Distinct Neural Representations of Hunger and Thirst in Neonatal Mice before the Emergence of Food- and Water-seeking Behaviors

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SUMMARY

Hunger and thirst are two fundamental drives for maintaining homeostasis, and elicit distinct food- and water-seeking behaviors essential for survival. For neonatal mammals, however, both hunger and thirst are sated by consuming milk from their mother. While distinct neural circuits underlying hunger and thirst drives in the adult brain have been characterized, it is unclear when these distinctions emerge in neonates and what processes may affect their development. Here we show that hypothalamic hunger and thirst regions already exhibit specific responses to starvation and dehydration well before a neonatal mouse can seek food and water separately. At this early age, hunger drives feeding behaviors more than does thirst. Within neonatal regions that respond to both hunger and thirst, subpopulations of neurons respond distinctly to one or the other need. Combining food and water into a liquid diet throughout the animal's life does not alter the distinct representations of hunger and thirst in the adult brain. Thus, neural representations of hunger and thirst become distinct before food- and water-seeking behaviors mature and are robust to environmental changes in food and water sources.

INTRODUCTION

Hunger and thirst drive feeding and drinking to maintaining energy and water homeostasis, respectively. Neural circuits regulating hunger and thirst are well distinguished in the adult mammals. For hunger, a well-studied hypothalamic region is the arcuate nucleus (ARC)[1]. Agouti-related protein (AgRP)-expressing neurons in ARC are activated by hunger (we refer to these as hunger neurons hereafter) and necessary for feeding in adult mice[2]. Stimulating AgRP hunger neurons is sufficient to drive feeding in fed animals[3]. AgRP neurons project to a wide array of regions both within and outside of the hypothalamus, which have been described in regulating various aspects of feeding behavior[4–7]. For thirst, well-studied brain regions include the

subfornical organ (SFO), vascular organ of the lamina terminalis (OVLT), and median preoptic nucleus (MnPO)[1]. In thirsty animals, specific excitatory neuron types in the SFO and OVLT that express an angiotensin receptor (Agtr1a) are activated by an increase in blood osmolarity and in the level of hormone angiotensin II[8,9], and project to and activate specific population of Agtr1a+ MnPO excitatory neurons (for simplicity, we refer to these dehydration-activated SFO, OVLT, and MnPO neurons as thirst neurons hereafter). Stimulating SFO or MnPO thirst neurons is sufficient to drive drinking behavior in hydrated animals[8–11].

While hunger and thirst circuits have been well characterized in the mature brain, their functions in neonatal stages are much less clear. Neonatal mammals do not have sources of food and water outside of maternal milk, and thus obtain both nutrition and hydration through the same source. Given that mice cannot independently seek food or water, it is unclear if neonatal thirst- or hunger-regulating hypothalamic circuits respond distinctly to these needs or if they overlap in their response given their common behavioral output is increased attachment to the mother and suckling for milk. Furthermore, some studies have suggested that instead of promoting need-specific behaviors, neonatal hypothalamic populations such as the AgRP hunger neurons drive maternal-seeking behaviors rather than milk consumption[12]. Other studies have suggested that hunger-regulating hormones primarily play a role in the maturation of hypothalamic circuits in neonatal stages rather than in the direct regulation of feeding[13–16]. Thus, it is unclear whether adult hunger and thirst neurons are distinct in their responses in this early neonatal stage, or if their activity corresponds to other developmental functions.

Much of this lack of clarity has largely been due to the inability to isolate hunger and thirst in neonates as neonates satiate both drives by consuming maternal milk. In addition, given that the neonates cannot independently seek food or water, it also remains an open question if hunger or thirst alone would be sufficient to drive feeding. Here, we developed a continuous feeding platform for neonatal mice separate from the dam to isolate hunger and thirst response in the neonatal brain. We found that neural responses were already need-specific by the first postnatal week, well before the age at which mice can independently seek water or food. As in adult, ARC neurons responded specifically to hunger while SFO and OVLT neurons responded specifically to thirst in the neonate. Behaviorally, induced hunger or optogenetic activation of AgRP neurons drove feeding behaviors more than did induced thirst or activation of Agtr1a+ neurons in neonatal mice. Furthermore, different subpopulations within MnPO, a region previously described in the thirst-responding circuit[17], were active under hunger and thirst conditions in neonatal stages. Across ARC, SFO, OVLT, and MnPO, distinct neural representations of different need states were maintained in adulthood regardless of whether mice were introduced to food and water separately

or if they were fed a liquid diet throughout their lifetime. Together, our findings demonstrate that hunger and thirst neurons become active in a need-specific manner before animals can independently seek food and water, and suggest that the specificity of their response properties arises innately.

RESULTS

Distinct neural responses to starvation and dehydration in neonatal mice

To isolate hunger and thirst drives in neonatal mice, we developed a continuous feeding platform to selectively induce hunger or thirst in neonatal mice (**Figure 1A, B**). Mice were cannulated with a thin microurethane tube into the upper esophagus and isolated in a warmed chamber containing their homecage bedding, with body temperature maintained at 32°C. Using this platform, mice could be fed with various liquids while isolated from the dam. Using a continuous pump to deliver these liquids, we found that feeding a mouse milk substitute[18] 70–100 µl per hour for approximately 16 hours led to a similar weight gain as homecage mice who fed from their dam for a similar duration of time (**Figure 1C**). Thus, we used this feeding rate and duration for the remainder of experiments using this feeding platform.

