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Feeding state-dependent neuropeptidergic modulation of a sensorimotor circuit biases non-feeding related decisions in *Drosophila* larva

La modulation neuropeptidergique d'un circuit sensorimoteur en fonction de l'état alimentaire biaise les décisions non liées à l'alimentation chez la larve de Drosophile

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ÉCOLE DOCTORALE

Signalisations et réseaux
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(BIOSIGNE)



Titre : La modulation neuropeptidergique d'un circuit sensorimoteur en fonction de l'état alimentaire biaise les décisions non liées à l'alimentation chez la larve de *Drosophila*

Mots clés : Larve de *Drosophila*, Décisions sensorimotrices, Neuropeptides, Circuits neuronaux, Etat interne, Neuromodulation

Résumé : La flexibilité comportementale, soit l'adaptation du comportement aux changements de l'environnement ou de l'état interne, est essentielle pour la survie et la reproduction des animaux. L'état alimentaire, en particulier, exerce une forte influence sur le comportement, même en dehors des décisions liées à la nourriture. Cependant, la manière dont l'information sur l'état alimentaire affecte les circuits de décision non directement liés à l'alimentation reste un sujet de recherche en neurosciences. Notre projet vise à éclairer ces relations entre états physiologiques, circuits neuronaux et comportement, cruciales pour comprendre le fonctionnement cérébral en santé et en maladie.

Nous étudions les mécanismes de modulation des circuits neuronaux dépendant de l'état alimentaire chez les larves de *Drosophila*. Nous utilisons les outils génétiques de ce modèle et son système nerveux simple pour examiner un circuit impliqué dans leur prise de décision sensorimotrice. Ce circuit orchestre le choix entre deux actions mutuellement exclusives (la rétraction de la tête, ou "hunch", une réponse de sursaut et le balancement de la tête, ou "head-cast", une réponse d'évitement actif) en réponse à une stimulation mécanique. Des interneurons inhibiteurs réciproquement connectés engagés dans plusieurs sous-motifs pilotent l'état de sortie de ce circuit, avec une inhibition réciproque de l'inhibition mettant en œuvre le choix comportemental et une désinhibition rétroactive consolidant le "head-cast". Une telle architecture de circuit permet une sélection flexible du comportement de sortie, qui est probabiliste à la fois entre et au sein des individus. Les larves affamées ou nourries uniquement de sucrose orientent leurs décisions vers plus de "head-cast" au détriment du "hunch" en modulant l'activité de deux interneurons inhibiteurs interconnectés.

Pour étudier ce mécanisme, nous combinons des techniques telles que la manipulation de neuropeptides dans des cellules individuelles, l'imagerie calcique, l'analyse comportementale et la connectomique. Nous avons ciblé les neuropeptides NPF et sNPF, liés au NPY mammifère et connus comme régulateurs des comportements alimentaires. Alors que les récepteurs des deux neuropeptides sont exprimés dans les neurones mécanosensoriels, nos résultats montrent que leur modulation n'est pas essentielle pour orienter les décisions de la larve. Au contraire, sNPF et NPF modulent différemment les interneurons inhibiteurs interconnectés de manière dépendante de l'état alimentaire, influençant les calculs du circuit. Nous montrons que les neurones de projection du circuit n'expriment pas de récepteurs pour sNPF ou NPF, tandis que les interneurons inhibiteurs expriment différentes combinaisons de récepteurs. Cela suggère que les interneurons sont des cibles de modulation tandis que les neurones de projection transmettent la décision au côté moteur. En effet, la réduction de la signalisation sNPF sur ces interneurons oriente le choix des larves vers moins de "hunch" et plus de "head-cast" en régulant leur activité de manière opposée, reproduisant partiellement un phénotype "sucrose-like" ou "starvation-like". D'autre part, l'activité des neurones NPF est augmentée lors de l'ingestion de sucrose et la mise à jeun, et le NPF lui-même est nécessaire pour la modulation dépendante de l'état alimentaire de la locomotion de base des larves. De plus, le NPF est responsable d'une augmentation de l'activité spécifiquement de l'un des interneurons, qui inhibe le "hunch" et favorise le "head-cast".

En conclusion, nos données suggèrent que ces interneurons inhibiteurs servent d'intégrateurs de l'information sur l'état physiologique, orientant ainsi le comportement en fonction de l'état alimentaire. Ces résultats posent les bases pour comprendre les mécanismes de neuromodulation dans des cerveaux complexes, avec des implications potentielles pour le traitement des troubles neuropsychiatriques, dont les troubles alimentaires.

Title : Feeding state-dependent neuropeptidergic modulation of a sensorimotor circuit biases non-feeding related decisions in *Drosophila* larva

Keywords : *Drosophila* larva, Sensorimotor decisions, Neuropeptides, Neural circuits, Internal state, Neuromodulation

Abstract : Behavioral flexibility, the capacity to adapt behavior in response to changes in the environment or internal state, is a fundamental aspect of animal behavior, essential for survival and reproductive success. Notably, an animal's feeding state strongly influences its behavior, even beyond feeding-related choices and impact even decisions unrelated to feeding. While feeding state-dependent modulation of feeding related behaviors have been extensively studied, how feeding state information modulates decision circuits not directly tied to feeding behaviors is an ongoing question in neuroscience. This project aims to shed light on the intricate relationships between physiological states, neural circuits, and behavior, crucial for comprehending brain function in health and disease.

We delve into the mechanisms of feeding-state dependent neural circuit modulation in *Drosophila* larvae, leveraging this model organism's genetic tools and relatively simple nervous system to scrutinize the mechanisms of modulation of a neural circuit involved in larval sensorimotor decision-making. Specifically, this circuit orchestrates the choice between two mutually exclusive actions (hunching, a startle response and head-casting, an active avoidance response) in response to an aversive mechanical stimulation. Early in sensory processing, reciprocally connected inhibitory interneurons engaged in several sub-motifs drive the output state of this circuit, with reciprocal inhibition of inhibition implementing behavioral choice and feedback disinhibition providing positive feedback to consolidate the head-cast. Such circuit architecture allows for a flexible selection of the output behavior, which is probabilistic both between and within individuals. Starving larvae or feeding them only on sucrose, biases their decisions towards more head-casting at the expense of hunching by modulating the activity of two interconnected inhibitory interneurons.

In order to study the mechanisms of modulation of this circuit, we employ a combination of techniques including cell-specific neuropeptide manipulations, calcium imaging, behavioral analysis and connectomics. We focused on the neuropeptides NPF and sNPF, related to the mammalian NPY and known regulators of feeding behaviors. While both neuropeptides' receptors are expressed in mechanosensory neurons, our findings show their modulation is not essential to bias larva decisions to an air puff. Instead, sNPF and NPF differentially modulate interconnected inhibitory interneurons in a feeding state-dependent manner, influencing circuit computations. We show that the projection neurons of the circuit do not express receptors for either sNPF or NPF, while the inhibitory interneurons express different combinations of receptors. This suggests that the interneurons are targets of modulation while the projection neurons relay the decision to the motor side. Indeed, reduction of sNPF signaling on these interneurons biases the choice of the larvae towards less hunching and more head-casting by oppositely regulating their activity, partially recapitulating a "sucrose-like" or "starvation-like" phenotype. Furthermore, NPF-neurons activity is increased upon sucrose feeding and starvation, and that NPF itself is necessary for the feeding state-dependent modulation of larval baseline locomotion. Moreover, NPF is responsible for an increase in the activity of specifically one of the interneurons, which suppresses hunches and promotes head-casts.

In conclusion, our data propose that this pair of interconnected inhibitory interneurons serves as an integration hub for physiological state information conveyed by neuropeptides, thus biasing behavioral choice based on the feeding state. These insights lay the groundwork for understanding state-dependent neuromodulation mechanisms in more complex brains, specifically those that hold potential implications for treating neuropsychiatric disorders, including eating disorders.

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Table of contents

Remerciements	7
Table of contents.....	Erreur ! Signet non défini.
Abbreviations	10
I. Introduction.....	11
I. A. Neural circuits for decision-making.....	12
I. A. 1. Connectivity Motifs in Neural Circuits	14
I. A. 2. Examples of circuit motifs in decision-making circuits	15
I. B. Encoding behavioral flexibility through neural circuits modulation	16
I. B. 1. Gene expression dependent circuit adaptation	17
I. B. 2. Cellular mechanisms of circuit modulation	19
I. C. Feeding state modulation of behaviors	28
I. C. 1. Modulation of peripheral sensory neurons	30
I. C. 2. Modulation of central neurons in circuits.....	31
I. C. 3. Neuropeptides involved in feeding state-dependent modulation of neural circuits in <i>Drosophila</i>	32
I. D. <i>Drosophila</i> larva model system for the study of neural circuit modulation ..	36
I. D. 1. Brain simplicity.....	36
I. D. 2. Genetic tools.....	38
I. D. 3. Neuron manipulation and recording	40
I. D. 4. Behavioral assays.....	43
I. D. 5. Relevance to other species	45
I. E. Description of the sensorimotor decision circuit.....	45
II. Aims.....	48
III. Methods and Results.....	51
III. A. Feeding stat-dependent neuropeptidergic modulation of reciprocally interconnected inhibitory neurons biases sensorimotor decisions in <i>Drosophila</i>	53
III. A. 1. Article summary.....	53
III. A. 2. Article manuscript.....	54

III. B. Effects of neuropeptides on mechanosensory neurons	125
III. B. 1. Expression of sNPF and NPF receptors on mechanosensory chordotonal neurons	125
III. B. 2. sNPF effect on mechanosensory chordotonal neurons	127
III. C. Decision-making in conflicting sensory gradients	131
III. C. 1. Methods.....	132
III. C. 2. Results	136
IV. Discussion.....	143
IV. A. Neuropeptidergic action on the mechanosensory chordotonal neurons	145
IV. B. Modulation of sensorimotor decisions by NPF.....	148
IV. C. Opposite effects of sNPF on Griddle-2 and Handle-B.....	150
IV. D. Origin of sNPF state-dependent action on the circuit.....	151
IV. E. Difficulties of internal states characterization.....	152
IV. F. Studying decision-making in conflicting sensory gradients in <i>Drosophila</i> larva	154
IV. G. Relevance of the feeding state-dependent modulation of non-feeding-dependent behaviors and its study	155
V. References	157
VI. Synthèse en Français	194

Abbreviations

LTP: Long-term potentiation

LTD: Long-term depression

GPCR: G-protein coupled receptor

NO: Nitric oxide

CO: Carbon monoxide

CREB: cAMP response element-binding protein

CRE: cAMP response element

BDNF: Brain-derived neurotrophic factor

NPY: Neuropeptide Y

IPC: Insulin-producing cells

DM-NPF: Dorsomedial pair of NPF-releasing neurons

DL-NPF: Dorsolateral pair of NPF-releasing neurons

SEZ: Subesophageal zone

APC: Adipokinetic hormone-producing cells

AKH: Adipokinetic hormone

CCK: Cholecystokinin

PDF: Pigment dispersing factor

C. elegans:
Caenorhabditis elegans

CNS: Central nervous system

mAChRs: Muscarinic acetylcholine receptors

fMRI: Functional magnetic resonance imaging

EM: Electron microscopy

L1: First instar

L3: Third instar

VNC: Ventral nerve cord

DBD: DNA-binding domain

AD: Activation domain

GECIs: Genetically encoded calcium indicators

GEVIs: Genetically encoded voltage indicators

GFP: Green fluorescent protein

PNs: Projection neurons

AgRP: Agouti-related protein

POMC: Pro-opiomelanocortin

CART: Cocaine- and amphetamine-regulated transcript

ILP: Insulin-like peptide

sNPF: short Neuropeptide F

NPF: Neuropeptide F

sNPFR: sNPF-receptor

NPFR: NPF-receptor

NPYR: NPY-receptor

Lk: Leukokinin

Crz: Corazonin

DP7: Dorsal pair of ILP7 producing neurons

I. Introduction

On a day-to-day basis, animals are faced with constant changes in their environment that require a behavioral adaptation so as to fulfill the animal's biological needs while avoiding dangers (Wong and Candolin 2015). This adaptation requires that the animal's nervous system integrates information about the environment, which is continuously sampled using different sensory modalities at the disposal of the animal, with both its immediate physiological state and its past experiences (Fu, Horng, and Chou 2022). Numerous parameters can influence the internal state of an animal such as hunger, stress, weariness, age, etc (Devineni and Scaplen 2022). The balance between the environment and the internal state is very important for the selection of behaviors that ensure the best chances of survival (Jiang and Pan 2022). For instance, feeding is essential for survival and an animal may take more risks to feed when it is hungry, especially if it has not been able to eat in several days (Herberholz and Marquart 2012). However, if an immediate threat arises, such as a predator attack, while feeding, the animal must be able to decide to abandon this food source and escape to save its life (Herberholz and Marquart 2012). This context-dependent behavioral expression requires flexibility in neural circuits involved in selecting and triggering behaviors so their activity can be modulated to timely adapt their output to new conditions (Devineni and Scaplen 2022; Jiang and Pan 2022; Fu, Horng, and Chou 2022).

I. A. Neural circuits for decision-making

Decision-making in animals, from a biological perspective, refers to the process by which organisms evaluate available options based on sensory inputs, internal states, and past experiences to select and execute appropriate behavioral responses (Budaev et al. 2019; Adams et al. 2012; Jovanic 2020; Barron et al. 2015; Brembs 2014; Kristan 2008). This cognitive process involves the integration of sensory information, such as visual, auditory, olfactory, and tactile cues, with internal physiological states, including hunger, thirst, and reproductive needs (Bargmann 2012; Marder 2012; Bargmann and Marder 2013). Decision-making often entails weighing potential risks and benefits associated with different choices, considering environmental factors and past outcomes (Budaev et al. 2019; Adams et al. 2012; Jovanic 2020; Barron et al. 2015; Brembs 2014; Kristan 2008). Such decisions tend to facilitate the selection of behaviors that optimize survival, reproduction, and adaptation to changing environmental conditions (Budaev et al. 2019; Adams et al. 2012; Jovanic 2020; Barron et al. 2015; Brembs 2014; Kristan 2008). For instance, hunger may enhance sensitivity to food-related cues, prompting increased foraging behavior to satisfy metabolic demands (Bargmann 2012; Slankster et al. 2020; Root et al. 2011; Ko et al. 2015; Chao et al. 2004; Ezcurra et al. 2011; Chalasani et al. 2010; Inagaki, Panse, and Anderson 2014; Wosniack et al. 2022).

At the level of neural circuits, decisions have been thought to be implemented through circuit architecture capable of “winner-take-all” computations that orchestrate the selection between competing behaviors (Kristan 2008; Mysore and Kothari 2020). The activity of individual neurons can be closely correlated to the exact moment when a decision is made (Kristan 2008; Cui et al. 2013; M. R. Cohen and Newsome 2004). However, growing evidence suggest that decisions depend on the covariance of several neurons distributed throughout all levels of sensorimotor neural circuits, instead of the activity of single neurons in specialized circuits (Kristan 2008; Briggman, Abarbanel, and Kristan 2005; Cisek and Kalaska 2010; Cisek 2012).

In parallel, internal states such as hunger, thirst, and reproductive needs induce the release of a multitude of signals that can influence sensory processing regions to modulate the sensitivity of sensory pathways and bias decision-making processes based on the organism's current physiological needs (Sayin et al. 2018; Vogt et al. 2021; Münch et al. 2020).

Furthermore, past experiences and learned associations also play a critical role in decision-making, shaping neural circuits through synaptic plasticity mechanisms such as long-term potentiation and long-term depression (Leslie and Nedivi 2011; Brini et al. 2014; Bading 2013; Destexhe and Marder 2004; Jovanic 2020). Neural circuits involved in decision-making exhibit dynamic changes in synaptic strength and connectivity in response to learning and memory processes, allowing animals to adapt their behavior based on previous outcomes and experiences (Leslie and Nedivi 2011; Brini et al. 2014; Bading 2013).

Ultimately, the integration of sensory information, internal states, and past experiences occurs at the level of complex patterns of connectivity, such as mutual inhibition of feedforward inhibition, and activity dynamics within neural circuits (Liquan Luo 2021; Koyama and Pujala 2018; Mysore and Kothari 2020; Jovanic 2020; Grunwald Kadow 2019). Such motifs, described in the following section of this manuscript and at the core of the neural circuit studied in the article in section III.A and described in section I.E, provide the framework for flexible information processing, allowing neural circuits to generate diverse behavioral responses from a limited set of inputs (Jovanic et al. 2016; Herberholz and Marquart 2012; Koyama et al. 2016; Gaudry and Kristan 2009; Palmer and Kristan 2011). This dynamic interplay between sensory, physiological, and cognitive factors enables animals to adaptively navigate their surroundings, optimizing behavior to maximize survival and reproductive success in diverse environmental contexts.

I. A. 1. Connectivity Motifs in Neural Circuits

The concept of connectivity motifs in neural circuits refers to recurring patterns of synaptic connections among neurons that serve as fundamental building blocks for computations within the brain (Mysore and Kothari 2020; Koyama and Pujala 2018; Liqun Luo 2021). These motifs, characterized by specific arrangements of synaptic connections, play a crucial role in shaping the functional properties of neural circuits and their ability to process information.

Connectivity motifs provide a framework for understanding how neural circuits perform computations and generate behavioral responses. By identifying common patterns of connectivity, researchers can dissect the underlying principles governing information processing in the brain (Mysore and Kothari 2020; Koyama and Pujala 2018; Liqun Luo 2021). These motifs serve as architectural templates that govern the flow of neural activity and determine the emergence of complex behaviors.

One of the key roles of connectivity motifs is to enable specific types of computations within neural circuits. For example, feedforward motifs, where signals propagate from sensory neurons to higher-order processing regions, are well-suited for feature detection and sensory integration (Liqun Luo 2021; Mysore and Kothari 2020). Conversely, feedback motifs, where signals loop back from higher-order regions to lower-level processing areas, facilitate context-dependent modulation and feedback control (Liqun Luo 2021; Mysore and Kothari 2020).

A well-known example of circuit motifs is lateral inhibition, which is involved in the inhibition of neighboring neurons by an activated neuron (Liqun Luo 2021). Commonly observed in sensory processing circuits, lateral inhibition sharpens sensory responses and enhances contrast in neural representations. For instance, in the visual system, lateral inhibition highlights edges and enhances contrast sensitivity by suppressing the activity of adjacent photoreceptors (Kuffler 1953; Liqun Luo 2021). This selective amplification of relevant sensory cues and suppression of irrelevant information improves spatial resolution and discrimination capabilities, facilitating the extraction of pertinent sensory details and the generation of contextually appropriate responses.

Overall, connectivity motifs serve as the building blocks for computations within neural circuits by shaping the flow of information and organizing neural activity patterns.

I. A. 2. Examples of circuit motifs in decision-making circuits

Inhibition has been proposed to implement selection in various circuits across species (Kovac and Davis 1977; 1980; Gaudry and Kristan 2009; 2012). Theoretical work on "winner-take-all" models capable of selecting one option and suppressing the competing ones proposed different network organizations involving different combinations of recurrent connectivity motifs with either reciprocal or lateral inhibitory connections, capable of achieve this computation.

Over recent years, the circuit motif of mutual inhibition of inhibition (Figure I.A-1) has been shown in multiple species and brain areas to perform similar computations that involve competitive selection (Koyama and Pujala 2018; Jovanic et al. 2016; Koyama et al. 2016; Mysore and Kothari 2020).

Schematic of an example motif of mutual inhibition of inhibition

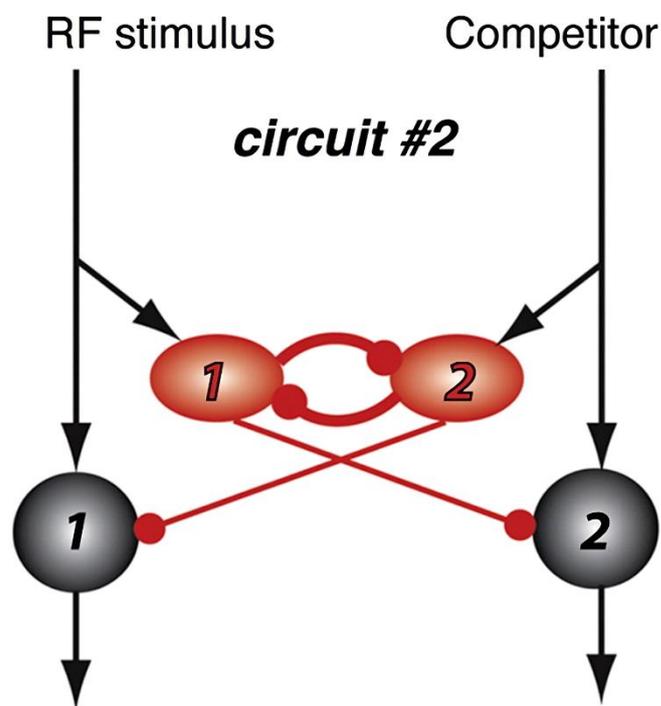


Figure I.A-1 Schematic of an example of mutual inhibition of inhibition circuit motif. Image from (Mysore and Knudsen 2012), figure 4.A. Red oval represent inhibitory neurons, red circled arrows are inhibitory connections. Black circles represent output units of the circuit and black arrows illustrate the flow of excitatory activity. RF stimulus refers to a stimulation at the center of the receptive field for the output 1, which is in competition with the competitor stimulus

Mutual inhibition motifs are characterized by reciprocal connections between two populations of neurons, where activation of one population inhibits the activity of the other, and vice versa (Liqun Luo 2021; Mysore and Knudsen 2012; Mysore and Kothari 2020). Theoretical work suggests that this motif alone allows for the selection between competing behavioral outputs but is not capable of generating flexible selection in

response to varying intensities of stimulation (Mysore and Knudsen 2012; Mysore and Kothari 2020), limiting its biological relevance. Indeed, in this motif, the strength of inhibition of a specific output relies solely on the strength of competing stimuli, which implies that a strong enough inhibition will always prevent its selection, no matter the strength of the stimulus (Mysore and Knudsen 2012; Mysore and Kothari 2020).

To achieve a switch in the neural representation that is flexible to changes in stimulus intensity, there needs to be feedback on the inhibitory neurons (Mysore and Knudsen 2012). Reciprocal inhibition of inhibition was shown to be the most structurally simple and efficient motif able to drive adaptation to the stimulus strength. Indeed, by inhibiting each other, the inhibitory neurons provide information from all stimuli, making the neural representation dependent on the relative strength of the different stimuli (Mysore and Knudsen 2012; Mysore and Kothari 2020). For instance, in circuits determining approach or avoidance behaviors, one neuron population may promote approach while simultaneously inhibiting avoidance, and vice versa (Spielberg et al. 2012; Koyama et al. 2016). By dynamically inhibiting each other, these populations ensure the selection of a single behavioral option in a process resembling a “winner-take-all” computation (Prescott et al. 2006) while suppressing competing alternatives, enhancing behavioral specificity and adaptability.

Overall, the mutual inhibition of inhibition implements flexibility in the decision-making process (Liqun Luo 2021; Koyama and Pujala 2018; Mysore and Kothari 2020). Mutual inhibition of inhibition resolves conflicting signals and biases circuits towards specific actions. By regulating activity patterns, this motif plays a crucial role in mediating adaptive behaviors in biological systems (Liqun Luo 2021; Koyama and Pujala 2018; Mysore and Kothari 2020). These principles illustrate how neural circuits, by their very structure, can lead to flexible behavioral outputs. The modulation of such circuits thus provides a supplementary layer of flexibility to adapt their outputs to various conditions.

I. B. Encoding behavioral flexibility through neural circuits modulation

Understanding neural circuit modulation is crucial for elucidating the adaptation of behavioral responses to specific situations across different organisms. Neural circuits are formed by neurons that communicate with each other to give rise to a specific function. The connections between neurons are an anatomical backbone for the circuits and form what is referred to as the connectome. Anatomically defined circuits exhibit plasticity, reforming over time or in response to specific conditions. Moreover, even when the circuitry linking the neurons together does not change, modulators can change the way they interact with each other to introduce flexibility to what would

otherwise be a fixed network, for instance through feeding state-dependent neuropeptide release (Marder 2012; Ezcurra et al. 2016; Jin et al. 2023; Jing and Gillette 2003; Bargmann 2012; Nässel and Zandawala 2022; Nässel 2002). Therefore, the study of neural circuit modulation provides valuable insights into the processes underlying behavioral decisions and responses to changes in both the environment and internal state. The following section will describe the mechanisms of modulation of neural circuits and their activity.

I. B. 1. Gene expression dependent circuit adaptation

Activity-regulated gene expression serves as a pivotal mechanism through which the functioning and connectivity of neural circuits are shaped in response to experiences, stimuli, and internal states. Upon neuronal activation, particularly at the synapse, intracellular signaling cascades are activated to convey signals to the nucleus. These cascades often involve the activation of various kinases, phosphatases, and second messengers (Leslie and Nedivi 2011). For example, the influx of Ca^{2+} from the synapse into the postsynaptic cell through receptors and ion channels, such as glutamate receptors and voltage-sensitive Ca^{2+} channels, triggers the activation of Ca^{2+} -dependent signaling pathways (Bading 2013; Brini et al. 2014). These pathways can include the activation of protein kinases, such as Ca^{2+} /calmodulin-dependent protein kinase (CaMK), and the subsequent phosphorylation of downstream targets involved in gene expression regulation (Leslie and Nedivi 2011; Hardingham et al. 1997; Bading 2013).

Specific transcriptional activators are targeted by the intracellular signaling cascades to initiate gene expression. One of the well-studied transcription factors involved in activity-dependent gene expression is cAMP response element-binding protein (CREB). CREB is activated by phosphorylation in response to elevated intracellular Ca^{2+} levels and subsequent activation of Ca^{2+} /calmodulin-dependent kinases (Leslie and Nedivi 2011; Bading 2013; Brini et al. 2014). Once phosphorylated, CREB binds to cAMP response elements (CREs) in the promoter regions of target genes, thereby initiating their transcription. The regulated genes in turn impact circuit plasticity through the modification of synaptic strength or number, thereby leading to changes in the connectivity of neuronal circuits (Leslie and Nedivi 2011; Brini et al. 2014; Bading 2013).

One such gene is the Brain-derived neurotrophic factor (BDNF) gene. The protein it encodes, BDNF, is involved in synaptic plasticity (Miranda et al. 2019; Wardle and Poo 2003; Leal, Comprido, and Duarte 2014) as its *Drosophila* homolog, neurotrophin 1 (Zhu et al. 2008). BDNF expression is upregulated by neuronal activation and can in turn regulate local synaptic expression of numerous proteins (Leal, Comprido, and Duarte 2014). Among other things, BDNF influences the strength of synapses by reducing the

release of GABA from presynaptic neurons and downregulating the expression of postsynaptic potassium/chloride co-transporter (Wardle and Poo 2003). Furthermore, BDNF influences the morphology of dendritic spines by promoting actin polymerization (Miranda et al. 2019; Rex et al. 2007). Additionally, BDNF can regulate the expression and activity of miRNAs, which are non-coding RNAs that are able to repress translation of target transcripts and were themselves linked to synaptic plasticity (Chekulaeva and Filipowicz 2009; Leal, Comprido, and Duarte 2014; Konecna et al. 2009).

In addition to the immediate activation of transcription factors, activation of the synapse can be relayed to the nucleus where it can also involve epigenetic regulation. Epigenetic modifications of DNA and chromatin, such as DNA methylation and histone acetylation, can influence the accessibility of gene promoters and enhancers, thereby regulating the transcriptional response to synaptic activity (Leslie and Nedivi 2011).

Activity-regulated genes are thus at the root of neuronal plasticity, which can be broadly categorized as structural, involving morphological changes, and functional, involving changes in synaptic strength. Structural plasticity encompasses the physical alterations in the structure of neurons and their synaptic connections, while functional plasticity refers to the modifications in synaptic strength and efficacy, influencing the efficiency of communication between neurons and the overall functional properties of neural circuits (Leslie and Nedivi 2011; Yamada and Kuba 2016; Gipson and Olive 2017). These distinctive forms of plasticity play integral roles in shaping the adaptability and stability of neural circuits in response to sensory experience, learning, and environmental stimuli.

I. B. 1. a. Neuronal plasticity

Long-term potentiation (LTP) and long-term depression (LTD) are at the core of synaptic plasticity, encompassing mechanisms crucial for adaptive changes in neuronal circuits. Both these phenomena correspond to a lasting increase and decrease, respectively, in the strength of a synapse resulting from specific patterns of neural activity (Leslie and Nedivi 2011; Destexhe and Marder 2004). Modulating the strength of synapses leads to alterations in the communication between neurons. Indeed, increasing the strength of a synapse means that the excitatory or inhibitory effect (depending on the properties of the pre-synaptic neuron) on the post-synaptic neuron will be increased. This way, it is possible, without modifying the anatomical structure of a circuit, to tune its activity to specific conditions. Both LTP and LTD are largely considered as the main mechanisms driving learning and memory (Leslie and Nedivi 2011; Sweatt 2016; Destexhe and Marder 2004), which makes them ideal candidates to carry information from past-experiences to circuits associated with flexible behaviors. One example illustrating the relevance of LTP in neural circuit modulation is its

implication in the modulation of innate social behaviors in mice. Indeed, LTP was shown to be essential for aggression priming in a circuit linking the amygdala, the hypothalamus and the striatum (Wei, Talwar, and Lin 2021).

LTP and LTD are often accompanied with formation of new synapses or pruning of irrelevant synapses respectively (Leslie and Nedivi 2011). Similarly to modulating the strength of synapses, this changes in synaptic numbers leads to an alteration of the communication between partner neurons and thus to an adaptation of the neural circuit to past-experiences. An example of structural plasticity in neural circuits is the phenomenon of adult neurogenesis in the hippocampal dentate gyrus and the olfactory bulb of the mammalian brain. This process involves the generation of new neurons and their integration into existing neuronal networks, requiring synaptic rewiring and leading to significant structural changes in the circuitry. Adult neurogenesis has been implicated in learning and memory consolidation, highlighting the crucial role of structural plasticity in shaping neural circuits and influencing cognitive functions (Butz, Wörgötter, and van Ooyen 2009).

Such gene expression dependent mechanisms of neural circuit alteration are relevant to the flexibility of behaviors, they are however not immediate as they require repeated expositions to specific conditions and protein synthesis (Leslie and Nedivi 2011; Destexhe and Marder 2004; Sweatt 2016; Gipson and Olive 2017; Hogan, Hamilton, and Horner 2020). It is of paramount importance that other processes are able to modulate behaviors to respond to immediate threats and needs. Furthermore, such conditions requiring immediate adaptation of behavior, such as hunger, are also fleeting and should not introduce irreversible or long-lasting neural circuit modulation.

I. B. 2. Cellular mechanisms of circuit modulation

The vast majority of communications between cells in an organism happen through the release of a molecule that will bind to a specific receptor as its ligand to trigger a cascade of intracellular processes responsible for the cellular response (synaptic communication, muscle contraction, hormone release, etc...). The enormous multitude of molecules and receptors used in cell communications is essential to signal countless combinations of internal states and sensory environments that an organism can be subjected to and lead to a quick and reversible adaptation of circuit activity and behavior.

Neuromodulators are signaling molecules that play a crucial role in modulating the function of synapses and neural circuits. They can include various classes of molecules such as neuropeptides, biogenic amines, gases, and other signaling molecules. Neuromodulators can induce rapid, dynamic, and reversible changes in the activity

patterns within fixed neural circuits, enabling the generation of diverse patterns of neural circuit activity (Bargmann 2012; Marder 2012). Neuromodulators typically exert their actions by binding to receptors coupled to G-proteins (GPCRs), whose activation triggers a cascade of intracellular signaling that is responsible for the cellular effects exerted by the neuromodulator. The ability of neuromodulators to induce such dynamic changes in circuit activity enables the nervous system to quickly adapt to varying physiological and environmental conditions, ultimately shaping the behavioral responses of the organism.

1. B. 2. a. Neuropeptides

Neuropeptides are highly diverse signaling molecules found across various animal species. The repertoire of neuropeptides is gigantic, with several hundred different neuropeptides identified to date (Elphick, Mirabeau, and Larhammar 2018; DeLaney et al. 2018). Structural and functional similarities are often observed among neuropeptides from different species. These similarities may arise due to evolutionary conservation from a common ancestor or as a result of convergent evolution, where the production of similar neuropeptide structures represents an optimal evolutionary response to ensure specific physiological and behavioral functions (Muneoka et al. 2000; O'Shea and Schaffer 1985). The diverse array of neuropeptides and their receptors allows for precise and context-dependent modulation of neural circuits underlying a wide range of behaviors, including feeding, reproduction, stress responses, and social interactions (Nässel and Zandawala 2022; Devineni and Scaplen 2022; Jékely et al. 2018).

(1) Neural circuits modulation by neuropeptides

Unlike classical neurotransmitters, neuropeptides can be released in a non-synaptic manner (Figure I.B-1) and can exert both acute and long-term effects on neuronal properties and network activity (van den Pol 2012; Jékely et al. 2018). The release of neuropeptides is tightly regulated and can occur under specific conditions, including neuronal activity, stress, and various physiological states (Marder and Bucher 2007; Bargmann and Marder 2013; Jékely et al. 2018). Upon release, neuropeptides bind to specific receptors, predominantly GPCRs, expressed on target neurons, initiating a cascade of intracellular signaling events (Jékely et al. 2018; Nässel and Zandawala 2022). This activation leads to diverse effects on neuronal properties, including the modulation of synaptic transmission, neuronal excitability, and network activity (Cardin 2019).

Comparison of the properties of neuropeptides and classical neurotransmitters

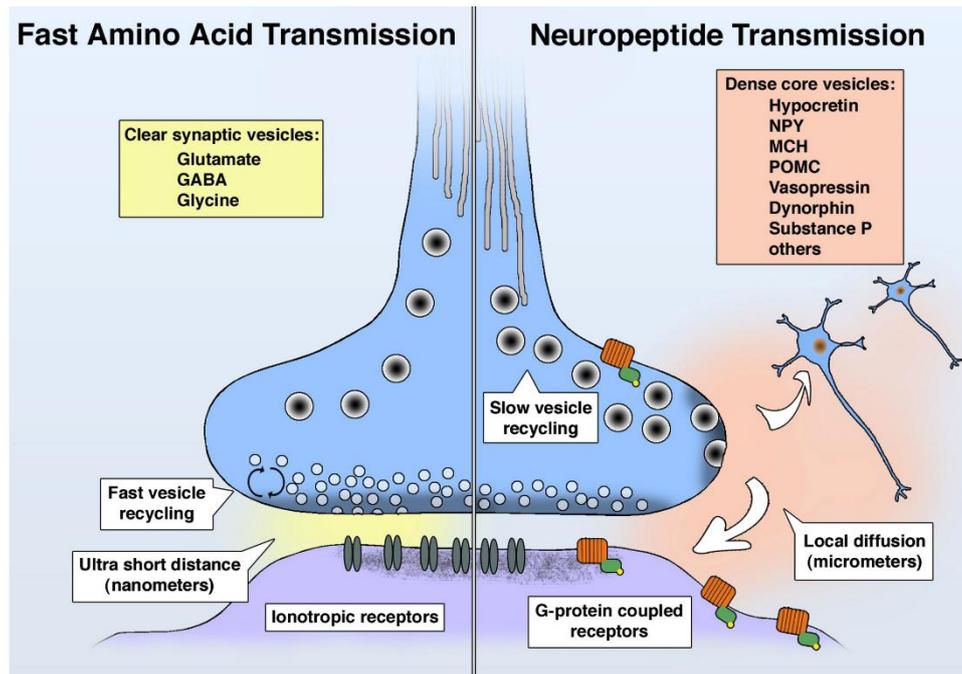


Figure I.B-1 Schematic illustrating the differences between the properties of neuropeptides (right) and classical neurotransmitters (left). Image from (van den Pol 2012), figure 3. Classical neurotransmitters are released at synapses where they bind to ionotropic receptors and are quickly recycled in the presynaptic neuron. Neuropeptides can be released outside of synapses and diffuse over longer distances to bind to G-protein coupled receptors.

The effects of neuropeptides on neural circuits and behavior are context-dependent and can vary based on the specific neuropeptide-receptor interactions, the cellular and circuit-level properties of the neurons involved, and the overall network dynamics (Jékely et al. 2018; Devineni and Scaplen 2022). Importantly, the neuromodulatory actions of neuropeptides contribute to the flexibility and adaptability of neural circuits, enabling the integration of internal states, environmental cues, and behavioral demands to appropriately adapt behavioral responses to the immediate context (Su and Wang 2014).

Each neuron comprising the nervous system possesses intrinsic characteristics that are specific to it and result from the ensemble of ion channels and receptors expressed on its membrane. The action of a neuropeptide on a neuron can modify the number of receptors or channels expressed on its membrane, their type, and their kinetic properties (Harris-Warrick and Marder 1991).

In most cases, the modulation of the intrinsic properties of a neuron is the result of the action of several neuropeptides. The binding of different neuropeptides to their receptors on a neuron's membrane can have an additive, synergistic, or opposing effect on the activity or expression of a receptor or channel. Furthermore, the same neuropeptide can lead to a modification of the expression or activity of several different receptors or channels, either in the same direction or in opposite directions. Thus, it would be imperative to know all the neuropeptides acting on a neuron and all the cellular mechanisms involved in the activation of their GPCRs to fully understand their effect on the targeted neuron and its impact on the neural circuit to which it belongs (Nadim and Bucher 2014). The development of single-cell transcriptomics provides hope that such a comprehensive understanding of all the intracellular processes could be achieved in every neuron of a circuit, in different conditions, in the near future (Heumos et al. 2023).

The modification of the intrinsic properties of neurons that connect to other neurons through a specific synapse leads to the modulation of the efficiency of that synapse. Indeed, the modulation of the presynaptic neuron can influence the amount of neurotransmitter released into the synaptic cleft. Phenomena of feedback on the presynaptic neuron, either through retrograde messengers or through the action of autoreceptors, can also be the target of modulation. Complementarily, the sensitivity of the postsynaptic neuron to the neurotransmitter can be modulated, altering the strength of the connection between the two neurons (Harris-Warrick and Marder 1991; Nadim and Bucher 2014).

Through these mechanisms, it is possible that certain neurons in the network, usually inactive, are recruited under certain conditions only, transiently reshaping the circuit in a process referred to as information re-routing (Bräcker et al. 2013; Crossley, Staras, and Kemenes 2018; Lewis et al. 2015). Moreover, the release and action of a neuropeptide can themselves undergo modulation through other neuropeptides targeting neurons that release them or exerting a competing action on their target neurons. Finally, electrical synapses can also be modulated by modifying the strength of the electrical coupling between the two neurons (Harris-Warrick and Marder 1991; Nadim and Bucher 2014).

Moreover, neuropeptides can also induce long-lasting changes in neuronal function through mechanisms such as synaptic plasticity and gene expression (Leslie and Nedivi 2011). These long-term effects contribute to the adaptive nature of neuropeptide modulation, allowing neural circuits to undergo persistent changes in response to specific stimuli or behavioral states (Harris-Warrick and Marder 1991).

Altogether, the release of a single neuropeptide can have a multitude of consequences on the activity of one or more neurons composing a circuit and thus drastically change its functionality. However, the significant number of neuropeptides found in an animal brain and the dynamics of their secretion and diffusion mean that neurons are constantly in an unstable equilibrium that depends on the integration of all the signals they receive at a given moment. Understanding the conditions leading to changes in this balance is essential for understanding the generation and modulation of behaviors from neuronal circuits but remains to this day impossible to study in humans at the scale of neuron circuits due to the invasiveness of the necessary techniques and the too large number of neurons and synaptic connections.

(2) Water and food related neuropeptides

The intricacies of neuropeptidergic signaling are perfectly illustrated by the multitude of neuropeptides involved in the regulation of food and water intake (Woods et al. 1998; Nijenhuis, Oosterom, and Adan 2001; Lambert et al. 1998; Blevins, Schwartz, and Baskin 2004; Nieuwenhuizen and Rutters 2008; Posovszky and Wabitsch 2015; Latorre et al. 2016; Wren and Bloom 2007; Larhammar 1996; Chambers, Sandoval, and Seeley 2013). Moreover, most of these neuropeptides have been shown to also regulate other processes, as illustrated by the examples below. Overall, the involvement of a single molecule in the regulation of different biological processes and of several molecules in the regulation of any individual biological process show the essential role of neuropeptides and the challenge of studying their action in diverse neural circuits.

As a lot of neuropeptides are evolutionarily conserved (Muneoka et al. 2000; O'Shea and Schaffer 1985), comparative studies across species are especially interesting. I give below examples of mammalian neuropeptides, as I will develop more on *Drosophila* neuropeptides later in the manuscripts.

Neuropeptide Y (NPY) is a widely distributed mammalian neuropeptide that plays a role in regulating a variety of physiological processes, including promoting feeding and reducing stress response, and anxiety (Bale and Doshi 2023; Brain and Cox 2006; Pedrazzini, Pralong, and Grouzmann 2003; Sánchez, Rodríguez, and Coveñas 2023). In the brain, extrinsic application of NPY leads to a regulation of synaptic transmission and neuronal excitability (Bacci, Huguenard, and Prince 2002). NPY has also been implicated in the impairment of memory acquisition and stimulation of memory retention (Gøtzsche and Woldbye 2016).

Substance P serves as both a neurotransmitter and neuromodulator in the central and peripheral nervous systems, playing crucial roles in regulating pain perception, stress response, and mood. Its involvement in various neurological and psychiatric disorders underscores its significance in neural function and dysfunction (Brain and Cox 2006; Mantyh 2002; Mashaghi et al. 2016).

Orexins, another group of neuropeptides, play a key role in promoting arousal and feeding, thus regulating energy homeostasis (Sakurai and Mieda 2011). They act as neuromodulators to regulate synaptic transmission and neuronal excitability and have been implicated in the modulation of sleep-wake cycles and reward processing (Harris and Aston-Jones 2006).

Vasopressin, functioning as both a hormone and neuromodulator, regulates physiological processes such as water balance, stress and blood pressure (Landgraf and Neumann 2004; Lozić et al. 2018). In the brain, vasopressin acts as a neuromodulator to regulate reshape inhibitory and excitatory signals (Kombian et al. 2000; Ramanathan et al. 2012), and has been implicated in the modulation of social behavior (Engelmann and Landgraf 1994).

Cholecystokinin (CCK) acts as a neurotransmitter and neuromodulator in the central and peripheral nervous systems. It is involved in the inhibition of food intake, anxiety, and pain perception, and has been implicated in the pathophysiology of various neurological and psychiatric disorders (Dockray 2012; Bernard et al. 2021).

I. B. 2. b. Amines, gases and other molecules

Biogenic amines identified as playing a neuromodulatory role are few (<20) and highly conserved across different species in the animal kingdom, both in vertebrates and invertebrates (Muneoka et al. 2000; O'Shea and Schaffer 1985; Blenau and Baumann 2001; Sloley and Juorio 1995). Among them, dopamine, serotonin, and norepinephrine are prominent examples, synthesized from amino acids and serving as both neurotransmitters and neuromodulators (Masek et al. 2015; Berridge and Waterhouse 2003; Peters, Cheer, and Tonini 2021). These monoamines regulate a wide range of physiological processes, including mood regulation, attention, and reward. Dopamine and serotonin have been particularly extensively studied (Ligneul and Mainen 2023; Peters, Cheer, and Tonini 2021; Speranza et al. 2021).

Dopamine acts as a multifaceted neuromodulator involved in a range of functions, including reward processing, motor control, learning, and motivation (Speranza et al. 2021; Peters, Cheer, and Tonini 2021). Dopaminergic neurons project widely throughout the brain, influencing diverse neural circuits. In reward-related pathways, dopamine plays a central role in reinforcement learning, facilitating the encoding of reward

prediction errors and guiding decision-making processes (Speranza et al. 2021; Peters, Cheer, and Tonini 2021; Sayin et al. 2018; Eichler et al. 2017). In motor circuits, dopamine modulates movement initiation, coordination, and execution, with dysfunction in dopaminergic pathways leading to movement disorders such as Parkinson's disease (Speranza et al. 2021).

Serotonin is another crucial neuromodulator implicated in mood regulation, emotional processing, sleep-wake cycles, appetite, and cognition (Ligneul and Mainen 2023; Peters, Cheer, and Tonini 2021). Serotonergic neurons project extensively throughout the brain, modulating the activity of diverse neural circuits. In mood regulation, serotonin plays a key role in modulating affective states, with alterations in serotonergic signaling implicated in mood disorders such as depression and anxiety (Ligneul and Mainen 2023; Peters, Cheer, and Tonini 2021). Additionally, serotonin contributes to cognitive functions such as learning, memory, and decision-making, shaping behavioral responses to environmental stimuli (Chao et al. 2004; Ligneul and Mainen 2023; Peters, Cheer, and Tonini 2021; Gaudry and Kristan 2009). Moreover, serotonin has been associated with the regulation of feeding both in vertebrates and invertebrates (Tierney 2020; van Galen, ter Horst, and Serlie 2021), increasing the hedonic value of food and its dysregulation being involved in the pathophysiology of obesity (van Galen, ter Horst, and Serlie 2021).

Gases, such as nitric oxide (NO) and carbon monoxide (CO), also exert neuromodulatory effects, in both vertebrates and invertebrates (Ueno et al. 2020; Jeong 2024; Esplugues 2002; Garthwaite 2008; Heinemann et al. 2014; Aso et al. 2019). These small molecules are synthesized by neurons and diffusing across cell membranes to activate intracellular signaling pathways. NO is involved in a wide range of physiological processes, including learning and memory (Esplugues 2002; Garthwaite 2008; Aso et al. 2019; Jeong 2024), while CO is involved in hypertension and inflammation (Heinemann et al. 2014; Ueno et al. 2020).

Furthermore, lipids such as endocannabinoids can also act as neuromodulators. These molecules are synthesized by neurons and act on cannabinoid receptors to modulate synaptic transmission. Endocannabinoids are involved in a wide range of physiological processes, including appetite, sleep and arousal (Peters, Cheer, and Tonini 2021).

Other notable neuromodulatory molecules encompass adenosine (Fredholm et al. 2005), acetylcholine (Picciotto, Higley, and Mineur 2012), and histamine (W. Hu and Chen 2017), each integral to diverse physiological processes such as sleep regulation, attention, and memory.

The examples above were taken mostly from mammalian studies where the neuromodulatory effects of these molecules have been first characterized. Nonetheless, very similar properties have been observed in invertebrates, and especially *Drosophila*, for most of these molecules, suggesting a conserved role across species (Tierney 2020; Aso et al. 2019; Jeong 2024; Ueno et al. 2020; Dacks et al. 2009; Van Damme et al. 2021; Inagaki et al. 2012; Eriksson et al. 2017; Sampson et al. 2020; Driscoll et al. 2021; Malloy et al. 2019; Bielopolski et al. 2019; Rosikon, Bone, and Lawal 2023).

I. B. 2. c. *Examples of decision-making circuits modulation*

(1) *Behavioral states in C. elegans*

In (Flavell et al. 2013), the authors discuss dwelling and roaming states in *C. elegans*. These are long-lasting behavioral states that last for several minutes and are regulated by a neuromodulatory circuit involving serotonin and the pigment dispersing factor neuropeptide (PDF).

They were able to establish that serotonin promotes dwelling states through the MOD-1 serotonin-gated chloride channel. The spontaneous activity of serotonergic neurons correlates with dwelling behavior, and optogenetic modulation of the critical MOD-1-expressing targets induces prolonged dwelling states. On the other hand, PDF promotes roaming states through a G α s-coupled PDF receptor. Optogenetic activation of cAMP production in PDF receptor-expressing cells induces prolonged roaming states. The neurons that produce and respond to each neuromodulator form a distributed circuit orthogonal to the classical wiring diagram. This suggests that the neuromodulatory circuit is a distinct and independent system that operates in parallel with the classical synaptic wiring diagram to regulate long-lasting behavioral states in *C. elegans*. As *C. elegans* central nervous system (CNS) is only composed of 302 neurons, forming a few thousand synapses (White 1986; Ringstad 2017), the additional layer of regulation of neural activity provided by neuromodulation is especially important for the generation, selection and regulation of different behaviors and their adaptation to external context and internal states (Ringstad 2017).

(2) *Acetylcholine modulation of locomotor circuit in zebrafish*

In (Bertuzzi and Ampatzis 2018) the researchers explored how cholinergic modulation affects the swimming ability of zebrafish at different speeds. Although the conditions leading to the release of acetylcholine by spinal interneurons are not clearly elucidated, it seems likely to be tuned to the behavioral state of the animal (Zagoraïou et al. 2009; Bertuzzi and Ampatzis 2018).

They found that cholinergic interneurons differentially control the excitability of distinct classes of motoneurons through the release of acetylcholine and the activation

of m2-type muscarinic acetylcholine receptors (mAChRs). The study reveals that m2-type mAChRs expression is different depending on the type of motoneurons. Since m2-type mAChRs activation decreases neuronal firing, this differential expression of mAChRs allows acetylcholine to control the locomotor output of the animal through a selective action on different motoneurons.

(3) *Octopamine modulation of aggressive behavior in Drosophila*

Drosophila flies engage in fighting behaviors to establish dominance and gain access to resources. Aggression is innate and stereotypical, and can be influenced by factors such as age, body size, residency status, prior experience, and genetic components (Fernandez, Trannoy, and Certel 2023; Zwarts, Versteven, and Callaerts 2012; Asahina 2017).

(Zhou, Rao, and Rao 2008) found that octopamine, the insect equivalent of norepinephrine, is crucial for modulating aggressive behavior in both male and female flies. Reduction of octopamine levels decreased aggression, leading to a decrease in the initiation of fighting and a reduced ability to compete for copulation with females. Conversely, enhanced octopaminergic signaling increased aggression in socially grouped flies. This octopamine-dependent promotion of aggression is triggered by the activation of Gr32a-expressing sensory neurons that are sensitive to pheromones and synapse onto octopamine neurons (Andrews et al. 2014), thus initiating aggressive interactions.

(4) *Dopamine modulation of exploration/exploitation trade-off*

In decision-making, individuals must balance the need to exploit familiar, potentially rewarding choices with the desire to explore new alternatives that may yield even greater benefits in the long run (J. D. Cohen, McClure, and Yu 2007; A. R. Walker et al. 2022). Too much exploitation can lead to inflexibility and missed opportunities, while excessive exploration may result in inefficient decision-making (J. D. Cohen, McClure, and Yu 2007; A. R. Walker et al. 2022). This trade-off is crucial for adaptive behavior in dynamic environments and has been studied in various contexts, including neuroscience, psychology, and animal behavior (J. D. Cohen, McClure, and Yu 2007; A. R. Walker et al. 2022; Ferecatu and De Bruyn 2021). Studies have shown that dopamine plays a crucial role in the exploration-exploitation trade-off (Cinotti et al. 2019; Chakroun et al. 2020).

