Dorsomedial hypothalamic prodynorphin neuron is crucial for expression of food anticipation in mice.

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To my family & Sharon
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Tzu-wei Kuo

Abstract

DMH prodynorphin neuron (Pdyn\textsuperscript{DMH}) is robustly activated when mice consume a large amount of food. The role of Pdyn\textsuperscript{DMH} in feeding behaviors, however, is unknown. Here we show that Pdyn\textsuperscript{DMH} activity arises before the incoming food intake, and exhibits sustained activity during feeding process. Optogenetically stimulating Pdyn\textsuperscript{DMH} leads to autonomic activation that increases brown adipose fat (BAT) thermogenesis, blood pressure and locomotor activity. Silencing Pdyn\textsuperscript{DMH} abolishes the anticipatory BAT thermogenesis and locomotor activity to food in both fed and scheduled feeding conditions. Together, these results demonstrate that Pdyn\textsuperscript{DMH} is critical for mice to express anticipatory responses to food.
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Chapter I: Introduction

Through billions of years of evolution, animals have developed various adaptive strategies to optimize energy retrieval. The cephalic phase responses to food, such as salivation and gastric acid secretion before feeding, enable animals to quickly prepare for imminent food intake and nutrient digestion (Power & Schulkin, 2008). As an important component of these anticipatory responses, autonomic activation, including thermogenesis, cardiovascular arousal and locomotor activity, also arises quickly in a time course of seconds to minutes before feeding is initiated (Blessing, Mohammed, & Ootsuka, 2012; Kontos, Menezes, Ootsuka, & Blessing, 2013; Matteo et al., 2006). These physiological changes allow animals to meet the quick uprising metabolic demands from the increased internal organ activities, the potential prey-hunting actions or competing for food with other animals.

Interestingly, these anticipatory responses to food can also be expressed intensively over a longer time scale up to hours in certain conditions. In 1922, Curt Richter described rats rapidly increased their spontaneous activity several hours prior to meal time when he fed rats every day at noon for 25 mins (Hunter & Richter, 1922; Patton & Mistlberger, 2013). This is the first discovery of food-anticipatory activity (FAA) in mammals, and later this phenomenon was found broadly across different animal species (for review, see Mistlberger, 1994; Stephan, 2002). In fact, food has known to be a strong entrainer to entrain animals to express a circadian change along with meal time, which is defined as food-entrainable circadian rhythm. This circadian changes is not only limited to gross activity, but also include body temperature, cardiovascular output and serum corticosterone level (Ralph E. Mistlberger, 2011; Patton & Mistlberger, 2013).
1.1 The substrate of food-entrainable oscillator(s)?

In mammals, light-entrainable circadian rhythm is controlled by suprachiasmatic nucleus (SCN), the major light-entrainable oscillator (LEO). However, food-entrainable rhythm can be regulated but does not depend on SCN or any light cues (Stephan, Swann, & Sisk, 1979b, 1979a; Storch & Weitz, 2009), suggesting that central food-entrainable oscillator (FEO), if existing, may be located outside SCN.

Despite decades of intensive lesion studies aiming to identify the location of FEO, including ablating of sensory nerve, numerous hypothalamic, hindbrain and forebrain nuclei, all the studies failed to provide solid evidences to reveal a single brain structure or gene as the essential FEO (For comprehensive reviews, see Davidson, 2009; Pendergast & Yamazaki, 2018). One currently accepted view, based on numerous negative findings in the past, is proposing a working model that networks of interconnected brain regions cooperate together to entrain food-entrainable circadian rhythm (Carneiro & Araujo, 2009; Ralph E Mistlberger, 2011), while the concluding evidence to support such working model is still lacking.

1.2 Dorsomedial hypothalamus controversies and my research focus

Dorsomedial hypothalamus (DMH) plays important roles in regulating brown adipose fat (BAT) thermogenesis, cardiovascular output, and various behavioral circadian rhythms (Chou et al., 2003; Enriori, Sinnayah, Simonds, Garcia Rudaz, & Cowley, 2011; Matteo et al., 2006; Zaretskaia, Zaretsky, Shekhar, & Dmicco, 2002). It is known to mediate quick cardiovascular arousal to food (Matteo et al., 2006) and expresses robust activation during after large amount of food intake (Knight et al., 2012; Wu et al., 2014). Furthermore, DMH is one of the few brain regions that activate during FAA period (Gooley, Schomer, & Saper, 2006; Knight et al., 2012; Poulin & Timofeeva, 2008). Considering the evidences above, the hypothesis proposing dorsomedial hypothalamus (DMH) as a putative FEO was once popular (Chou et al., 2003;
Fuller, Lu, & Saper, 2008; Gooley et al., 2006; Mieda, Williams, Richardson, Tanaka, & Yanagisawa, 2006). However, this hypothesis has not yet been supported by solid evidences; conflicting results had been presented by different groups (Fuller et al., 2008; Gooley et al., 2006; Landry, Simon, Webb, & Mistlberger, 2006; Mieda et al., 2006; Ralph E Mistlberger, 2009). Still, knowledge about DMH’s role in feeding behaviors remains limited.

Compared to other DMH neurons, DMH prodynorphin neuron (Pdyn^{DMH}) is strongly activated by and expresses high percentages of ribosome subunit phosphorylation after large amount of food intake (Knight et al., 2012). We therefore decided to use cell type-specific approaches to investigate the role of Pdyn^{DMH} in feeding behaviors.