To mimic a starvation condition, mice were fed water (distilled water with 35mM NaCl, a hypotonic solution but reduces the chance of significant hyponatremia) for 16 hours before perfusion. To mimic a dehydration condition at the end of the experiment, mice were fed milk[18] for 16 hours but was injected with 2M NaCl at hour 15, 1 hour prior to perfusion. These were compared to “sated” mice who were fed milk without NaCl injection, but who were also isolated in the warmed chamber for 16 hours to control for isolation from the dam and nest as a stimulus. In sated and thirsty mice, feeding resulted in similar weight gains as homecage mice who were not removed from the dam and nest, whereas water feeding resulted in a smaller increase in weight likely due to lack of caloric intake (**Figure 1C**). Mice that were cannulated but not fed with milk or water, mimicking a starvation-and-dehydration condition, showed a marked decrease in weight over the same period.

Using the expression of immediate early gene product Fos as a surrogate for active neurons, we found that mice at this age already demonstrate distinct neural responses to starvation and dehydration. Starvation induced activity in ARC but not OVLT or SFO, whereas dehydration induced activity in OVLT and SFO but not ARC (**Figure 1D, E**). This demonstrates that these putative hunger and thirst regions have distinct activation in this early developmental stage. Interestingly, MnPO, a region that receives input from OVLT and SFO and has been

implicated in the thirst but not hunger response[10,17], showed substantial Fos+ cells under not only dehydration but also starvation conditions (**Figure 1D, E**). As will be described later, these likely represent distinct subpopulations of neurons.

To confirm that starvation-activated neurons in ARC correspond to AgRP neurons, we used the TRAP2 mouse[10,19] to permanently label neurons activated by starvation in neonatal mice. AgRP is known to be co-expressed with neuropeptide Y (NPY) in the ARC hunger neurons[2]. Crossing TRAP2 with *NPY-GFP* mice, we found that starvation-activated neurons labeled in P8 mice fed only water had high correspondence to NPY+ neurons when examined at P14-16 (**Figure 1F, G**). Co-staining with pro-opiomelanocortin (POMC), which is expressed in neurons that respond to satiation rather than starvation, revealed low correspondence with TRAPed neurons in hungry mice at a similar age (**Figure 1F, G**). These findings suggest that the starvation-activated neurons in neonatal ARC largely represent AgRP hunger neurons.

Together, these findings suggest that the neural correlates of hunger and thirst are already present even before animals can perform distinct behaviors in response to starvation and dehydration.

Neonatal feeding behaviors are driven more strongly by hunger than by thirst

Although neural responses to starvation and dehydration were already distinct in neonates, it is unclear the degree to which these responses would promote downstream feeding behavior. Thus, we next asked whether hunger or thirst states were sufficient to induce feeding behaviors. First, using our feeding platform, we induced hunger, thirst, or both in neonates (**Figure 2A**). We then assayed feeding motivation by measuring latency to attach to an anesthetized dam, total time attached to the dam, and weight change after free feeding. For behavioral experiments, thirst was induced by feeding salty milk (See Methods) to remove the need to inject NaCl solutions shortly before the behavior session, which may cause disruptions in behavior.

Following induction of starvation and/or dehydration, we find no significant difference in latency to attach or total time attached to the dam compared to the sated pup (**Figure 2B, C**). Interestingly, pup behaviors for latency to suckling to mother demonstrated a bimodal distribution, where most mice attached immediately across all conditions. This may suggest that for suckling behavior, isolation from the mother may play a more significant role than hunger or thirst states. Of note, mice that were isolated for an extended period of time from the mother have been known to demonstrate reductions in maternal-seeking behaviors such as ultrasonic vocalizations, and thus a lack of a statistically significant difference here could be masked by this reduction[12]. It is possible that this occurred for the subset of mice who did not demonstrate any feeding behaviors.

Regardless, these data suggest that attachment to the dam does not differ across hunger and thirst states when induced using our cannula feeding platform (**Figure 2A**, *behavior 1 & 2*).

Because suckling may be a readout of a bonding behavior in addition to hunger and thirst, we next used a more direct measure of consummatory behavior by assaying weight change over free feeding after starvation and/or dehydration states were induced (**Figure 2A,** *behavior 3*). We found that sated or dehydrated-only mice consumed significantly less milk than starved-and-dehydrated mice (**Figure 2D**). Starved-only mice, however, did not have significantly reduced milk consumption compared to starved-and-dehydrated mice (**Figure 2D**). This suggests that hunger drives feeding more similarly to a pup with both hunger and thirst. Interestingly, the fact that the magnitude of feeding was greater in starved-and-dehydrated mice than starved-only mice while dehydration only did not elicit much consumption suggests that hunger and thirst may still act synergistically. Together, these findings suggest that feeding may be driven more strongly by hunger than thirst in neonatal stages.

One potential issue with this approach is that the salt imbalance caused by prolonged salty milk or water feeding may differentially influence sensory processing or motor output. In addition, prolonged isolation in our feeding platform also likely affects attachment to the dam. To address these concerns, we used the step-function opsin SOUL to activate AgRP or Agtr1a neurons to artificially induce a state of hunger or thirst, respectively[11,20]. Previous studies have shown that transcranial optogenetic stimulation in neonatal mice was sufficient to activate SOUL-expressing neurons, likely owing to the relative translucency of the skull at this early developmental stage[21]. We found that brief transcranial stimulation of neonates was indeed sufficient to activate the majority of AgRP or Agtr1a neurons expressing SOUL in ARC and SFO, respectively (**Figure 2E–-G**). Thus, we used SOUL activation in these mice as a method to artificially induce hunger and thirst states in neonates without the need for extended isolation from the dam and nest.