In a study by (Cinotti et al. 2019), rats were given a task that required them to choose between a known, safe option and a risky, uncertain option. The researchers found that blocking dopamine receptors impaired the rats' ability to balance exploration and exploitation, leading to an increase in random choices without affecting learning

capacities. This suggests that dopamine may adapt the exploration-exploitation trade-off in decision-making when facing changing environmental contingencies, with higher levels of dopamine favoring exploitation over exploration.

Furthermore, in a double-blind, placebo-controlled study involving the administration of L-dopa (a dopamine precursor) and haloperidol (a dopamine antagonist) and functional magnetic resonance imaging (fMRI), (Chakroun et al. 2020) showed that boosting central dopamine with L-dopa specifically attenuated exploratory choice patterns, while marginally increasing exploitative choices. Additionally, the neural signatures of exploration, exploitation, and prediction error were unaffected by L-dopa, while it attenuated neural representations of overall uncertainty in specific brain regions.

Altogether, dopamine seems to play a pivotal role in modulating the exploration-exploitation trade-off by influencing decision-making processes, reward learning, and the balance between exploring new options and exploiting known ones. Interestingly, a similar function of dopamine was observed in *Drosophila* where it promotes food-odor tracking in hungry flies (Sayin et al. 2019).

I. C. Feeding state modulation of behaviors

Internal states play a pivotal role in shaping an organism's behavioral repertoire, allowing it to adapt and respond dynamically to changing physiological conditions (Palmer and Kristan 2011). Among these internal states, the feeding state is of paramount importance to ensure an organism's survival and its effect on the modulation of behaviors and neural circuits is extensively studied. The decision to feed involves various key points such as locating a food source, initiating feeding, responding to external stimuli while feeding, and determining when to stop. Understanding how animals make decisions related to feeding can provide insights into broader decision-making processes within the nervous system. By studying feeding behaviors, it is possible to investigate how animals prioritize different stimuli, make choices between competing options, and regulate their behavior based on internal states (Gaudry and Kristan 2012). Moreover, the feeding state can also modulate decisions that are not related to feeding, for instance increasing risk-taking (Figure I.C-1) across species, (Moran et al. 2021; Smith and Grueter 2022), but also influencing social and cognitive processes in human such as gambling decisions or ethical judgments (van Swieten, Bogacz, and Manohar 2023; Vicario et al. 2018; H. Brown, Proulx, and Stanton Fraser 2020; Hamelin et al. 2022). The mechanisms by which feeding state information is integrated in decision-making neural circuits not related to feeding decisions remains elusive.

Illustration of behavioral prioritization depending on the hunger state

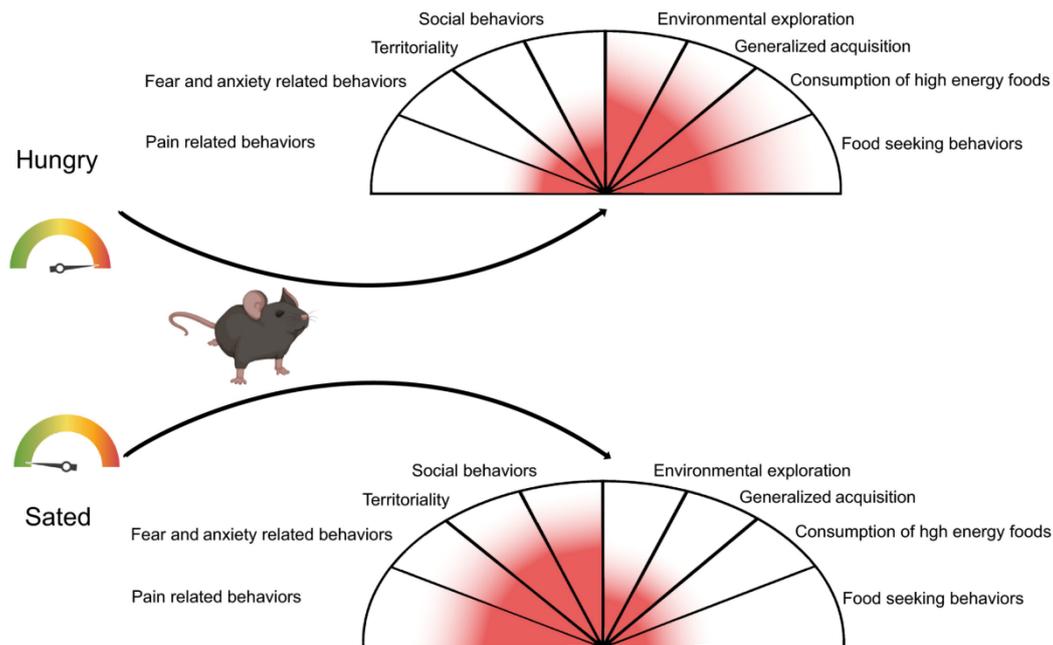


Figure I.C-1 Schematic representation of social behavioral modulations caused by hunger in mice. Image from (Smith and Grueter 2022), figure 6. When hungry, mice neglect pain, fear and social behaviors to promote exploration, food seeking and consumption.

The initiation of feeding itself is strongly dependent on the environmental context and the internal state of the organism (Woods et al. 2000). Moreover, once feeding is initiated, very numerous molecular signals are released, both in the gut and the brain to regulate the amount of food consumed (Woods et al. 1998; Nijenhuis, Oosterom, and Adan 2001; Lambert et al. 1998; Blevins, Schwartz, and Baskin 2004; Nieuwenhuizen and Rutters 2008; Posovszky and Wabitsch 2015; Latorre et al. 2016; Wren and Bloom 2007; Larhammar 1996; Chambers, Sandoval, and Seeley 2013). In humans, the hypothalamus is instrumental in both the initiation and regulation of feeding through several populations of neuropeptidergic neurons. In particular, two populations of neurons found in the arcuate nucleus of the hypothalamus are instrumental in the regulation of food intake: the first co-expresses the neuropeptide Y (NPY) and the Agouti-related protein (AgRP), which both increase food intake, and the second co-expresses proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which decrease it (K. A. Simpson, Martin, and Bloom 2008; Meister 2007; Parker and Bloom 2012; Guillod-Maximin et al. 2009).

The gastrointestinal tracts of both *Drosophila* larvae and adult flies are remarkably similar in their structures to that of humans (Chopra, Kaushik, and Kain 2022). Interestingly, many of the neuropeptides identified as involved in the regulation of food intake in *Drosophila* are analogs to human neuropeptides that bear similar functions

(Chopra, Kaushik, and Kain 2022; Lin, Senapati, and Tsao 2019; Yoshinari et al. 2021; Oh et al. 2019; K. A. Simpson, Martin, and Bloom 2008; Parker and Bloom 2012), suggesting that many of the mechanisms responsible for the regulation of food intake are evolutionarily conserved.

A good example of the influence of the feeding state on behavioral decisions is the leech or hard ticks that suppress behavioral responses to external stimulations while feeding (Gaudry and Kristan 2012; Sonenshine et al. 2014). However, feeding does not only have an immediate impact on other behaviors but also long-lasting effects. Indeed, leeches have been shown to their locomotion preference towards crawling after feeding (Gaudry and Kristan 2012) as well as their temperature preference, as is also the case for various reptiles (Gaudry and Kristan 2012; Sievert, Jones, and Puckett 2005; Tsai and Tu 2005).

Many of the molecules involved in the regulation of feeding are neuropeptides. As previously described, neuropeptides are key actors of neuromodulation and the feeding state of an individual is a strong regulator of behavior. It is thus likely that at least some of the neuropeptides involved in the regulation of feeding can carry information about the feeding state of the animal to tune different behavior-related neural circuits.

I. C. 1. Modulation of peripheral sensory neurons

One way for the feeding state to alter behaviors is by modulating the sensitivity of sensory neurons to specific stimuli involved in sensing stimuli that induce said behaviors (Bargmann 2012). Such mechanisms have been extensively studied in the context of feeding state-dependent modulation of chemosensory neurons involved in olfaction (Sengupta 2013; Shanahan and Kahnt 2022). Studies have shown in different species including *C. elegans* (Chalasani et al. 2010), rats (Aimé et al. 2007), *Drosophila* larva (Slankster et al. 2020) and adult fly (Root et al. 2011; Ko et al. 2015) and even humans (Albrecht et al. 2009), that the detection threshold of odors, especially food-related odors, are modulated by the feeding state, leading to a facilitation of feeding. Several neuromodulators and neuropeptides have been implicated in such processes such as serotonin (Chao et al. 2004), dopamine (Ezcurra et al. 2011), insulin-like peptides (ILP) (Root et al. 2011; Chalasani et al. 2010), short neuropeptide F (sNPF) (Root et al. 2011). The modulation of sensitivity to odors is implemented through both increased responses of the sensory neurons via presynaptic facilitation (Root et al. 2011; Chao et al. 2004; Ezcurra et al. 2011) and facilitated responses in their postsynaptic targets (Chao et al. 2004; Ezcurra et al. 2011; Root et al. 2011; Chalasani et al. 2010).

Chemosensory neurons are not the only type of sensory neurons that can be modulated by the feeding state. Indeed, studies showed modulation of thermosensory neurons by ILP in *C. elegans* (Takeishi et al. 2020) or of sweet and bitter taste sensitivity by several neuropeptides in *Drosophila* (Inagaki, Panse, and Anderson 2014).

I. C. 2. Modulation of central neurons in circuits

In addition to modulating sensory neurons to lead to the modulation of circuits activity, the feeding state can also modulate central neurons to tune the output of circuits to a change in internal state (Sengupta 2013; Bräcker et al. 2013; Crossley, Staras, and Kemenes 2018). By influencing the activity of central neurons, the feeding state can dynamically adjust the processing of sensory information within neural circuits to shape their behavioral outputs. By integrating signals from both peripheral and central sources, these central neurons serve as key orchestrators in coordinating complex behavioral outcomes in fluctuating environments. This is the case for instance in *Drosophila* where neurons expressing the neuropeptide gene *hugin* (Bader, Wegener, and Pankratz 2007) have been shown to control the initiation of food intake depending on previous food exposure through connections to central neurons in the protocerebrum (Melcher and Pankratz 2005).

The modulation of central neurons can go as far as recruiting alternate pathways under different conditions, awakening or putting to sleep some neurons of the circuit, a process referred to as information rerouting (Bräcker et al. 2013; Crossley, Staras, and Kemenes 2018; Lewis et al. 2015). For instance, it was shown that the mushroom body of *Drosophila* flies is recruited specifically when larvae are starved or presented with food odors to diminish their avoidance of carbon dioxide, promoting feeding even on suboptimal food sources (Bräcker et al. 2013; Lewis et al. 2015)

However, unlike for peripheral neurons, studying the modulation of circuits at the level of central neurons necessitates to extensively map the circuits of interest. Consequently, investigating this aspect presents greater challenges and fewer studies are currently available on this mechanism of modulation. Particularly, there is a notable gap in our understanding of how feeding state influences behaviors unrelated to feeding (Smith and Grueter 2022; Moran et al. 2021; van Swieten, Bogacz, and Manohar 2023; Vicario et al. 2018; H. Brown, Proulx, and Stanton Fraser 2020). For instance, *Drosophila* has been shown to increase aggression upon food deprivation but likely as a consequence of the modulation of their feeding behavior (Edmunds, Wigby, and Perry 2021).

I. C. 3. Neuropeptides involved in feeding state-dependent modulation of neural circuits in *Drosophila*

As previously described, the feeding state is both controlled by and responsible for the release of numerous molecules, including various neuropeptides, that may bear the neuromodulatory effects triggered by changes in the feeding state. In this section, I will describe some feeding state-related neuropeptides found in *Drosophila* and their neuromodulatory actions, with a focus on NPF and sNPF that were at the center of my PhD work.

I. C. 3. a. sNPF & NPF

NPF and sNPF, despite their nomenclature, do not belong to the same neuropeptide families (Fadda et al. 2019; Mirabeau and Joly 2013). Only NPF shares a common ancestor with the vertebrate NPY, while sNPF seems to be specific to protostomes. However, both sNPF-receptor (sNPFR) and NPF-receptor (NPFR) are GPCRs that share structural similarities with the vertebrate NPY-receptor (NPYR), and especially with NPY2R (Fadda et al. 2019; Nässel and Wegener 2011; Larhammar and Salaneck 2004). Importantly, and despite their evolutionary distance, sNPF and NPF both share some functional roles with NPY. Most notably, all three peptides are deeply involved in the regulation of feeding and metabolism: their expression increases with food deprivation, and they increase food intake (Nässel and Wegener 2011; Fadda et al. 2019; Yeom et al. 2021; K.-S. Lee et al. 2008; 2004; K. S. Kim et al. 2021). Different neuronal populations that express either NPF or sNPF have been shown to respond to changes in the physiological state of the animal by tuning their activity with respect to the glucose concentration in the hemolymph (Ping Shen and Cai 2001; Nässel and Wegener 2011; Oh et al. 2019)

NPF and sNPF strongly interact with ILP to regulate feeding (Lin, Senapati, and Tsao 2019; Yoshinari et al. 2021; Oh et al. 2019; Sudhakar et al. 2020; Kapan et al. 2012). In the adult *Drosophila* fly, after a short starvation period, a positive feedback loop takes place between sNPF and insulin-producing cells (IPC) in the brain, that promotes the expression of both sNPF and ILP and trigger food intake (Sudhakar et al. 2020; Kapan et al. 2012; Oh et al. 2019; Manière et al. 2016). Similarly, NPF was shown to stimulate the production and release of ILP in the IPC, although this effect might originate more from gut-derived NPF (Yoshinari et al. 2021). Moreover, increase in feeding tolerance caused by NPF was shown to require ILP signaling on NPFR-expressing cells (Wu et al. 2005). Nonetheless, the regulation of feeding is not the only function of these neuropeptides, as suggested by their and their receptors' expression pattern.

Indeed, both sNPFR (Mertens et al. 2002; Nässel and Wegener 2011) and NPFR (Garczynski et al. 2002; Nässel and Wegener 2011; Fadda et al. 2019) are expressed in

numerous neurons of the *Drosophila* larva CNS (Figure I.C-2C-D), both in the brain lobes and the VNC, suggesting their implication in a wide range of different circuits. While NPF is only expressed consistently by a two pairs of neurons in *Drosophila* larva brain lobes (Figure I.C-2-B), one of which, named DM-NPF for dorsomedial NPF-releasing neuron, sends descending projections into the VNC, and in one pair of neurons in the subesophageal zone, named DL-NPF for dorsolateral NPF-releasing neuron (SEZ) in response to sweet taste exposition (Ping Shen and Cai 2001; Nässel and Wegener 2011), sNPF is very widely expressed (Figure I.C-2-A) in neurons of the brain lobes and VNC (Nässel et al. 2008; Nässel and Wegener 2011).

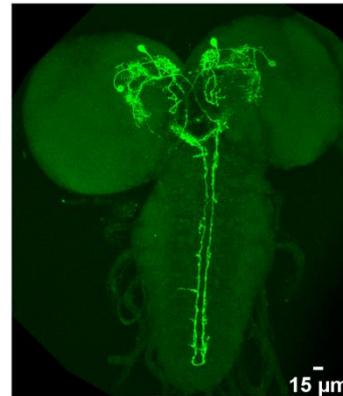
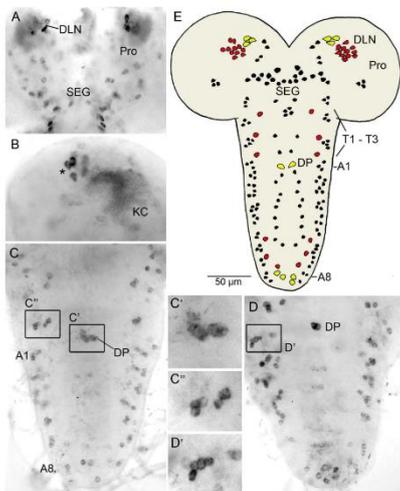
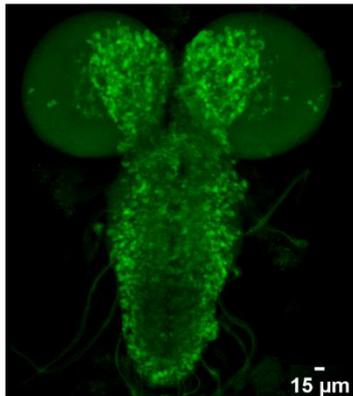
A Expression of sNPF in *Drosophila* larva brainB Expression of NPF in *Drosophila* larva brainC Expression of sNPFR in *Drosophila* larva brainD Expression of NPFR in *Drosophila* larva brain

Figure I.C-2 Expression patterns of sNPF and NPF neuropeptides and their receptors in *Drosophila* larva central nervous system. A. Image from (Nässel et al. 2008), figure 1. Expression pattern of sNPF in *Drosophila* larva CNS determined by *in situ* hybridization. B. Light microscopy image of NPF-Gal4>UAS-GFP larva. C. Light microscopy image of sNPFR T2A-Gal4>UAS-GCamP6s, stained with an antibody directed against GCamP6s. D. Light microscopy image of NPFR T2A-LexA>LexAop-jRGECO1a, stained with an antibody directed against jRGECO1a.

The expression of NPF is influenced by several factors. During development of the *Drosophila* larva, a NPF expression level drop is correlated with cessation of feeding upon entering wandering stage (Wu et al. 2003; Nässel and Wegener 2011). Additionally, both food deprivation (Wu, Zhao, and Shen 2005) and sweet taste (Ping Shen and Cai 2001) were shown to increase the activity of NPF neurons. A decrease in NPF activity has been linked to a decrease in tolerance of noxious food (Wu, Zhao, and Shen 2005), in sensitivity to food-related odors (S. Lee, Kim, and Jones 2017) and cold-resistant feeding (Lingo, Zhao, and Shen 2007), and an increase water intake (Landayan et al. 2021). NPF has also been implicated in behaviors such as courtship and circadian clock (G. Lee, Bahn, and Park 2006), aggression (Dierick and Greenspan 2007) and ethanol intoxication sensitivity (Wen et al. 2005).

Apart from its action on feeding and metabolism regulation, sNPF was shown to be involved in osmotic homeostasis (Kahsai et al. 2010), locomotion (Kahsai, Martin, and Winther 2010), circadian clock (Johard et al. 2009), mechanonociception (Imambocus et al. 2022), and possibly learning (Johard et al. 2008). These effects of sNPF are mediated by different neuronal populations that all express sNPF in the protocerebrum (Kahsai et al. 2010), central complex interneurons (Kahsai, Martin, and Winther 2010), ventral and dorsal clock neurons (Johard et al. 2009) and mushroom body (Johard et al. 2008). Interestingly, in *Drosophila* larva VNC, one pair of dorsal neurons co-express sNPF and ILP to orchestrate behavioral response respectively to noxious touch and light (Imambocus et al. 2022).

Collectively, the characteristics exhibited by sNPF and NPF underscore their significance as mediators that instigate feeding state-dependent modulation across a myriad of processes, in various neural circuits. Consequently, they emerge as prime candidates for investigation within the scope of my PhD research, as will be described in the scientific article in section III.A of this manuscript and the additional results presented in section III.B.

I. C. 3. b. *Other neuropeptides*

As mentioned previously, the feeding state is a complex state that involves a wealth of signaling molecules. Furthermore, beyond feeding-related processes, internal states such as thirst, which reflects the body's physiological need for water and drives behavior aimed at seeking and consuming water to restore hydration levels and maintain homeostasis, are also regulated by neuropeptide signaling (Landayan et al. 2021). It is thus unsurprising that several neuropeptides, other than sNPF and NPF, would be involved in the feeding state-dependent modulation of neural circuits (Bhumika and Singh 2018).

I briefly introduced the role of ILP in the regulation of feeding while presenting its interactions with the sNPF and NPF. In both the *Drosophila* larva and adult fly brain, insulin-producing cells produce and release three of the total eight different ILP identified in that species (Lin, Senapati, and Tsao 2019; Nässel and Vanden Broeck 2016). Overexpression or downregulation of ILP respectively lead to a decrease and an increase in feeding in both the adult fly and the larva (Lin, Senapati, and Tsao 2019). Unlike for the adult *Drosophila*, larval IPC do not directly sense the glucose level in the hemolymph (Nässel and Vanden Broeck 2016). Instead, adipokinetic hormone-producing cells (APC) sense glucose and their release of adipokinetic hormone (AKH) triggers secretion of ILP by the IPCs (Nässel and Vanden Broeck 2016). Apart from regulating feeding, ILP has been shown to suppress starvation-induced hyperactivity in the adult fly through the inhibition of neurons expressing the receptor for AKH in the SEZ (Lin, Senapati, and Tsao 2019; Selcho and Pauls 2019).

For instance, leucokinin (Lk) plays a significant role in regulating feeding behavior across insect species, influencing meal size and food preference (Nässel 2021). In *Drosophila* larvae, Lk is only expressed by 20 neurons, located in the brain lobes, SEZ and VNC (de Haro et al. 2010; Nässel 2021). Notably, a subset of Lk-expressing neurons in the VNC co-expresses the DH44 neuropeptide (Zandawala, Marley, et al. 2018). This particular subset of Lk neurons has been involved in the regulation of water homeostasis (Zandawala, Yurgel, et al. 2018), food intake (Zandawala, Yurgel, et al. 2018; Zandawala, Marley, et al. 2018; Yiting Liu et al. 2015), pre-ecdysis behavior (D.-H. Kim et al. 2015) and turning behavior in response to light or touch (Okusawa, Kohsaka, and Nose 2014). Lk neurons located in the brain lobes of *Drosophila* contribute to state-specific recall of water and sugar memories (Senapati et al. 2019), and sleep regulation (Murphy et al. 2016).

Another neuropeptide, Corazonin (Crz), is expressed by 24 neurons in *Drosophila* larva CNS, both in the brain lobes and VNC (Khan et al. 2021). Crz expression is increased upon food deprivation (Megha, Wegener, and Hasan 2019) and has been involved in the regulation of water homeostasis and food intake (Khan et al. 2021; Kubrak et al. 2016; Johnson et al. 2005). Furthermore, extensive interactions between Crz and other neuropeptidergic systems have been reported, including ILP, DH44 and sNPF (Khan et al. 2021).

These examples highlight the significant advancements in comprehending the role of neuropeptides in state-dependent modulation of neural circuits and behavior. Additionally, they underscore the complexities and redundancies inherent in feeding-related neuropeptides, posing challenges in accurately characterizing internal feeding states, and thus their influence of neuronal circuits, due to the need to consider interactions among all molecular and neuronal components involved in behavior adaptation.

I. D. *Drosophila* larva model system for the study of neural circuit modulation

Drosophila larvae are an ideal model organism for studying neural circuits and behavior due to their relatively simple nervous system and the availability of sophisticated genetic tools for manipulation and analysis (I. Hunter et al. 2021; Martín and Alcorta 2017). The *Drosophila* larval locomotor network, in particular, has been highlighted as a model circuit for understanding mammalian circuit development and function (I. Hunter et al. 2021). The use of genetic techniques, such as UAS-Gal4, has further enhanced the ability to visualize, map, and manipulate behavioral circuits in *Drosophila* (Sivanantharajah and Zhang 2015; Jenett et al. 2012; Pfeiffer et al. 2008; 2010; Li et al. 2014). These tools have been instrumental in providing insights into the organization and function of neural circuits in *Drosophila*, unraveling mechanisms underlying neural processes such as learning and memory (Keene and Waddell 2007; Eschbach et al. 2020; Weber et al. 2023), sensorimotor transformations (Ohyama et al. 2013; Jovanic et al. 2016; Imambocus et al. 2022; Masson et al. 2020), navigation (Linjiao Luo et al. 2010; Jovanic et al. 2019; Gomez-Marin, Stephens, and Louis 2011), etc...

I. D. 1. Brain simplicity

The relatively small number of neurons in the larval *Drosophila* brain (~10000) provides a unique opportunity to comprehensively map and understand the connectivity and function of neural circuits in an organism that is capable of a wide repertoire of behaviors (Figure I.D-1). This simplicity facilitates the identification of individual neurons and their connections with other neurons, enabling researchers to dissect the mechanisms of neural circuits modulation at a level of detail that is challenging in more complex nervous systems (I. Hunter et al. 2021; Ohyama et al. 2015; Jovanic et al. 2016).

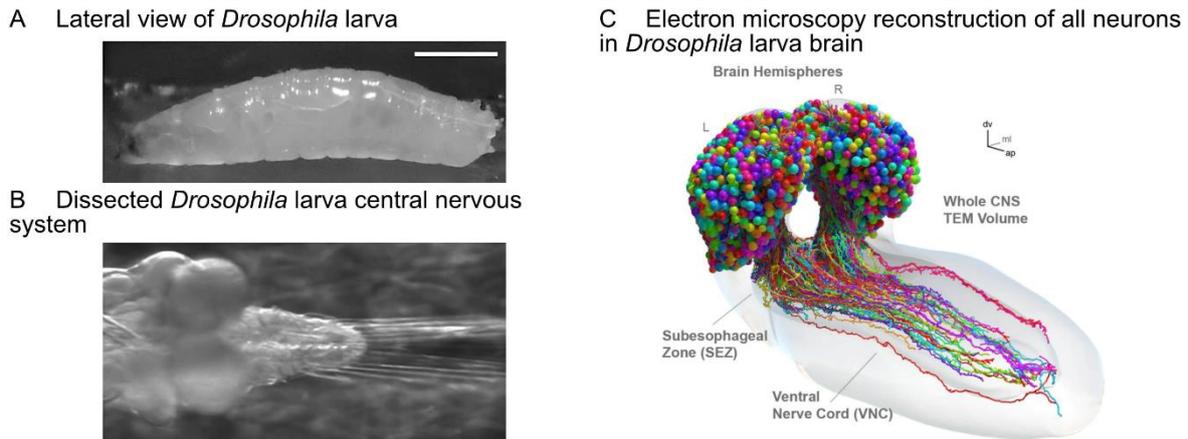


Figure I.D-1 Anatomy of *Drosophila* larva and its central nervous system. A. Image from (Kohsaka, Guertin, and Nose 2017), figure 1A. Lateral image of a third instar larva. Scale bar: 1 mm. B. Image from (Kohsaka, Guertin, and Nose 2017), figure 1C. Image of a dissected *Drosophila* larva brain. From left to right, we can see the brain lobes, the VNC and the peripheral nerves. C. Image from (Winding et al. 2023), figure 1A. Morphology of all *Drosophila* larva neurons whose cell bodies are located in the brain lobes, reconstructed from an electron microscopy dataset spanning the entire CNS of a first instar larva.

The simplicity of the larval *Drosophila* nervous system enabled researchers to map the connectome (or wiring diagram) of the entire brain of the *Drosophila* larva (Figure I.D-1-C) at the level of individual neurons (Winding et al. 2023). This was done using the dataset obtained by sequentially imaging the whole central nervous system of a first instar (L1) female larva using electron microscopy (EM) (Ohyama et al. 2015). The use of an L1 larva is especially practical since their brains are smaller, and thus easier to image and less time-demanding to reconstruct, while having very similar structural properties to later stages of larvae (Gerhard et al. 2017). EM images give nanometer-resolution images that allow to clearly see the neurons, their organelles, projections and synapses. This enormous dataset was made available through an online tool for a collaborative effort of manual annotation of neurons through the whole stack of EM images to reconstruct their anatomy and label their synapses (number and partners) (Schneider-Mizell et al. 2016). After years of effort exerted by many research laboratories, the whole brain connectome was published in 2023 (Ohyama et al. 2015; Jovanic et al. 2016; 2019; Zarin et al. 2019; Heckscher et al. 2015; Zwart et al. 2016; Miroschnikow, Schlegel, and Pankratz 2020; Winding et al. 2023; Lehman et al. 2023; C. Hu et al. 2017; Imambocus et al. 2022; Burgos et al. 2018; Yingtao Liu et al. 2023). At the time of writing this thesis manuscript, the reconstruction of the ventral nerve cord (VNC) of that larva is well on its way, albeit still ongoing (Ohyama et al. 2015; Jovanic et al. 2016; 2019; Zarin et al. 2019; Heckscher et al. 2015; Zwart et al. 2016;

Miroschnikow, Schlegel, and Pankratz 2020; Winding et al. 2023; Lehman et al. 2023; C. Hu et al. 2017; Imambocus et al. 2022; Burgos et al. 2018; Yingtao Liu et al. 2023).

Knowledge of the connectome of an organism is extremely precious for the study of the neural circuit mechanisms underlying its behaviors and their modulation. Indeed, it provides a detailed map of the synaptic connections between all of its neurons, allowing the identification and characterization of specific neuronal circuits associated with behaviors. Mapping the connections between neurons in a circuit allows to uncover specific connectivity motifs that give information about how information is processed and transmitted within the circuit (Jarrell et al. 2012; Jovanic et al. 2016; 2019; D. Lee et al. 2017; Liqun Luo 2021; Winding et al. 2023), thus providing researchers with candidate neurons to study in their experiments. Furthermore, it is possible to use the information from the wiring diagram of a circuit to create a computational model that can predict how changes in properties of neurons in the circuits might affect its associated behavior, which is valuable for designing experiments and better understand the intricacies of complex circuits (Jovanic et al. 2016; 2019; Masson et al. 2020; Ohyama et al. 2015; Schneider-Mizell et al. 2016; Eichler et al. 2017; Saumweber et al. 2018).

I. D. 2. Genetic tools

The *Drosophila* model organism provides access to a uniquely powerful genetic toolbox that allows for single-cell manipulations of neurons (protein expression, cell activation or inhibition) (Sivanantharajah and Zhang 2015; Eschbach and Zlatic 2020). This genetic approach is essential in the dissection of neural networks and their modulation. Out of the numerous tools available to *Drosophila* researchers, I will only describe a subset that I used during my PhD.

The most widely used genetic tool available to *Drosophila* researchers is the Gal4-UAS dual expression system. This tool allows for the expression of any transgene, targeted to specific cell populations. In this system, the Gal4 protein, derived from yeast, serves as a transcriptional activator. The tissue-specific promoter that drives Gal4 expression is known as the "driver" line, while the DNA sequence containing Gal4 binding sites, called the Upstream Activating Sequence (UAS), is present in the "responder" line along with the gene of interest. When a Gal4 driver line is crossed with a responder line containing the gene of interest downstream of the UAS element, the Gal4 protein binds to the UAS sequence and activates transcription of the gene specifically in the cells where the Gal4 driver is active. This results in spatially controlled expression of the gene of interest, allowing researchers to manipulate gene expression in a targeted manner (Brand and Perrimon 1993).

Additional tools have been developed to refine the temporal and spatial transgene expression driven by the Gal4-UAS system. (Luan et al. 2006) designed a version of the Gal4 protein that takes advantage of the fact that yeast transcriptional activators have separable functional domains that must both be present in order to drive transcription: a DNA-binding domain (DBD) and an activation domain (AD). They were able to split the DBD and AD of the Gal4 protein and use different genetic drivers to express each domain independently, in different cells of an individual, using two promoters (Luan et al. 2006). This leads to the Gal4 protein being complete and functional only in cells at the intersection of the drivers used to express the AD and DBD domains and thus allows for a great spatial refinement of the expression of a UAS-regulated transgene.

Another yeast-derived transcription regulator, Gal80, has become a powerful tool to refine the Gal4 driven transgene expression, both spatially and temporally. Gal80 is a repressor of the expression of the Gal4 protein. Expression of Gal80, similarly to that of Gal4, can be targeted to specific cells using different genetic promoters and will prevent the expression of the Gal4 protein in these cells (T. Lee and Luo 1999; Suster et al. 2004). It is thus possible to refine the expression profile of a Gal4 driver line by expressing Gal80 under the control of a different promoter, in which case UAS-regulated transgene will be expressed only in cells of the Gal4 driver profile that do not overlap with the Gal80 driver profile. Furthermore, (McGuire et al. 2003) developed a temperature sensitive version of the Gal80 protein that can be deactivated by heat exposure (30 °C) to time-selectively induce expression of the Gal4 protein, in cells targeted by its promoter, and thus the UAS-regulated transgene.

An alternative to the Gal4-UAS system is the LexA-LexAop dual expression system. Similarly to Gal4-UAS, the LexA-LexAop, transgene expression of controlled by the LexAop promoter which is activated by the binding of the LexA protein whose expression can be targeted to specific cells using different promoters (Lai and Lee 2006). To make it a more powerful tool, different versions of the LexA protein have been developed, for instance making it resistant or sensible to Gal80 inhibition or splitting it in a similar manner to the split-Gal4 (Lai and Lee 2006; Ting et al. 2011). The biggest strength of the LexA-LexAop system resides in that it can used in combination with the Gal4-UAS system to drive the expression of different transgenes in different cell populations, in the same individuals.

Such dual expression systems have become particularly valuable tools when used conjointly with the connectome. Indeed, it is now possible to precisely match the neurons targeted by a specific driver line to the reconstructed neurons in the connectome by expressing a fluorescent protein allowing imaging of the anatomy of all neurons targeted by the driver line in light microscopy. This permits functional

exploration of connectivity patterns found in the wiring diagram of circuits (Jovanic et al. 2016; 2019; Jovanic 2020; Eschbach et al. 2020; J. H. Simpson and Looger 2018).

I. D. 3. Neuron manipulation and recording

Taking advantage of the tools previously described, we can identify neurons putatively involved in a specific behavior and selectively express transgenes in these neurons. The tremendous catalog of available transgenes, and the relative ease of creating new ones for specific purposes, give *Drosophila* researchers the opportunity to exquisitely manipulate and monitor neuronal functions at different scales (from molecular to network scale).

I. D. 3. a. Monitoring neurons

Monitoring the activity of neurons in *Drosophila* is crucial for understanding neural circuits and their role in behavior. Various techniques have been developed to monitor neuronal activity, providing valuable insights into the functioning of the nervous system.

Calcium imaging is a widely used method to monitor neuronal activity in *Drosophila*. Genetically encoded calcium indicators (GECIs), such as the green GCaMP or red RGECO, allow the visualization of changes in intracellular calcium levels in real-time. When neurons are active, there is an influx of calcium ions, leading to an increase in fluorescence signal from the GECIs (Akerboom et al. 2012; A. S. Walker, Burrone, and Meyer 2013; Tian et al. 2009). This makes it possible to track neuronal activity at single-cell resolution and study the dynamics of neural circuits in a variety of contexts and in response to various stimulations. GECIs of different colors have been developed, allowing the simultaneous monitoring of different neurons to study their communication, by driving each GECI expression with a different genetic driver. This kind of imaging is particularly easy to implement in *Drosophila* larvae since their translucent cuticle allows both excitation and emission light through, permitting imaging of neurons in intact larvae.

The more technically challenging technic of electrophysiological recording remains a gold standard for monitoring neuronal activity with the highest temporal precision possible. Electrophysiological techniques such as voltage clamp, current clamp, and patch clamp recordings provide insights into the functional connectivity and activity patterns of neural circuits by measuring the electrical activity of neurons. The small size of the neurons in *Drosophila* made the use of electrophysiology on central neurons *in vivo* particularly challenging. (Rohrbough and Broadie 2002), such that it only appeared at the beginning of the 2000s and remains seldom used both in adult and larval *Drosophila* (Rohrbough and Broadie 2002; Wilson 2004; Gouwens and Wilson 2009;

Jovanic et al. 2016; Zwart et al. 2016; Tuthill et al. 2014). These technics allow the investigation of intrinsic electrical properties of neurons, their expression of ion channels, action potentials, and communications within neural circuits (Rohrbough and Broadie 2002; Baines and Bate 1998; Bykhovskaia and Vasin 2017; Goel, Li, and Dickman 2019). These information are crucial for the elaboration of computational models of neural circuits (Augustin, Zylbental, and Partridge 2019; Shiu et al. 2023).

Similarly to GECIs, genetically encoded voltage indicators (GEVIs) can be expressed in specific neuronal populations for voltage imaging. GEVIs allow to directly monitor membrane potential in neurons and even to visualize action potentials and subthreshold events with high spatial and temporal resolution (Pu et al. 2018; Borden et al. 2017; Cao et al. 2013). Voltage imaging is particularly complementary to electrophysiology as it provides a non-invasive way to record membrane potential, facilitating long-term imaging.

The extensive genetic toolbox, available for *Drosophila* studies, provides several ways to study protein expression in specific neurons of the *Drosophila* CNS using *in vivo* fluorescence imaging. To this aim, different libraries of *Drosophila* can be used to label neurons that express a protein of interest. The T2A-Gal4 library (P.-T. Lee et al. 2018) takes advantage of the self-cleaving T2A peptide whose sequence can be fused to a gene of interest, consequently cueing the ribosome to cleave the protein it is translating before resuming transcription. The T2A-GAL4 library for *Drosophila* was developed by inserting a T2A-GAL4 sequence into individual genes using CRISPR/Cas9 technology, leading to the Gal4 protein being expressed specifically in cells expressing that gene of interest, which can thus be labeled using a UAS line containing a fluorescent protein transgene to assess expression of a gene in specific cells. However, to get more information about the function and localization of the proteins, one needs to be able to precisely visualize the proteins themselves during their action. (Sarov et al. 2016) developed a *Drosophila* library allowing this by inserting a sequence coding for a green fluorescent protein (GFP) at the end of the coding sequence of genes of interest, leading to the production of a chimeric protein tagged with a GFP that can be followed using classical *in vivo* imaging technics.

I. D. 3. b. *Manipulating neurons*

In order to decipher the workings of neural circuits and behaviors, it is necessary to manipulate individual neurons while monitoring the activity of the circuit to discern their specific roles. Out of the extensive catalog of transgenes available to *Drosophila* researchers, several can be used to either ablate or enhance the activity of a specific neuron in a circuit (Venken, Simpson, and Bellen 2011).

One approach to study the activity of a neural circuit without a specific neuron is to genetically kill this neuron, by selectively expressing either toxins, diphtheria or ricin for instance (H. J. Bellen et al. 1992; Moffat et al. 1992), or pro-apoptotic genes, such as grim or reaper (Jaffri et al. 2020). However, killing a neuron is an extreme manipulation that can give rise to unwanted side effects, especially if the transgenes used are expressed throughout development. A more subtle approach is to inhibit the activity of the neuron either by blocking synaptic transmission using tetanus toxin (Sweeney et al. 1995) or shibire (Kitamoto 2001) or by preventing its depolarization which will not only block synaptic but all communication from the target neuron, for instance with the expression of inward-rectifying K⁺ channel Kir2.1 (Hodge 2009). On the other side of the spectrum, some transgenes facilitate the activation of neurons by increasing their excitability (Marella et al. 2006; Nitabach et al. 2006).

The most exquisite control over the manipulation of neurons is achieved using optogenetics, which consists in the genetic expression of a light activated ion channel. Different channels have been developed to allow for either the activation (Klapoetke et al. 2014) or the inhibition (Govorunova et al. 2015) of the target neuron. Depending on the tool used, shining a light of the appropriate wavelength will open the ion channel and consequently allow ionic movements that will either hyper- or depolarize the neuron. In absence of light, the channel remains closed, allowing a very precise temporal control over the manipulation of the activity of the neurons. Furthermore, taking advantage of the genetic toolbox of *Drosophila* allows to precisely select the neurons that will express the channel and thus to have a great spatial resolution for the manipulation. Finally, this technic is of particular interest for the *Drosophila* larva since their translucent cuticle allow optogenetic stimulation in intact behaving larvae (Murawski, Pulver, and Gather 2020).

I. D. 3. c. *Functional connectivity studies*

By combining both the monitoring of a neuron and the manipulation of one of its presynaptic partners (based on EM reconstruction data), it is possible to experimentally probe the effect of one neuron on another (whether it is excitatory or inhibitory) and the effectors responsible for this effect. Indeed, the combination of different dual expression systems allows the manipulation of one neuron's activity, for instance with optogenetics, while simultaneously monitoring the activity of a partner neuron, with GECIs for example (J. H. Simpson and Looger 2018; Jovanic 2020). This is a handy way to investigate the causal relationship between the activity of the manipulated neuron and the response of its partner neuron and is essential in confirming the functional role of anatomical connections discovered in the EM connectome dataset. By selectively manipulating individual neurons within a circuit and monitoring the downstream effects

using imaging techniques, it is possible to elucidate the flow of information and the functional roles of different neurons within the network.

The critical parameter to take into account for such experiments is compatibility of the excitation and emission wavelengths of all the different effectors used for manipulation and imaging, the selection of the effectors among the available catalog is thus of capital importance (Murawski, Pulver, and Gather 2020).

Similar approaches have been used in *Drosophila* to study various circuits including circuits controlling grooming (Hampel et al. 2015), courtship (Zhou et al. 2015), aggression (Hoopfer et al. 2015), backward locomotion (Takagi et al. 2017), etc...

I. D. 4. Behavioral assays

Drosophila larvae offer the advantage of a remarkably short developmental time, with the entire life cycle from egg to pupa lasting only a few days under laboratory conditions (Ormerod et al. 2017). This rapid development allows for efficient experimental design of high-throughput experiments taking advantage of the genetic manipulations possible in this model organism.

Despite their relatively simple CNS, *Drosophila* larvae exhibit a diverse repertoire of ethologically relevant behaviors including locomotion (M. Q. Clark et al. 2018), foraging (Wosniack et al. 2022), learning and memory (Weber et al. 2023), stimuli induced behaviors (Ohyama et al. 2013; Jovanic et al. 2016; Gershow et al. 2012), etc... Importantly, these behaviors are highly amenable to quantitative analysis and can be studied with specific high-throughput automated behavioral assays (Ohyama et al. 2013; Jovanic et al. 2016; Neckameyer and Bhatt 2016). This allows for the detection of even slight behavioral changes in response to external interventions, in combination with genetic manipulations.

I. D. 4. a. *Taxis behaviors*

A specific type of behavior that is deeply studied in *Drosophila* larvae is taxis. The term taxis refers to a directed movement or orientation of an organism or cell in response to a stimulus. This movement can be either towards (positive taxis) or away from (negative taxis) the stimulus. Taxis allow organisms to navigate their environment and locate favorable conditions or resources essential for survival and reproduction. The stimulus that elicits taxis can be of various types, including light (phototaxis), chemicals (chemotaxis), temperature (thermotaxis), wind (anemotaxis), etc...

Drosophila larvae are able to perform taxis behaviors in different types of sensory gradients, including phototaxis (Kane et al. 2013; Gepner et al. 2015), thermotaxis (Linjiao Luo et al. 2010; Klein et al. 2015), chemotaxis (Gomez-Marin and Louis 2014;

Gomez-Marin, Stephens, and Louis 2011; Davies, Louis, and Webb 2015; Gepner et al. 2015) and anemotaxis (Jovanic et al. 2019). The locomotor strategies used by the larvae to orient themselves and navigate in such gradients and their dynamics have been extensively characterized and are similar under different types of sensory stimulation (Gomez-Marin and Louis 2014).

When exposed to a sensory gradient, in order to navigate towards more favorable conditions, larvae regulate the probability of initiating a turn depending on the direction they are currently running in by decreasing it they are currently running in a favorable direction, and increasing it otherwise (Linjiao Luo et al. 2010; Jovanic et al. 2019; Gomez-Marin, Stephens, and Louis 2011). This is controlled by a steady increase (or decrease) in the strength of the signal as the larva is moving in a specific direction (Gomez-Marin, Stephens, and Louis 2011). When a run is interrupted, the larva casts its head laterally to sample its environment and detect small differences in the sensory gradient to determine which direction is more favorable (Gomez-Marin and Louis 2014). The direction of the first head sweep is random but the likelihood of the turn event ending with the acceptance of a particular head sweep is biased toward head sweep pointing in the more favorable direction (Linjiao Luo et al. 2010; Jovanic et al. 2019; Gomez-Marin, Stephens, and Louis 2011). Moreover, the size of the head sweep, and thus the angular amplitude of the turn, is increased if the larva was crawling towards the less favorable condition before the turn event (Linjiao Luo et al. 2010; Jovanic et al. 2019; Gomez-Marin, Stephens, and Louis 2011).

Despite this extensive characterization of the strategies used by *Drosophila* larvae to navigate sensory gradients, little is known on the neural processes involved in the transformation of the sensory inputs into the appropriate behavioral output. Most importantly, this description of taxis behaviors only accounts for one sensory gradient at a time while animals are constantly exposed simultaneously to a multitude of sensory gradients, which may have conflicting valences. Part of my PhD project focused on the simultaneous study of anemotaxis and chemotaxis in *Drosophila* larvae in a complex conflicting multisensory environment.

(Jovanic et al. 2019) described, within *Drosophila* larva, a network of sensory neurons, nerve cord neurons, and central neurons that collectively orchestrate the larvae's response to air currents. Chordotonal and multidendritic class III sensory neurons serve as key players in detecting and relaying information about air current speeds to local interneurons, first and second order SEZ neurons, and brain projection neurons. This connection to brain neurons makes it a likely center for the integration of wind sensory information with other, potentially conflicting, sensory modalities.

I. D. 5. Relevance to other species

Despite the simplicity of its nervous system, *Drosophila* exhibits a number of neurobiological similarities with vertebrates and even humans. Studies have shown that key molecular pathways essential for complex animal development, such as organogenesis, nervous system wiring, and cell proliferation, are highly conserved between *Drosophila* and vertebrates (Reiter et al. 2001; Bier and Bodmer 2004; Hugo J Bellen, Tong, and Tsuda 2010). This conservation extends to various complex behaviors that actually use similar neural substrates between *Drosophila* and vertebrates, such as circadian rhythms (Hugo J Bellen, Tong, and Tsuda 2010; Takahashi et al. 2008; Konopka and Benzer 1971; Sun et al. 2022) and learning and memory (Hugo J Bellen, Tong, and Tsuda 2010; Eschbach et al. 2020; Barco, Bailey, and Kandel 2006).

Moreover, (Reiter et al. 2001) showed that a very important proportion of human disease-associated genes (more than 75 % of the 929 studied genes) share significant sequence homology with *Drosophila* genes. Considering the extensive genetic toolbox available to drosophilists, *Drosophila* emerges as a highly pertinent model for acquiring novel insights that are both relevant and beneficial to human research.

Furthermore, neuropeptides are ubiquitous across the animal kingdom (Nässel 2002). Of particular interest regarding my PhD work, it appears that many of the genes coding for neuropeptides and their GPCRs in invertebrates are ancestrally related to mammalian genes (Nässel and Wegener 2011; Taghert and Veenstra 2003; Vanden Broeck 2001; Claeys et al. 2002; Hauser et al. 2006; 2008; Hewes and Taghert 2001; Hill et al. 2002; Hummon et al. 2006; Husson et al. 2007; McVeigh et al. 2008; Gard et al. 2009). This makes *Drosophila* larva an ideal model organism to study the neuropeptidergic modulation of neural circuits that can provide relevant information for understanding similar processes in vertebrates.

I. E. Description of the sensorimotor decision circuit

As described previously, the characterization of neural circuits is essential for the study of their modulation. (Jovanic et al. 2016) laid out the foundation of my PhD project when they characterized a three-layer recurrent sensorimotor neural network in *Drosophila* larvae VNC that determines their behavioral response to a mechanical stimulation (Figure I.E-1).

Schematic of the neuronal circuit orchestrating the decision between two mutually exclusive actions in response to a mechanical stimulation in *Drosophila* larva

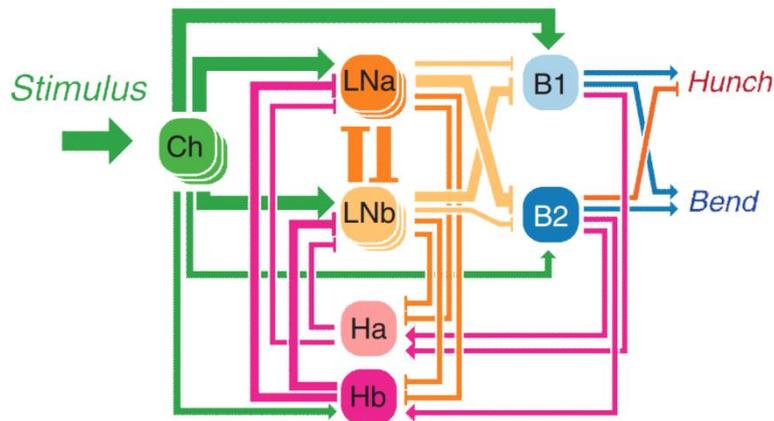


Figure I.E-1 Decision-making circuit orchestrating the choice between hunching or head-casting (bend) in *Drosophila* larva in response to an air puff. Image from (Jovanic et al. 2016), figure 2E. Sharp arrowheads represent excitatory connections, flat arrowheads are inhibitory connections. The width of the arrows are proportional with the number of synapses between the neurons. Green Ch are mechanosensory chordotonal neurons. Blue neurons are Basin projection neurons (B1 = Basin-1, B2 = Basin-2) at the output of the circuit. Orange and magenta neurons are reciprocally interconnected inhibitory interneurons implementing the decision between hunch and head-cast. LNa and LNb are feedforward inhibitory neurons and Ha (Handle-A) and Hb (Handle-B) are feedback inhibitory neurons.

At the entry of this circuit are mechanosensory chordotonal neurons that sense the mechanical stimulation and trigger the cascade of neural activity that leads to the selection of specific behavioral responses (Jovanic et al. 2016). These comprise eight subtypes of segmentally repeated peripheral sensory neurons whose cell bodies are attached to the cuticle (Okabe and Okano 1997; Singhania and Grueber 2014). The mechanosensory chordotonal neurons can sense different types of sensory modalities including wind (Jovanic et al. 2016; 2019; Yorozu et al. 2009; Ohyama et al. 2013), sound (Yorozu et al. 2009; W. Zhang et al. 2013; Ohyama et al. 2013), touch (Caldwell et al. 2003), temperature (Y. Kwon et al. 2010) and proprioception (Caldwell et al. 2003; Fushiki, Kohsaka, and Nose 2013). It appears that different subtypes of chordotonal neurons are differently tuned to different types of stimuli (Y. Kwon et al. 2010; Ohyama et al. 2013), we however lack genetic drivers that would allow to individually target chordotonal neurons and further characterize their specificities.

This network consists of two excitatory projection neurons (PNs), Basin-1 and Basin-2 that receive inputs from mechanosensory neurons and contribute to the generation of behavioral responses such as hunching (head retraction) and head-casting. Basin-1 promotes hunching when it is active alone, while the co-activation of Basin-1 and Basin-2 inhibit hunching to promote head-casting (Jovanic et al. 2016).

