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海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人日本学術振興会 理事長　殿

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受 付 番 号　2011960208
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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）　ワシントン大学　（　米国）
2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
   メスマウスの内的状態依存的に不安状態を調節する神経基盤の解析
3. 派遣期間：平成　31年　4 月　15 日　～　令和　3 年　4 月　14 日
4. 受入機関名及び部局名
   受入機関名：　University of Washington
   部局名：Department of Anesthesiology and Pain Medicine, Department of Pharmacology
5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

Abstract

Feeding is critical for the survival of an animal. Interestingly, feeding is heavily impacted by circulating hormones such as estrogens. Estrogen depletion in post-menopause women leads to high risk of obesity, while estrogen replacement therapy suppresses body weight (Svendsen et al., 1995). While previous studies have started to reveal the neural substrate of feeding behavior control, how they are modulated by estrogen action is not well understood. In this study, I focus on a brain area highly involved in feeding behavior control, the paraventricular hypothalamus (PVH) (Timper and Brüning, 2017). Implantation of estrogen into the PVH reduced food intake and body weight in ovariectomized rats, suggesting that estrogen action on PVH can have a negative impact on feeding (Silvestre et al., 2018). The goal of this study is to reveal how estrogen modulates neural activity in PVH neurons that control feeding behavior in mice.

Results

1. Anatomical characterization of estrogen receptor expressing neurons in the PVH

First, to anatomically characterize estrogen sensitive neurons in the PVH, I analyzed the expression of estrogen receptor mRNA transcripts in the PVH. Among the two well-known estrogen receptors, *Esr1* and *Esr2*, *Esr2* was dominantly expressed in the PVH (*Esr2*: 1012 ± 406, n = 33, *Esr1*: 406 ± 262, n = 30). Interestingly, in the PVH, Esr2 and Esr1 were mostly expressed in different neurons. The PVH is a highly heterogeneous brain area containing multiple cell types that express unique neuropeptide and receptor genes. Further characterization of *Esr2* expression revealed that *Esr2* was heavily expressed in *Cholecystokinin receptor alpha (Cckar)* neurons and in *Prodynorphin* neurons in the posterior portion of the PVH (**Figure 1**, >75% of *Esr2*+ neurons in the posterior part of PVH had co-expression of *Cckar* or *Pdyn*). The co-expression ratio of *Esr2* and *Cckar* and *Pdyn* was the two highest among other genes tested (*Oxt, Avp, Glp1r, Mc4r, Sst, Crh, Npy1r*). Cckar is a receptor target of cholecystokinin, a neuropeptide known to induce satiety. Pdyn+ PVH cells has been shown to regulate feeding behavior (Li et al., 2019). This further supports the hypothesis that *Esr2* neurons in the PVH control estrogen-mediated feeding behavior modulation. Interestingly, co-expression of *Esr2* and *Cckar* was confirmed in a different dataset obtained in the lab to characterize the transcriptional property of PVH neurons using sc-RNAseq technology.

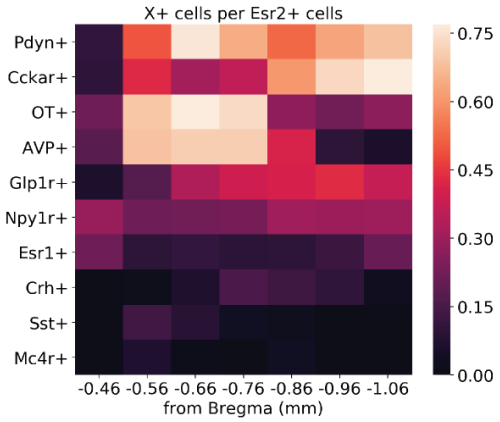

Figure 1. Gene expression in PVH^{Esr2}

Quantification of co-expression of genes in *Esr2*+ cells in the PVH. *Esr2*+ neurons in the PVH show high co-expression of *Ot* and *Avp* in the anterior part of the PVH, and *Pdyn* and *Cckar* in the posterior part of the PVH.