Using this approach, we found that inducing hunger but not thirst significantly increased both attachment behaviors (**Figure 2H, I**) as well as consummatory behavior (**Figure 2J**). Of note, mice across all conditions had weaker attachment and consummatory behaviors following optogenetic activation compared to those tested using our feeding platform, likely because these mice were not subjected to prolonged isolation. However, the findings between our feeding platform and optogenetic stimulation consistently demonstrate a preferential increase in feeding behaviors in hunger states. Taken together, these findings suggest that hunger drives feeding behaviors more than does thirst in this early developmental stage.

Neural representation of hunger and thirst is distinct by neonatal stages and does not depend on separation of food and water source

Given MnPO contained Fos-expressing neurons in both hungry and thirsty neonatal mice (**Figure 1D, E**), we asked whether distinct subpopulations of neurons within MnPO respond to starvation and dehydration in neonatal mice. To accomplish this, we used TRAP2 to permanently label dehydration-activated (thirst-TRAPed) populations in MnPO in neonatal mice. 2–3 days following TRAPing, we induced starvation or dehydration and performed Fos staining to quantify the proportion of thirst-TRAPed neurons that were reactivated (**Figure 3A**).

We found significantly higher reactivation of thirst-TRAPed MnPO populations by dehydration than by starvation (**Figure 3B, C**). Similar results were found in thirst-TRAPed neurons in OVLT and SFO (**Figure 3C**). Likewise, we found significantly higher reactivation of hunger-TRAP neurons by starvation than by dehydration (**Figure 3C**). Taken together, these data suggest that neuronal populations within hypothalamic regions responding to starvation and dehydration are already distinctly responsive to their given modality before mice can seek food and water separately.

Lastly, we asked whether these distinct neural responses in hunger and thirst could be altered by modifying the adult mouse diet such that food and water was obtained through the same source (**Figure 3D**). To do so, we created a liquid diet that was near-isotonic and nutritionally complete. Adult mice exclusively on the liquid diet were similar in weight to mice on normal diets (**Figure 3E**) and had very few Fos-expressing cells in the hunger and thirst regions, suggesting this diet was sufficient to sate both needs (**Figure 3F**).

We then examined neuronal activation patterns using hunger-TRAP followed by Fos staining under thirst condition in the same control mice or mice on liquid diet (**Figure 3D**). To induce hunger, we replaced liquid with water (for mice on the liquid diet) or removed food pellets (for control mice on normal diet consisting of solid food pellet and water) 36 hours prior to performing TRAP. Animals were subsequently returned to either liquid diet or normal diet. Later, thirst was induced by removing water (for control mice) or replacing liquid diet with solid food (for mice on the liquid diet) for 36 hours before perfusion and Fos staining. Mice on the liquid diet were also provided a small amount of salty liquid food (See Methods) to facilitate the switch in diet. As expected, hunger-TRAP predominantly captured neurons in ARC (**Figure 3G**), and thirst-Fos predominantly labeled neurons in OVLT and SFO (**Figure 3H**). Although some differences are seen in the total number of TRAPed or Fos+ cells between mice on liquid and normal diet, mice in both conditions had little overlap between neurons activated under the hunger and thirst conditions (**Figure 3G, H**). In particular, MnPO, which had active neurons in response to both

starvation and dehydration, had no increase in overlap in response to starvation and dehydration in mice that were only fed liquid diet (**Figure 3I**). This suggests that the distinction between neural correlates of hunger and thirst states does not depend on having the experience of separate food and water intakes in adult life.

DISCUSSION

Proper neural responses to starvation and dehydration are vital for survival across the lifespan of an animal. Here, we develop a novel cannulation platform that allows prolonged feeding of neonatal mice isolated from the mother to distinguish the hunger and thirst states. Using this platform, we demonstrated that activation of hunger and thirst regions is specific to the respective needs in neonatal stages before the animal can separately seek food or water. Hunger states, whether induced optogenetically or starving but not dehydrating the animal, drives feeding behaviors more than does thirst in neonates. Lastly, in MnPO where cells are active in both hunger and thirst states, distinct subpopulations of neurons are activated in each state by early postnatal stages, and these distinct responses are not altered if an animal does not have separate access to food and water throughout their lifespan. These findings demonstrate that brain regions involved in responding to starvation and dehydration and regulating hunger- and thirst-driven behavior are established early and are robust to environmental changes later in life.

The early developmental specificity in neural correlates of hunger and thirst is significant given it is unclear at what stage these neurons develop the activation profiles seen in adults. For example, a previous study suggested that the activity of AgRP neurons in neonates correlate more to maternal interaction than starvation or feeding[12]. Given that mice in both the hunger and thirst conditions had similar warming in the chamber and isolation from the dam, our data demonstrates that neurons in ARC did indeed respond to prolonged starvation at this stage. In another example, studies have shown that hunger and satiety hormones in development influence axonal growth of AgRP neurons rather than inducing changes in activity as seen in adult[14,15]. The switch to adult-like responses to hormones occurs around the time animals become independent and can seek food and water separately[16]. Additionally, axonal projections from AgRP neurons in ARC only reach a subset of their eventual target regions within the first postnatal week[13]. Early-maturing AgRP networks may be responsible for neonatally-relevant behaviors, while a fully mature AgRP network in the adult may be required to drive the complete repertoire of hunger-driven behaviors. Knowledge of when certain regions first become active under hunger and thirst conditions would thus help define critical windows during which alterations in hormonal signaling may affect maturation of such circuits. These possibilities highlight how future studies with more

complete spatiotemporal map of hunger- and thirst-driven brain-wide activity across development would provide insight into the physiological maturation of circuits driving diverse behaviors across development, as well as how stage-dependent perturbations may lead to alterations at different connections within these networks.