Two types of reciprocally connected local feedforward inhibitory interneurons that receive input from the mechanosensory chordotonal were identified in the circuit. These inhibitory interneurons preferentially target Basin-1 or Basin 2 and shape their activity by inhibiting and disinhibiting them. They are thus involved in the flexible implementation of the selection of a behavioral state through the reciprocal inhibition motif of inhibition motif (Jovanic et al. 2016).

Two types of disinhibitory feedback interneurons reciprocally connected with the feedforward inhibitory interneurons were identified downstream of the PNs. They play a crucial role in biasing behavioral choice and preventing sequence reversals: input from Basin-1 on these neurons leads to lateral disinhibition of Basin-2, thus promoting sequence transition from hunching to bending (Figure I.E-2). Once both PNs are activated, feedback disinhibition from these interneurons stabilizes the selected behavioral state and prevents reversals to previous behaviors (Jovanic et al. 2016).

Illustration of connectivity motifs found in the circuit and their role in the decision

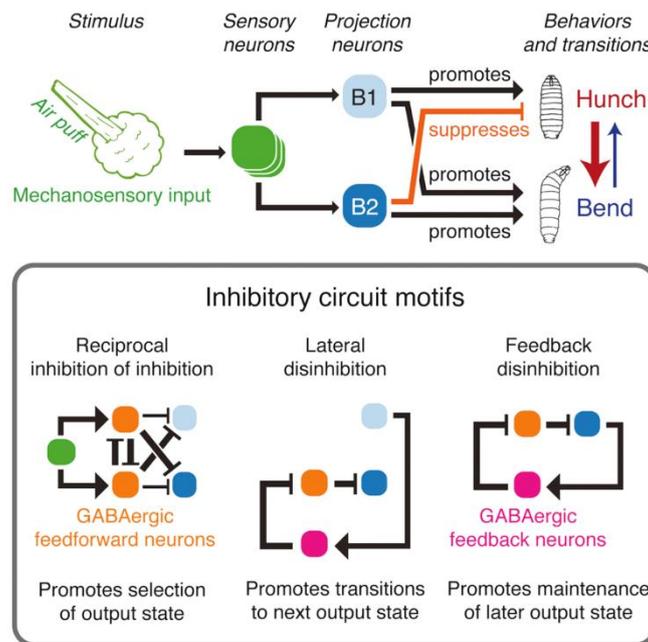


Figure I.E-2 Schematic representation of motifs in the sensorimotor decision circuit and their role in the decision. Image from (Jovanic et al. 2016), graphical abstract. The Basin-1 projection neuron (light blue) promotes both the hunch and head-cast (bend), while if Basin-2 (dark blue) is activated, it suppresses hunching to promote head-casting. The output state of the Basin projection neurons depends on the relative level of activation of the inhibitory interneurons involved in a reciprocal inhibition motif.

II. Aims

Despite the well-established impact of nutrition on brain function (Takeishi et al. 2020; Miroschnikow, Schlegel, and Pankratz 2020; Zandawala, Yurgel, et al. 2018; Bhumika and Singh 2018; Sengupta 2013), the causative link between nutrients and the changes in non-feeding-related neural circuit function remains poorly understood. To be able to characterize the neural circuit changes that occur upon changes in feeding state, one needs to establish causal relationships between the activity of individual neurons and distinct, measurable behaviors. Additionally, mapping the connections among all neurons within the circuit with synaptic precision is essential. Furthermore, the ability to manipulate the activity or protein expression in individual neurons of a circuit while simultaneously monitoring their activity (e.g., through calcium imaging) or the behavior of the organism is crucial to be able to study the modulation of the circuit caused by changes in the feeding state of the organism.

My supervisor, Tihana Jovanic, and colleagues have in the past characterized at synaptic resolution a neural circuit that controls the choice between two competing sensorimotor responses to following a mechanosensory stimulus: a “startle” Hunch or an “escape” Head cast (Jovanic et al. 2016). They reconstructed the complete network from the sensory input to the output at the level of projection neurons, up to the synaptic level. Then, they characterized the interactions between different neurons in the network: two types of projection neurons, B1 and B2, drive exclusive behaviors, and different types of inhibitory feedforward and feedback interneurons shape the competition between these mutually exclusive behaviors and control their dynamics. Moreover, research from my host laboratory, parallel to my own, has shown a crucial role of two interconnected inhibitory interneurons of the circuit (G2 and Hb) in the feeding state-specific modulation of the behavioral response of the larvae, as described in the scientific article in section III.A of this manuscript.

The main aim of my PhD project was to use this knowledge as a starting point to study the neural circuit mechanisms underlying feeding state-dependent modulation of non-feeding-related neuronal circuits and the effectors involved, from single neuron activity to behavior. To address this, I took advantage of the uniquely powerful *Drosophila* larva model which allowed me to combine *in vivo* single-cell manipulation of neuropeptides and receptors expression in a target neuron type while monitoring its activity with calcium imaging, or automated behavioral monitoring, all informed by the connectome reconstructed from an electron-microscopy dataset of the entire central nervous system (Jovanic et al. 2016; Ohyama et al. 2015; Masson et al. 2020; Schneider-Mizell et al. 2016).

My work mainly focused on the modulatory action of two neuropeptides (sNPF and NPF) on this circuit. sNPF and NPF are functional homologs of the mammalian NPY, instrumental in the regulation of feeding behavior. Their expression increases in fasted larvae (Nässel and Wegener 2011; Fadda et al. 2019; Yeom et al. 2021; K.-S. Lee et al. 2008; 2004; K. S. Kim et al. 2021; Wu, Zhao, and Shen 2005). Incidentally, the inhibition of the sNPF-R suppresses eating (K.-S. Lee et al. 2004), and the release of NPF reduces aversion to low-quality food (Wu, Zhao, and Shen 2005). Nevertheless, sNPF was shown to be involved in non-feeding-related behaviors such as response to mechanonociception as well (C. Hu et al. 2017; Imambocus et al. 2020). I sought to investigate how the feeding state information is relayed to the sensorimotor decision circuit (between Hunch and head-cast), identify the effectors and the neural circuit mechanisms by which they exhibit a modulatory action on larval decisions. To achieve this goal, I examined the role of these neuropeptides in transmitting feeding state-dependent information to the circuit (Eloïse de Tredern et al. 2023).

Additionally, as a side project during my PhD, I aimed to enhance our understanding of the state-dependent modulation of behavioral decisions in more complex paradigms. To this aim, I adapted our behavioral setup to be able to present larvae with multiple sensory stimulations at the same time.

III. Methods and Results

I will report the methodology and results of the majority of the work performed during my thesis in the format of a scientific article, for which a preprint is available on BiorXiv at <https://www.biorxiv.org/content/10.1101/2023.12.26.573306v2> (Eloise de Tredern et al. 2024), in the section III.A of this manuscript. The article is currently in revision at Nature Communications. In this scientific article, my work uncovered a feeding state-dependent neuropeptidergic modulation of sensorimotor decisions in *Drosophila* larva. For this article, I performed and analyzed all calcium imaging experiments related to sNPF and NPF neuropeptides and to the mechanosensory chordotonal neurons. I also performed the behavioral experiments upon sNPFR knockdown in Handle-B and Griddle-2 and the immunostainings for sNPFR and NPF, with the help of Sandra Autran. Finally, I was strongly involved in the refinement of the machine learning classification method used to generate all the behavioral quantifications in the article, as well as in the production of the figures.

In addition to the results presented in the article, experiments were performed to investigate the possibility that the feeding state of the larvae could exert part of its modulatory action by targeting the mechanosensory chordotonal neurons involved in the sensing of the wind stimulation used in our research. Indeed, altering the sensitivity of sensory neurons to stimuli is a known mechanism by which the feeding state can modulate feeding-related behavioral decisions (Bargmann 2012; Sengupta 2013; Shanahan and Kahnt 2022; Takeishi et al. 2020; Inagaki, Panse, and Anderson 2014). While these findings were not included in the original publication, they contribute valuable insights that complement and enrich our understanding of feeding state-dependent modulation of sensorimotor decisions. These supplementary results are presented in section III.B of this manuscript.

A secondary aim of my PhD project was to develop a behavioral assay that would allow the study of multisensory integration and how it is modulated by the feeding state of the larvae. To address this question, we decided to couple mechanical stimulation with food-related olfactory cues to put larvae in a conflicting sensory environment that would force them to choose which stimulus to follow. Over the course of my PhD, I adapted our behavior tracking apparatus to be able to combine a gradient of odor concentrations with a gradient of air speeds and supervised several interns who tested the apparatus. The apparatus and results gathered by interns that I co-supervised will be described in section III.C of this manuscript.

III. A. Feeding state-dependent neuropeptidergic modulation of reciprocally interconnected inhibitory neurons biases sensorimotor decisions in *Drosophila*

III. A. 1. Article summary

My work focused on the characterization of the role of the neuropeptides NPF and sNPF in the feeding state-dependent modulation of a *Drosophila* larva sensorimotor decision circuit (Jovanic et al. 2016). Combining a genetic co-labeling of target neurons and neuropeptide receptors with immunostaining, we show that both sNPF and NPF receptors are expressed in mechanosensory neurons. However, optogenetic stimulation of these mechanosensory neurons in larvae exposed to different diets show that their modulation is not necessary to bias larva decisions in response to a mechanical stimulation.

Moreover, we show that the projection neurons of the circuit do not express receptors for either sNPF or NPF, suggesting that a feeding state-dependent bias of the behavioral decision of the larvae carried by neuropeptides would be targeted to the interconnected inhibitory interneurons of the circuits. We show expression of sNPF in the two interconnected inhibitory interneurons that orchestrate the behavioral choice in the circuit, and expression of NPF in only one of them, suggesting that the interneurons are indeed targets of modulation while the projection neurons relay the decision to the motor side.

We further show that a reduction of sNPF signaling on these interneurons biases the choice of the larvae towards less hunching and more head-casting by oppositely regulating their activity, partially recapitulating a "sucrose-like" or "starvation-like" phenotype.

Moreover, we found, in the EM-connectome dataset (Ohyama et al. 2015; Jovanic et al. 2016), synaptic connections between one pair of NPF-releasing neurons and one of the inhibitory interneurons that promotes head-casting at the expense of hunching. We show that the activity of this NPF-releasing neuron is increased upon sucrose feeding and starvation. Finally, we show that NPF is required for the modulation of larval baseline locomotion by the feeding, as well as the increase in the intensity of the responses of the inhibitory interneuron, which suppresses hunches and promotes head-casts, to mechanical stimulation upon sucrose feeding and starvation.

Altogether, sNPF and NPF differentially modulate interconnected inhibitory interneurons in a feeding state-dependent manner, influencing circuit computations and thus leading to a bias in the behavioral choice of the larvae in response to a mechanical stimulation.

III. A. 2. Article manuscript

Feeding-state dependent neuropeptidergic modulation of reciprocally interconnected inhibitory neurons biases sensorimotor decisions in *Drosophila*

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III. A. 2. a. Abstract

Animals' feeding state changes behavioral priorities and thus influences even non-feeding related decisions. How is the feeding state information transmitted to non-feeding related circuits and what are the circuit mechanisms involved in biasing non-feeding related decisions remains an open question. By combining calcium imaging, neuronal manipulations, behavioral analysis and computational modeling, we determined that the competition between different aversive responses to mechanical cues is biased by feeding state changes. We found that this is achieved by differential modulation of two different types of reciprocally connected inhibitory neurons promoting opposing actions. This modulation results in a more frequent active type of response and less frequently a protective type of response if larvae are fed sugar compared to when they are fed a balanced diet. The information about the internal state is conveyed to the inhibitory neurons through homologues of the vertebrate neuropeptide Y known to be involved in regulating feeding behavior.

III. A. 2. b. *Introduction*

Physiological states like hunger and thirst are powerful regulators of behavior across the animal kingdom due to strong homeostatic drives that are critical for survival (Allen et al. 2019; Anderberg et al. 2016; Burnett et al. 2016; Eiselt et al. 2021; Jourjine 2017; Jourjine et al. 2016; Smith and Grueter 2021). For example, across model systems, food-deprivation was shown to modulate responsiveness to stimuli by influencing sensory neurons and sensory pathways (Inagaki, Panse, and Anderson 2014; Ko et al. 2015; Loch, Breer, and Strotmann 2015; Sayin et al. 2018), suggesting that food-deprivation can alter the perceived value of a stimulus, which in turn affects the behavioral decisions. Various studies have implicated changes in central processing (Bräcker et al. 2013; Crossley, Staras, and Kemenes 2018; Filosa et al. 2016; Padilla et al. 2016) which lead to changes in behavioral decisions in hungry animals. However, the detailed neural circuit mechanisms of this state-dependent flexibility of behaviors remain largely unknown.

Internal drives (e.g., hunger and thirst) need to be balanced by environmental demands such as the need to avoid dangers. Avoiding dangers is a critical instinctive behavior that needs to be balanced with finding and consuming food in order to ensure survival. Avoidance behaviors tend to be robust, which makes them excellent systems for studying neural bases of behavior (Card 2012; Evans et al. 2019; Imambocus et al. 2022; Peek and Card 2016); yet they also need to be flexible in order for animals to adapt their behavioral strategies to different contexts and according to different internal states (Evans et al. 2019; Ache et al. 2019; Devineni and Scaplen 2022; Oram and Card 2022). Feeding states and contexts can for example influence both the tolerance to the level of threat and action selection during threat avoidance (Card 2012; Evans et al. 2019; Imambocus et al. 2022; Schadegg and Herberholz 2017).

At the neural circuit level, such behavioral flexibility is thought to be implemented by neuromodulation (modulation of existing synaptic connections by neuropeptides, for instance) that could bias the outcome of competition between diverse behaviors. The outcome could in that case differ depending on the neuropeptide released (Dickinson 2006; Eriksson et al. 2017; Krasne and Edwards 2002; Lin, Senapati, and Tsao 2019; Marder 2012; Taghert and Nitabach 2012; Tierney 2020). Alternatively, information rerouting (using alternative circuit pathways) (Bräcker et al. 2013; Crossley, Staras, and Kemenes 2018; Lewis et al. 2015) where information is processed differently depending on the context or state, could alter behavior choice in a context-dependent manner. The types of circuit motifs underlying competitive selection must allow for such flexible processing of information. However, the detailed neural circuit mechanisms underlying the state-dependent modulation of behavior, the neuromodulators

involved, and their mechanism of actions on specific circuits, especially those that pertain directly to non-feeding or non-water seeking behavior, are not well understood.

This paper addresses the understudied aspect of the influence of physiological drives on competitive selection during avoidance behaviors and investigates the neural circuit mechanisms involved in a powerful model organism for neural circuit analysis: the *Drosophila* larva (Jovanic 2020). *Drosophila* larvae are ideally suited for combining comprehensive, synaptic-resolution circuit mapping in electron microscopy (EM) across the nervous system (Eschbach and Zlatic 2020; Ohyama et al. 2015; Jovanic et al. 2016; Schneider-Mizell et al. 2016) with targeted manipulation of uniquely identified circuit motifs at the individual neuron level, which makes it possible to establish causal relationships between circuit structure and function in a brain-wide manner. In addition, evolutionarily conserved neuropeptidergic and hormonal pathways in *Drosophila* have been shown to regulate its diverse behaviors (Sayin et al. 2018; Imambocus et al. 2022; Lin, Senapati, and Tsao 2019; C. Hu et al. 2017; Nässel and Zandawala 2019).

Previous studies have described larval avoidance response to a mechanical stimulus (air-puff) and detailed the neural circuit underlying the competition between “Hunching”, a protective or “startle-like” type of behavior, and “Head Casting”, an “active” exploratory type of behavior that can lead to escape (Jovanic et al. 2016; Lehman et al. 2023; Gershow et al. 2012; Jovanic et al. 2019; Gomez-Marin and Louis 2014; Klein et al. 2015). We identified circuit motifs underlying competitive interactions between behavioral actions (reciprocal inhibition of inhibition) and sequence transitions (lateral and feedback disinhibition) between the two behaviors. These types of motifs based on disinhibition would allow for flexible behavioral selection in a context/state dependent manner. By combining calcium imaging, neuronal and neuropeptide manipulation at the single cell level with automated tracking, behavior classification and computational modeling, this work shows that larval responses to the air-puff are biased towards less protective actions and towards more active, exploratory actions upon changes in their feeding condition. We determine that this bias is due to the differential modulation of two reciprocally connected inhibitory neurons that drive competing behaviors (Hunching and Head Casting): the activity of the neuron that promotes the protective Hunching is decreased and the activity of the neuron that inhibits Hunching is increased. We also show that the modulation at the level of reciprocally interconnected inhibitory neurons results in a bias at the level of the network output, towards a state that will lead to Head Casting at the expense of Hunching upon feeding on sucrose. Finally, we determine that NPF and sNPF modulate the activity of the reciprocally interconnected inhibitory neurons to bias the behavioral output in a feeding state-dependent manner.

III. A. 2. c. *RESULTS*

(1) *Changes in feeding conditions for short periods of time affect larval feeding and locomotion*

To study the effect of diet on behavioral decisions as a result of cognitive control and prioritization of needs and motivations rather than to long-term physiological changes that would affect circuit properties, we sought to establish a food-deprivation protocol of short duration. We determined the shortest duration of food deprivation that was sufficient to induce quantifiable changes in behavior. Depriving larvae of food completely (by putting them on a water-soaked filter paper) or feeding them on 20% sucrose only (and therefore depriving them of proteins and increasing their sugar intake) for 90 mins was sufficient to alter larval locomotion. Starved larvae and larvae fed on sucrose moved at a higher speed and spent more time Crawling than normally-fed larvae, eventually dispersing faster in less-curved trajectories (Fig. 1D, Extended Data Fig. 1A-B). These changes in locomotion are consistent with increased exploration and are likely due to an increased drive to find nutrients caused by deprivation. In order to determine that the 90 minutes feeding deprivation protocol induced changes in behavior that were reversible, we monitored locomotion in these larvae upon refeeding them for 15 minutes. Upon refeeding, larvae were slower and their locomotion phenotype was similar to the one of larvae that were constantly fed (Extended Data Fig. 1C).

To determine whether the larval need for nutrients was affected by the feeding protocols that they were subjected to, the consumption rate of different foods was quantified in different feeding conditions. Starved larvae significantly increased their intake of standard *Drosophila* food and yeast (rich in amino acids) compared to both normally-fed larvae and larvae that were fed on sucrose (Fig. 1A), which is consistent with a deficit in nutrients in starved larvae. Larvae fed on sucrose significantly increased their intake of yeast, which is consistent with a deficit in amino acids (Fig. 1A). These larvae, however, did not increase their intake of standard *Drosophila* food, suggesting that the increased sugar consumption might suppress their intake of carbohydrate-rich food. To test whether larvae fed on sucrose were repulsed by sugar, a choice assay was performed: larvae were added to the middle of an agar plate where half of the plate was covered in agar and the other half in agar mixed with sucrose. The larvae were then monitored for 15 minutes (Fig. 1C). After the 15 minutes, more larvae were found on the agar+sucrose half of the arena in normally-fed and starved larvae, with a preference index increasing over time, while larvae fed on sucrose showed a decrease in preference for agar supplemented with sucrose. Larvae fed on only 20% sucrose for 90 minutes significantly decreased their sucrose intake compared to fed and starved larvae, and their sucralose intake was similar to that of normally-fed and starved larvae (Fig. 1A4), suggesting that their avoidance of sugar was mediated by energy sensing pathways and not taste.

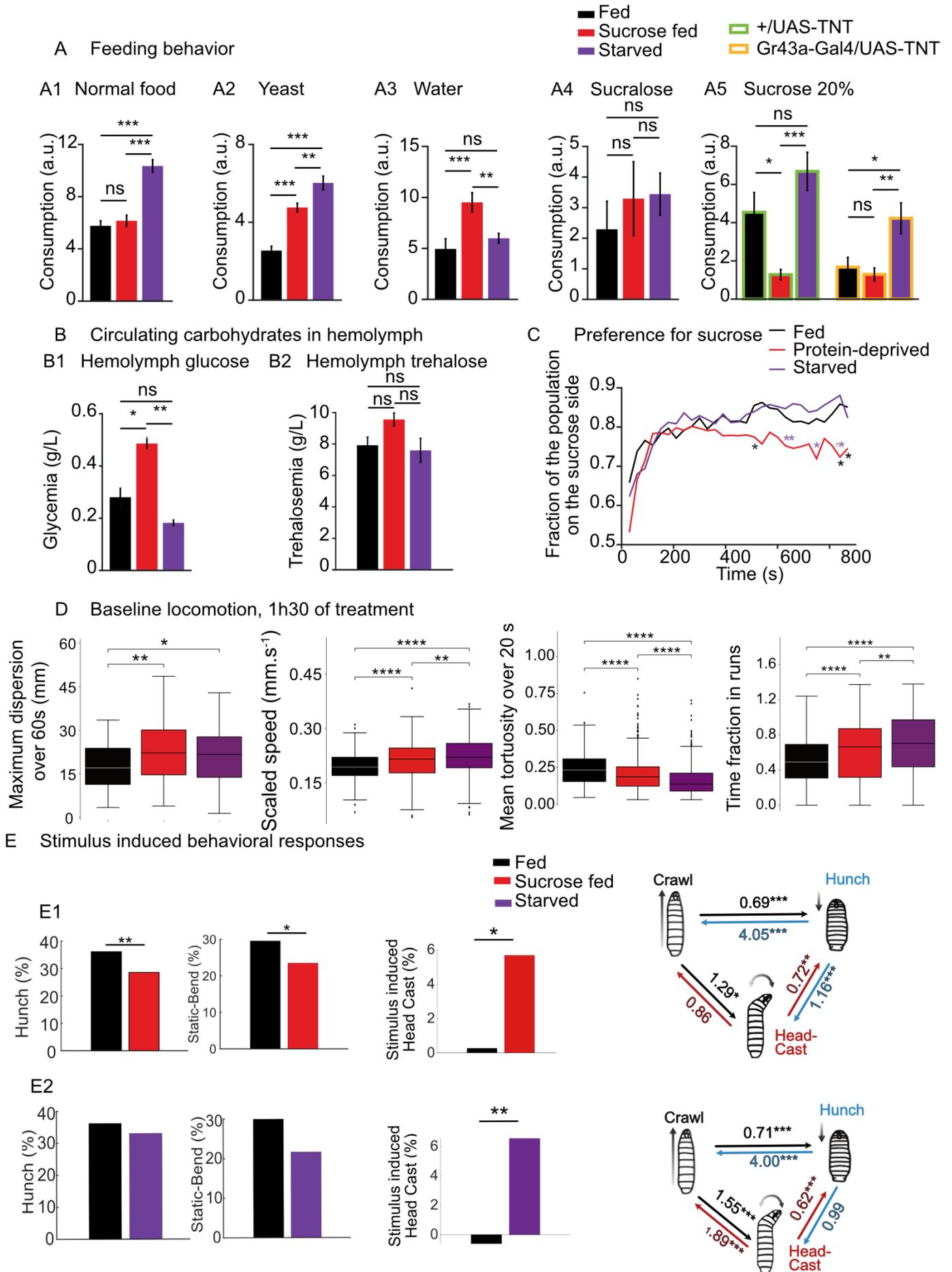


Fig. 1. Changes in physiology and behavior of food-deprived larvae. A: Larval feeding on different substrates was quantified, in control animals fed ad-libitum, in animals fed on sucrose or in animals subjected to complete starvation during 90 min. A1: Starved animals increased their feeding on a standard food medium as compared to fed animals, while animals fed on sucrose only did not (ANOVA, $n = 35-41$ larvae, Tukey post-hoc test ***: $p < 0.001$). A2: Both animals fed on sucrose only and starved animals increase their yeast feeding as compared to normally fed larvae (ANOVA, $n = 51-59$ larvae, Tukey post-hoc test ***: $p < 0.001$, **: $p < 0.01$). A3: While starved animals consume a similar amount of water as fed ones, animals fed on sucrose only double their water consumption as compared to fed and starved larvae (ANOVA, $n = 52-54$ larvae, Tukey post-hoc test ***: $p < 0.001$, **: $p < 0.01$). A4: Consumption of the non-energetic sweetener sucralose was similar among conditions ($n = 25-32$ larvae). A5: Larvae that had fed on a 20% sucrose solution for 90 min decreased their sucrose intake as compared to fed and starved larvae (ANOVA, $n = 29-33$ larvae, Tukey post-hoc test ***: $p < 0.001$, *: $p < 0.05$). Gustatory neurons inhibition (Gr43a-Gal4>TNT) decreases sucrose intake in fed larvae to the level of larvae fed on sucrose, while the consumption of starved larvae remains higher (ANOVA, $n = 24-33$ larvae, Tukey post-hoc test **: $p < 0.01$, *: $p < 0.05$). B: Concentration of glucose and trehalose in larval hemolymph. B1: Glucose concentration is increased in the hemolymph of larvae that were fed on 20% sucrose solution for 90 min compared to larvae fed on a standard diet and to starved animals (ANOVA, $N = 3$ samples from 5 larvae each, Tukey post-hoc test **: $p < 0.01$, *: $p < 0.05$). B2: Trehalose concentration is similar in the hemolymph of larvae fed on the different diets ($N = 6$ samples from 10 larvae each). C: Place preference assay for sucrose. Larvae that fed on a 20% sucrose solution for 90 min exhibit a decreased glucose preference compared to normally fed and starved larvae ($n = 79-133$ larvae, Chi-2 test **: $p < 0.01$, *: $p < 0.05$). D: Analysis of larval locomotion in the absence of sensory stimulus. After 90 min of sucrose feeding or complete starvation, larval dispersion, time spent crawling and speed are significantly increased, while the tortuosity of the trajectory is decreased compared to larvae fed on a standard diet (Mann-Whitney test, ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$). E: Manipulating the feeding state modulates behavioral responses to mechanosensory stimuli. Behavior in response to air-puff during the first five seconds uoin stimulus onset, for, E1 in larvae fed on sucrose only ($n = 592-629$ larvae) and E2 starved larvae compared to larvae fed on standard food ($n = 704-771$ larvae, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$). See also Extended Data Fig. 1 and Supplementary Tables 2, 3, 4 and 5.

To determine whether the 90-minute food-deprivation or sucrose diet protocols caused changes in glucose levels, the hemolymph glucose levels were quantified in the different feeding conditions. Glucose levels were slightly but not significantly decreased in starved larvae compared to the fed larvae. In larvae that were fed on 20% sucrose, the glucose level was significantly increased compared to both normally-fed and starved larvae (Fig. 1B). This high glucose level suggests that larvae fed on sucrose suppress consumption of carbohydrates due to high circulating levels of glucose. Finally, the water consumption of larvae in different feeding conditions was quantified.

Larvae fed on 20% sucrose increased their water consumption significantly compared to both normally-fed and starved larvae (Fig 1A3), likely due to changes in extracellular osmolality as a result of high sugar intake (Jourjine et al. 2016). We therefore tested whether rehydration would reduce the increase in exploration and locomotion observed in larvae fed on sucrose. Indeed, after larvae fed on sucrose only were put on water for 15 minutes, their locomotion was similar to the ones of normally fed larvae (Extended Data Fig. 1D).

Altogether, these results suggest that depriving larvae of nutrients for 90 minutes is sufficient to alter feeding and larval locomotion and that these changes are due to lack of nutrients and lower energy levels in starved larvae and protein hunger and thirst in larvae fed on sucrose. The observed increase in locomotion in larvae deprived of nutrients would thus likely result from an increase in motivational drive to find the missing nutrients.

(2) *Changes in physiological states affect sensorimotor decisions in response to an air-puff*

To determine whether starvation and a sucrose-only diet can affect non-feeding-related behaviors, we monitored *Drosophila* larva sensorimotor decisions in response to an aversive mechanical stimulus, the air-puff. In response to an air-puff, larvae perform probabilistic sequences of five mutually exclusive actions that we have characterized in detail in the past (Jovanic et al. 2016; Masson et al. 2020): Hunch, Bend, Stop, Back-up, and Crawl. We have also identified circuit motifs and characterized the neural circuit mechanisms underlying the competitive interactions between the two most prominent actions (i.e. the Hunch and the Bend) that occur in response to an air-puff (Jovanic et al. 2016). The model in that study predicts that the different activation levels of inhibitory neurons determine which actions will take place: the Hunch, Bend, or the Hunch Bend sequence.

With characterized neurons and synaptic connectivity between the neurons, as well as the availability of driver lines that label neurons of interest, this circuit provides an excellent system to study whether the activity of the neurons is modulated by the changes in an animal's physiological state.

To determine whether the feeding state modulates larval behavior in response to the mechanosensory stimulus and, by extension, whether sensorimotor decision-making in response to an air-puff is an adequate behavioral paradigm to study the effects of food deprivation on neural circuit activity, we compared larval behavioral responses to an air-puff after subjecting them to the different feeding protocols (as described in the first section of the Results). For this purpose, we used automated tracking (Ohyama et al. 2013) to monitor larval behavior in response to an air-puff and updated the machine-learning-based classification method developed in our previous work (Masson et al. 2020) to compute probabilities of the different actions that occur

in response to an air-puff. The new classifiers were trained on larvae fed on different diets, thus taking into account a broader range of behavioral dynamics, and separated the bend behavioral category into two types of bending behavior: Static Bends, a form of protective action where the larva responds to the stimulus by immobilizing in a curved position for a period of time, and the exploratory active Head Casts that can lead to escape (Lehman et al. 2023).

Our analysis showed that larvae fed on sucrose Hunch less (and perform less of Static Bends) and Head Cast more (Fig. 1E1). Similarly, starved larvae performed less Static Bends and more Head Casts (Fig. 1E2). Transitions to Hunching were decreased in larvae fed on sucrose and starved larvae, while transitions to Head Cast were increased (from Hunching to Head Casting in larvae fed on sucrose only and from Crawl to Head Casting in both starved larvae and larvae fed on sucrose) (Fig. 1E). Increasing duration of sucrose feeding and starvation to 5h resulted in similar phenotypes (Extended Data Fig. 1E, F).

Using our machine-learning based classification to monitor internal state induced behavioral changes revealed that changing feeding conditions for short periods of time alters sensorimotor decisions in response to an aversive mechanical cue and results in less protective type of actions, and more actions consistent with active exploration and escape.

(3) *The feeding state does not affect the responses of chordotonal sensory neurons to a mechanical stimulus*

To test whether changes in feeding conditions alter the perceived value of a stimulus, which in turn would affect the behavioral decisions, calcium responses in sensory neurons that sense the air-puff, the chordotonal sensory neurons (Jovanic et al. 2016; 2019; Masson et al. 2020; Ohyama et al. 2013) were monitored in response to a moderately strong mechanical stimulus in the three different feeding conditions: normally fed, fed on sucrose and starved (Extended Data Fig. 2B). We found no significant differences in chordotonal calcium responses in the different feeding states (Fig. 2B, Extended Data Fig. 2A), although the responses were slightly higher in starved larvae compared to the larvae fed on standard food and on sucrose only. To ensure that the changes in feeding conditions did not influence protein expression and, by extension, GCAMP expression levels, GFP was genetically expressed in the neurons and its expression level was quantified by comparing the fluorescence in larvae exposed to all three feeding conditions. No differences were found in GFP fluorescence in the three feeding states (Extended Data Fig. 2C). The results therefore suggest that chordotonal responses are not significantly affected by starvation or sucrose feeding and that the behavioral changes observed in the different feeding conditions are not due to the changes in stimulus sensitivity at the level of sensory neurons. We further confirmed this by optogenetically activating chordotonal sensory neurons in larvae in all three feeding states using a driver that labels all eight subtypes of chordotonal sensory

neurons: R61D08. We found that although the activation of chordotonal sensory neurons in all three feeding states was the same due to optogenetic stimulation, larvae performed fewer Hunches and more Head Casts when they were fed only on sucrose than when they were normally fed (Fig. 2C). Similarly, the modulation of behavior when they were starved could be observed upon chordotonal optogenetic activation (Extended Data Fig. 2D). Altogether, these results show that the feeding state-dependent modulation could target neurons downstream of the chordotonal sensory neurons.

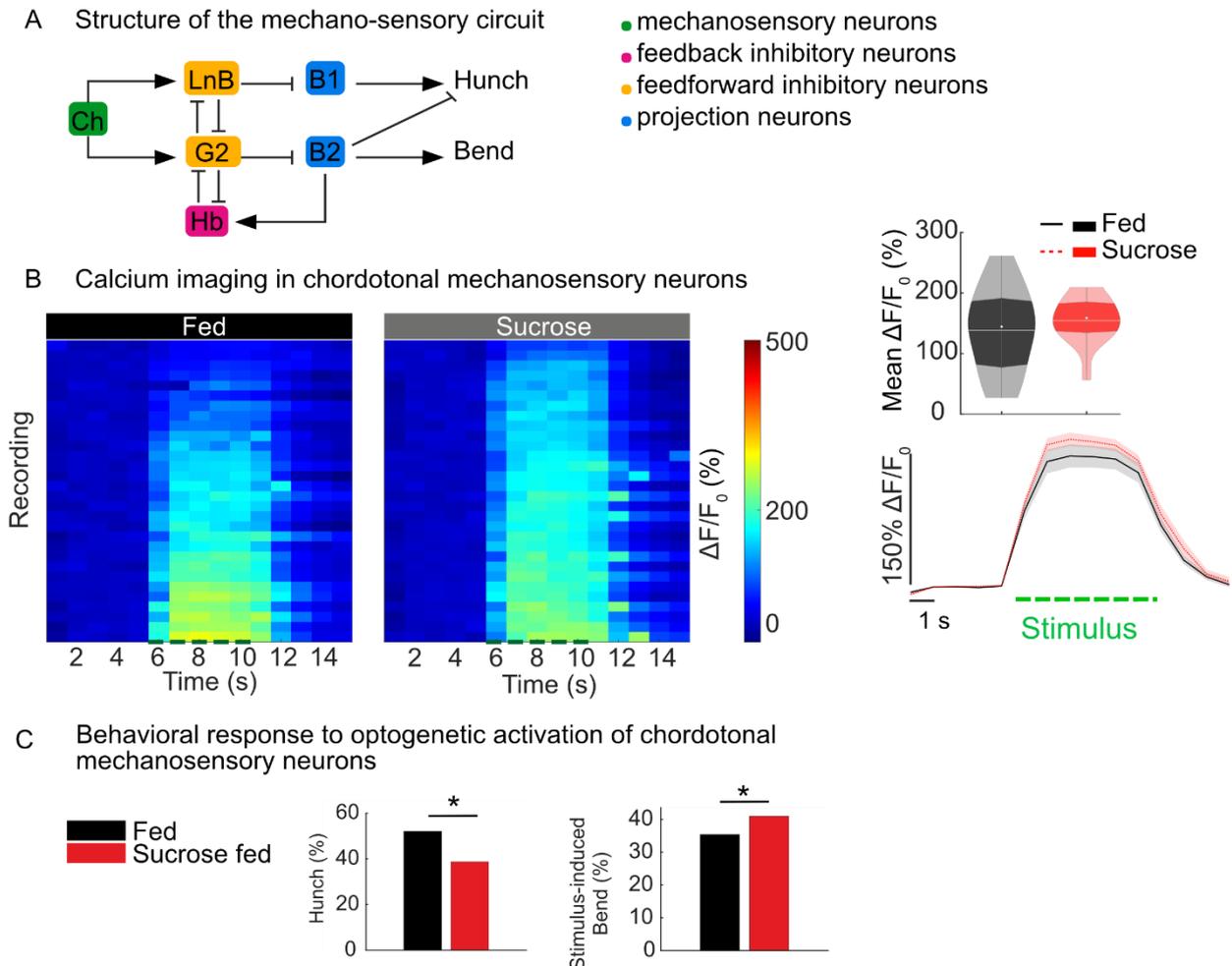


Fig. 2. Feeding state dependent bias in sensorimotor responses does not come from the modulation of sensory neurons. A: Organization of the circuit. B: Calcium responses to mechanical stimulations in chordotonal neurons larvae fed on sucrose only and on standard food (R61D08-Gal4/UAS-GCaMP6s). Left panel: calcium responses of chordotonal neuron projections in the VNC from different individuals fed on different diets. Lower right panel: mean calcium response of chordotonal neurons over time. The green dashed line corresponds to stimulus duration. Upper right panel: calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of chordotonal mechanosensory neurons is unchanged in animals fed on sucrose as compared to larvae fed on standard food ($n = 10$ larvae, 3 trials per larva, t -test $p = 0.249$). C: Optogenetic activation of cho in larvae fed only on sucrose. Hunch probability is computed during the first 2 seconds from stimulus onset. Bend is the mean probability during the first 10 seconds from stimulus onset, corrected by the baseline recording prior to the stimulus. ($n = 192$ larvae for fed, 168 for sucrose fed, $p = 0.011$ for Hunch, 0.028 for Bend). See also Extended Data Fig. 2 and Supplementary Tables 4 and 6.

(4) *Feeding states modulate the output of the circuit for the choice between Hunch and Bend*

To determine whether the changes in feeding state influence the output of the network for the selection between the Hunch and the Bend, characterized in previous work (Jovanic et al. 2016), calcium responses of projection neurons Basin-1 and Basin-2 were monitored at different intensities of mechanical stimulation in the three feeding states. Basin-1 and Basin-2 are differentially involved in Hunching and Bending, as shown in (Jovanic et al. 2016): Basin-1 is required for both Hunching and Bending, while Basin-2 promotes Bending and inhibits Hunching. Responses in Basin-2 neurons can thus be used as a read-out of the Bend state: if Basin-2 is ON, the larva will Bend, and if it is OFF, the larva will Hunch. We found that for most stimulus intensities, the Basin-1 responses remain only mildly affected by the sucrose-only diet (Fig. 3A, Extended Data Fig. 3C). Only for the very weak stimulus intensity do we observe a decrease in Basin-1 response (Extended Data Fig. 3C). In the starved condition, the effect on Basin-1 neurons was similar to the sucrose-only feeding condition (Extended Data Fig. 3A, C). The activity of Basin-2, on the other hand, was moderately increased in larvae fed only sucrose at all intensities except at the lowest stimulus intensity (Fig. 3B, Extended Data Fig. 3D), where there was no difference between Basin-2 responses in different feeding conditions (Extended Data Fig. 3D). However, the activity of Basin-2 in starved larvae was slightly (but not significantly) decreased compared to fed larvae at lower intensities of stimulation, whereas it was increased at higher intensities of stimulation (Extended Data Fig. 3B,D).

We then compared the distributions of Basin-2 responses in larvae fed on either standard food or sucrose to a moderately strong mechanical stimulus (5V applied to piezo at 1000 Hz) and observed a higher probability of absence of neuronal response in normally-fed larvae (Extended Data Fig. 3E). The previous study has recorded depolarization responses simultaneously from Basin-1 and Basin-2 to a mechanical stimulus and has shown that Basin-1 neurons always respond while Basin-2 responses are probabilistic (Jovanic et al. 2016). In this study, we computed the probability of Basin-2 responses to the mechanical stimulus and observed a significantly higher probability of responses that cross the threshold (see methods) in larvae fed on sucrose, which is consistent with higher Head Casting and lower Hunching probabilities in these larvae (Extended Data Fig. 3E). In starved larvae, similar trends were observed at higher stimulus intensities, while at medium intensity (5V), there was no difference between Basin-2 response probability in fed and starved larvae (Extended Data Fig. 3E). This difference in Basin-2 responses between larvae fed only sucrose and starved larvae could explain why Hunching is not consistently significantly reduced in starved larvae.

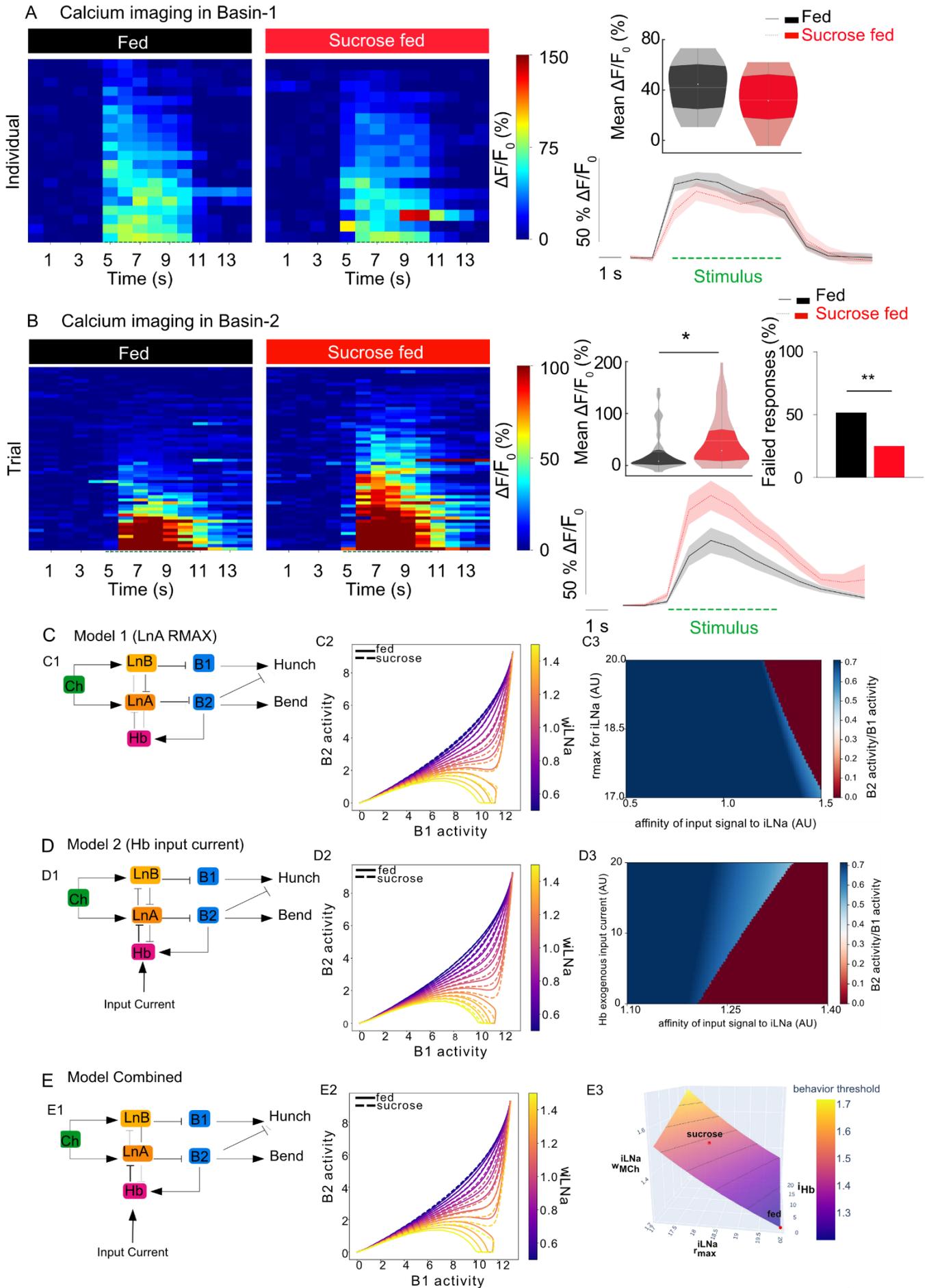


Fig. 3. The feeding status affects the output of the sensorimotor circuit. A: Basin-1 calcium responses to mechanical stimulations in larvae fed on standard food and on sucrose only larvae (20B01-lexA; LexAop-GCaMP6s, UAS-CsChrimson-mCherry). Left panel: calcium responses of Basin-1 from different individuals fed on different diets. Lower right panel: mean calcium response of Basin-1 over time. The green dashed line corresponds to stimulus onset. Upper right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Basin-1 neurons is similar in larvae fed on sucrose only compared to larvae fed on a standard food ($n = 17/20$ larvae, 1 trial per larva, t-test $p = 0.2577$). B: Basin-2 calcium responses to mechanical stimulations in different states (SS00739/UAS-GCaMP6s). Left panel: calcium responses of Basin-2 from different individuals fed on different diets. Lower right panel: mean calcium response of Basin-2 over time. The green dashed line corresponds to stimulus onset. Upper right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Low panel: percentage of trials that failed to elicit a calcium response in Basin-2. Stimulus-induced activity of Basin-2 neurons is enhanced in animals fed on sucrose only compared to larvae fed on standard food ($n = 12$ larvae, 5 trials per larva, mean response comparisons t-test $p = 0.0118$, percentage of failed responses comparison Chi-2 test $p = 0.0027$). C-E: Simple connectome-based rate model of the decision circuit for the selection Hunch and Bend/Hedcast C: Model 1: version of the model where the sucrose state is modeled by a decreased maximum rate of the LNa node. C1. schematic of the circuit where the influence of LNa on downstream targets is weaker C2. state space trajectories of Basin-1 (B1) and Basin-2 Activity as a function of coupling from MCh to LNa for model 1. C3. phase ratio phase diagram for model 1 D: Model 2: version of the model where the sucrose state is modeled by an input current to Hb) Handle B neuron D1. schematic of the circuit where the influence of Hb on downstream targets is stronger D2. state space trajectories of Basin-1 (B1) and Basin-2 Activity as a function of coupling from MCh to LNa for model 2. D3. phase ratio phase diagram for model 2 E: Combined model: version of the model where the sucrose state is modeled by both an input current to Handle B (Hb) neuron and decreased Maximum rate of LNa E1. schematic of the circuit where the influence of Hb on downstream targets is stronger and the one of Lna weaker E2. state space trajectories of Basin-1 (B1) and Basin-2 Activity as a function of coupling from MCh to LNa for the combined model. E3. behavior phase diagram for the combined model. See also Extended Data Fig. 3 and Extended Data Fig. 5 and Supplementary Table 6.

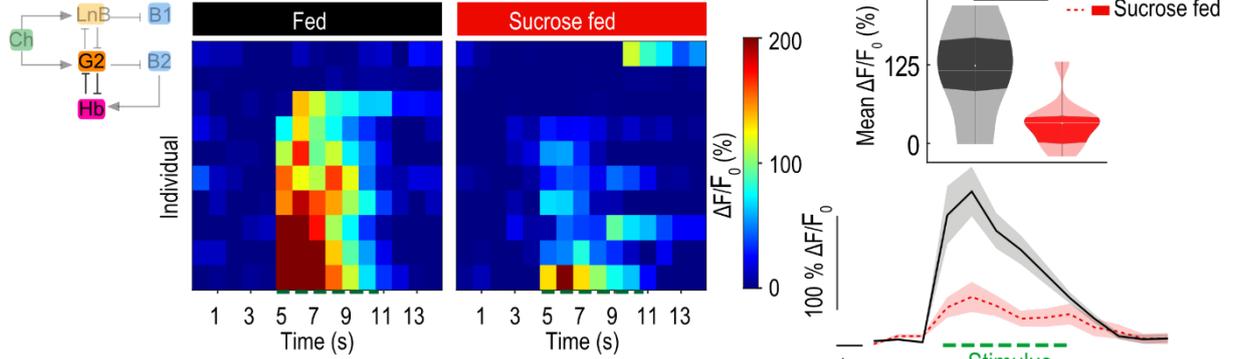
(5) *Reciprocally connected Inhibitory interneurons in the decision circuit are differentially modulated by the feeding state*

Based on the simulation of the model of the circuit for a choice between two air-puff-induced actions (Hunch and Bend) (Jovanic et al. 2016), it is predicted that the relative level of activation of the reciprocally connected feedforward inhibitory interneurons determines the outcome of the competition: Basin-1 only state (Hunch) or Co-active state (Bend). In this study, we investigated if changes in the feeding state could affect the level of activation of two different classes of the inhibitory neurons: feedforward and feedback inhibitory neurons.

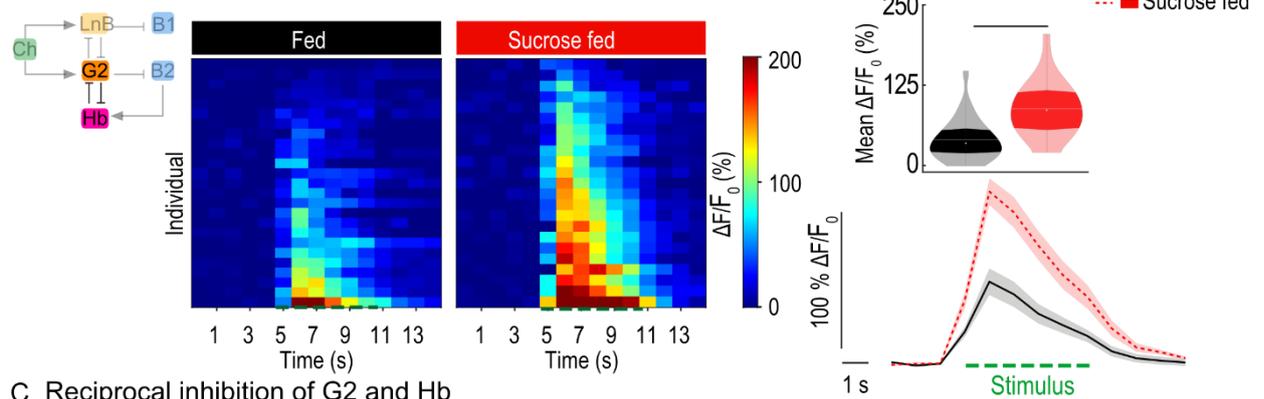
To address this, we started by building a simple rate model, that, as in the previous study (Jovanic et al. 2016), is based on the observed connectivity between neuron classes from the EM reconstruction data, and where weights of excitatory or inhibitory connections between neurons were proportional to the number of synapses (Fig. 3C-E). As previously described, the model predicts that the level of activation of the two classes of feedforward inhibitory neurons will determine the output state of the network at the level of the Basin-1 and Basin-2 neurons (Fig. 3C-E, Extended Data Fig. 5A). The "sucrose state" was modeled in one version of the model as the decreased maximum intensity of LNa neuron activity to explore the role of the feedforward inhibitory neurons in the feeding state modulation (Fig. 3C). In order to explore the contribution of the feedback inhibitory neurons, in another version of the model, the sucrose state was represented by adding an input to the Handle-b neurons (Fig. 3D). Finally, we made a combined model where both the LNa and Handle-b were modulated in the sucrose state (Fig. 3E, Extended Data Fig. 5A-D). In all of the versions of the model the Hunching decreased and Head Casting increased in the sucrose state compared to normal fed state (Fig. 3C3, D3, E3).