In this study, I will combine phosphoTRAP (TRAP: translating ribosome affinity purification), fiber photometry, optogenetics and neuron-silencing experiments to show that Pdyn^{DMH} participates in feeding behaviors and is critical for mice to express food anticipatory behaviors.
Chapter II: Results

2.1 PhosphoTRAP from micro-dissected DMH reveals a list of DMH cell marker candidates associated with scheduled feeding.

Dorsomedial hypothalamus is known to be robustly activated and express extensive c-Fos signals after large amount of food intake (Wu et al., 2014b). However, DMH neurons haven’t been systematically characterized based on their cell types and functions. Utilizing phosphoTRAP techniques (see Chapter IV: Materials and Methods), Knight et al 2012 profiled the whole hypothalamus and characterized prodynorphin neurons in dorsomedial hypothalamus (Pdyn\textsuperscript{DMH}), which is robustly activated in scheduled feeding (Fig. 1B, S1A, S1B).

To further characterize DMH neurons based on their activity in feeding behaviors, we trained mice with time-restricted scheduled feeding (feed animal only at circadian time 4-7) for 7 to 10 days (Fig. 1A, C), and performed phosphoTRAP on micro-dissected DMH (Fig. 1C). In addition to Pdyn\textsuperscript{DMH}, we found a list of DMH neuron populations, such as CCK, GSBS, GRP and Rorb neurons (Fig. 1E, S1E), are all located in the same compact DMH region as Pdyn\textsuperscript{DMH}. We also found CCK\textsuperscript{DMH} and Pdyn\textsuperscript{DMH} have high percentage of overlapping. (Fig. 1B, S1A)

This result also revealed numerous activity-dependent immediate early genes (Fig. S1C, S1D) and CLOCK genes-expressing neurons (PER1, PER2, Rorb and Cry, Fig. S1C, S1D) are involved in scheduled feeding, compatible with some previous studies studying CLOCK genes and time-restricted schedule feeding (Fuller et al., 2008; Gooley et al., 2006)

2.2 Pdyn\textsuperscript{DMH} activity arises before the incoming food intake, and exhibits sustained activity during feeding process.

To what capacity and how is Pdyn\textsuperscript{DMH} involved in feeding behaviors? To answer the question, we selectively expressed genetically-encoded calcium indicator (GCaMP6s) on Pdyn\textsuperscript{DMH} by injecting adeno-associated virus (AAV) carrying Cre recombinase-dependent GCaMP6s into the
DMH of Pdyn-Cre mice (Fig. 2A). Each mouse was implanted with an optical fiber above DMH to read GCaMP6s fluorescent signals from Pdyn$^{\text{DMH}}$ (Fig. 2A).

In object presentation experiment, we recorded Pdyn$^{\text{DMH}}$ GCaMP6s responses to food (chow or peanut butter) versus non-food objects (a piece of LEGO building block or water (in a 35mm culture dish). We found that Pdyn$^{\text{DMH}}$ exhibits a ubiquitous transient peak activation (~30-40 seconds) to the presenter’s initial hand-approaching action regardless of the object’s properties (Fig. 2B-C). Surprisingly, in fasted condition, food presentation always generates a robust, minutes-lasting sustained Pdyn$^{\text{DMH}}$ activity along with food intake after the initial peak signals, comparing to almost zero responses to non-food objects (Fig. 2C, 2G). While in fed state no sustained responses were observed for all objects (Fig. 2B, 2F).

To determine whether the sustained Pdyn$^{\text{DMH}}$ activation to food in fasted state is caused by the immediate voracious eating, we presented the objects covered with a cage, so that mice could smell and see the objects but were unable to access them. The sustained Pdyn$^{\text{DMH}}$ activation to food was then completely abolished by cage-blocking (Fig. 2E, 2F). This indicates that actual food intake is required to maintain the sustained Pdyn$^{\text{DMH}}$ activation.

Interestingly, caged food objects in fed state induced small but significant Pdyn$^{\text{DMH}}$ responses compared to LEGO (Fig. 2D, 2F), suggesting the food odor could illicit partial Pdyn$^{\text{DMH}}$ activation.

To avoid the non-specific activation by human interference and synchronize GCaMP signals with the precise timing of food ingestion, we recorded Pdyn$^{\text{DMH}}$ activity during liquid diet and water consumption, which is reported with precise licking time by 2 separated lickometers (Fig. 3A). Specifically, we found that Pdyn$^{\text{DMH}}$ activation is ‘phase-locked’ with liquid diet consuming, but not with water in both fed and fasted condition (Fig. 3B-D). Of note, the GCaMP6s signal
arises 3 - 5 seconds prior to Ensure licking, and quickly decays within 10 seconds after the licking bout ends (Fig. 2E-H).

**2.3 Stimulating Pdyn^{DMH} induces autonomic activation and locomotor activity.**

To investigate effects of Pdyn^{DMH} activation, we then expressed channelrhodopsin2 (ChR2) on Pdyn^{DMH} for *in vivo* optogenetic stimulation (Fig. 4A). Photo-stimulation immediately induces locomotor activity and autonomic activation, including elevated blood pressure and BAT thermogenesis (Fig. 4B-D). In fed state, Pdyn^{DMH} stimulation induces strong locomotor activity and suppresses food intake. (Fig. 4E). In fasted condition, food intake is not affected by Pdyn^{DMH} stimulation (Fig. 4F).

To reveal the postsynaptic targets of Pdyn^{DMH}, recombinant synaptophysin-GCaMP6 molecule was selectively expressed on Pdyn^{DMH} (Fig. 4G, left) by AAVDJ-EF1a-DIO-synaptophysin-GcaMP6s (made by Stanford vector core, titer:5.6X10^{12}, 35 nL in right DMH) injection, which is supposed to label Pdyn^{DMH}'s presynaptic axonal puncta. The labeled axonal terminals are only distributed within DMH and Arcuate nucleus (Fig. 4G, right), consistent with a previous report (Garfield et al., 2016).