Our novel platform for inducing starvation and dehydration as well as our use of SOUL in AgRP and Agtr1a neurons provides the first isolation of hunger and thirst states in neonates. While this facilitated the finding that hunger drives feeding behaviors more robustly than does thirst, there are two main caveats to these findings. First, while feeding water through our cannulation system ensures starvation, injecting NaCl or feeding salty milk may not activate all thirst neurons given some thirst neurons respond to hypovolemia[9]. However, the substantial activation of thirst regions with NaCl injection suggests a thirst condition is likely induced. Second, SOUL activation in AgRP and Agtr1a neurons may drive downstream behaviors to different degrees, given Fos expression is a binary rather than continuous measure of activity. However, given that consummatory behaviors are not significant different between starved mice and mice with both starvation and dehydration, we suspect that hunger does drive feeding more strongly than does thirst in neonates.

While one might expect circumventricular regions such as ARC, SFO, and OVLT to develop state-specific activation with starvation and dehydration early, it is surprising that downstream regions such as MnPO also have state-specific activation. This raises the question of what distinguishes these largely non-overlapping populations of dehydration- and starvation-activated neurons in MnPO. Recent work in thirst circuits of adult mice has identified transcriptomically distinct cell types that play different roles in the thirst-sensing process[8,9]. These studies show that different cell types respond to hypovolemic thirst, which promotes drinking to replenish fluid volume, and hyperosmotic thirst, which promotes drinking to normalize plasma osmolarity. Further work investigating when and how these cell types begin to separate into their distinct roles will provide more granular insight into the development of these otherwise early-established and robust circuits regulating hunger and thirst. For example, we found that mice on a liquid diet had decreased activation of SFO in response to dehydration and increased activation of MnPO in response to starvation. Reliance on a liquid diet likely alters baseline plasma osmolarity or volume, and investigations into which cell types may have decreased responses to dehydration will provide insight on how these circuits flexibly adapt to environmental changes.

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AUTHOR CONTRIBUTIONS

D.C.W., Y.W. and L.L. designed the experiments. D.C.W. and C.M. performed the experiments. D.C.W. and L.A.E. analyzed the data. D.C.W. and L.L wrote the manuscript with help from Y.W. All the authors read and edited the final version of the manuscript.

MATERIALS AND METHODS

Animals

All animal procedures adhered to animal care guidelines from Stanford University's Administrative Panel on Laboratory Animal Care.

To generate *TRAP2;Ai14* and *TRAP2;Ai32* mice, we crossed *Fos2A-iCreER* mice (FosTRAP2, Jackson Laboratory, Stock 030323)[19] with tdTomato Cre reporter mice (*Ai14*, Jackson Laboratory, Stock 007914)[22] or ChR2-eYFP Cre reporter mice (*Ai32*, Jackson Laboratory, Stock 012569)[22]. These mice were then backcrossed to wild-type CD1 mice twice, and bred to homozygosity for all alleles, resulting in *TRAP2/TRAP2;Ai14/Ai14* or *TRAP2/TRAP2;Ai32/Ai32* mice. Backcrossing to CD1 improved litter size and pup size to facilitate cannulation. To generate *AgRP-Cre;SOUL* and *Agtr1a-Cre;SOUL mice*, *SOUL-P2A-tdT* Cre reporter mice (*SOUL*, Jackson Laboratory 032301) were crossed to *AgRP-Cre* (AgRP, Jackson Laboratory 012899)[23] or *Agtr1a-Cre* (*Agtr1a*, Jackson Laboratory 031487)[24] mice. To generate *TRAP2/+;Ai14/+;NPY-hrGFP/+* mice, *TRAP2;Ai14* mice were crossed with *NPY-hrGFP* mice (NPY, Jackson Laboratory 006417)[25].

Tamoxifen preparation, administration, and neonatal mouse fostering post-administration

4-hydroxytamoxifen (4-OHT; Sigma H6278) at 20 mg/ml in ethanol was added to a 1:4 mixture of castor oil:sunflower seed oil (Sigma 259853 and Sigma S5007) to give a final concentration of 10 mg/ml 4-OHT. Ethanol was evaporated by vacuum centrifugation. Neonatal 4-OHT administration was 15mg/kg of animal body weight; adult 4-OHT administration was 50 mg/kg of animal body weight. All 4-OHT was administered intraperitoneally (IP).

Histology

Animals were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were then post-fixed overnight at 4°C in 4% PFA. Coronal sections were then cut at 60-µm thickness using a Leica Vibratome system. Immunostaining was performed with primary antibodies for c-Fos (Synaptic Systems 226 008), tdTomato (Rockland 600-401-379), or POMC (Phoenix Pharmaceuticals 27-52) in PBST (PBS + 0.1% Triton) at 4°C for two nights, washed 3 x 10 min with PBST, secondary antibodies in PBST overnight at room temperature, washed 3 x 10 min with PBST, and mounted in Vectashield (Vectorlabs).

