Decoding the control of food intake

Insights from the habenula and hypothalamus

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GENERAL DISCUSSION

In this thesis, we investigated how the lateral hypothalamus (LH) and lateral habenula (LHb), which are involved in homeostatic and reward-related feeding behavior, modulate caloric intake, and contribute to the development of diet-induced obesity. Caloric intake is largely driven by energy demand, which is termed homeostatic feeding. In contrast, reward-related feeding can occur in addition to homeostatic feeding and can drive overconsumption of calories. Mechanistic understanding of the brain circuits that promote or inhibit caloric overconsumption is crucial for the prevention and/or treatment of obesity.

Brain circuits involved in homeostatic feeding receive information regarding the energy balance via diverse routes – including but not limited to nutrients, hormones and direct neural information from the vagal nerve [1, 2]. Hypothalamic areas, including arcuate nucleus (ARC), paraventricular nucleus (PVN) but also LH are specialized in integrating this energy balance-related information to regulate caloric intake according to metabolic needs [2, 3]. This homeostatic-like circuitry interacts with dopaminergic circuits from the ventral tegmental area (VTA) and substantia nigra (SN), which drive reward-related feeding by altering the reinforcing value of food and increase motivation for food-seeking behavior beyond homeostatic needs [4-6]. Dopamine signaling is involved in three psychological components of reward processes: liking (hedonics), wanting or incentive salience, and learning via the formation of cue associations [4, 7-10]. While homeostatic and reward-related feeding are often discussed as separate entities, these circuits overlap and continuously interact, and it is very complicated to completely disentangle these circuits and related processes. For example, ventricular nuclei like the ARC and PVN are purely homeostatic regulators of feeding, whereas the VTA and SN (which produce dopamine) and dorsal raphe (DR; which produces serotonin) are monoaminergic nuclei that can modulate feeding, but also have more general functions, including reward arousal, movement, and motivation [11]. There are also intermediate nuclei, including the LH, LHb, and central amygdala (CeA), which have a role in reward in general as well as in feeding behavior specifically, and are also connected with ventricular and monoaminergic nuclei [11]. This uniquely positions them to process food-related signals and integrate these with internal or external inputs, to optimize behavior. For example, specific LH neuronal subpopulations balance competing social and nutritional needs [12]. In this thesis, I have studied the role of glutamatergic and GABAergic populations in the LH (**chapters 3 and 4**), and LHb circuits in rats (**chapters 5, 6 and 7**) and humans **(chapter 8)** related to caloric intake and the development of obesity.

Why did I focus on the LH and the LHb? The LH is a brain region that is composed of heterogeneous cell populations and is well-described for its role in caloric intake, via the integration of homeostatic and reward-related information. The LH receives dopaminergic input from the midbrain, and both direct and indirect feeding-related information via several routes, including nutrients, hormones, and neuronal projections from the brainstem and other hypothalamic nuclei. Upon a positive stimulus, such as a cue predicting a (food) reward, the VTA will release dopamine in many brain regions, including the nucleus accumbens (NAc), which projects to the LH and

stimulates subsequent (reward-driven) feeding (**Figure 1A**). In contrast, the LHb is a small nucleus that can bidirectionally modulate dopaminergic activity, but predominantly inhibits VTA dopamine neurons. Therefore, it has been hypothesized that the LHb is a negative regulator of food intake, as it negatively impacts caloric intake by inhibiting dopamine release from the VTA, thereby decreasing caloric intake (**Figure 1B**). Additionally, we also studied the CeA, which receives dopaminergic input from the VTA, and through its connections with NAc is important for both positive and negative valence encoding (**Figure 1C**).

Glutamatergic and GABAergic LH neurons respond differentially to consumption of a fcHFD

While neurons in the LH express a great variety of neuropeptides and receptors, most neurons can be divided into having either excitatory (glutamatergic, defined by *Vglut*) or inhibitory (GABAergic, defined by *GAD* or *Vgat*) neurotransmission [13]. In this thesis, we showed that glutamatergic and GABAergic neurons in the LH respond differentially to consumption of a free-choice high-fat diet (fcHFD). In **chapter 4**, consumption of a fcHFD for two weeks lowered the overall neuronal activity of GABAergic LH neurons, without altering responses to sucrose

