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The role of central amygdala neuronal types in drug-related and appetitive behaviors

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Publication date
2022

[Link to publication](#)

Citation for published version (APA):

Bouhuis, A. L. (2022). *The role of central amygdala neuronal types in drug-related and appetitive behaviors*. [Thesis, fully internal, Universiteit van Amsterdam].

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CHAPTER 4. THE ROLE OF CENTRAL AMYGDALA NEURONAL TYPES IN APPETITIVE AND HOMEOSTATIC BEHAVIORS

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Abstract

Obesity is a significant health problem around the world, and it is of great importance to understand the neuronal mechanisms involved in feeding behaviors. Even though several studies have described a role for the CeA in feeding behaviors, its exact function is not known and has been ambiguous. In this chapter we examine the role of a newly identified CeA neuronal population in homeostatic feeding behaviors. We inhibited both VIPR2-expressing and Prkcd-expressing CeA neurons while assessing continuous access feeding, as well as feeding after food deprivation. In addition, we also looked at homeostatic processes such as body weight regulation and locomotor activity. We showed that permanent inhibition of VIPR2-expressing CeA neurons increased food intake in males, while having no significant effect on females. These results might indicate that baseline VIPR2 activity could play a role in homeostatic feeding behaviors. Interestingly, the increase in food intake did not affect body weight or locomotor activity in these males. To assess which neuronal circuit could underlie these behavioral effects, we assessed the projection pattern of the VIPR2-expressing neurons we inhibited, and we identified a strong projection from VIPR2-expressing CeA neurons to the lateral PBN. Further research is necessary to examine if this projection is indeed involved in the feeding effects we see in males.

4.1 Introduction

Obesity is a significant health problem across the world, making it of huge importance to understand the mechanisms and circuits that underlie obesity. In healthy situations, body weight and fat content are pretty stable over time, which is caused by homeostatic processes that involve the lateral hypothalamus (LH), the arcuate nucleus of the hypothalamus (ARC), the ventral tegmental area (VTA) and the nucleus accumbens (NAcc)¹⁰⁵. In addition to homeostatic food intake, these brain areas regulate reward seeking and consummatory behavior as well, combining the homeostatic need for food with the hedonic value and the motivation to work for said food. Different agents are involved in consummatory behavior, including agouti-related protein (AGRP). AGRP neurons are situated in the hypothalamic arcuate nucleus (ARC) and play a vital role in food homeostasis; when these neurons are activated they drive food consumption¹⁰⁶. AGRP neurons are inhibited by leptin, a hormone that is released from adipose tissue after feeding, and they are activated by ghrelin, a gastric hormone that is released when hungry¹⁰⁷.

Apart from these key players in these brain areas, the CeA has also been implicated in feeding behaviors. The CeA can receive food related information through a direct input from AGRP neurons in the ARC¹⁰⁸. Even though several studies have shown a role for the CeA in food behaviors, its exact function is not known and has been ambiguous. Some studies point towards a role for the CeA in the initiation of feeding behavior, while others show an association of CeA activation with a reduction of feeding behavior^{49,53,54,109}. Others show effects of CeA manipulations only on drinking behavior¹⁷. These seemingly contradictory results can partly be explained by differences in neuronal types, neurotransmitter signaling and differing inputs.

This study aims to provide a deeper understanding of the role of a newly identified CeA neuronal marker: vasoactive intestinal peptide receptor 2 (VIPR2), which is a receptor that responds to VIP as well as pituitary adenylate cyclase-activating peptide (PACAP)^{110,111}. PACAP has been shown to be involved in feeding behaviors, including hedonic feeding, making the VIPR2-expressing CeA neurons an interesting candidate to be implicated in feeding¹¹²⁻¹¹⁴.

4.2 The role of VIPR2-expressing CeA neurons in continuous access feeding and feeding after food deprivation

To assess the role of VIPR2-expressing CeA neurons in continuous access feeding and feeding after food deprivation, 24-hour food and water intake in the home-cage was measured, as well as food intake after food deprivation, in animals in which activity of CeA VIPR2-expressing neurons was inhibited (Figure 4.1). We used a cre-dependent virus expressing tetanus toxin-light chain (TeLC), which blocks neurotransmitter release, to permanently inhibit neurons in which the virus is expressed. Control animals were injected with a cre-dependent GFP virus. Viruses were injected in the CeA of either VIPR2-cre or Prkcd-cre animals as a comparison. After sufficient viral expression, food and water was measured in their home-cage at two timepoints 24 hours apart, and intake was calculated. After this, food intake was measured after 18-hour food deprivation, at 30, 60, 90, 120 and 180 min timepoints. While 24-hour food and water intake measurements give an indication of baseline homeostatic feeding processes, food intake after food deprivation shows the ability of animals to re-establish feeding patterns after a challenge. Through focusing on both these feeding paradigms, we can examine multiple aspects of feeding behavior in the same animals.