Isolation of hunger and/or thirst states in neonatal mice

To selectively induce hunger or thirst states in neonatal mice, esophageal cannulation was performed to provide continuous feeding of liquids. Mice were removed from the homecage, weighed, and briefly anesthetized with 3% isofluorane to facilitate implanting the feeding tube.

The feeding tube, a 0.025" OD polyurethane tubing (Braintree MRE025), was attached to a 27G needle on a 3 ml syringe containing milk (Organic Valley Half & Half), salty milk (half & half with solid NaCl added to a final concentration of 100 mM), or water (with 35 mM NaCl). The end of the tube was briefly heated over flame to create a curve in the tubing. The curved tip of the tube was dipped in milk and inserted into the pup's oral cavity until the end of the tube just entered the esophagus (a slight increase in insertion resistance occurred when the tube hit the epiglottis, and entrance into the esophagus provided relief of that resistance). Shallow esophageal cannulation (located to upper esophagus) was necessary for feeding over many hours, as mice at this age do not reflexively ingest liquid directly placed in the oral cavity, and cannulation deeper than this compresses thoracic structures leading to respiratory difficulty and high rates of mortality.

After cannulation, a small dab of superglue was applied to adhere the curved part of the tubing to the cheek of the pup, and the pup was allowed to wake from isoflurane anesthesia. Pups were then placed in isolated chambers on a heat pad and kept at 32°C. Milk, salty milk, or water was then pumped at a rate of 70–110 µl per hour (New Era Pump Systems NE-1200) for approximately 16 hours before 4-OHT administration for TRAP, or perfusion for Fos staining.

To induce both hunger and thirst in neonatal mouse mice, pups taken from homecage were cannulated as described above and kept at 32°C in an isolated chamber for approximately 16 hours but without any milk or water infusion prior to TRAPing or perfusion.

To induce thirst in neonatal mouse mice, fully sated mice received IP injections of 2 M NaCl (5 ul / g bodyweight). For TRAP, 15 mg/kg 4-OHT was administered 90 min after NaCl

injection. For mice that were perfusion for Fos staining, perfusion occurred 60 min after NaCl injection. For experiments in which previously TRAPed neurons were compared to Fos staining in P10-12 mice, thirst was induced by feeding milk followed by 2 M NaCl injection 60 min prior to perfusion for Fos staining.

Feeding behavioral experiments in neonates

Dams were anesthetized with IP injections of ketamine (100 mg/kg) and xylazine (10 mg/kg), followed by IP injection of oxytocin (4 IU/kg) to facilitate milk letdown. Prior to each experiment, milk letdown was manually confirmed. Each litter used within a behavioral session contained mice that were sated, hungry, thirsty, or both, typically 1–2 mice per condition.

Anesthetized dams were placed supine in a small chamber over nesting and homecage bedding, and mice were placed approximately 2 cm from a nipple on the dam's ventrum. Mice were not manually held during behavioral assays. Latency to attach was measured as the time elapsed before the pup began suckling from a nipple. Mice were then observed for 3 min, and the total time attached within these 3 min was recorded. Mice who did not attach were given a latency of 3 min. Mice were then removed from the mother, and the nipple was covered with tape before placing the next pup 2 cm in front of a different nipple.

After attachment behaviors were completed for each pup, dams received a second dose of ketamine, xylazine, and oxytocin. All mice were then returned to the anesthetized dam by placing them directly on top of a nipple (to promote attachment and feeding), and allowed to freely feed for 1 hour. Pup weights were recorded before and after 1 hour of feeding, and mice that were stressed and urinated significantly during handling or feeding were removed from analysis.

SOUL activation in neonatal mice

P8–10 mice were isolated with their littermates from dam for approximately 45 minutes prior to behavioral assay. SOUL was then activated in AgRP > SOUL or Agtr1a > SOUL mice via transcranial stimulation for 30 s using 20-mW 488-nm laser by placing a bare optic fiber over the skin covering over lambda. Transcranial optogenetic stimulation has been previously demonstrated to be sufficient to activate SOUL-expressing neurons[21]. No surgery was performed on these animals. Following stimulation, mice were allowed to rest with littermates isolated from dam for approximately 5 minutes prior to behavioral assays. Behavioral assays were then executed as described above.

Liquid diet composition and delivery

To maintain animals on a completely liquid diet, Ensure Complete Nutrition was mixed with whole milk at a 1:2.5 ratio. This yields a solution of approximately 350 mOsm, similar to the osmolality of many electrolyte replacement solutions. A plastic container containing 100 ml of this liquid mixture was placed in the mouse cage and replaced daily. Feeding of liquid began at P13–14 (soon after eye opening and before mice begin to explore food and water sources outside of maternal milk). Litters were weaned at P21, and half the litter continued on this liquid diet while the other half of the litter was given access to water and solid food pellets. Liquid diets were continued until mice were >P35 at which point the mice were used in experiments.

Isolation of hunger or thirst states in adult mice

To induce hunger in adult mice, food was removed from the cage for 36 hrs, and mice were given *ad libitum* access to regular water. To induce thirst in adult mice, water was removed from the cage for 36 hrs, and mice were given ad libitum access to regular food pellets. Mice previously on a liquid-only diet were also given 2 ml / mouse of the liquid (Ensure + whole milk) with 500 mM NaCl at the beginning of the thirst-induction period to facilitate the switch to solid food. 50 mg/kg 4-OHT was then administered for TRAPing. Food or water was returned 6–12 hrs later, or animals were perfused for Fos staining.