**2.4 Silencing Pdyn^{DMH} blocks anticipatory behaviors to food intake.**

To understand the role of Pdyn^{DMH} activation in feeding behaviors, we selectively silenced Pdyn^{DMH} by injecting AAV that carries Cre-dependent tetanus neurotoxin (TeNT) (AAVDJ-CMV-DIO-eGFP-TeNT (Stanford vector core, titler:1.4X10^{10}) into the DMH of Pdyn-Cre mice (Figure 5A). Since Pdyn^{DMH} activation promotes BAT thermogenesis and locomotor activity, we implanted a telemetry transponder (G2-Emmitter, Starr Life Sciences, Fig. 5B) into the subcutaneous interscapular BAT (iBAT) region of the mouse for continuous monitoring BAT temperature and locomotor activity.
After 3-4 weeks' recovery, each mouse is singly housed in a cage with a programmable automatic feeding device (FED; each time deliver a 20mg food pellet, Fig. S2) and a water bottle (with an optical lickometer to measure the lick counts) inside (Fig. 5B). A transceiver plate (ER-4000, Starr Life Sciences) which reads signals from the iBAT transponder was placed underneath the cage. All data were transmitted to a computer for synchronous recording (Figure 5B, Arduino recording interface, Figure S3). Each single recording setup (for one mouse), as shown in figure 5B, is placed in one individual sound-attenuating box (Med-Associates). Light and dark cycle was kept at 12:12.

In previous studies, whether DMH plays a role in food-entrainable circadian rhythm is unclear (Gooley et al., 2006; Landry et al., 2006; Mieda et al., 2006; Ralph E Mistlberger, 2009). Given the closely relation between $P_{dn}^{DMH}$ activity and food intake (Knight et al., 2012), we hypothesized $P_{dn}^{DMH}$ could also be involved in food-entrainable circadian behaviors. To test the hypothesis, we let the mice subject to ad lib feeding (habituation from day -7- -1, then ad lib feeding from day 0-7 in Fig. 5A, 6A) and then trained animals to adapt to scheduled feeding (food only be supplied at Z T3-6. ZT: zeitgeber time) for at least 3 weeks (day 8 - 28, Fig. 5A, 6A).

Surprisingly, food-anticipatory activity (FAA) was abolished in $P_{dn}^{DMH}$ silencing group under scheduled feeding condition (Fig. 6A bottom, 6C), while the dark phase locomotor activity was only slightly weaker than that of GFP control group (Fig. 6C). Moreover, BAT temperature rising during FAA period was also abolished in $P_{dn}^{DMH}$ silencing group during scheduled feeding protocol (Fig. 6D, 6E. Day 22-25). Both results show that $P_{dn}^{DMH}$ is necessary for food-entrainable rhythm to develop.

Of note, in ad lib condition, BAT temperature in $P_{dn}^{DMH}$ silencing group in the dark phase is slightly lower than the GFP control group (Fig. 5C). Holding at a lower baseline, and lack of anticipatory BAT temperature rising before food intake compared to control group was also noted in ad lib state (Fig. 6C, 5C). However, in fast-refeeding condition (scheduled feeding day
1), the mice had no clues of when food would supply, and the overall BAT temperature profiles between 2 groups do not differ significantly (Fig. 5D). These results suggesting Pdyn<sub>DMH</sub> silencing only affects the anticipatory BAT temperature rising before feeding, but does not affect postprandial thermogenesis (Fig. 5C. 5D). Finally, the body weight and food intake of Pdyn<sub>DMH</sub> silencing group were not significantly different from the control group (Fig. 5E, 5F).

### 2.5 Conclusion

In sum, Pdyn<sub>DMH</sub> is readily activated when mouse anticipates the incoming food intake, and food intake results in sustained Pdyn<sub>DMH</sub> activation. Optogenetically stimulating Pdyn<sub>DMH</sub> leads to quick arousal and autonomic activation. Silencing Pdyn<sub>DMH</sub> specifically abolished FAA and anticipatory BAT temperature rising before food intake. These suggesting Pdyn<sub>DMH</sub> is crucial for mice to develop food anticipatory behaviors.
Chapter III: Discussion

The pursuit of physical substrate of food-entrainable oscillator (FEO) has persisted for decades. However, the anatomical and molecular substrate of FEO remains mysterious (Patton & Mistlberger, 2013; Pendergast & Yamazaki, 2018). In this study, we applied phosphoTRAP techniques on micro-dissected DMH tissue to confirm that Pdyn^{\text{DMH}} activates during scheduled feeding. Utilizing cell type-specific manipulation, we showed that Pdyn^{\text{DMH}} activates a few seconds earlier than food intake initiates, and exhibits sustained activation during food intake until food intake stops for 5-10 seconds. Silencing Pdyn^{\text{DMH}} abolishes food-entrainable rhythm in both fed and scheduled feeding condition. Thus, Pdyn^{\text{DMH}} can be viewed as a putative FEO that readily senses incoming food intake and is responsible for food-entrainable circadian rhythm to develop. Future experiment using wheel-running activity, which was shown to strengthen FAA (Flôres, Bettilyon, Jia, & Yamazaki, 2016), to assess Pdyn^{\text{DMH}} silencing effects should be also performed in the future, as a cross-examination the necessity of Pdyn^{\text{DMH}} for entraining food-entrainable rhythm.