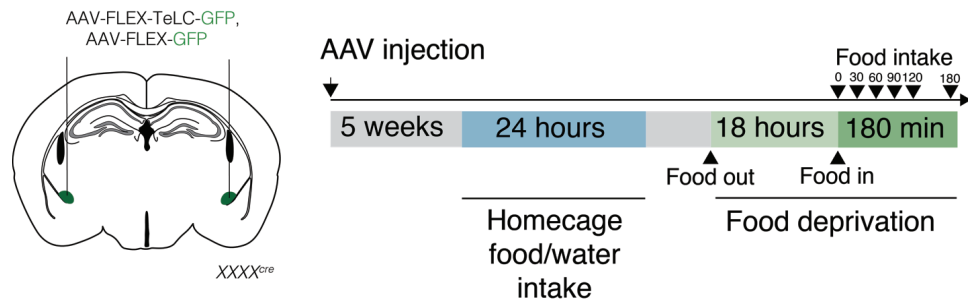


Figure 4.1 Experimental setup

4.2.1 The role of VIPR2-expressing CeA neurons in feeding behaviors

To assess the role of VIPR2-expressing CeA neurons in feeding behaviors, VIPR2-cre animals were injected in the CeA with cre-dependent TeLC or cre-dependent GFP (Figure 4.2A,B). After viral expression, 24-hour food and water intake was measured. Inhibition of VIPR2-expressing CeA neurons caused an increase in 24-hour food intake in males, while having no effect on their water intake (Figure 4.3A; unpaired t-test, $p=0.0118$, $p>0.05$). VIPR2 inhibition did not affect 24-hour food and water intake in females (Figure 4.3A; unpaired t-test, all $p>0.05$). When animals were challenged with food deprivation, followed by reintroduction of food, no main effects were observed

in their food intake between TeLC and GFP animals in either females or males (Figure 4.3B; two-way ANOVA, $p>0.05$). Interestingly, an interaction effect was found in females between Time x GFP/TeLC (two-way ANOVA, $p=0.0088$), with no significant effects found with post-hoc tests (Sidak's multiple comparison tests, all $p>0.05$). This indicates that TeLC females are affected differently by the food challenge than GFP females. It is possible that sex differences in responses to stressors can account for this interaction effect.