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FIGURES

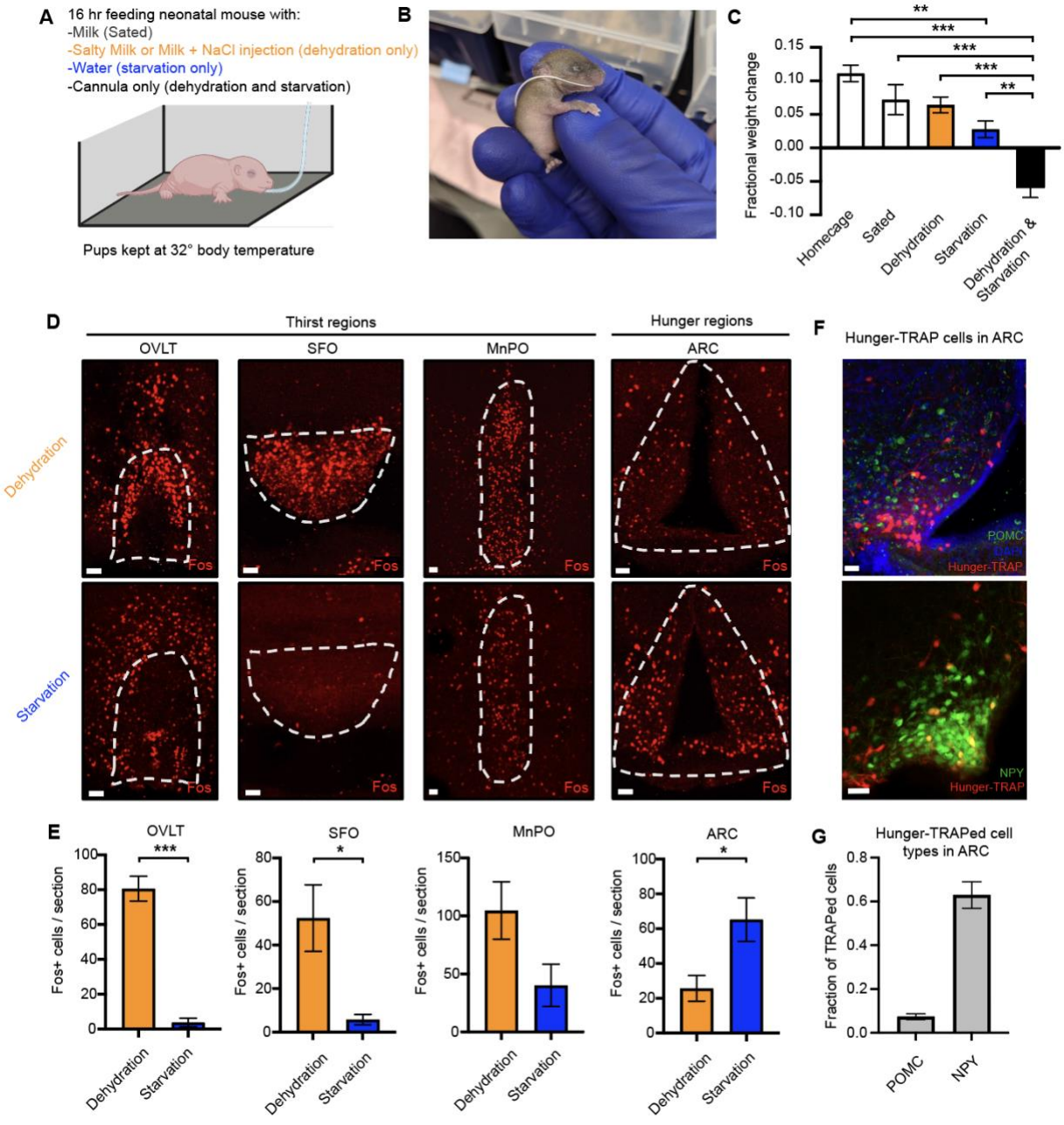

Figure 1. Distinct activation of hunger and thirst regions in neonatal mice by starvation and dehydration.

(A) Schematic of neonatal mouse with an implanted esophageal cannula in a warmed chamber.

(B) Photo of a cannulated P7 neonatal mouse.

(C) Weight change of neonates after 16 hours of feeding in the cannula system, shown as fractional body weight change. n = 4–5 per condition, one-way ANOVA with post-hoc Tukey HSD.

(D) Example images of Fos staining in hunger and thirst regions in a dehydrated (top row) or a starved (bottom row) neonatal mouse. Scale bar, 20 μm.
(E) Quantification of Fos-expressing cells in hunger and thirst regions in neonatal mice subjected to dehydration or starvation. n = 4–5 per condition, unpaired t-test.
(F) Example image showing overlap of P8 hunger-TRAPed cells with POMC (left) or NPY (right) stained at P14–16 in sections of the arcuate nucleus (ARC); NPY and AgRP are normally co-expressed in the ARC.
(G) Quantification of fraction of P8 hunger-TRAPed cells that express POMC or NPY at P14–16. n = 3 per condition.
Error bars, SEM.

