Neuromodulatory mechanisms underlying the regulation of feeding drive in zebrafish

A Thesis

Submitted in partial fulfilment of requirements

Of the degree of

Doctor of Philosophy

By

Devika S Bodas

Roll no.- 20133252

Indian Institute of Science Education and Research

Pune

(2021)

CERTIFICATE

Certified that the work incorporated in the thesis entitled “Neuromodulatory mechanisms underlying the regulation of feeding drive in zebrafish” submitted by Devika S Bodas was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

(Supervisor)

Date: 29-12-2021

Dr. Aurnab Ghose

Declaration

I declare that this written submission represents my ideas in my own words and where others' ideas have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

Signature

Date: 29/12/2021

Devika S Bodas
Roll No. 20133252

Acknowledgments

I joined IISER as a Ph.D. student. Since then, it has been an incredible learning experience, both personally and professionally. I would like to take this opportunity to thank all the people who were a part of my journey in IISER.

Firstly, I would like to express my gratitude towards my thesis supervisor Dr. Aurnab Ghose. He has been an excellent mentor throughout the course of my Ph.D., and I am grateful for the training I have received from him. He always provided the freedom to explore new ideas that made me grow as a researcher. He has been an inspiration during my work and will continue to be so later. He has provided the vision and critical guidance during thesis writing and manuscript preparations.

I would especially like to thank Prof. Nishikant Subhedar, who has been an important part of our lab. I am grateful to him for constantly inspiring me with his tremendous passion for neuroscience and providing critical inputs during lab and annual research meetings.

My sincere thanks to my research advisory committee members Dr. Raghav Rajan and Dr. Suhita Nadkarni, for bringing a new perspective during meetings and prompt communication of ideas and otherwise. Their advice and suggestions have been critical in shaping the work presented in this thesis. I am also grateful to Dr. Mayurika Lahiri and her lab members for assisting in the animal cell culture facility.

I would like to thank my past and present lab members Abhishek, Sampada, Ketakee, Tanushree, Dhriti, and Aditi, for helping with the research techniques in the initial months and providing a nourishing environment for the exchange of scientific, non-scientific ideas. I am also grateful to my batchmates friends at IISER for the constant support, treat, and celebrations that made these years memorable.

A special thanks to the non-academic staff at IISER and the IISER biology staff- Mahesh, Piyush, Kalpesh, Dr. Mrinalini, who took care of all the administrative work.

I am grateful to all the people in the IISER microscopy facility, which was an integral part of this work. Special thanks to Vijay and Santosh for their help regarding microscopy issues and image analysis.

I would also like to thank animal house facility members- Shraddha, Sameer, Prakash for taking care of feeding schedules and overall maintenance of the fish facility. This work would not have been possible without their assistance.

Last but not least, I would like to thank my family and friends for standing by my side during the ups and downs of the Ph.D. journey.

Contents

Abstract

Feeding, one of the innate, survival-oriented behaviours, is intricately controlled by conserved neural mechanisms within vertebrates. The feeding behaviour is composed of two alternating states-hunger and satiety, which are predominantly regulated by the internal energy needs of the body. Interoceptory neurons in the brain sense the internal energy conditions and regulate the release of orexic (e.g., NPY) and anorexic (e.g., CART) neuropeptides. These neuropeptides reconfigure the activities of feeding circuit elements to tune food intake with the body's metabolic needs. In mammals, the role of these neuropeptides in the establishment of and transition between hunger and satiety states is well documented. However, the complexity of neural mechanisms in higher vertebrates limits the understanding of circuit-level modulatory mechanisms regulating feeding behaviour.

Using a simple vertebrate system, zebrafish, this study elucidates molecular level modulatory mechanisms regulating food intake and characterizes a novel neuroanatomical region that is correlated with the regulation of hunger-satiety bistable states. We demonstrate that antagonistic actions of orexic neuropeptide NPY and anorexic neuropeptide CART regulate the activity of dorsomedial telencephalon (Dm) to establish hunger-satiety states. CART facilitates the activation of Dm neurons to induce anorexia. In contrast, NPY leads to decreased activation of Dm neurons to increase food intake.

To identify the plausible molecular candidates involved in feeding regulatory actions of CART and NPY, we performed pharmacological interventions and monitored their effect on feeding drive and Dm activity. Our data suggest that antagonistic activities of CART and NPY activate opposing biochemical signaling pathways that converge on NMDA receptor (NMDAR) function in Dm. Opposing actions of CART and NPY differentially phosphorylate serine 897 on the NR1 subunit of NMDARs. Phosphorylation of this residue is known to increase the calcium permeability of the receptor and thereby enhance NMDAR function. We find CART treatment leads to increased phosphorylation NR1 subunit by Protein kinase A (PKA) and Protein kinase C (PKC) activation to increase NMDAR activity and thereby increase the excitability of Dm neurons. On the other hand, NPY activates the protein phosphatase calcineurin and downregulates PKA activity to reduce NR1 phosphorylation, consequently de-potentiates NMDAR activity and reduces Dm excitability neurons.

The modulatory configuration of biochemical signaling allows Dm to exhibit bi-stable excitability, which forms a neural representation of the energy state of the body and can be correlated with the modulation of feeding drive in zebrafish.
Based on these results, we propose that antagonistic actions of CART and NPY tune the excitability of Dm neurons by modulating the NMDA receptor activity to regulate hunger-satiety behavioural outputs in zebrafish.

Parallelly, we have undertaken the work to identify novel receptor/s for CART peptide. This study has identified a candidate CART responsive cell line- GH3, which shows receptor-like activity and produces a robust response to the CART treatment by upregulating intracellular calcium levels. We have optimized the conditions for the Ligand-based Receptor Capture method (LRC), which will be further used in the future to isolate and identify CART receptor/s from GH3 cells.

1 Chapter 1 : Introduction

1.1 Neural regulation of animal behaviour

Animals need to constantly adapt to the changes in internal or external stimuli and thereby mount an appropriate behavioural response to ensure their survival and reproductive success. At a given time, the animal behavior is composed of a stable behavioural state which can either be extended or switched to an alternating state depending on environmental factors or homeostatic stimuli. For example, a hungry animal is likely to continue foraging even in the presence of a predator. In contrast, the same animal in a sated state would be less willing to take the risk for food (Sutton and Krashes, 2020; Lima and Dill, 1990; Mobbs et al., 2018). Making rapid switches between different behavioural states by evaluating internal and external conditions is the key to the survival of an animal.

Neural mechanisms play a pivotal role in the context-dependent modulation of animal behaviour, ranging from simple, instinctive behaviours like feeding to complex cognitive tasks. The brain continuously evaluates internal and external cues and accordingly modulates the activities of underlying neural networks to dictate appropriate alterations in behavioural states.

The individual behavioural state is a result of the distinct neural activity pattern within the specific regions of the brain. Context-dependent modulation of the activity of this network drives either extension of current behavioural state or transition to the alternating state. For example, the signature activity patterns of a distinct population of neurons within the basal amygdala are associated with exploratory and non-exploratory (defensive) states of behaviour and are known to be modulated by internal states in mice. Looking at these population signatures, one can predict the present state of the mice as well as the transitions between the two states (Gründemann et al., 2019).

The major questions that need to be answered to understand the neural basis of animal behaviour are: How do neural circuits sense the internal/external cues and thereby coordinate changes in behavioural states? Specifically, what drives context-dependent modulation of the neural activity that ultimately results in maintenance or transition into the alternating behavioural state? Understanding cellular and molecular mechanisms involved in the modulation of neural activity and the establishment of behavioural states continue to pose a challenge for neuroscientists.

1.1.1 Neuromodulation

Neural circuits must possess intrinsic flexibility to tune the behavioural outcomes with changing internal and external cues. Neuromodulatory mechanisms provide functional flexibility to neural circuit elements wherein a single anatomical circuit can produce multiple behavioural outcomes. Neuromodulators enable neural circuits to reconfigure their activities in a context-dependent manner to produce appropriate behavioural output.

Neuropeptides, a major class of neuromodulators, have been shown to be involved in the regulation of behavioural circuits (van den Pol, 2012). These peptides, often produced by neurons in response to the changes in internal-external conditions, modulate the activities of component circuit elements to dictate alterations in the behavioural states. For example, orexic neuropeptide- Neuropeptide Y (NPY), produced by energy-sensing neurons in the hypothalamus under energy-deprived conditions, has been shown to be critical for elicitation of feeding and establishing a hunger state (Loh et al., 2015).

As demonstrated in Fig A1, unlike fast acting neurotransmitters, neuropeptidergic release is not restricted to presynaptic active zones and can be released from non synaptic sites. They can diffuse over longer distances to influence larger populations of neurons or even recruit the neurons in the circuit that are not anatomically connected (Bargmann, 2012; Marder and Bucher, 2007). Neuropeptides typically act via G- protein-coupled receptors (GPCRs) that have slower kinetics as compared to ionotropic receptors and mediate long-lasting biochemical changes over component neurons. These long-lasting modulations of biochemical properties of neural circuit elements are important for maintenance and the transition between alternate behavioural states (van den Pol, 2012; Marder, 2012).

1.1.2 Mechanisms of neuromodulation

The mechanisms of neuromodulation are complex- one neuropeptide can affect the activity of multiple channels and various types of neurons whereas, multiple neuropeptides can target a single neuron (Nadim and Bucher, 2014). In vitro studies in non-vertebrate systems like lobsters have provided insights into the neuromodulatory mechanisms that modulate the functioning of neural circuits (Marder and Bucher, 2007). Neuromodulators, working via GPCRs, activate biochemical signaling cascades to mediate sustainable but reversible modifications in circuit elements and generate alternate activity patterns (Warrick, 1991). The mechanisms by which neuromodulators modulate circuit functioning can be divided into two

broad categories- They work via modulating synaptic efficacy of the network elements or altering the intrinsic properties of the component neurons.

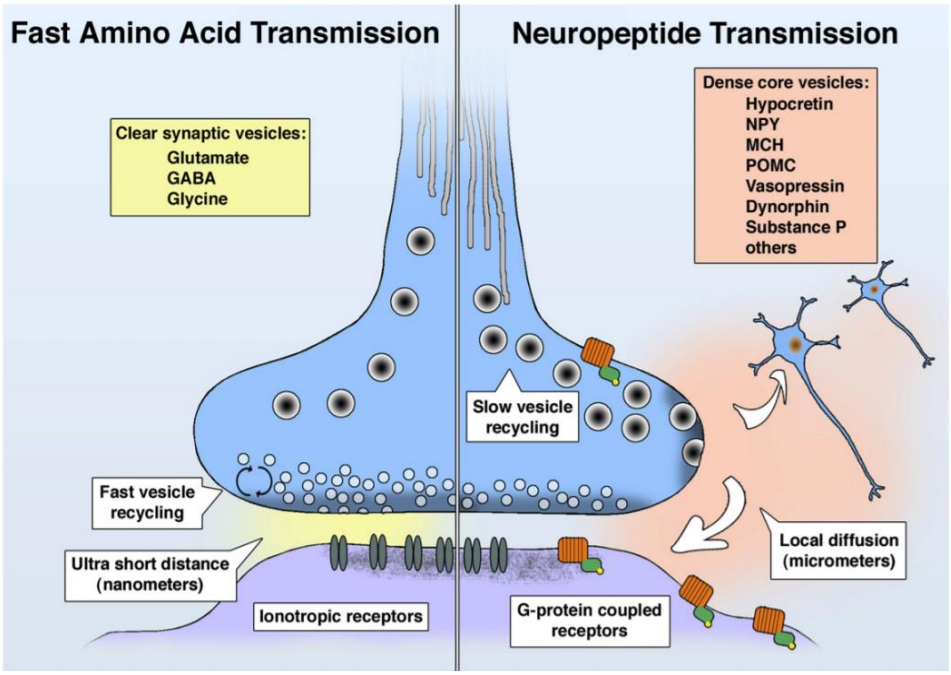
The common example of modulating synaptic efficacy is to modulate the synaptic strength. Neuromodulators can strengthen or weaken the synaptic strength of anatomically connected neurons in the circuit. Dopamine has been shown to strengthen the particular synaptic connection in a pyloric circuit in lobster by increasing the voltage-gated calcium accumulation in the presynaptic neuron (Johnson et al., 2003).

Similarly, neuromodulators can also affect synaptic dynamics. Serotonin has been demonstrated to reduce presynaptic potassium currents via activating PKA resulting in increased duration of action potential and neurotransmitter release (Klein et al., 1982).

Another way by which neuromodulators influence the circuit functioning is by dynamically modifying the intrinsic properties of component neurons. The intrinsic response properties of a neuron can be attributed to the activities of ion channels expressed on the membrane. Neuromodulators can change the type, expression levels (Jin et al., 2011), and the response kinetics of these ion channels (Chiu et al., 2010) to dynamically regulate the excitability of component neurons in response to changing conditions.

For example- neuromodulators acting via GPCRs activate secondary messenger systems like kinases and phosphatases, which alter the gating properties of ion channels to regulate responsiveness of a given neuron to the excitatory neurotransmitters like glutamate that are often coreleased with the modulator (Ismailov and Benos, 1995; Swope et al., 1999). Different neuromodulators can affect different ion channels or have an opposite modulatory effect on the same ion channel on the same neuron, leading to context-dependent modulation of excitability of the target neuron (Nadim and Bucher, 2014).

In summary, neuromodulators influence circuit functioning by modifying synaptic efficacy and/or alteration in neuronal excitability, allowing for context-dependent behavioural flexibility.



Reproduced from van den Pol, 2012

Figure 1. 1 Comparison between mechanisms of transmission by fast-acting neurotransmitters and slow-acting neuromodulators

Fast-acting neurotransmitters like glutamate and GABA act synaptically and influence postsynaptic targets by activating ionotropic receptors (Left side). In contrast, slow-acting neuropeptides (a class of neuromodulators) diffuse over a longer distance and target a larger population of neurons. They mediate their action via activating GPCRs to modulate the biochemical properties of target neurons.

1.1.3 Neuromodulation of behaviour

Neuromodulators have been shown to play crucial role in the establishment and transition between distinct behavioural states. Context driven long-lasting but reversible biochemical modifications of circuit function is critical for maintenance of long lasting behavioral states as well as allows rapid transition between them. For example, opposing actions of PDF and Serotonin have been shown to be important in maintaining alternating behavioural states roaming and dwelling in *c.elegans*. Serotonin establishes dwelling state by activating serotonin gated chloride channel, whereas PDF promotes roaming state by activating cAMP-mediated secondary messenger systems (Flavell et al., 2013). Studies in *Drosophila* also showed that hunger-mediated activation of sNPF signaling increases olfactory and gustatory sensitivity to food related cues to facilitate feeding (Su and Wang, 2014).

Studies in central pattern generators (CPGs) across animals best describe the functional consequences of neuromodulation on animal behaviour. CPGs are the neuronal circuits that regulate rhythmic motor behaviours like walking, breathing etc. in invertebrates and vertebrates. CPGs produce rhythmic circuit outputs without any input and are susceptible to the action of various neuromodulators, altering the circuit outputs according to changing conditions (Bucher et al., 2015). The neuronal composition of CPGs is fixed, however, the subset of neurons which participate in producing particular motor output depends on the modulatory state. Different modulators activate or inhibit different subcircuits within CPGs to tune motor outcome with changing behavioural needs. In rodents, respiratory CPGs are reconfigured during normal respiration, sigh, and gasping. The switch between normal respiration and sigh, for example, requires activation of different ionic channels in different subgroups of neurons to provide different rhythm to the breathing (Harris-Warrick, 2011).

The cellular and network level mechanisms of neuromodulation have been well understood in crustacean somatogastric ganglion (STG). Pyloric CPGs in this network are subjected to various neuromodulators which are released from modulatory projections in paracrine manner or can be intrinsically released by a group of neurons. Neuromodulators have been shown to affect the synapses and/or intrinsic excitability of neurons to produce different rhythmic patterns in isolated pyloric network (Marder and Thirumalai, 2002). For example, all the neurons in the pyloric network express dopamine receptors but the subsets of ion channels affected by dopamine and sign of the effect varies across cell types. These cell type specific divergent effects of dopamine are able to produce multiple activity patterns. Moreover, multiple neuromodulators might have convergent actions and can target a single ion channel, however each neuromodulator affects a subset of neurons in the network which express their receptor leading to divergent network effects (Marder and Bucher, 2007). These studies in CPGs provide global insight into the modulatory principles that enable animals to adapt their behaviours according to changing conditions.

In conclusion, a multitude of convergent and divergent modulatory actions at cellular and network level allow neural circuits to exhibit remarkable degree of flexibility to dictate context dependent changes in behavioural outcomes.

1.2 Feeding behaviour

Feeding behaviour is one of the intricately regulated, innate behaviours associated with the survival of an animal. Specific actions involved in the expression of this behaviour may vary between different species or even within single species, but the common principle which drives this behvaiour is the ability of an animal to sense the energy needs and influence the choice of actions towards the fulfillment of those needs (Johannsen, 1933). Feeding behaviour can be subdivided into two states: 1) Hunger state represents energy deficit 2) Satiety state represents energy surfeit.

In a hunger state, energy deficiency creates a motivational state, 'Feeding drive,' which directs an animal's actions towards food intake and encourages it to work for the food (Atasoy et al., 2012a). The feeding drive and resultant food intake gradually decrease as the animal fulfills the metabolic energy requirements and achieves a satiety state. Enhanced feeding drive under hunger state not only influences food intake but also modulates the sensory perception of food-related cues (Kaniganti et al.,2021; Sternson et al., 2013). For example, hunger sensitizes other sensory modalities such as smell and taste to food-related cues. Under energy-deprived conditions, NPY increases the sensitivity of olfacto-sensory neurons to food-related cues in zebrafish (Kaniganti et al., 2021) and dopamine enhances gustatory responses to sugar in flies. (Inagaki et al., 2014a). Neural circuits, activated by energy deficit, have been shown to impart motivational components to the process of feeding, but their mechanism of action is poorly understood.

Apart from homeostatic energy needs, the hedonic properties of food can also drive feeding even in sated conditions (Johnson, 2013). However, this study only deals with the homeostatic regulation of feeding behaviour.