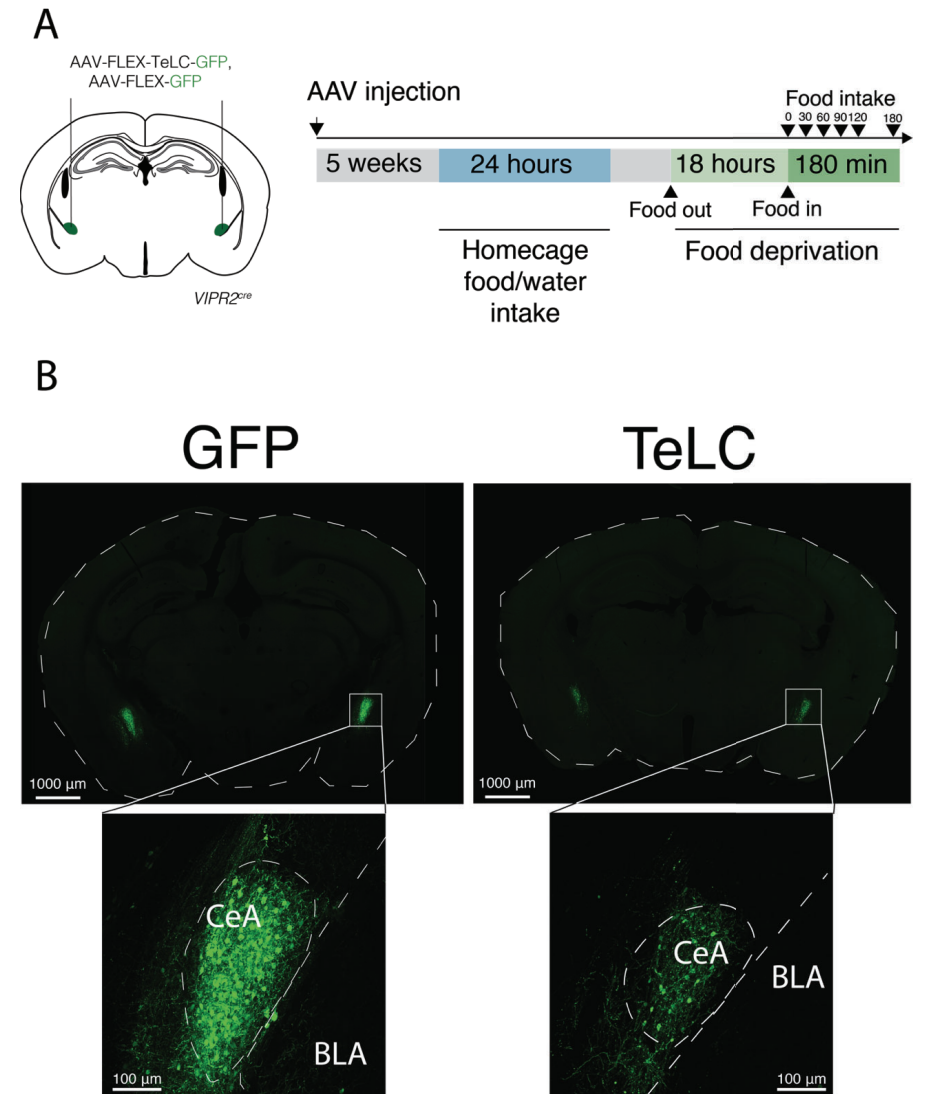


Figure 4.2 Expression of TeLC and GFP virus in VIPR2-expressing CeA neurons. (A) Schematic of experimental methods. (B) Representative histology images of a GFP (left) and TeLC (right) injected animal.

The increase in food intake in males in which VIPR2-expressing CeA neurons were inhibited, might indicate that baseline VIPR2-expressing CeA neuronal activity could play a role in regulating homeostatic feeding behaviors. Inhibition of these neurons might cause the animals to increase their eating, leading to an increase in bodyweight. To address this, weight of males was compared pre-surgery to weight at the start of behavioral experiments. In addition, VIPR2-expressing CeA neurons might affect locomotor activity, causing a change in feeding behaviors.