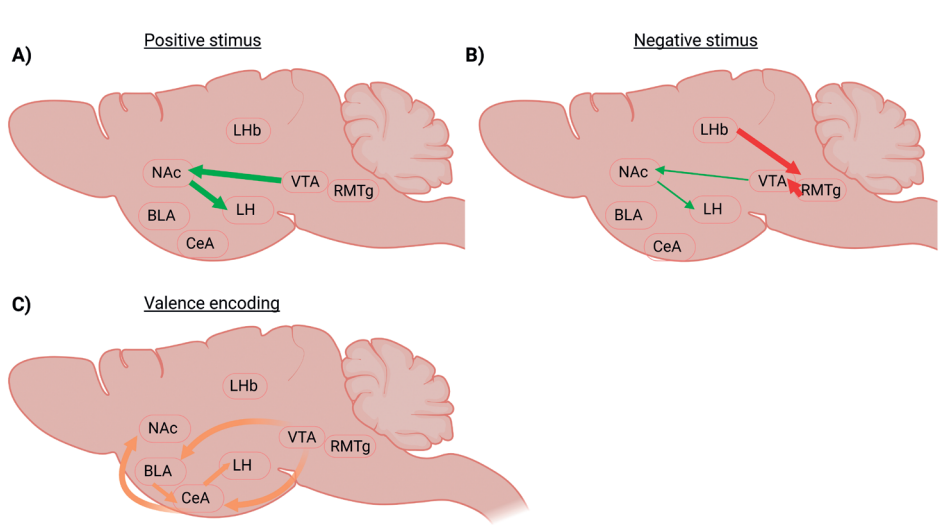

Figure 1. Brain circuitry involved in positive and negative stimuli, and valence encoding. **A)** Upon a positive stimulus (like a reward-predicting cue), the VTA releases dopamine to the NAc, which in turn drives food-seeking behavior through the LH. **B)** The LHb is a negative regulator of food intake and is activated through aversive or negative stimuli. Through complex indirect and direct projections, it predominantly inhibits VTA dopamine, and in turn, reduces NAc-LH-mediated appetitive behaviors. **C)** Both the BLA and Cea receive dopaminergic input from the VTA. The BLA is considered the main input area of the amygdala, while the CeA is considered the main output nucleus. In line, the BLA projects to the CeA and the CeA projects to LH and NAc (among others) to encode both negative and positive valence and drive or reduce appetitive behaviors. Green = positive stimulus, red = negative stimulus, orange = bidirectional valence.

consumption. In contrast, in **chapter 3,** we reported that consumption of a fcHFD disturbed the response of LH^{Vglut} neurons to sucrose consumption, indicating a dysregulated nutrient-sensing ability. In **chapter 3**, to determine if these LH neuron populations were responsive to calories or nutrients, we included sucralose, a low-calorie sweetener, to determine whether the responses were specific to the sweet taste or the post-ingestive effects of sucrose. During consumption of a standard chow diet, of LH^{Vglut} neurons responded similarly to sucralose or water. However, during consumption of a fcHFD, calorie sensing was disturbed, and LH^{Vglut} neurons only responded to sucralose, whereas sucrose and water elicited a similar response. In LH^{Vgat} neurons, our small preliminary dataset showed that the neural response was similar to sucralose or water, and that consumption of a fcHFD did not alter this response (**Figure 2A, B**). However, we were unable to perform additional experiments to verify these findings, therefore the sucralose effects are not included in **chapter 4**. Collectively, these data suggest that LH^{Vgat} neurons specifically respond to sucrose drinking and that this sucrose response is unaltered by consumption of a fcHFD. For LH^{Vglut} neurons, fcHFD consumption disturbs the nutrient-sensing abilities, shifting the response from sucrose to sucralose.

The combined observations from **chapters 3 and 4** suggest that both GABAergic and glutamate LH neurons can distinguish sucrose and sucralose, which have a similar sweet taste but differ in their post-ingestive effects. While both sucrose and sucralose activate sweet taste receptors expressed throughout the gastrointestinal tract and pancreas [14-17], sucrose results in a far greater dopamine release in the brain compared to sucralose [18-22]. This seems to be driven by the cue predicting either sucrose or sucralose, as a sucrose-predicting cue elicits a larger dopamine release than a non-nutritive sweetener-predicting cue [23]. A similar observation has