1.2.1 Neural regulation of homeostatic feeding

Neural circuits underlying survival behaviours like feeding are directly under selective pressure and hence expected to have some hardwired components which can be developmentally identified (Sohn et al., 2013). These circuits have been shaped throughout evolution, providing redundant and robust mechanisms for adapting and responding to changes in nutrient availability.

Tuning food intake to changing energy demands of the body is critical for the survival of an animal. Hence, underlying neural circuits need to be flexible to regulate feeding according to energy requirements. Neuropeptides, a class of neuromodulators, are predicted to provide such

functional flexibility to hardwired feeding circuits to dictate energy state-dependent feeding. However, the mechanisms underlying peptidergic modulation remain poorly understood.
To understand the neural basis of feeding behaviour, the major questions that need to be answered are - How does the brain sense the internal energy states? How are these internal states represented at the level of neural circuits? What drives changes in neural activities that ultimately result in energy state-dependent regulation of food intake.

1.2.1.1 Sensing energy needs of the body

Circulating neuroendocrine molecules act as signals for the energy status of the body. Some of these peripheral signaling molecules are leptin, ghrelin, insulin, and cholecystokinin. Leptin is a satiety signal produced in the adipocytes, the circulating levels of which parallel the body fat stores (Takahashi and Cone, 2005). Insulin is also a satiety signal secreted by the pancreatic islet ß cells in response to food intake and enhances glucose uptake and glycogen synthesis. Both leptin and insulin can cross the blood-brain barrier and serve as peripheral indicators of energy status. Ghrelin and cholecystokinin are peptides released from the stomach and intestinal endocrine cells, respectively, which have opposing actions. Cholecystokinin is released in response to a meal and helps to terminate the feeding, whereas ghrelin is involved in stimulating the food intake (Barsh and Schwartz, 2002).
In mammals, the arcuate nucleus (ARC) of the hypothalamus and nucleus tractus solitarius (NTS) of the hindbrain contain interoceptory populations that sense these neuroendocrine signals of energy condition of the body (Andermann and Lowell, 2017).
As represented in Fig A2, the arcuate nucleus of the hypothalamus contains two intermingled populations of interoceptory neurons- **Agouti-related protein (AGRP) / Neuropeptide Y (NPY) neurons** and **proopiomelanocortin (POMC)/ cocaine and amphetamine-regulated transcript (CART) neurons** that are activated by hunger and satiety signals respectively. AGRP/NPY neurons are activated by signals of energy deficiency such as ghrelin and enhance food intake by activating hunger circuits (Cowley et al., 2003). These neurons produce orexic (hunger-promoting) neuropeptides like NPY, which modulate feeding circuit activities to promote feeding. In contrast, POMC/CART neurons are activated by energy surfeit signals like leptin and release anorexic (hunger-suppressing) neuropeptides like POMC and CART to suppress feeding by activating satiety circuits (Ollmann et al., 1998; Haskell-Luevano and Monck, 2001). Additionally, AGRP/NPY neurons inhibit POMC/CART neurons under hunger conditions by sending GABAergic projections to promote feeding (Atasoy et al., 2012b).

Ghrelin, a hunger hormone produced by the stomach, upregulates the activity of AGRP/NPY neurons, increasing the glutamatergic drive from presynaptic terminals. (Yang et al., 2011). Leptin, a satiety hormone produced by adipocytes, acts by modulating the activity of both AGRP and POMC neurons. Cowley et al. demonstrated that leptin directly activates POMC neurons and reduces GABAergic synaptic inputs onto POMC neurons by acting on NPY/AGRP neurons. Both of these actions lead to a resultant increase in the firing of POMC neurons.

Another energy-sensing region has been identified in rodents- an area in the hindbrain called NTS integrates peripheral satiety signals from the gastrointestinal tract and the vagal nerve. A distinct subpopulation of neurons in NTS also responds to leptin (Neyens et al., 2020) and releases anorexic neuropeptide CCK, nor-epinephrin, etc., resulting in the termination of the meal and the suppression of food intake (Fan et al., 2004; Katsurada et al., 2014).

In summary, rodent studies have demonstrated that interoceptory neurons in the hypothalamus and hindbrain sense the neuroendocrine signals of energy status and respond by releasing orexic (e.g.-NPY) /anorexic (e.g., CART) neuropeptides. These neuropeptides, acting as molecular signals of energy conditions, modulate the activity of feeding circuit elements to dictate energy state-dependent regulation of food intake.

1.2.1.2 Integration of neuropeptidergic signals of energy status

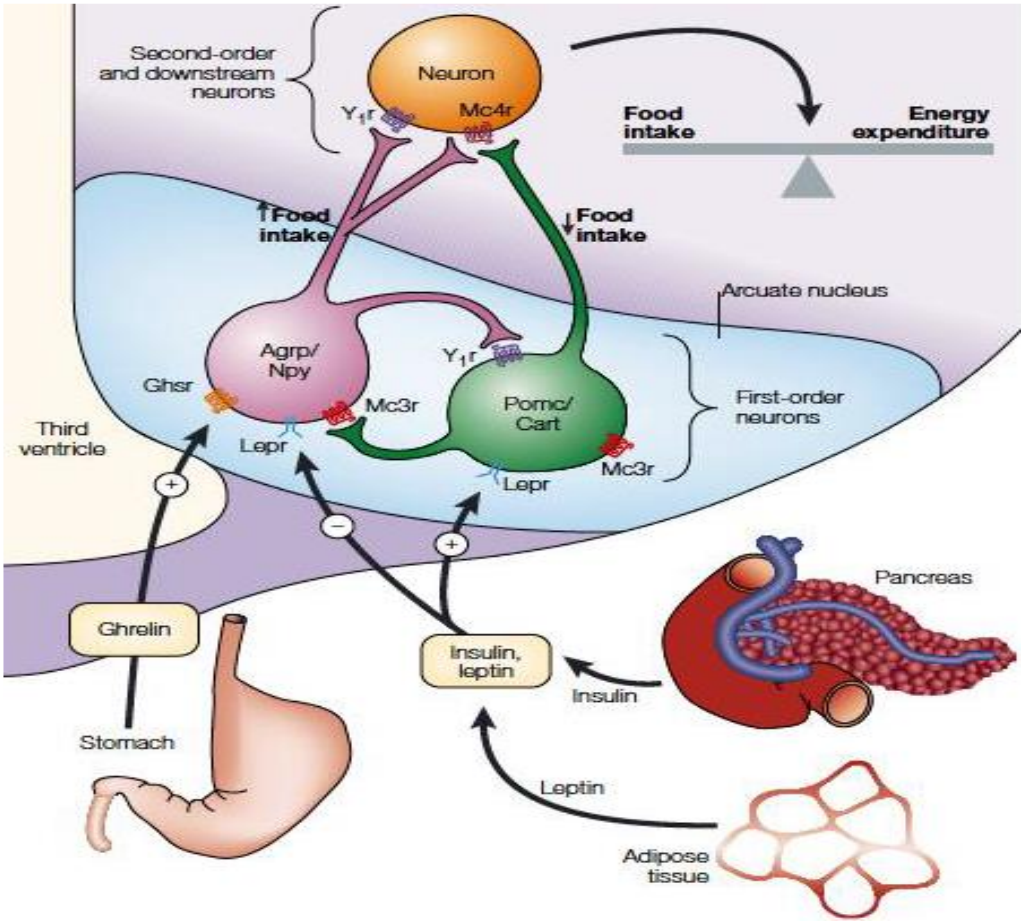
In mammals, energy-sensing mechanisms by the hypothalamus and hindbrain are well studied, but there is very little knowledge about downstream neuroanatomical regions involved in the integration and processing of this information.

AGRP/NPY and POMC/CART neurons are known to have overlapping targets within and outside the hypothalamus. Both these populations project to paraventricular (PVN), ventromedial, dorsomedial, and lateral hypothalamus and to the nucleus of tractus solitarii (NTS), the parabrachial nucleus (PBN), the amygdala, and the bed of the stria terminalis (BNST), which lie outside the hypothalamus (Broberger et al., 1998). In these regions, AGRP and NPY containing neurons are found in close apposition to POMC-containing neurons (Sohn et al., 2013; Blevins and Baskin, 2009; Morton et al., 2006; Zigman et al., 2014). This suggests that some of the above-mentioned brain centers might have a role in integrating hunger and satiety signals.

One well-studied example of integration of orexic /anorexic signals is a melanocortin system in PVH. Opposing activities of AGRP/NPY and POMC/CART neurons converge on

melanocortin receptors in PVH. AGRP antagonizes melanocortin receptor 4 (MCR4), which is a principal effector of POMC action. In turn, activation of MCR4 by POMC cleavage product αmelanocyte-stimulating hormone (α-MSH) inhibits AGRP neuron activity (Vergoni and Bertolini, 2000). This bistable circuit activity allows rapid behavioural switch from hunger to satiety or vice versa. Local circuits further amplify antagonistic actions of AGRP/NPY and POMC/CART neurons in the hypothalamus for robust and stable representation of the energy state of the body (Andermann and Lowell, 2017).

In the hindbrain satiety circuit, a population of neurons in NTS activate parabrachial nucleus (PBN) CGRP neurons and terminate feeding via projecting to the central amygdala (CEA) (Carter et al., 2013). Additionally, a subpopulation of NTS neurons producing CCK and GLP1 project to the hypothalamus to establish a satiety state (D'Agostino et al., 2016; Gaykema et al., 2017).



Reproduced from Barsh and Schwartz, 2002

Figure 1. 2 Hypothalamic regulation of energy homeostasis

AGRP/NPY and POMC/CART containing neurons in the arcuate nucleus are regulated by the circulating hormones released from the periphery. Leptin and insulin stimulate POMC/CART neurons and inhibit AgRP/NPY neurons. Ghrelin activates AGRP/NPY neurons and stimulates food intake.

1.2.2 Energy state-dependent modulation of feeding circuits

The actions of multiple neuromodulators reconfigure a single anatomical neural network to produce different behaviours in response to changing conditions (Harris-Warrick and Marder, 1991). Studies in mammals suggest that food intake is also regulated by such polymorphic networks, which integrate signals of physiological energy state and accordingly modulate their activity to enable rapid transition between hunger and satiety states (Lee and Blackshaw, 2014). Interoceptory neurons in the brain produce feeding-related neuropeptides, which reconfigure the activities of downstream circuit nodes to regulate hunger-satiety behavioural states. For example, NPY, released from interoceptory neurons in the arcuate nucleus, modulates the synaptic strength between melanocortin receptor-positive PVH neurons and interoceptory

arcuate neurons by enhancing inhibitory presynaptic currents on MCR4+ PVH neurons. Inhibitory GABAergic connection between AGRP and PVH shows an extraordinary synaptic property that can be linked to neuromodulation. One millisecond of the light pulse to AGRP neurons led to a barrage of delayed asynchronous inhibitory IPSCs that lasted for about 1 sec. Prolonged GABA release reduced spontaneous as well as induced excitation of PVH neurons for hundreds of milliseconds which can be correlated with increased food intake (Aponte et al., 2011; Atasoy et al., 2012b)

A report by Yang et al. demonstrated that modulatory mechanisms not only influence the activity of higher-order neurons but also modulate the activity of sensory neurons. AGRP neuronal activity is shown to be modulated presynaptically in response to changing energy conditions. AGRP neurons switch between high and low activity states in response to hunger and satiety signals, respectively (Yang et al., 2011). Food deprived state or ghrelin treatment upregulates the activity of AGRP neurons which is mediated by glutamate release from a subset of PVH neurons (Krashes et al., 2014). Ghrelin acts on a subset of PVH neurons and leads to activation of AMPK through the calcium/calmodulin-dependent protein kinase (CAMKK) pathway. AMPK and ryanodine receptor-mediated calcium release from internal stores in presynaptic sites endows the excitatory synaptic inputs onto AGRP neurons. Ghrelin-mediated synaptic activity persists for hours even after the removal of ghrelin. The hysteretic nature of this signaling is likely due to the positive feedback loop in which calcium release, in turn, leads to additional AMPK activity, which would trigger the release of more calcium from internal stores. The activity is switched off by leptin, an anorexigenic adipocyte-derived hormone that directly activates POMC neurons (Cowley et al., 2001).

The modulatory mechanisms involved in the regulation of food intake are far from understood. Still, a few studies in mammals that show evidence for such phenomenon suggest that neuromodulation operates at every level of feeding circuitry, from sensory perception to motor output, to ensure robust and stable behavioural response which will satisfy physiological needs.

1.2.3 Neuropeptides involved in regulating food intake

Neuropeptides are the principal modulatory agents that play a crucial role in energy state-dependent regulation of food intake. Orexic /anorexic neuropeptides are produced centrally in response to changes in energy states wherein they are predicted to reconfigure the activities of feeding circuit elements to tune feeding with prevailing energy conditions.

Multiple orexic/ anorexic neuropeptides have been identified in vertebrates (Table 1). Their expression profiles and behvioural effects have been well characterized. However, the circuit-level mechanisms and molecular players involved in their modulatory actions remain poorly understood.

Table 1. 1 CNS factors involved in the regulation of feeding behaviour

Orexigenic Factors	Anorexigenic Factors
NPY	α-MSH
AgRP	CART
Orexin A and B	CRH
MCH	TRH
Galanin	Neurotensin
GHRH	Galanin-like peptide
β-endorphin	Oxytocin
Dynorphin	GLP-1
GABA	Serotonin
Noradrenaline	Prolactin

1.2.3.1 Cocaine and Amphetamine Regulated Transcript in the regulation of feeding behavior

CART was identified as a transcript whose expression was significantly increased in rat striatum upon cocaine and amphetamine treatment (Douglass et al., 1995). After its discovery in 1995, the transcript sequence was found to match the previously identified somatostatin-like peptide from the ovine hypothalamus (Spiess et al., 1981). In rodents, CART has two biologically active forms, CART (55–102) and CART (62–102), which share strong homology with human CART (49–89) and the long-form CART (40–89), respectively (Douglass and Daoud, 1996).

Early studies in rodents have shown that CART is expressed in brain regions and peripheral organs, which are involved in the regulation of feeding behavior. CART peptide is expressed in multiple areas of the hypothalamus, including the arcuate nucleus (Arc), lateral hypothalamus (LHA), dorsal medial hypothalamus (DMH), ventral medial hypothalamus (VMH), and paraventricular nuclei (PVN), where energy states of the body regulate its expression levels(Choudhary et al., 2018; Farzi et al., 2018; Hunter et al., 2004; Singh et al., 2021). Apart from the hypothalamus, CART peptide expression in the amygdala is sensitive to

the energy status of the body. In the central nucleus of the amygdala (CeA), the differential expression of CART peptide has been shown to modulate homeostatic feeding in mice (Cai et al., 2014).

Exogenous application of CART in 3^{rd,} 4th and lateral ventricles reduces food intake in mice and rats, signifying its anorexic function in rodents. On the other hand, immunoneutralization of endogenous CART function by administration of CART antibody increases feeding, suggesting it's a potent endogenous anorexic factor in the brain. Exogenous application of CART is known to activate Arc, PVN, and DMH of the hypothalamus and the NTS, AP, and PBN of the hindbrain (Zheng et al., 2002; Vrang et al., 1999; Smedh et al., 2015). These areas are known to be involved in the regulation of feeding, suggesting that they are the primary targets for CART-mediated feeding regulation. Further studies are required to investigate the mechanism of CART action in these areas to deduce their role in CART-mediated anorexia.

Although the behavioural effects of CART have been extensively studied, the lack of well-characterized receptor/s for the peptide limits the investigation of the mechanism of CART action and the development of pharmacological agents to specifically manipulate CART signaling in the brain.

1.2.3.2 Neuropeptide Y in the regulation of feeding behaviour

NPY, a 36 amino acid peptide, was first identified from porcine brain extracts (Tatemoto et al., 1982). Later its role in the regulation of feeding as one of the most potent orexigenic agents was demonstrated (Stanley and Leibowitz, 1984). It is abundantly expressed in hypothalamic feeding centers, predominantly in the arcuate nucleus, where its expression is upregulated under a hunger state (Sainsbury and Zhang, 2010). NPY positive neurons in ARC integrate peripheral signals of energy conditions like leptin and ghrelin to control feeding (Kohno and Yada, 2012). The feeding regulatory action of NPY is mediated via NPY Y1 and Y2 receptors. Administration of Y1 receptor antagonist in hypothalamus leads to significant decrease in food intake and body weight suggesting that in hypothalamus NPY works via Y1 receptor to induce orexia. (Danielsa et al., 2001). NPY has been shown to inhibit PVH neurons via activation of the Y1 receptor, an inhibitory GPCR to promote food intake in rodents. (Barsh and Schwartz, 2002). Apart from this, there is no clear evidence for downstream mechanisms of NPY action that induce orexia in rodents. Further studies should identify downstream targets and elucidate modulatory mechanisms that mediate NPY's orexic action.

In summary, studies in mammals have demonstrated that feeding behaviour is intricately regulated by coordinated actions of multiple neural networks that integrate neuroendocrine signals of energy state and accordingly modulate food intake. Although the circuitry is far from understood, the role of the hypothalamus in sensing and integrating energy status-related signals has been well established. The energy sensing neurons in the hypothalamus produce orexic/ anorexic neuropeptides that dynamically modulate the activity of feeding circuits at various levels to establish hunger and satiety states and coordinate transition between them. To understand how neural circuits and modulatory systems interact to generate hunger-satiety behavioural outcomes, it is crucial to elucidate the mechanisms of neuropeptidergic actions and identify neuroanatomical correlates which mediate their effects to regulate feeding.

1.3 Objectives of the study

This study is aimed at elucidating circuit-level modulatory mechanisms that are involved in the regulation of feeding drive. Here, we focus on two neuropeptides- CART and NPY, which are known to potently regulate feeding in vertebrates, and characterize their role in regulating satiety/hunger behvaioural states, respectively.

The complexity of neural systems in higher vertebrates like mammals limits the investigation of modulatory mechanisms involved in regulating hunger and satiety states. Invertebrate systems like Drosophila and C.elegans have been used to elucidate the molecular mechanisms of neuromodulators and identify neuroanatomical substrates involved in regulating distinct behavioural states- hunger and satiety (Ben Arous et al., 2009; Eriksson et al., 2017; Inagaki et al., 2014b; Pool and Scott, 2014; Su and Wang, 2014; Yu et al., 2016). However, the translational value of the knowledge gained from invertebrates systems is very limited as their neural systems differ substantially from vertebrates.