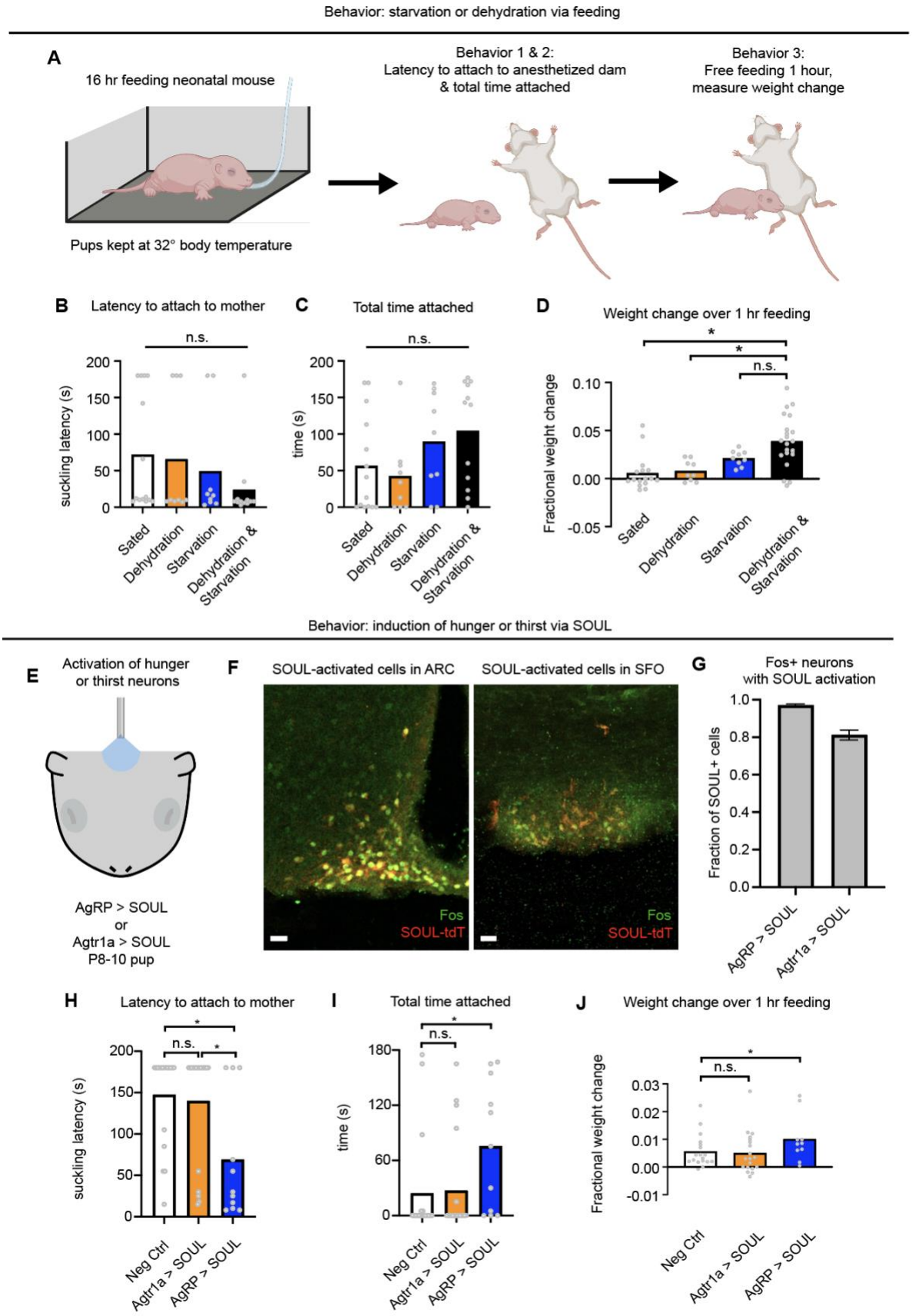

Figure 2. Attachment and feeding behaviors in thirst- or hunger-induced neonatal mice
(A) Schematic of attachment and feeding behaviors in a neonatal mouse after induction of hunger and/or thirst.
(B) Quantification of latency to attach to mother after induction of hunger and/or thirst in neonatal mice via feeding.
(C) Quantification of total time a neonate was attached to the mother for a 3-minute period after induction of hunger and/or thirst.
(D) Quantification of fractional body weight change after a neonate was allowed to freely feed from the mother for 1 hour after induction of hunger and/or thirst.
(E) Schematic showing transcranial activation of SOUL in *AgRP*- or *Agtr1a*-expressing neurons in a neonatal mouse. AgRP > SOUL, *AgRP-Cre/+;SOUL/+.* Agtr1a > SOUL, *Agtr1a-Cre/+;SOUL/+.*
(F) Example image showing Fos expression in AgRP > SOUL mice (left) and Agtr1a > SOUL mice (right) after transcranial activation of SOUL. Scale bar, 20 μm.
(G) Quantification of fraction of SOUL+ neurons that express Fos after activation of SOUL. For all experiments, n = 3 per condition. Error bars, SEM.
(H) Quantification of latency to attach to mother after induction of hunger or thirst in neonatal mice via SOUL.
(I) Quantification of total time a neonate was attached to the mother for a 3-minute period after induction of hunger or thirst in neonatal mice via SOUL.
(J) Quantification of fractional weight change after a neonate was allowed to freely feed from the mother for 1 hour after induction of hunger or thirst in neonatal mice via SOUL.
For (C,D,I,J), n = 9–15 per condition, one-way ANOVA with post-hoc Tukey HSD; *p<0.05.
For (B,H), n = 9–15 per condition, Kruskal-Wallis test with post-hoc Dunn's test; *p<0.05.