Our results also provide a possible explanation for the previous conflicting results of DMH ablation, that is, the controversies could be caused by non-specific ablation of various types of DMH cells. One possible condition is that DMH may consist of different cell types that have antagonizing effects in regulating food-entrainable rhythm. In this case, the final result would be inconsistent and depends on the net effect of the remaining neurons. In addition, in previous DMH ablation studies, DMH-ablated animals lost majority of their locomotor activity, while our approaches of selective Pdyn^{\text{DMH}} silencing provides much higher specificity in blocking FAA while preserving most locomotor activity.
Although Pdyn$^{\text{DMH}}$ expresses a sustained activity during food intake, Pdyn$^{\text{DMH}}$ silencing only affects anticipatory BAT thermogenesis and the postprandial BAT thermogenesis remains intact. A possible explanation is that from a recent study (Li et al., 2018) showing that the majority of postprandial BAT thermogenesis is mediated by secretin, a circulating gut hormone. So Pdyn$^{\text{DMH}}$ may not be necessary for the postprandial thermogenesis. On the other hand, Pdyn$^{\text{DMH}}$ has a dense axonal arborization locally distributed in entire ipsilateral DMH. Thus, Pdyn$^{\text{DMH}}$ may have versatile functions as its activation could universally influence the whole DMH, which project broadly to multiple brain regions that control autonomic nervous system and internal organs (Greenwood & DiMicco, 2017; Matteo et al., 2006; Thompson, Canteras, & Swanson, 1996).

Finally, this study identifies Pdyn$^{\text{DMH}}$ as a putative FEO target with a defined anatomical and molecular structure. It can be viewed a starting point for future research to study regulatory mechanisms of circadian rhythm, metabolism and autonomic nervous system by continuing exploring Pdyn$^{\text{DMH}}$ and the associated neural circuits.
Chapter IV: Material and Methods

4.1 Animal maintenance and experimental models

Pdyn-IRES-Cre (B6;129S- Pdyn\textsuperscript{tm1.1(cre)Mjkr}/LowlJ, Jackson # 027958) and ROSA26-EGFP-L10 (B6.129S4- Gt(ROSA)26Sor\textsuperscript{tm1(CAG-EGFP/Rpl10a,-birA)Wtp}/J, Jackson # 022367) mice were used in this study. All animals were maintained under a 12h:12h light/dark cycle schedule (Light: 5AM-5PM; dark: 5PM-5AM) Ad libitum access to chow (PicoLab Rodent Diet 5053) and water were provided. All experimental protocols were approved by the University of California, San Francisco IACUC following National Institutes of Health guidelines for the care and use of Laboratory Animals.

4.2 Stereotaxic surgery

Mice were anesthetized with isoflurane in the induction chamber and then mounted on a stereotaxic frame (Kopf Inc.) for surgery. Bupivacaine, Caprofen and Buprenophen were then administered for analgesia following UCSF IACUC guidelines. The scalp was shaved and disinfected with betadine and 70% ethanol. A longitudinal incision is made on the scalp to expose the skull surface. Skull holes (0.3-0.5mm in diameter) were made through a top-mounted drill to expose the brain surface. For virus injection, a borosilicate glass pipette (Sutter Instrument) pulled by a micropipette puller (Sutter P-1000) with a 10-uL Hamilton syringe were used for virus injection. The infusing volume and speed are controlled by a micropump (Ultra Micro Pump III, World Precision Instruments). For all DMH virus injection, the following injection coordinates and infusion rate were applied: -1.75 mm A/P, +-0.35mm M/L, -5.4mm below bregma; infusion rate: 30nL/min. After virus infusion, the glass pipette was left in situ for 5-10 mins to allow virus to diffuse. For optical fiber implantation for optogenetic and fiber photometry
experiment, a fiber optic implant was implanted to the following coordinates above right DMH:
for optogenetic- angled at 4 degree, -1.75 mm A/P, +0.35mmM/L, -4.9 mm below bregma. For
fiber photometry- angled at 4 degree, -1.75 mm A/P, +0.58mmM/L, -5.4 mm below bregma.
Implants were then secured using dental cement.

After surgery, Vetbond (SCB, sc-361931) or surgical suture was used to close the incisions.
Mice were then transferred back to a new clean cage for recovery.

4.3 PhosphoTRAP (modified from Zachary Knight 2013 pS6 IP protocol)

For more detailed protocol, please visit UCSF knight lab website
(https://knightlab.ucsf.edu/phosphotrap-protocol/)

4.3.1 Preparation

At the previous day before experiment, a sufficient quantity Protein A dynabeads was loaded
to eppendorf tube for total number IPs to be performed (100 uL beads/IP). The beads were then
washed 2 times with 0.15 wash buffer using magnet, and suspend beads in 0.15 M buffer. Anti-
pS6 244/247 antibody (4 ug per IP) + BSA (~0.1% final concentration) were added and mixed
over at 4 C overnight.

Brain dissection tools, homogenization tubes and pestles were cleaned with soap and water.
RNaseZap was applied on these items for 10 minutes and then rinse extensively with MilliQ
water to remove the RnaseZap. Then we used clean paper towels or blue diaper were used to
dry them and place them in refrigerator to chill on day of experiment.

On experiment day

Dissection buffer (50 mL per person doing dissections), homogenization buffer (2 mL per IP)
and 0.35 M wash buffer (4 mL per IP) were prepared and put on ice. Mice were killed by cervical
dislocation and dissect brain region of interest in 10 cm dish that contains dissection buffer and
cooled on ice. Hypothalamus or other tissue sample was then transferred to 2 cm dish on ice with dissection buffer.