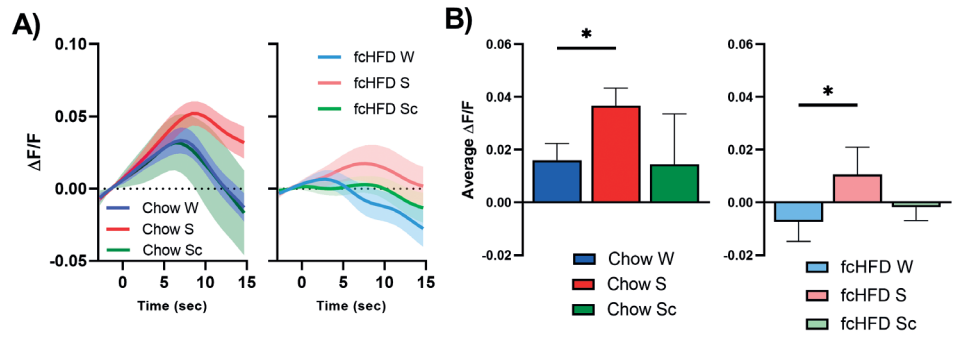

Figure 2. Consumption of a fcHFD lowers LH^{Vgat} activity but does not disturb nutrient sensing. **A)** Change in fluorescence (ΔF/F) over time after delivery of water, sucrose or sucralose at t=0 in chow-fed mice (left) or fcHFD-fed mice (right). **B)** Average ΔF/F after delivery of water (chow W, n = 187 neurons; fcHFD W, n = 221 neurons), sucrose (chow S, 172 n = neurons; fcHFD W, n = 212 neurons) or sucralose (chow Sc, n = 71 neurons; fcHFD Sc, n = 126 neurons). Statistics were performed using a mixed effects model as described in chapters 3 and 4.

been made in humans, where the matching of the sweetness with the calories in the beverage is crucial in determining striatal reactivity [24]. *Pmch*-expressing glutamatergic LH neurons have been implicated in this process, as optogenetic stimulation of this neuronal population during consumption of sucralose increased striatal dopamine, which occurred via connections to the VTA dopamine neurons [25]. In our experiments in **chapters 3 and 4**, a tone was used to signal the start of the trial, which acts as a cue. Therefore, it would be interesting to time-lock the fluorescence signal to the cue instead of the consummatory lick. In addition, future studies should repeat the same experiment in *Pmch*-Cre mice, to assess how much of the effects of the glutamatergic population is driven by *Pmch*-expressing neurons.

While consumption of a fcHFD lowered overall LH^{Vgat} activity, it disturbed the sensing of caloric density in the glutamatergic population. The LH also contains orexin-expressing neurons with glucose-sensing properties [26, 27], which are affected by consumption of a fcHFD [28]. We could hypothesize that *Pmch* and orexin subpopulations are underlying the effects observed in LH glutamatergic neurons. How the nutrient-sensing abilities of these neurons are disturbed when consuming a fcHFD needs to be investigated in more detail. Furthermore, as we observed very inconsistent responding in individual neurons over time (**chapters 3, 4),** it is likely that interactions with other (glutamatergic) subpopulations are also involved, again warranting future investigations. Besides subpopulations, different projection areas could also be important to consider in explaining the inconsistent responses.

In both studies **(chapters 3, 4**), fcHFD induced changes independent of changes in body weight, suggesting that the dietary fat itself drives the observed changes in LH neuronal activity. This can be mediated by direct effects of triglycerides on the LH neurons, or indirectly through hormones or changes in vagal input to the brain. While it is known that triglycerides can cross the blood-brain barrier (BBB)[29], how this could modulate neurons within the LH directly is not fully understood. Glutamatergic LH orexin neurons have been shown to receive increased excitatory neurotransmission already after one day of HFD feeding, thus most likely resulting from the dietary fat itself [28]. During prolonged HFD feeding, but before changes in weight gain, also LH MCH neurons received enhanced excitatory input [28]. These results suggest that glutamatergic LH populations might be affected by dietary fat consumption, however, whether GABAergic LH populations are similarly affected is yet unknown. Direct effects of triglycerides have been observed for dopaminergic neurons in the striatum [30], which could be an upstream target controlling LH neuronal activity. Another route through which triglycerides could affect LH activity is through hormones, such as ghrelin and insulin. Circulating triglycerides can increase the transport of ghrelin into the brain [31], which acts on orexin, but not MCH neurons within the LH [32-34]. Additionally, triglycerides have been associated with changes in central insulin receptors[29, 31, 35], but how this could affect LH neuronal activity is unknown. Finally, the dietary fat from fcHFD feeding might be altering vagal input to the brain [36-43], and could subsequently affect LH neuronal activity[44-47]. In conclusion, there are several pathways

allowing dietary fats to directly modulate LH neuronal activity. However, for most routes, effects on MCH and orexin neurons have been described, and effects on GABAergic or other glutamatergic subpopulations are less known. More research is needed on responses of specific subpopulations, for example, mRNA and gene expression analysis of these neuropeptides after fcHFD feeding.