Zebrafish, a non-mammalian vertebrate, serves as an ideal model for neurobehavioural research. It retains the advantages of simpler nervous systems like small and less complex nervous systems with conserved neuroanatomical structures and neuromodulatory systems as in higher vertebrates. Smaller brain size and ease of genetic manipulations provide advantages over mammalian systems. Many transgenic lines have been developed to label the specific neuropeptide-producing neurons, making tracing their downstream targets easier (Förster et al., 2017; Kawakami et al., 2010). Optogenetic and chemogenetic tools have been developed for specific activity manipulation to test the role of neuropeptides in the regulation of distinct behavioural states (Friedrich et al., 2010; Wyart and Del Bene, 2011). Moreover, imaging and

analyzing the activity dynamics of a large number of neurons in response to different cues have made an investigation of circuit-level modulatory mechanisms easier (Mu et al., 2020; Randlett et al., 2015).

Evidence suggests feeding circuits in fish show structural and functional similarities with higher mammals (Soengas et al., 2018). The function of feeding-related neuropeptides like AgRP, CART, NPY, and orexin is conserved in fish and mammals (Volkoff et al., 2005). These peptides are expressed in the hypothalamus, a well-conserved region involved in integrating energy state-related signals (Delgado et al., 2017). Moreover, the expression of CART, NPY and AgRP is regulated by energy status in zebrafish (Jeong et al., 2018; Opazo et al., 2019; Yokobori et al., 2012). For example- CART levels decreased in starvation in NLT of the hypothalamus (Akash et al.,2014), a fish homolog of the arcuate nucleus, a well-known interoceptory region in mammals. In addition to the hypothalamus, the entopeduncular nucleus (EN) of the ventral telencephalon has been shown to be involved in sensing energy conditions of the body in fish. CART expression in this region is upregulated by the central administration of glucose in zebrafish (Mukherjee et al., 2012) and leptin in catfish (Subhedar et al., 2011). Altogether, these data suggest that zebrafish would serve as an excellent model system to study mechanisms of peptidergic actions regulating feeding behaviour.

The thesis work presented here envisioned to elucidate modulatory mechanisms of CART and NPY action that led to the regulation of feeding drive in adult zebrafish. In this study, we assessed the role of CART and NPY in the regulation of food intake, identified molecular players, and characterized circuit-level mechanisms involved in antagonistic actions of CART and NPY leading to regulation of feeding drive in adult zebrafish.

2 Chapter 2: Materials and methods

2.1 Animal handling and sampling procedures

Wild-type indian strain zebrafish (*Danio rerio*) of both the sexes were used in the present study. The fish were housed in multiplexed standalone systems (Aquatic Habitats and Techniplast, USA) and maintained at 28.5°C under controlled light-dark conditions (14L/10D). Regular water quality checks were undertaken to maintain optimum water quality (hardness 100 - 300 mg/L of $CaCO_3$; alkalinity 50 - 300 mg/L of $CaCO_3$; nitrate < 20 mg/ml; pH 6-8; conductivity 180 – 350 µS). Fish were fed Ziegler feed and live artemia three times a day. Adult Tg (NeuroD:GcAMP6f) in nacre background were used for calcium activity imaging experiments, which were maintained in the same conditions. For behavioural experiments, long-finned wild-type fish were used. All behaviour assays and activity imaging experiments were performed between 10 am and 5 pm. Unless specified otherwise, the study used adult fish of either sex. All procedures were performed according to the guidelines of the Institutional Animal Ethics Committee (IAEC) of IISER, Pune.

2.2 Chemicals and antibodies used

CART peptide (55-102) (10 ng) used was provided by Thim lab (Novo Nordisk, Bagsvaerd, Denmark) and (Phoenix), **NPY peptide** (10pmol) (Sigma Aldrich, St. Louis, MO), **BIBP-3226** (100pmol) (Sigma Aldrich, St. Louis, MO), **AP5** (0.1nmol), (Sigma Aldrich, St. Louis, MO), **KT5720** (1.39pmol) (Sigma Aldrich, St. Louis, MO), GF109206X (1.5pmol) (Tocis biosciences)**, FK506** (0.13nmol) (Sigma Aldrich, St. Louis, MO), **Forskolin** (1.2nmol) received from NCCS (Sigma Aldrich, St. Louis, MO), **2-PhenoxyEthanol** (1:2000) (Sigma-Aldrich), **Glucose** (8000ng/ul), **Paraformaldehyde** (4%), **Primary polyclonal antibody for phospho-p44/42MAPK (Thr202/Tyr 204 pERK1/2)** (1:700, Cell Signaling Technology, Beverly), a primary monoclonal antibody for phospho-p44/42MAPK (Thr202/Tyr 204 pERK1/2), **Anti-CART antibody** (1:1500) were received as gift from Dr.Thim (Novo Nordisk, Bagsvaerd, Denmark), **Anti- pNR1 (S897) antibody** (1:70) (Millipore Cat.No.-ABN99). **Secondary antibodies, anti-mouse 568 and anti-rabbit 488** (1:500, Molecular Probes, Invitrogen, USA), **DAPI** (Molecular Probes, Invitrogen, USA).

2.3 Intracerebroventricular (ICV) injections

The fish were anesthetized using 2-phenoxy ethanol (1: 2000) in distilled water. The anesthetized fish were put on a cotton bed in a 50 mm Petri plate with its head submerged in anesthesia. 1-4 µl volume of the drug was delivered in the ventricular space between skull and brain, at the mid-sagittal plane, between the two eyes without touching the brain. The injection was done using an insulin needle (Becton Dickinson Insulin Syringe U40 - 31G) attached via a catheter to a 10 µl Hamilton microsyringe. This method of ICV administration had been previously standardized in our lab. After injection, the fish were returned to their tanks and allowed to recover before proceeding for either behavioural recordings or immunohistochemistry. Unless otherwise indicated, all the behavioural and immunofluorescence studies were undertaken 15 mins following injection.

2.4 Immunohistochemistry

As represented in Fig 2.1, fish were ICV injected with a drug or a vehicle and allowed to recover for 15 mins. After recovery, fish were anesthetized, and their brain was exposed via the dorsal window and fixed in 4% PFA overnight at 4^{o}C. After fixation for 12-14hrs (10 hrs for pNR1 staining), the brain was dissected and cryoprotected in 25% sucrose before sectioning. Serial 20 µm thick coronal sections of the entire telencephalon were collected onto lysine-coated slides and stored at -40^{o}C till further processing. Sections were allowed to dry for 2 hrs and washed with 0.5% Triton-X100 in 1X PBS (PBST) 3 times for 10 min each. Sections were then blocked using 5%BSA-PBST for 1hr prior to addition of primary antibody and incubated overnight at 4^{o}C. The next day, washes were repeated, and sections were blocked in 5%BSA/ 5% HIGS for 1 hr. A secondary antibody was added and incubated at RT for 2.5 hrs in the dark. Sections were washed three times and mounted in mounting media (N propyl gallate, 70% glycerol in 1XPBS) containing DAPI. The pERK positive neurons in Dm were counted manually under the Zeiss Apotome microscope. The total number of PERK positive cells in the 400µm region of Dm - 250 µm before AC, 100 µm spanning AC and 50 µm caudal to AC was considered for the analysis. The mean pNR1 intensity was calculated from the 350 µm region of Dm - 250 µm before AC, 100 µm spanning AC.

The representative images were acquired using Leica SP8 confocal microscope. ImageJ was used to adjust the size, contrast, and brightness of the micrographs. Inkscape (ver. 0.91) was used to prepare the panels and diagrammatic representations.

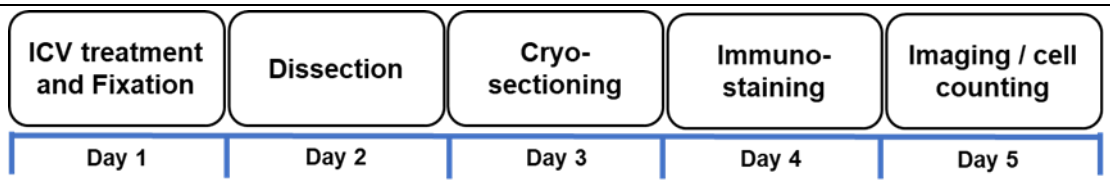


Figure 2. 1 Schematic for the immunohistochemistry protocol

2.5 Quantification of feeding drive- feeding behavioural protocol

As represented in Fig. 2.2, fish were isolated from home tanks and habituated to the experimental tank for three days. During this period, the fish were singly housed and were fed food pellets for 1 hour in the recording chamber. From the second day, fish were habituated to experimental conditions like recording setup, handling stress, and insoluble, floating food pellets. On the fourth day, the fish were habituated to injection stress by performing a saline (0.9% NaCl) injection followed by a mock injection the next day. Fish were observed on all days of the protocol for any anxious/abnormal behaviour. Any fish that exhibited these signs was excluded from the study. On the day of the experiment, fish were anesthetized and ICV injected with appropriate drugs. Following 15-30 mins of incubation and recovery period, the food pellets (~10-15) were added to the tank. The behaviour of the fish was recorded using a video recorder (Sony Handycam) for an hour. The number of biting attempts was counted manually from the recorded video and plotted as the number of cumulative biting attempts in 60 mins. In all the experiments, animals that were food-deprived for three days are referred to as 'starved,' and those that received food as per regular feeding schedule, and used for experiments within one hour of feeding, are referred to as 'fed.'

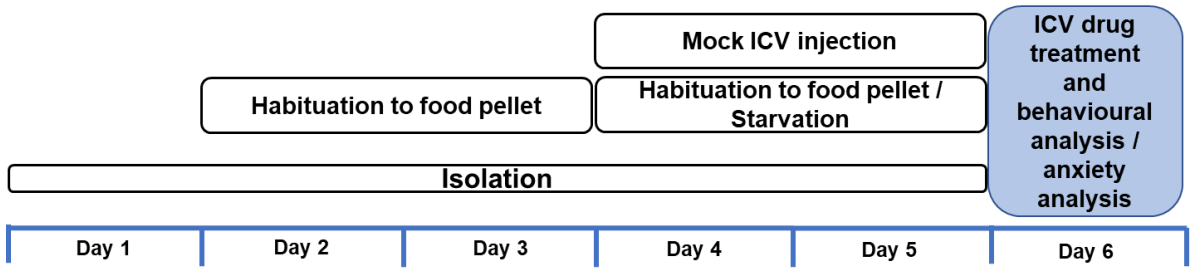


Figure 2. 1 Schematic for the feeding behavioural protocol

2.6 Calcium imaging

As shown in Fig 2.3 and 2.4, adult Tg(NeuroD:GcAMP6f) fish were injected with a pahrmacological agent via the ICV route. After 15 min, the fish were anesthetized in ice-cold HEPES-based Ringer's solution (NaCl, 134 mM; MgCl2, 1.2 mM; CaCl2, 2.1 mM; KCl, 2.9 mM; HEPES, 10 mM; Sucrose/glucose, 10 mM, pH 7.2) bubbled with 100% O_2 and craniotomised. Intact telencephalon/ whole brain was dissected and mounted in 4% low-melt agarose (in 50%Ringers) onto the RC-26 GLP chamber from Warner Instruments. The imaging chamber was mounted onto an upright Leica SP8 MP and continuously perfused with HEPES-based Ringer's solution via VC 8 perfusion system (Warner Instruments) triggered using recording software (Leica). The activity imaging was performed in resonant bi-directional scanning mode on Leica SP8 MP with 25x water-immersion objective (N.A 0.95) and binned to 512x300 pixels at 9.8 Hz using the confocal laser (488nm) and PMT detector.

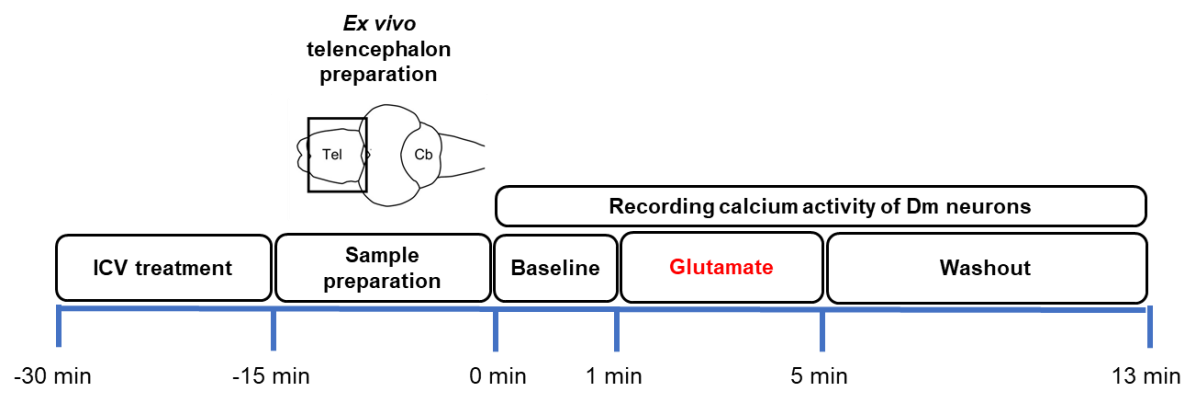


Figure 2. 2 The experimental time line for calcium imaging in *ex-vivo* telencephalon preparation

For the calcium imaging in *ex-vivo* telencephalon preparation, the basal activity of the Dm region (around 250 µm rostral to and 50 µm on the AC) was calculated by imaging for 1 min without any stimulation. After baseline recording, 4 mins of glutamate stimulation was given, followed by 8 mins of washout.

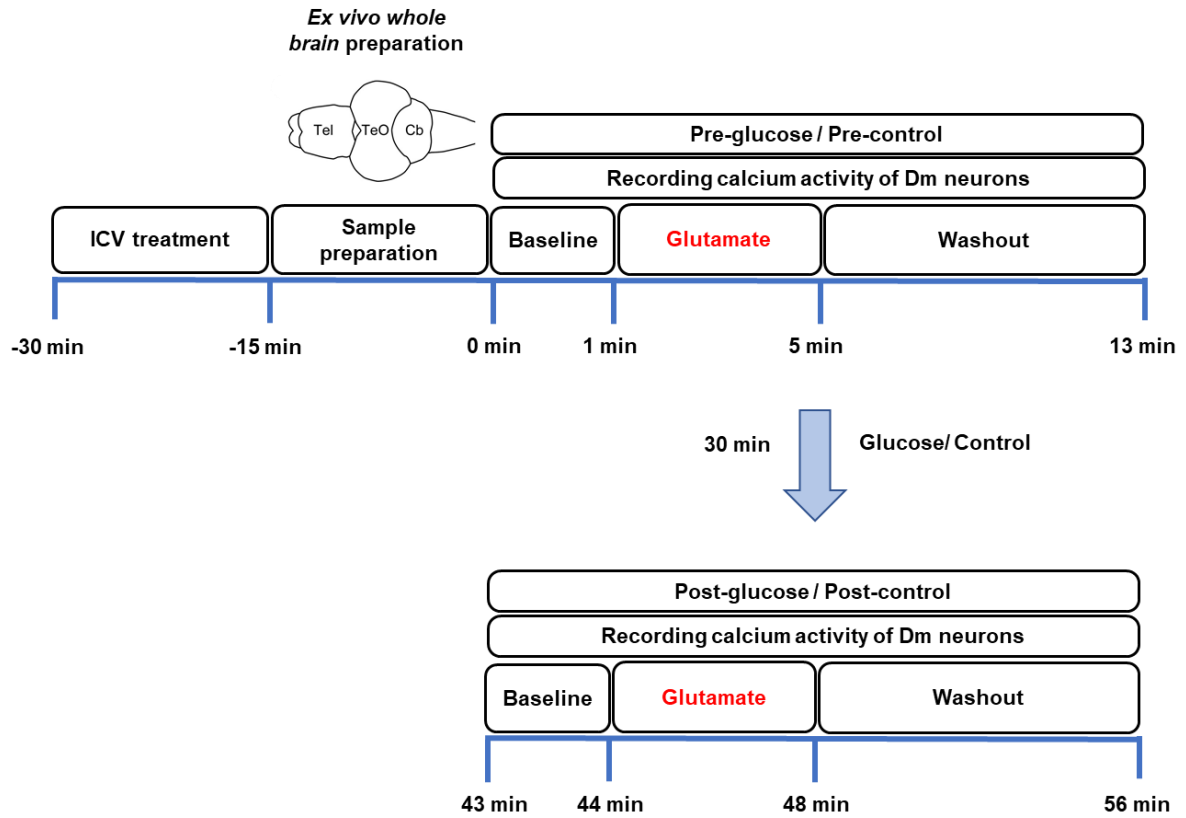


Figure 2. 3 The experimental time line for calcium imaging in *ex-vivo* whole preparation

As shown in fig 2.4, for the calcium imaging in *ex-vivo* whole-brain preparation, the response of Dm neurons to glutamate was recorded twice. First, the response of Dm neurons to glutamate in these starvation mimicked conditions was recorded. Next, the same brain preparation was treated with glucose for 30 mins to mimic satiety conditions, and the response of Dm neurons to the same dose of glutamate was re-evaluated. For control experiments, the brain was continuously maintained starvation mimicked conditions, and the response was recorded twice.

2.7 Data Analysis

2.7.1 For IHC experiments:

The tissue slices were observed under Zeiss Apotome microscope (20x), and the number of p-ERK positive cells in Dm were scored manually. To avoid over-estimating cell count due to sectioning, the cell numbers were corrected using Abercrombie's method.

Average pNR1 intensity per cell in Dm was calculated using IMAGE J. For these experiments, the exposure time and other imaging conditions were kept constant for every sample processed.

Each neuron in Dm was selected using manual thresholding on the DAPI filter, and the mean intensity of pNR1 per neuron was calculated using IMAGEJ in-built algorithms.