Brain tissue was then mixed with 1.0 mL of homogenization buffer and homogenized by the automated tissue homogenizer in the 4 degree C cold room. The homogenized lysate was transferred to new Rnase-free microcentrifuge tube. Spin at 2000xg at 4C for 10 minutes. Supernatant was transferred to a new eppendorf tube. Discard the pellet were discarded.

Supernatant was clarified by adding approximately 70 μL of 10% NP40 and 70 μL of DHPC (from 300 mM stock) per 1.0 mL of supernatant. The clarified supernatant was spinned at maximum speed for 10 min in cold room. After spin, take supernatant and transfer to a new eppendorf tube and discard the small pellet. This supernatant is the Input.

4.3.2 Immunoprecipitation & sample analysis

Immunoprecipitation was performed to the cleared supernatant using pS6 244/247 antibody loaded magnetic beads. 0.35 M wash buffer was used for repetitive washes up to 4 times. After washes, 350 uL buffer RLT from the Qiagen RNeasy Micro kit were added to the beads. The IP RNA was eluted using RNeasy micro and the quality was assessed using pico-chip read by Agilent Bioanalyzer (Agilent Technology). RNA samples with RIN value> 8.0 were sent to NGS sequencing (Illumina Inc.) for further analysis.

4.3.3 Buffers and reagents (All buffers need to be stored at 4C and maintained RNase-free)

Basic Reagents:

Antibody (~4 ug per IP): pS6 244/247 antibody (#44-923G) from Invitrogen/ Thermo Fisher

Cycloheximide Stock (1000x): 100 mg/mL solution prepared in MeOH. Store at -20 C

DHPC: 100 mg DHPC in 0.69 mL H2O. Store at -20 C

10% NP40: 5 grams of NP40 in 45 mL Milli-Q buffer.

Glucose (1M, 500mL) solution
Buffers:

1. **Homogenization Buffer (500mL):** 5 mL of 1M HEPES solution; 37.5 mL of 2M KCl solution; 2.5 mL of 1M MgCl$_2$ solution; water 455 mL. Prepare a 2 mL aliquot per IP, and then before brain homogenization add the following per 2 mL: 5 μL of 1 M DTT solution; Protease Inhibitors (EDTA Free): 1/2 mini tablet; 5 μL RNAsin; 5 μL of 100 mg/mL Cycloheximide stock in MeOH; 10 μL Phosphatase inhibitor cocktail; 2 μL Calcyculin (1000x)

2. **0.15 M KCl IP Wash Buffer (500mL):** 5 mL of 1M HEPES solution; 37.5 mL of 2M KCl solution; 2.5 mL of 1M MgCl$_2$; 50 mL of 10% NP40 solution; 405 mL water

3. **0.35 M KCl IP Wash Buffer (500 mL):** 5 mL of 1M HEPES solution (pH 7.4); 87.5 mL of 2M KCl solution; 2.5 mL of 1M MgCl$_2$; 50 mL of 10% solution; 355 mL water. Immediately before use, prepare an aliquot with 4 mL per IP, and supplement with the following additives per 4 mL: 5 μL of 1 M DTT solution; 10 μL RNAsin; 5 μL of 100 mg/mL Cycloheximide stock in MeOH; 2 μL Calcyculin (1000x)

4. **Dissection Buffer (500mL):** 50 mL of 10x HBSS solution; 1.25 mL of 1M HEPES solution; 2 mL of 1M NaHCO$_3$ solution; 429.25 mL water. Immediately before dissection, prepare one 50 mL aliquot on ice for each person doing a dissection. Add to this 50 mL aliquot the following. 35 mM Glucose 1.75 mL of 1M solution Cycloheximide (100 μg/mL) 50 μL of 100 mg/mL stock in MeOH.

4.4 **In vivo fiber photometry experiment**

Pdyn-Cre mice (age 8-12 weeks) were used for virus injection and optical fiber implantation surgery. 35 nl AAV1-Syn-Flex-GCaMP6s (UPenn, titer:1.8×10$^{12}$) was stereotaxically injected into right DMH and an optical fiber (Thorlabs catalog # FP400URT) was implanted right above right DMH and secured by dental cement on the skull surface. Animals are allowed to recovery for 3 weeks before photometry recording experiment. RZ5P photometry system (Tucker-Davis
technologies, TDT) and a LED offering dual wavelength (405nm UV and 473 nm blue light) were used for fiber photometry recording. During recording, an optical fiber (Thorlabs) would be tethered on the head fiber implant to transmit GCaMP fluorescent signal. The green fluorescence signals, which elicit by 473 nm blue light, were normalized to the UV-elicited background fluorescence and down-sampled to 10Hz for data processing. For all animals used in photometry experiment.

4.4.1 Object presenting experiment

Each mouse was habituated in the test cage (Coulbourn Instrument) for 20 mins before object presentation. Two different presenting sequences were used: 1. Present LEGO or water (in a 3 cm-wide culture dish) in the cage for 20 minutes. Then the previous object was removed and wait for 30 mins. A 3 - 4 grams chow pellet was then presented. 2. Present LEGO or water (in a 3 cm-wide culture dish) in the cage for 20 minutes. Then the previous object was removed and wait for 30 mins. Then 3 - 4 grams peanut butter (on a 3 cm-wide culture dish) was presented. Each mouse was tested using the above protocol for fed or fasted condition.

4.4.2 Liquid diet licking experiment

Ensure (1K cal/ml) and water were loaded in two separated bottles with lickometers in the test cage (Coulbourn Instrument). The access to Ensure and water were blocked by a plastic panel. At 5PM mice were transferred to the test cage and habituated for 20 mins. The access blocks were then removed and mice are allowed to have free access to water and Ensure.