Balance of glutamate and GABA in the LH

Before the utilization of genetically modified animal models, investigators heavily relied on pharmacological approaches to understand how different receptor-expressing neurons in a brain area influenced behavior. As such, it was demonstrated that the administration of glutamate or a glutamatergic agonist in the LH of satiated, non-stressed rats increased caloric intake [48-51]. This was mediated via NMDA receptors, as an NMDA receptor, but not AMPA/kainate receptor, antagonist inhibited feeding in both food-deprived and satiated animals at the beginning of their normal feeding burst at the beginning of the dark phase [48, 52]. The LH effect on food intake depends on the energy state of the animal, as hungry rats will readily work for self-stimulation [53]. In contrast, satiated rats sometimes avoid LH stimulation [54]. While glutamate in the LH elicits feeding when rats are sated, GABA decreases food intake [55]. For example, a GABA antagonist stimulated caloric intake, whereas a GABA agonist blunted caloric intake [56, 57]. Accordingly, during caloric intake, extracellular GABA levels increase, and eating stops when these extracellular GABA levels peak [58]. This led to the hypothesis that LH GABA drives postprandial satiety [58]. This balance between GABA and glutamate input to the LH might be an important regulator of caloric intake [55]. However, it is currently unknown if simultaneous administration of GABA and glutamate in the LH affects caloric intake, and whether this is a physiologically relevant situation *in vivo*.

While pharmacological studies administered glutamate and GABA separately to different populations of LH neurons, genetically modified animal models provide the opportunity to activate or inhibit such neuron populations directly and specifically. As previously described, pharmacological experiments demonstrated that glutamatergic inputs to the LH drive caloric consumption and GABAergic inputs blunt caloric consumption [55]. Now genetically modified animal models are available to directly target glutamatergic or GABAergic neuron populations to determine their involvement in regulation of caloric consumption. Importantly, GABAergic and glutamatergic LH neurons are largely non-overlapping populations [11, 59]. As glutamatergic LH neurons are important for suppressing caloric intake [59-61], and GABAergic LH neurons for stimulating caloric intake (in non-challenging conditions) [62, 63], it does seem that GABAergic LH neurons receive glutamatergic input and that glutamatergic neurons receive GABAergic input. However, specific in- and outputs have been described to date for very few subpopulations. Next, we will discuss the LH to VTA and NAc to LH projections in more detail.

GABAergic and glutamatergic LH -> VTA pathways and their role in caloric intake

LH to VTA projecting neurons release both glutamate and GABA [64-68]. The LH^{Vglut}->VTA pathway is reported to encode aversion and avoidance behavior [66, 67, 69] [70], and does not respond to feeding hormones [60], while the GABAergic LH->VTA pathway is implicated in feeding regulation in a non-stressed state [66, 67], which is in line with the modulatory role of leptin on this projection [71]. Additionally, optogenetic stimulation drives caloric intake in a frequency-dependent manner[68]. Interestingly, LH^{Vglut} neurons, including those projecting to the VTA, are increasing their activity upon sucrose consumption (**chapter 3**) [60, 70, 72], however, VTA projecting LH^{Vglut} neurons are typically active during unexpected unpleasant stimuli intake [69]. This suggests that LH^{Vglut} neurons blunt caloric intake and are sensitive to internal state and experience [60, 72]. Indeed, we showed in **chapter 3** that short-term consumption of a fcHFD feeding (without inducing body weight gain) can increase LH^{VGlut} activity upon sucrose drinking, whereas others have shown that diet-induced obesity decreased LH^{VGlut} activity upon sucrose drinking [72]. A recent study revealed that the excitability of the LH->VTA projection is sensitive to social stress, and drives stress-induced palatable food intake [73]. Thus, the GABAergic and glutamatergic LH->VTA projections are both involved in regulation of caloric intake; however, the *VGlut* pathway might be more state- and context-dependent. Collectively, combined GABAergic and glutamatergic input from LH->VTA likely modulates dopaminergic output, can be modulated by leptin, and subsequently drives reward-seeking and motivational behavior.

The LH receives GABAergic input from the NAc

Dopamine neurons in the VTA release dopamine in, among others, the NAc, which in turn sends GABAergic projections to the LH [74-76]. For example, the NAc shell (NAcSh) is a major source of GABAergic input to the LH [77]. Optogenetic activation of the NAcSh, and specifically *Drd1*-expressing NAcSh neurons, decreases consummatory behavior [77, 78]. This observation is in line with the effects of infusion of GABA agonists in the LH [79, 80]. Even during a hungry state, activation of NAcSh^{Drd1+} neurons was sufficient to decrease consummatory behavior [77]. Conversely, inhibition of the NAcSh promotes consummatory behavior [81] and increases activity of orexin and MCH neurons within the LH [82]. Thus, GABAergic input from the NAcSh to the LH can bi-directionally modulate consummatory behavior, likely via its effects on LH orexin and MCH neurons. Dopamine is released in the NAc during consumption of food or upon a cue predicting food, which in turn activates NAcSh neurons relative to the amount of consumed palatable food [83]. Subsequently, via the *Drd1*+ LH projection, these neurons could signal satiety and halt further consummatory behavior. While orexin and MCH LH populations are likely involved[82], the involvement of specific subpopulations of LH neurons that are innervated by the NAcSh is not yet known.