2.7.2 For calcium imaging

Population-level calcium activity measurements in Dm were performed using IMAGEJ, where the average GCaMP6f fluorescence in Dm was calculated for each time point. The Dm was selected based on the landmarks and GCaMP expression pattern. F0 was obtained by calculating the average of fluorescence values in the baseline recording. The relative change in fluorescence (dF/F0), calculated using the formula F-F0/F0, where F stands for the fluorescence value at a given time and maximum response amplitude, i.e., max dF/F0, was calculated using Microsoft Excel. The total response elicited was calculated using the area under the curve (AUC) function in Prism 8.

2.7.3 For feeding behaviour assay

Videos were analyzed manually at 1.5x-2x speed, and the number of biting attempts was scored. The data was recorded in bins of 15 mins, and cumulative biting attempts were plotted using GraphPad Prism software.

2.8 Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test in GraphPad Prism 8. Behavioural and immunohistochemical data analyses and calcium imaging data analyses were performed using t-test with Welch's correction (for single comparison) and One or Two-way ANOVA, with Bonferroni's posthoc analysis (for multiple comparisons). All values are expressed as mean ± SEM of the group, and differences were considered significant at $p<0.05$. Graphs were plotted using the GraphPad Prism 8.0 statistical software.

3 Chapter 3: Antagonistic modulatory activities of CART and NPY regulate feeding drive in zebrafish

Background

Regulation of food intake according to changing energy demands of the body is critical for the survival of an organism. Therefore, the underlying neural networks, though hardwired, need to be flexible enough to modulate feeding depending on the body's energy needs. The neuropeptidergic signaling provides functional flexibility to the feeding circuit elements, allowing the modulation of food intake according to the body's energy requirements (Jékely et al., 2018). Multiple such neuropeptides have been identified in vertebrates. Their expression profiles and role in the regulation of feeding have been extensively studied in rodents (Andermann and Lowell, 2017). However, molecular, and network-level mechanisms of peptidergic modulation underlying the regulation of food intake are poorly understood.

CART is one such anorexic neuropeptide widely expressed in vertebrates, including mammals and fish. Early studies revealed that CART acts as a potent anorexic factor and endogenous satiety factor in rodents (Kristensen et al., 1998; Larsen et al., 2000). The function of CART seems to be conserved within non-mammalian vertebrates like teleosts (Volkoff et al., 2005). The CART expression in the entopeduncular nucleus (EN) and the hypothalamus, well-known energy-sensing regions in teleosts (Otero-Rodiño et al., 2018; Aguilar et al., 2011; Delgado et al., 2017; Lin et al., 2000b), is regulated by nutritional status. In response to glucose treatment, CART levels were upregulated in EN of catfish, zebrafish (Subhedar et al., 2011; Mukherjee et al., 2012), and the hypothalamus of rainbow trout. In contrast, starvation resulted in the downregulation of CART transcripts in EN and hypothalamus of zebrafish (Akash et al., 2014). Furthermore, intracerebroventricular injection of CART peptide inhibited food intake in goldfish (Volkoff and Peter, 2000).

NPY is another feeding-related neuropeptide that acts as a potent orexigenic agent in vertebrates. NPY is often expressed with CART in energy sensing regions of the brain like the hypothalamus and has been shown to interact with CART to exert opposing effects on food intake (Rogge et al., 2008). Consistent with mammals, NPY expression is upregulated in the hypothalamus under the starved state in zebrafish (Yokobori et al., 2012). Moreover, intracerebroventricular (ICV) administration of NPY stimulates food intake in goldfish (Narnaware and Peter, 2001) and zebrafish (Yokobori et al., 2012).

These reports suggest the endogenous levels of CART and NPY are increased in response to satiety (ICV treatment with glucose) and hunger condition, respectively, in energy sensing regions of the fish brain. Moreover, exogenous administration of these peptides is sufficient to influence food intake in teleosts.

Despite the well-conserved role of CART and NPY as potent anorexic and orexic agents in vertebrates, the mechanisms of modulatory action are not known. The complexity of the neural system in higher vertebrates like mammals limits the understanding of mechanistic principles operating at the molecular and cellular levels.

This study aimed to elucidate circuit-level mechanisms of modulatory action of CART and NPY underlying the regulation of feeding drive using a non-mammalian vertebrate, zebrafish, as a model system.

The primary objectives of the study are to:

1. Establish the role of CART and NPY in the regulation of feeding drive.

2. Investigate biochemical mechanisms of modulatory activities of these peptides that lead to energy state-dependent regulation of food intake.

3. Elucidate cellular effects of CART and NPY mediated neuromodulation that can be correlated with alteration in feeding drive.

Results

Previous studies in our lab have demonstrated that CART and NPY expression changes according to internal energy states in the energy-sensing regions: the hypothalamus and entopeduncular nucleus (EN) of the zebrafish brain.

CART transcripts levels are shown to be downregulated in the starved state (Akash et al.,2014), and peptide expression is significantly increased in these energy-sensing regions upon central administration of glucose (Mukherjee et al., 2012) in zebrafish. In contrast, NPY transcript levels in the hypothalamus are shown to be upregulated in starved conditions (Yokobori et al., 2012), and peptide levels are increased in EN and hypothalamus under energy-deficient conditions (Kaniganti, 2014) in the zebrafish brain.

Based on these findings, we hypothesized that CART and NPY signaling is involved in regulating feeding under satiety and hunger conditions, respectively, in zebrafish.

To probe the role of CART and NPY signaling in regulating feeding drive in zebrafish, we performed pharmacological interventions to block endogenous activities of these peptides and tested their effects on food intake. The pharmacological agents were administered via the ICV route in the ventricular space above the brain, and the resultant impact on the feeding drive was monitored. ICV administration was performed to restrict the manipulation centrally and avoid any peripheral effects.

Quantification of feeding drive in zebrafish

To quantify the feeding drive in adult zebrafish, we have developed a behavioural paradigm (Fig. 3.1A), described in detail in the material and methods chapter. In the behavioural experiment, we count the number of biting attempts made by the fish upon delivery of insoluble, floating, pellet food. The 'cumulative number of biting attempts' made by the fish throughout 60 mins is used as a measure of feeding drive. We demonstrated that starved fish made a significantly higher number of cumulative biting attempts (794±57.46) as compared to recently fed fish (173±58.39) (Fig. 3.1 B). However, there was no difference in locomotion or anxiety-like behaviours between the two groups of animals (Fig 3.2.2 and unpublished data by Aditi Maduskar). Thus, this assay can be reliably used to quantify the feeding drive in the adult zebrafish.

3.1 CART regulates satiety behaviour in zebrafish

Since CART expression is upregulated under satiety mimicked conditions in energy sensing regions, we tested if endogenous CART signaling is required to reduce feeding drive in zebrafish. To upregulate endogenous CART levels in the brain, we administered glucose (satiety mimic) via the ICV route in a starved fish (Mukherjee et al., 2012, Kaniganti, 2014). The endogenous CART signaling was inhibited by immunoneutralizing CART action using a CART antibody. The antibody used has been shown to block the CART receptor activation in rats (Choudhary et al., 2018; Upadhya et al., 2013) and in cultured GH3 cells. (Refer to Fig 4.1.2). We found that ICV administration of CART antibody in glucose-treated starved fish abolished the satiety-inducing effect of glucose. A two-way ANOVA was performed to evaluate the effect of CART Ab and glucose treatments in starved fish over time on feeding drive of the starved fish. There was a statistically significant interaction between these treatments and time on feeding drive ($F(9, 48) = 5.79$, $p < 0.0001$). Therefore, the effect of each treatment was analyzed at each time point using Bonferroni's posthoc analysis. Our analysis showed that CART Ab reversed glucose induced satiety effect as indicated by the significant increase in the cumulative number of biting attempts (765.6 ± 81.58 for CART Ab + glucose Vs. 329.4±46.11 for glucose). Whereas treatment with CART antibody alone did not affect the feeding drive in the starved fish (658.4±67.13 for CART Ab treated starved fish Vs. 651.4± 31.40 for starved controls) (Fig. 3.1 C). These data indicated that increased endogenous CART signaling in satiety conditions is required for reducing feeding drive in zebrafish.

Further, we tested if exogenously added CART can influence feeding drive in starved conditions where endogenous CART levels are low. Results showed that ICV administration of Rat-CART (55-102) was sufficient to induce anorexia in starved fish (Fig. 3.1 D).

This data demonstrated that CART is a major regulator of satiety behaviour in adult zebrafish.

3.2 NPY regulates hunger behaviour in zebrafish

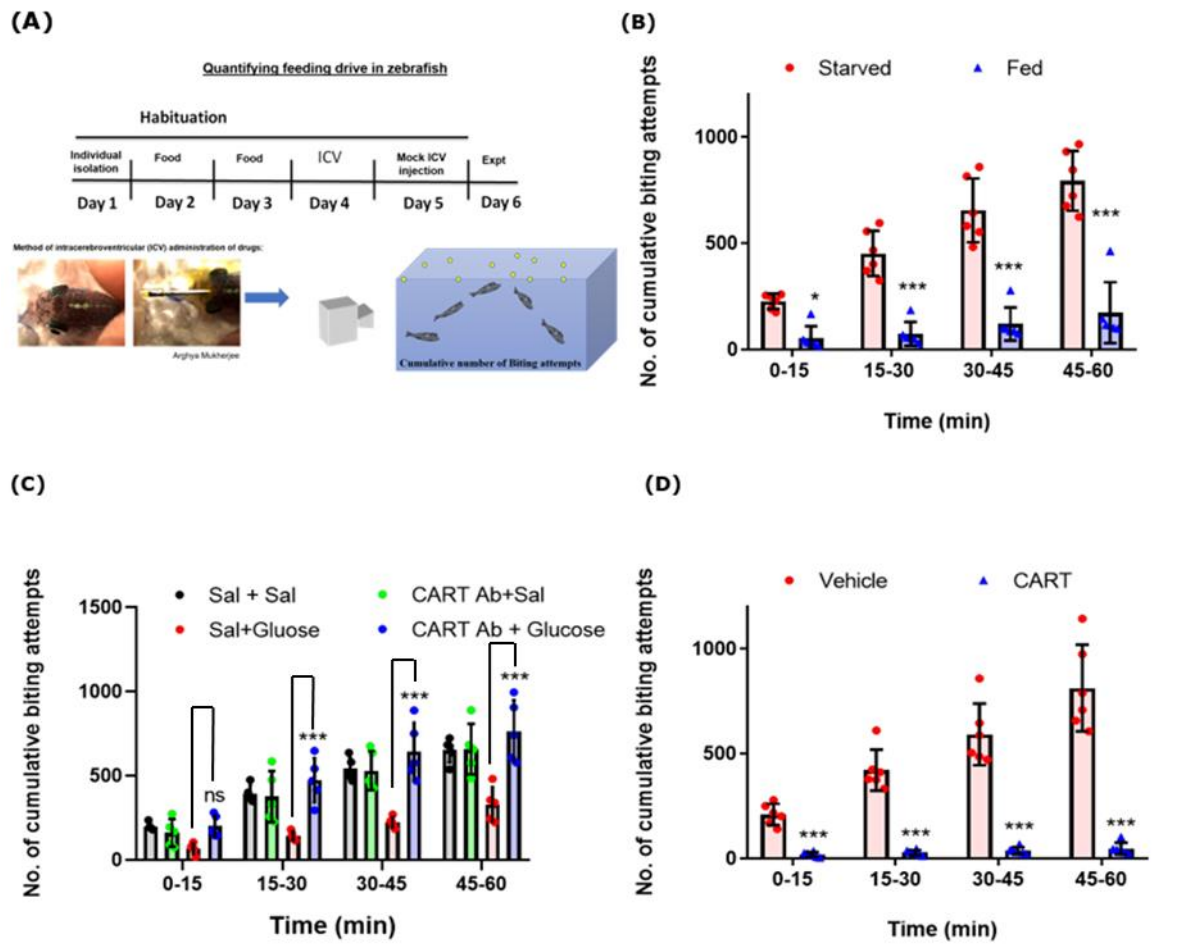
In contrast to CART, NPY expression is shown to be upregulated under starved conditions in energy sensing regions of the zebrafish brain. Hence, we tested if endogenous NPY signaling is required to regulate feeding drive under hunger conditions.

NPY is known to act via the NPY-Y1 receptor to regulate feeding in zebrafish (Yokobori et al., 2012). Therefore, we blocked endogenous NPY activity in starved fish using a specific Y1 receptor antagonist, BIBP 3226. Behavioural results showed that abrogating endogenous NPY

signaling by BIBP 3226 significantly decreased the feeding drive in starved fish (144.7±21.57 for BIBP treated starved fish Vs. 789±69.46 for Starved controls) (Fig. 3.2.1 A), indicating that increased endogenous NPY signaling under starved conditions is required for increasing feeding drive in fish.

Next, we tested if exogenous administration of NPY can influence feeding drive in fed fish wherein endogenous NPY levels are low. We found that ICV administration of NPY led to a significant increase in the number of biting attempts in the fed fish (983.7±75.84 for NPY treated fed fish Vs. 338.8±70.4 for fed controls) (Fig. 3.2.1 B).

These data demonstrated that NPY is a major regulator of hunger behaviour in adult zebrafish.

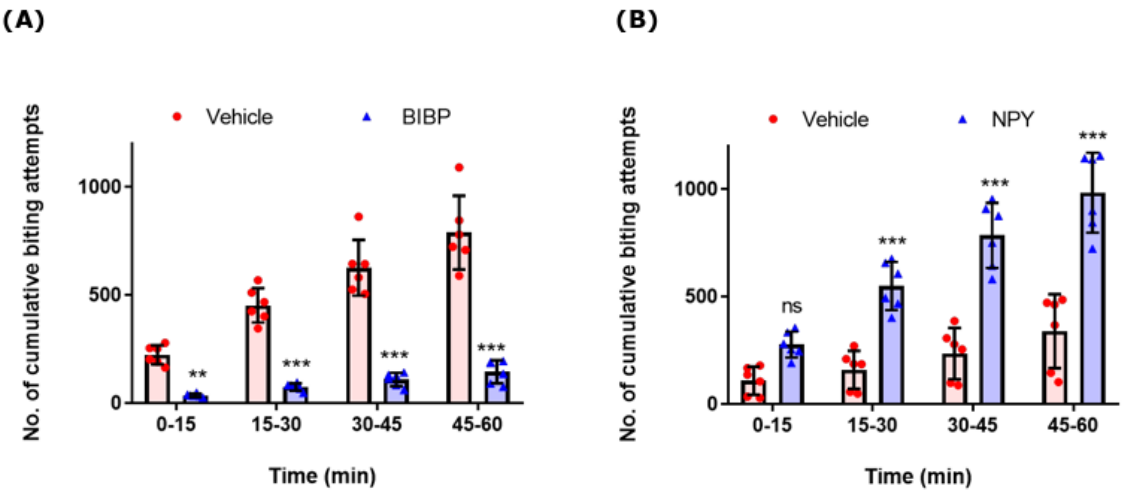


3.1CART regulates satiety behaviour in zebrafish

(A) Schematic of feeding behavioural assay for quantifying feeding drive (details in the methods section) **(B)** Feeding drive of fed animals is higher as compared to starved ones (n=6 animals per group, ***p<0.001) Data are sown as mean +/- SEM and means were compared using unpaired t-test with Welch's correction **(C)** Treatment with CART antibody reduces glucose-induced anorexia in starved fish (n=5 animals per group, ***p<0.001) Data are represented as cumulative biting attempts in 15 min bins over 1 hour and compared using Two-way ANOVA, with Bonferroni's posthoc analysis (error bars represent +/-SEM) **(D)**Treatment with CART peptide induces anorexia in starved fish (n=6 animals per group, ***p<0.001) Data are shown as mean +/- SEM and means were compared using unpaired t-test with Welch's correction.

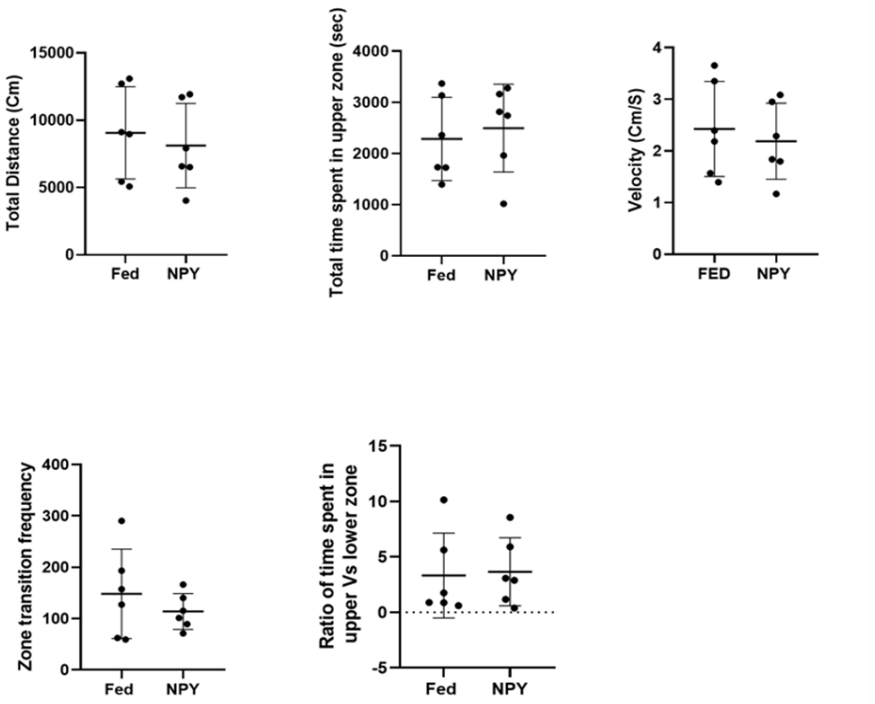
In summary, we found that endogenous CART and NPY signaling is required to regulate food intake under satiety and hunger conditions, respectively. Moreover, exogenous administration of these peptides is sufficient to modulate the feeding drive in zebrafish. These data suggested that the modulatory activities of these peptides are crucial for the regulation of feeding in zebrafish.