4.5 In vivo optogenetic stimulation of Pdyn

Pdyn-Cre mice (age 8-12 weeks) were used for virus injection and optical fiber implantation surgery. 35 nl AAV5-EF1a-DIO-ChR2(h134R)-eYFP (UNC, titer: 4.2X10^{12}) virus was stereotaxically injected into right DMH. An optical fiber (Thorlabs catalog#: FT200EMT) with plastic sleeve was then implanted right above right DMH and secured by dental cement on the
skull surface. Animals are allowed to recovery for 3 weeks before photometry recording experiment. *Post hoc* histology verification was performed after behavior experiments were completed.

**4.5.1 Measuring food intake in fed condition**

Initial habituation: At 5PM, each mouse was transferred to a feeding chamber (Coulbourn Instrument, habitest system) with an optical fiber tethered to its head implant to habituate the chamber and the feeding device (Coulbourn Instrument, habitest system, deliver a 20mg food pellet (Biopac Systems Inc.) for 2 nights, and was removed from the chamber in the next morning. After initial habituation each mouse was then subjected to the following experiment protocol for 4 consecutive days: At 5PM, mice were transferred to the same feeding cage and tethered with an optical fiber. After 30 mins, mice either received continuous light stimulation (473nm blue light, 10 Hz/10 millisecond; 1 second on /1 second off; power: 10-15 mW/cm$^2$) or sham stimulation for 3 hours (stimulation sequence - day 1: sham stimulation; day 2: light stimulation, day 3: sham stimulation; day 4: light stimulation). At 8:30 PM animals were removed from the chamber. Data from day 1 and day 3 were averaged for sham stimulation result; Data from day 2 and day 4 were averaged for stimulation result.

**4.5.2 Measuring food intake in fast-refeeding condition**

Food was removed from the home cage at 5PM the previous day before fast-refeeding experiment. Next day each mouse was transferred to the feeding chamber at 5PM, with an optical fiber tethered and habituation for 30 mins. After habituation, food pellets were then supplied with light stimulation (473nm blue light, 10 Hz/10 millisecond; 1 second on /1 second off; power: 10-15 mW/cm$^2$) or sham stimulation at the same time. The test condition sequence is: week 1: sham stimulation; week 2: light stimulation; week 3: sham stimulation; week 4: light stimulation. Each fast-refeeding experiment was performed after 5 - 7 days from the previous
one to allow mice to recover to the baseline body weight. Data from week 1 and week 3 were averaged for sham stimulation result; Data from week 2 and week 4 were averaged for light stimulation results.

4.5.3 Locomotor activity measurement

Each mouse was kept in a white acrylic box (15cmX15cmX15cm) from 8AM and habituation for at least 3 hours before light stimulation. A webcam mounted on the floor was used to record the video from the top. The locomotor activity is calculated by using MATLAB program to analyze the mouse position in video frame by frame (5-10 fps).

4.5.4 Blood pressure measurement

Ketamine (75 mg/kg) + Medetomidine (0.5 mg/kg) was intraperitoneally injected for sedation. Each mouse was then gently put into an animal holder for habituation for 10 minutes. A tail cuff was mounted on the root of the tail to measure blood pressure and heart rate.

4.5.5 Short-term BAT temperature recording

An IPTT 300 (BioMedic Data Systems) passive temperature transponder was implanted subcutaneously under the interscapular region. A hand-held transceiver was used to read BAT temperature by approaching the mouse. Temperature measurement was carried out least 14 days after implantation surgery to allow mice to recover.

4.6 Tetanus neurotoxin silencing of Pdyn<sub>DMH</sub> and long-term recording experiment

For Pdyn<sup>DMH</sup> silencing, 35-45 nL of AAVDJ-CMV-DIO-eGFP-2A-TeNT (Stanford, titer: 1.4X10<sup>10</sup>) was injected (infusing rate: 30nL/min) into bilateral DMH of Pdyn-Cre mice (8-12 weeks). For control, the virus was shifted to 20nL of AAV2-EF1a-DIO-GFP-L10 (UNC, 6.9X10<sup>12</sup>). After surgery, mice were single-housed for 3 weeks before experiments were conducted. Post hoc histological quantification of hypothalamic GFP (+) cells were performed after experiments were completed.
4.6.1 Long-term continuous monitoring of feeding and drinking

Three weeks after surgery, each mouse was transferred to a cage inside a sound-attenuating chamber (Med Associates) for habituation for at least 7 days. Each of them was singly housed. Inside the cage, a programmable feeding device ('FED', Nguyen, O’Neal, Bolonduro, White, & Kravitz, 2016; a 20mg food pellet is delivered each time) was modified to record food intake in the cage. An optica llickometer (modified from photointerrupter (# GP1A57HRJ00F) with a 3D-printed adaptor mounted) was mounted on the licking tube of the water bottle to measure numbers of licks. Data were transmitted to an Arduino mega recording device and a computer for storage.

4.6.2 Long-term continuous monitoring of BAT temperature and locomotor activity

A transponder (G2 Emitter, Starr Life Sciences) was implanted to the interscapular region for BAT temperature and locomotion recording. A transceiver (ER-4000, Starr Life Sciences) was placed underneath the home cage for detecting transponder signals for each mouse at a sampling rate of 0.5 Hz; the data were then transmitted to a computer in real time.

4.7 Histology

Animals were anesthetized under isoflurane and then perfused with 10% formalin (Sigma-Aldrich). Brains were kept in 10% formalin overnight and then shifted to 30% sucrose in PBS for 48 hours. Brains were embedded in O.C.T. Compound (Tissue-Tek) and frozen to -20 °C for cryosection (50mm per slice).

4.8 Data quantification and statistical analysis

Data were analyzed using MATLAB 2015b and Prism 7.0
References


dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker.