2. Manipulation of PVH^{Esr2+} neurons

Next, to study whether PVH^{Esr2+} neurons have a causal role on feeding behavior, I manipulated the activity of these neurons. To investigate the sufficiency of PVH^{Esr2+} activity on feeding behavior, a Cre-dependent viral vector was bilaterally injected into the PVH of Esr2-Cre mice to induce expression of ChR2, a light-gated ion channel, in the PVH^{Esr2+} neurons. ChR2-expressing animals, and eYFP -expressing animals as control, went through a behavior paradigm to test feeding behavior. As result, light stimulation of PVH suppressed feeding behavior in ChR2-expressing behaviors (**Figure 2A**).

Next, to investigate the necessity of PVH^{Esr2+} activity on feeding behavior, a Cre-dependent viral vector was bilaterally injected into the PVH of Esr2-Cre mice to induce expression of hM4Di, a pharmacogenetic tool to inhibit neurons, in the PVH^{Esr2+} neurons. Clozapine-N-oxide (CNO) which selectively activates hM4Di to suppress neural activity. hM4Di -expressing animals, and eYFP-expressing animals as control, went through a behavior paradigm to test feeding behavior. As result, CNO administration suppressed feeding behavior in hM4Di-expressing behaviors (**Figure 2B**).

Thus, this result indicates that PVH^{Esr2+} neurons have a causal role in feeding behavior control.

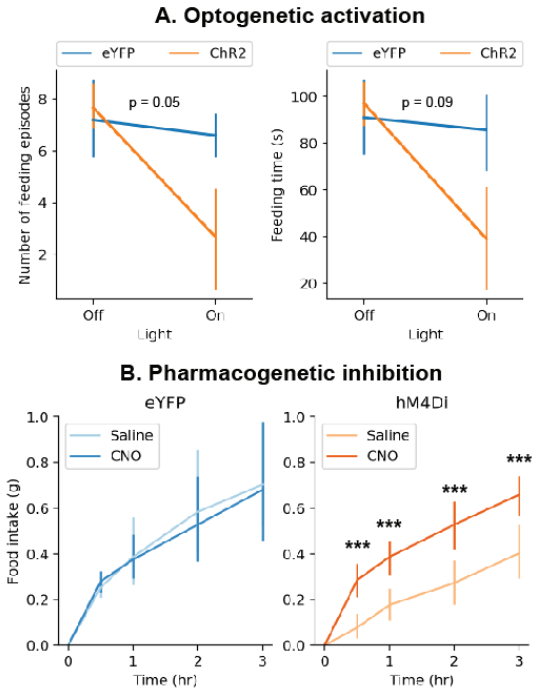

Figure 2. Manipulation of PVH $^{Esr2+}$ neurons modulate feeding.
A. Quantitative analysis of number of feeding episodes and feeding time during a feeding behavior test. Animals showed reduced feeding behavior during light stimulation period. Each period was 10 minutes. Mean ± std.
B. Quantitative analysis of amount of food intake during a feeding behavior test. hM4Di-animals showed increased feeding behavior during CNO administration trial compared to saline administration trial. Mean ± std.

3. Two-photon calcium imaging from PVH^{Esr2+} neurons

To further study how the neural activity of the PVH changes during feeding, and how it is modulated by estrogen, I conducted calcium imaging from these neurons using two-photon microscopy. This technology enabled stable imaging from the neurons across different days when the animal was in different estrogen conditions (**Figure 3**). In brief the animals were imaged on either a low or high estrogen condition (diestrus or proestrus stage).

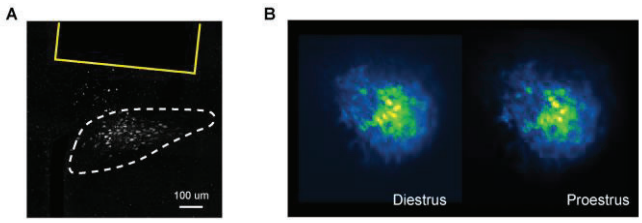

Figure 3. Two-photon microscopy enables stable imaging of the same neurons under different hormonal condition. Example standard deviation projection of activity across time. Images on each row were acquired from the same animal in different hormonal condition.