These model results suggest that modulating both feedforward and feedback inhibitory neurons of the circuit could result in the observed behavioral changes upon sucrose feeding. To test this experimentally, calcium transients in response to a mechanical stimulus were imaged in the two different types of inhibitory neurons to which we had genetic access. An LNa neuron type, Griddle-2 (G2), that promotes Hunching (Jovanic et al. 2016), showed significantly lower responses to a mechanical stimulus in larvae fed on sucrose compared to the normally fed larvae (Fig. 4A). The decrease in response was observed at different intensities of mechanical stimulation (Extended Data Fig. 4C). Similarly, in starved larvae, the responses of Griddle-2 neurons were also decreased across different intensities of stimulation (Extended Data Fig. 4A, C).

A Calcium imaging in Griddle-2

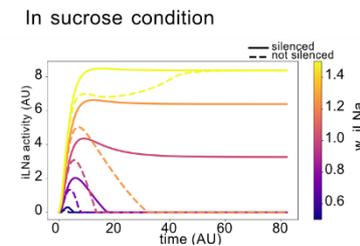
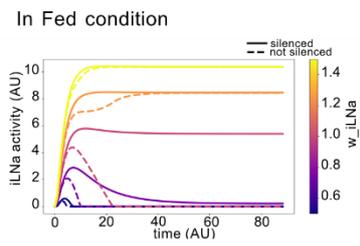


B Calcium imaging in Handle-B

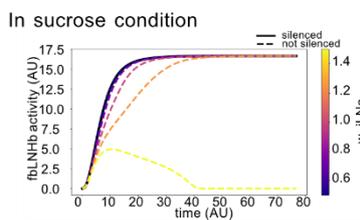
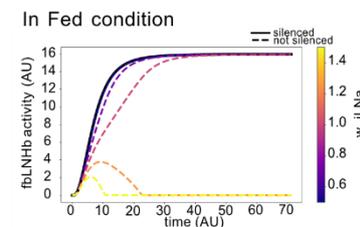


C Reciprocal inhibition of G2 and Hb

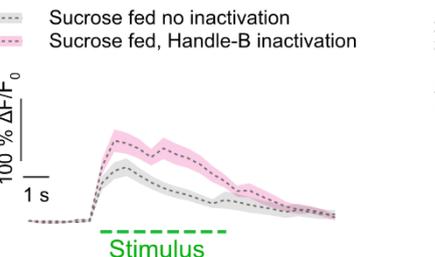
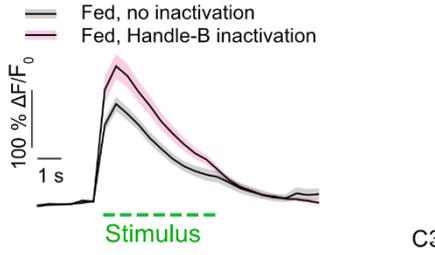
C1 iLNa activity upon silencing Hb



C4 Hb activity upon iLNa silencing



C2 Hb inactivation and Ca imaging in G2



C5 G2 inactivation and calcium imaging in Hb

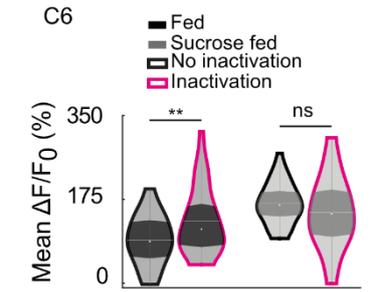
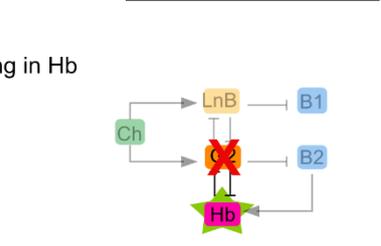
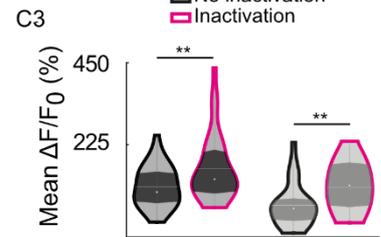
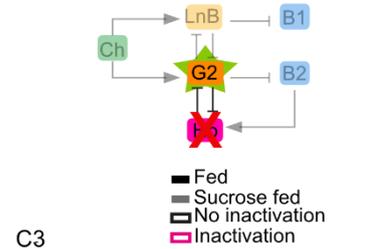
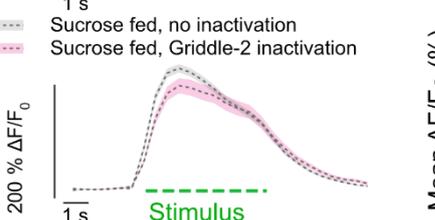
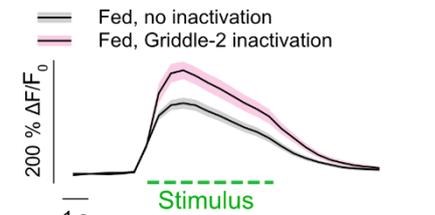


Fig. 4. Two reciprocally connected interneurons are oppositely modulated by the feeding state. A: Griddle-2 calcium responses to mechanical stimulations in larvae fed on standard food and sucrose only (SS00918/UAS-GCaMP6s). Left panel: calcium responses of Griddle-2 from different individuals, fed on standard food or on sucrose only. Lower right panel: mean calcium response of Griddle-2 over time. The green dashed line corresponds to stimulus onset. Upper right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Griddle-2 is decreased in animals fed on sucrose only compared to larvae fed on a standard food (n = 10 larvae, 1 trial per larva, t-test p = 0.0068). B: Handle-b calcium responses to mechanical stimulations in larvae fed on standard food or on sucrose only (SS00888/UAS-GCaMP6s). Left panel: calcium responses of Handle-b from different individuals, fed on standard food or on sucrose only. Lower right panel: mean calcium response of Handle-b over time. The green dashed line corresponds to stimulus onset. Upper right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Handle-b is increased in animals fed on sucrose only compared to larvae fed on a standard food (n = 23/25 larvae, 1 trial per larva, t-test p = < 0.0001). C: Reciprocal inhibition between Handle-b and Griddle-2. C1: model simulation, trajectories of LNa activity upon Hb inactivation in fed (top panel) and sucrose condition (bottom panel) C2: Left panel: mean calcium response of Griddle-2 over time, with (SS0888-Gal4>UAS-TNT 55C05-LexA>LexAop-GCaMP6s) or without Handle-b inactivation (+/UAS-TNT 55C05-LexA>LexAop-GCaMP6s), in larvae fed on standard food or on sucrose only. The green dashed line corresponds to stimulus onset. C3: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Griddle-2 responses to mechanical stimuli are increased after Handle-b inactivation, both in larvae fed on standard food (top panel) or on sucrose (bottom panel) (n = 7-9 larvae, 4 trials per larva, t-test after inactivation in larvae fed on standard food p = 0.009, fed on sucrose only p = 0.002). C1. model simulation, trajectories of Hb activity upon iLNa inactivation in fed (top panel) and sucrose condition (bottom panel) C5. mean calcium response of Handle-b over time, with (55C05-LexA>LexAop-TNT 22E09-Gal4>UAS-GCaMP6s) or without Handle-b inactivation (+/LexAop-TNT 22E09-Gal4>UAS-GCaMP6s), in larvae larvae fed on standard food or on sucrose. The green dashed line corresponds to stimulus onset. C6: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Handle-b responses to mechanical stimuli are increased after Griddle-2 inactivation only in fed animals (n = 6-10 larvae, 5 trials per larva, t-test after inactivation in fed p = 0.0027, sucrose condition p = 0.065). See also Extended Data Fig. 4 and Extended Data Fig. 5 and Supplementary Table 6.

Based on the model, the decrease in the activity of LNa would result in less Hunching and more Head Casting which is consistent with the behavioral changes we observed after feeding on sucrose. The model has also shown that another motif in the circuit, feedback disinhibition, promotes Bending by amplifying the activity of Basin-2. We recorded Handle-b responses to the mechanical stimulus in the different feeding states and found that the Handle-b neurons, that inhibit Hunching (Jovanic et al. 2016), show stronger responses to a mechanical stimulus in larvae fed on sucrose and in starved larvae compared to normally fed larvae (Fig. 4B, Extended Data Fig. 4B,D). This was true for all the different intensities of stimulation we tested (Extended Data Fig. 4D). Therefore, the decreased response of Griddle-2 neurons (Fig. 4A) and increased responses of Handle-b neurons (Fig. 4B) in larvae fed on sucrose could bias the behavioral outcome, consistent with the behavioral changes observed in larvae upon sucrose feeding (less Hunching and more Head Casting) (Fig. 1E1).

EM analysis shows that Griddle-2 and Handle-b neurons are reciprocally interconnected (Fig. 4A) (Jovanic et al. 2016). The circuit and the model predict that silencing Handle-b results in an increase in Griddle-2 activity and vice-versa (Fig. 4C). Further, the reciprocal connections between Griddle-2 and Handle-b were probed functionally and the model predictions tested experimentally; calcium responses to a mechanical stimulus were monitored in Griddle-2 neurons while inactivating Handle-b using tetanus-toxin in both larvae fed on standard food and on sucrose only. The responses were increased compared to control larvae in both feeding states (Fig. 4C1-C3, Extended Data Fig. 4E), which is consistent with the Handle-b inhibition of Griddle-2. However, the increase in Griddle-2 responses upon Handle-b inactivation in larvae fed on sucrose did not reach the levels of Griddle-2 responses upon inactivation in larvae fed on standard food (Fig. 4C2-C3, Extended Data Fig. 4E). This could suggest that the increase in Handle-b activity after sucrose feeding is insufficient to reduce the Griddle-2 responses in larvae fed on sucrose and that both Handle-b and Griddle-2 neurons could be independently modulated. Indeed, this is in line with model 1 and 3 predictions where Griddle-2 is modulated extrinsically (Fig. 3C,E, Fig. 4C, Extended Data Fig. 5E).

The converse experiments were also performed, i.e., inactivating Griddle-2 and imaging the activity in Handle-b (Fig. 4C4-C6). Handle-b responses to the mechanical stimulus were increased in normally fed larvae upon Griddle-2 inactivation (Fig. 4C5-C6, Extended Data Fig. 4F), which is consistent with Griddle-2 inhibition of Handle-b. In larvae fed on sucrose, the activity of the Handle-b neuron was not significantly different upon Griddle-2 inactivation and was similar to the activity upon Griddle-2 inactivation in larvae fed on standard food (Fig. 4C5-C6, Extended Data Fig. 4F). This lack of increase in Handle-b activity upon Griddle-2 inactivation in larvae fed on sucrose could be due to the different state of the network in the larvae fed only sucrose: the activity of Griddle-2 is already low in these larvae (Fig. 4A, Extended Data Fig. 4C), and removing

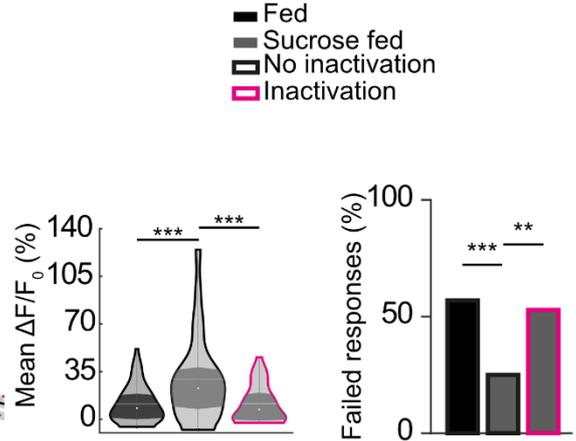
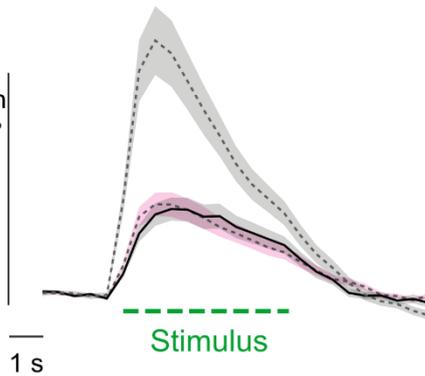
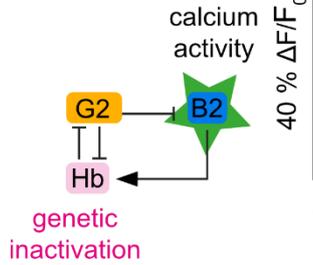
it may not impact its inhibition of Handle-b. Moreover, when silencing the LNa type Griddle-2, other two LNa type neurons remain intact and may continue to inhibit Handle-b. In addition, removing Griddle-2 in this condition could impact the disinhibition of Basin-1 and/or the breaking of the positive feedback Basin-2-Handle-b-Basin-2.

(6) The feeding state-dependent increase in Basin-2 responses depends on the changes in activity of the inhibitory interneurons

Our previous work has shown that Basin-2 (B2) neurons receive input directly from the inhibitory neuron LNa and are disinhibited by the feedback inhibitory neuron Handle-b that makes inhibitory connections onto LNa and LNb neurons; Handle-b receives input primarily from Basin-2 neurons. The feedback disinhibition of Basin-2 creates positive feedback that stabilizes the Basin-2 ON state (Jovanic et al. 2016). To determine whether the increase in Basin-2 responses in larvae fed on sucrose depends on feeding state-dependent changes in the activity of the inhibitory neurons, Handle-b neurons were optogenetically activated, and the activity of B2 neurons was recorded in response to a mechanical stimulus. We found that activating Handle-b in normally fed larvae increases the levels of Basin-2 responses to a level similar to that observed in larvae fed on sucrose (Fig. 5B, Extended Data Fig. 6B). We further silenced Handle-b neurons and monitored calcium responses in Basin-2 neurons in different feeding states (Fig. 5A, Extended Data Fig. 6A). Silencing Handle-b neurons in larvae fed on sucrose decreased Basin-2 responses and erased the difference in Basin-2 response levels between larvae fed on standard food and on sucrose (Fig. 5A, Extended Data Fig. 6A). This result is consistent with previous characterization of the Handle-b disinhibition of Basin-2. Because Handle-b disinhibits Basin-2, silencing Handle-b therefore increases the inhibition of Basin-2 by LNa, and thus reduces Basin-2's responses to mechanical stimuli. In addition, in normally fed larvae where the activity of LNa is high, Basin-2 responses were almost completely abolished (Extended Data Fig. 6A).

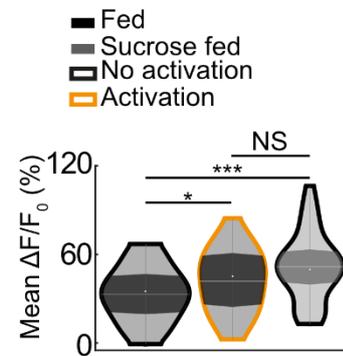
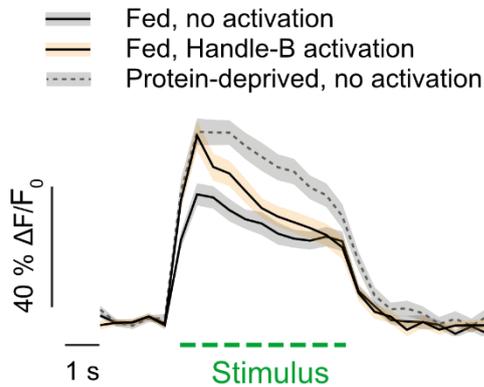
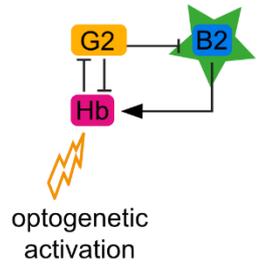
A Handle-B inactivation and calcium imaging in Basin-2

— Fed, no inactivation
 - - - Sucrose fed, no inactivation
 - · - · Sucrose fed, Handle-B inactivation



B Handle-B activation and calcium imaging in Basin-2

— Fed, no activation
 — Fed, Handle-B activation
 - - - Protein-deprived, no activation



C Griddle-2 inactivation and calcium imaging in Basin-2

— Fed, no inactivation
 — Fed, Griddle-2 inactivation
 - - - Sucrose fed, no inactivation
 - · - · Sucrose fed, Griddle-2 inactivation

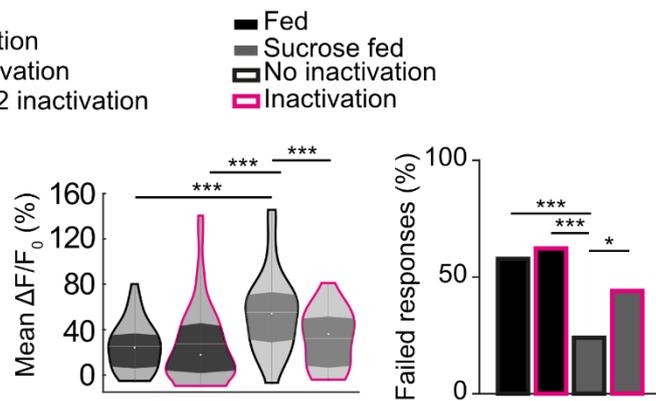
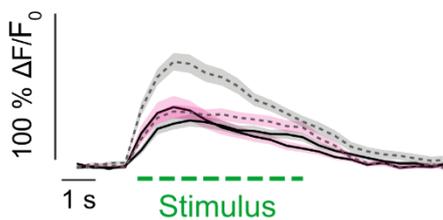
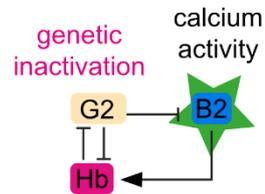


Fig. 5. The modulation of inhibitory interneurons is required for the feeding state dependent changes in the circuit output. A: Left panel: mean calcium responses of Basin-2 over time from individuals fed on different diets, with (38H09-LexA>LexAop-GCaMP6s 22E09-Gal4>UAS-TNT) or without (38H09-LexA>LexAop-GCaMP6s +/->UAS-TNT) Handle-b inactivation. The green dashed line corresponds to stimulus onset. Middle panel: calcium response of Basin-2 averaged during the stimulus. White line represents the mean, white dot represents the median. Right panel: percentage of trials that failed to elicit a calcium response in Basin-2. Inactivating Handle-b in larvae fed on sucrose only prevents the effect of sucrose feeding on Basin-2 activity (n = 12-14 larvae, 5 trials per larva ; ANOVA with post-hoc tests: ***: p < 0.001; chi-2 test ***: p < 0.001, **: p < 0.01). B: Left panel: mean calcium responses of Basin-2 over time from individuals fed on different diets, with or without optogenetic activation of Handle-b (22E09-Gal4>UAS-CsChrimson::tdTomato 38H09-LexA>LexAop-GCaMP6s) during the first second of mechanical stimulus (red box). The green dashed line corresponds to stimulus onset. Right panel: calcium response of Basin-2 averaged during the first second of the stimulus. White line represents the mean, white dot represents the median. Activating Handle-b in fed larvae phenocopies the effect of sucrose feeding on Basin-2 activity (n = 8 larvae, 5 trials per larva; ANOVA with post-hoc tests: ***: p < 0.001, *: p < 0.05). C: Left panel: mean calcium responses of Basin-2 over time from individuals fed on different diets, with (38H09-LexA>LexAop-GCaMP6s 55C05-Gal4>UAS-TNT) or without (38H09-LexA>LexAop-GCaMP6s +/->UAS-TNT) Griddle-2 inhibition. The green dashed line corresponds to stimulus onset. Middle panel: calcium response of Basin-2 averaged during the stimulus. White line represents the mean, white dot represents the median (ANOVA with post-hoc tests: ***: p < 0.001). Right panel: percentage of trials that failed to elicit a calcium response in Basin-2 (chi-2 test ***: p < 0.001, *: p < 0.05). See also Extended Data Fig. 6 and Supplementary Table 6.

Griddle-2 neurons were then inactivated and the activity of Basin-2 was imaged in the two feeding states. As expected, silencing Griddle-2 abolished the difference in Basin-2 responses in the two states. This result is consistent with the effect of Griddle-2 on Handle-b activity (Fig. 5). Silencing Griddle-2 in fed larvae resulted in small (but not significant) increase in Basin-2 responses (Fig. 5C, Extended Data Fig. 6C). However, the mean responses of Basin-2 in larvae fed only with sucrose unexpectedly and significantly decreased upon Griddle-2 silencing. We also computed Basin-2 response probabilities. While inactivating Griddle-2 did not affect Basin-2 response probabilities in normally fed larvae, it did so in larvae fed on sucrose; the non-response probabilities are significantly increased upon Griddle-2 inactivation in sucrose-fed larvae. (Fig. 5C, Extended Data Fig. 6C). The mean responses of trials with only Basin-2 ON responses reveal that the level of responses of Basin-2 was increased in normally fed larvae to the level found in larvae fed on sucrose in the control. The mean responses of trials with only Basin-2 ON responses also show that the level of Basin-2 response was decreased in larvae fed on sucrose (Extended Data Fig. 6C) and similar to the level of activity in fed control larvae.

These findings suggest that there are two mechanisms that control Basin-2 response probabilities and amplitudes, respectively. The two mechanisms are decoupled upon Griddle-2 inactivation. Inactivating Griddle-2 may decrease Basin-2 response probabilities by decreasing Basin-1 responses (that then disinhibits less Basin-2). At the same time, inactivating Griddle-2 increases Handle-b responses and thus amplifies Basin-2 responses more once Basin-2 responses are triggered.

Griddle-2 is thus required for state-dependent modulation of Basin-2, possibly by mediating the disinhibition of Basin-2 by Handle-b. Inactivating Griddle-2 in normally fed larvae disinhibits Basin-2. On the other hand, inactivating Griddle-2 in larvae fed on sucrose when the activity of Griddle-2 is already low (and Griddle-2 is not inhibiting Basin-2 strongly) may thus favor other inhibitory pathways and result in lower Basin-2 responses.

These results show that two types of reciprocally connected inhibitory neurons that promote competing actions inhibit each other during responses to a mechanosensory cue and are differentially modulated by the change in feeding states (starvation and feeding on sucrose). The activity of the Griddle-2 that promotes Hunching is decreased, while the activity of Handle-b that promotes Bending is increased. This differential modulation of the inhibitory neurons, in turn, has an impact on the state of the network in a feeding state-dependent manner and biases the behavioral responses towards less Hunching and more Head Casting by increasing the activity of the Basin-2 neurons when larvae are fed on sucrose only. In addition, it reveals that two different layers of the network are modulated by the changes of the feeding state: the reciprocally connected feedforward inhibitory neurons, as well as the feedback inhibitory neurons that stabilize the state of the network, resulting in the co-activation of both Basin-1 and Basin-2.

(7) The feedback inhibitory neuron receives input from an NPF-releasing neuron, that senses the changes in the feeding state

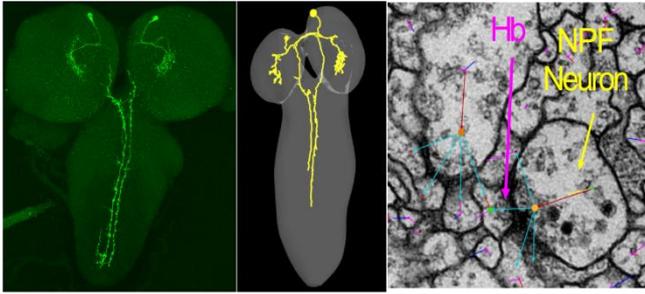
In order to determine the sources of feeding state-dependent modulation, we looked in the connectome for upstream partners of Griddle-2 and Handle-b neurons. Previous work has shown that the different neurons in the circuit receive input from long-range projection neurons that, as suggested by their morphology and connectivity, could bias the output of the network based on contextual/internal state information (Jovanic et al. 2016). Interestingly, by matching light microscopy images to electron microscopy reconstruction images, we found one of these neurons is an NPF-expressing neuron that synapses on Handle-b. NPF (neuropeptide F), a homolog of the mammalian neuropeptide Y (M. R. Brown et al. 1999), is a hunger signal, and its expression promotes feeding in both adult and larval *Drosophila* (Lin, Senapati, and Tsao 2019). We, therefore, sought to investigate the implication of NPF neurons in modulating the activity of the inhibitory neurons in a state-dependent manner. In the larva, there are two pairs of NPF-expressing neurons. Both of them have cell bodies in

the brain; the dorsolateral pair (DL-NPF) projects ipsilaterally within the brain lobes and the dorsomedial pair (DM) sends descending projections via the suboesophageal zone (SEZ) through the ventral nerve cord (VNC) (Extended Data Fig. 7A).

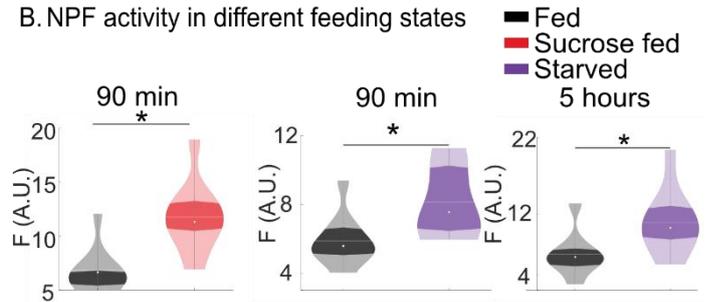
To study the influence of NPF neurons on Handle-b activity, the activity of the DM-NPF descending neuron was monitored in the different feeding states. To this end, we drove GCAMP6s expression in the NPF descending neurons with NPF-GAL4 and measured its intensity of fluorescence in the VNC projections of larvae fed either on standard food (fed), on 20% sucrose (sucrose fed) or completely starved (given only water for 90 minutes). We showed that starvation or feeding on sucrose both increase the activity of the NPF descending neuron (Fig. 6B).

NPF was shown to be involved in promoting feeding (Wu et al. 2003). To investigate whether the increase in activity of NPF descending neurons upon 90-minute sucrose feeding or starvation had an influence on NPF release and behavior, NPF was knockdown selectively in the NPF descending neuron using a split-GAL4 driver that labels only the descending pair of NPF neurons (Fig. 6A) and larval locomotion monitored in larvae fed on standard food, larvae fed on sucrose and starved larvae (Fig. 6C, Extended Data Fig. 8A). Downregulating NPF in the DM-NPF neurons descending into the VNC impaired the increase in exploration observed in larvae fed on sucrose and starved larvae: it abolished the difference in tortuosity over 20 s, time fraction in runs, mean scaled speed and maximum dispersal over 60 s between larvae fed on standard food and starved larvae. Moreover, NPF downregulation abolished the difference between larvae fed on standard food and larvae fed on sucrose or even decreased exploration in larvae fed on sucrose compared to larvae fed on standard food as observed by increased tortuosity over 20 s and decreased maximum dispersal and time fraction in runs compared to larvae fed on standard food (Fig. 6C, Extended Data Fig. 8A).

A. NPF neuron connects to Handle-B

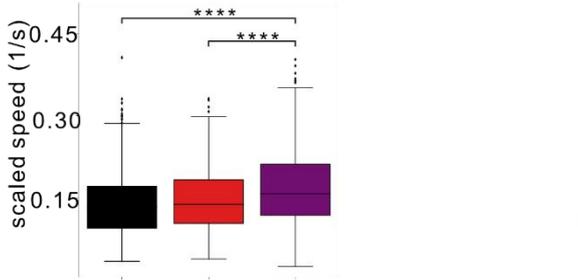


B. NPF activity in different feeding states

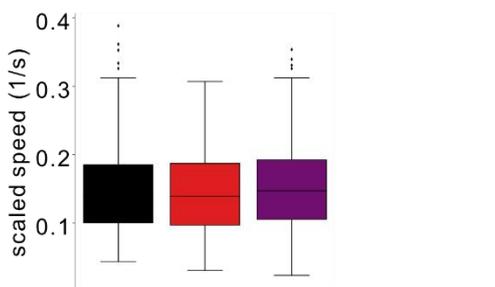


C. Locomotion of larvae upon NPF knockdown

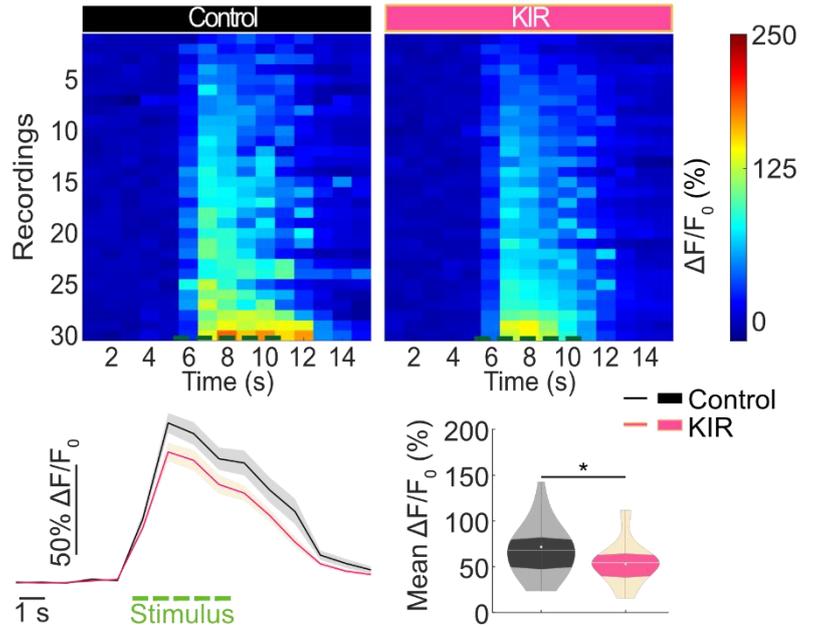
C1. Control



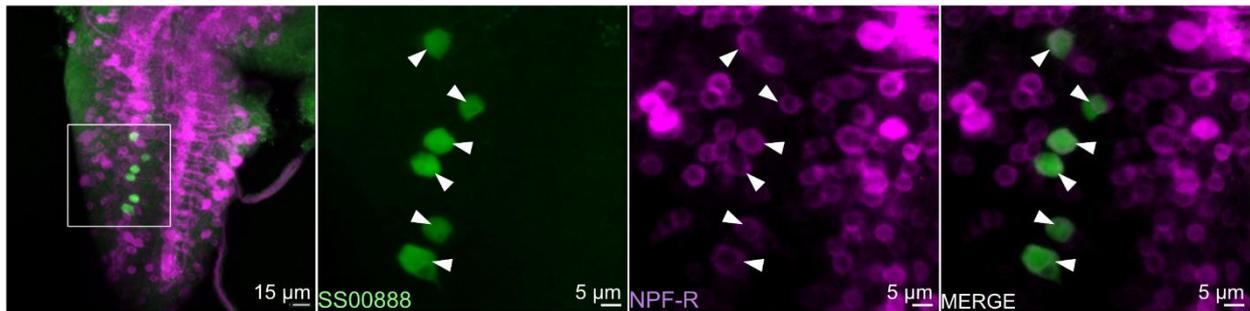
C2 NPF knockdown in DM-NPF neuron



D. Ca-imaging in Hb upon silencing of NPF neurons



E. Immunolabeling SS00888-NPFR T2A-LexA



F. Ca-imaging in Hb upon NPFR knockdown

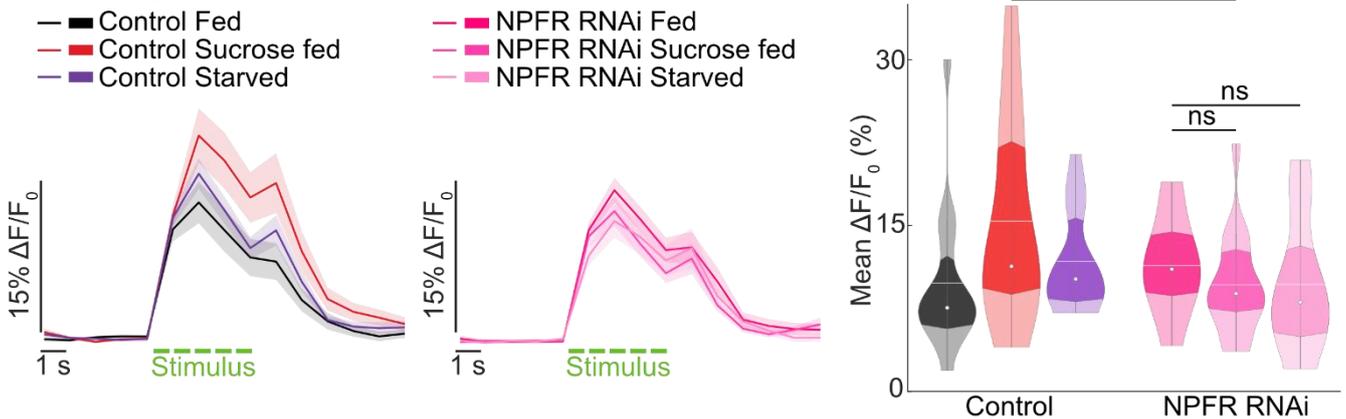


Fig. 6. NPF-releasing neurons convey internal state information to the feedback inhibitory neuron. A: Expression profile characterization of GMR_SS01635>UAS-GFP stained with a GFP-specific antibody. GMR_SS01635 split-Gal4 line selectively targets one pair of NPF neurons. Comparison of light microscopy images with electron microscopy (EM) reconstruction images indicates that a descending neuron synapses on Handle-b (Jovanic *et al*, 2016) is the DM-NPF descending neuron. EM image shows neuropeptide-containing dense core vesicles in the NPF descending neuron near one of its synapses with Handle-b. B: Baseline calcium fluorescence measured in the projections of NPF descending neurons in the VNC (ventral nerve cord) of larvae fed on sucrose only for 90 minutes (N = 10 larvae), starved for 90 minutes (N = 10 larvae) or 5 hours (N = 8-9 larvae). White line represents the mean, white dot represents the median. Starvation or feeding only on sucrose only increase NPF descending neuron activity (two-tailed T-test: *: $p < 0.05$). C: Analysis of larval locomotion in the absence of sensory stimulus. Top row shows control larvae (C1). Bottom row shows larvae with NPF knockdown in DM- NPF descending neurons (C2). NPF knockdown in DM-NPF descending neuron abolishes differences in locomotion mean between larvae in different states; as measured by the average movement speed (Mann-Whitney test, ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$) D: Calcium responses in Handle-b with (UAS-GCamp6s; 22E09-Gal4, NPF-LexA>LexAop-KIR) or without (UAS-GCamp6s; 22E09-Gal4, NPF-LexA;+) NPF neurons silencing. Left panel: calcium responses of Handle-b, in each trial of mechanosensory stimulation. Right lower panel: mean calcium response of Handle-b over time. The green dashed line corresponds to the stimulus. Top right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Handle-b is decreased upon Coaster silencing (n = 10 larvae, 3 trials per larva, t-test $p = 0.045$). E: Immunohistochemical labeling for NPFR in Handle_B UAS-GCamp6s is expressed in Handle-b using the GMR_SS00888 split-Gal4 line (green) and LexAop-jRGeco1a is expressed under the control of the NPFR promoter using a T2A-LexA construct (magenta). Antibodies against GFP and dsRed were used to increase detection sensitivity. Co-localization of LexAop-jRGeco1a and GCamp6s show that Handle-b expresses the NPFR. F: Calcium responses in Handle-b upon NPFR knockdown (GMR_SS00888>UAS-NPFR-RNAi; UAS-GCamp6s) compared to a control (GMR_SS00888>UAS-GCamp6s). Left panel: mean calcium response of Handle-b over time in control larvae fed, fed on sucrose or starved. The green dashed line corresponds to the stimulus. Middle panel: mean calcium response of Handle-b over time upon NPFR knockdown in larvae fed, fed on sucrose or starved. The green dashed line corresponds to the stimulus. Right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median (n = 14-15 larvae). Handle-B responses are increased in control larvae upon feeding on sucrose (Mann-Whitney test, $p = 0.04846$) or starvation (Mann-Whitney test, $p = 0.02807$). NPFR knockdown erases this modulation (Mann-Whitney test, $p = 0.09853$ and $p = 0.121$). NPFR knockdown significantly decreased Handle-B responses in larvae fed on sucrose (Mann-Whitney test, $p = 0.03754$). See also Extended Data Fig. 7 and Extended Data Fig. 8 and Supplementary Tables 2, 3 and 6.

To understand the influence of NPF neurons on Handle-b activity, the interactions between the two neurons were tested functionally by hyperpolarizing the NPF neurons with the inwardly rectifying potassium channel Kir2.1 using an NPF-LexA driver line that labels both pairs of NPF neurons and monitoring calcium responses of Handle-b to a mechanical stimulation. Upon NPF neurons silencing, we observed a decrease in Handle-b responses to mechanical stimulations (Fig. 6D). The NPF descending neuron could thus facilitate responses of Handle-b neurons. Upon changes in the feeding state, the NPF neurons whose activity increases upon starvation and sucrose feeding, could enhance Handle-b responses to a mechanical stimulus, resulting in higher Handle-b responses (Fig. 6B, D) that bias behavioral choice towards less Hunching and more Head Casting.

In the connectome, we found large dense core vesicles in the NPF neurons in the proximity of the Handle-b neuron (Fig. 6A). Moreover, we showed that Handle-b expresses the NPFR1 using a T2A-LexA method (P.-T. Lee et al. 2018) where we drove the expression of one reporter in all NPFR1 neurons using the NPFR1 T2A-Lexa and of another reporter with a Handle-b specific split-GAL4 driver. The intersection of the two expression patterns revealed that the Handle-b neurons express the NPFR1 (Fig. 6E). Having established that Handle-b and Griddle-2 are instrumental in the feeding state-dependent modulation of the behavioral choice of larvae in response to mechanical stimulation and that NPF modulates Handle-b, given that the model and functional connectivity experiment predict that the Handle-b and Griddle-2 could be independently modulated (Fig. 4C, Extended Data Fig. 5E-F), we investigated whether Griddle-2 could be also influenced by NPF. We found that Griddle-2 does not receive synaptic input from the NPF descending neurons. Since neuropeptides can act outside of synaptic sites and we found dense core vesicles characteristic for neuropeptide release along the NPF axon in the proximity of Griddle-2 neurons, we also examined the NPFR1 expression in Griddle-2 neurons, as well as in Basin-1 and Basin-2 using a similar approach as for Handle-b and didn't find any NPFR1 positive cells in these neurons (Extended Data Fig. 7D).

We then downregulated the expression of NPFR1 in the Handle-b neurons and imaged their calcium responses to mechanical stimulation in larvae fed on standard food, larvae fed only on sucrose and in starved larvae (Fig. 6F, Extended Data Fig. 8B). Downregulation of NPFR1 in Handle-B abolished the feeding-state dependent modulation of Handle-b as there was no significant increase in Handle-B responses upon sucrose feeding or starvation when NPFR1 was downregulated (Fig. 6F, Extended Data Fig. 8B).

Altogether, these results show that NPF neurons sense the changes in feeding state and their activity increases upon starvation or sucrose only feeding. The changes in NPF activity influence Handle-b activity that increases upon starvation or sucrose only

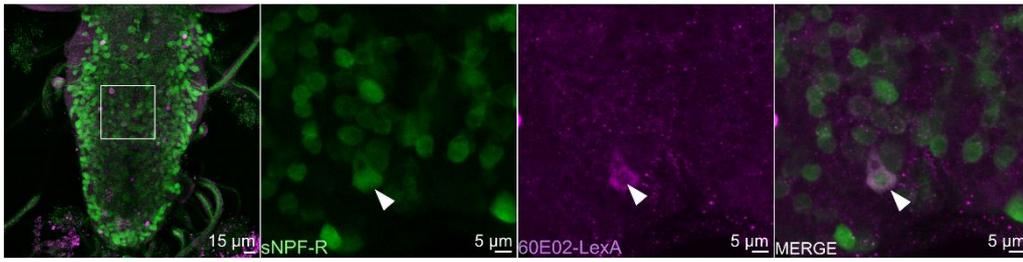
feeding due to a facilitating effect of NPF. This increase in activity then biases the sensorimotor decisions towards less Hunching and more Head Casting.

(8) *sNPFR in the interconnected inhibitory interneurons mediates the state-dependent modulation of larval behavioral responses to a mechanical stimulus*

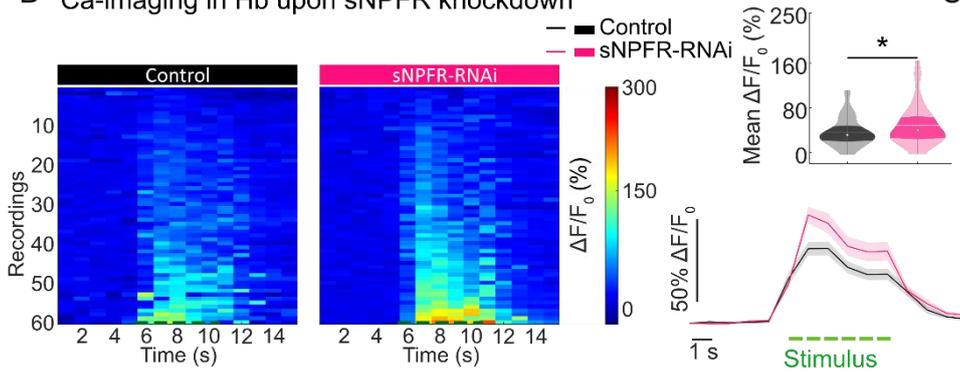
The model and calcium imaging suggests that Griddle-2 and Handle-b are independently modulated by the feeding state (Fig. 4). Since Griddle-2 does not express the NPFR1 receptor, other neuropeptides could influence Griddle-2 and the circuit. We therefore investigated the effect of another neuropeptide on the circuit: sNPF (the short neuropeptide F). sNPF is a second homolog of the mammalian NPY whose receptor is widely distributed in the larval nervous system, including the VNC (Carlsson, Enell, and Nässel 2013), and was shown to be involved in regulating hunger-driven behaviors (Lin, Senapati, and Tsao 2019), and in facilitating mechano-nociceptive responses (C. Hu et al. 2017) among other functions. Therefore, we sought to examine whether the interneurons in the circuits express the receptor for sNPF. By genetically co-expressing a red fluorescent reporter (jRGeco1a) under the control of LexA drivers that label either Handle-b, Griddle-2, Basin-1 or Basin-2 neurons we identified in the literature and existing Gal4 databases (see Methods for details), and a green fluorescent reporter (GCaMP6s) under the control of the sNPFR1 promoter with a T2A-Gal4 construct, we showed that both Handle-b (Fig. 7A) and Griddle-2 (Fig. 7D) express the sNPFR1, while the two Basin neurons do not (Extended Data Fig. 7E).

To determine whether sNPF signaling was involved in responses to a mechanical stimulus in these two neurons, their responses were monitored upon sNPFR1 downregulation. Calcium imaging recordings show that genetically downregulating sNPFR1 expression in Handle-b increases its responses to mechanical stimulation (Fig. 7B), suggesting that sNPFR1 has an inhibitory effect on Handle-b. On the other hand, responses of Griddle-2 were decreased upon sNPFR1 downregulation (Fig. 7E), suggesting that sNPF facilitates Griddle-2 responses to a mechanical stimulus. Since Handle-b inhibits Hunching and promotes Head Casting, while Griddle-2 promotes Hunching and inhibits Head Casting, downregulating the sNPFR1 in these neurons would inhibit Hunching and promote Head Casting. Indeed, the behavior experiments showed that sNPFR1 knockdown in either of these two neurons leads to less Hunching and more transitions to Head Casting (Fig. 7C, F) in response to an air-puff. The sNPFR1 knockdown in Handle-b and Griddle-2 leads to a modulation of their activity similar to that caused by feeding on only sucrose (lower Griddle-2 and higher Handle-b activity) and yields the same behavioral outcome. This suggests that sNPF signaling targeting these neurons could be downregulated in larvae fed on sucrose and at least partially responsible for the modulation of the behavioral choice of the larvae in response to a mechanical stimulation. Giving stock to this hypothesis, the downregulation of sNPFR1 in Handle-b or Griddle-2 did not impact their calcium responses to mechanical stimulation in larvae fed on sucrose or starved (Extended Data Fig. 9).

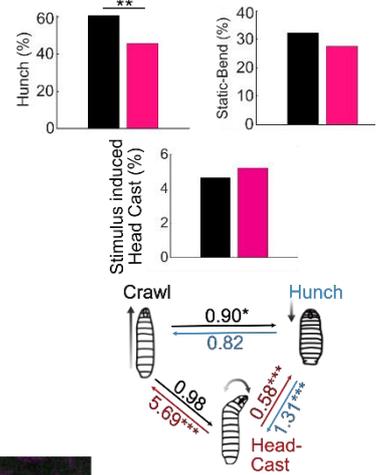
A sNPFR expression in Handle-b



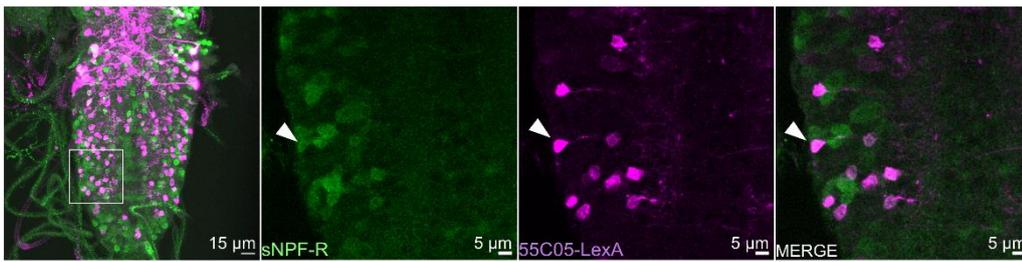
B Ca-imaging in Hb upon sNPFR knockdown



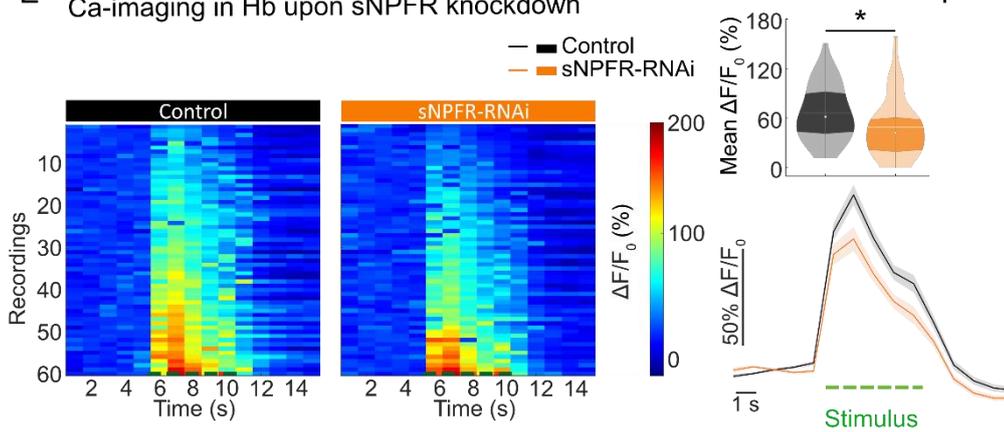
C



D sNPFR expression in Griddle-2



E Ca-imaging in Hb upon sNPFR knockdown



F

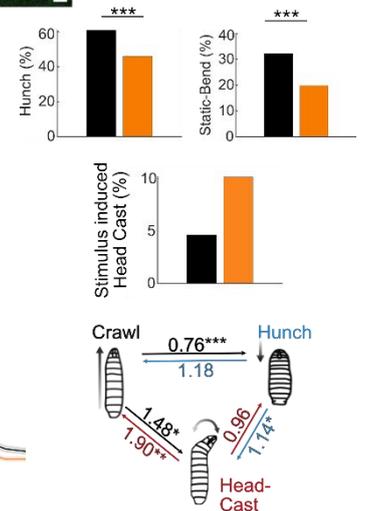


Fig. 7. sNPFR signaling inhibits Handle-b and facilitates Griddle-2 mechanosensory responses. A: Immunohistochemical labeling for sNPFR in Handle-b. LexAop-jRGeco1a is expressed in Handle-b using the 60E02-LexA line (magenta) and UAS-GCamp6s is expressed under the control of the sNPFR promoter using a T2A-Gal4 construct (green). Antibodies against GFP and dsRed were used to increase detection sensitivity. Co-localization of antibodies against jRGeco1a and GCamp6s shows that Handle-b expresses the sNPFR. B: Calcium responses in Handle-b upon sNPFR knockdown (GMR_SS00888>UAS-GCamp6s; UAS-sNPFR-RNAi) compared to a control (GMR_SS00888>UAS-GCamp6s) in Handle-b. Left panel: calcium responses of Handle-b from different individuals and trials. One line is a trial. Lower right panel: mean calcium response of Handle-b over time. The green dashed line corresponds to the stimulus. Top right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Handle-b is increased by sNPFR knockdown (n = 15 larvae, 4 trials per larva, t-test p = 0.035). C: Behavior in response to air-puff during the first five seconds upon stimulus onset, for larvae in which sNPFR was knocked down in Handle-b neurons (SS00888>sNPFR-RNAi) compared to the control (n = 185-260 larvae) (***: p < 0.001, **: p < 0.01, *: p < 0.05). D: Griddle-2 immunostaining for sNPFR expression. LexAop-jRGeco1a is expressed in Griddle-2 using the 55C05-LexA line (magenta) and UAS-GCamp6s is expressed s expressed under the control of the sNPFR promoter using a T2A-Gal4 construct (green). Antibodies against GFP and dsRed were used to increase detection sensitivity. Co-localization of antibodies against jRGeco1a and GCamp6s show that Griddle-2 expresses sNPFR. E: Calcium responses in Griddle-2 upon sNPFR knockdown (SS_TJ001>UAS-GCamp6s; UAS-sNPFR-RNAi) compared to the control (SS_TJ001>UAS-GCamp6s). Left panel: calcium responses of Griddle-2 from different individuals and trials. One line is a trial. Lower right panel: mean calcium response of Griddle-2 over time. The green dashed line corresponds to the stimulus. Top right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Griddle-2 is decreased by sNPFR knockdown (n = 10 larvae, 3 trials per larva, t-test p = 0.003). F: Behavior in response to air-puff during the first five seconds upon stimulus onset, for larvae in which sNPFR was knocked down in Griddle-2 neurons (SS_TJ001>sNPFR-RNAi) compared to the control (n = 243-438 larvae, ***: p < 0.001, **: p < 0.01, *: p < 0.05). See also Extended Data Fig. 7, Extended Data Fig. 9 and Supplementary Tables 4, 5 and 6.

Altogether these results show that Handle-b and Griddle-2, reciprocally interconnected inhibitory neurons that drive opposing actions, are differentially modulated by the sNPF signaling pathway to bias the response to air-puff towards less Hunching and more Head Casting.