Since CART and NPY are also known to be involved in regulation anxiety behaviour in rodents, we tested if central administration of these peptides induces any anxiety like behviours in zebrafish using a novel tank diving test as described in Assad et al. We evaluated behavioural paradigms like time spent in the upper zone, the ratio of time spent in the upper Vs. the lower zone, zone transition frequency (upper to lower), total distance covered, and average velocity and demonstrated that values do not significantly differ between NPY Vs. fed fish (Fig 3.2.2) and CART Vs. starved fish (unpublished data by Aditi Maduskar) validating that the modulation of food intake upon central administration of CART and NPY are solely dependent on the changes in feeding drive of the fish.



3.2. 1 NPY regulates hunger behvaiour in zebrafish

(A) Blocking NPY signaling reduces feeding drive in starved fish (n=6 animals per group, **p<0.01, ***p<0.001) **(B)** Treatment with NPY induced orexia in fed fish. (n=6 animals per group, ***p<0.001) Data are shown as mean +/- SEM, and means were compared using unpaired t-test with Welch's correction**.**

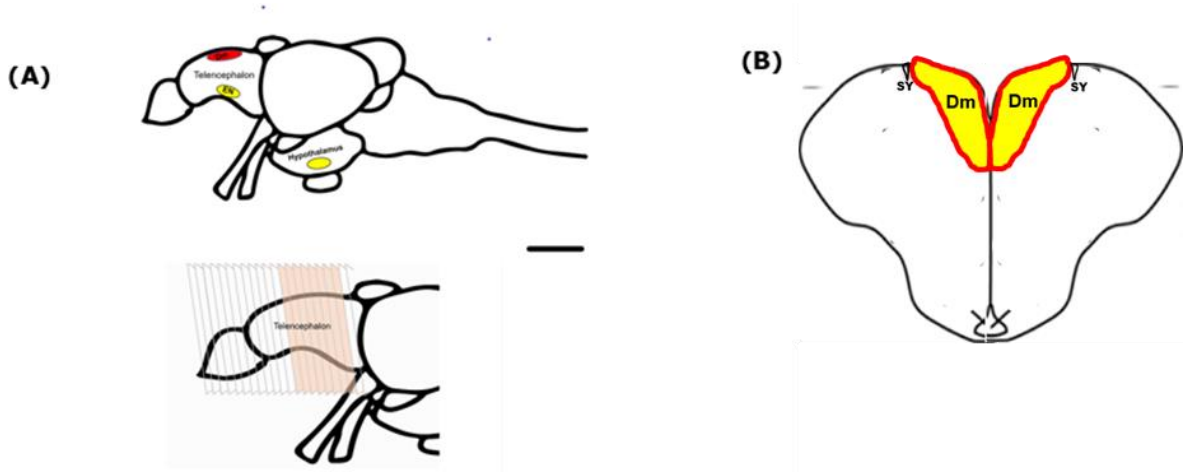


3.2. 2 Behavioural paradigms to assess anxiety

Behavioural paradigms used to assess anxiety in fed fish treated with NPY Vs. vehicle (n=6 animals per group). Data are shown as mean +/- SEM, and means were compared using unpaired t-test with Welch's correction.

3.3 CART and NPY differentially modulate the activity of a specific region of the Dorsomedial telencephalon

To probe the molecular mechanisms of the peptidergic actions, we sought to identify neural substrates for CART and NPY action that underlie the regulation of feeding behaviour in zebrafish. To identify the brain regions that respond to CART, the activity-based screening was performed previously in the lab using immunohistochemical analysis of pERK, a well-established neural activity marker in zebrafish (Randlett et al., 2015b). The screen identified a specific region of dorsomedial telencephalon (Dm) which showed significantly higher number of pERK positive neurons in response to exogenous application of CART in a starved fish (282.5±22 for CART Vs. 126.7±13.4 for vehicle) (Kaniganti, 2014) and (Fig 3.3.2A-C). The region that showed differential activation is a subregion of dorsomedial telencephalon that spans 400 µm along the rostrocaudal axis. The area covers 250 µm of dorsomedial telencephalon rostral to the anterior commissure (AC), around 100 µm on the AC, and 50 µm caudal to AC (Fig3.3.1 A). This region is the focus of this study and is referred to as Dm in the entire thesis.



3.3. 1 The specific region of dorsomedial telencephalon that shows differential activation upon CART and NPY treatment

(A) Schematic overview of adult zebrafish brain with the shaded area shows region differentially activated by CART and NPY. The region of our interest spans 400 µm across the anteroposterior axis with the last 100 µm caudal to the anterior commissure. [Scale bar 500 µm] **(B)** Coronal section of telencephalon at anterior commissure with yellow shaded area depicting dorsomedial telencephalon. SY: sulcus ypsiloniformis anatomical landmark for lateral spread of Dm.

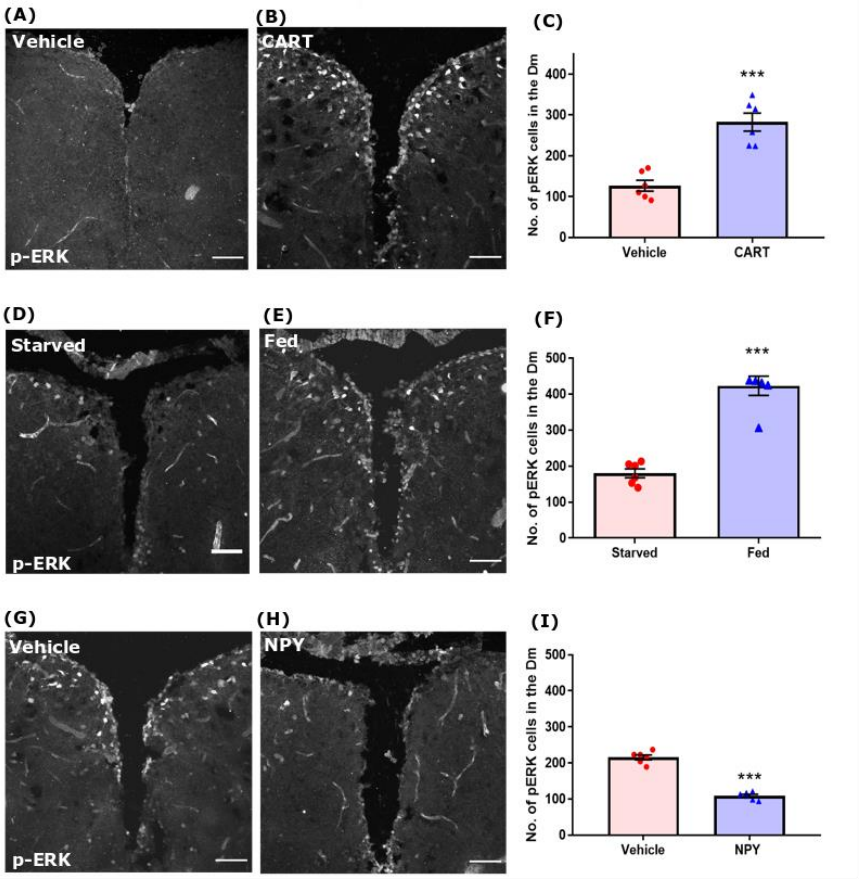
Further, we tested if this region responds to changes in physiological energy states by comparing the activity patterns of Dm in alternating energy states. Recently fed fish showed increased pERK positive neurons in Dm compared to starved fish (423.2±26.61 for fed Vs. 180.0±12.42 for starved), demonstrating that Dm is differentially activated in satiety and hunger states (Fig 3.3.2D-F).

We reasoned that increased activation of Dm in fed conditions could be attributed to increased CART signaling. In zebrafish, CART expression was shown to be upregulated under satiety mimicked conditions fish (Mukherjee et al., 2012; Kaniganti, 2014), and exogenous CART application anorexia (Fig 3.3.2A-C) which is indicative of increased CART signaling under energy-rich induced (fed) conditions.

Since we have earlier demonstrated that NPY is necessary for regulating hunger behaviour in zebrafish and its expression is upregulated in starved conditions (Fig 3.2.1; Kaniganti, 2014), we questioned whether increased NPY signaling is responsible for the decrease in neuronal activity of Dm. To test this possibility, we injected NPY via the ICV route in fed fish and compared the changes in the activity of Dm. Since endogenous NPY levels in the fed fish are low (Yokobori et al., 2012; Kaniganti, 2014), it would serve as a control for the experiment. Results showed that NPY administration decreased the number of pERK positive neurons in Dm compared to the control fed animals (108.7±4.35 for NPY treated fed fish Vs. 215.3±6.85

for fed controls) (Fig 3.3.2G-I), demonstrating that NPY signaling at Dm leads to decreased neuronal activation.

In summary, these findings demonstrated that CART and NPY signaling antagonistically regulate the activity of Dm neurons, resulting in the differential activation of Dm under alternating energy states- Satiety and Hunger, respectively.



3.3. 2 CART and NPY differentially modulate the activity of a specific region of the Dorsomedial telencephalon

(A-C) Treatment with CART leads to a significant increase in activation of Dm Representative transverse sections showing the immunoreactivity of p-ERK (p-ERK –ir) in starved fish treated with either vehicle (**A)**or CART **(B)** with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(C)**
(D-F) Dm shows increased activation in fed fish as compared to starved fish. Representative transverse sections showing the immunoreactivity of p-ERK (p-ERK –ir) in starved fish **(D)** and fed fish **(E)** with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(F).**
(G-I) Treatment with NPY leads to decreased activation of Dm. Representative transverse sections showing the immunoreactivity of p-ERK (p-ERK –ir) in fed fish treated with either vehicle **(G)** or NPY **(H)** with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(I)**. (n=6 animals per group; *, $p< 0.05$). Data are shown as mean +/- SEM, and means were compared using unpaired t-test with Welch's correction. [Scale bar 50 µm]

Previous tracing studies from that lab have indicated that Dm is directly connected with energy sensing regions of the zebrafish brain- EN and Hypothalamus. CART and NPY positive

neurons in EN and the hypothalamus send peptidergic inputs to Dm (Kaniganti, 2014), possibly leading to modulation of Dm activity according to internal energy conditions.

To elucidate molecular mechanisms of peptidergic actions underlying the regulation of feeding drive, we performed pharmacological interventions coupled with behavioural and immunohistochemical analysis. Different pharmacological agents were administered via the ICV route, and after 15 mins, the effects were tested on the feeding drive and Dm activity to unravel the biochemical signaling activated by CART and NPY.

3.4 Mechanisms of CART signaling

3.4.1 CART induced anorexia and increased activation of Dm requires NMDAR function

A research group studying the role of CART in nociceptive transmission (Chiu et al., 2009, Lin et al., 2005) and a previous report from our lab describing the function of CART in processing innate fear responses (Rale et al., 2017) identified the involvement of NMDAR in CART induced signaling in rodents. Since NMDARs have also been implicated in feeding regulation in different vertebrate systems (Burns and Ritter, 1997; Guard et al., 2009; Haberny and Carr, 2005; Lee and Stanley, 2005; Liu et al., 2012; Neyens et al., 2020; Resch et al., 2014; Wright et al., 2011; Wu et al., 2013), we tested the involvement of NMDA receptors in mediating CART's anorexic action. A two-way ANOVA was performed to evaluate the effect of blocking NMDAR activity in CART treated starved fish on feeding drive over time. There was a statistically significant interaction between drug treatments and time on feeding drive ($F(9, 60) = 19.36$, $p < 0.0001$). Therefore, the effect of each treatment was analyzed at each time point using Bonferroni's posthoc analysis. For these experiments, we used starved fish where endogenous CART levels are low. Exogenous application of CART in starved fish induced extreme anorexia with a drastic decrease in the number of cumulative biting attempts (812.8± 84.38 for starved controls Vs. 47.33±11.40 for CART). Blocking NMDAR signaling using AP5 (competitive NMDAR antagonist) in CART-treated starved fish allowed us to assess if NMDAR is involved in mediating CART-induced anorexia. We found that pretreatment of AP5 along with CART in starved fish alleviated CART induce anorexia. (488.7±42.03 for AP5+CART Vs. 47.33±11.40 for CART). However, application of antagonist AP5 alone showed no significant difference in biting attempts as compared to vehicle controls (845±89.82

for AP5 treated starved fish Vs. 812.8± 84.38 for starved controls), suggesting that NMDAR signaling is required for CART induced anorexia (Fig 3.4A)

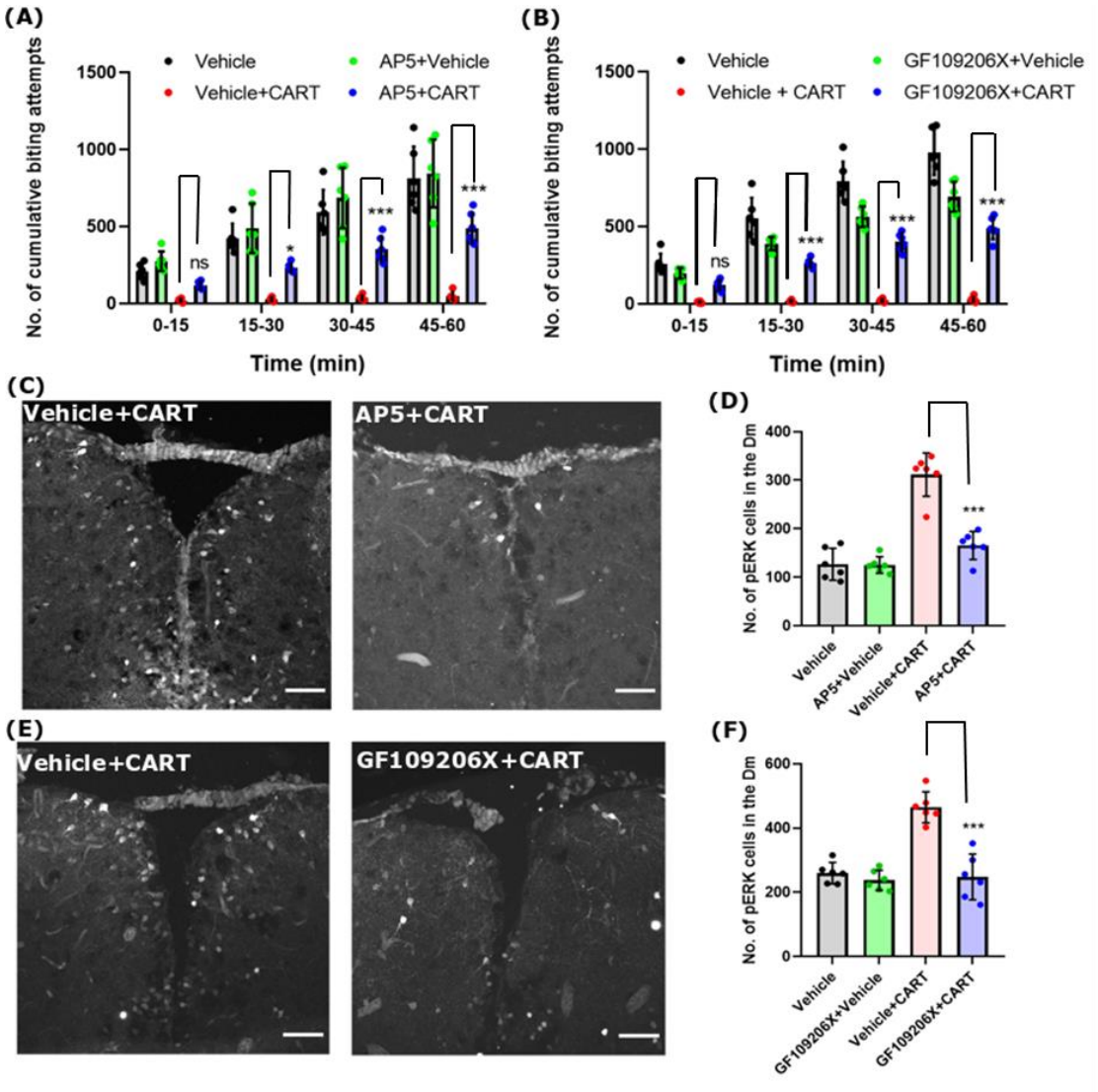
Next, we tested if NMDAR antagonism also influences the CART-induced activation of Dm neurons. Starved fish where endogenous CART levels are low (Akash et al.,2014) were used for these experiments. Exogenous CART administration in starved fish led to a significant increase in the the number of pERK positive neurons in Dm (311.5±18.17 for CART Vs. 126.7±13.40 for starved controls). To test if NMDAR antagonism also inhibited CART mediated upregulation of pERK positive neurons in Dm, we co-administered AP5 with CART in starved fish via the ICV route. One way ANOVA ($F(3, 20) = 44.52$, $P<0.0001$) and post hoc Bonferroni test showed that blocking NMDAR activity reduced CART-induced increase in the number of pERK positive neurons in Dm (165.5±11.85 for AP5+CART Vs. 311.5±18.17 for CART), as seen from the representative micrographs in Fig 3.4C and the corresponding quantitation in Fig 3.4D. However, treatment with AP5 alone did not affect the basal number of pERK positive neurons in Dm in starved fish (125.2±6.96 for AP5 treatment in starved fish Vs. 126.7±13.40 for starved controls), suggesting that NMDAR activation is required for CART induced increased activation of Dm neurons.

Together these data demonstrated that NMDAR activity is involved in mediating CART's modulatory effect on the feeding drive and the activity of Dm neurons.

3.4.2 CART induced anorexia and increased activation of Dm requires Protein Kinase C activity

NMDAR activity is modulated by different kinase/ phosphatase systems (Chatterjea et al., 2010a, 2010b; Chen and Roche, 2007; Chen et al., 2006), including Protein kinase C (PKC)(Chen and Roche, 2007; Huang, 1993; Lau et al., 2009). PKC has also been identified as a downstream effector of CART signaling (Chiu et al., 2009). Therefore, we tested if CART-induced anorexia requires PKC activation. For these sets of experiments, GF109206X, a selective PKC inhibitor, was administered via the ICV route to inhibit PKC activity in CART treated starved fish. The effects of inhibition of PKC activity over time was analysed by two-way ANOVA. There was a statistically significant interaction between drug treatments and time on feeding drive ($F(9, 60) = 52.16$, $p < 0.0001$). Therefore, the effect of each treatment was analyzed at each time point using Bonferroni's posthoc analysis. In behavioural experiments, co-treatment of GF109206X with CART rescued CART induced anorexia (489.8± 29.38 for GF109206X+CART Vs. 29.83±6.76 for CART), while treatment

with GF109206X alone had no significant effect on the feeding drive as compared to the starved control animals (693.7±39.52 for GF109206X treated starved fish Vs. 978.8±59.26 for starved controls) (Fig 3.4B). These experiments suggest that PKC activity is involved in the regulation of CART induced anorexia.