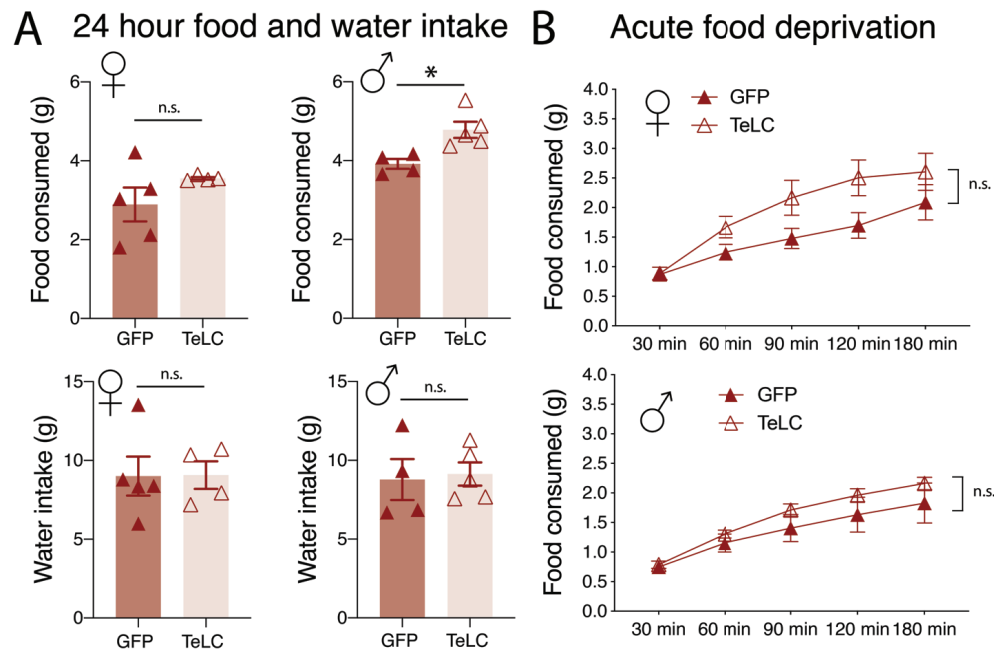


Figure 4.3 VIPR2 neurons inhibit home-cage feeding behavior in males. (A) No difference was found between GFP and TeLC females on 24 hour food or water intake (unpaired t-test, $p > 0.05$). TeLC males had significantly higher food intake compared to GFP males (unpaired t-test, $p = 0.0118$), while no differences were found on water intake ($p > 0.05$). (B) No significant differences were found between GFP and TeLC females or males on food intake after acute food deprivation (two-way ANOVA, $p > 0.05$). However, a significant interaction effect of Time \times GFP/TeLC was found in females (two-way ANOVA, $p = 0.0088$), with no significant effects on post-hoc tests (Sidak's multiple comparison test, all $p > 0.05$). Data represented as mean \pm s.e.m.

4.2.2 The role of VIPR2-expressing CeA neurons in homeostatic processes in males

To examine if VIPR2-expressing CeA neurons play a role in homeostatic processes, body weight and locomotor activity was recorded. Body weight was measured pre-surgery and before start of behavioral experiments (>5 weeks after surgery), and weight change was calculated (post / pre weight). Home-cage locomotor activity was recorded during 23 hours, with an 11 hr light and 12 hr dark cycle. Velocity (cm/s) was recorded and ultimately separated in average velocity during light and dark cycle.

When body weight change was recorded, both GFP and TeLC animals showed an increase in body weight from the start of behavioral experiments vs pre-surgery (Figure 4.4A; > 1.0 ratio), this increase is expected when taking into account the age of animals at surgery (two months) and normal body weight gain with aging. No differences were found in body weight change between GFP and TeLC males, indicating that inhibition of VIPR2-expressing CeA neurons does not cause a change in body weight (Figure 4.4A; unpaired t-test, $p > 0.05$). When home-cage locomotor activity was assessed, no differences were found in average total velocity between GFP and TeLC animals (Figure 4.4B; unpaired t-test, $p > 0.05$), as well as no difference in velocity between GFP and TeLC animals during light and dark cycles (figure 4.4B; two-way ANOVA, $p > 0.05$). These results show that even though inhibition of VIPR2-expressing CeA neurons causes an increase in feeding behavior, no effects are seen on body weight or locomotor activity. This suggests that VIPR2-expressing CeA inhibition can alter food intake without affecting other homeostatic metabolism processes.

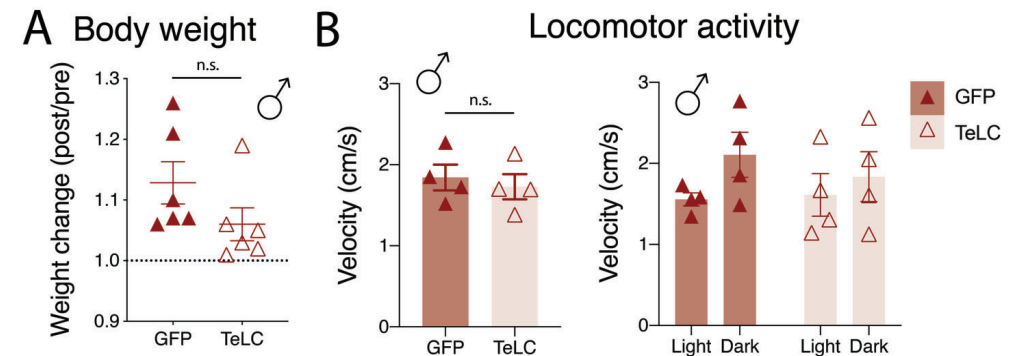


Figure 4.4 Inhibition of VIPR2-expressing CeA neurons does not alter body weight or velocity in males. (A) No significant difference was found between GFP and TeLC males in body weight change (unpaired t-test, $p > 0.05$). (B) No difference was found on locomotor activity between GFP and TeLC males (unpaired t-test, $p > 0.05$), as well as no differences when velocity was separated between light and dark cycles (two-way ANOVA, $p > 0.05$). Data represented as mean \pm s.e.m.

4.3 The role of Prkcd-expressing CeA neurons in feeding behaviors and homeostatic processes

To examine if the effects seen of VIPR2-expressing CeA neuron inhibition on feeding behaviors, are selective to the VIPR2 population, the experiments were repeated with Prkcd-expressing CeA inhibition. VIPR2-expressing and

Prkcd-expressing neurons seem to be two largely non-overlapping group of neurons (unpublished data from our lab) allowing for the examination of the importance of VIPR2-expressing neurons in these behavioral effects.