On the imaging day, animals were head-fixed under the two-photon microscope. Animals were presented with 20 trials of sucrose presentation with random inter trial intervals. The fluorescence changes of each neuron after sucrose presentation was analyzed as "response". After collecting data from 143 neurons, I used spectral clustering method to classify the response into 4 categories (**Figure 4)**. As result, there was one cluster of cells (Cluster 2) that responded positively to sucrose in the low estrogen condition. To sum, a subset of PVH^{Esr2} neurons positively change their response to sucrose in low estrogen conditions.

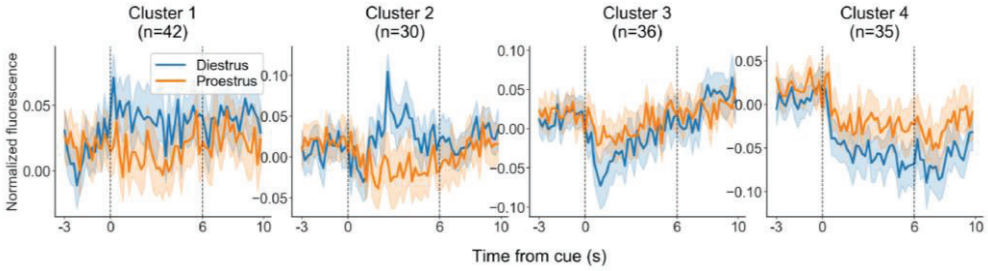

Figure 4. Diverse sucrose response patterns of PVH^{Esr2+} neurons in low and high estrogen conditions.

Classification of neurons into 4 response clusters based on their trial-averaged response to sucrose. Blue line represents low estrogen condition, orange line represents high estrogen condition. Total of 143 neurons from n = 5 animals.

Discussion

The initial goal of this project was to characterize the role of Esr2 neurons in the bed nucleus of stria terminalis (BNST) in anxiety-related behavior control. However, in the first year of the study, I identified that the expression of *Esr2* was stronger in PVH compared to the expression in BNST. Additionally, *Esr2* and *Esr1* was highly co-expressed in the BNST, while it was not in PVH, suggesting that *Esr2* and *Esr1* signaling have unique roles in the PVH. Considering these data, I decided to focus on the PVH where it was more likely to observe a novel function of Esr2. Since previous studies had suggested that PVH contain cells that control various behaviors such as stress response, social behavior and feeding, I conducted behavior tests, such as open field test, elevated-plus maze test and social interaction test, while activating PVH^{Esr2+} neurons. As result, activation of PVH^{Esr2+} neurons had the strongest impact on feeding behavior as shown in result 2. For this reason, I decided to focus on feeding behavior rather than anxiety-related behavior.

The data shown above suggest that PVH^{Esr2+} neurons may play a critical role in modulating animals feeding behavior in response to changes in estrogen level. In rodents, increase of estrogen is correlated with decrease of food intake (Olofsson et al., 2009). Furthermore, direct application of estradiol into PVH induced loss of food intake as well as loss of body weight in ovariectomized rats (Butera and Beikirch, 1989). We found in an *ex vivo* electrophysiological experiment that PVH^{Esr2+} cells increase their action potential threshold in high estrogen condition, which suggests that PVH^{Esr2} neurons suppress their activity in high estrogen condition (data not shown). Thus, we hypothesized that PVH^{Esr2} neurons suppress their activity during high estrogen conditions which leads to suppressed food intake. As result, we found that a subset of PVH^{Esr2+} show weaker response to sucrose in high estradiol condition than in low estradiol condition (result 2), which supports our hypothesis. To further validate whether this effect is directly caused by estrogen receptors in the PVH, it will be critical to conduct a loss-of-function experiment to knock-down *Esr2* gene expression in the PVH.

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