III. A. 2. d. *Discussion*

Whereas many studies showed that various behaviors are affected by an animal's physiological state, especially hunger, this work goes further by describing the neural circuit mechanisms by which transient changes in the physiological status impact behaviors that are not directly related to feeding itself. To determine whether and how changes in the feeding state affect behaviors unrelated to feeding, we took advantage of a well characterized behavioral response of *Drosophila* larva to a mechanical stimulus for which we had in the past dissected the circuit mechanisms underlying the selection between two main types of responses: Hunch and Head Cast. The current study reveals the neural circuit mechanisms of modulation of these sensorimotor decisions by the feeding conditions. Slight changes in feeding conditions affect an animal's motivational state and bias responses to an air-puff towards less Hunching and more Head Casting. Reciprocally interconnected inhibitory neurons that drive competing actions are differentially modulated by the changes in the feeding state: the activity of the neuron that promotes a Hunch is decreased and the activity of the neuron that inhibits a Hunch is increased. This modulation at the level of inhibitory neurons involving NPF and sNPF signaling systems biases the output of the network towards promoting Head Casting and inhibiting Hunching, a modulation consistent with the state-dependent behavioral changes we observed.

Hungry animals behaving differently in non-feeding related contexts have been reported in various studies and across the animal kingdom (Smith and Grueter 2021; Moran et al. 2021). However, the underlying logic and neural mechanisms have not been well understood. These changes could be linked to overall changes in behavioral strategies in hungry individuals (Smith and Grueter 2021) to ensure survival. Risk-taking has been shown to be increased by hunger (P. Hunter 2013a; Moran et al. 2021; Sandhu et al. 2018), even in social-related decisions (Smith and Grueter 2021; Levy, Thavikulwat, and Glimcher 2013), further raising questions about how food-deprivation signals are integrated into the neural computations underlying non-feeding related behaviors. Animals need to be able to make any decisions flexibly depending on the need that is most critical at a given moment. Our study shows that a circuit underlying sensorimotor decisions in response to a mechanical stimulus located at the early stages of sensory processing is influenced by the changes in diet. This finding supports the idea that state dependent flexibility of behavior could be achieved by the physiological state acting on circuits throughout the nervous system and thus reorganizing its activity in a distributed manner. In various ecological contexts, survival often entails a trade-off between avoiding dangers and pursuing food and water-seeking behaviors (Moran et

al. 2021). For example, increased exploration will increase the likelihood of finding food but also of encountering a dangerous situation (Moran et al. 2021). If animals' need for food or water increases either due to food deprivation or thirst, they might be more likely to explore intensively despite an increasing risk of threat. Similarly, food-deprived and thirsty animals might take more risks and ignore aversive cues to increase their chances of getting near food or water sources. Hungry animals use different strategies when escaping predators compared to satiated ones (Evans et al. 2019; Schadeegg and Herberholz 2017).

While various studies have shown that hunger affects behaviors by altering the responses of sensory neurons (Ko et al. 2015; Sayin et al. 2018; Inagaki et al. 2012; Root et al. 2011), others have implicated central mechanisms in feeding state dependent behavioral flexibility (Crossley, Staras, and Kemenes 2018; Lewis et al. 2015; Petzold et al. 2023). We found that the activity of chordotonal mechanosensory neurons that sense the air-puff was not significantly altered upon feeding on sucrose or starvation, contrary to the activity of the downstream neurons. The changes at the level of sensory pathways tunes animals' perception in order to increase their likelihood of finding food and feeding by increasing responsiveness to appetitive and decreasing responsiveness to aversive food-related stimuli. The implication of central mechanisms, on the other hand, may suggest that hunger acts as a global regulator of behavior, i.e. hunger may change brain activity in such a way to reevaluate goals and behavioral strategies in order to increase animals' chances of survival (Smith and Grueter 2021).

Our experiment monitors calcium responses of all the chordotonal sensory neurons together. The different inhibitory and projection neurons in the circuit receive inputs from different chordotonal subtypes. This could also explain the differential modulation of the different neuron subtypes in the circuit if some subtypes of chordotonal are modulated by the changes in the internal state while others are not. However, optogenetic activation of all the chordotonal neurons in the different feeding conditions still resulted in decreased Hunching and increased Head Casting upon starvation or sucrose diet, strongly suggesting that downstream neurons are involved. Even if chordotonals are involved in altering behavior in a state-dependent manner, their modulation is not required to alter the behavior in response to the mechanical stimulus due to the contribution of the downstream circuitry.

Calcium imaging combined with neuronal manipulations revealed that, in the circuit for selecting between the Hunch and the Head Cast, inhibitory neurons (and not the projection neurons) are the target of modulation by the changes in the feeding conditions. Inhibitory neurons were shown to be the target of contextual modulation in other systems (Hsu et al. 2021; Morozova, Newstein, and Marder 2022; Pakan et al. 2016).

Previous work has identified reciprocal inhibition of inhibition as a motif underlying the competition between a startle-type action and an exploratory action (Jovanic et al. 2016). Such a motif was shown to underlie similar computations in different species and brain areas (Jovanic et al. 2016; Chen et al. 2016; Fadok et al. 2017; Koyama et al. 2016; Koyama and Pujala 2018; Mysore and Knudsen 2012; Mysore and Kothari 2020) and was proposed to provide flexibility to the selection process (Jovanic et al. 2016; Koyama and Pujala 2018; Mysore and Kothari 2020). The current study determines that one of the reciprocally connected inhibitory neurons within this motif, LNa type Griddle-2, is modulated by the changes in the feeding conditions and that this modulation contributes to biasing sensorimotor decisions. These results confirm theoretical predictions that such a motif confers the sensorimotor circuit the capacity to be tuned to other types of information, in this case, internal state information (starvation and thirst). Moreover, these findings are in line with predictions that shaping the output of the network through disinhibition by reciprocally connected inhibited neurons allows for flexible, competitive selection (Jovanic et al. 2016; Koyama and Pujala 2018; Mysore and Kothari 2020).

This work shows that another type of inhibitory neuron, Handle-b, which is a feedback inhibitory neuron that provides positive feedback to the Hunch inhibiting Basin-2 neuron through feedback disinhibition, is also modulated by changes in internal physiology. Handle-b is also reciprocally connected to the LNa neurons that participate in the reciprocal inhibition of inhibition motifs. This connectivity pattern suggests that, in addition to competition within the reciprocal inhibition of inhibition motifs, the competition between the two layers of the circuits is also modulated by the changes in internal physiology. Similar to recurrent excitation, the feedback disinhibition motif provides positive feedback that stabilizes the selected output (Jovanic et al. 2016). Using inhibitory rather than excitatory connection may have the advantage of allowing the decisions to be influenced by contextual and state information. In addition, in this circuit architecture, the feedback inhibitory neuron Handle-b contacts both reciprocally connected inhibitory neurons in the circuit and can thus shape the circuit activity at the level of the site of competition. It is thus well suited to be the site of integration of mechanosensory information and information about an animal's state.

The behavioral response of the larvae to a mechanical cue depends on the state of the circuit at the level of reciprocally interconnected inhibitory neurons, which will shape the activity of the Basin projection neurons to either give rise to a state where Basin-1 only is active or a state where Basin-1 and -2 are co-active (Jovanic et al. 2016). We showed that the feeding state-dependent modulation of the Basin-2 neuron is dependent on the modulation of the activity of Handle-b and Griddle-2. Accordingly, genetic co-labeling of Basin-1 or Basin-2 neurons with either sNPFR1 or NPFR1 using the T2A GAL4/LexA technology showed no expression of these neuropeptide receptors in the two Basin neurons. This is in line with the finding that the state-dependent

changes in the air-puff induced sensorimotor decisions are caused by the modulation that acts on the reciprocally interconnected inhibitory neurons that integrate mechanosensory information with current internal state needs.

Previous work revealed that the inhibitory neurons in the mechanosensory circuit are contacted by long-range projection neurons (Jovanic et al. 2016). These long-range projection neurons may carry contextual or state information to the mechanosensory circuit. We found that among these long-range projection neurons is a pair of NPF-releasing neurons that contacts the Handle-b. NPF is a homologue of the mammalian neuropeptide Y that is a hunger signal. NPF neurons have indeed been shown to be involved in hunger dependent behaviors in both adult flies and larvae (Lin, Senapati, and Tsao 2019; Wu et al. 2003). We found that the activity of the NPF descending pair of neurons changes as a function of the feeding state. These neurons could thus convey the information about the satiation state to the mechanosensory circuit and bias the sensorimotor decisions to mechanosensory cues by modulating the activity of the Handle-b. Additionally, the release of NPF at the proximity of the circuit by the NPF neurons (as suggested by the existence of dense core vesicles) could modulate Handle-b that expresses the NPFR1 (while other neurons in the circuit do not). The fact that the feedback inhibitory neuron is directly influenced by the NPF neurons supports the idea that it could gate circuit activity in a state dependent manner. Our results indicate that both the feeding state induced changes in locomotor strategy (motivational locomotor state and exploration persistence) and the acute response to transient environmental stimuli are dependent on NPF signaling. The descending NPF neurons could convey information about the internal state to diverse neuronal populations in the VNC to regulate exploratory locomotion and stimulus-dependent motor responses, thus adjusting behavioral interactions with the environment according to the current animal needs. The NPF could serve as an internal state signal that couples various sensorimotor behaviors to the motivational/exploratory state of the animal and its physiological needs, thus regulating behavior across different timescales.

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III. A. 2. f. *Author contributions*

E.T. behavioral and physiology experiments and analysis, Calcium-imaging experiments and analysis; Writing: figures, edits and revisions; D. M. behavioral experiments and analysis, Calcium-imaging experiments and analysis, immunohistochemistry experiments, Writing: original draft, figures; A.B. modeling; P.S. Data analysis; C.B. behavioral classification and analysis, statistical analysis, writing, methods; F. V. behavioral experiments; V. S. behavioral and physiology experiments; A.H. and S. A immunohistochemistry experiments; M.N. supervision, funding acquisition; J.B. Methodology, supervision, funding acquisition, writing- edits and revision; T.J. Conceptualization, analysis, supervision, funding acquisition and project administration; writing: original draft, figures, edits and revision.

III. A. 2. g. *Declaration of interests*

The authors declare no competing interests.

III. A. 2. h. *Material and methods*

(1) *Drosophila rearing and handling*

Flies (*Drosophila Melanogaster*) were raised on a standard food medium (ethanol 2%, methylhydroxybenzoate 0.4%, yeast 8%, cornmeal 8%, and agar 1%) at 18°C. Third instar larvae were collected as follows: male and female flies from the appropriate genotypes were placed together for mating, then transferred at 25°C for 12-16 h on a petri dish containing a fresh food medium for egg laying. The petri dish was then placed at 25°C for 72 h. Foraging third instar larvae were collected from the food medium by using a denser solution of 20% sucrose, scooped with a paint brush into a sieve and gently and quickly washed with water. Larvae used for optogenetic experiments were raised at 25°C in complete darkness, on standard food supplemented with all-trans retinal at 0.25 mM (R240000, Toronto Research Chemicals). The full list of genotypes used in the study can be found in the Supplementary Table 1 Resource table.

(2) *Dietary treatments*

For dietary treatments, larvae were placed in 60x15 mm circular petri dishes that contained a 45 mm circular Whatman paper. Larvae were subjected to different diets: standard food without agar for "fed" larvae (as described in the *Drosophila rearing* section), 20% sucrose solution for "protein deprived" larvae, and water for "starved" larvae. The Whatman paper was soaked with 0.6 mL MilliQ water ("starved"), sucrose

solution ("fed on sucrose"), or soaked with 0.6 mL water and 1 mL of standard food medium was added on top ("fed"). Larvae were collected after the appropriate amount of time (90 or 300 min) and rinsed in water before behavioral, imaging, or biochemistry experiments. For the behavioral experiments with rehydration, larvae were collected after 90 minutes, rinsed in water, and placed in a petri dish with a Whatman paper soaked with 0.6 mL water for 15 minutes. Likewise, for the refeeding experiments, starved larvae were collected after 300 minutes, rinsed, and placed for 15 minutes in a petri dish with standard food medium and water as in the "fed" condition. After the treatment, larvae were once more collected and rinsed before the experiment.

(3) Behavioral tracking

We used an apparatus previously described (Masson et al. 2020; Ohyama et al. 2013). Briefly, the apparatus comprises a video camera (Basler ace acA2040-90 μ m) for monitoring larvae, a ring light illuminator (Cree C503B-RCS-CW0Z0AA1 at 624 nm in the red), a computer and a hardware module for controlling air-puff, controlled through multi worm tracker (MWT) software (<http://sourceforge.net/projects/mwt>) (Ohyama et al. 2013; Swierczek et al. 2011). The arena consisted of a 25625 cm² square of 3% Bacto agar gel (CONDALAB 1804-5) with charcoal (Herboristerie Moderne, 66000 Perpignan) in a plastic dish, and was changed for each experiment. For optogenetic experiments, plates without charcoal were used, and larvae were tracked thanks to IR light. Collected third instar larvae were washed with water, moderately dried and spread on the agar starting from the center of the arena. We tested approximately 30–100 larvae at once during each experiment. The temperature of the behavioral room was kept at 25°C.

(4) Locomotion analysis

To assess larval locomotion in the absence of stimulation, larvae were placed on top of the agar in the arena inside the tracker, and either tracked for 5 min continuously (intact attP2>UAS-TNT larvaewild type larvae) or for 60s. The coordinates of the 11 points along the central spine and the outline of each larva were computed as described previously (Masson et al. 2020; Ohyama et al. 2013). These 2D X-Y coordinates, structured as time series of irregular framerate, labeled by the instantaneous tracking time of the recording, comprise the raw datasets which have been subsequently analyzed to derive all secondary metrics.

Analysis was performed in python using the [larvaworld](https://pypi.org/project/larvaworld/) behavioral analysis and simulation platform (<https://pypi.org/project/larvaworld/>). The 3-step analysis pipeline included preprocessing, computation of secondary angular, translational and temporal metrics and behavioral epoch detection to annotate strides, runs and pauses, as described previously (Sakagiannis, Jürgensen, and Nawrot 2021). During preprocessing the raw time series were adjusted to a 10 Hz constant framerate by interpolating them

at a 0.1 second timestep. Noise reduction was achieved by applying a low-pass filter with a 2Hz cut-off frequency, a threshold high enough not to alter the crawling-related dynamics around the dominant ~ 1.4 Hz crawling frequency.

For trajectory-based spatial metrics such as pathlength and dispersal, to avoid the cumulative effect of body micromovements, the position of the 9th point along the midline was used as a proxy for the larva's position, a relatively stable rear point unaffected by lateral and translational jitter. To correct for different larval sizes, any metric measured in absolute spatial units (m or mm) can be scaled to body-length, measured in dimensionless body-length units. As the instantaneous body-length of an individual larva fluctuates during crawling due to subsequent stretching and contraction, individual larva length is defined as the median of the midline length across time (total length of the line connecting all 11 midline points). A trajectory's pathlength is the cumulative displacement of the larva during the entire track. Dispersal is the instantaneous straight-line distance relative to its initial position. Track tortuosity was quantified by the straightness index (S.I), computed by advancing a fixed time window (20 seconds in this study) along the track and calculating at each point the ratio of the dispersal to the actual distance traveled. This index, which varies from 0 (no movement) to 1 (straight line movement), can capture very well the complexity of the movement at various scales (set by the window time frame) throughout the track.

For the detection of peristaltic strides and crawl-pauses, the scaled crawling speed time series were used. To this end the dominant crawling frequency for each track was extracted by applying a Fourier analysis and its inverse was used as the expected duration of a peristaltic cycle. A stride was therefore defined as the epoch between two local speed minima, that included a local maximum of at least 0.3 body-lengths/s and lasted between 0.7 and 1.5 times the expected cycle duration. A run was defined as an uninterrupted sequence of consecutive strides and a crawl-pause as an epoch lacking any strides during which the scaled speed was constantly below the 0.3 body-lengths/s threshold.

(5) *Air-puff stimulation during behavioral tracking*

Air-puff was delivered as described previously (Jovanic et al. 2016; Masson et al. 2020; Ohyama et al. 2013) to the 25625 cm² arena at a pressure of 1.1 MPa through a 3D-printed flare nozzle placed above the arena, with a 16 cm x 0.17 cm opening, connected through a tubing system to plant supplied compressed air. The strength of the airflow was controlled through a regulator downstream from the air amplifier and turned on and off with a solenoid valve (Parker Skinner 71215SN2GN00). Air-flow rates at 9 different positions in the arena were measured with a hot-wire anemometer to ensure consistent coverage of the arena across experimental days. The air-current relay was triggered through TTL pulses delivered by a Measurement Computing PCI-CTR05 5-channel, counter/timer board at the direction of the MWT. The onset and duration of the stimulus were also controlled through the MWT. Larvae were left to crawl freely on the agar plate for 60 seconds prior to stimulus delivery. air-puff was delivered at the 60th second and applied for 30 seconds.

(6) *Behavior classification and analysis*

Behaviors were detected thanks to a custom-made machine learning algorithm that was previously described (Masson et al. 2020). Behaviors were defined as mutually exclusive actions. Larvae were tracked using MWT software, all the time series of the contours and the spine of individual larvae are obtained using Choreography. From these times series some features are computed, center of the larva, velocities, etc., all key features are presented in (Masson et al. 2020). Behavior classification consists of a hierarchical procedure that were trained separately based on a limited amount of manually annotated data. Here, we required a more detailed definition of behavior. Hence, we extended the hierarchy with another layer to separate some Bends and Hunches, between different behaviors. Bends were separated into Static Bend, Head-Cast (see the description of each behavior below). We take all the Bends, Hunches and Back-ups obtained by the first classification algorithm, to reclassify with new annotated data on new lines.

(7) *Action definition*

Head Cast: dynamic bends in which the head moves laterally from one side to the other. There are two exits from Head-Cast in both the head moves strongly, but one that is slower and the tail moves at the same speed as the center of mass, and on the contrary a second one where the tail moves a lot (fast Head-Cast).

Static Bend: Low speed turning movement, and where the head moves little, and the angle between the segment between the center of mass and the head and the center of mass and the tail remains constant.

(8) *New annotated data.*

We required new annotated data to ensure the classifier matched the phenotype of the larva used in these experiments. The sets of, Hunch, Bends, Backs and Crawls were manually tagged from actions selected using the (Masson et al. 2020) old behavior classification pipeline. A few numbers of tags are used for the model.

(9) *New features.*

To train the new layer of the pipeline, we combined features that were previously evaluated in the classifier's preceding layer with new features. Each characteristic is calculated for the time step we are examining and the three time steps before and after.

- The three velocities include the head velocity, the motion velocity, and the tail velocity, all normalized by the length of the larva. The motion velocity is the velocity of center of mass return by the MWT software. Head and tail are the terminal point of the spine; The averaging along the spine curve and its derivative, $S = \frac{1}{2}(3 \langle \cos^2 \theta \rangle - 1)$ with $\cos \theta$ the scalar product between normalized vectors associated to a segment of the spine and the direction of the larva body.
- The shape factor $\lambda = \frac{\lambda_1 - \lambda_2}{\lambda_1 + \lambda_2}$ with λ_i the eigenvalues of the mean covariance matrix of movement which characterizes the shape of the larva and takes value between 0 and 1.

We have also introduced new features:

- The ratio between the length of the head-center of mass and the tail-center of mass $\frac{||\overrightarrow{HG}||}{||\overrightarrow{TG}||}$ with H, T and G respectively coordinate points of the head, the tail and the center of the masse
- The projection of the head and tail velocity on the spine of the larva. If we note the velocity vector of the head HV_h with H coordinate point of the head and V_h the coordinate point at the end of the velocity vector, the projection point satisfies the basic relationship: $||V_h - V'_h|| = \min ||V_h - x||$ with $x \in \overrightarrow{r_{st}}$.
- The cosine of the angle between the vector of the head (tail) velocity and the first (last) segment of the larva. $\cos(\theta) = \frac{\overrightarrow{r_{s1}} \cdot \overrightarrow{v_{head}}}{||\overrightarrow{r_{s1}}|| ||\overrightarrow{v_{head}}||}$ with $\overrightarrow{v_{head}}$ the vector velocity of the head and $\overrightarrow{r_{s1}}$ the first vector of the spine.

All features are normalized by the length of the larva to ensure scale-free properties.

(10) *New classifications*

We employed a Random Forest algorithm for classification, distinguishing each time step of original Bends, Hunches, and Backs into new categories. We conducted ten random forests on all Hunch and back tags, along with a random selection of a thousand bends, utilizing balanced weights. The predicted behavior represents the most probable outcome, with each random forest's confusion matrix exceeding 80% accuracy for each behavior.

To address the issue of having a behavior span only two time steps, which is not biologically plausible, we introduced a preventive measure by adding five time steps before and after the behavior. Additionally, to mitigate noisy results, we implemented smoothing. This involved excluding behaviors with fewer than three time steps, logically categorizing them as the behavior before or after (based on the length of the behavior and behaviors $N+2$ and $N-2$). According to our knowledge, Hunch behavior initiates at the beginning of stimulation, around 60 second (Jovanic et al. 2016; Masson et al. 2020). Larvae typically do not exhibit multiple Hunches. In cases where they do, we classify the second Hunch as a cast, a classification verified through ground truthing. We applied the same threshold as outlined in (Masson et al. 2020) to the effective length change during the behavior. If a Hunch fails to surpass this threshold, the time window is assigned to the small behavior. The threshold is not the same depending on the line, some lines were slower or smaller than others (threshold between 0.6 and 0.3). The validation of these thresholds was performed through ground truthing, contributing to the enhancement of classification, particularly in cases where performance may be suboptimal for certain lines.

The distinction between Static Bends and Head-Cast is determined by applying a threshold to the head velocity. If bending occurs over 'n' time steps, the motion velocity normalized by the length of 'P%' of those 'n' time steps must be below 'p' times the mean head velocity of the larva before the stimulation. The values of the two thresholds, 'P' and 'p', are line-specific, contingent upon the statistical characteristics of the velocity for that particular line; some lines are slower than others.

We computed the cumulative probabilities of actions (Stop, Hunch, Back-up) during five seconds after stimulus onset (as described in Masson *et al*, 2020), only in larvae that were tracked during the entire time window. For actions that occur during baseline locomotion and at high frequency (Crawl and Head Cast), we computed the mean probability over five seconds after stimulus onset and over three seconds starting one second after stimulus onset. We corrected the mean probabilities for these actions by the mean probability computed over twenty seconds of recording prior to stimulus

onset. For optogenetic activation of the mechanosensory neurons, because the dynamic of the response was different to that of air-puff experiments, the time window used for computing the cumulative probability of Hunching was two seconds after the stimulus onset and that for bending probability was ten seconds after stimulus onset, by which time bending probability reached baseline levels in larvae fed on standard food. Transition probabilities were computed as the frequency of transition from one action to another over five seconds after stimulus onset, for larvae that were tracked throughout this entire duration.

(11) *Food intake quantification*

In order to measure food intake, we quantified the amount of fluorescent food inside the digestive tract of larvae that were allowed to feed ad libitum for 15 min on fluorescent feeding media. To this end, Rhodamine B (Sigma R6626-25G) was diluted in different feeding media (water, yeast extract, sucrose solution or normal food medium, see in media section) to a final concentration of 0.20 $\mu\text{mol/L}$. 0.8 ml of each food medium was poured on top of a circular Whatman paper (Fisher scientific, Cytiva 1001-045) placed into a petri dish. 10 larvae were placed into each petri dish. After 15 min of ad libitum feeding on the fluorescent medium, larvae were collected, rinsed in ethanol and in water, and immediately mounted under a coverslip for imaging. Intact larvae were imaged thanks to a fluorescent binocular Zeiss Discovery.V12. The surface of the digestive tract stained by the fluorescent dye was quantified for each larva thanks to a custom-made Fiji script. This script quantified the number of pixels whose intensity was above background.

(12) *Sucrose preference*

To measure the preference of larvae between a sucrose-containing agar and a water-containing agar, we performed a place-preference assay. To this end, we prepared petri dishes filled with 0.3% agar diluted in water, divided each agar in two halves, and transferred one half into a new petri dish. Then, we filled the missing half in each petri dish with 0.3% agar diluted in a 20% sucrose solution. Therefore, each petri dish finally contained one side with 20% sucrose agar and one side with agar only.

After cooling down, about 20 third instar larvae were put on the midline of a petri dish and the dish was imaged every 30 s in a behavioral tracker. The number of larvae on each side was then counted over time, excluding the larvae touching the limit between the two media. The preference index was then calculated as: $PI = (N_s - N_a) / N_{tot}$, where N_s is the number of larvae on the sucrose side, N_a the number of larvae on the agar side, and N_{tot} the total number of larvae.

(13) *Carbohydrate measurements*

To measure carbohydrates concentrations inside larval hemolymph, we combined and adapted different methods already published (Matsuda et al. 2015; Matsushita and Nishimura 2020). Glucose measurements - Groups of 5 third instar larvae were rinsed in water and placed on a parafilm layer and their cuticle was cut with forceps. 2 μ L of the bleeding hemolymph was collected from each group, and 1 μ L of 0.05 g/L N-phenylthiourea diluted in PBS was added to avoid darkening of the samples. Samples were heat-inactivated by a 10 min incubation at 90°C and centrifuged 10 min at 10 000 rpm. 1 μ L of the supernatant was then mixed with 4 μ L of glucose assay kit (Sigma GAHK20-1KT) and incubated for 1 h at 37°C. Absorbance at 340 nm was measured against the blank thanks to a NanoDrop following the manufacturer's instructions. Hemolymph glucose concentration was finally calculated thanks to a standard curve of glucose concentration.

Trehalose measurements - Groups of 10 third instar larvae were rinsed in water and placed on a parafilm layer and their cuticle was cut with forceps. 3 μ L of the bleeding hemolymph was collected from each group, and 97 μ L of 0.05 g/L N-phenylthiourea diluted in trehalase buffer (Tris pH 5.5 5 mM, NaCl 137 mM, KCl 2.7 mM) was added to avoid darkening of the samples. Samples were heat-inactivated at 70°C for 10 min, centrifuged for 10 min at 10 000 rpm, and 5 μ L of the supernatant were either mixed to 5 μ L of trehalase (Sigma T8778-1UN) diluted in trehalase buffer (described above) to a 500 dilution. Samples were incubated at 37°C overnight. 1 μ L of each sample was then mixed with 4 μ L of glucose assay kit (Sigma GAHK20-1KT) and incubated for 1 h at 37°C. Absorbance at 340 nm was measured against the appropriate blank thanks to a NanoDrop following the manufacturer's instructions. Trehalose concentration was finally calculated thanks to a standard curve of glucose and trehalose concentrations and by additionally subtracting the concentration of glucose in the sample without trehalase.

(14) *Histochemistry labeling*

To determine the neurotransmitter identification in the interneurons, immunolabeling was performed from the split lines or Gal4 lines crossed to UAS-myr::GFP, or LexA lines crossed to LexAop-myr::GFP. The VNC was dissected out from 3rd instar larvae and fixed with 4% PFA for 45 min at room temperature. After rinsing in PBS, ten minutes permeabilization in PBS-T and two hours blocking in PBS-T-BSA 1%, the CNS preparations were incubated at 4°C (one to three nights) in the first antibodies raised against neurotransmitter and GFP in PBS-T. Then they were incubated at 4°C (one to two nights) in fluorophore-coupled secondary antibodies in PBS-T raised against

species of the first antibodies. After rinsing, the preparations were mounted in an anti-bleaching mounting medium (SlowFade Gold, ThermoFisher S36939) under a cover slip. The confocal images were captured with a Leica SP8 confocal laser microscope. Alexa Fluor 488 was excited with a laser light of 488 nm, Cy3 with a laser light of 561 nm, Alexa Fluor 647 with a light of 633 nm wavelength.

(15) *Neuropeptide receptor characterisation*

In order to characterize the expression pattern of sNPFR and NPFR in the circuit, expression of two genetically encoded reporter proteins of two different colors was targeted to two different subsets of neurons. To this aim, a T2A Gal-4 (P.-T. Lee et al. 2018) or LexA (for sNPFR and NPFR respectively) was used to express LexAop-jRGeco1a or UAS-GCaMP6s (for sNPFR and NPFR respectively) under the control of the promoter of the gene coding for that receptor, thus tagging all neurons which express the receptor transcript. A second genetic driver (LexA for sNPFR and Gal4 for NPFR) was used to individually label target neurons of the circuit with a second reporter protein (UAS-GCaMP6s for sNPFR and LexAop-jRGeco1a for NPFR). The VNC was then dissected and stained with antibodies as described in the previous section.

To the best of our knowledge, no clean or sparse LexA line exists to selectively target the Handle-B and Griddle-2 inhibitory interneuron. For sNPFR expression in Griddle-2, the LexA line L55C05 used targets many neurons in addition to Griddle-2. Griddle-2 could nevertheless be identified by comparing the cell body position and projections in the cross-section of the anterior abdominal segments of the CNS of the R55C05 LexA line and the sparse R55C05 GAL4 line that selectively labels griddle-2 in the VNC (see Extended Data Fig. 7B).

For Handle-B we used a L60E02-LexA line for which a neuron with a cell body in the midline resembling Handle-B is part of a very dense expression pattern. In order to confirm that the candidate neuron was indeed Handle-B, we used the selective split-Gal4 line GMR_SS00888 that specifically labels only Handle-B neurons. We expressed two reporters of different colors (LexAop-GCaMP6s and UAS-Chrimson-mCherry under the control of GMR_SS00888 and L60E02 in the same larva. The colocalization confirmed the line 60E02-LexA to target Handle-B (Extended Data Fig. 7C).

(16) *In vivo imaging of intact larvae*

Because opening the cuticle might affect the larval internal state, we developed a simple preparation for the imaging of intact larvae. For this purpose, third instar larvae were rinsed in water, and mounted between a 2 cm circular coverslip and a custom-made device that delivers mechanical stimulations in low melting point agarose 4% (melted in phosphate buffer saline), ventral side facing up. Larvae were gently squeezed

in this position until agar cooled down, so that the ventral nerve cord could be imaged through the cuticle.

All Gal4 and LexA drivers used for *in vivo* imaging are listed in the figure legends. The imaging plane was restricted to the location of the projections of neurons of interest, in particular when sparse lines that lacked specificity towards a unique neuronal type were used (R22E09).

Mechanical stimulations were generated by a waveform generator (Siglent sdg1032x) connected to a quick-mount extension actuator (Piezo Systems, Inc.), which was embedded in the sylgard-coated recording chamber (Sylgard Silicone Elastomer, WPI). The stimulation was set at 1000 Hz, with an intensity of 1 to 20 V applied to the actuator. The amplitude of the acceleration produced by the actuator was measured thanks to a triple axis accelerometer (Sparkfun electronics ADXL313) connected to a RedBoard (Sparkfun electronics) and bound to the sylgard surface thanks to high vacuum grease. Acceleration was $1.14 \text{ m}\cdot\text{s}^{-2}$ at 20 V, and $0.61 \text{ m}\cdot\text{s}^{-2}$ at 10 V. Mechanical stimulations were precisely triggered by the Leica SP8 software thanks to the Leica "Live Data Mode" and to a trigger box branched to the scanning head of the microscope. A typical stimulation experiment consisted in 5 s of recording without stimulation, then 5 s of stimulation, and 5 s of recording in the absence of stimulation.

For optogenetic activation during *in vivo* imaging, larvae were mounted in the dark, with the least intensity of light possible in the room, to avoid nonspecific activation of the targeted neurons. Optogenetic stimulation of CsChrimson was achieved by a 617-nm wavelength LED (Thorlabs, M617F2), controlled by a LED driver (Thorlabs, LEDD1B) connected to the waveform generator, and conveyed through a \varnothing 400 μm Core Patch Cable (Thorlabs) to the imaging field. Optogenetics stimulations were triggered at 50 Hz, 50% duty during 1 s, concomitantly to mechanical stimulations thanks to the waveform generator. Irradiance was measured at the level of the imaging field at 500 μW using a PM16-130 THORLABS photometer.

Imaging was achieved with 1-photon or 2-photon scanning Leica SP8 microscope, at 200 Hz, with a resolution of 512 x 256 pixels or 512 x 190 pixels. The rate of acquisition was 1 frame/s or 2 frames/s depending on the experiment. For Basin-2 recordings, the stimulation was repeated 5 times with resting intervals of 60 s in order to calculate a frequency of response. Recordings where the dF/F averaged over the whole stimulus duration did not exceed 10% were considered as failed responses. Optogenetic experiments were conducted with 2-photon imaging.

When the projections of the neurons were not visible before stimulation (imaging of Handle-b upon NPFR knockdown), we used resonance scanning with 10 line

accumulation and 6 frame averaging to increase the signal. This resulted in one image being taken each second and in the dF/F being lower than usual. With these settings one recording was acquired per larva.

Neuronal processes were imaged in the VNC at the axonal level and fluorescence intensity was measured by manually drawing a region of interest (ROI) in the relevant areas using custom Fiji macros. Data were further analyzed using customized MATLAB scripts. F_0 was defined as the mean fluorescence in the ROI during baseline recording, in the absence of mechanical stimulus or optogenetic activation. $\Delta F/F_0$ was defined at each time step t in the ROI as: $\Delta F/F_0 = (F(t) - F_0)/F_0$.

For recording the baseline activity level of NPF neurons, one frame was recorded each second for 20 seconds and, for each larva, the frame showing the most intense fluorescence in the neuronal projections was used to evaluate its raw fluorescence level.

For imaging neurons in different feeding conditions, the effect of food treatment on the expression level of fluorescent reporter proteins was assessed by expressing GFP in the chordotonal neurons. Fluorescence was measured after exposing larvae to different food treatments.

(17) *Statistical analysis*

(a) *Locomotion analysis*

For all boxplots and histograms pairwise Mann-Whitney tests were used to evaluate significant differences between larva groups, with Bonferroni correction for multiple comparisons. Significance was illustrated according to the p-value by asterisks in histograms (*: <0.05 , **: <0.01 , ***: <0.001 , ****: <0.0001) and by pairs of colored semicircles in boxplots, the left always corresponds to the group with highest mean value. Non-significant tests were omitted for visual clarity.

(b) *Behavior probabilities*

Chi² tests were used for statistical analysis of behavioral and transition probabilities.

To assess the effects of different states/neuronal manipulations on Head Casting in response to air-puff, we calculated an estimator designed to identify the emergence of behaviors at the population scale. We aim to determine the probability induced by the stimulation, so we need to subtract the stationary probability without the stimulus. We calculate the probability of larvae bending 5 seconds after the stimulus, on tracking larvae throughout this time window (denoted as p_A). For the probability of bending before the stimulus, we consider all larvae tracked continuously for 20 seconds prior to the stimulus, between 30 and 50 seconds (denoted as p_B). A probability is defined as $p_k = N_k/N_{k,all}$ with $k \in \{A, B\}$ and $N_{k,all}$ the total number of larva taking to compute

probabilities. In order to quantify the effect of the stimulus we defined $\chi = p_A - p_B$ as the difference in the ratio after and before the stimulus.

In order to compare test line our estimator was defined as

$\theta(p, q) = \chi(p) - \chi(q)$ with p and q the ratios of the lines and the control respectively. $\theta(p, q)$ takes value in $[-1, 1]$. The null hypothesis is $\theta(p, q) = 0$, if there are no differences between the line tested and the control. Positive or negative values indicate an effect of neuron silencing when compared to the control.

We use numerical simulations to conduct a statistical test where $\{p_k, q_k\}$ are generated from a hypergeometric distribution, $X \sim \text{Hypergeometric}(N_{k,all}, N_k, 1)$. We perform $N_{sim} = 10^3$ repetitions, computing $\theta(p, q)$ each time. The p-value is determined by the number of instances when the hypothesis is not verified, divided by the total number of repetitions, (pseudo-code in Jovanic *et al*, 2016).

Note that this estimator, $\theta(p, q)$, has the advantage of being able to detect the non-synchronous emergence of a behavior at a population scale. For example, head casting can either emerge as an immediate response to the puff or as the second response after Hunching. The statistics of start time of Head Casting is thus widely distributed at the population scale. Time evolution of the instantaneous ratio of larva performing bending would not exhibit a strong increase after stimuli because larvae are not all going to bend immediately after stimuli. $\theta(p, q)$ by accumulating events during a time window allows efficient detection of a behavior even if it is widely distributed in time.

(c) Calcium imaging

All data in line plots are presented as mean \pm SEM. Violin plots show the first and third quartiles, the average of all recordings as a white line and the median as a white dot. Comparisons of the data series between two conditions were achieved by a two-tailed unpaired t-test. Comparisons between more than two distinct groups were made using a one-way ANOVA test, followed by Bonferroni pairwise comparisons between the experimental groups and their controls.

(18) Mathematical model

We reproduced and extended the rate-based system model of the circuit that was published in a previous publication (Jovanic *et al*. 2016). The circuit is described as a rate model with a connection matrix derived from the larva connectome. Each neuron population (mechano-ch, iLNa, iLNb, fbLN-Ha and fLN-Hb) was modeled by a single node (Fig. 3C-E). The dynamics read:

$$\tau \frac{d\mathbf{r}}{dt} = -\mathbf{V}_0 - \mathbf{r} + \mathbf{s} + \mathbf{i} + k_{ex} (\mathbf{r} - \mathbf{r}^{\max}) (\underline{\mathbf{A}}^{\text{ex}} \mathbf{r} - \underline{\mathbf{A}}^{\text{in}} \mathbf{r})$$

(1)

with τ representing the vector of the characteristic time constants of the neurons, \mathbf{r} the rate vector, \mathbf{V}_0 the threshold vector, \mathbf{s} the sensory stimulus input vector, \mathbf{i} the vector of inputs from other brain regions, k_{ex} a sensitivity factor to overall input, \mathbf{r}^{\max} the maximal rate vector, $\underline{\mathbf{A}}^{\text{ex}}$ and $\underline{\mathbf{A}}^{\text{in}}$ respectively the excitatory and inhibitory coupling matrices.

Values in $\underline{\mathbf{A}}^{\text{in}}$ and $\underline{\mathbf{A}}^{\text{ex}}$ were directly extracted from synaptic counts (see Jovanic *et al.*, 2016).

In order to represent the variety of stimuli larvae are subjected to, and thus the variety of behavior they elicit, we follow the approach in (Jovanic *et al.* 2016) and vary the connection strength between Mch and iLNa populations. In the original paper, the connection strength between Mch and iLNb populations is also varied. We decided to fix the value of this connection strength at 2 in order to reduce the number of parameters to explore.

We used the `solve_ivp` routine of the integration package from SciPy, which internally calls the LSODA solver, able to switch between the Adams method and the BDF method, based on the stiffness of the equation. We used relative and absolute tolerances of 10^{-3} . Additionally, the solution vector is constrained to stay positive. This is obtained by replacing r by $\max(r, 0)$, and the components of r' by those of $\max(r', 0)$ whenever the corresponding component of r is smaller than 10^{-9} .

The behavior is defined based on the end state of the network and, more specifically, the rates of the neurons B1 and B2. We used a k-means clustering with $k=2$ to separate the values of the output neurons for an ensemble of simulations corresponding to connection strengths spanning $[0.5, 1.5]$ between MCh and iLNa, and $[1.5, 2.5]$ between iLNa and iLNb. The output activations cluster strongly in a coactive state ($\text{rate}(B1) > 0$, $\text{rate}(B2) > 0$) corresponding to bends, and a monoactive state ($\text{rate}(B1) > 0$, $\text{rate}(B2) = 0$) corresponding to Hunches. The two behaviors can also be distinguished by the single scalar $\text{rate}(B2)/\text{rate}(B1)$, which is positive for bends and zero for Hunches. This ratio is sometimes plotted instead of the discrete category.

We explore two models for neuromodulation, which modify the behavior output of the network without altering its connectivity.

The first model hypothesizes that in the sucrose state, Hb receives an additional input current, modeled by a nonzero entry to the vector \mathbf{i} . We show that as this input current increases, the range of stimuli evoking bends increases, allowing us to claim that an additional input current to Hb increases the likelihood of bends. We further fix the value of the input current to 10 a.u. in the sucrose state and 0 a.u. in the fed state, to perform silencing analyses.

The second model hypothesizes that in the sucrose state, the maximum rate for the iLNa neuron population is decreased, representing a saturation of the response to external stimuli. We show that as the r_{\max} parameter for iLNa decreases, the range of stimuli evoking bends increases, consistent with experimental observations and once again despite the use of arbitrary units. For silencing analyses, we define the sucrose state as $r_{\max} = 18$ a.u. and the fed state as $r_{\max} = 20$ a.u.

Finally, we also consider a model combining both hypotheses. In this model, every combination of a decrease in r_{\max} for iLNa and an increase in input current to Hb results in more bend. For silencing analyses, we fix the exact values of those parameters. In the combined model, we define the sucrose set by setting each parameter to the value defining the sucrose state in the single hypothesis models.

We provide here the list of parameters used in the simulations.

k_{ex}	2.5
\mathbf{i}	[0, 0, 0, 0, 0, 0, 0] in fed state [0, 10, 0, 0, 0, 0, 0] in sucrose state for the combined model
r_{\max}	[20, 20, 20, 20, 20, 20, 20] in fed state [20, 20, 20, 20, 20, 20, 18] in sucrose state for the combined model
V_0	[0, 20, 20, 20, 20, 20, 20]
\square	[1, 35, 35, 35, 35, 35, 35]

The excitatory and inhibitory matrices read

$$A_{\text{ex}} = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1.5 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0.75 & 0 & 0 & 0 & 0 & 0 & 0 \\ 2 & 0 & 0 & 0 & 0 & 0 & 0 \\ W_{iLNa} & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0.2 & 0.2 & 0 & 0 & 0 & 0 \\ 0.4 & 0 & 0.5 & 0 & 0 & 0 & 0 \end{bmatrix}$$

$$A_{\text{in}} = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1.7648 & 1.3841 & 0 & 0 \\ 0 & 0 & 0 & 1 & 5.9167 & 0 & 0 \\ 0 & 0 & 0 & 0 & 3.3744 & 1.6659 & 2.191 \\ 0 & 0 & 0 & 2.7133 & 0 & 1.1010 & 3.3031 \\ 0 & 0 & 0 & 1.8411 & 1.1158 & 0 & 0 \\ 0 & 0 & 0 & 1.7331 & 2.2145 & 0 & 0 \end{bmatrix}$$

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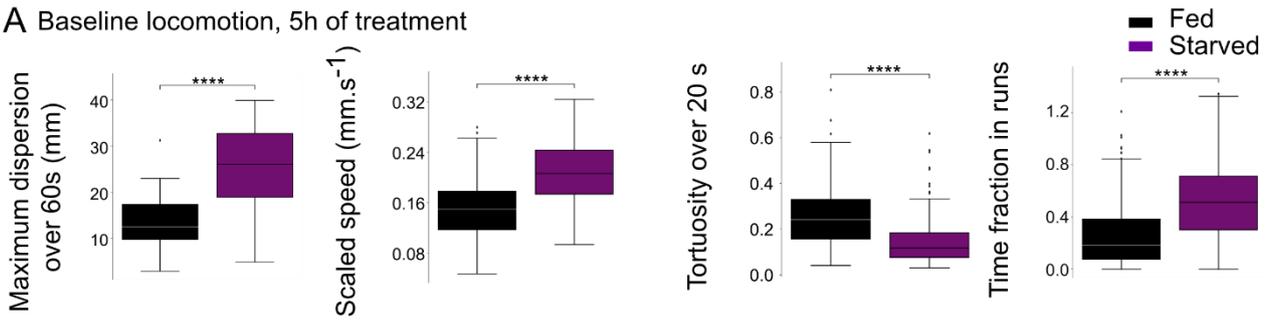
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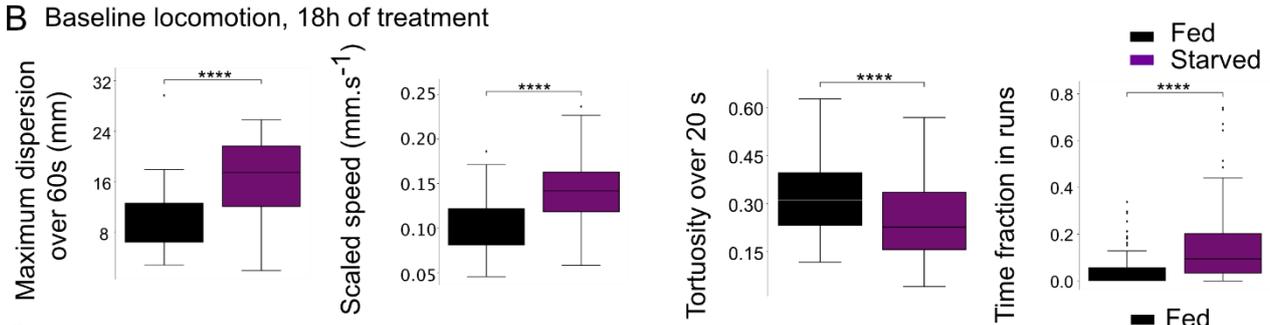
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III. A. 2. j. Supplementary figures

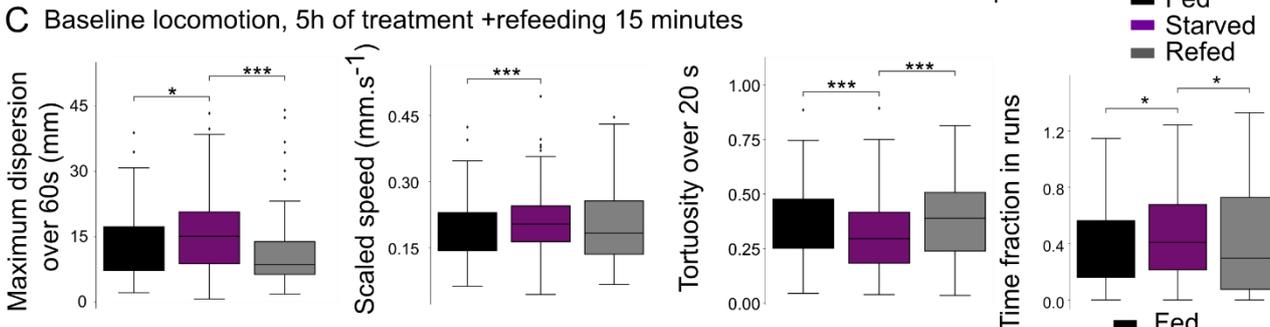
A Baseline locomotion, 5h of treatment



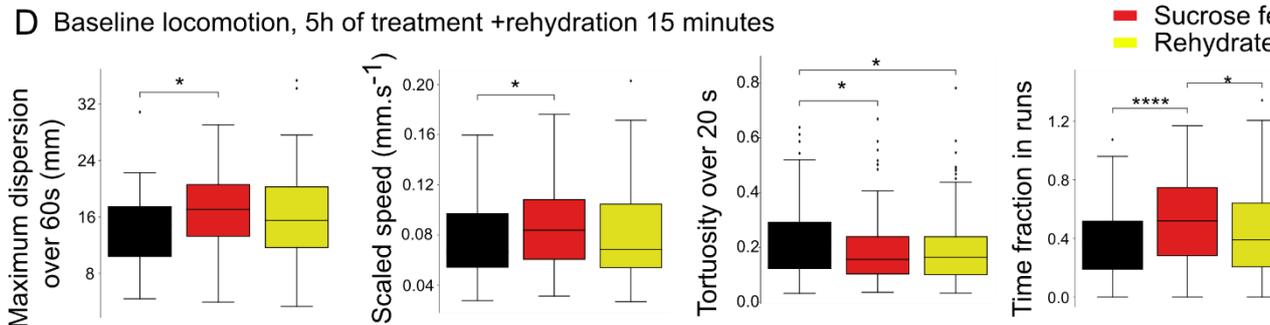
B Baseline locomotion, 18h of treatment



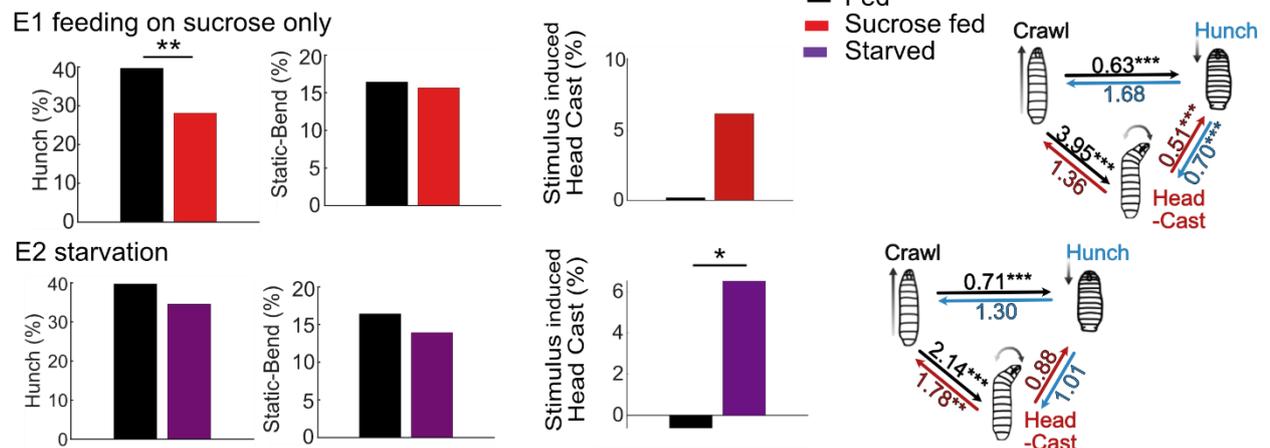
C Baseline locomotion, 5h of treatment +refeeding 15 minutes