Figures

Figure 1. PhosphoTRAP results of micro-dissected DMH from mice subjected to scheduled feeding.

(A) Schematic of time-restricted schedule feeding experiment. Food is only supplied from CT4-7 under a 12h:12h light/dark cycle. CT: circadian time. IHC: immunohistochemistry

(B) Prodynorphin (Pdyn, green; fluorescent in situ hybridization and antibody staining) expression in the dorsomedial hypothalamus (DMH), lateral hypothalamus (LH) and arcuate nucleus (Arc), with abundant colocalization with pS6 signal (red) in DMH. The mouse is subjected to CT4-7 scheduled feeding and sacrificed at CT6 (Images from Knight et al., 2012)

(C) The schematic of phosphoTRAP procedures for micro-dissected DMH tissues from mice subjected to scheduled feeding.

(D) Multiple enriched neuropeptide/receptor genes (green dots) revealed in the phosphoTRAP result. Prodynorphin (Pdyn) is marked in red in the left RPKM plot. RPKM: Reads Per Kilobase of transcript, per Million mapped reads.

(E) Among enriched genes from phosphoTRAP, CCK, GSBS, GRP and Rorb are commonly expressed in the similar compact DMH region (Images from Allen Brain Institute).
Figure 2. Fiber photometry recording of Pdyn<sub>DMH</sub> GCaMP6 in different object presentation scenarios.

(A) A schematic of fiber photometry experiments from (B) to (E). Right: a representative brain slice of an animal in the photometry experiments. Blue: DAPI. Green: GCaMP6s. Dotted white line: contour of the optic fiber implant. Scale bar: 400um.

(B & C) Group average of Pdyn<sub>DMH</sub> GCaMP6s fluorescent signals when presenting with different objects in fed and fasted state. F: fluorescence. Zero second represents the presenting timing of the object. PB: peanut butter. (n=7, 3M4F)

(D & E) Group average of Pdyn<sub>DMH</sub> GCaMP6s fluorescent signals when presenting with different caged objects in fed and fasted state. (n=7,3M4F)

(F & G) Statistics of area under curve (AUC) of GCaMP6s fluorescent signals from 30 to 300 seconds after different object presentation in (B) to (E) experiment. WD: water-deprived. Statistical method: Repeated measure 1-way ANOVA, multiple comparison with Tukey correction. *p<0.05, **p<0.01.
Figure 3. Fiber photometry recording of Pdyn<sup>DMH</sup> in liquid diet experiment.
(A) Schematic of fiber photometry with liquid-drinking experiments in (B) to (H)
(B, C, D) Representative traces of Pdyn<sup>DMH</sup> GCaMP6s fluorescent signal aligned with licking bouts. (B), (C) and (D) are from the same animal. Yellow tick: Ensure. Blue tick: water. F: fluoresce. A.U.: arbitrary unit.
(E & F) Group average of peri-lick GCaMP6s fluorescent signals of Pdyn<sup>DMH</sup> in fed animals. (n=6, 3M3F). Water (WD): Water licking bouts under water-deprived state. (E) Time zero represents the timing when lick bouts begin. Red star: significant difference between Ensure and water for the averaged ΔF/F percentage change over the 10 second period after the lick bouts started. Blue star: significant difference between drinking water in fed state and water-deprived state. (F) Time zero represents the timing of the lick bouts end. Red star: significant difference between Ensure and water for the averaged ΔF/F percentage change over the 10 to 20 second period after licks ended. Statistical method for (E) and (F): repeated measure 1-way ANOVA, multiple comparison to water group with Dunnett correction, comparing to licking water. *p<0.05
(G & H) Group average of peri-lick Pdyn<sup>DMH</sup> GCaMP6s fluorescent signals in fasted animals. (n=6,3M3F). The symbols and the statistical method are the same as (G) & (H)
Figure 4. Effects of optogenetic stimulation of Pdyn\textsuperscript{DMH} and its local axonal arborization.

(A) Schematic of AAV delivery of ChR2 into Pdyn\textsuperscript{DMH}. Stimulation protocol: 10Hz/10ms, 1 sec on/1 sec off, 10-15 mW/cm\textsuperscript{2}

(B) Locomotor activity rapidly increases along with and phase-locked to light stimulation (10 minutes for twice, blue area). Statistical method: repeated measure 2-way ANOVA, Dunnett's multiple comparisons test for comparing locomotor activity in stimulation and non-stimulation phases to that in the initial 10-minute baseline. *p<0.05, **p<0.01, ChR2 (n=5), GFP ctrl (n=6)

(C) BAT temperature increases rapidly along with light stimulation (10 minutes, blue area). Statistical method: repeated measure 2-way ANOVA, Dunnett's multiple comparisons test comparing mean BAT temperature of last 4-minute of stimulation and non-stimulation phases to that of the initial 10-minute baseline. ****p<0.0001. ChR2 (n=5), GFP ctrl (n=6)

(D) Blood pressure increases rapidly along with light stimulation (10 minutes, blue area) and also quickly returned to baseline after light stimulation stopped. Statistical method: repeated measure 2-way ANOVA, Dunnett's multiple comparisons test comparing mean blood pressure in stimulating and non-stimulating phases to that in initial 10-minute baseline**p<0.01. ChR2 (n=5), GFP ctrl (n=4)

(E & F) Accumulated food intake in fed and fasted state. Blue: ChR2 group; Black: GFP ctrl. Open circle (GFP-NS/ChR2-NS): no stimulation; Filled circle (GFP-Stim/ChR2-Stim): Continuous light stimulation for 3 hours (protocol as in (A)). Statistical method: repeated measure 2-way ANOVA, Sidak's multiple comparisons test comparing accumulated food intake between stimulation and non-stimulation at each time point within ChR2 and control animal group. **p<0.01, ***p<0.001. ****p<0.0001.