Interactions between LH subpopulations: a complex local circuitry

The LH is a heterogeneous brain area with several subsets of neurons that express various neuropeptides, which can overlap and have complex interactions [13, 84](**Figure 3**). For example, somatostatin (*Sst*) and *Nts* neurons are mostly GABAergic [13], although a small subset seems to be glutamatergic neurons [85]. MCH neurons are mostly glutamatergic [13], with a small amount co-expressing GABA [13, 60, 72, 86]. The overall populations of GABAergic and glutamatergic neurons have opposing roles in feeding behavior, as LH^{VGlut} neurons suppress consummatory behavior [61, 62, 72, 87] and LH GABA neurons stimulate consummatory behavior [63, 88], even beyond edible items [89] or when there are negative consequences [66]. Similarly, LH orexin and MCH neurons have opposing roles; glutamatergic orexin neurons increase arousal and motivation [84, 90], likely via their connections with the VP [91, 92], whereas LH MCH neurons promote rest [93]. Of note, while MCH neurons are considered mainly glutamatergic, they do express both glutamatergic and GABAergic markers[13, 60, 94], and a LH MCH subpopulation has been observed releasing either glutamate or GABA. Interestingly, MCH, and likely also orexin LH neurons, can release their neuropeptides directly in the CSF, enabling more brain-wide effects [95]. Orexin activity is affected by *Nts* neurons, as ablation of LH^{Nts} neurons decreases orexin expression [96]. Both orexin and *Nts* LH neurons are also sensitive to leptin [26], although this effect in orexin neurons is likely mediated via *Nts* neurons, because orexin neurons do not express leptin receptors [71, 97].

In conclusion, both GABAergic and glutamatergic neuron populations within the LH play a role in consummatory behavior. Both of these populations respond to sucrose consumption, where LH^{Vglut} neurons are important for processing of the post-ingestive effects of glucose and overall

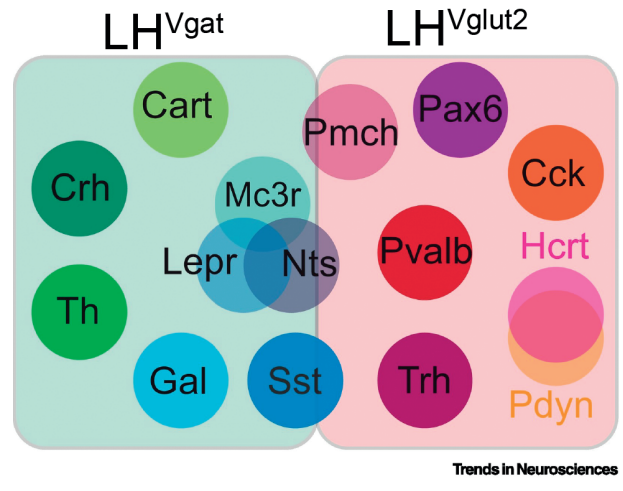

Figure 3. Subpopulations of LH *Vgat* and *Vglut2* expressing neurons. Adapted from Rossi (2023).

LH^{Vgat} neuron activity is reduced by consumption of a fcHFD (**chapters 3 and 4**). LH outputs can promote consummatory behavior by altering incentive salience (motivation), for example by interaction with VTA dopamine neurons by LH orexin neurons [90, 91, 98-101]. Another way of LH outputs to change consummatory behavior, is by altering the hedonics, for instance through projections to hedonic hotspots in the VP [91, 92]. Finally, the LH outputs may assist in learned associations by altering dopamine signaling [66, 67]. Specific subpopulations in the LH might be playing a role in these reward-related processes, and they can show overlap or interactive effects. While GABAergic and glutamatergic neuron populations seem to be mostly non-overlapping within the LH, MCH neurons are known to express both glutamatergic and GABAergic markers, suggesting there might be a balance in GABA and glutamate release, which in turn can promote certain behavioral states. Subpopulations can also counteract each other; therefore bulk experiments targeting multiple subpopulations might not yield clear results. Therefore, it is important to investigate genetically specified subpopulations and their projections in a specific context, as LH activity is both context- and state-dependent. Next to the genetic targeting of specific subpopulations, spatial transcriptomics to spatially define subpopulations might be important to answer several outstanding questions [90].