3.4 CART induced anorexia and p-ERK upregulation at Dm requires NMDAR and PKC activity

Treatment with **(A)** competitive NMDAR antagonist AP5 **(B)** selective PKC inhibitor GF109206X abrogates CART-induced anorexia. Data are represented as cumulative biting attempts in 15 min bins over 1 hour and compared using Two-way ANOVA with Bonferroni's posthoc analysis (n=6 animals per group; error bars represent +/-SEM; *p<0.05, ***, p< 0.001). **(C-D)** CART-induced increased activation of Dm requires NMDAR activity. Representative transverse sections showing the immunoreactivity of p-ERK (p-ERK –ir) in starved fish treated with either vehicle or AP5 before CART treatment **(C)** with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(D)**.
(E-F) CART-induced increased activation of Dm requires PKC activity. Representative transverse sections showing p-ERK (p-ERK –ir) immunoreactivity in starved fish treated with either vehicle or GF109206X prior to CART treatment. **(E)**with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(F)** (n=6 animals per group; ***p< 0.001) Data are shown as mean +/- SEM and compared using One-way ANOVA, with Bonferroni's posthoc analysis. [Scale bar 50 µm]

We next tested if the increase in Dm neuronal activity following treatment with CART also requires PKC activity. One way ANOVA ($F(3, 20) = 29.94$, $P<0.0001$) and post hoc Bonferroni test showed that co-injection of GF109206X with CART in starved fish suppressed CART-induced activation of Dm neurons. (247.7±29.03 for GF109206X+CART Vs. 464.5±19.62 for CART) as illustrated in the representative micrographs in Fig 3.4E and the corresponding quantitation in Fig 3.4F. However, PKC inhibitor alone did not affect Dm activity compared to starved control animals (237.2±12.85 GF109206X treated starved fish Vs. 259.5±13.74 for starved controls) (Fig 3.4F), suggesting that PKC activation is required for CART induced increased activity of Dm neurons.

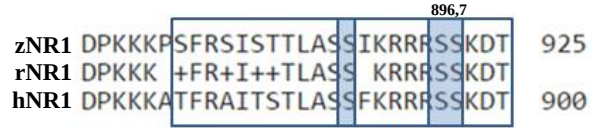
In summary, these data demonstrated that PKC signaling mediates CART's modulatory effect on feeding drive and Dm activity. Another parallel study in the lab has identified the involvement of PKA in CART's anorexic action. (Unpublished work by Aditi Maduskar).

These data, therefore, suggested that CART signaling is mediated via activation of PKA, PKC and results in decreased feeding drive and increased activity of Dm neurons.

Next, we evaluated if PKC and PKA influence NMDAR activity to mediate CART-induced activation of Dm neurons.

3.5 CART signaling increases phosphorylation of NR1 subunit of NMDARs in Dm

PKA and PKC belong to a class of Serine/Threonine kinases known to phosphorylate the NR1 subunit of NMDARs at serine 897 and serine 896, respectively in rodents (Chen and Roche, 2007; Chiu et al., 2009). PKA-PKC mediated phosphorylation enhances NMDAR functioning and is often involved in the regulation of behavioural plasticity. (Zou et al., 2002, 2004). The serine residues on NR1 subunits that PKA and PKC phosphorylate are well conserved within zebrafish, rodents, and humans (Fig 3.5.1).



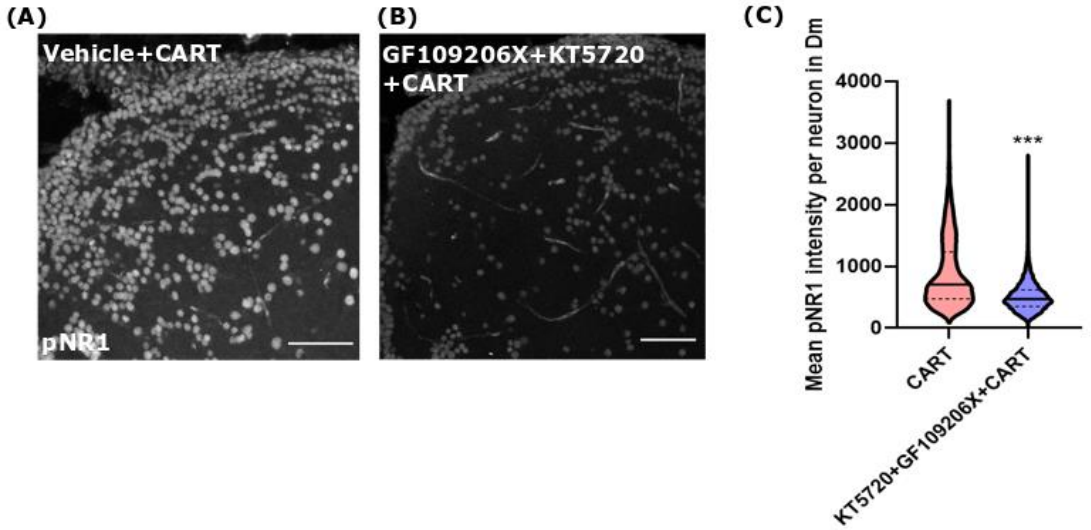
3.5.1 Multiple sequence alignment using BLAST for the NR1 subunit domain-containing serine residues phosphorylated by PKA and PKC are well conserved within zebrafish (z*NR1*) and rat (r*NR1*).

Hence, we predicted that PKA -PKC activation would lead to phosphorylation of serine 897 and 896 respectively on NR1 subunit to increase NMDAR function in zebrafish.

To assess if CART-mediated activation of PKC and PKA (unpublished data by Aditi Maduskar) leads to increased phosphorylation of NR1 subunit of NMDAR in the Dm, we performed immunofluorescence experiments to quantify levels of phospho-NR1 in Dm neurons directly. We tested the anti-phospho-NR1 Serine 896, Serine 897 antibodies in brain slices to detect PKC and PKA mediated phosphorylation of NR1 subunits, respectively. However, we could not observe any specific reaction with the anti-phosphoNR1 Serine 896 antibody in the zebrafish brain slices. Hence, only the anti-phosphoNR1 Serine 897 antibody was used for further studies.

The mean pNR1 fluorescence per neuron in Dm was compared between the starved fish treated with CART and the starved fish coinjected with KT5720 (selective PKA inhibitor), GF109206X (selective PKC inhibitor), and CART.

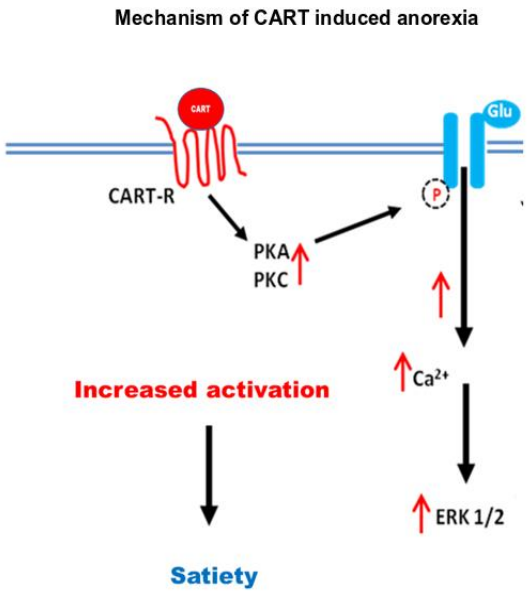
The results showed that inhibiting PKA-PKC activity in CART treated fish led to downregulation of CART-induced pNR1 levels in Dm (892.1±5 for CART Vs. 505.9±2.02 KT5720+ GF109206X+CART), as demonstrated in representative micrographs (fig 3.5.2A-B) and corresponding quantification (fig 3.5.2C).



3.5 .2 CART signaling increases phosphorylation of NR1 subunit of NMDARs in Dm

(A-C) CART treatment leads to increased levels of pNR1 in Dm. Representative micrographs showing PNR1 staining in the brains treated with either vehicle **(A)** or KT5720 and GF109206X **(B)** before CART treatment with corresponding quantification of mean pNR1 intensity per neuron across Dm **(C)** (n=12253 for vehicle +CART and n= 12015 for KT5720 + GF109206X+ CART) (N=3; ***p< 0.001) Data are shown as mean +/- SEM and means were compared using unpaired t-test with Welch's correction. [Scale bar 50 µm]

To sum up, we have demonstrated that CART signaling increases the phosphorylation of NMDARs in Dm via activating PKA and PKC that leads to increased activity of Dm neurons, and can be correlated with anorexic behavioural output.

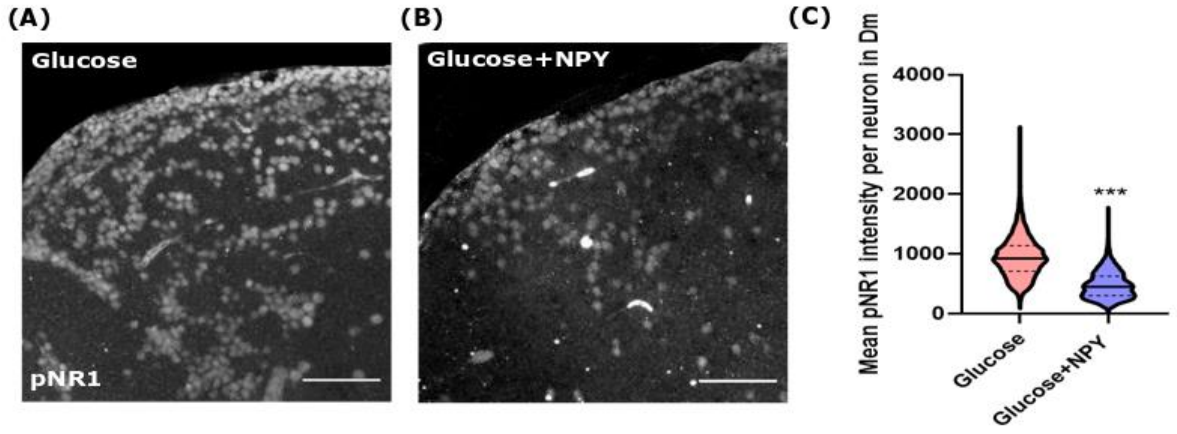


Summary of modulatory mechanisms of CART signaling that lead to anorexia and increased activation of Dm

3.6 NPY signaling decreases phosphorylation of NR1 subunit of NMDARs in Dm

As previously discussed, NPY and CART have opposing effects on food intake and Dm neuronal activity in zebrafish. We have demonstrated that the anorexic action of CART is mediated by the facilitation of Dm activity via increasing phosphorylation of NMDARs. Hence, we hypothesized that NPY opposes CART action by reducing phosphorylation of NR1 subunit of NMDARs in Dm. To test this hypothesis, we compared pNR1 levels in the Dm of glucose treated starved fish (satiety mimicked) with low endogenous NPY (Kaniganti et al., 2021) and glucose treated starved fish exogenously injected with NPY. Co-administration of NPY with glucose in starved fish significantly reduced the mean intensity of pNR1 per Dm neuron compared to glucose injected (satiety mimic) animals as shown in representative micrographs (Fig 3.6A and 3.6B) and corresponding quantification (944.3±2.98 for Glucose Vs. 478.2±2.03 for Glucose+NPY) (Fig 3.6C).

These results demonstrated that modulatory activity of NPY is mediated via reducing phosphorylation of NR1 subunit of NMDARs on Dm.



3.6 NPY signaling decreases phosphorylation of NR1 subunit of NMDARs in Dm

(A-C) NPY treatment leads to a reduction in pNR1 levels in Dm. Representative micrographs showing PNR1 staining in the brains treated with either vehicle **(A)** or NPY **(B)** before glucose treatment with corresponding quantification of mean pNR1 intensity per neuron across Dm **(C)** (n=12253 for vehicle+ glucose and n= 11901 for NPY + glucose) (N=3; ***p< 0.001) Data are shown as mean +/- SEM and means were compared using unpaired t-test with Welch's correction. [Scale bar 50 µm]

3.7 NPY induced orexia and reduction in Dm activation is exerted by activating Protein phosphatase 2b

Results in the previous section indicated that CART activates PKA and PKC to increase the phosphorylation of NMDARs. Hence, we hypothesized that NPY might antagonize CART signaling by opposing PKA and PKC activities, thereby reducing NR1 phosphorylation in Dm neurons.

The protein phosphatase calcineurin (also known as PP2b) is known to be activated by NPY (Leitermann et al., 2012). Calcineurin dephosphorylates the NR1 subunit of NMDA receptors at the same site where PKC phosphorylates them (Salter et al., 2009). Hence, we investigated if the orexic effect of NPY is mediated via the activation of calcineurin in Dm neurons.

First, we tested if calcineurin signaling is involved in the regulation of feeding drive under hunger conditions (where endogenous NPY activity is high) in zebrafish. Calcineurin inhibitor FK506 was administered in starved fish, and its effect was monitored on the feeding drive. Behavioural results demonstrated that treatment with FK 506 substantially reduced the feeding drive in the starved fish (528.1±123.4 for vehicle-treated starved Vs. 253.2±49.62 for FK506 treated starved) (Fig 3.7A), indicating that calcineurin signaling regulates feeding drive in starved fish.

To test if inhibiting calcineurin signaling also affects Dm activation in starved conditions, we injected FK506 in starved fish and evaluated its effect on the activity of Dm neurons. Treatment with FK506 significantly increased the number of pERK positive neurons in Dm as compared to starved fish as depicted in representative micrographs and corresponding quantification (216.5±43.03 for starved Vs. 731.8±92.06 for FK506) (Fig 3.7C-E). These experiments demonstrated that calcineurin signaling reduces the activation of Dm neurons in starved animals.

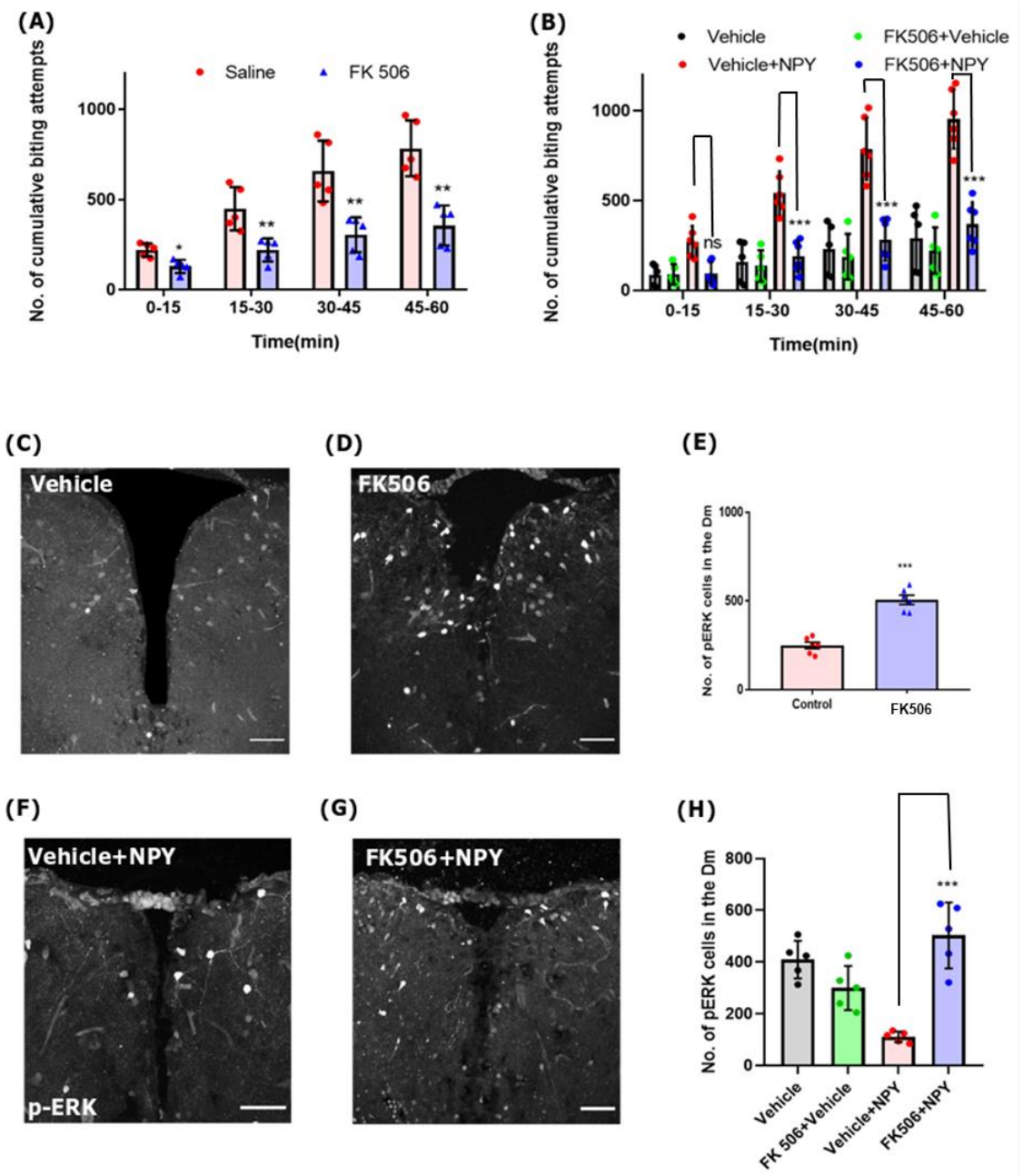
Collectively these experiments implicate calcineurin signaling in regulating hunger behaviour in zebrafish. Since we have shown that NPY is the major regulator of hunger behaviour in zebrafish, we speculated that NPY employs calcineurin to induce orexia and reduce activation of Dm.

Fed fish with low endogenous NPY levels (Kaniganti, 2014) exogenously injected NPY via the ICV route were used to test this hypothesis. Calcineurin signaling was blocked in these fish, and the feeding drive and Dm activity was evaluated.