4.3.1 The role of Prkcd-expressing CeA neurons in feeding behaviors and homeostatic processes

Prkcd-cre animals were injected in the CeA with cre-dependent TeLC to selectively inhibit Prkcd-expressing CeA neurons, or with cre-dependent GFP as a control virus (figure 4.5A). After 5 weeks of viral expression, 24-hour food and water intake was measured, as well as food intake after food deprivation (figure 4.5B).

Prkcd inhibition did not affect 24-hour food and water intake in either females or males (Figure 4.6A; unpaired t-tests, all $p > 0.05$). In addition, no main significant effects were found between GFP and TeLC animals on food intake after food deprivation (Figure 4.6B; two-way ANOVA, all $p > 0.05$). However, an interaction effect between Time x GFP/TeLC was found in males only, with post-hoc tests revealing no significant effects (Figure 4.6B; two-way ANOVA, $p = 0.035$; Sidak's multiple comparison test, $p > 0.05$). When body weight and locomotor activity was compared between GFP and TeLC males, no differences were found on either body weight change (Figure 4.7A; unpaired t-test, $p > 0.05$), or total velocity and velocity separated by cycle (Figure 4.7B; unpaired t-test, $p > 0.0$; two-way ANOVA, $p > 0.05$). These results show that Prkcd inhibition does not alter home-cage feeding in the same way VIPR2 inhibition in males does, suggesting that the effect we see in the VIPR2 inhibition is specific to this neuronal type. However, we do see an interaction effect from Prkcd inhibition in feeding behavior after food deprivation, in males only. This effect seems similar to the effect we see in VIPR2 females. Even though we do not understand the exact mechanisms that are underlying these interaction effects, it might be an intricate interplay of different factors, including stress, which causes these interaction effects.