Fat and sugar interaction in humans

As discussed in **chapter 2**, nutrients such as fat and sugar can interact with each other, resulting in more profound weight gain when consumed simultaneously in the same diet compared to diets containing either fat or sugar. These effects are possibly encoded by the LH. In **chapter 3**, we have shown that glutamatergic LH neurons indeed show an interactive effect between consumption of a fcHFD and sucrose. Functional magnetic resonance imaging (fMRI) in mice revealed that the LH response to a glucose bolus was impaired in animals on a high-fat high-sugar diet (HFHSD) [102]. Fats and carbohydrates are believed to recruit separate gut-brain pathways, which can then interact and have supra-additive effects [103-106]. This is supported by the finding that dietary fat consumption increased subsequent sucrose intake. This can be possibly mediated by NAc opioid signaling [107], as the NAc sends inhibitory projections to the LH. This fat and sugar interaction is also observed in humans, with lean individuals showing increased willingness to pay more for foods with a combination of fat and carbohydrates, than equally liked and energetic foods predominately containing either fat or carbohydrates [103]. This was accompanied by increased activity in the striatum [103]. It is postulated that this supra-additive effect originates from interactions between separate gut-brain circuits processing fat and carbohydrates [105].

Glucose intake is regulated by rapid, post-ingestive signals from the gut to release dopamine, and these signals are most likely mediated via a neural route [108]. Similarly, increases in blood plasma glucose after consumption of a sugar drink in humans correlated with the striatal response to the sight and taste of the drink [109]. Indeed, intragastric infusion of glucose caused significant DA release, and decreased NAc blood-oxygen level dependent (BOLD) response

in lean individuals [110]. In addition, actual, but not estimated, energy density predicted willingness to pay for foods and striatal responses [103, 111]. Thus, the neural representation of these nutrient signals seems to be independent of conscious perceptions. In a similar manner, intragastric infusion of lipids, although differently metabolized, releases dopamine in the striatum [110]. However, this dopamine release happens through a different pathway, via the nervus vagus [112] and depends on peroxisome proliferator–activated receptor α (PPARα) function [113]. In humans, intragastric infusion of both lipids and glucose decreased NAc BOLD response in lean individuals, and increased dopamine release [110]. Recently, van Galen *et al.* (2023) showed that these post-ingestive effects of glucose and lipids were dysregulated in obesity, as individuals with obesity (BMI > 30) did not show changes in NAc BOLD upon lipid or glucose infusion, and no clear DA release after lipid infusion [110]. Thus, in obesity, post-oral nutrient sensing is dysregulated [110], and furthermore, under obese circumstances there is no supra-additive effect of combining fat and carbohydrates, as has been observed in lean individuals [106].

Although many human feeding-related fMRI studies focus on the striatum, a few focus on other brain regions. For example, consumption of a glucose solution lowered the BOLD response in the LH after an overnight fast [114]. Furthermore, glucose-induced hypothalamic activation as measured with arterial spin labeling (ASL) was associated with sweet taste preference, and with longitudinal increases in BMI [115]. However, to date, no experiments have been performed to investigate if hypothalamic activity in response to glucose or lipids is dysregulated during obesity or consumption of a HFD. In order to translate the findings from preclinical rodent LH studies to the human LH, it is important to focus on the LH, and other sub-regions, instead of the hypothalamus as a whole in human imaging. Imaging of hypothalamic areas in humans can be challenging due to its small size, and its proximity to CSF and blood vessels, structures that can create artifacts in the signal. While identifying the hypothalamic subnuclei is still difficult, using high-resolution 7T protocols, some anatomical boundaries can be used to create subregions [116]. And, unfortunately, it is not possible to disentangle GABAergic and glutamatergic circuits, which are known to have opposing roles in consummatory behavior in the LH, with the current available (f)MRI techniques.

Glutamatergic transmission onto the LHb has minor effects on food intake

Similarly to the LH, the habenula, which consists of a medial and a lateral part, is highly connected to the VTA and influences dopamine release. The lateral habenula (LHb) is an important regulator of VTA dopamine neurons and responds predominantly to negative stimuli (**Figure 1B**). Historically, this response has been named a negative reward prediction error, reported in primate and human habenula neurons [117, 118]. This negative reward prediction error is most likely encoded by input from the endopeduncular nucleus (EPN) to the LHb [119,