(G) Schematic of AAV delivery of synaptophysin-GCaMP6 into Pdyn\textsuperscript{DMH} and the local synaptic arborization pattern in DMH.
Figure 5. \(\text{Pdyn}^{\text{DMH}}\) silencing and the long-term experiment for synchronous recording of BAT temperature, locomotor activity, food and water intake.

(A) Schematic of AAV delivery of Cre-dependent tetanus neurotoxin (TeNT) into \(\text{Pdyn}^{\text{DMH}}\). Right: a representative brain slice from a mouse infected with AAVDJ-CMV-DIO-eGFP-TeNT. Green: GFP signal. Bottom: the long-term recording protocol for continuous experiments.

(B) Illustration of the recording environment: the mouse was singly housed in a cage with a feeding device (FED) and a water bottle (with a lickometer) inside. A transponder is implanted into the interscapular region of the mouse to record BAT temperature. A
transceiver plate (Grey color in the graph) receiving transponder signals is placed underneath the cage. Data of pellet retrieval, water-licking, BAT temperature and locomotor were integrated and transmitted to a computer for storage.

(C) BAT temperature(temp.) change at food bout start in fed state. Blue trace: GFP control animal. Red trace: TeNT-silencing group. Right: Statistics of comparing BAT temperature rising speed (deg. C/min) from -5 to 0 min v.s. 0 to 5 min in the 2 groups. Repeated measure 2-way ANOVA with Sidak's multiple comparison test. NS: not significant; **p<0.01.

(D) BAT temperature(temp.) change at the first food bout start in fast-refeeding condition. Blue trace: GFP control animal. Red trace: TeNT-silencing group. Right: Statistics of comparing BAT temperature rising speed (deg. C/min) from -5 to 0 min v.s. 0 to 5 min in the 2 groups. Repeated measure 2-way ANOVA with Sidak's multiple comparison test.

(E) Food intake in ad lib and scheduled feeding state. No significant difference in the amount of food intake between 2 groups (GFP:3M1F; TeNT: 2M2F). Statistical method: unpaired t-test.

(F) Body weight in ad lib fed and scheduled feeding state. No significant difference in the body weight between 2 groups (GFP:3M1F; TeNT: 2M2F). Statistical method: unpaired t-test.
Figure 6. Pdyn\textsuperscript{DMH} silencing abolishes anticipatory locomotor activity and BAT temperature rising before feeding starts during schedule feeding.

(A) Actograms of a representative GFP and TeNT mouse respectively. Black ink dots were plotted according to activity counts. Grey area: dark phase. White blocks from day 7-28: scheduled feeding food supply time (ZT6-9). Red blocks: the 3-hour period (ZT3-6) before food supply, when most food anticipatory activities develop in the period. ZT: zeitgeber time.

(B & C) Hourly average of locomotor activity in ad lib (panel B) and scheduled feeding condition (panel C). Light : dark = 12:12, Blue trace: GFP control group. Red trace: TeNT group. Data were plotted as mean \pm s.e.m. Grey area (ZT12-24): dark phase. Green area (ZT6-9): food supply time. Statistical method: 2-way ANOVA with Sidak’s multiple comparison test between 2-hour or 3-hour-average of activities at different time period between the 2 group (ZT0-2, ZT2-4, ZT4-6, ZT6-9, ZT9-11, ZT12-14, ZT14-16, ZT16-18, ZT18-20, ZT20-22, ZT22-24). **p<0.01.

(C & D) Hourly average of BAT temperature in ad lib and scheduled feeding condition. Graph settings and statistical method are as in (D) ****p<0.0001

(E) Group average of BAT temperature traces in GFP control and TeNT group during scheduled feeding. Time course: from 3 hours before to 1 hour after feeding start. Time zero represents feeding starts. Sampling rate: 0.5Hz. Statistics of AUC from hour -3 to 0: ****p<0.0001, Unpaired t-test.
Supplementary Figure S1. Additional phosphoTRAP results of micro-dissected DMH.
(A) Majority of Pdyn^{DMH} (82%) express pS6 compared to other Pdyn neurons in lateral hypothalamus, paraventricular nucleus, and arcuate nucleus (modified from Knight et al 2012)
(B) Enriched transcripts in phosphoTRAP results of micro-dissected DMH (from mice subjected to scheduled feeding.) (C) Activity-dependent immediate early genes (red dots). (D) CLOCK genes (blue dots) (E) Overlapping of CCK, Pdyn and pS6 signals in DMH
Supplementary Figure S2. The wiring diagram and parts of FED device.
The wiring diagram and parts of FED device. VCC: voltage at the common collector; GND: ground.

Parts:
- 1 stepper motor (28BYJ-48 ULN2003 5V Stepper Motor)
- 1 stepper motor control board (ULN2003 Driver Board)
- 1 Arduino Nano
- 1 Omron photo interrupter (GP1A57HRJ00F)
- 1 GP1A57HRJ00F breakboard (SparkFun #09322)
- 1 DIP relay (EDR201A0500)
- 1 30 pins breadboard
- 1 4.7K resistor
- 7 breadboard steel jumpers
- 9 female-male jumper wires
- 12 male-male jumper wires
- 1 RJ11 adapter
- 1 7 feet RJ11 6P6C telephone wire
Supplementary Figure S3. The wiring diagram and parts of Arduino recording device
The wiring diagram and parts of an Arduino recording device (example of a recording device for two animals).
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