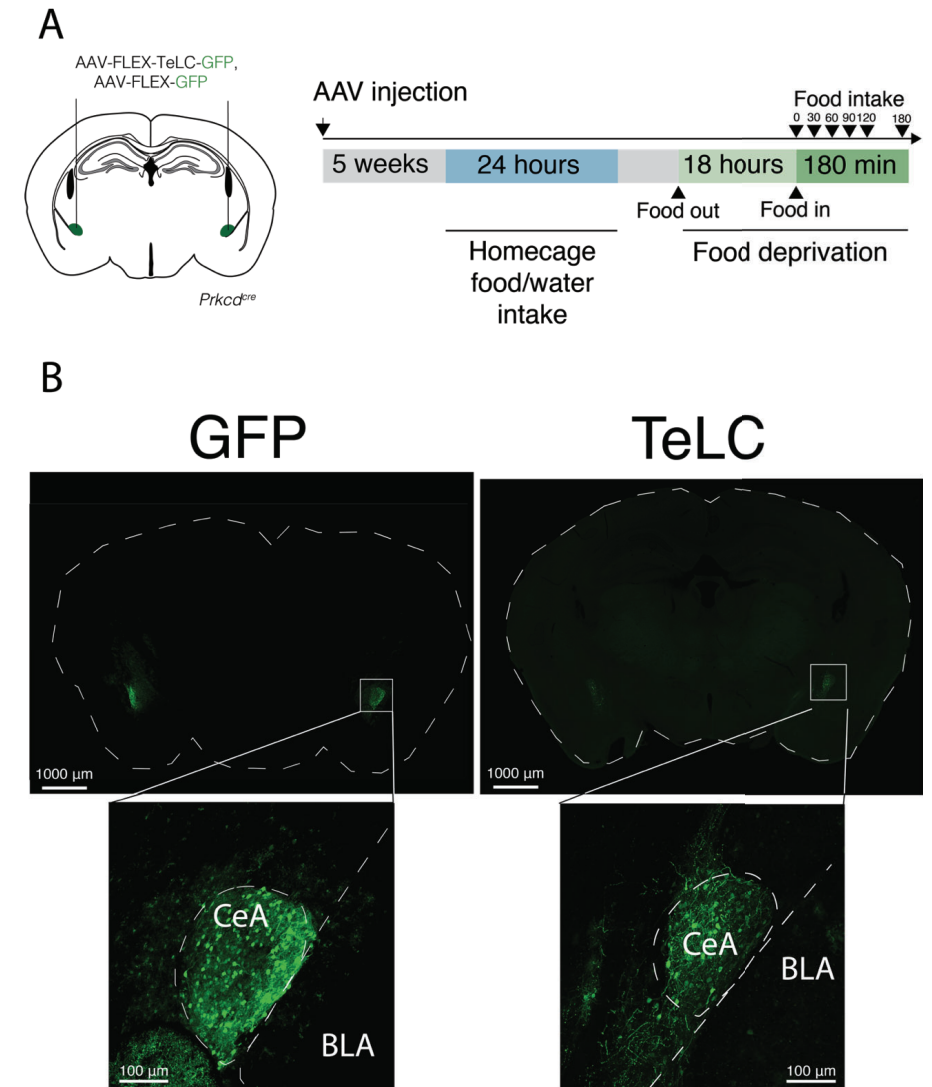


Figure 4.5 Expression of TeLC and GFP virus in Prkcd-expressing CeA neurons. (A) Schematic of experimental methods. (B) Representative histology images of a GFP (left) and TeLC (right) injected animal.

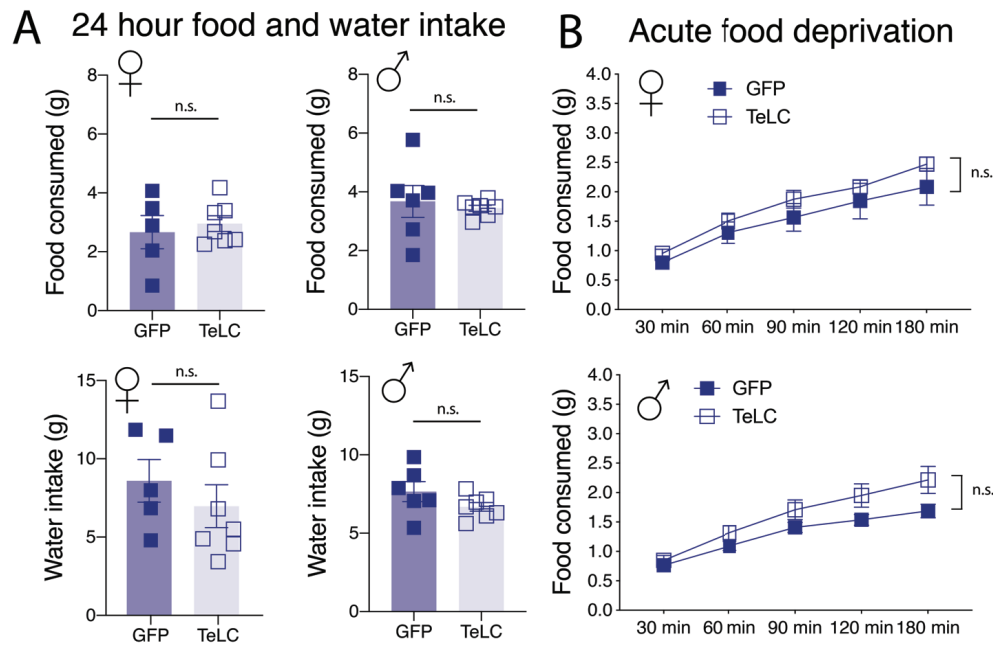


Figure 4.6 Inhibition of prkcd-expressing CeA neurons does not affect home-cage feeding behavior, or feeding behavior after a food deprivation challenge. (A) No differences were found in food or water intake in either males or females, between GFP and TeLC animals (unpaired t-tests, all $p > 0.05$). (B) No differences were found in food intake between GFP and TeLC animals in males or females, after food deprivation (two-way ANOVA, all $p > 0.05$). However, a significant interaction effect was found between GFP and TeLC males of Time x GFP/TeLC (two-way ANOVA, $p = 0.035$). Data represented as mean \pm s.e.m.