D Baseline locomotion, 5h of treatment +rehydration 15 minutes

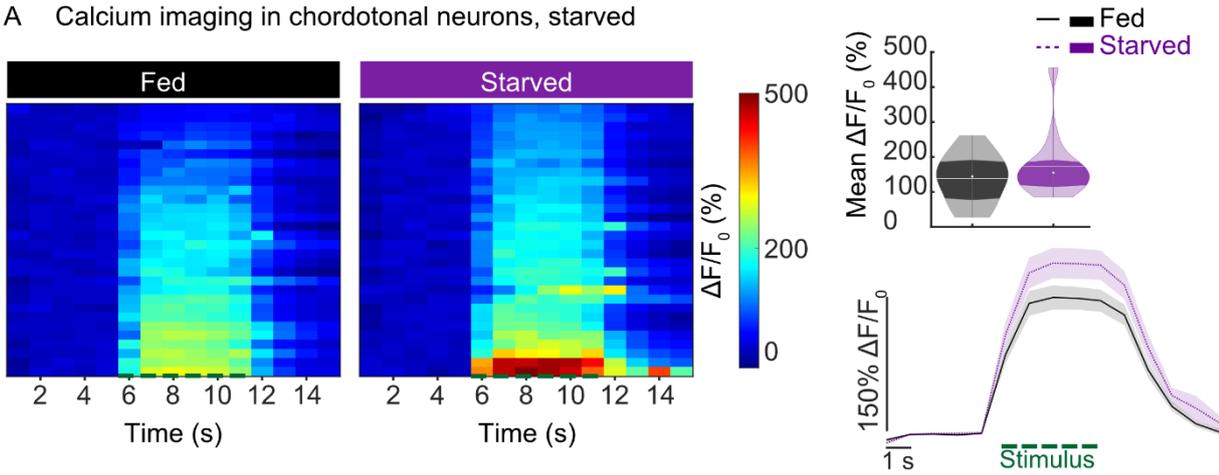


F Stimulus induced behavioral responses, 5h of treatment

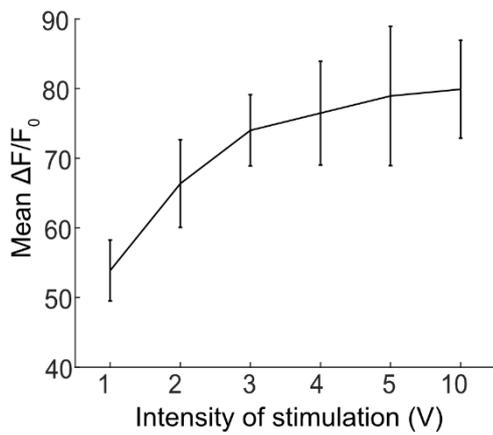


Extended Data Fig. 1. Locomotion of food-deprived larvae. A-D: Analysis of larval locomotion in different feeding states. A-B: Larvae starved for 5h (A, n = 283, 211, 309 larvae) or 18h (B, n = 173, 141, 139 larvae) disperse further, at a higher movement speed, allocating more time to crawling (time fraction in runs) with lower-tortuosity trajectories compared to larvae fed on a standard diet. C: Refeeding larvae on standard food for 15 min after a period of 5h starvation rescues the normal locomotion as dispersion distance, time allocation to crawling and crawling speed are no longer significantly increased, nor the trajectories' tortuosity decreased compared to larvae fed on a standard diet (n= 244, 160, 223 larvae) D: Rehydration by putting larvae for 15 minutes on water after 90 minutes of feeding of sucrose restores normal locomotion similar to larvae fed on standard food, since dispersion distance, time allocation to crawling and movement speed are no longer significantly increased, nor the trajectories' tortuosity decreased. (Mann-Whitney test, ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$) (n = 102, 109, 123 larvae) E-F: Behavior in response to air-puff during the first five seconds upon stimulus onset, Behavioral and transition probabilities upon 5h of sucrose feeding (E) and starvation (F) (n=233-301 larvae, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$)

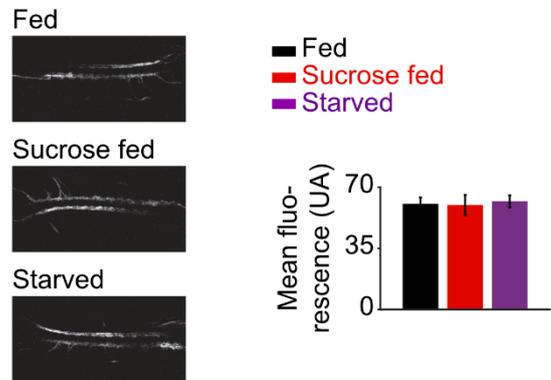
A Calcium imaging in chordotonal neurons, starved



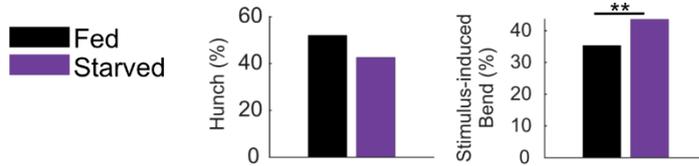
B Calcium imaging in chordotonal neurons



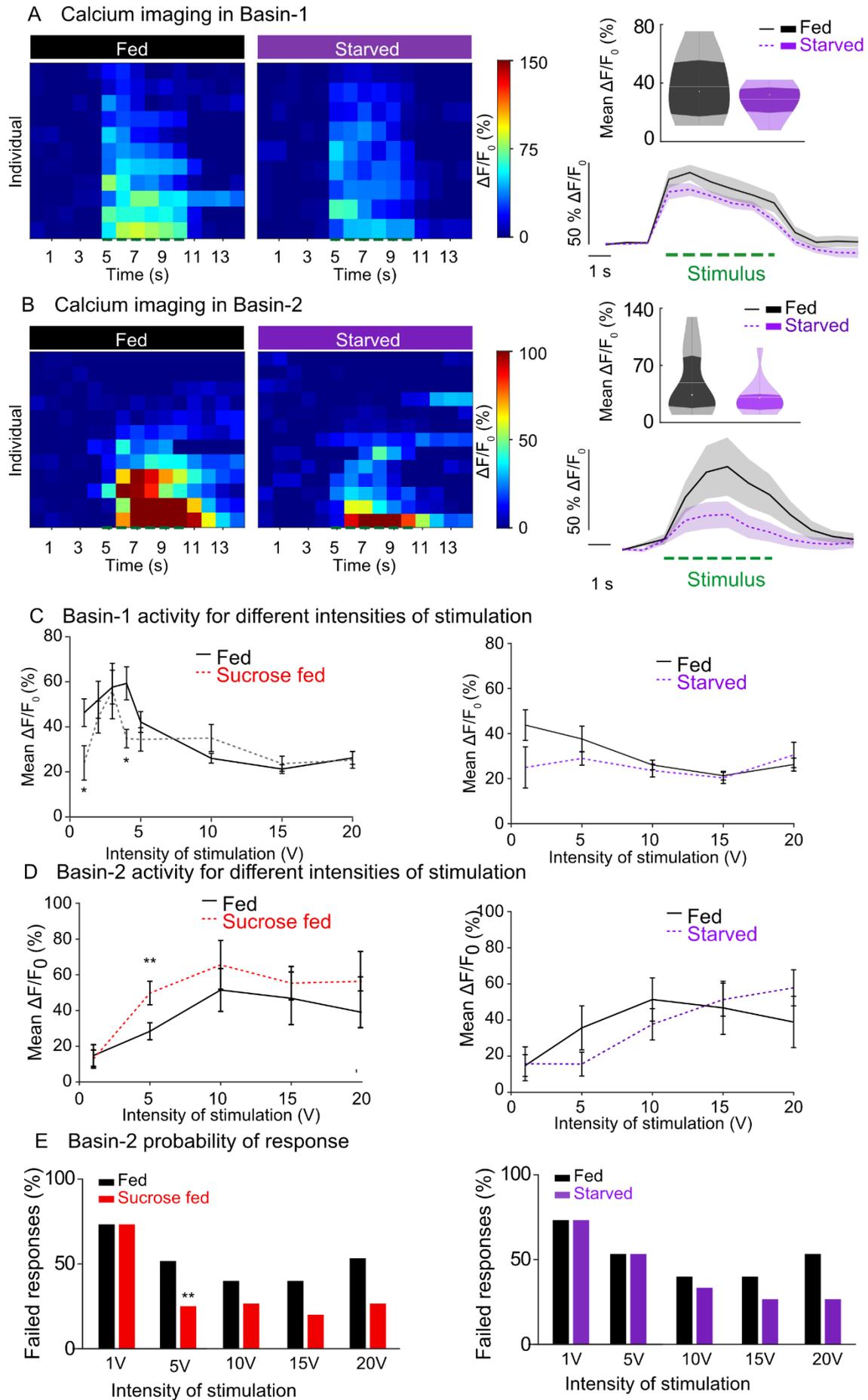
C GFP expression in chordotonal neurons



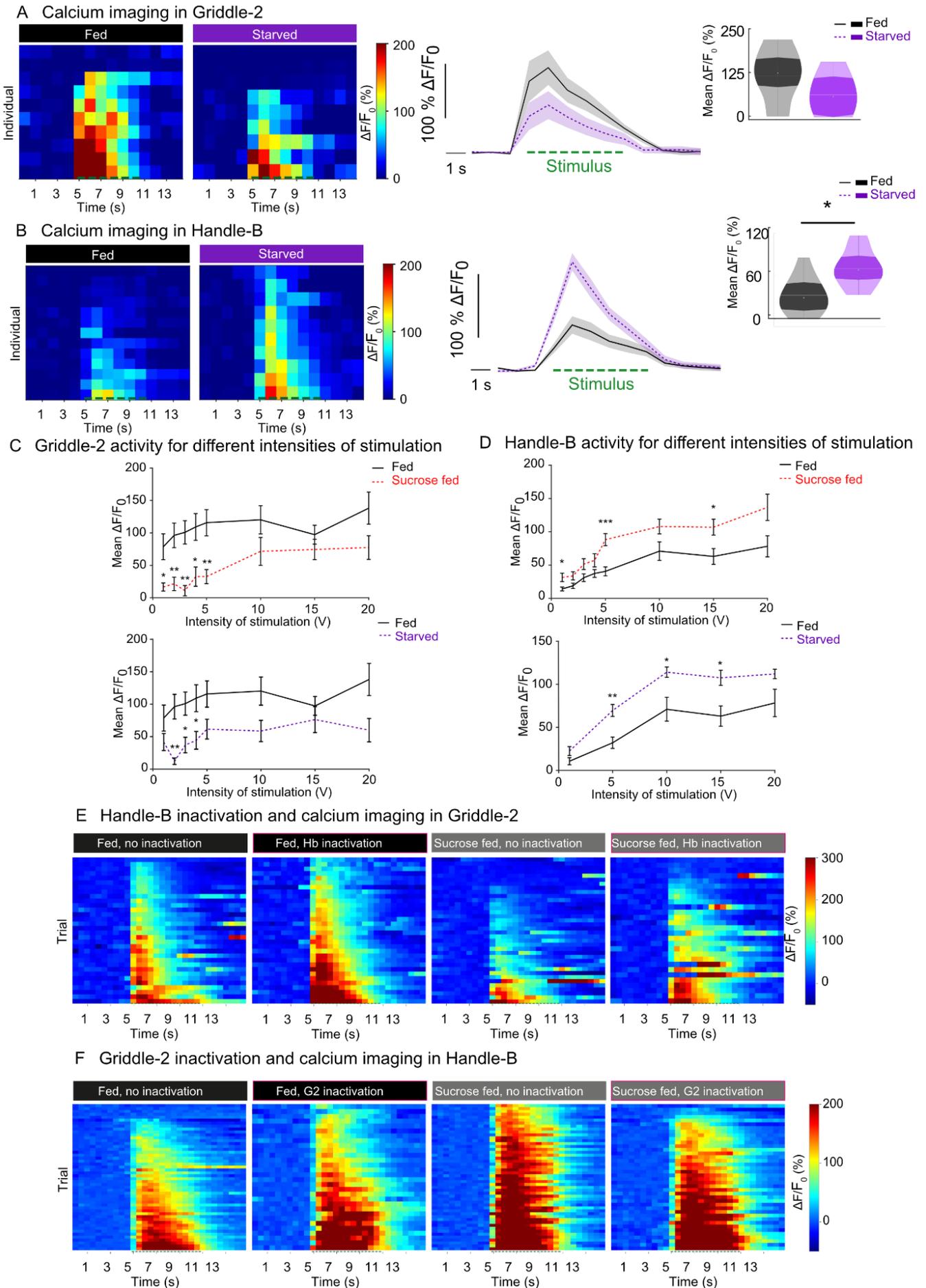
D Behavioral response to optogenetic activation of chordotonal mechanosensory neurons



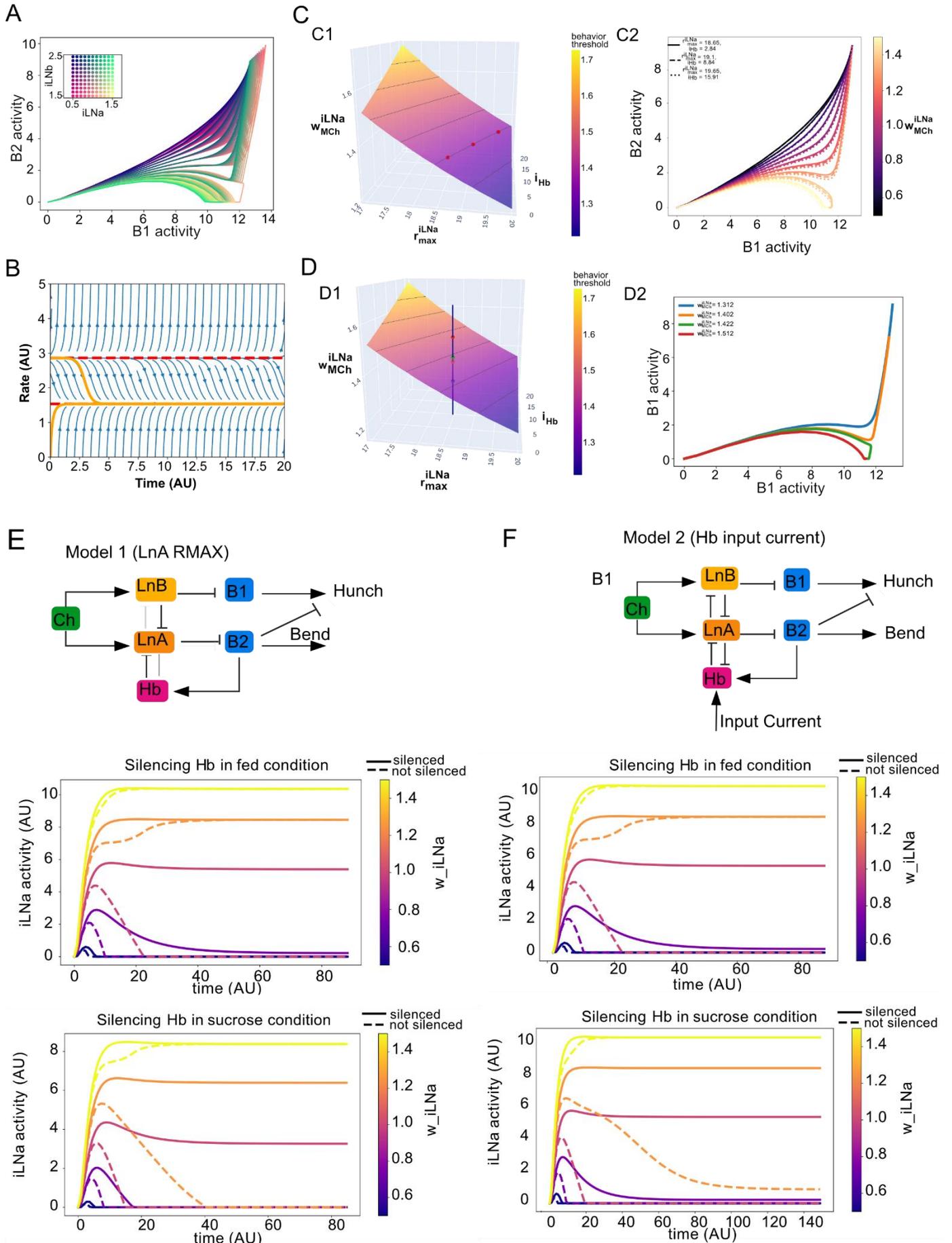
Extended Data Fig. 2. Feeding state dependent bias in sensorimotor responses does not come from the modulation of sensory neurons. A: Calcium responses to mechanical stimulations at 5V in chordotonal neurons in fed and starved larvae (R61D08-Gal4/UAS-GCaMP6s). Left panel: calcium responses of chordotonal neurons from different individuals fed on different diets. Lower right panel: mean calcium response of chordotonal neurons over time. The green dashed line corresponds to stimulus duration. Upper right panel: calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of chordotonal mechanosensory neurons is not significantly increased in starved animals as compared to larvae fed on a standard food (n = 10 larvae, 1 trial per larva, t-test p = 0.092). B: Calcium responses of chordotonal neurons to different intensities of mechanical stimulations, in larvae fed on standard food medium (n = 5 larvae). C: GFP expression levels in chordotonal neurons R61D08>GFP larvae fed on different food media: standard food, 20% sucrose and water (n = 4 larvae per condition). D: Optogenetic activation of cho in starved larvae. Hunch is cumulative probability during the first 2 seconds from stimulus onset. Bend is the mean probability during the first 10 seconds from stimulus onset, corrected by 40 seconds of recording prior to the stimulus. Hunching and stimulus-induced Bending (n = 192 larvae for fed, 157 for starved, p = 0.080 for hunch, 0.003 for bend).



Extended Data Fig. 3. Effect of starvation and different stimulus intensity on responses of projection neurons to mechanical stimulations. A: Basin-1 calcium responses to mechanical stimulations in fed and starved larvae (20B01-lexA ; LexAop-GCaMP6s, UAS-CsChrimson-mCherry). Left panel: calcium responses of Basin-1 from different individuals fed on different diets. Lower right panel: mean calcium response of Basin-1 over time. The green dashed line corresponds to stimulus onset. Upper right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Basin-1 neurons is similar in starved animals compared to larvae fed on a standard food (n = 11/9 larvae, 1 trial per larva, t-test p = 0.2892). B: Basin-2 calcium responses to mechanical stimulations in different states (SS00739/UAS-GCaMP6s). Left panel: calcium responses of Basin-2 from fed and starved animals. Lower right panel: mean calcium response of Basin-2 over time. The green dashed line corresponds to stimulus onset. Upper right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Basin-2 is not significantly decreased in starved animals compared to larvae fed on a standard food (n = 12/13 larvae, 1 trial per larva, t-test p = 0.1804). C: Basin-1 calcium responses for different intensities of stimulation, in larvae fed on standard food, on sucrose only or completely starved larvae (n = 8-20 larvae, 1 trial per larva). Average neuronal activities over time for different intensities of stimulation are plotted. D: Basin-2 calcium responses for different intensities of stimulation, in larvae fed on standard food, on sucrose only or completely starved larvae (n = 11-13 larvae, 1 trial per larva, t-test **: p < 0.01). Averaged neuronal activity over time for different intensities of stimulation are plotted. E. percentage of failed responses comparison Chi-2 test p = 0.0027).

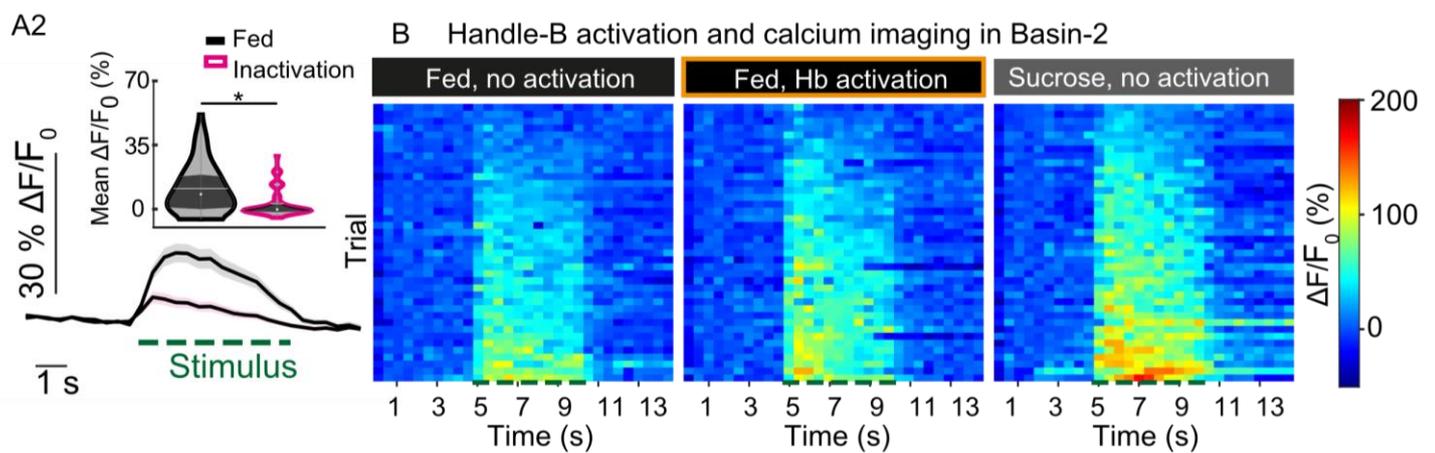
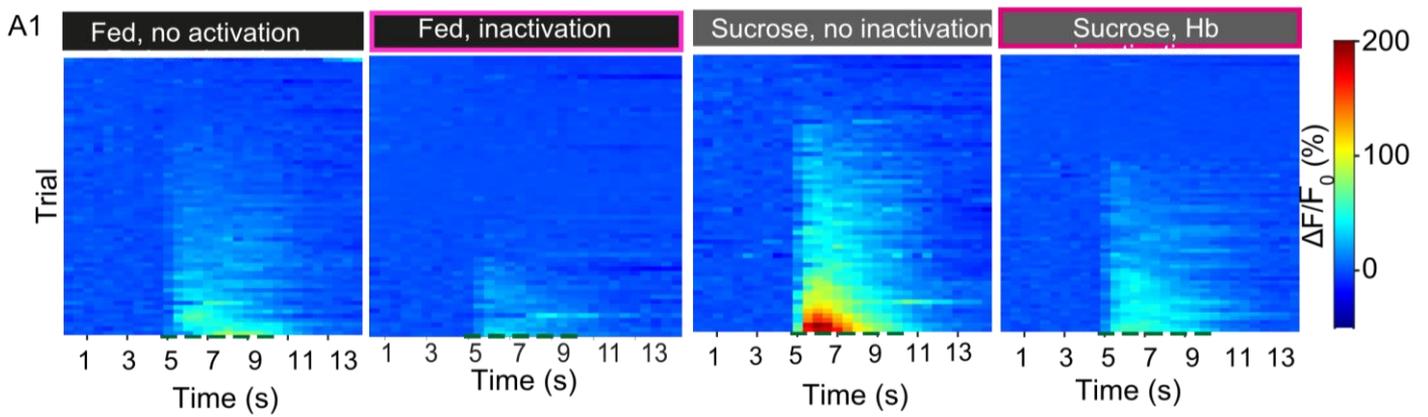


Extended Data Fig. 4. Two interneuron subtypes are oppositely modulated by the feeding state. A: Griddle-2 calcium responses to mechanical stimulations in fed and starved larvae (SS00918/UAS-GCaMP6s). Left panel: calcium responses of Griddle-2 from different individuals fed on different diets. Lower right panel: mean calcium response of Griddle-2 over time. The green dashed line corresponds to the stimulus. Upper right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Griddle-2 neurons is not significantly decreased in starved animals compared to larvae fed on a standard food (n = 10/9 larvae, 1 trial per larva, t-test p = 0.0979). B: Handle-b calcium responses to mechanical stimulations in different states (SS00888/UAS-GCaMP6s). Left panel: calcium responses of Handle-b from fed and starved animals. Lower right panel: mean calcium response of Handle-b over time. The green dashed line corresponds to stimulus onset. Upper right panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. Stimulus-induced activity of Handle-b is significantly increased in starved animals compared to larvae fed on a standard food (n = 10/13 larvae, 1 trial per larva, t-test p = 0.019). C: Griddle-2 calcium responses for different intensities of stimulation, in larvae fed on standard food, on sucrose only and starved larvae (n = 9-10 larvae, 1 trial per larva, t-test **: p < 0.01, *: p < 0.05). Average neuronal activities over time for different intensities of stimulation are plotted. D: Handle-b calcium responses for different intensities of stimulation, in larvae fed on standard food or on sucrose only and starved larvae (n = 9-25 larvae, 1 trial per larva, t-test ***: p < 0.001, **: p < 0.01, *: p < 0.05). Responses averaged during the stimulus for different intensities of stimulation are plotted. E: Calcium responses in Griddle-2 with (55C05-LexA>LexAop-GCaMP6s SS00888-Gal4>UAS-TNT) or without Handle-b inactivation (55C05-LexA>LexAop-GCaMP6s +/-UAS-TNT), in each trial of mechanosensory stimulation (n = 7-9 larvae, 4 trials per larva). F: Calcium responses in Handle with (55C05-LexA>LexAop-TNT 22E09-Gal4>UAS-GCaMP6s) or without Handle-b inactivation (+/LexAop-TNT 22E09-Gal4>UAS-GCaMP6s), in each trial of mechanosensory stimulation (n = 6-10 larvae, 5 trials per larva).

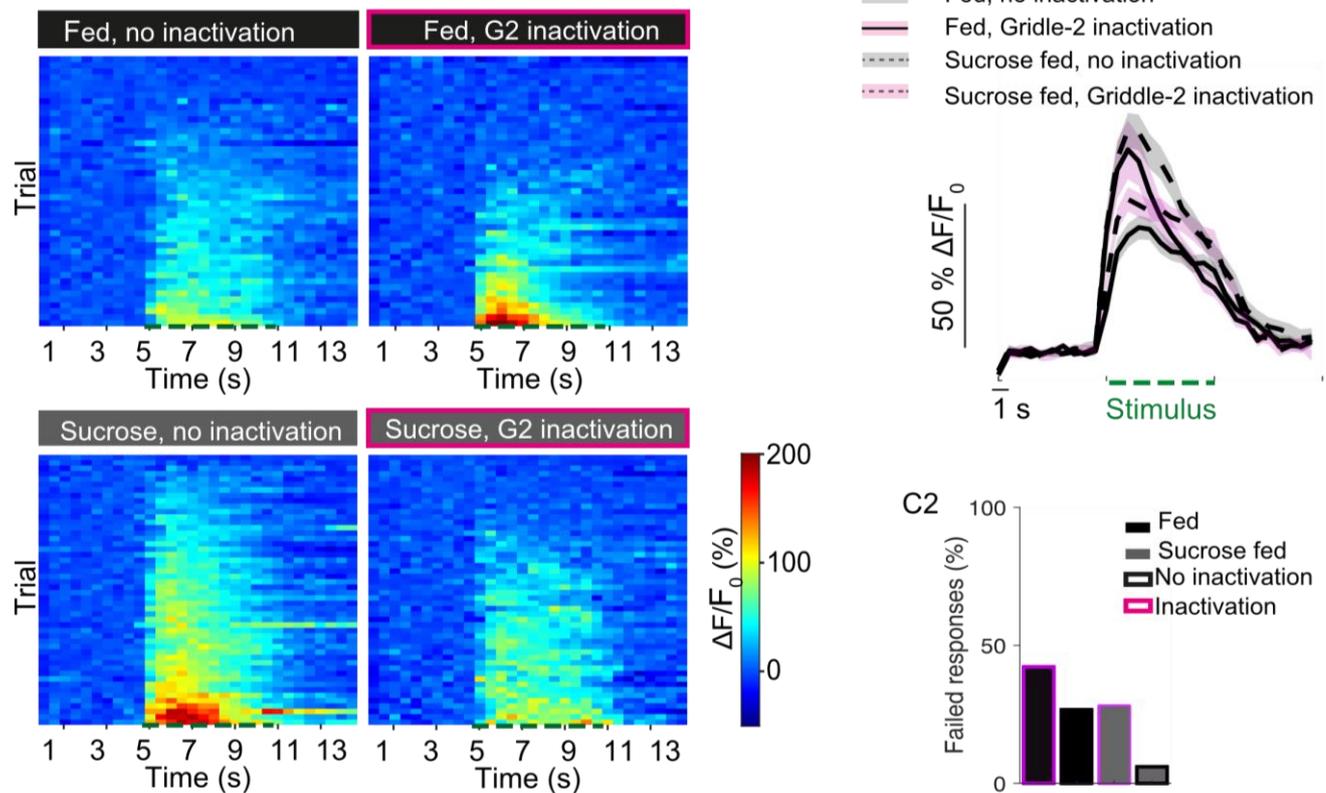


Extended Data Fig. 5. Circuit model. A: State space trajectories of B1 and B2 Dynamics as a function of inputs to LNa and LNb neurons in a simple rate model (as in Jovanic *et al.*, 2016). B: Dynamics of a single model neuron with self inhibition shows two equilibria, only one of which is stable C: Level-set for threshold in combined model. C1. Points on lines at the intersection of the surface and horizontal planes represent sets of parameters with identical behavior response. C2. State-space trajectories of the models parameterized by the three points in C1 do not differ qualitatively D: Combined model D1. Points on a vertical line correspond to the same model parameter, with different inputs to iLNa. Points above the surface converge to the monoactive state, while points below the surface converge to the coactive state. D2. Trajectories corresponding to the points in D1, color-coded. E: Silencing Handle-B in model 1, where the sucrose state is modeled as a decrease in LNa max. F: Silencing Handle-b in model 2 where the sucrose state is modeled as increase input in Handle-b.

A Handle-B inactivation and calcium imaging in Basin-2



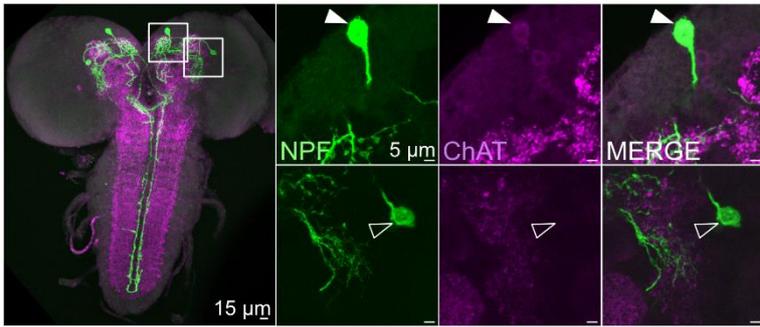
C Griddle-2 inactivation and calcium imaging in Basin-2 C1



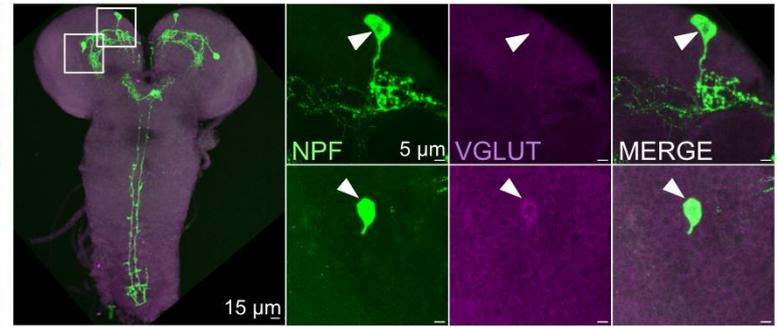
Extended Data Fig. 6. Inhibitory interneurons are required for the feeding state dependent modulation of projection neurons. A1: Calcium responses in Basin-2 with (38H09-LexA>LexAop-GCaMP6s 22E09-Gal4>UAS-TNT) or without (38H09-LexA>LexAop-GCaMP6s +/->UAS-TNT) Handle-b inactivation in each trial of mechanosensory stimulation, in larvae fed on different diets (n = 12-14 larvae, 5 trials per larva). A2: calcium response of Basin-2 averaged during the stimulus. White line represents the mean, white dot represents the median with (38H09-LexA>LexAop-GCaMP6s 22E09-Gal4>UAS-TNT) or without (38H09-LexA>LexAop-GCaMP6s +/->UAS-TNT) (n = 12-14 larvae, 5 trials per larva ; ANOVA with post-hoc tests: *: p < 0.05. B: Individual calcium responses in Basin-2 from individuals fed on different diets, with or without optogenetic activation of Handle-b (22E09-Gal4>UAS-CsChrimson::tdTomato 38H09-LexA>LexAop-GCaMP6s) during the first second of mechanical stimulus (n = 8 larvae, 5 trials per larva). In all plots, the green dashed line corresponds to stimulus onset. C: Calcium responses in Basin-2 with (38H09-LexA>LexAop-GCaMP6s 55C05-Gal4>UAS-TNT) or without (38H09-LexA>LexAop-GCaMP6s +/->UAS-TNT) Griddle-2 inhibition, in each trial of mechanosensory stimulation, in larvae fed on different diets (n = 6-10 larvae, 5 trials per larva).

A Neurotransmitter expression in NPF neurons

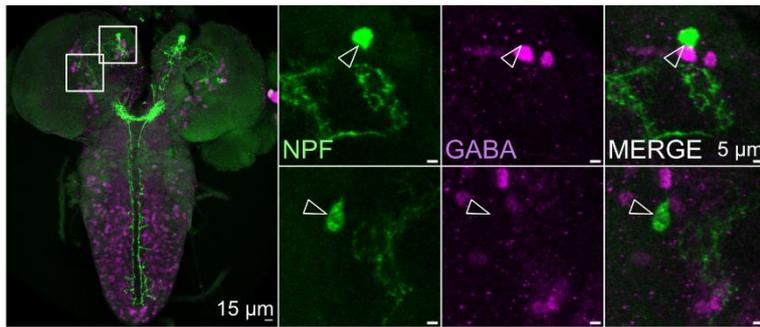
A1 ChAT expression



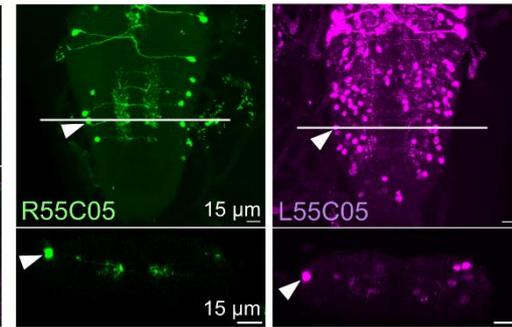
A2 VGLut expression



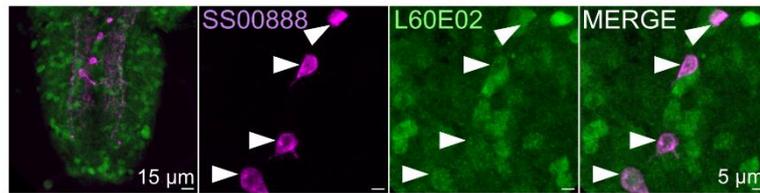
A3 GABA expression



B Identifying Griddle-2 in L55C05

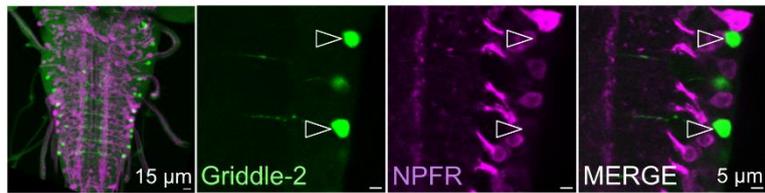


C Identifying Handle-b in L60E02

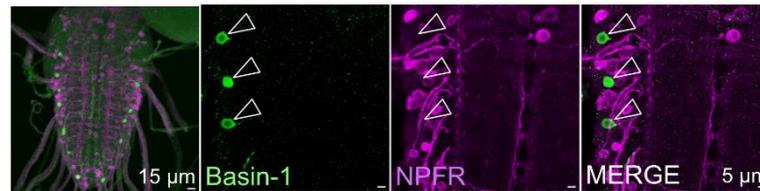


D NPFR expression

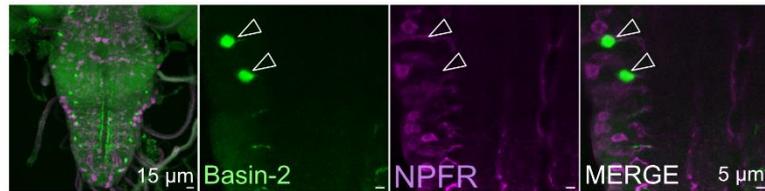
D1 NPFR expression in Griddle-2



D2 NPFR expression in Basin-1

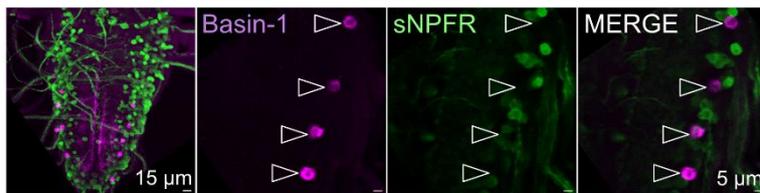


D3 NPFR expression in Basin-2

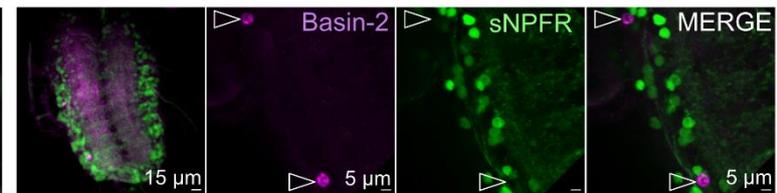


E sNPFR expression

E1 sNPFR expression in Basin-1



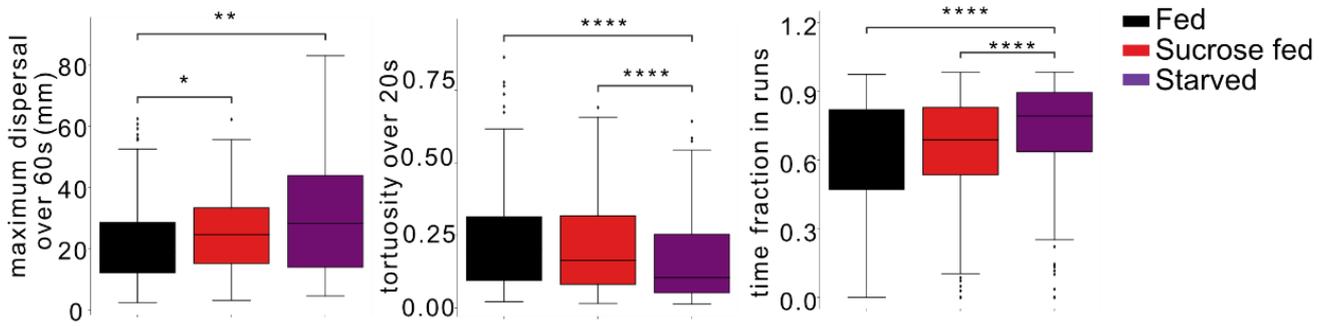
E2 sNPFR expression in Basin-2



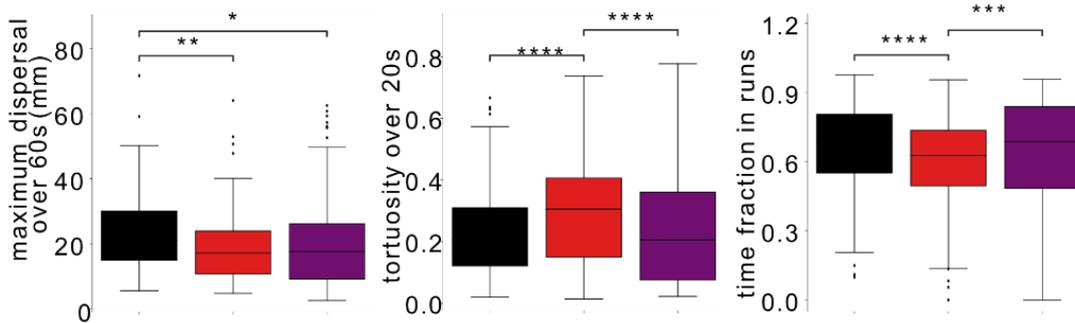
Extended Data Fig. 7. Neurotransmitter identity of NPF neuron and NPF and sNPF receptor expression in neurons of the circuit. A.: Immunohistochemical labeling of NPF neuron neurotransmitters (NPF-Gal4>UAS-GFP) labels both pairs of NPF expressing neurons: dorsomedial (DM) and dorsolateral (DL). Brains were then stained with antibodies against either ChAT as a proxy of acetylcholine (A1), V-Glut as a proxy of glutamate (A2) and GABA (A3). Co-localization shows expression of acetylcholine in the DL NPF neurons and glutamate in both pairs of NPF descending neurons. Both pairs were negative for GABA antibody labeling. B: identifying griddle-2 in 55C05. The position of cell bodies and projections was compared between neurons in R55C05 (GAL4) (previously identified as Griddle-2) and L55C05 (LexA) that had a much broader expression pattern. C: Identification of Handle-b in line L60E02. L60E02 is driving the expression of LexAop-GCamp6s and GMR_SS00888 the expression of UAS-Chrimson-mCherry (60E02-LexA; UAS-Chrimson-mCherry, LexAop GCamp6s>GMR_SS00888). Specific antibodies against GFP and mCherry were used to increase detection sensitivity. Co-localization of jRGeco1a and GCamp6s show that the neuron with the cell body in the midline labeled by the 60E02-LexA is Handle-b. D1. Immunohistochemical labeling for NPFR in Griddle-2. UAS-GCamp6s is expressed in Griddle-2 using the SS_TJ001 split-Gal4 line (green) and LexAop-jRGeco1a is expressed under the control of the NPFR promoter using a T2A-LexA construct (magenta). Antibodies against GFP and dsRed were used to increase detection sensitivity. No expression of NPFR could be detected in Griddle-2. D2-D3: Basin-1 and -2 immunostaining for NPFR expression. UAS-GCamp6s is expressed in Basin-1 or Basin-2 using the R20B01 or GMR_SS00739 driver lines (green) respectively and LexAop-jRGeco1a is expressed instead under the control of the NPFR transcript using a T2A-LexA construct (magenta). Antibodies against GFP and dsRed were used to increase detection sensitivity. No expression of NPFR could be detected in Basin-1 or -2. E: Basin-1 and -2 immunostaining for sNPFR expression. LexAop-jRGeco1a is expressed in Basin-1 or Basin-2 respectively using the L20B01 or L38H09 lines (magenta) and UAS-GCamp6s is expressed under the control of the sNPFR promoter using a T2A-Gal4 construct (green). Specific antibodies against GFP and dsRed were used to increase detection sensitivity. No expression of NPFR could be detected in Basin-1 or -2.

A. Locomotion of larvae upon NPF knockdown

A1. Control

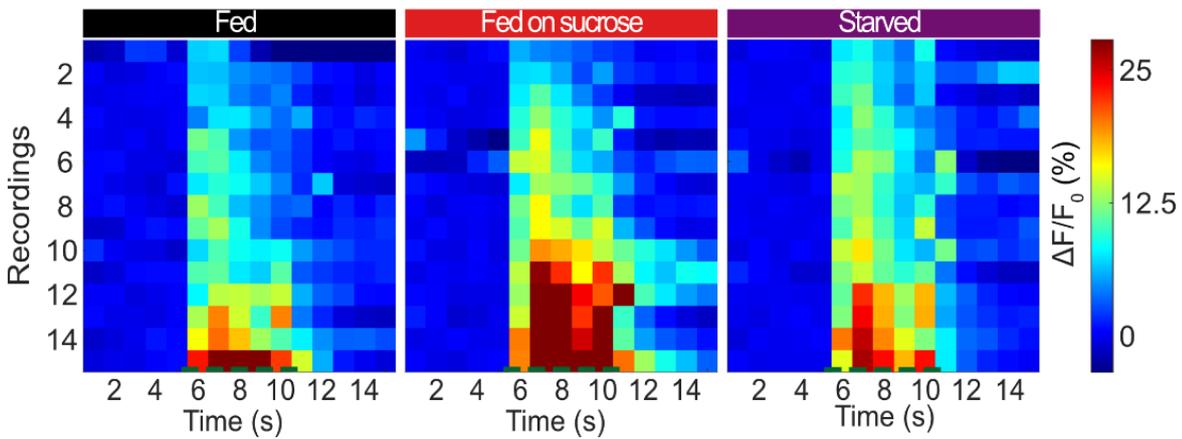


A2 NPF knockdown in DM-NPF neuron

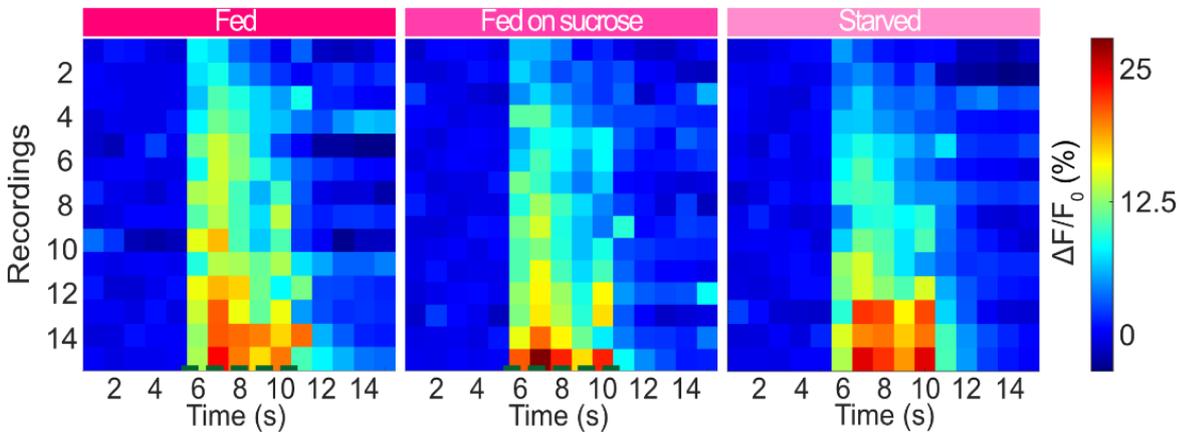


B. Ca-imaging in Hb upon NPF knockdown

B1. Control

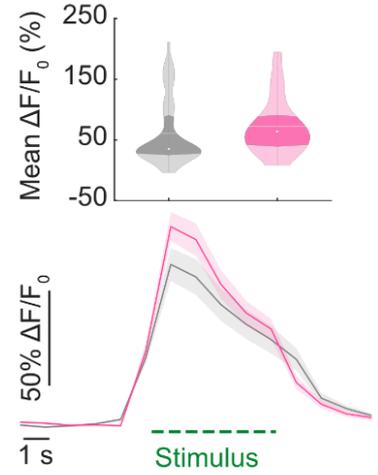
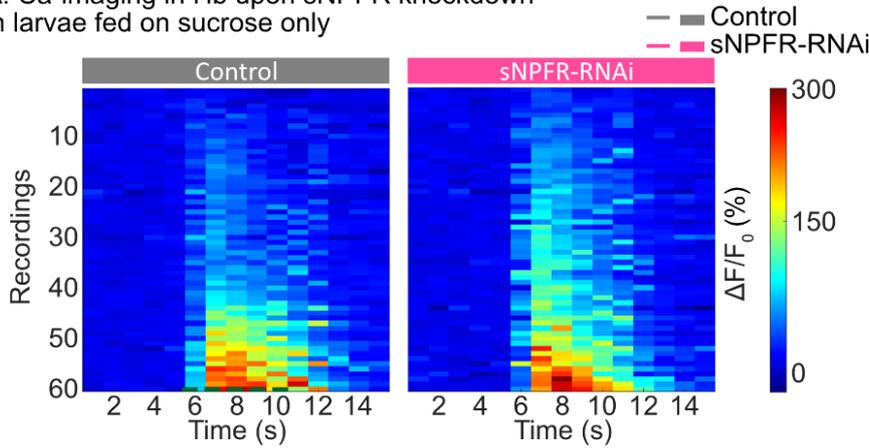


B2. NPF knockdown in Hb

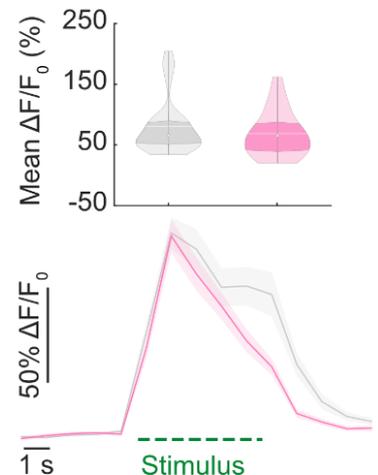
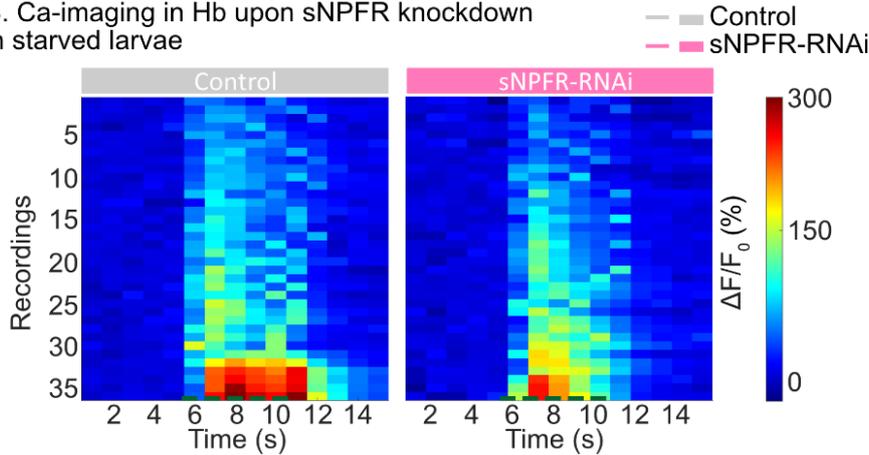


Extended Data Fig. 8. NPF regulates feeding state dependent changes in locomotion and Handle-b activity. A: Analysis of larval locomotion in the absence of sensory stimulus. Top row shows control larvae (A1). Bottom row shows larvae with NPF knockdown in DM- NPF descending neurons (A2). NPF knockdown in DM-NPF descending neuron abolishes differences in locomotion mean between larvae in different states; it abolishes the difference in trajectory tortuosity, time allocation in exploration (time fraction in runs), and dispersal between larvae fed on standard food and starved larvae. In larvae fed on sucrose the increase in exploration compared to larvae fed on standard food was also abolished (Mann-Whitney test, ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$) B: Calcium responses in Handle-b upon NPFR knockdown ($GMR_SS00888 > UAS-NPFR-RNAi; UAS-GCamp6s$) compared to a control ($GMR_SS00888 > UAS-GCamp6s$). Top row: calcium responses of Handle-b in control larvae fed, fed on sucrose or starved, in each trial of mechanosensory stimulation (n = 15 larvae per condition). Bottom row: calcium responses of Handle-b upon NPFR knockdown in larvae fed, fed on sucrose or starved, in each trial of mechanosensory stimulation (n = 14-15 larvae per condition).

