, element-specific appetite has been well documented although the precise feedback and feed-forward mechanisms remain to be discovered [4,82-85]. Further studies will clarify whether and how peripheral detection of individual nutrient components modulates brain appetite circuits.

This review exclusively focused on feed-forward aspects of appetite modulation. It is, however, important to note that homeostatic feedback signals also play crucial functions for ingestive termination on a longer time scale. For instance, intravenous infusion of water or caloric contents can alleviate thirst and hunger, respectively, without apparent feed-forward signals[86,87]. Similarly, gastric sodium load provides little satiation effects in the order of minutes. But a few hours after loading, sodium intake is significantly suppressed due to feedback signals[21,40]. Therefore, nutrient absorption

itself also carries satiation signals to inhibit central appetite circuits. In reality, sensory-based feed- forward signals and homeostatic-based feedback signals work together to regulate animals' daily ingestive behavior. How these two sources of signals modulate each other is an important area of future study.

## 4.5. Need-based neural circuit architecture for appetite and satiation

Different appetite circuits drive ingestion over distinct time scales, and receive unique sensory modulations from the periphery. What is the evolutionary benefit of having dedicated regulations for each appetite instead of a single mechanism? We propose that operating time of appetite neurons and feed-forward signals are specifically evolved to meet the nutrient need. The requirement of nutrient factors widely varies. Humans roughly consume 2-5 liters of water and 1500-3000 kcal of energy while we approximately need only 1.5 g of sodium daily[88-90]. Laboratory mice also consume widely variable amounts of nutrients[91,92]. How does the brain adjust ingestive behavior in order to meet variable and unique nutrient needs of the body? Compared to other major nutrients, sodium requirement is minimal due to sodium retention in the kidney [93]. Correspondingly, sodium appetite neurons are fast-operating: their activity is precisely time-locked to ingestive behavior[40]. The activation is rapidly quenched upon sodium detection in the oral cavity. We speculate that the combination of acute appetite induction and satiation allows ingestion of a minimum but necessary amount of sodium. Conversely, animals need to consume a larger quantity of food through slow chewing or biting processes. This would require longer behavioral control by the brain after the onset of eating. In fact, major hunger satiation signals arise from the

gut, which are much slower to take effect compared to taste signals. Consequently, this delayed signals allow animals more time to consume food. Importantly, as mentioned above, AgRP neurons drive persistent feeding even after the activity is quenched. This slow and persistent behavioral regulation may contribute to maximizing food consumption. Thirst is somewhere in-between because thirst neuron activity corresponds well with drinking behavior, but major satiation signals derive from oropharyngeal and gut areas[25,29,33,67]. This combination may allow animals to consume enough water before satiation signals terminate the behavior. Altogether, these functional analyses of major appetite circuits suggest that feed-forward signals associated with ingestion are a key factor to determine the amount of ingestion. If these speculations are correct, then interrupting feed-forward signals should affect ingestive behavior. Recent studies on the thirst circuit addressed the functional relevance of feed-forward signals to drinking. When thirst satiation neurons (GLP1r-positive neurons) in the LT are functionally silenced, animals spend an unusually long amount of time in drinking[25,67]. Apart from the LT, several candidate neurons that may encode feed-forward signals have been identified. GLP1r-positive neurons in the paraventricular hypothalamus are activated by food cues and caloric contents[94]. Stimulation of these neurons bidirectionally regulates feeding behavior. Oxytocin receptor-positive neurons in the parabrachial nucleus are acutely activated by fluid intake, suggesting their roles in fluid satiation[95]. Broader analysis of satiation circuits will provide further insights into the roles of pre-absorptive satiation signals in ingestive control.

Our natural instinct, when we get thirsty, is to look for liquid water and gulp it down. However, nutrient availability is not equal for all species. A typical example is water sources: although some species like laboratory mice or humans drink liquid water, it is rarely available for animals in arid climates. Indeed, these animals generally eat instead of drink to gain water[96-98]. To deal with this nutrient pressure, desert species have adapted their body (e.g., kidney morphology and function) for better water conservation[99]. In the nutrient-specific feed-forward model, we predict that central appetite and satiation circuits have co-evolved to deal with nutrient availability in the environment. More specifically, 1) liquid-based feed-forward satiation mechanisms (i.e., gulping) may have been replaced by other mechanisms, and 2) thirst circuit activity may be innately linked to eating behavior in arid species. In this regard, it would be interesting to compare appetite and satiety circuits in laboratory mice and desert rodents that rarely drink water throughout life, and investigate how their behavior and neural functions adapted to their living environment.

## 4.6. Conclusion

In the past decade, cell-type-specific analysis has seen great success in unveiling the function of interoceptive neurons and their immediate downstream neural circuits[1,12,14,17,56] These studies have revealed appetite regulation mechanisms for major nutrient factors: water, sodium and energy. First, they have Thirst and sodium appetite circuits are fast operating in that neural activity drives appetite and ingestive behavior rapidly. Conversely, need-based hunger circuits seem to have slower behavioral kinetics after neural firing. Second, all appetite neurons receive feed-forward satiation

signals before nutrient absorption. However, the source of these signals are quite different. As summarized in Figure 2, thirst, sodium appetite, and hunger circuits are modulated by distinct combinations of pre- and post-consumption signals form the periphery. Recent studies suggested that a unique appetite induction and satiation mechanism may contribute to optimal nutrient ingestion and fluid/energy homeostasis.