In the behavioural experiments, a two-way ANOVA was performed to evaluate the effect of FK506 in NPY treated fed fish on feeding drive over time. There was a statistically significant interaction between drug treatments and time on feeding drive ($F(9, 72) = 4.572$, $p < 0.0001$). Therefore, the effect of each treatment was analyzed at each time point using Bonferroni's posthoc analysis. We found that the co-treatment of FK506 with NPY reversed NPY induced orexia in fed fish (368±50.97 for FK506+NPY Vs. 954±67.55 for NPY). However, the application of FK 506 alone did not significantly alter the number of biting attempts compared to control-fed animals (222±57.39 for FK506 treated fed fish Vs. 292.4±79.26 for vehicle-treated fed fish) (Fig 3.7B).

To test if inhibiting calcineurin activity interferes with NPY induced reduction of Dm activity, we injected FK506 along with NPY in fed fish. One way ANOVA ($F(3, 16) = 19.40$, $P<0.0001$) and post hoc Bonferroni test demonstrated that blocking calcineurin activity in NPY treated fish abrogated NPY induced reduction in pERK levels in Dm (503±56.97 for FK506+NPY Vs. 111±9.34 for NPY) as seen from the representative micrographs in Fig 3.7F&G and the corresponding quantitation in Fig 3.7H. Treatment with FK506 alone did not affect the basal number of pERK positive neurons in the Dm compared to fed fish (298.8±38.1 for FK506 treated fed fish Vs. 409.6±32.64 fed controls).

Collectively, these data indicate that NPY signaling activates calcineurin to induce orexia and reduce the activation of Dm neurons.



3.7 NPY induced orexia and reduction in Dm activation is exerted by activating Protein Phosphatase 2b

(A) Treatment with Calcineurin inhibitor FK506 decreases feeding drive in starved fish.(n=5 animals per group, **p< 0.05, **p< 0.01) means were compared using unpaired t-test with Welch's correction. **(C-E)** FK506 treatment in starved fish increased neuronal activation in Dm Representative transverse sections showing the immunoreactivity of p-ERK in starved fish treated with either vehicle **(C)** or FK506 **(D)** with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(E)** (n=5/6 animals per group; ***p< 0.001) Data are shown as mean +/- SEM and compared using unpaired t-test with Welch's correction. **(B)** Treatment with Calcineurin inhibitor FK506 alleviates NPY induced orexia. Data are represented as cumulative biting attempts in 15 min bins over 1 hour and compared using Two-way ANOVA with Bonferroni's posthoc analysis (n=5/6 animals per group; error bars represent +/-SEM; ***p< 0.001). **(F-H)** NPY induced reduction in Dm activation is mediated via activation of calcineurin signaling. Representative transverse sections showing the immunoreactivity of p-ERK in starved fish treated with either vehicle **(F)** or FK506 **(G)** prior to NPY treatment with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(H)**. (n=5/6 animals per group; **p< 0.01) Data are shown as mean +/- SEM and compared using one-way ANOVA, with Bonferroni's posthoc analysis [Scale bar 50 µm]

3.8 NPY induced orexia and reduction in Dm activation is exerted by inhibiting adenylyl cyclase activity

We have previously reported that NPY engages NPY Y1 receptors to induce orexia (Fig 3.2.1A). Since the Y1 receptor is an inhibitory G-protein receptor that inhibits adenylyl cyclase activity, Y1 receptor activation is expected to reduce cAMP levels and, consequently, PKA activation. The resultant reduction in PKA activity would lead to decreased phosphorylation of NR1 in Dm, which is in line with NPY mediated signaling at Dm (Fig 3.6). Hence, we hypothesized that NPY signaling is mediated via reducing cAMP levels.

First, we tested if downregulation of cAMP is required to regulate feeding drive under hunger conditions in zebrafish. Adenylyl cyclase activator Forskolin was administered via the ICV route in a starved fish, and the effect on the feeding drive was monitored. Behavioural results demonstrated that treatment with Forskolin reduced feeding drive in the starved fish (494.9±110.7 for starved Vs. 266.3±66.91 for Forskolin) (Fig 3.8A), indicating that downregulation of cAMP is required for the regulation of the feeding drive in starved fish.

To test if activation of adenylyl cyclase also affects Dm activation in starved conditions, we injected Forskolin in starved fish and looked at its effect on the activity of Dm neurons. Treatment with Forskolin significantly increased the number of pERK positive neurons in Dm compared to starved fish as depicted in representative micrographs and corresponding quantification (248.7±18.63 for starved Vs. 506±26.42 for Forskolin) (Fig 3.8C-E), demonstrating that downregulation of cAMP is required for the decreasing Dm neuronal activity in starved animals.

In summary, we have demonstrated that downregulation of adenylyl cyclase activity is required to regulate hunger behaviour in zebrafish. Since NPY is the major regulator of hunger behaviour in zebrafish, we speculated that NPY employs this pathway to induce orexia and reduce the activation of Dm.

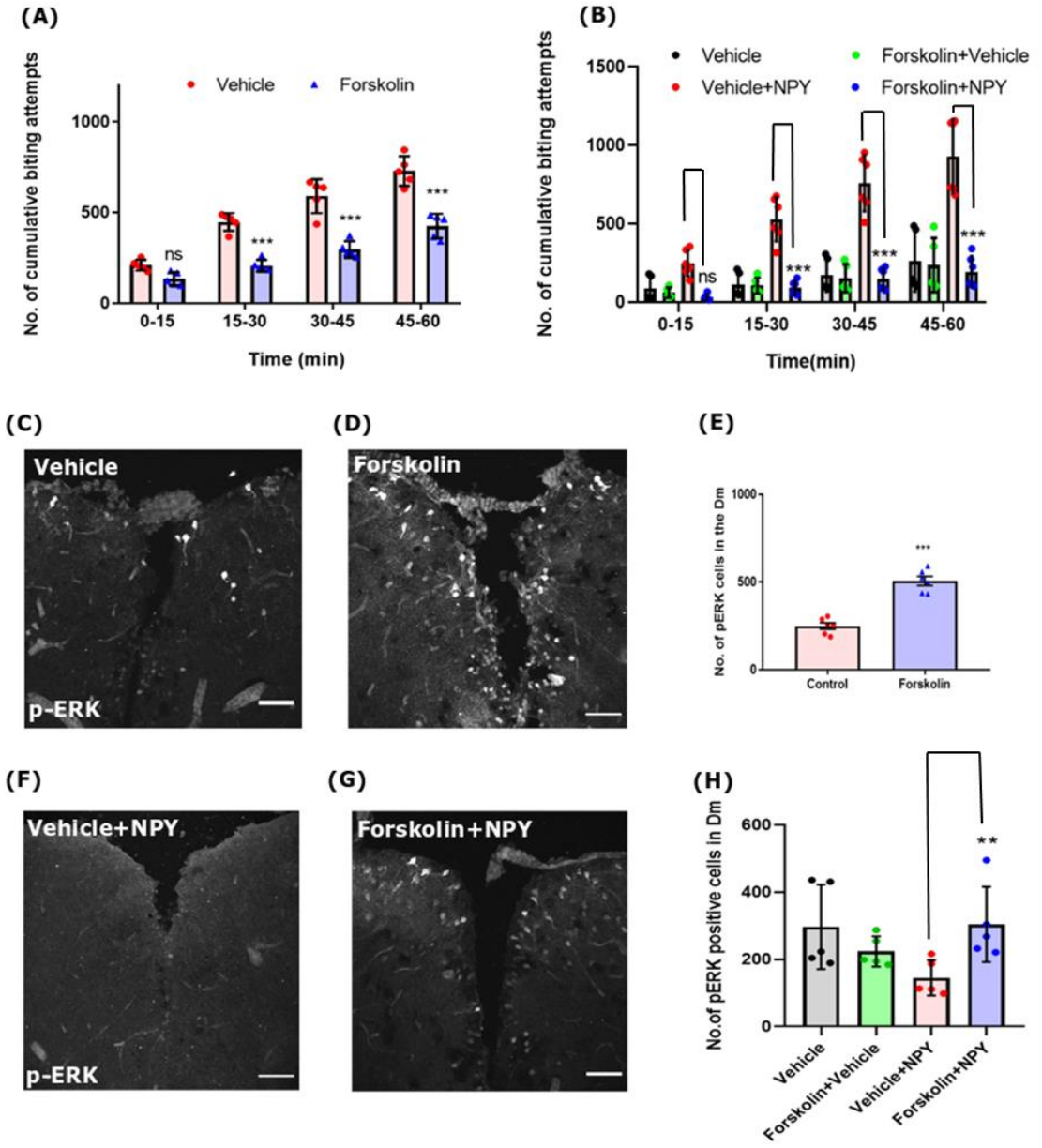
To test if activation of adenylyl cyclase interferes with NPY induced orexia, Forskolin (adenylyl cyclase activator) was coinjected with NPY in a fed fish, and its effect was tested on the feeding drive. A two-way ANOVA was performed to evaluate the effect of Forskolin treatment in NPY treated fed fish on feeding drive over time. There was a statistically significant interaction between drug treatments and time on feeding drive ($F(9, 72) = 4.9$, $p < 0.0001$). Therefore, the effect of each treatment was analyzed at each time point using Bonferroni's posthoc analysis. Results showed that application of Forskolin alone did not display any significant effect on the number of biting attempts as compared to vehicle controls

(236±77.40 for Forskolin treated fed fish Vs. 261.8±86.78 fed controls); however, application of Forskolin in NPY treated fed fish reversed NPY induced orexia (194.7±40.7 for Forskolin+NPY Vs. 929±96.86 for NPY) (Fig 3.8B).

To test if adenylyl cyclase activation also reverses NPY induced reduction in Dm activity, we co-administered Forskolin and NPY in fed fish via the ICV route. One way ANOVA ($F(3, 16) = 3.342$, $P=0.45$)and post hoc Bonferroni test showed that adenylyl cyclase activation in NPY treated fish reversed NPY induced reduction in pERK levels of Dm (304±49.92 for Forskolin+NPY Vs. 145±23.66 for NPY) as seen from the representative micrographs in (Fig 3.8F&G) and the corresponding quantitation in (Fig 3.8H).While, treatment with the Forskolin alone did not affect the basal number of pERK positive neurons in the Dm compared to fed controls (223.8±20.07 for Forskolin treated fed fish Vs. 296.6±56.15 for fed controls). However,. These data indicate that NPY signaling reduces adenylyl cyclase activity to induce orexia and reduce the activity of Dm neurons.

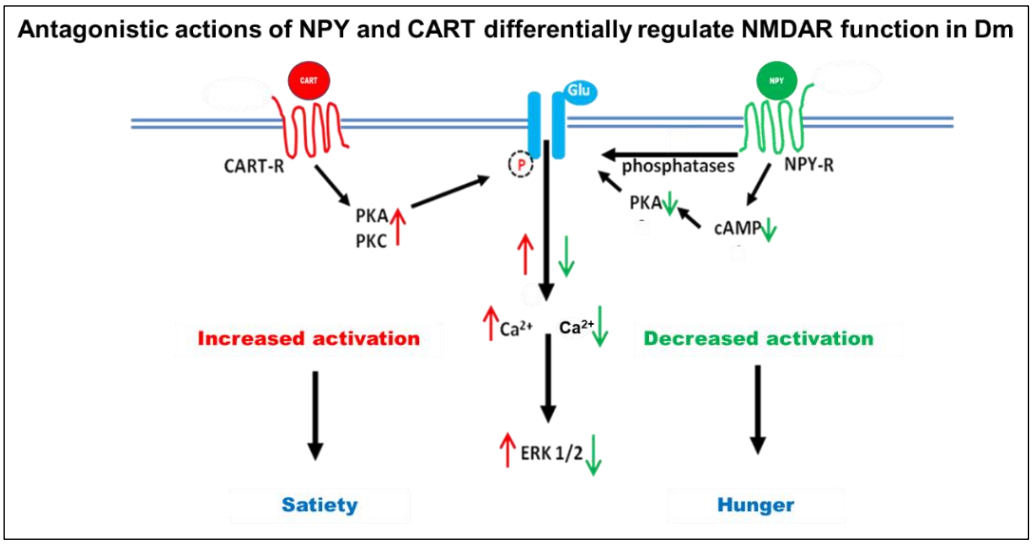
Collectively, we show that NPY activates protein phosphatase calcineurin and downregulates cAMP to induce orexia and reduce activation of Dm neurons.

In summary, we find that CART and NPY are the major regulators of satiety and hunger behaviour in zebrafish. Their antagonistic activities converge on Dm neurons, where they differentially phosphorylate NMDARs to regulate food intake according to the body's energy requirements.



3.8 NPY induced orexia and reduction in Dm activation is exerted by inhibiting adenylyl cyclase activity

(A) Treatment with adenylyl cyclase activator forskolin decreases feeding drive in starved fish.(n=5 animals per group, ***p< 0.001) means were compared using unpaired t-test with Welch's correction. **(C-E)** Forskolin treatment in starved fish increased neuronal activation in Dm Representative transverse sections showing the immunoreactivity of p-ERK in starved fish treated with either vehicle **(C)** or forskolin **(D)** with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(E)** (n=5/6 animals per group; ***p< 0.001) Data are shown as mean +/- SEM and compared using unpaired t-test with Welch's correction **(B)**Treatment with adenylyl cyclase activator forskolin alleviates NPY induced orexia. Data are represented as cumulative biting attempts in 15 min bins over 1 hour and compared using Two-way ANOVA with Bonferroni's posthoc analysis (n=5/6 animals per group; error bars represent +/-SEM; ***p< 0.001). **(F-H)** NPY induced reduction in Dm activation is mediated via a downregulation of adenylyl cyclase activity. Representative transverse sections showing the immunoreactivity of p-ERK in starved fish treated with either vehicle **(F)** or Forskolin **(G)** prior to NPY treatment with the corresponding quantification of the total number of p-ERK positive neurons in Dm **(H)**. (n=5 animals per group; **p< 0.01) Data are shown as mean +/- SEM and compared using one-way ANOVA, with Bonferroni's posthoc analysis [Scale bar 50 µm]



Summary of neuromodulatory mechanisms regulating feeding drive in zebrafish

Antagonistic activities of CART and NPY differentially modulate the function of NMDARs in Dm neurons by regulating PKA and PKC activities. Opposing neuromodulatory actions of these peptides leads to differential activation of Dm neurons that can be correlated with modulation of feeding drive.

3.9 Modulation of Dm neuronal excitability by CART and NPY

Antagonistic activities of CART and NPY differentially phosphorylate NR1 subunit of NMDARs by regulating PKA and PKC activities in Dm. PKA phosphorylates the NR1 subunit at serine 897 and has been shown to increase the Ca2+ permeability of the receptor (Skeberdis et al., 2006). PKC Phosphorylates NR1 subunit at serine 890 and 896, leading to the increased opening probability of the NMDARS (Lan et al., 2001). Together these reports suggest that PKA and PKC mediated phosphorylation of NR1 subunits results in enhanced functioning of NMDARs. We found that NR1 of NMDARs in Dm shows elevated phosphorylation upon CART treatment (Fig 3.5.2) and under satiety mimicked (Glucose injected) conditions (Fig 3.6). Hence, we hypothesized that CART-induced increased functioning of NMDARs under energy-rich conditions would lead to increased neuronal excitability, which is manifested as increased activation of Dm neurons, as seen in Fig 4E.

To test if Dm neurons exhibit increased NMDAR activity and hence show enhanced sensitivity to excitatory cues under satiety conditions, we performed real-time calcium imaging of Dm neurons in the whole brain *ex-vivo* preparation and assessed their activation in response to glutamate. We performed preliminary experiments with different doses of glutamate ranging from 25 µM to 100 µM and settled upon 75 µM glutamate for stimulation in further experiments since it provided a broad dynamic range to resolve control and test responses.

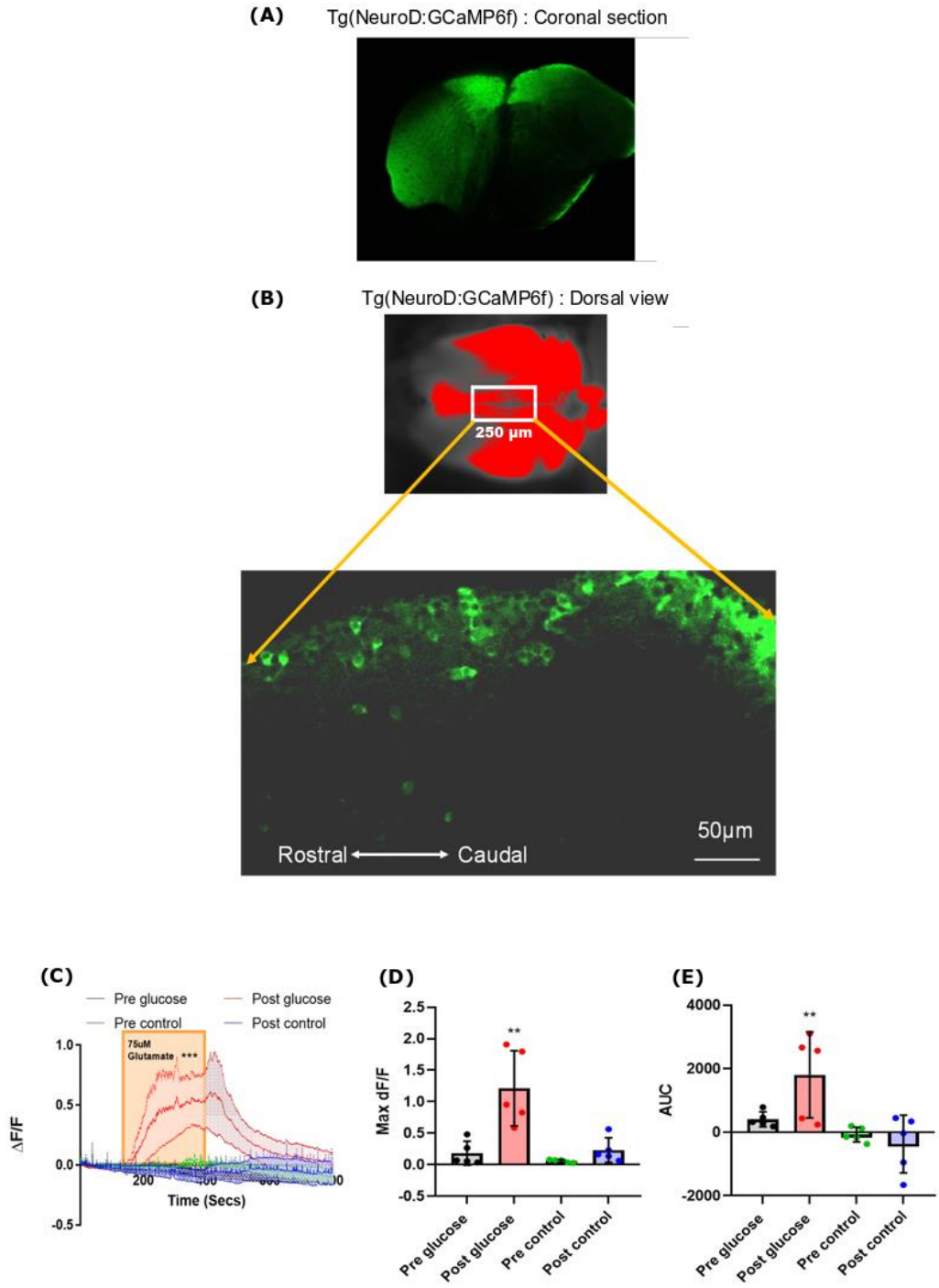
Calcium imaging experiments were performed using the Tg (NeuroD: GCaMP6f) line, which expresses genetically encoded calcium indicator GCaMP6f in the Dm of the adult zebrafish brain (Fig 3.9.1A-B).