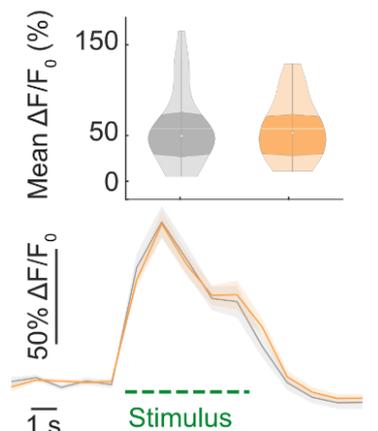
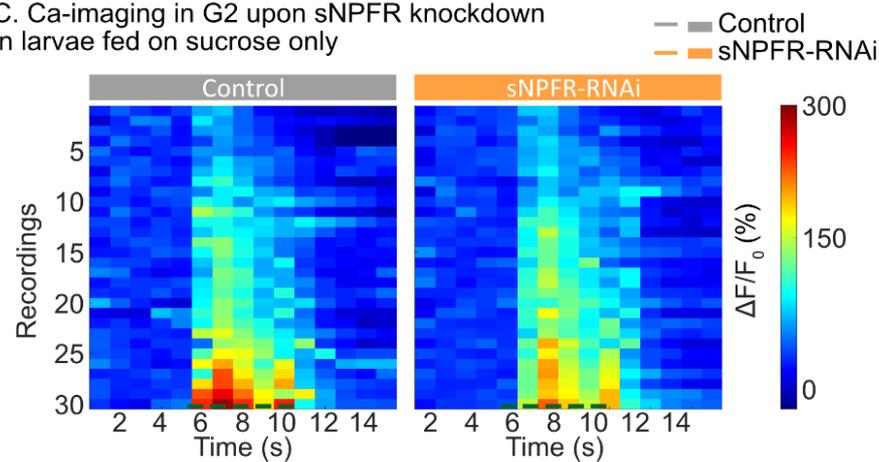
A. Ca-imaging in Hb upon sNPFR knockdown in larvae fed on sucrose only



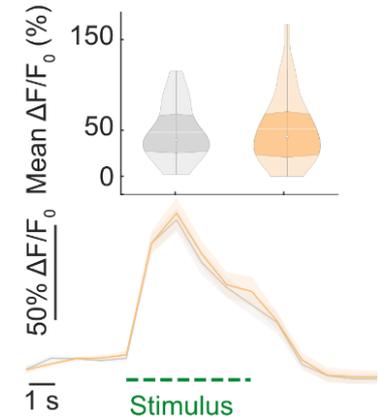
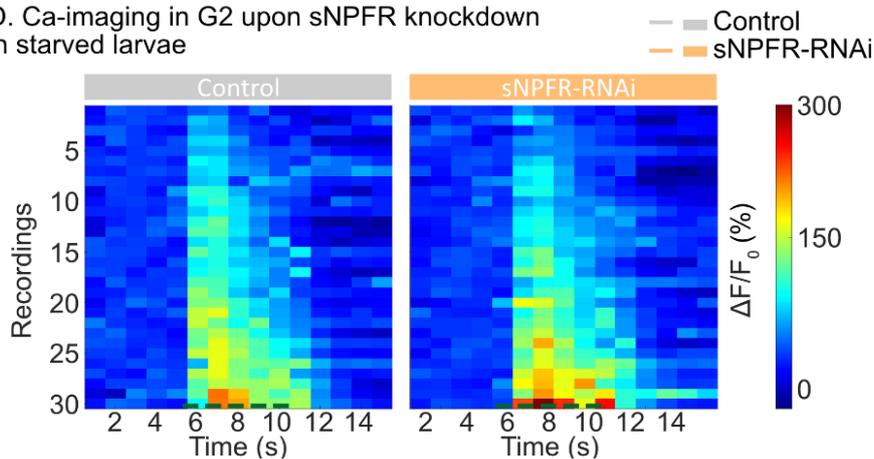
B. Ca-imaging in Hb upon sNPFR knockdown in starved larvae



C. Ca-imaging in G2 upon sNPFR knockdown in larvae fed on sucrose only



D. Ca-imaging in G2 upon sNPFR knockdown in starved larvae



Extended Data Fig. 9. sNPFR knockdown does not impact interneurons responses in larvae fed on sucrose or starved larvae. A: Calcium responses in Handle-b sNPFR knockdown in Handle-b ($GMR_SS00888 > UAS-GCamP6s; UAS-sNPFR-RNAi$) compared to the control ($GMR_SS00888 > UAS-GCamP6s$) in larvae fed on sucrose only (top row) or starved (bottom row). Left panel: calcium responses of Griddle-2, in each trial of mechanosensory stimulation. Lower right panel: mean calcium response of Handle-b over time. The green dashed line corresponds to stimulus onset. Top right panel: response averaged during the stimulus. White line represents the mean, white dot represents the median. sNPFR knockdown does not influence the stimulus-induced activity of Handle-b in larvae fed on sucrose only or starved ($n = 9-15$ larvae, 4 trials per larva). B: Calcium responses in Griddle-2 with ($SS_TJ001 > UAS-GCamP6s; UAS-sNPFR-RNAi$) or without ($SS_TJ001 > UAS-GCamP6s$) sNPFR knockdown in Griddle-2 in larvae fed on sucrose only (top row) or starved (bottom row). Left panel calcium responses of Griddle-2, for each trial Lower right panel: mean calcium response of Griddle-2 over time. The green dashed line corresponds to stimulus onset. Lower top panel: mean calcium response averaged during the stimulus. White line represents the mean, white dot represents the median. sNPFR knockdown does not influence the stimulus-induced activity of Griddle-2 in larvae fed on sucrose only or starved ($n = 10$ larvae, 3 trials per larva).

III. B. Effects of neuropeptides on mechanosensory neurons

In order to allow behavioral flexibility, mostly two modes of neuromodulation have been described: modulating the sensitivity of sensory neurons to external stimuli (Slankster et al. 2020) or modulating the activity of downstream (central) neurons involved in the computations of the circuit output (Melcher and Pankratz 2005; Crossley, Staras, and Kemenes 2018). In the scientific article in section III.A of this manuscript, we showed that the modulation of the behavioral choice between hunching and head-casting in response to an air puff by the feeding state of *Drosophila* larvae is implemented through the latter: modulation of interconnected inhibitory interneurons is sufficient to trigger a shift in behavioral choice, while modulation of the mechanosensory neurons is not required. We showed that sNPF and NPF are involved in the feeding-state-dependent modulation of the neural circuit described in (Jovanic et al. 2016) and they target a pair of reciprocally connected inhibitory interneurons. Furthermore, this work showed that modulation of the mechanosensory neurons is not necessary to the feeding-state dependent modulation of sensorimotor decisions governed by this circuit. However, while characterizing the expression of sNPFR and NPFR in the circuit, we showed that they are expressed by the mechanosensory chordotonal neurons (as described below). We thus tried to understand the role of neuropeptidergic signaling on the mechanosensory neurons.

This section will not include a separate Methods sections because most of the technics used are identical to the ones used in the article manuscript in section III.A and have already been described there. I will however detail some aspects and highlight any novelty in the experimental protocols.

III. B. 1. Expression of sNPF and NPF receptors on mechanosensory chordotonal neurons

As the gating of sensory inputs is a known mechanism giving rise to state-dependent modulation of behavior (Bargmann 2012), we checked whether neuropeptidergic input on the mechanosensory neurons plays a role in modulating the choice between startle and escape upon changes in diet.

To this aim, we genetically expressed fluorescent proteins of different colors (namely the green GCaMP6s and the red jRGeco1a) either in the studied neuron using a specific genetic driver (R61D08 or L61D08) or all neurons expressing the studied receptor using a T2A construct (T2A-Gal4_sNPFR or T2A-LexA_NPFR). T2A is a self-cleaving peptide that was inserted in the sequence of various genes in combination with the Gal4 sequence (P.-T. Lee et al. 2018; Brand and Perrimon 1993) to create a library of *Drosophila* flies in which we can target the expression of any UAS transgene (Brand and

Perrimon 1993) under the same regulatory constraints as the gene containing the T2A-Gal4 construct (see section I.D.3.a of this manuscript for a more in depth description of T2A). This strategy allowed us to replace the expression of the receptor transcript with that of a fluorescent protein and to label neurons that express the neuropeptide receptor of interest. We then stained the preparation with specific antibodies to increase sensitivity. Since the chordotonal mechanosensory neurons are peripheral sensory neurons, a different technique of dissection was used for this preparation. Indeed, their cell bodies are located in the cuticle of the larva, it was thus essential to keep the whole cuticle for the staining in order to identify the different chordotonal subtypes (Parton et al. 2010; Schrader and Merritt 2000). In this preparation, the cuticle of the larva is cut open along the longitudinal axis on the dorsal side and then spread out and pinned down. The whole digestive tract of the larva is then removed, with extra care given to not damaging the central nervous system and the peripheral nerves radiating from it, preserving the chordotonal neurons for the staining. After staining, we used a confocal microscope (see methods of the article in section III.A) to localize the cell bodies of the mechanosensory chordotonal neurons, labeled either in green (R61D08) or in red (L61D08) in the cuticle. Because part of the sample could be damaged during the dissection, some of the neurons were sometimes missing after staining. We looked across 3-5 individuals and several body segments to find one where all the eight different subtypes of mechanosensory neurons (Parton et al. 2010; Schrader and Merritt 2000) were visible and recorded a 3D stack of their entire cell bodies and their most proximal projections. Staining in body segments where some neurons were missing showed comparable results to those presented in the Figure III.B-1 for the neurons that could be seen. During image analysis, we browsed the 3D stack searching for co-localization of the green and the red staining, which equates to a neuron expressing the studied receptor. For illustration purposes, we created a Z-projection over the cell bodies, as shown in Figure III.B-1. Altogether, we determined that out of the eight subtypes of chordotonal neurons, all express NPFR (Figure III.B-1-B) and all but two lch neurons express sNPFR (Figure III.B-1-A). This suggests that the activity of the mechanosensory neurons could be modulated by NPF and sNPF, possibly also depending on the feeding state of the larvae.

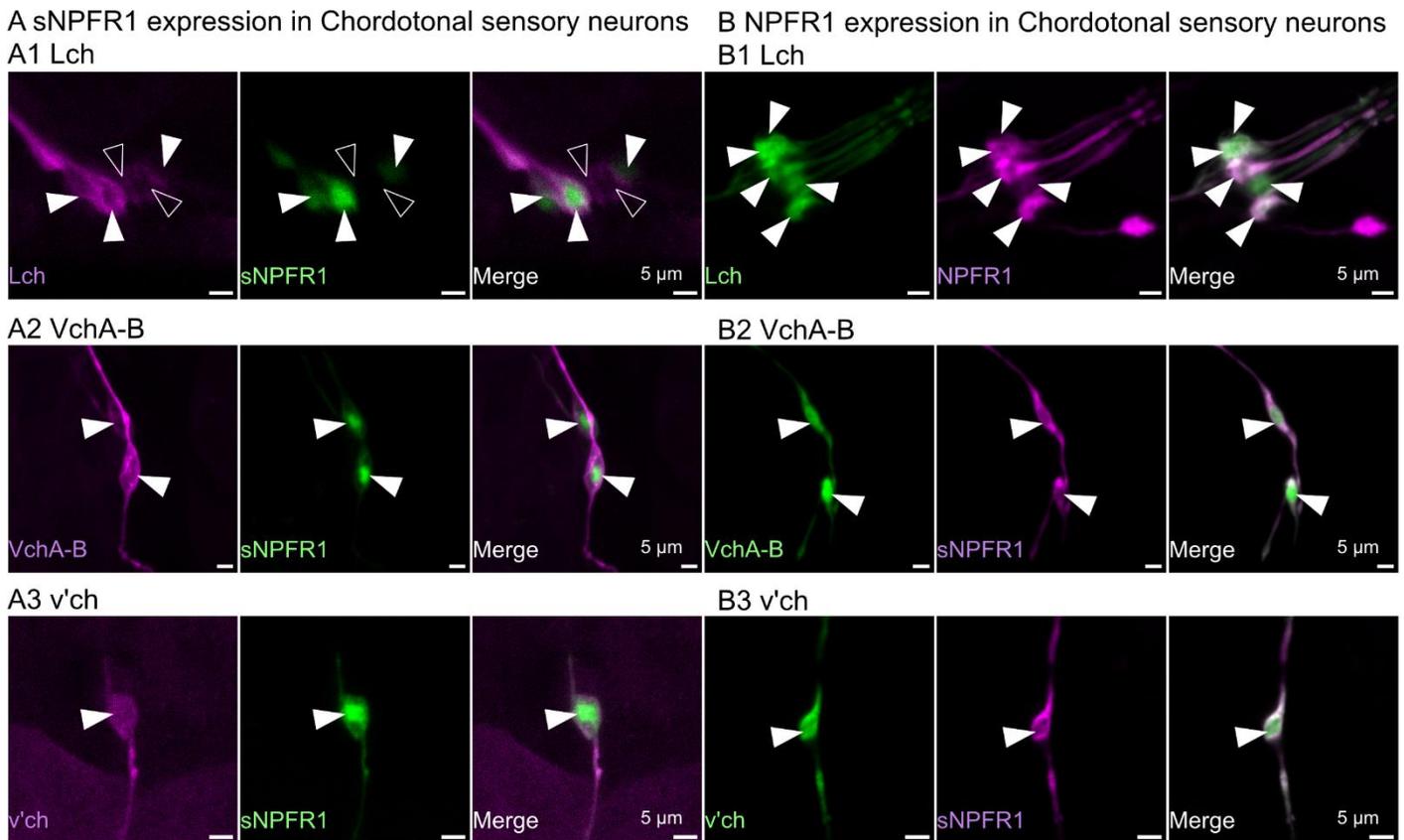


Figure III.B-1 Neuropeptide receptors expression in Chordotonal sensory neurons.

A. Immunohistochemical labeling for sNPFR in Chordotonal sensory neurons. LexAop-jRGeco1a is expressed in Chordotonal neurons using the 61D08-LexA line (magenta) and UAS-GCamP6s is expressed under the control of the sNPFR promoter using a T2A-Gal4 construct (green). Antibodies against GFP and dsRed were used to increase detection sensitivity of GCamP and jRGeco respectively. Co-localization of antibodies against jRGeco1a and GCamP6s shows that all Chordotonal neurons but 2 Lch express the sNPFR. B. Immunohistochemical labeling for NPFR in Chordotonal sensory neurons. UAS-GCamP6s is expressed in Chordotonal neurons using the R61D08-Gal4 line (green) and LexAop-jRGeco1a is expressed under the control of the NPFR promoter using a T2A-LexA construct (magenta). Antibodies against GFP and dsRed were used to increase detection sensitivity. Co-localization of LexAop-jRGeco1a and GCamP6s show that all Chordotonal neurons express the NPFR.

III. B. 2. sNPF effect on mechanosensory chordotonal neurons

As described in the Methods of the article manuscript in section III.A, we recorded in live larvae the responses of the mechanosensory neurons to mechanical stimulations using two-photon calcium imaging with GCamP6s genetically targeted to the mechanosensory neurons (R61D08>UAS-GCamP6s) after transiently feeding larvae either standard food (fed), 20 % sucrose (sucrose), or only water (starved) for 90 minutes. These changes in diet did not introduce a difference in the intensity of response of the mechanosensory neurons, suggesting that their amplitude of response

is not involved in the modulation of the behavioral choice of the larvae by their feeding state (Figure III.B-2-A).

Ca-imaging in Chordotonal sensory neurons in different feeding conditions

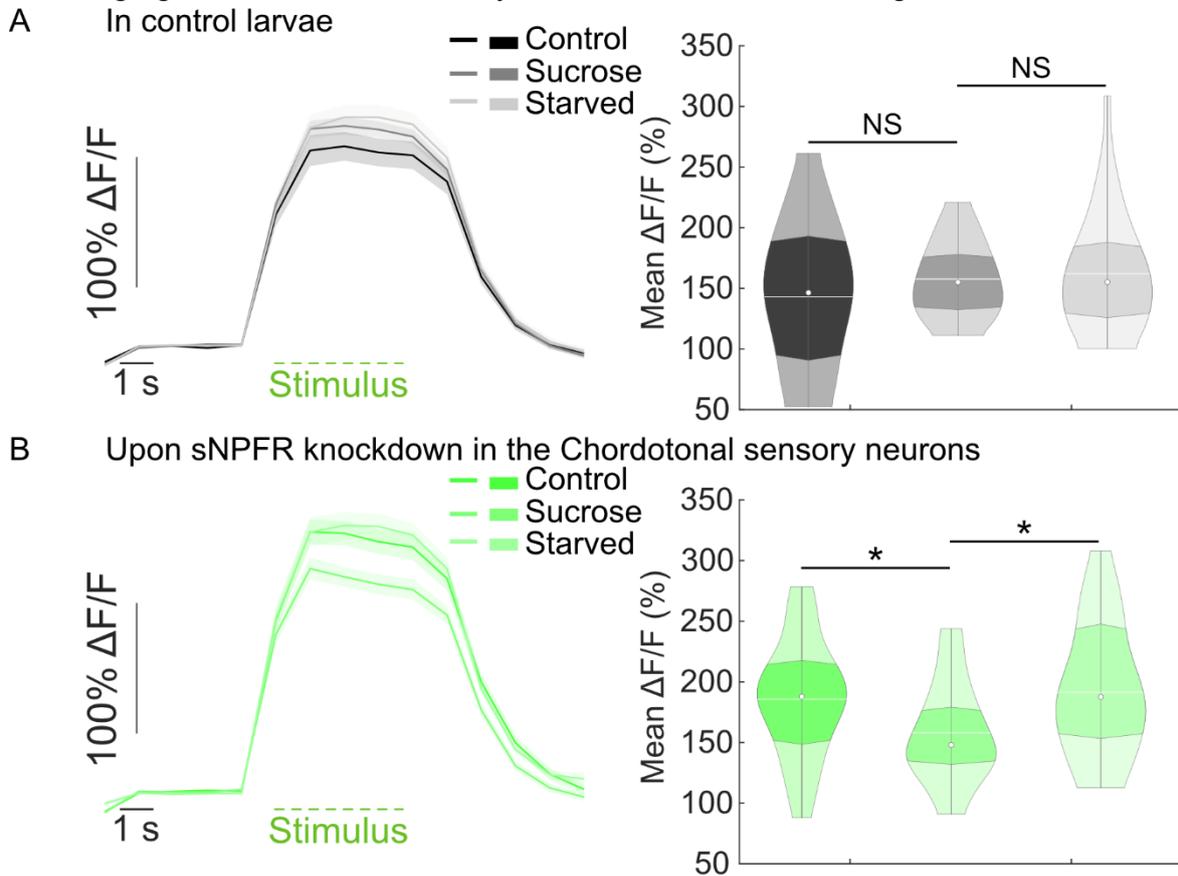


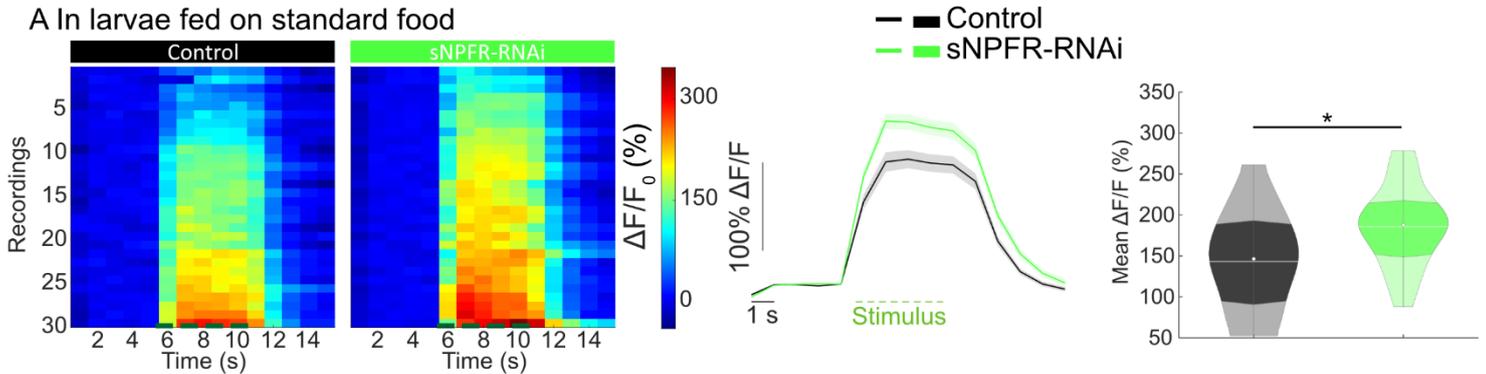
Figure III.B-2 Calcium responses of the Chordotonal mechanosensory neurons in larvae fed on standard food, sucrose only or starved. A. In control larvae. N = 6 larvae in each condition. B. Upon sNPFR knockdown in the Chordotonal sensory neurons. N = 6 larvae in each condition. The violin plots represent the quantification of the mean calcium response (dF/F_0) during 5 s after the beginning of the stimulus. White line represents the average, white dot the median. * = t-test with Bonferroni correction for two comparisons $p < 0.05$. NS = t-test with Bonferroni correction for two comparisons $p > 0.05$.

However, as we showed expression of the sNPFR in these mechanosensory neurons, we studied the effect of sNPF on their activity. To this aim, we downregulated the expression of sNPFR in the mechanosensory neurons using an RNAi (R61D08>UAS-GCamp6s, UAS-RNAi_sNPFR) and recorded their responses to mechanical stimulation using GCamp6s as a reporter. Upon knockdown of sNPFR, the intensity of the responses of the mechanosensory neurons were significantly lower in larvae fed on sucrose only than in larvae fed on standard food or starved (Figure III.B-2-B).

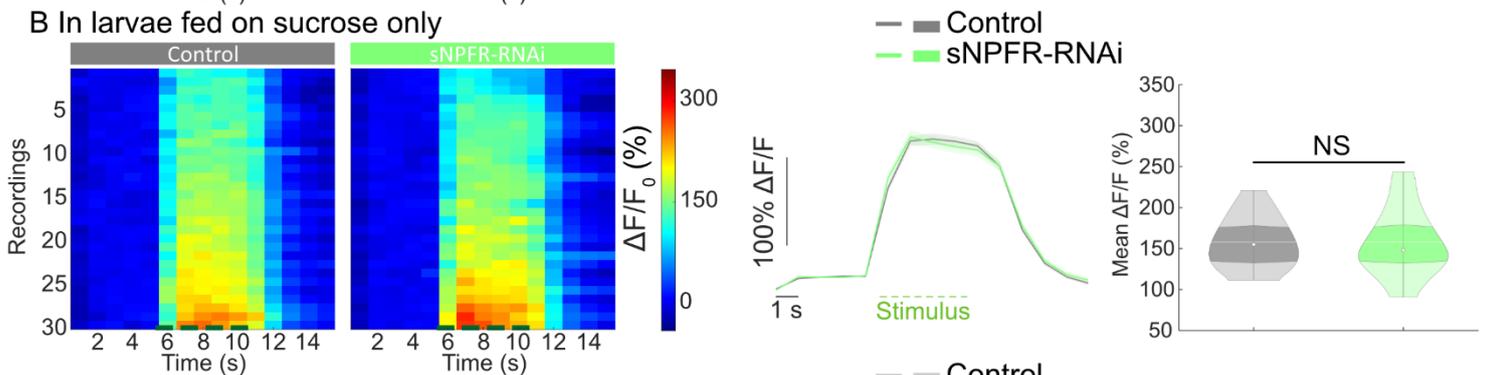
Moreover, we showed that sNPFR knockdown in the mechanosensory neurons increases their responses compared to control larvae in larvae fed on standard food or in starved larvae (Figure III.B-3). Interestingly, the responses of the mechanosensory neurons in larvae fed on sucrose only were not different between control larvae or larvae with sNPFR knockdown (Figure III.B-3).

Ca-imaging in Chordotonal sensory neurons upon sNPFR knockdown

A In larvae fed on standard food



B In larvae fed on sucrose only



C In starved larvae

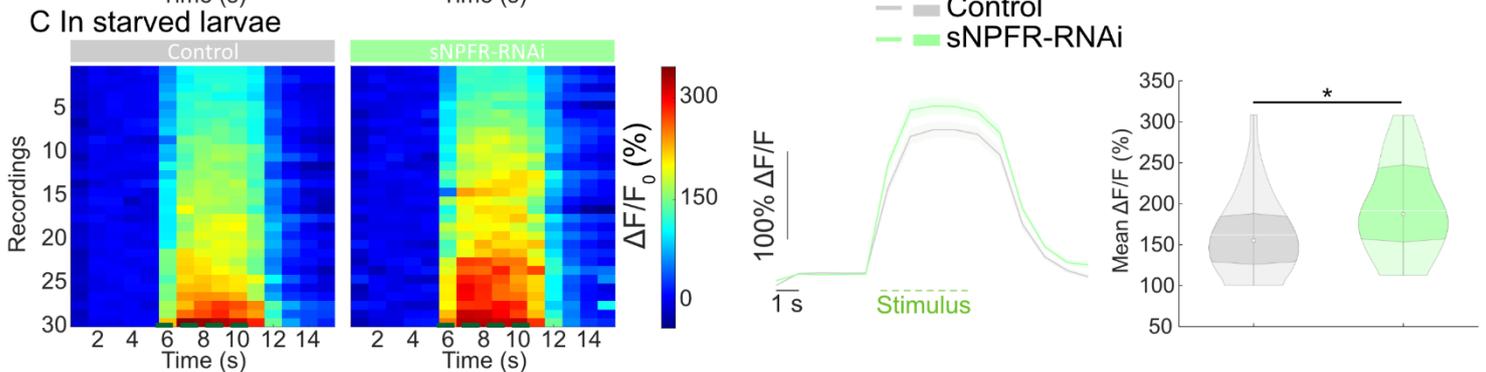


Figure III.B-3 sNPF effect on Chordotonal sensory neurons in different feeding states. Average traces of calcium activity following a mechanosensory stimulus in mechanosensory neurons in larvae A. Fed on standard food, B. Fed on 20 % sucrose, C. Starved. Shades of grey are control larvae. Green shades are larvae in which sNPFR has been downregulated in the mechanosensory neurons. The violin plots represent the quantification of the mean calcium response (dF/F_0) during 5 s after the beginning of the stimulus. White line represents the average, white dot the median. N = 6 larvae in each condition. * = t-test $p < 0.05$. NS = t-test $p > 0.05$.

These results show that sNPF has an inhibitory effect on the mechanosensory chordotonal neurons. This inhibitory effect is however absent when larvae are fed on sucrose only. Nonetheless, because no modulation of the mechanosensory neurons responses can be seen upon changes in the feeding state of control larvae, we argue that the state-dependent inhibitory effect of sNPF on the mechanosensory chordotonal neurons is not driving the bias of the behavioral decision that comes from sucrose feeding or starvation. This is in accordance with the persistence of the feeding state induced bias in behavioral decision that we describe in the article in section III.A of this manuscript upon optogenetic activation of the mechanosensory chordotonal neurons with the same intensity in different feeding conditions.

Because knocking down the sNPF in the mechanosensory neurons in larvae fed on sucrose only does not alter their response compared to control larvae, it is likely that sNPF signaling on the mechanosensory chordotonal neurons is decreased in this condition. This could come from a decrease in the expression of the sNPF in the mechanosensory chordotonal neurons or a decrease in the sNPF that is released to them. Several physiological parameters of the larvae are altered after they feed on sucrose only. Namely they have a high glucose concentration in the hemolymph, they are thirsty and have a specific hunger for proteins. Any of these parameters, or their combination, could be responsible for the alteration of the sNPF signaling pathway that we uncovered.

The relevance of the state-dependent inhibitory effect of sNPF on the mechanosensory chordotonal neurons for larval behavior is unclear. Since no difference in the responses of the mechanosensory chordotonal neurons is seen upon changes in the internal state in control larvae, we can expect that several actors, including sNPF, cooperate to maintain their response stable in spite of the alteration of the feeding state of the larvae. It would for instance be possible that some other neuropeptide could exert an excitatory action on the mechanosensory chordotonal neurons in larvae fed on standard food or starved but would be absent in sucrose-fed larvae, to which the circuit would respond by relaxing its sNPF-mediated inhibition. Alternatively, another inhibitory neuropeptide could be recruited by sucrose-feeding, suppressing the need for the sNPF-mediated inhibition. Such a push-pull regulation of the mechanosensory chordotonal neurons responses could allow for an easy bidirectional fine-tuning of their responses to different intensity of stimulation in different contexts.

III. C. Decision-making in conflicting sensory gradients

In the real world, outside of the laboratory, animals are constantly subjected to a combination of ever-changing sensory signals, that may include a complex range of sensory modalities (Ohyama et al. 2015), to which they need to adapt their behavior and decisions. For this purpose, a value is attributed to each sensory information, which is constantly updated depending on the context, internal state and experience (Kato et al. 2023; Eschbach and Zlatic 2020). When multiple sensory cues reach an animal concomitantly, each is weighted against the other in order to make a decision (Eschbach and Zlatic 2020; W.-H. Zhang et al. 2016; Lewis et al. 2015). As such, the addition of a second sensory modality to a task can alter the behavioral responses of the animal, as it was shown in *Drosophila* flies which can switch from avoidance to approach of a visual cue when presented with an appetitive odor (Cheng, Colbath, and Frye 2019).

In the brain, multisensory integration is known to arise in higher-order areas as well as at early stages of sensory processing (Ohyama et al. 2015; Eschbach and Zlatic 2020; W.-H. Zhang et al. 2016). The weighting of sensory cues is especially important when they have opposing valences, making the decision to approach or avoid the stimuli a matter of balance between risk and gain (Lewis et al. 2015). Across species, hunger has been associated with an increase in risk-taking (Lin, Senapati, and Tsao 2019; Tsao et al. 2018; Moreno-Padilla, Fernández-Serrano, and Reyes del Paso 2018; Steinhoff et al. 2020; P. Hunter 2013b; Levy, Thavikulwat, and Glimcher 2013; Moran et al. 2021; Sandhu et al. 2018). In order to study the influence of the feeding state of the larvae on risk-taking, we designed an assay to present conflicting sensory information to the larvae.

Indeed, we can present a gradient of appetitive odor (such as ethyl acetate) that has an attractive effect on the larvae (Khurana and Siddiqi 2013), at the same time as an air speed gradient that is aversive to the larvae (Jovanic et al. 2019), thus forcing larvae to make a decision between escaping or approaching. When navigating sensory gradients, larvae orient by modulating their frequency of turning and increasing the probability to accept a turn in the direction of the most favorable sensory environment (Gomez-Marin and Louis 2014; Linjiao Luo et al. 2010; Jovanic et al. 2019; Gomez-Marin, Stephens, and Louis 2011). Very little is known about navigation strategies adopted by larvae in conflicting gradients. We argue that in such contexts, larval decisions are not binary but rather arise from the accumulation of probabilistic decisions of turn initiation and direction.

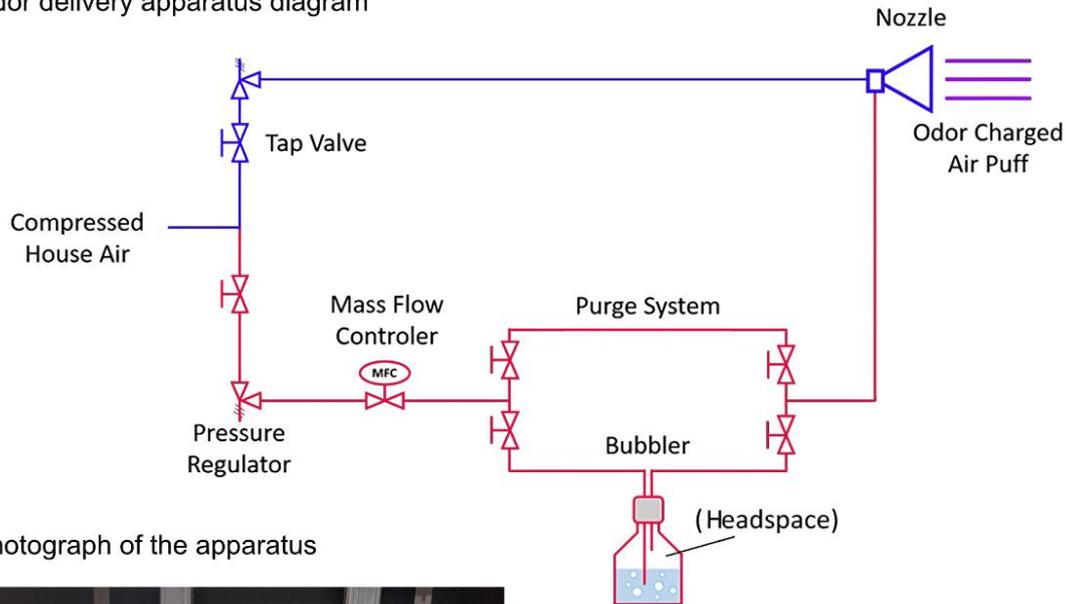
To this aim, we develop an assay allowing us to study the effect that combining odor and air speeds gradients could have on the navigational decisions that *Drosophila* larvae make during anemotaxis (Jovanic et al. 2019). Once this assay is fully established, it would allow us to investigate whether the changes in larval feeding state will increase their probability of moving towards the odor, despite the presence of strong wind, which is aversive and represents a risk for the larva. We could thus study the influence of the feeding state of the larvae on the integration of these conflicting sensory information and uncover the neural substrates underlying this integration.

III. C. 1. Methods

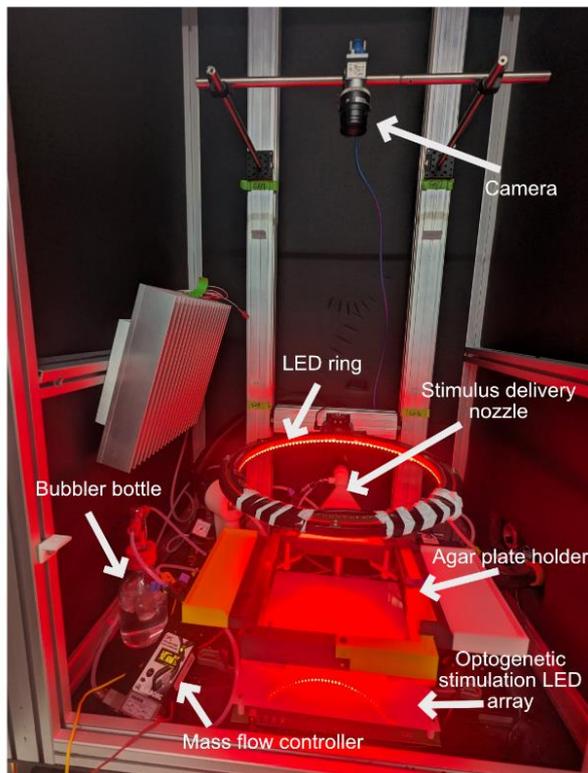
III. C. 1. a. *Description of the apparatus*

To be able to mix an odor into the air puff, I added a bubbler to our behavioral apparatus described in (Ohyama et al. 2013) and the material and methods section of the article in section III.A of this manuscript. Briefly, a camera allows the real-time tracking of larvae spread on an agar plate and illuminated by a ring of LEDs (Figure III.C-1-B). Compressed air is injected into a bottle (DWK Life Sciences 257040101) containing a solution of the odorant. Air entering the bottle will create bubbles inside the solution and thus be charged by the odor before exiting the bottle. The air will then go through a tubing to the delivery nozzle (3D printed by Sculpteo® using Nylon PA12 and SLS) which will present it to the larvae (Figure III.C-1-A). The apparatus allows us to easily perform experiments with only airspeed gradient, only odor concentration gradient, or both gradients simultaneously. The concentration of odor that will be presented to the larvae depends on three factors: the vapor pressure of the odorant molecule, the concentration of the solution inside the bottle, and the dilution of the odor-charged air into the air puff when it is used simultaneously, which is dependent on the ratio of the flow rates of both air streams. We used ethyl acetate as an attractive odor so that the valences of both gradients are opposite, and the larvae need to make a choice between these conflicting sensory information. Indeed, the airspeed gradient is aversive (Jovanic et al. 2019) while the odor concentration gradient (ethyl acetate) is attractive (Khurana and Siddiqi 2013) and both gradients will be strongest at the nozzle.

A Odor delivery apparatus diagram



B Photograph of the apparatus



C MiniPID quantification of odor delivery

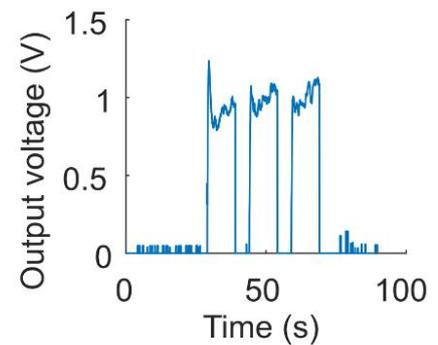


Figure III.C-1 Description of the setup for studying locomotion of larvae in conflicting multisensory gradients. A. Diagram representing the odor delivery apparatus. The red part is the addition I made to the previously existing blue part. B. Photograph of the behavioral apparatus. C. MiniPID voltage output in response to three 10 s pulses of air going through the bubbler bottle.

III. C. 1. b. *Experiments with larvae*

These experiments were performed by Ivain Raslain, under my supervision. We used solutions of concentrations ranging from 10^{-4} mol/L to 10^{-2} mol/L, with the mass flow controller set to its maximum flow output (12 L/min), to empirically determine settings that would allow us to record significant behavioral changes in larvae. To avoid evaporation of the odorant, a new solution was prepared each experimental day. A similar protocol as the one described in the article manuscript was used, except that only 20 to 30 wild type larvae (CantonS) were put in the arena and they were placed in a straight line in the middle of the agar plate, perpendicular to the gradients of airspeed and odor concentration so that we could quantify their displacements in the gradients. Moreover, as in (Jovanic et al. 2019), air speeds gradients were made steeper so larvae could sense the directionality of the air (either from 5 m/s near the nozzle to 2 m/s at the end of the arena, for strong wind speed gradient or from 3 m/s to 1 m/s for a weak wind speed gradient). The behavior of the larvae was monitored for 10 minutes while continuously exposed to either the odor alone, the air puff alone, or a combination of the air puff and the odor.

The displacement of the larvae along the gradients axis was computed as the change in the average coordinate of the population of larvae on that axis compared to their starting position. Positive values represent larvae moving closer to the source of the stimuli. A navigation index was computed as the ratio of the average velocity of the population of larvae along the gradient axis and the average speed of the population, over the whole duration of the experiment (Jovanic et al. 2019). Positive values represent larvae moving away from the source of the stimuli. We averaged the navigational index over the whole duration of the recording (600 s) for the air puff gradient alone and air puff gradient combined with odor. For the odor only condition, the navigational index was averaged over the first half of the recording only (300s) as several larvae had reached the odor source by that time.

III. C. 1. c. *Concentration quantification*

In order to quantify the concentration of ethyl acetate delivered to the larvae and characterize the odor concentration gradient in our experiments, we used a miniPID (Aurora scientific 200B) that was graciously lent to us by Brice Bathelier. We were able to show that the bubbler system is capable of timely delivery of odors to the arena (Figure III.C-1-C). However, quantifying the concentration of odor mixed with the air at high speeds delivered through the nozzle requires a standardized concentration sample and, to my knowledge, no standard concentration sample of ethyl acetate can be purchased. Thus, we were not able to experimentally measure the concentration of ethyl acetate that reaches the larvae. We then switched to a theoretical approach that would

allow us to compute a close approximation of the concentration needed in the solution in order to reach the desired concentration of odor in the air exiting the nozzle. Following is the description of this approach, applied to ethyl acetate.

Compressed air is injected into a solution of 99.8% anhydrous ethyl acetate that we approximated as pure for our calculations. The flow rate of air injected into the system is controlled by a mass flow controller (Aalborg DPC17) and is equal to the flow rate of vapors exiting the bubbler. The gas volume of the container above the liquid will be referred to as "headspace".

In order to determine the concentration of ethyl acetate in the headspace (C_{hs}), we first calculated the saturated vapor pressure (SVP) of ethyl acetate using Antoine's law $SVP = 10^{A - \frac{B}{C+T}}$, where A , B and C are component-specific constants in $mmHg$ and T is the temperature in $^{\circ}C$.

At a temperature of $25^{\circ}C$, $A = 7.10179 mmHg$, $B = 1244.95 mmHg$, $C = 217.9 mmHg$. Thus the saturated vapor pressure of ethyl acetate is $94.7174 mmHg$ or $0.124628 atm$.

Assuming that ethyl acetate acts as an ideal gas under $25^{\circ}C$ and atmospheric pressure, the ideal gas law tells us that $P \times V = n \times \frac{R}{T}$, with temperature in K and R being the ideal gas constant.

$$R = 0.082057366080960 \frac{L \times atm}{K \times mol}$$

By rearranging the equation and setting the previously calculated SVP as P , we get a concentration of ethyl acetate in the headspace of $\frac{n}{V} = \frac{SVP}{R \times T}$ where $\frac{n}{V} = C_{hs}$ in mol/L .

$$C_{hs} = \frac{0.124628}{0.082057366080960 \times 298.15} = 5.094 \times 10^{-3} mol/L$$

We defined a goal concentration $C_{odor} = 1.03 \times 10^{-4} mol/L$ according to concentrations used in (Gershow et al. 2012) where they created an ethyl acetate odor gradient ranging from 2 to 2500 ppm . In order to convert ppm concentration values to mol/L , we use the ideal gas law to compute the volume taken by 1 mol of gas at the temperature used in the paper of $23^{\circ}C$ and under a pressure of 1 atm .

$$V = \frac{1 \times R \times 295.15}{1} = 24.2192316 L$$

This gave us that 1 mol of gas at $23^{\circ}C$ and under 1 atm fills 24.2192316 L . Since gas ppm are $\mu L/L$, it follows that 2500 ppm are equal to $C_{odor} = \frac{2500 \mu L}{1 L} \times \frac{1 mol}{24.2192316 L} \approx 1.03 \times 10^{-4} mol/L$.

Since the gas exiting the bubbler will be diluted in the air flow of the air puff, the final concentration of odor delivered to the larvae (C_{odor}) is equal to $C_{odor} = \frac{Q_{odor}}{Q_{total}} \times C_{hs}$ where Q_{odor} is the flow of air going through the bubbler and Q_{total} is the total air flow at the end of the air puff nozzle. In order to be consistent with our experiments, Q_{total} was set at 5 m/s or 6.28 L/s since the tubing of the air puff has a radius of 2 cm.

By rearranging the equation, it follows that $Q_{odor} = \frac{C_{odor}}{C_{hs}} \times Q_{total} = 0.127 \text{ L/s}$ or 7.62 L/min. Consequently, the mass flow controller needs to be set at 7.61 L/min in order to deliver a concentration of ethyl acetate $C_{odor} = 1.03 \times 10^{-4} \text{ mol/L}$.

Finally, when no air puff is added, a different solution needs to be used because no dilution of the odor-charged air will occur. To determine the concentration of the solution we need to use in the bottle, we use Henry's law which states that $H^{cc} = \frac{C_{aq}}{C_g} = H^{cp} \times R \times T$, where H^{cc} is the dimensionless Henry solubility, C_{aq} is the concentration of ethyl acetate in the solution, C_g the desired concentration of ethyl acetate in the gas, H^{cp} is Henry's solubility which is constant for each molecule at a specific temperature, R is the gas constant and T the temperature in Kelvin. Rearranging the equation gives $C_{aq} = C_g \times H^{cp} \times R \times T$.

For ethyl acetate, Henry's solubility value is $H_{ethyl\ acetate}^{cp} = 6 \times 10^{-2} \frac{\text{mol}}{\text{m}^3 \times \text{Pa}}$ (Fenclová et al. 2014). We can thus compute the concentration of ethyl acetate required in the solution in order to reach the desired concentration in the gas:

$$C_{aq} = 1.03 \times 10^{-4} \times 6 \times 10^{-2} \times 8.314 \times 298.15 = 1.53 \times 10^{-2} \text{ mol/L}$$

III. C. 2. Results

The setup was first tested with each stimulus separately. We were able to reproduce anemotaxis behavior (Jovanic et al. 2019) with the air puff gradient alone, in which condition the larvae moved away from the nozzle (Figure III.C-2-C). When presented with only ethyl acetate, larvae moved towards the nozzle (Figure III.C-2-B). This behavior was stronger when using a solution of ethyl acetate concentrated at 10^{-3}M (Figure III.C-3-A1).

Examples of tracks for different stimulations

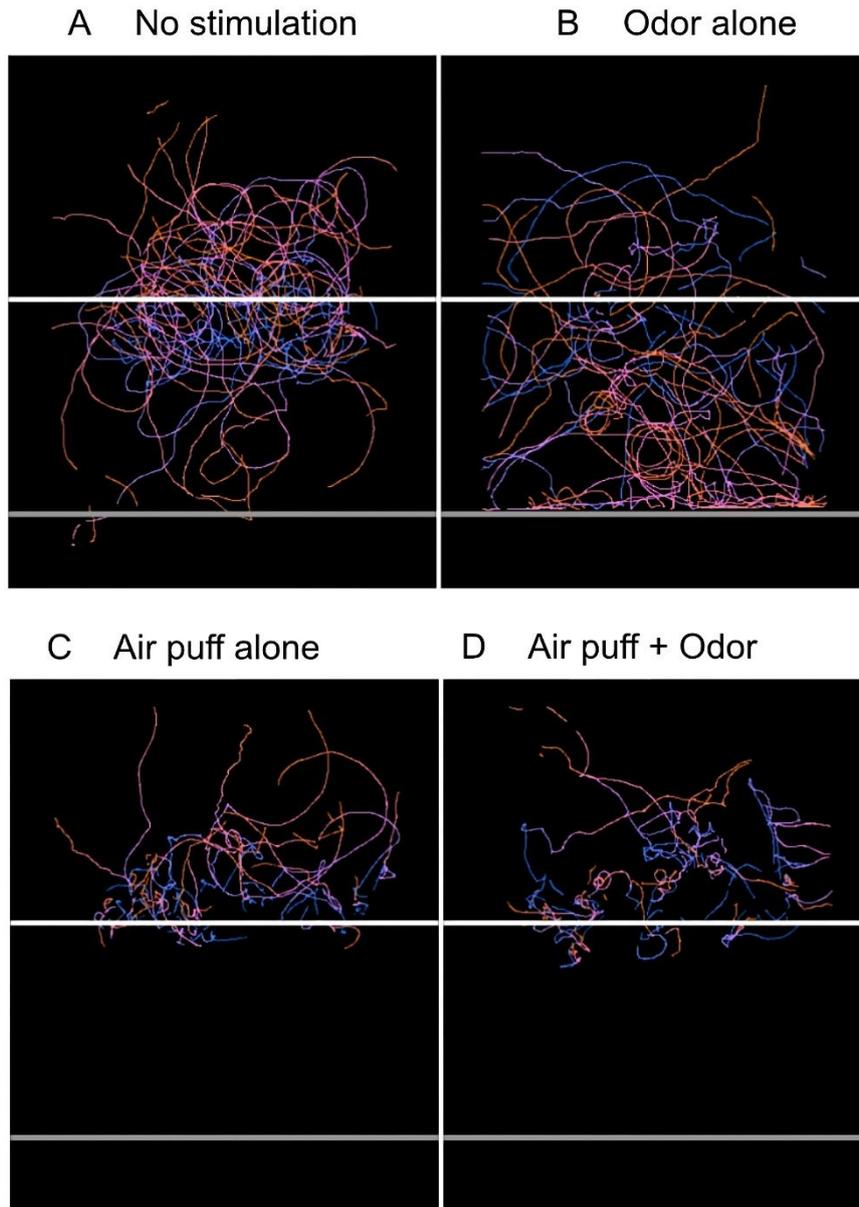


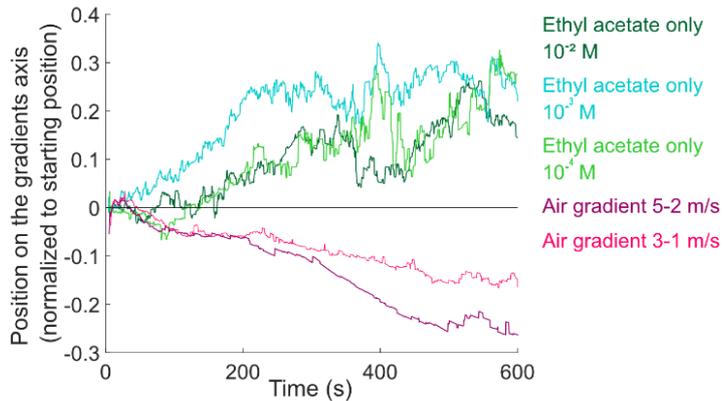
Figure III.C-2 Example recordings of larvae during 10 min with different stimulation modalities. Each line represents the track traveled by a larva. The color of the lines change from blue to orange to show the passing of time during the recording. White line is the starting position for that recording. Grey line is the stimuli delivery nozzle. A. No stimulation delivered. Larvae disperse randomly. B. Continuous delivery of only odor. Larvae agglutinate close to the nozzle. C. Continuous delivery of only air puff. Larvae move down the wind gradient. D. Continuous delivery of both odor and air puff. Larvae behave similarly to the air puff only modality, with a slight reduction in the displacement away from the air puff.

In conflicting gradients, larvae did not move towards the nozzle, regardless of the concentration of ethyl acetate used (Figure III.C-3-A2). We observed a slight reduction in the displacement of larvae away from the nozzle when presented with an odorant stimulus with a concentration of ethyl acetate of 10^{-3}M or 10^{-4}M but not 10^{-2}M (Figure

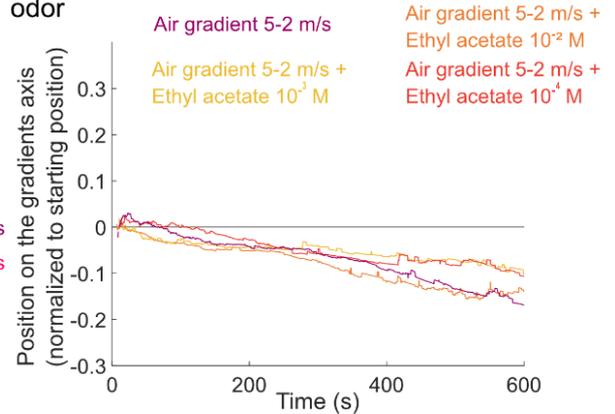
III.C-3-A2). The addition of ethyl acetate to the air gradient induced a decrease in navigation index averaged over the whole 10 min of recording, which did not reach significance (Figure III.C-3-B).