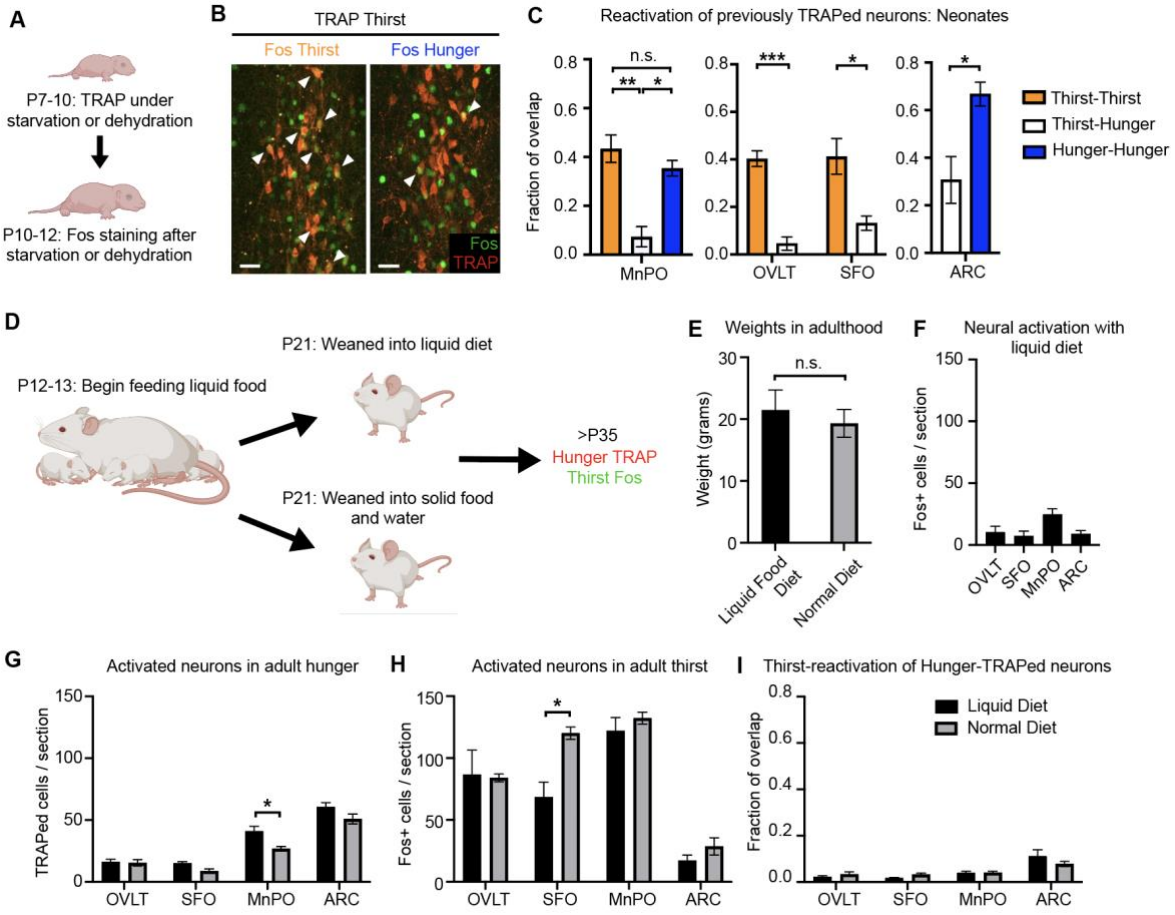

Figure 3. Starvation and dehydration activate distinct neuronal populations in neonates and adults fed a liquid diet.

(A) Schematic showing thirst- or hunger-TRAP in a neonatal mouse followed by thirst- or hunger-Fos several days after TRAP.

(B) Example image of Fos expression following thirst or hunger induction co-stained with thirst-TRAPed neurons, in neonatal MnPO. Arrowheads indicate double-labeled cells. Scale bar, 20 μm.

(C) Quantification of Fos expression (indicating activation in thirst or hunger) in previously thirst- or hunger-TRAPed neurons in neonatal brain regions. Overlap is calculated as Fos+TRAP+ / TRAP+ neurons (n = 3–5 per condition. For OVLT, SFO, and ARC, unpaired t-test, *p<0.05, ***p<0.001. For MnPO, two-way ANOVA with post-hoc Tukey HSD; *p<0.05, **p<0.005). Error bars, SEM.

(D) Schematic of timeline for maintaining mice on a liquid diet.

(E) Weights of P35 mice on a liquid diet and normal diet consisting of solid food and water (n = 5 per condition, unpaired t-test). Error bars, SEM.

(F) Baseline Fos expression in hunger and thirst regions for mice on a liquid diet (n = 5).

(G, H) Quantification of activated neurons in adults fed a liquid diet or normal diet under induction of hunger (G) or thirst (H) (n = 5 per condition, two-way ANOVA with post-hoc Tukey HSD; *p<0.05). Error bars, SEM.

(I) Quantification of overlap of Fos expression (indicating activation in thirst) and TRAP labeling (indicating activation in hunger). Overlap is calculated as Fos+TRAP+ / Fos+ neurons (n = 5 per condition, two-way ANOVA with post-hoc Tukey HSD). Error bars, SEM.