Despite better understanding of appetite regulation, there are still many missing pieces of puzzles to understand the basis of ingestive regulation. For one thing, it remains unknown how signals from interoceptive neurons modulate our perception of nutrient, motivational state, and behavioral output. For another, the precise neural pathways/mechanisms underlying feed-forward appetite regulation waits further investigation. Expanding neurotechnologies including transcriptomics and gene editing should provide a springboard to tackle these key questions.

## 4.7. Acknowledgements

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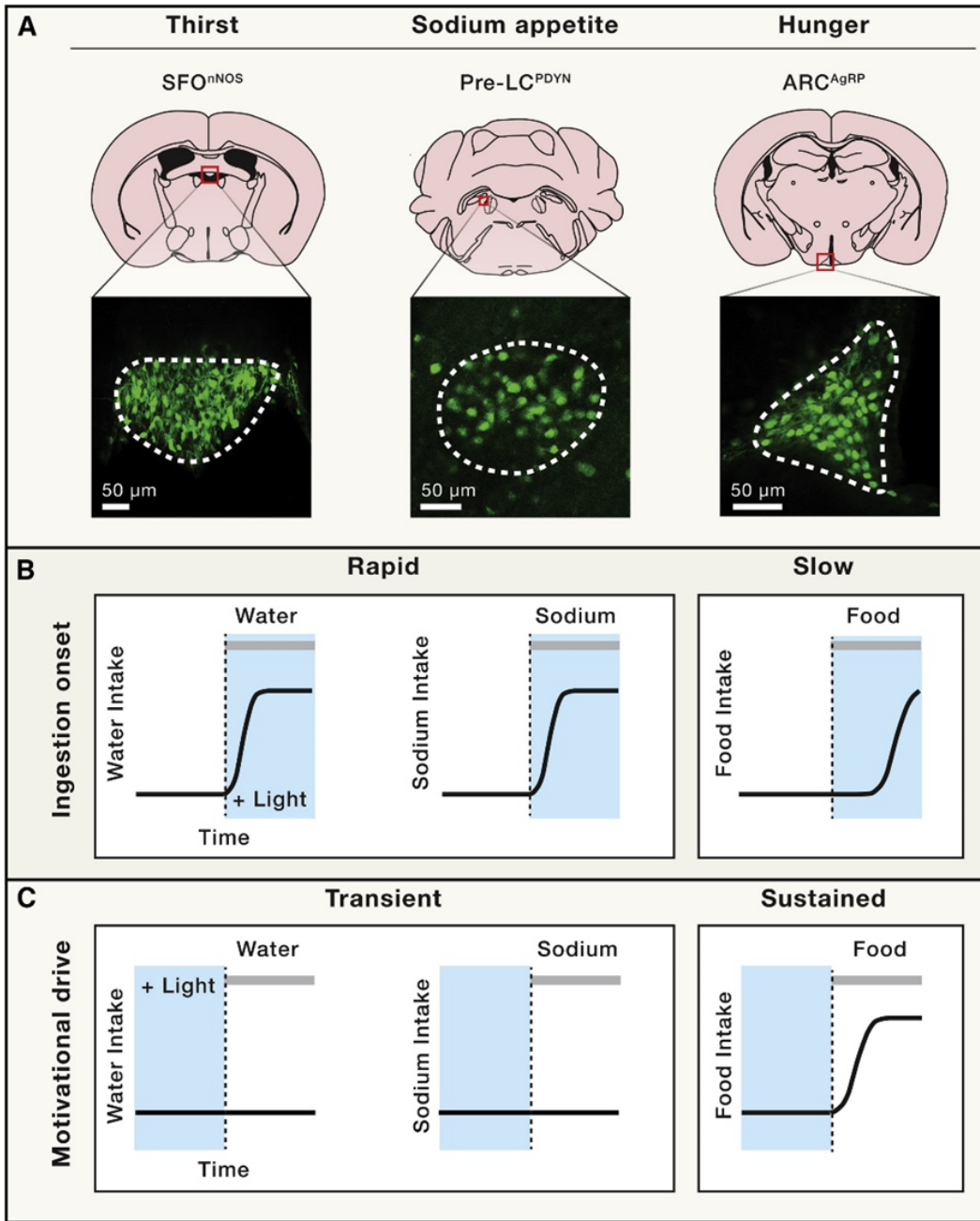
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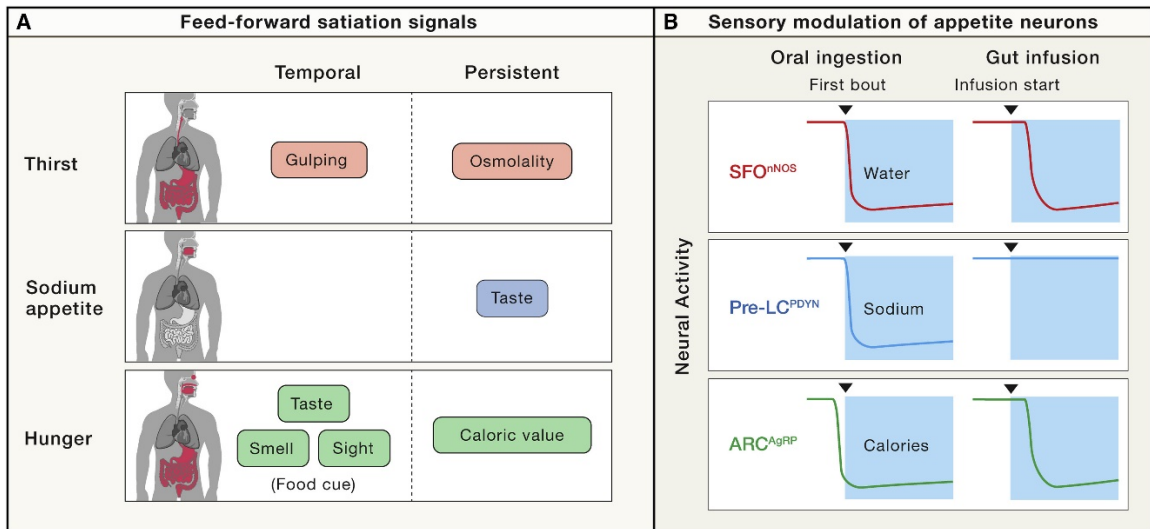
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**A**