A Displacement of larvae along the gradients axis

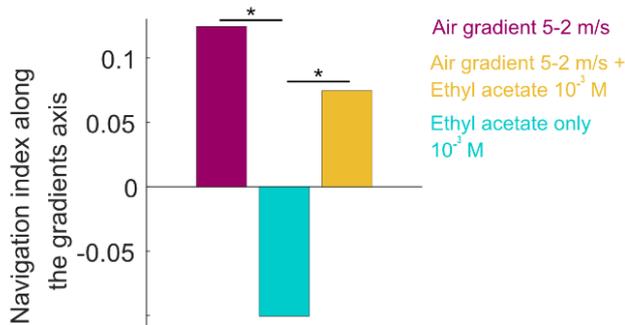
A1 Stimulation with either air puff alone or odor alone



A2 Stimulation with a combination of air puff and odor



B Navigation index along the gradients axis



C Connectivity between mechanosensory neurons and food-related sensory neurons in the brain

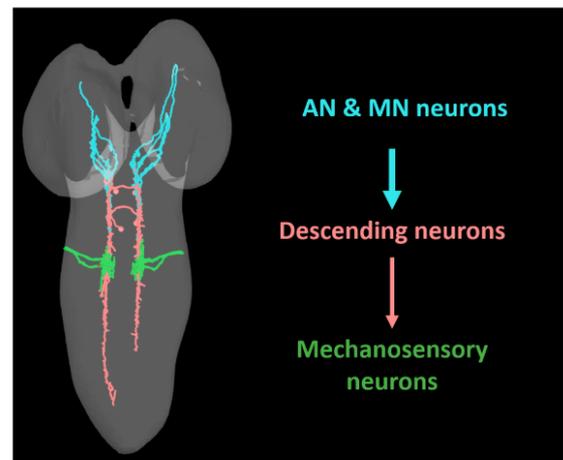


Figure III.C-3 Locomotion analysis of larvae in conflicting multisensory gradients.

A. Displacement of larvae along the gradients axis, corrected by the starting position and recorded over 10 min. Positive values correspond to larvae moving towards the source of stimuli. A1. Larvae were stimulated either with ethyl acetate alone or an air puff alone. A2. Larvae were monitored in a conflicting sensory gradient of wind speeds and ethyl acetate concentration. B. Navigation index computed over the entire duration of the recording for different stimulation modalities. Positive values correspond to larvae moving away from the source of stimuli. C. 3D reconstruction of neurons from electron-microscopy dataset. The mechanosensory Chordotonal neurons (green) are connected to food-related sensory neurons (blue) via two pairs of descending neurons (red).

Altogether, these results suggest that the aversion from the air puff is too strong for the addition of ethyl acetate to alter the choice of the larvae to move down the airspeed

gradient. However, developing a more precise analysis of the recording may reveal subtle changes in locomotion that we could not detect here. For instance, the probability of Head-casting, their direction and their acceptance rate could be modulated by the addition of odors. This new assay is promising in allowing us to study the neuronal circuitry involved in the integration of the olfactory and mechanosensory environment.

This is especially interesting regarding a connection between the chordotonal mechanosensory neurons and some sensory neurons, located in the head of the larva (Figure III.C-3-C), that we uncovered in the electron-microscopy dataset (Ohyama et al. 2015) using the collaborative online tool Catmaid (Saalfeld et al. 2009). Indeed, these neurons, labelled AN and MN (or MxN), are part of a collection of nerves, the pharyngeal nerves, which innervate the muscles that drive food intake (Miroschnikow, Schlegel, and Pankratz 2020; J. Y. Kwon et al. 2011). More precisely, AN neurons are part of the antennal nerve and MN are part of the maxillary nerve. Both these nerves relay projections of sensory neurons respectively from the dorsal organ ganglion, and from terminal and ventral organs ganglions (J. Y. Kwon et al. 2011). The dorsal contains a combination of thermosensory, olfactory and gustatory neurons (Klein et al. 2015; Kreher, Kwon, and Carlson 2005), the terminal organ contains thermosensory neurons and gustatory neurons (L. Liu et al. 2003; Vosshall and Stocker 2007) and the ventral organ contains gustatory and mechanosensory neurons (Apostolopoulou, Rist, and Thum 2015).

We found that these AN and MN neurons are connected to the chordotonal mechanosensory neurons through two pairs of descending neurons (T1 and T2 descending neurons), that send projections all along the ventral nerve chord or the larva. Because the electron microscopy dataset does not span the peripheral nervous system of the larva (Ohyama et al. 2015; Jovanic et al. 2016; 2019; Zarin et al. 2019; Heckscher et al. 2015; Zwart et al. 2016; Miroschnikow, Schlegel, and Pankratz 2020; Winding et al. 2023), the exact nature of the AN and MN neurons involved in this connection is currently unknown. However, (Miroschnikow et al. 2018) anatomically characterized in great details the Antennal nerve which divides into three distinct bundles (B1-3) upon entering the larval brain (Figure III.C-4). The three bundles can be easily recognized anatomically since B1 is the most medial, B3 the most lateral and B2 falls between the other two.

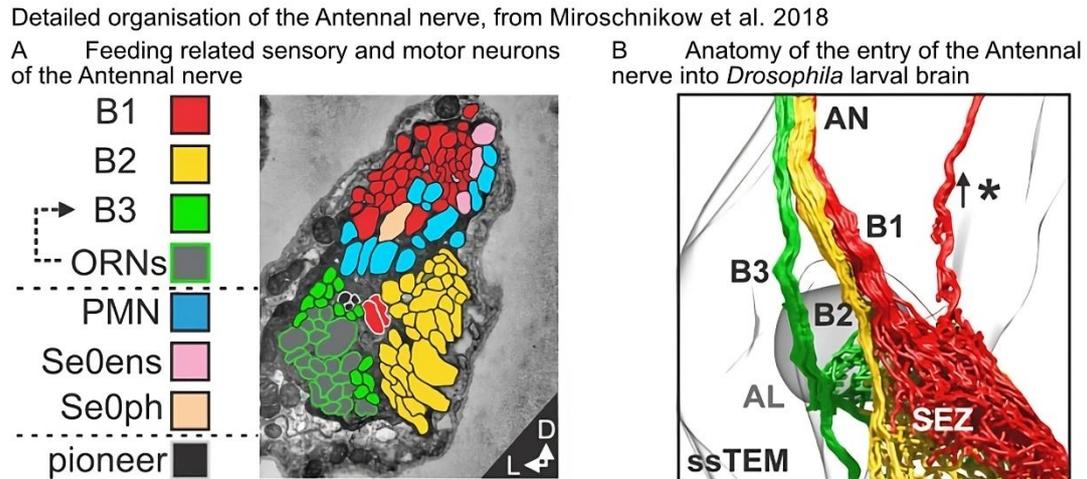


Figure III.C-4 Organization of the Antennal nerve in *Drosophila* larva from (Miroshnikow et al. 2018). Anatomical characterization of the Antennal nerve by (Miroshnikow et al. 2018), A is from figure 5A and B from figure 5B. Upon entry in the brain, the Antennal nerve divides into three bundles (B1 to B3). Only B3 contains axons from olfactory neurons.

Interestingly, axons from the olfactory neurons of the dorsal organ are exclusively part of the 3rd bundle (Miroshnikow et al. 2018). A close comparison of the anatomy of the Antennal nerve bundles with the anatomy of the AN neurons reconstructed in the electron microscopy connectome dataset (Ohyama et al. 2015; Jovanic et al. 2016; 2019; Zarin et al. 2019; Heckscher et al. 2015; Zwart et al. 2016; Miroshnikow, Schlegel, and Pankratz 2020; Winding et al. 2023) allowed us determine that one of the three AN neurons that synapses onto the T1 and T2 descending neurons could be olfactory since it belongs to the most lateral bundle, corresponding to B3 (Figure III.C-5). The other two neurons are part of B2, the intermediate bundle between B1 and B3. We argue that these two neurons are likely to be gustatory sensory neurons, since a large portion of neurons from B2 were shown to express gustatory receptors (Miroshnikow et al. 2018).

Considering the strong multisensory role of the pharyngeal nerves, which carry information from all the terminal, ventral, and dorsal organs (Klein et al. 2015; Kreher, Kwon, and Carlson 2005; L. Liu et al. 2003; Vosshall and Stocker 2007; Apostolopoulou, Rist, and Thum 2015), it seems a reasonable assumption that these T1 and T2 descending neurons could relay feeding-related sensory information to this pair of descending neurons. If this were confirmed, the involvement of T1 and T2 descending neurons in conveying feeding-related sensory information, sensed by the sensory organs of the head, to the chordotonal mechanosensory neurons would be very likely. Our assay will allow for the determination of a possible role of these neurons in the decision of the larvae in conflicting multisensory gradients. Eventually, manipulation of the feeding state of the larvae in this assay will uncover the influence of the internal

state on multisensory integration and decision-making that may involve weighing risks and benefits in the conflicting scenario of opposing olfactory and wind speed gradient. It will further allow us to identify the neuronal and signaling pathways involved in the transmission of state-dependent information.

Localization of AN neurons connected to the mechanosensory neurons

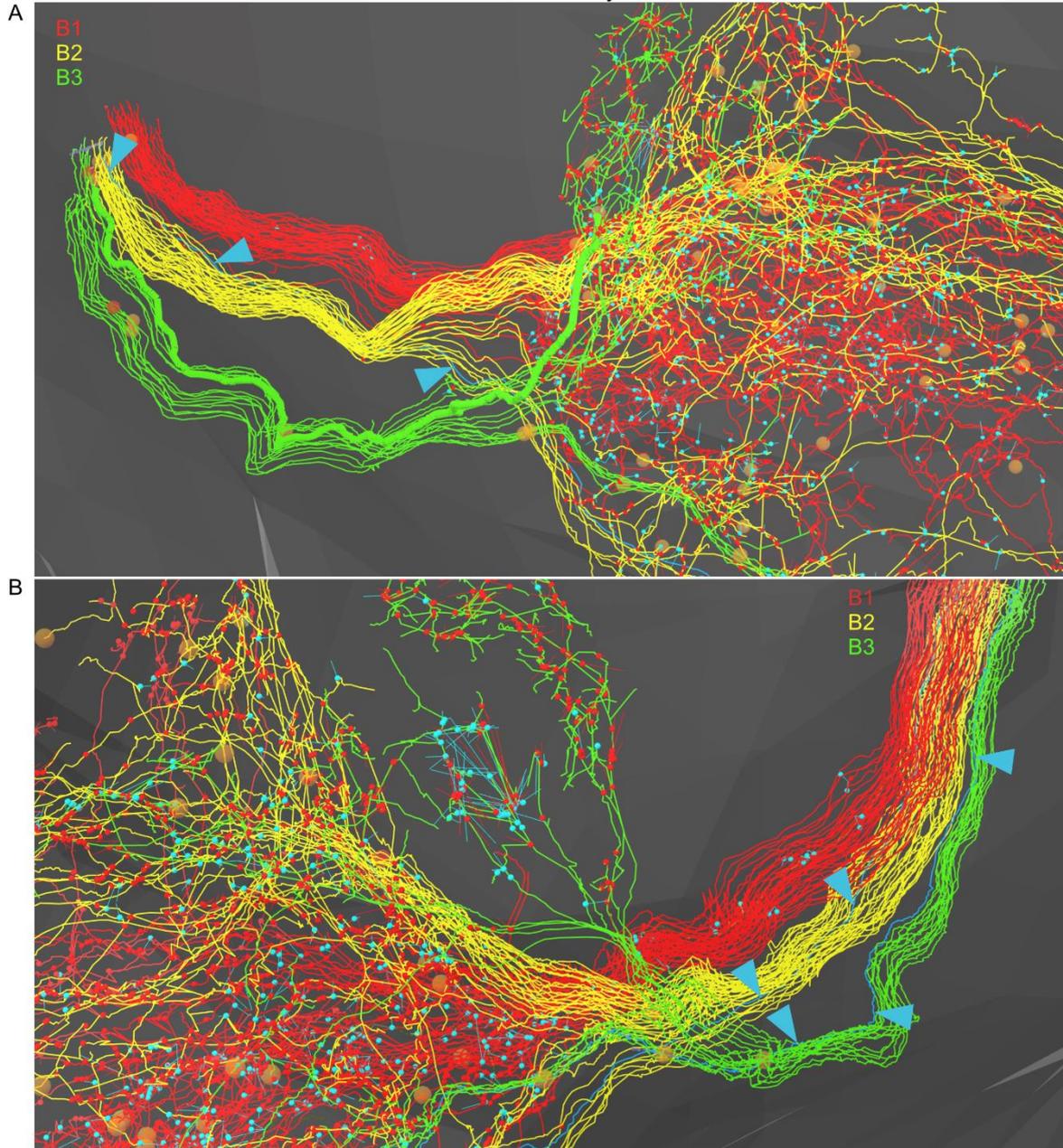


Figure III.C-5 Identification of the Antennal nerve neurons upstream of the T1 and T2 descending neurons. Localization of Antennal nerve sensory neurons that synapse on the T1 and T2 descending neurons reconstructions from the electron microscopy connectome dataset from the left (A) and right (B) side of the body. Each bundle from the Antennal nerve is in a different color (B1 = red ; B2 = yellow ; B3 = green). The neurons that synapses onto T1 and T2 descending are colored in blue, blue arrows highlight their belonging to bundles 2 and 3. Images made in Catmaid (<https://neuophyla.mrc-lmb.cam.ac.uk/catmaid/drosophila/l1/seymour/>), Dataset hosted by Cardona lab (LMB-MRC, Cambridge University)

IV. Discussion

Throughout the work presented in this manuscript, our objective was to uncover the neural circuit mechanisms underlying how an animal's feeding state can influence decisions unrelated to feeding. Leveraging a well-characterized neural circuit in *Drosophila* larvae responsible for choosing between hunching and head-casting behaviors in response to mechanical stimulation (Jovanic et al. 2016), we explored how this circuit is modulated under varying feeding conditions. Our findings demonstrate that changes in the feeding state bias larval behavior towards increased head-casting at the expense of hunching, achieved by regulating the activity levels of two reciprocally connected inhibitory interneurons crucial for determining the circuit's output state. Interestingly, we identified the two feeding-related neuropeptides, NPF and sNPF (Fadda et al. 2019), known to possess neuromodulatory properties (Wu, Zhao, and Shen 2005; S. Lee, Kim, and Jones 2017; Lingo, Zhao, and Shen 2007; Landayan et al. 2021; G. Lee, Bahn, and Park 2006; Dierick and Greenspan 2007; Wen et al. 2005; Kahsai, Martin, and Winther 2010; Kahsai et al. 2010; Johard et al. 2009; Imambocus et al. 2022; Johard et al. 2008), as key players in this modulation process. We show that the modulatory action of NPF and sNPF on larval behavioral decisions does not rely on the direct modulation of the projection neurons at the output of the circuit which do not express their receptors. Moreover, although the mechanosensory neurons at the origin of the activation of the circuit do express the sNPF and NPF, we show that their modulation is not required for biasing the decisions of the larvae. Instead, both sNPF and NPF are involved in the modulation of the interconnected inhibitory interneurons which are responsible for the feeding state-dependent modulation of larval behavioral decisions in response to an air puff. The following figure (Figure IV.1) summarizes the state-dependent modulatory actions of sNPF and NPF on the inhibitory interneurons Handle-B and Griddle-2 that were uncovered throughout my PhD project.

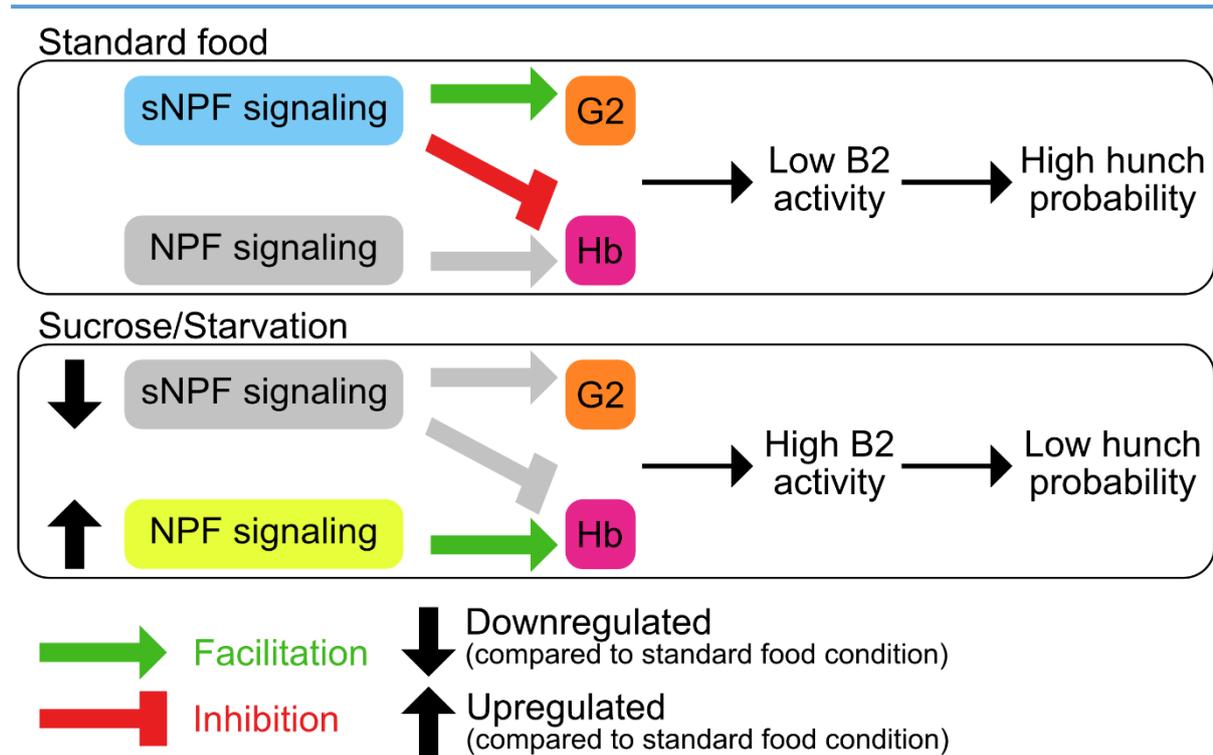


Figure IV.1 Summary of feeding state-dependent action of sNPF and NPF on Handle-B and Griddle-2 interneurons. Red flat arrows represent inhibition of the target neuron activity, green pointed arrows represent facilitation of the target neuron activity, and grey elements represent pathways that are weak and have no significant effect in the corresponding feeding state. In larvae fed on standard food, sNPF inhibits Handle-B and facilitates Griddle-2, which results in lower Basin-2 responses and leads to a higher hunching probability. In larvae starved or fed on sucrose, sNPF is downregulated and NPF facilitates Handle-B activity, promoting Basin-2 responses, which results in a decreased hunching probability.

IV. A. Neuropeptidergic action on the mechanosensory chordotonal neurons

We showed expression of both NPFR and sNPFR in most subtypes of the mechanosensory chordotonal neurons. However, our calcium imaging results show that their activity remains similar in the different feeding states that we studied, suggesting that the modulation of the decision of the larvae happens downstream in the circuit. Accordingly, imposing an activation of the mechanosensory chordotonal neurons of constant strength in different feeding states via optogenetic stimulation led to a normal bias in larval behavioral choice (an increase in head-casting responses at the expense of hunching), confirming that the feeding state-dependent modulation of the circuit does not require the modulation of the mechanosensory neurons but instead is implemented downstream in the circuit. We thus demonstrated that the action of NPF and sNPF on the mechanosensory neurons is not to alter the decisions of the larvae about how to respond to an air puff in different feeding states.

What is then function of neuropeptides receptors involved in feeding regulation expressed in chordotonal neurons if the activity of the mechanosensory neurons is not modulated by the feeding state? By knocking-down sNPFR expression in the mechanosensory neurons and monitoring their responses to mechanical stimulation using calcium imaging, we show that sNPF has a feeding state-dependent inhibitory effect on their responses, found in larvae fed on standard food or starved, but inexistent in larvae fed on sucrose only. The role of this state-dependent inhibition of the mechanosensory neurons remains to be elucidated, as we showed that it is not necessary for biasing the behavioral decision of the larvae.

We can hypothesize that the response probability of the larvae does not exhibit a smooth transition across a range of mechanosensory neurons activation amplitudes but instead evolves stepwise when thresholds of mechanosensory neurons' activity are crossed. The inhibitory effect of sNPF that we recorded in larvae fed on standard food or starved could be small enough to keep the activity of the mechanosensory neurons in the same range as that of control larvae, thus not leading to any change in the behavioral response of the larvae. Such a mechanisms could lead to a binary response probability of the larvae, allowing them to ignore weaker stimuli and respond in a stereotyped manner to strong enough stimuli, as was shown for their response to thermo-nociceptive stimulation for instance (Y. Hu et al. 2020).

It is also possible that sNPF, decreases the sensitivity to the mechanical stimulation, thus reducing the probability for larvae to respond to the stimulation, without biasing the behavioral choice between hunching and head-casting when they do respond. (Masson et al. 2020) showed that a strong reduction of the air puff intensity (from 6 m/s to 3 m/s) indeed leads to more larvae ignoring the stimulation as shown by an increase in the probability of larvae to crawl in response to a weaker stimulation. However, this strong reduction in the stimulus strength did lead to an alteration of the proportion of the different actions performed in response to the air puff, with a notable increase in the probability of larvae stopping, at the expense of hunching and bending for example (Masson et al. 2020).

To test these hypotheses, it would thus be interesting to compare the stopping probability between control larvae and larvae in which expression of the sNPFR in knockdown in the mechanosensory chordotonal neurons, from the same intensity of air puff stimulation. We could also easily characterize the response regiment of the larvae to a wide range of stimuli intensities (from weak to strong) and quantifying the proportion of larvae that respond to the stimulation, as well as the proportion of hunching, head-casting, and stopping responses.

We unfortunately did not have the time to explore the effect of NPF on the mechanosensory neurons. Investigating the consequences of NPF signaling on the mechanosensory neurons will provide valuable information about the role of their neuropeptidergic modulation. Indeed, considering that the activity of the mechanosensory neurons remains similar across the tested feeding states in control larvae and that the sNPF has a state-dependent inhibitory effect on them, it is likely that some other effector, possibly NPF, would compensate the inhibitory action of sNPF. Under this hypothesis, the integration of several state-dependent signals in the mechanosensory neurons would lead to a homeostatic balancing of their amplitude of response. For this purpose, other neuropeptides could affect the activity of the mechanosensory neurons, including ILP whose expression is regulated by and can regulate sNPF expression (Kapan et al. 2012; Yeom et al. 2021) and acts oppositely to sNPF in a neural circuit orchestrating the behavioral response to noxious touch and light (Imambocus et al. 2022).

What is then the purpose of modulating the mechanosensory responses in a state-dependent manner if their amplitude is eventually homeostatically tuned to remain in a specific range? The expression of receptors to several neuropeptides would allow the mechanosensory neurons to integrate information about several internal states at once. It is possible that a different state different to the feeding states we studied, anxiety for instance, could exert a stronger modulatory effect on the responses of the mechanosensory neurons, allowing them to adapt to dangerous environments or the presence of predators (Min and Condrón 2005; Mohammad et al. 2016; Berne et al. 2023). Interestingly, the mammalian homolog of sNPF and NPF, NPY, has been implicated in reducing anxiety (Bale and Doshi 2023; Brain and Cox 2006; Pedrazzini, Pralong, and Grouzmann 2003; Sánchez, Rodríguez, and Coveñas 2023). Moreover, noxious light stimulation has been used to induce anxiety in *Drosophila* larvae (Min and Condrón 2005) and recruit insulin-like peptides to produce locomotor escape responses (Imambocus et al. 2022). It is thus possible that several neuropeptides converge on the mechanosensory neurons to homeostatically regulate their responses across different internal states while allowing this regulation to be overridden when critical conditions arise.

Alternatively, it could be that the effect of sNPF on the mechanosensory chordotonal neurons impacts a different function of theirs, proprioception, leading to adaptations in their locomotion (Agrawal and Tuthil 2022). Indeed, in *Drosophila* larva the chordotonal neurons were shown to also mediate proprioception (Agrawal and Tuthil 2022). The mechanosensors in the chordotonal organ which are activated during the propagation of the peristaltic wave generated when larvae crawl (Agrawal and Tuthil

2022; Orgogozo and Grueber 2005; Hughes and Thomas 2007). Alteration of this proprioceptive activation leads to qualitative changes in the locomotion of the larvae, modifying the dynamics of the peristaltic wave and overall, the crawling speed of the larvae and their probability of head-casting (Agrawal and Tuthil 2022; Gjorgjieva et al. 2013). Considering this, we can hypothesize that the state-dependent inhibitory action of sNPF on the mechanosensory neurons could modulate their proprioceptive function and be involved in the modulation of their crawling speed during baseline locomotion in different feeding states.

IV. B. Modulation of sensorimotor decisions by NPF

In the article in section III.A, we uncover that the one of the descending neurons that makes synaptic connections with the feedback inhibitory interneuron (Handle-B) that promotes head-casting at the expense of hunching in a sensorimotor circuit in the VNC (Jovanic et al. 2016) is a pair of NPF-releasing neurons (DM-NPF) in *Drosophila* larva brain (Jovanic et al. 2016). Using calcium imaging to record responses of Handle-B upon complete silencing of NPF neurons with KIR, we showed they have an excitatory action on Handle-B. Although we did not have tools to monitor the release of NPF directly, we showed that DM-NPF exhibits a constitutive basal calcium signal that increases if the larvae are fed on sucrose only or starved, suggesting an increased release of NPF. Consistent with an increase in the release of NPF in starved and sucrose-fed larvae, we show in calcium imaging that knocking-down the NPFR in the feedback inhibitory interneuron, that receives synaptic inputs from DM-NPF and expresses the NPFR, abolishes the increase in its responses to mechanical stimulation exhibited by control larvae after starvation or sucrose-feeding. Taken together, these results strongly suggest that DM-NPF increases its release of NPF in larvae fed on sucrose only or starved and that NPF consequently facilitates the responses of Handle-B to mechanical stimulations.

We have shown that an increase in the amplitude of responses of Handle-B is responsible for the bias in the behavioral choice of larvae fed on sucrose only or starved. We thus propose that the increased release of NPF by DM-NPF is instrumental in biasing the decision of the larvae. Experiments where the effect of NPFR knockdown specifically in Handle-B on the behavioral responses of larvae to an air puff after sucrose-feeding or starvation are currently being performed to confirm the necessity of NPF signaling on Handle-B for the bias in larval decisions.

Moreover, we show that knockdown of NPF in DM-NPF influences the modulation of basal locomotion by the feeding state of the larvae. Indeed, several locomotor features such as the crawling speed, the time spent in runs and the dispersal of the

larvae increase in control larvae starved or fed on sucrose only but are diminished or unchanged upon NPF knockdown in DM-NPF. Although we do not know the neurons responsible for this modulation of basal locomotion, this result shows that NPF is able to transmit information about the feeding state to circuits involved in locomotion located in the VNC.

To fully understand the mechanism of NPF-dependent modulation of sensorimotor decisions in different states, the question of whether DM-NPF senses directly the alteration of the feeding state or whether it receives inputs from other neurons that sense it remains. Indeed, it is surprising that the activity of DM-NPF increases both in larvae fed on sucrose only and starved larvae and we do not know the exact signal responsible for that increase. In the brain, several mechanisms have been discovered that allow individual neurons to tune their activity to the metabolic state of the organism depending on glucose levels (López-Gambero et al. 2019). It is possible that DM-NPF is one such neuron whose activity is regulated by glucose. However, if that were the case, we would expect starvation and sucrose feeding to have opposite actions on DM-NPF activity. Indeed, we have shown that our sucrose feeding condition increases the glucose levels in the hemolymph of the larvae, while starvation tends to decrease it.

It could be that different cellular pathways are recruited in these two conditions to lead to an increase in DM-NPF activity. The activation of sweet taste receptors on neurons by glucose has been shown to be able to trigger depolarization (López-Gambero et al. 2019). (P. Shen and Cai 2001) have shown that in *Drosophila* larva, upon sugar feeding, the sweet taste, but not the metabolic effect of glucose, is responsible for increasing the activity of NPF-producing neurons. It is thus possible that the increased activity we see in DM-NPF in sucrose-fed larvae is independent of the metabolic state of the larvae and subsequent to the activation of taste receptors. Whether or not DM-NPF receives input from neurons that carry sweet taste receptors directly, or indirectly, however, remains to be determined. On the other hand, the increase in activity of DM-NPF in starved larvae could be dependent on a decrease in DM-NPF access to glucose, a mechanism known to increase activity of some neurons, notably in the hypothalamus, where NPY neurons regulate feeding in mammals (López-Gambero et al. 2019; Tong et al. 2007; Parker and Bloom 2012).

It is also possible that DM-NPF does not directly sense changes in glucose levels and that satiety signals such as hormones or ILP could provide an inhibitory feedback to DM-NPF after food intake to normalize its activity (Meister 2007; Valassi, Scacchi, and Cavagnini 2008). Indeed, insulin-producing neurons in *Drosophila* brain were shown to sense glucose levels (Lin, Senapati, and Tsao 2019), increasing ILP release in

response to increased glucose levels. Moreover, (Wu et al. 2005) showed that ILP action on NPFR positive cells is necessary for the hunger-induced increase in food tolerance, showing a competitive action of these two neuropeptides, although no direct effect of ILP on NPF release has been as of yet (Lin, Senapati, and Tsao 2019; Wu et al. 2005; Nässel and Zandawala 2020).

IV. C. Opposite effects of sNPF on Griddle-2 and Handle-B

In this study, we have found that the modulation of two reciprocally connected inhibitory interneurons (Griddle-2 and Handle-B) is responsible for the feeding state-dependent bias in the behavioral responses of the larvae. Interestingly, we establish that both of these interneurons express the sNPFR and that knocking-down its expression in either of them leads to a bias in larval decision in response to an air puff towards less hunching and more head-casting. This behavioral bias is similar to the one observed upon sucrose-feeding in control larvae. By monitoring their activity in calcium imaging upon sNPFR knockdown, we showed that this bias is implemented by a facilitating effect of sNPF on Griddle-2 and an inhibitory effect on Handle-B.

Since only one receptor for sNPF is expressed in both inhibitory interneurons, our results raise the question of the cellular mechanisms leading to an opposite effect of sNPF on Griddle-2 and Handle-B. Most GPCRs are not selective of a single G-protein and can even couple G-proteins of different families (Harding et al. 2018; Okashah et al. 2019; Inoue et al. 2019). The effect exerted by their ligands thus depends strongly on the G-protein that is recruited upon activation of the receptor. In *Drosophila*, the sNPFR shares structural similarities with the mammalian NPY2R (Fadda et al. 2019; Nässel and Wegener 2011; Larhammar and Salaneck 2004), which has been shown to be able to couple both with G_i/G_o and G_q/G_{11} proteins (Harding et al. 2018; Misra et al. 2004). (Inoue et al. 2019) developed a predictor that reinforces this idea and suggests that the NPY2R could also couple with $G_{12/13}$ proteins. If we loosely extrapolate these properties to the sNPFR, for which no G-protein coupling study is available to the best of my knowledge, we can argue that the opposite effect of sNPF that we recorded on the Handle-B and Griddle-2 inhibitory interneurons could originate from the recruitment of different G-proteins following sNPFR activation in each of these neurons.

Supporting this hypothesis, (Oh et al. 2019) found an opposite effect of sNPF on insulin-producing cells (stimulation) and adipokinetic hormone-producing cells (inhibition) in the adult *Drosophila* fly. Interestingly, exposition to an inhibitor of the G_q pathway specifically abolished the sNPF-dependent activation of the insulin-producing cells, while exposition to an inhibitor of the G_i pathway specifically reduced the sNPF-dependent inhibition of adipokinetic hormone-producing cells (Oh et al. 2019).

Additionally, neuropeptides are known to modulate electrical properties of neurons' membranes through the regulation of ion channels expression and function (Wicher, Walther, and Wicher 2001; Cardin 2019). It is also possible that the compositions of ion channels on the membrane of Handle-B and Griddle-2 are different and that the activation of the sNPFR in each of these neurons exert a different effect by biasing the balance between the activation of cationic and anionic channels (Wicher, Walther, and Wicher 2001).

IV. D. Origin of sNPF state-dependent action on the circuit

We have uncovered a state-dependent modulation of a sensorimotor decision-making circuit by NPF via the activation of a pair of descending neurons whose cell bodies are located in the brain of *Drosophila* larva. However, we do not know the identity of the neurons that release sNPF to the circuit, nor the mechanisms responsible for the state-dependent regulation of its action. Indeed, numerous neurons express sNPF in *Drosophila* larva, both in the brain and VNC (Nässel et al. 2008; Nässel and Wegener 2011). (Imambocus et al. 2022) identified a dorsal pair of ILP7 producing neurons (DP7) that releases sNPF in response to noxious touch. These DP7 have their cell bodies in the VNC and send both local projections in the VNC and ascending projections into the brain lobes (Imambocus et al. 2022). The localization of these neurons in proximity to the circuit studied in our work and their involvement in the regulation of locomotor escape responses make them candidate neurons that could bring sNPF to our circuit.

However, as many other neurons in the VNC express sNPF (Nässel et al. 2008; Nässel and Wegener 2011) it seems difficult to precisely regulate the amount of sNPF reaching any individual neuron in a state-dependent manner. We thus propose that the most efficient mechanism of state-dependent modulation of sNPF effect on the circuit does not arise from modulation of its release but of that of its receptor expression in target neurons. Although we did not quantify the number of receptors expressed on each neuron of our circuit in different feeding states, our results seem to be in line with this hypothesis. Indeed, we showed that knocking-down sNPFR expression in either Griddle-2 or Handle-B in larvae fed on standard food modulates their responses to mechanical stimulation in calcium imaging towards a "sucrose-like" or "starved-like" phenotype, suggesting that sNPFR expression could be decreased in these neurons upon sucrose feeding and starvation. Moreover, knockdown of sNPFR expression in either inhibitory interneuron did not introduce any change in their responses in larvae fed on sucrose or starved, reinforcing the idea that sNPFR expression could be decreased in these conditions.

Supporting this idea, (Root et al. 2011) showed that sNPF signaling modulated food search behavior through a hunger-dependent presynaptic facilitation of olfactory neurons in *Drosophila* adult flies. Most interestingly, they showed that this state-dependent modulatory effect of sNPF signaling is mediated by the increase of the expression of the sNPFR in hungry flies, and not by changes in the expression of sNPF.

To confirm this hypothesis, quantification of the sNPFR expression via RT-qPCR would be very valuable. More easily, we have recently acquired a *Drosophila* line that would allow us to overexpress the sNPFR. We could thus use this line to investigate whether over-expression of sNPFR rescues the phenotype of larvae fed on standard food after feeding them sucrose only or starving them.

IV. E. Difficulties of internal states characterization

The opposite effect of sNPF on different neurons from a single receptor is a reminder that a specific role cannot be easily attributed to one molecule. One needs to investigate the entire signaling cascade triggered by the activation of a receptor in order to truly understand the effects of a molecule in a specific context, which may be different in another context. Expanding on this idea, at any given moment, one neuron does not only receive inputs from a single molecule. Instead, a combination of many molecules continuously flood neurons, the level of which varies depending on the immediate state of the organism. As such, any state must be defined by the immediate concentration of each molecule and the subsequent pattern of activity exhibited by their target neurons. Nevertheless, no any state exists on its own but the organism always exhibit a balance between several states (such as hunger, fatigue or stress) that all influence the amount of molecules reaching a neuron and its integration of all signals.

To my mind, combining neural circuits mapping as described in this manuscript and articles such as (Jovanic et al. 2016; 2019; Masson et al. 2020; Ohyama et al. 2015; Eschbach et al. 2020; Takagi et al. 2017; Saumweber et al. 2018; Eichler et al. 2017; Winding et al. 2023) with the use of single-cell transcriptomics (Heumos et al. 2023) is currently the best way to comprehensively characterize such state-dependent alterations. Indeed, single-cell transcriptomics can provide uniquely detailed information about the level of expression of all proteins in each cell of a circuit, including neuropeptides, membrane receptors, ion channels, etc... By comparing transcriptomics results in each cell in different states, one could identify changes in protein expressions that characterize that specific state and model the consequences in the circuit activity.

Additionally, technics allowing to image the activity of all the neurons in the brain at once with cellular resolutions are starting to be implemented in *Drosophila* research (Aimon et al. 2023; Mann, Gallen, and Clandinin 2017; Tainton-Heap et al. 2024). Such technics, though still technically challenging, would be extremely powerful to identify key neurons whose activity change in different states, eventually allowing to define a specific state as a precise pattern of activity of all the neurons whose activity is modulated in this state.

An illustration of this principle is our sucrose-fed condition. Indeed, we show that larvae fed sucrose only exhibit a combination of physiological alterations with thirst and a specific hunger for proteins. We believe that the thirst in these larvae arises from changes in their osmolality from the high concentration of glucose in their hemolymph (Jourjine et al. 2016; Jourjine 2017; González Segarra et al. 2023). It is thus to be expected that, on top of the regulation of sNPF and NPF that we uncovered, other neuropeptides involved in feeding and water regulation would be involved in the signaling of this particular state

As such, the role of abdominal leucokinin neurons, which are located in the larva VNC and co-express leucokinin and DH44, would be particularly interesting to study in the context of sucrose feeding. Indeed, these neurons are able to control different physiological processes, including increasing food ingestion and decreasing water retention, through DH44 and Lk respectively (Zandawala, Marley, et al. 2018). Moreover, knockdown of any of these neuropeptides in this neuronal population increases resistance to desiccation, starvation and ionic stress (Zandawala, Marley, et al. 2018), an effect opposite to the physiological changes we observe in the larvae after feeding on sucrose only. The first step to study the potential implication of DH44 and Lk in the modulation of sensorimotor decisions in larvae fed on sucrose will be to investigate whether their receptors are expressed by neurons in the decision circuit.

Moreover, neuromodulatory amines may be recruited on top of neuropeptides. Indeed, in flies, sweet taste produces reward signals through the activation of dopaminergic neurons (Burke et al. 2012; C. Liu et al. 2012; May et al. 2020). Moreover, dopamine has been associated with an increase in exploratory behaviors (Cinotti et al. 2019; Chakroun et al. 2020) which is in line with the behavioral bias we observe in larvae fed on sucrose only, as head-casting is dynamical action that promotes both escape and exploration of the environment by larvae. Plus, serotonin can promote feeding by increasing the hedonic value of food (van Galen, ter Horst, and Serlie 2021), which could collaborate with NPF effect on food tolerance (Wu, Zhao, and Shen 2005; S. Lee, Kim, and Jones 2017; Lingo, Zhao, and Shen 2007) to increase the motivation of the larvae to feed, leading to the modulation of their behavioral choices.

IV. F. Studying decision-making in conflicting sensory gradients in *Drosophila* larva

The modification of our behavioral apparatus allows us to put larvae in a gradient of odor concentrations, either alone or in combination with a gradient of air speeds. Our results show that this updated setup is able to reproduce anemotactic navigation as in (Jovanic et al. 2019), driving larvae to move away from the source of the air. Moreover, presenting ethyl acetate alone to the larvae led them to move towards the source of odor, showing that they are indeed able to sense the gradient of odor concentrations in that assay. Finally, combining the air speeds gradient with the gradient of ethyl acetate concentrations, we observe a slight non-significant reduction in the anemotactic performances measured by the navigational index, compared to presentation of the air gradient alone. This suggests that there may be alterations of the navigational strategies of the larvae when both gradients are combined that our analysis is not detailed enough to reveal.

However, the analysis displayed in this work is preliminary and does not extract detailed information about the navigation strategies of the larvae. Implementing the analysis of the probability and direction of head-casting depending on the orientation of the larva in the gradient (Linjiao Luo et al. 2010; Jovanic et al. 2019; Gomez-Marin, Stephens, and Louis 2011) is especially interesting to measure alterations of the navigation performance of the larvae. We expect that the addition of an appetitive sensory gradient to the aversive air gradient will decrease larval anemotactic performance, especially in food-deprived larvae, as both their risk-taking and their motivation to find food sources would increase. In practice, this would be reflected by an increase in the turning probability of larvae moving down the air speeds gradient and away from the odor. Ultimately, genetically inhibiting individual neurons in this assay will allow us to identify the sites responsible for the integration of the sensory stimuli presented to the larvae, and uncover the mechanisms leading to their modulation by the feeding state. We hypothesize that genetically inhibiting the neurons T1 and T2 descending, which we found may connect food-related sensory neurons (Klein et al. 2015; Kreher, Kwon, and Carlson 2005; L. Liu et al. 2003; Vosshall and Stocker 2007; Apostolopoulou, Rist, and Thum 2015) to the mechanosensory chordotonal neurons that sense the wind, may impair multisensory integration in this assay in a state-dependent manner.

A caveat with our assay is that we were not able to characterize the gradient of odor concentrations and we are unsure of its steepness. Importantly, when both gradients are combined, the odor is mixed into a very important volume of air that might lead to a saturation of the entire arena with the odorant. To circumvent this issue, it is possible to replace the gradient of odor concentrations with a gradient of red light intensity,

that will allow us to optogenetically activate olfactory neurons. Uncoupling the delivery of the sensory information in this way will help avoid the technical issues arising from mixing odors into the wind stimulus.

Moreover, by selecting the appropriate genetic driver, we could use the setup to combine other sensory modalities than olfaction with the air speeds gradient. Optogenetically activating gustatory sensory neurons in this way would be particularly interesting with regard to our discovery of two descending neurons linking the mechanosensory chordotonal neurons to sensory neurons from the antennal and maxillary nerves. Indeed, both nerves relay information from several sensory modalities, including gustation (Miroschnikow, Schlegel, and Pankratz 2020; J. Y. Kwon et al. 2011; Klein et al. 2015; Kreher, Kwon, and Carlson 2005; L. Liu et al. 2003; Vosshall and Stocker 2007; Apostolopoulou, Rist, and Thum 2015).

IV. G. Relevance of the feeding state-dependent modulation of non-feeding-dependent behaviors and its study

We believe the modulation of the behavioral decision of the larvae in response to an air puff by alterations of the feeding state that we uncover in the article in section III.A of this manuscript reflects an increase in risk-taking by the larvae. Indeed, head-casting is a dynamic action that allows larvae to explore their environment while hunching is a protective startle response (Jovanic et al. 2016; Gomez-Marin, Stephens, and Louis 2011; Ohyama et al. 2013). On the one hand, the reduction of the hunching response seen upon sucrose feeding or starvation indicates a reduction in risk aversion. On the other hand, the increase of head-casting promotes exploration (Berni 2015). Altogether, the bias of larval decisions may help larvae find food to fulfill the physiological needs brought about by alteration of their feeding state.

Although not characterized with neural circuit resolution at the same level of details as the circuit we studied in the article in section III.A of this manuscript, a lot of different behaviors have been shown to be altered by hunger across species, such as risk-taking, anxiety or social behaviors (Smith and Grueter 2022; Lin, Senapati, and Tsao 2019; Moreno-Padilla, Fernández-Serrano, and Reyes del Paso 2018; Burnett et al. 2016; Sutton and Krashes 2020; Grunwald Kadow 2019). Such a feeding state-dependent modulation of non-feeding-related behaviors illustrates how hunger can reshape the behavioral repertoire of an animal to prioritize behaviors fulfilling the current most critical need (Smith and Grueter 2022; Sutton and Krashes 2020; Burnett et al. 2016; 2019). We argue that most of these hunger-driven modulations of behaviors promote impulsivity, risk-taking and ignorance of dangers. In the context of nutrients deprivation, biasing the overall behavior of an individual towards more risk-taking and

impulsivity may lead to faster decisions, promoting exploration, even in dangerous environments. Indeed, as the pressure from hunger increases, the urgency to find food increases and may overcome the primal need to avoid dangers (Moran et al. 2021).

Elucidating the mechanisms underlying feeding state driven modulation of non-feeding-related neural circuits is particularly important to develop innovative treatments of eating disorders. Indeed, even if eating circuits are strongly dysregulated in patient suffering of conditions such as obesity, bulimia or anorexia (Morales 2022; Yeom et al. 2021; Bailer and Kaye 2003; Baranowska and Kochanowski 2018; van Galen, ter Horst, and Serlie 2021; Timper and Brüning 2017; L. Zhang, Bijker, and Herzog 2011), growing research efforts show impairment of brain processes not directly related to feeding in these conditions. Namely, eating disorders and obesity are associated with alterations in reward processing, salience detection and correlate with stress and addiction behaviors (Morales 2022; Fernández-Aranda, Granero, and Jiménez-Murcia 2023; Sinha 2018; Yau and Potenza 2013; P. C. Lee and Dixon 2017; Donofry, Stillman, and Erickson 2019). Notably, alterations in neuropeptidergic signaling, and especially of NPY signaling, are involved in the pathophysiology of obesity and eating disorders (Timper and Brüning 2017; L. Zhang, Bijker, and Herzog 2011; Bailer and Kaye 2003). Although it is not clear whether the alterations of neuropeptides are a source or a consequence of the development of these disorders, it is clear that they reinforce maladaptive behaviors, trapping patients in a vicious circle. Gaining a deeper understanding of the dysregulations affecting neuropeptides involved in the maintenance of these disorders will provide new therapeutic targets, notably to help fight obesity, the most prominent metabolic disorder in the world (Masood and Moorthy 2023). Furthermore, as we have seen throughout this manuscript, even feeding-related neuropeptides are not involved exclusively in the regulation of feeding processes. A better understanding of neuropeptides and their functions may also help in the treatment of chronic pain or neuropsychiatric disorders such as anxiety and depression (Smith and Grueter 2022; Rana et al. 2022).

V. References

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VI. Synthèse en Français

Afin de survivre dans un environnement en constante évolution, les animaux doivent adapter leurs comportements à chaque situation qu'ils rencontrent en fonction de l'état interne dans lequel ils se trouvent. L'état alimentaire, en particulier, est d'une importance critique pour la survie et exerce une grande influence sur nombre de décisions afin d'obtenir une régulation optimale de la prise alimentaire. Cependant, des comportements qui ne sont pas liés à la prise alimentaire sont également régulés par l'état alimentaire, comme la prise de risque ou les jugements éthiques et sociaux. Bien que l'influence de l'état alimentaire soit bien documentée dans les décisions liées à la nourriture, son impact sur les circuits neuronaux orchestrant d'autres comportements demeure un domaine de recherche en neurosciences. L'élucidation des mécanismes permettant à l'état interne d'influencer des circuits neuronaux qui ne sont pas directement reliés à sa régulation est essentielle pour comprendre le fonctionnement cérébral en santé et en maladie.

Nous utilisons la larve de *Drosophile* pour étudier les mécanismes de modulation des circuits neuronaux en fonction de l'état alimentaire. Le système nerveux compact de la larve de *Drosophile*, les puissants outils génétiques de ce modèle et la reconstruction de son connectome avec une résolution synaptique à partir d'un volume de microscopie électronique, nous donnent un accès unique à la cartographie et l'étude de circuits neuronaux des larves. Dans mon projet de thèse, j'ai examiné en particulier un circuit, précédemment décrit par ma directrice de thèse, impliqué dans la prise de décisions sensorimotrice des larves. Ce circuit orchestre le choix entre deux actions mutuellement exclusives (la rétraction de la tête, ou "hunch", qui est une réponse de sursaut et le balancement de la tête, ou "head-cast", qui est une réponse d'évitement actif) en réponse à une stimulation mécanique par un jet d'air comprimé. Des interneurones inhibiteurs réciproquement connectés engagés dans plusieurs sous-motifs pilotent l'état de sortie de ce circuit, avec une inhibition réciproque de l'inhibition mettant en œuvre le choix comportemental et une désinhibition rétroactive consolidant le "head-cast". Une telle architecture de circuit permet une sélection flexible du comportement de sortie, qui est probabiliste à la fois entre et au sein des individus est peut donc être considérée comme une forme de décision. Nous avons montré que des larves affamées ou nourries uniquement de sucrose orientent leurs décisions vers plus de "head-cast" au détriment du "hunch". Cette modulation de la

décision comportementale en réponse à la stimulation est provoquée par la modulation de l'activité de deux interneurons inhibiteurs interconnectés. Dans le but d'étudier les mécanismes à l'origine de la modulation de l'activité de ces interneurons par l'état alimentaire des larves, nous utilisons une combinaison de plusieurs techniques incluant la manipulation génétique de l'expression de neuropeptides ou de leurs récepteurs dans des cellules individuelles, l'imagerie fonctionnelle de neurones individuels par imagerie calcique, la quantification de la réponse comportementale de centaines de larves à l'aide d'une classification automatique de leurs comportements par machine learning. L'utilisation de ces techniques est éclairée par les informations extraites du connectome reconstruit à partir de microscopie électronique, nous permettant des neurones interagissant avec nos neurones d'intérêt.

Nous avons centré nos recherches sur les neuropeptides NPF et sNPF, tous deux liés au NPY des mammifères, et connus comme étant d'importants régulateurs dans la prise alimentaire. Alors que les récepteurs des deux neuropeptides sont exprimés dans les neurones mécanosensoriels, nos résultats montrent que leur modulation n'est pas essentielle pour modifier les décisions de la larve. Nous montrons également que les neurones de projection du circuit n'expriment pas de récepteurs pour sNPF ou NPF, tandis que les interneurons inhibiteurs expriment différentes combinaisons de récepteurs. Au total, cela implique que le sNPF et le NPF modulent différemment les interneurons inhibiteurs interconnectés, influençant les calculs du circuit et non les signaux d'entrée ou de sortie du circuit. En effet, la réduction de la signalisation sNPF sur ces interneurons inhibiteurs oriente le choix des larves vers moins de "hunch" et plus de "head-cast" en régulant leur activité de manière opposée, reproduisant partiellement un phénotype similaire à celui observé chez des larves à jeun ou des larves nourries avec du sucre. D'autre part, l'activité des neurones sécrétant le NPF est augmentée lors de l'ingestion de sucre et la mise à jeun, et le NPF lui-même est nécessaire pour la modulation dépendante de l'état alimentaire de la locomotion de base des larves. De plus, le NPF est responsable spécifiquement de l'augmentation de l'activité de l'un des interneurons, qui inhibe le "hunch" et favorise le "head-cast".

En conclusion, ces résultats, présentés sous la forme d'un article scientifique dans la section III.A de ce manuscrit, suggèrent que ces interneurons inhibiteurs servent d'intégrateurs de l'information sur l'état physiologique, orientant ainsi le comportement en fonction de l'état alimentaire.

Dans la section III.B de ce manuscrit, je présente des expériences montrant un effet dépendant de l'état du sNPF sur les neurones mécanosensoriels du circuit, bien que leur modulation ne soit pas nécessaire pour biaiser le choix comportemental des larves. Enfin, dans la section III.C de ce manuscrit, je présente le développement d'un setup expérimental permettant d'étudier la prise de risque chez les larves dans un contexte de navigation dans des gradients sensoriels conflictuels. Cet essai comportemental, testé sous ma supervision par des stagiaires, permettra d'étudier l'influence de l'état alimentaire sur les décisions prises par les larves au cours de la navigation dans ces gradients.