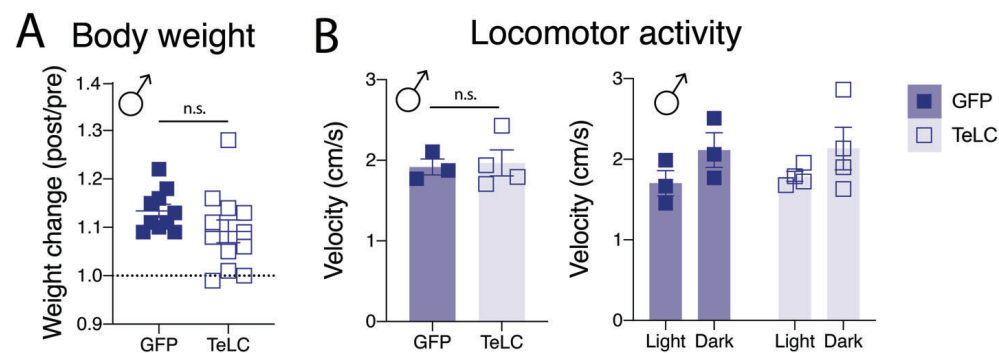


Figure 4.7 Prkcd-expressing CeA neuronal inhibition does not affect body weight or locomotor activity in males. (A) No significant difference was observed between body weight change in GFP and TeLC animals (unpaired t-test, $p > 0.05$). (B) No differences were observed between total velocity, or velocity during dark or light cycles, between GFP and TeLC animals (unpaired t-test, $p > 0.05$; two-way ANOVA, $p > 0.05$). Data represented as mean \pm s.e.m.

4.4 Projection pattern of VIPR2-expressing CeA neurons

To further understand the effect of VIPR2-expressing CeA neuronal inhibition on food intake, we examined the projections of VIPR2-expressing CeA neurons in male animals. VIPR2-IRES-cre animals were injected unilaterally with AAV-FLEX-GFP in the CeA and the brain was scanned for projection patterns (Figure 4.8A). We found a clear projection pattern from VIPR2-expressing CeA neurons to the bed nucleus of the stria terminalis (BNST) and the parabrachial nucleus (PBN) (Figure 4.8B,C). Projections to the lateral part of the PBN were strongest. These CeA to PBN projections could be possible pathways through which feeding signals are transmitted.

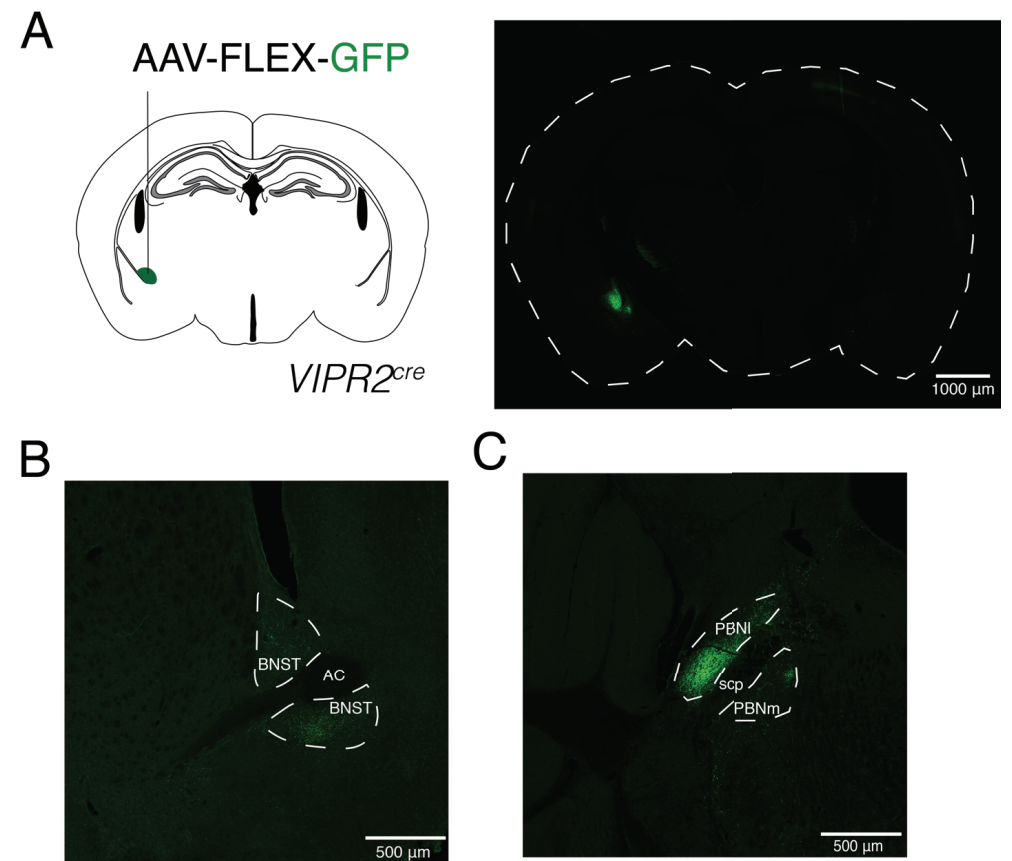


Figure 4.8 Projections from VIPR2-cre neurons to other brain areas. (A) Experimental methods and image of injection site. (B) Projection fibers in the BNST. (C) Projection fibers in the PBN. BNST – bed nucleus of the stria terminalis; AC – anterior commissure; PBNl – lateral part of the parabrachial nucleus; PBNm – medial part of the parabrachial nucleus; scp – superior cerebellar peduncle.