Thirst | Sodium appetite | Hunger

SFO^nNOS | Pre-LC^PDYN | ARC^AgRP

50 µm | 50 µm | 50 µm

**B**

Ingestion onset

Rapid | Slow

Water | Sodium | Food

Water Intake / + Light / Time | Sodium Intake | Food Intake

**C**

Motivational drive

Transient | Sustained

Water | Sodium | Food

+ Light / Water Intake / Time | Sodium Intake | Food Intake

**Figure 1. Operating Timescale of Distinct Appetite Neurons a,** Representative neuronal populations that regulate thirst (left), sodium appetite (middle), and hunger (right) visualized with fluorescence reporters. Shown are coronal sections of mouse brain containing individual neural populations (upper panels), and magnified images (lower panels). Scale bars, 50 μm. **b,** Temporal relationship between neural stimulation and nutrient consumption. The onset of ingestion upon optogenetic activation of appetite neurons. The x axis shows the time before and after neural stimulation, and the y axis shows nutrient consumption. Stimulation of thirst (left) and sodium appetite (middle) neurons induces rapid consumption. Conversely, hunger neurons (right) drive feeding with a longer latency. Photostimulation periods are shaded in blue, and nutrient access is indicated by gray bars. **c,** Motivational drive after continuous stimulation of appetite neurons. In the absence of ongoing thirst (left) and sodium appetite (middle) neuron activities, animals do not consume water and sodium, respectively. By contrast, robust food consumption is induced after the termination of hunger neuron stimulation (right). Although AgRP-related homeostatic feeding regulation is a slow process, not all feeding circuits are slow-operating. For example, stimulation of GABAergic neurons in a few brain areas drives acute feeding (Hao et al., 2019, Jennings et al., 2013, Zhang and van den Pol, 2017). Abbreviations are as follows: AgRP, agouti-related protein; ARC, arcuate nucleus; nNOS, neuronal NO synthase; PDYN, prodynorphin; pre-LC, pre-locus coeruleus; SFO, subfornical organ.

**A**    Feed-forward satiation signals

Temporal    Persistent

Thirst    Gulping    Osmolality

Sodium appetite    Taste

Hunger    Taste / Smell / Sight (Food cue)    Caloric value

**B**    Sensory modulation of appetite neurons

Neural Activity

Oral ingestion    Gut infusion
First bout    Infusion start

SFO$^{nNOS}$    Water
Pre-LC$^{PDYN}$    Sodium
ARC$^{AgRP}$    Calories

**Figure 2. Feed-Forward Regulation of Appetite Circuits. a,** Temporal and persistent feed-forward factors regulating thirst (top), sodium appetite (middle), and hunger (bottom). Thirst circuits are temporally inhibited by liquid gulping through signals derived from the oropharyngeal area, while persistent inhibition stems from the gut based on the osmolality of the ingested fluid. Sodium taste signals arising from the mouth persistently inhibit sodium appetite circuits. Various food-related cues like vision, smell, or taste contribute to temporal inhibition of hunger neurons in the ARC. Persistent modulation of hunger neurons is caused by caloric detection by the gut. **b,** Sensory modulations of appetite neurons. Shown are schematic calcium dynamics of thirst (red), sodium appetite (blue), and hunger neurons (green) after oral and intragastric nutrient administration. Each neural population receives unique sets of sensory modulation from the periphery after nutrient ingestion