120]. The relatively small LHb receives input from many brain regions, including the LH [61, 121, 122], basal forebrain [123], ventral pallidum [124, 125], (medial) prefrontal cortex (mPFC) [126], amygdala [127, 128] and reciprocal connections from VTA and DR/MnR [129-131]. Most of these projections are glutamatergic in nature, although some have been reported to co-release GABA and glutamate [119, 120]. Our hypothesis was that the LHb, as a negative regulator of reward processing, could very well be a negative regulator of consummatory behavior as well. Remarkably, the overall activity of the LHb seems to be decreased during consummatory behavior [132]. However, pharmacological inhibition of all glutamatergic inputs to the LHb did not acutely change consummatory behavior, either when consuming a standard diet or palatable food items (fat/sugar)**(chapter 5)**. Following infusion of the NMDA receptor antagonist AP5, fat intake was decreased at later time points, independently of whether the animals also had sugar available (fcHFHS/fcHF). The AMPA/kainate receptor antagonist CNQX did not have this effect. Taken together, this suggests that overall glutamatergic inhibition of the LHb does not have an acute effect on consummatory behavior, but it might have a delayed, modulatory effect on fat consumption. It could well be that the role of LHb in consummatory behavior is more dependent on specific inputs, or specific emotional or satiation states. Indeed, one of the early reports implicating the LHb in feeding regulation, focused on the glutamatergic input from the LH [61].

LH glutamatergic input to the LHb in relation to feeding

The LH projects extensively to the LHb, which could be the main input that modulates tonic inhibition of VTA dopamine release [133-135]. Indeed, these LH inputs are glutamatergic and project to the LHb neurons which subsequently project to the VTA/RMTg neurons [135] that have been observed to synapse predominantly on mPFC-projecting VTA neurons [136]. This suggests that a LH -> LHb -> VTA/RMTg -> mPFC circuit is involved in encoding aversion [135, 136]. Stress is an aversive stimulus, and social defeat stress has indeed been reported to activate both the LH and LHb [137]. In the LH, orexin neurons were predominantly activated and infusion of an orexin agonist into the LHb improved depressive-like behaviors induced by social stress [137]. Of note, the glutamatergic LH orexin neuronal connection to the LHb has also been implicated in the actions of anesthetics [138, 139]. Regarding consummatory behavior, glutamatergic LH to LHb input can negatively regulate palatable feeding [60, 61]. While the net effect of LH->LHb is excitatory, this input might also have a minor GABAergic component [61]. Interestingly, unpublished work from the Kenny lab suggests that the LH -> LHb circuit has opposing actions on chow (regular food) *versus* palatable (cheesecake) food intake in mice [132]. Furthermore, LHb neuronal activity decreased more pronounced during consumption of palatable foods than during chow, and this effect is exaggerated in obese mice compared to lean mice [132]. General LHb activity, but also LH->LHb projection activity, is energy state-dependent, with reduced activity during sated states [60, 132]. This is in line with increased LHb activity during stress, when we consider fasting to be stressful. These findings suggest a role for the LH ->LHb projection in regulation of consummatory behavior, which is

supported by the notion that this pathway is sensitive to leptin and ghrelin, which have opposing effects on activity of the LH -> LHb projection [60]. In conclusion, the glutamatergic LH -> LHb pathway seems to encode aversion, is sensitive to stress, satiety states, and feeding hormones, and is negatively impacted by consumption of palatable foods.

Central amygdala input to the LHb and consummatory behavior

As discussed above, the LHb receives a wide variety of input to integrate and modulate various signals, behaviors, and motivational states. Because the LHb mediates emotional states and aversion, input from the CeA likely plays a role in these processes. Indeed, a glutamatergic, somatostatin expressing, CeA->LHb projection was shown to be involved in pain and depression-like behaviors [127]. Furthermore, a GABAergic, NPY receptor 1 (*Npy1r*) or corticotropin-releasing factor (*Crf*)-expressing CeA -> LHb projection was shown to be involved in binge-like consumption of ethanol, but not sucrose [128, 140]. In **chapter 6**, we demonstrated that a predominantly glutamatergic *Drd1*-expressing CeA->LHb pathway is involved in satiety-state-dependent regulation of caloric intake. In contrast to the inhibiting effects of the LH->LHb projection on caloric intake, we observed that chemogenetic activation of this CeA$^{Drd1>}$->LHb projection in sated animals specifically increased fat consumption. Similarly, Ip *et al.* (2023) have recently demonstrated that chemogenetic inhibition of *Npy1r*-expressing neurons in the LHb, which receive GABAergic input from CeA, increased consumption of a palatable diet. Furthermore, this pathway was activated during consumption of a HFD, and this effect was abolished when combined with stress [141]. Collectively, these observations indicate that CeA glutamatergic (*Drd1*) and GABAergic (*Npy1r*) inputs have opposing roles in consummatory behavior compared to the LH->LHb projections. Additional studies are necessary to determine whether these opposite effects result from distinct projection routes onto LHb neuron (sub)populations.