4.5 Discussion

In this chapter we tried to examine the role of a newly identified CeA neuronal population in homeostatic feeding behaviors. We showed that permanent inhibition of VIPR2-expressing CeA neurons increased food intake in males, while having no significant effect on females. These results might indicate that baseline VIPR2 activity could play a role in homeostatic feeding behaviors. Interestingly, the increase in food intake did not affect body weight or locomotor activity in these males.

It has been shown that there are functional interactions between neurons within the CeA, with most of these connections being inhibitory^{16,115,116}. There have been neuronal types within the CeA that, when activated, could promote feeding behaviors, for example the Htr2a-expressing CeA neurons⁴⁹. Possibly, the inhibition of VIPR2-expressing CeA neurons in the current study, caused a disinhibition of these Htr2a-expressing CeA neurons, which lead to an increase in feeding behavior. However, most local connections within the CeA are between neurons of the same subtype, i.e. SST->SST and Prkcd->Prkcd¹¹⁶. This makes it less likely that VIPR2-expressing CeA neurons have inhibitory input onto Htr2a-expressing CeA neurons, although not impossible.

An alternative explanation for our behavioral effect might be found in the projections from the VIPR2-expressing neurons we inhibited. We identified a strong projection from VIPR2-expressing CeA neurons to the lateral PBN. It has been known that the PBN projects towards the CeA and that the activation of this projection reduces food intake¹³. A reciprocal projection from the CeA to the PBN has also been identified, and its role in pain processing has been explored¹¹⁷⁻¹¹⁹. In addition, activation of these CeA neurons could inhibit taste-neurons in the PBN^{120,121}. It has also been shown that this group of CeA neurons has partial overlap with SST-expressing neurons¹²²⁻¹²⁴. Based on these studies and our current results, it might be suggested that the VIPR2-expressing CeA neurons are the population in question. In this study, we permanently inhibited VIPR2-expressing CeA neurons, which disinhibited the input onto PBN neurons, causing a disruption of taste processing. Somehow this disruption in taste processing could have altered the feeding behaviors of these animals. Further research is necessary to elucidate these results and to pinpoint the exact mechanisms involved.

In line with our research, a previous study has shown that infusion of PACAP into the CeA could cause a reduction in feeding and loss of body weight¹¹⁴. Since VIPR2-expressing neurons can respond to PACAP, it is possible that this peptide is involved in the feeding effect we see when inhibiting VIPR neurons. Under normal conditions, PACAP might be necessary to integrate anorexic feeding signals. In our situation, the permanent inhibition of VIPR2-expressing neurons partly removed the ability of PACAP to control food intake, causing the animal to increase feeding. In our study, we do not see an effect on body weight, and the effect on feeding behavior is small. One possible reason for this could be that we did not inhibit all VIPR2-expressing CeA neurons, causing an underestimation of the effect.

Interestingly, we only saw an effect of VIPR2 inhibition on food intake in males. It would be interesting to further examine if there are inherent sex differences in feeding behaviors or in the activity of this neuronal circuit, to explain why we only see an effect of VIPR2 inhibition on food intake in males. Our current study only included males in our projection experiment, but this CeA-PBN projection is not sex-specific¹¹⁸. First of all, there are known sex differences between males and females and their responses to stress¹²⁵, which could be another explanation for a lack of VIPR2 inhibition effects in females. It is possible that the experiment was stressful for the animals since they needed to be single-housed in order to be able to measure their food intake individually. Animals were habituated for one day before the experiment, but it is possible that this caused stress that affected the behavior of the males and females differently. In addition to this, it is known that feeding behaviors differ between males and females, even in humans, with obesity being more prevalent amongst women^{126,127}.

We have shown a role for VIPR2-expressing CeA neurons in feeding behavior in males only in this study. It is of great importance to further examine what the exact mechanisms are that underlie this phenomenon.