Co-release of GABA and glutamate

While overall glutamatergic input to the LHb does not seem to have a significant effect on consummatory behavior **(chapter 5)**, specific inputs might play a role in consummatory behavior. We previously discussed LH and CeA inputs and their involvement in feeding, with a focus on CeA^{Drd1+} input in **chapter 6.** There are probably other relevant inputs, although knowledge on this topic has been limited to date. For example, a glutamatergic input from the basal forebrain has been described to decrease chow intake during a hungry state [142]. Activation of this basal forebrain->LHb projection drives aversive behaviors, thereby limiting appetitive behavior (feeding) without disturbing appetite [142]. At least for the LH and CeA^{Drd1+} input, electrophysiological observations in the LHb revealed that there are both GABAergic and glutamatergic components present **(chapter 6)** [61]. Co-release of GABA and glutamate

has been reported in the VTA [143] as well as in EPN input to the LHb [144]. Thus, both LH and CeA^{Drd1+} inputs contain GABAergic and glutamatergic components, however whether these inputs show GABAergic and glutamatergic co-release would be an interesting future direction. Such a GABA/glutamate balance within a synapse could be an effective way of regulating LHb activity, important for reward-seeking behaviors [120, 121, 144, 145].

Translational aspects

To curb the current obesity pandemic, the replication of fundamental findings in humans can help design new treatments that are desperately needed in the clinical setting. Despite the small size of the habenula, recent improvements in imaging techniques have facilitated studies that have investigated habenula function in humans. In line with its role in pain and mood disorders, similarly as in rodents, increased habenula activity was observed in humans with depression [146-149]. Successful pilots utilizing deep brain stimulation suggest a causal role between habenula hyperactivity and mood disorders [150, 151]. In relation to consummatory behavior, habenula functional connectivity (FC), as measured with fMRI, was associated with weight loss [152-154]. In **chapter 7**, we demonstrated that in individuals with a healthy long-term glucose level (HbA1c), resting state FC (rsFC) of the habenula with reward-related regions of interest (ROI), did not correlate with BMI. However, without correction for multiple comparisons, exploratory analyses suggested that in people with prediabetes, rsFC between the habenula and VTA negatively correlated with BMI, whereas it correlated positively in individuals with a healthy HbA1c (**chapter 7**). This is in line with a possible role of the habenula in glucose regulation.

For this chapter, we used data from the Human Connectome Project, which is of outstanding quality [155]. That we failed to find a statistically significant correlation could be due to BMI being not the most accurate measure to assess obesity. Another reason could be that the required sample size to reveal cross-sectional effects between phenotype and rsFC is much higher than what was available. A recent report demonstrated that sample sizes in the thousands (>10.000) are required to reveal meaningful correlations between rsFC and phenotype in cross-sectional designs[156]. This stresses the importance of large-scale consortia to reveal such correlations, which are relevant for the population and therefore can aid societal policymakers. Alternatively, focused studies, with a smaller sample size, maximized signal and minimal noise are important in answering individual, specific questions within people to aid clinical care[157]. The proper study design can improve statistics power, for example using an intervention in a within-subject design. Indeed, several studies that studied habenula FC in relation to weight loss used bariatric surgery as a weight-loss intervention, which increases the power to find significant results [153, 154] and might thus be a more reliable way than aiming for rsFC-phenotype associations. Finally, due to possible opposing roles of specific inputs to the LHb, without this input-specificity, it might be challenging to find correlations between habenular activity and phenotypes. This also includes the lack of ability to distinguish the medial and lateral habenula using current human

fMRI protocols; the ability to separate these areas would further improve our studies, as these divisions of the habenula are known to have differential functions.

Concluding remarks

In this chapter, I have discussed the role of the LH and LHb, two intermediate nuclei that integrate various inputs to guide reward-related behaviors, including consummatory behavior. Both are sensitive to previous experiences, including stressors, and satiety-related signals. The LH can be differentially affected by fat and sugar intake, with glutamatergic neurons responding preferentially to post-ingestive effects of sucrose intake, and GABAergic neurons responding preferentially to dietary fats. The balance between GABA and glutamate might be important for the regulation of consummatory behavior, a phenomenon also observed in the LHb. Various LHb inputs can release both GABA and glutamate and might use this balance to regulate habenular activity and guide (reward-related) behavior. While fundamental and translational studies are crucial to advance our mechanistic insights regarding obesity etiology, human fMRI studies are necessary to implement these findings in a clinical setting. However, imaging studies to the LH and LHb are hindered by the lack of input specificity, and the need for immense sample sizes to reliably determine rsFc-phenotype interactions. It is key to develop an optimal design, for example by performing within-subject comparisons, or by sharing and combining several datasets to achieve the best statistical power. In conclusion, consumption of a HFD affects both glutamatergic and GABA LH neuron populations. Specific inputs to the LHb, including the CeA, modulate consummatory behavior in a satiety-state dependent manner. These studies contribute to a better knowledge of the etiology of obesity and eventually will aid in development of treatment options.

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