3.9.1 **The excitability of Dm neurons is tuned to changes in energy states**

(Conducted in collaboration with Aditi Maduskar)

This set of experiments were performed in the whole brain *ex-vivo* preparation. The intact brain from starved fish was dissected out and held in the recording medium lacking glucose and supplemented with sucrose to maintain energy-deficient conditions. The response of Dm neurons to glutamate in these starvation mimicked conditions was recorded. Next, the same brain preparation was treated with glucose for 30 mins to mimic satiety conditions, and the response of Dm neurons to the same dose of glutamate was re-evaluated. While, under starvation-like conditions, Dm neurons failed to respond to glutamate, the same tissue showed heightened activation to glutamate in response to glucose treatment, as depicted by a significant increase in change in fluorescence intensity (dF/F), the maximum amplitude of the response (max dF/F), and the total response denoted by the area under the curve (AUC) after glucose treatment. As a control experiment, we recorded the response of the Dm neurons without glucose treatment by maintaining the brain in a glucose-free medium for the entire duration. In this experiment, Dm neurons failed to respond to second glutamate stimulation, indicating that the observed increase in activity was due to alterations in energy states and not influenced repeated treatments with glutamate. (Fig 3.9.1 C-E). In summary, these results indicated that the sensitivity of Dm neurons to the excitatory cue is modulated by changes in energy status wherein Dm neurons show increased excitability under satiety conditions compared to starved conditions.

Previous studies in the lab have demonstrated that CART expression is upregulated upon glucose treatment in energy sensing areas of the zebrafish brain: EN and hypothalamus (Mukherjee et al., 2012; Kaniganti, 2014). These areas are directly connected to Dm (Kaniganti, 2014). Brains isolated from starved animals have low endogenous levels of CART neuropeptide. The results in this section suggest that glucose treatment induces CART-release at the Dm from the energy-sensing neurons of the interoceptive centers and result in the increased excitability of Dm neurons.



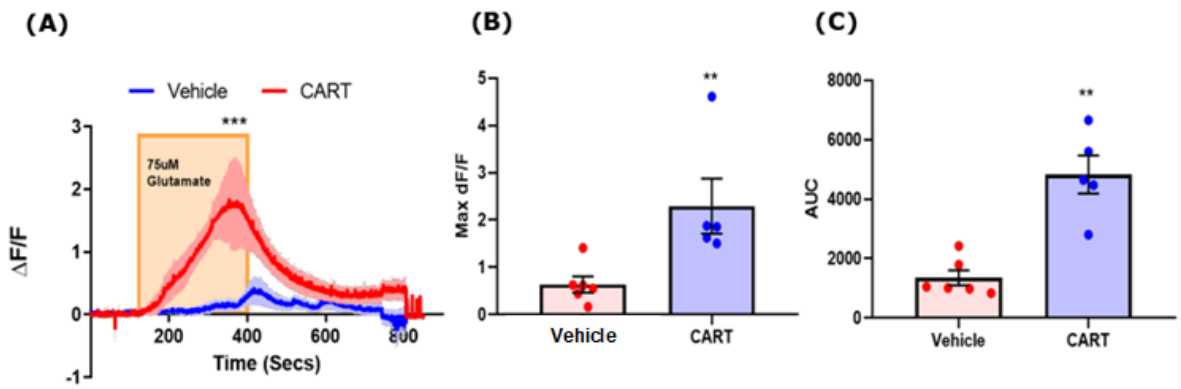
3.9.1 Excitability of Dm neurons is tuned to changes in energy states

(A) Coronal section of the telencephalon of Tg(NeuroD: GCaMP6f) fish showing GCaMP expression profile (Green). **(B)** Top view of Tg(NeuroD: GCaMP6f) telencephalon with the shaded region showing GCaMP expression. Inset shows the single optical plane of one lobe of the telencephalon, which is approx. 50 µm deep and around 250 µm along the rostrocaudal axis. **(C)**The fluorescence traces represent the average change in calcium activity (dF/F) over time in response to 75 µM glutamate. The dark lines represents mean+/- SEM , with the shaded area denoting the limits for standard error. The coloured box indicates the duration for which glutamate was present in the bath. Response of Dm neurons post glucose treatment (red trace) and pre-glucose treatment (Grey trace) was compared. As a control, the response of Dm neurons to 1st glutamate exposure (green trace) and second glutamate exposure (Blue trace) was recorded. **(D)** The max amplitude of response for test and control sets and **(E)** total response for test and control sets. The data were analyzed using One-Way ANOVA with Tukey's multiple comparisons test (n=5 brains each for glucose and sucrose controls, *** $p<0.001$,** $p<0.01$)

3.9.2 CART signaling increases the sensitivity of Dm neurons to glutamate

(conducted in collaboration with Aditi Maduskar)

To assess if increased CART signaling under satiety conditions increases the excitability of Dm neurons, we compared the glutamate-induced activity response of Dm neurons in CART treated starved fish with control starved fish. The following experiments were performed in *ex-vivo* telencephalon preparations to cut off primary neuropeptidergic inputs from the hypothalamus. The telencephalon was dissected from the whole brain 15 min after ICV treatment with CART/ Vehicle, held in the sucrose supplemented media to maintain energy-deprived conditions, and the response of Dm neurons to glutamate was recorded. CART treated Dm showed a heightened response to the glutamate as seen by a significant increase in the change in fluorescence intensity (dF/F) (Fig 3.9.2A), Max amplitude of response (max dF/F) (Fig 3.9.2B), and Total response (AUC) (Fig 3.9.2C) demonstrating that CART signaling increases excitability of Dm neurons.

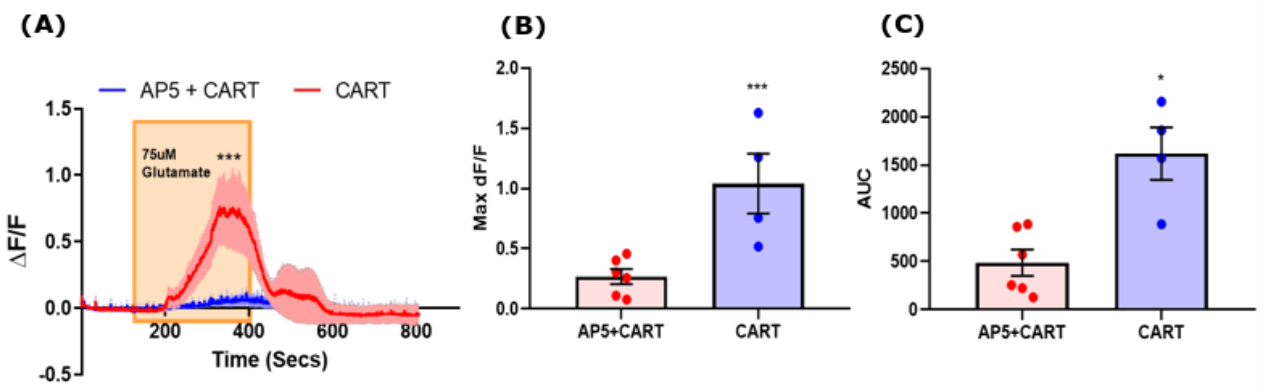


3.9.2 CART signaling increases the sensitivity of Dm neurons to glutamate

(A) The fluorescence traces represent the average change in calcium activity (dF/F) over time in response to 75 µM glutamate. The dark line represents mean, with the shaded area denoting the limits for standard error. The colored rectangular box indicates the duration for which glutamate was presented. The response of CART treated (Red) Dm neurons as compared to starved controls (Blue). **(B)** The max amplitude of response and **(C)** total response for CART treated Dm neurons compared to starved controls. The data were analyzed using a t-test with Welch's correction. (n= 5 for CART and n=6 for control telencephalons, **p<0.01, ***p<0.001).

3.9.3 CART signaling potentiates NMDAR activation to increase the sensitivity of Dm neurons to glutamate (Conducted in collaboration with Aditi Maduskar)

Next, we tested if CART signaling requires NMDAR activity to increase the excitability of Dm neurons. We blocked NMDAR signaling in CART-treated starved fish by co-administration of AP5 and CART and compared the response of Dm neurons to the ones that were only treated with CART. The telencephalon was dissected 15 mins after the ICV injection, and calcium activity of Dm was recorded in ex-vivo preparation. We found that while CART-treated starved fish showed increased activation in response to glutamate, blocking NMDAR activity in these fish abolished the stimulus-induced activation in Dm. (Fig 3.9.3A-C), confirming the involvement of NMDAR signaling in the sensitization of Dm neurons.

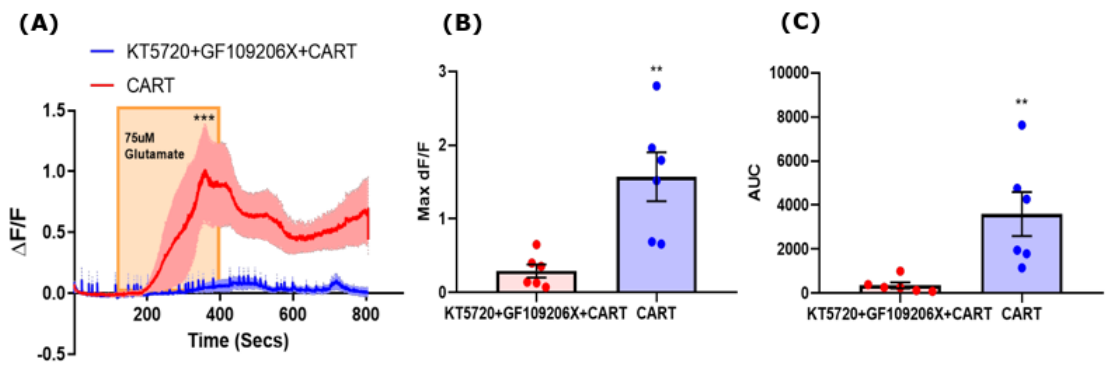


3.9.3 CART signaling potentiates NMDAR function to increase the sensitivity of Dm neurons to glutamate

(A) The fluorescence traces represent the average change in calcium activity (dF/F) over time in response to 75 µM glutamate. The dark line represents the mean, with the shaded area denoting the limits for standard error. The colored rectangular box indicates the duration for which glutamate was presented. The response of CART treated (Red) Dm neurons compared to AP5+CART treated fish (Blue). (B) The max amplitude of the response and (C) total response for CART treated Dm neurons as compared AP5+CART treated fish. (n= 4 for CART and n=6 AP5+CART telencephalons, ***p<0.001, *p<0.05) The data were analyzed using a t-test with Welch's correction.

3.9.4 CART signaling activates PKA and PKC to increase the sensitivity of Dm neurons to glutamate (Conducted in collaboration with Aditi Maduskar)

Since PKA and PKC have been implicated in the potentiation of NMDAR functioning (Chiu et al., 2009), and we have previously shown that CART activates these kinases to induce anorexia, we tested if PKA and PKC activity is required for enhancing the excitability of CART treated Dm neurons. For this experiment, we inhibited the activity of PKA and PKC in CART treated fish by co-administration of PKA and PKC inhibitors with CART and compared the response of Dm neurons to the ones that were only treated with CART. The telencephalon was dissected 15 mins after the ICV injection, and calcium activity of Dm was recorded in *ex-vivo* preparation. Results demonstrated that inhibiting PKA and PKC signaling abrogated CART induced an increase in sensitivity of Dm neurons to glutamate. (Fig 3.9.4 A-C).



3.9.4 CART signaling requires PKA-PKC activity to increase the sensitivity of Dm neurons to glutamate

(A) The fluorescence traces represent the average change in calcium activity (dF/F) over time in response to 75 µM glutamate. The dark line represents the mean, with the shaded area denoting the limits for standard error. The coloured rectangular box indicates the duration for which glutamate was presented.
The response of CART treated (Red) Dm neurons as compared to PKA, PKC inhibitors+CART treated fish (Blue) **(B)** The max amplitude of the response and **(C)** Total response for CART treated Dm neurons as compared to starved controls. The data were analyzed using a t-test with Welch's correction. (n=6 telencephalons per group, **p<0.01, ***p<0.001).

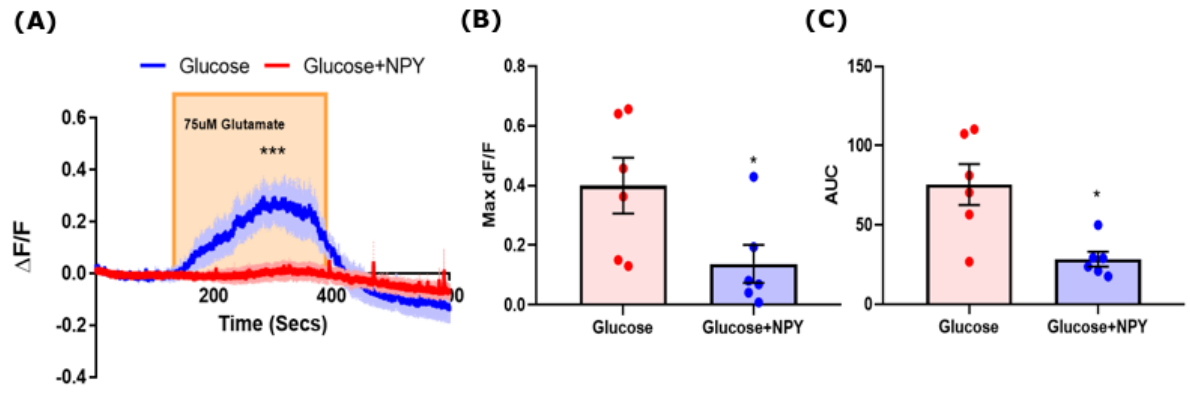
3.9.5 NPY signaling decreases the sensitivity of Dm neurons to glutamate

(Conducted in collaboration with Aditi Maduskar)

We have previously demonstrated that NPY signaling reduces Dm neuronal activation by reducing phosphorylation of the NR1 subunit of NMDARs in Dm neurons. It is known that dephosphorylation of the NR1 subunit leads to depotentiation of NMDAR function. (Salter et

al., 2009) and action of NPY via Y1 receptor has been shown to desensitize spinal nociceptive neurons via modulation of NMDAR activity. (Fu et al., 2019)

Hence, we hypothesized that increased NPY signaling would decrease the excitability of Dm neurons. To assess if increased NPY signaling reduces the excitability of Dm neurons, we compared the glutamate-induced activity response of Dm neurons in glucose injected starved fish (satiety mimic) with the glucose injected starved fish treated with exogenous NPY. The experiment was performed in *ex-vivo* telencephalon preparations. Telencephalon was dissected from the whole brain 15 min after treatment with NPY+glucose /glucose, held in the glucose supplemented media to maintain energy-rich conditions. The response of Dm neurons to glutamate was recorded. Dm neurons in NPY treated satiety mimicked fish showed very minimal response to the glutamate as compared to satiety mimicked fish as seen by a significant decrease in the change in fluorescence intensity (dF/F) (Fig 3.9.5A), Max amplitude of response (max dF/F) (Fig 3.9.5B), and Total response (AUC) (Fig 3.9.5C) demonstrating that NPY signaling reduces the excitability of the Dm neurons.



3.9.5 NPY signaling decreases the sensitivity of Dm neurons to glutamate

(A)The fluorescence traces represent the average change in calcium activity (dF/F) over time in response to 75 µM glutamate. The dark line represents mean +/- SEM, with the shaded area denoting the limits for standard error. The colored rectangular box indicates the duration for which glutamate was presented. The response of NPY treated (Blue) Dm neurons as compared to fed controls (Red). The data were analyzed using a t-test with Welch's correction. (n=6 telencephalons per group, ***p<0.001).) **(B)** The max amplitude of the response and **(C)** total response for NPY+Glucose treated Dm neurons as compared to glucose treated starved controls. The data were analyzed using a t-test with Welch's correction. (n=6 telencephalons per group, *p<0.05, ***p<0.001).

In summary, We have demonstrated that antagonistic actions of NPY and CART differentially modulate the excitability of Dm neurons to regulate hunger-satiety behavioural states in zebrafish.

3.10 Discussion

Feeding behaviour is one of the survival-oriented behaviours primarily driven by the internal energy needs of the body and can be divided into two alternating states -hunger and satiety. Hunger or energy deficiency creates a motivational state called 'feeding drive' that propels animals to look for and consume large amounts of food. The feeding drive and resultant efforts to consume food are gradually reduced as the body progressively achieves a state of satiety or energy surfeit. Although neuropeptides have been shown to play a critical role in the maintenance and transition between alternating behavioural states, the investigation of cellular and molecular level mechanisms of peptidergic actions remains poorly understood.

This study elucidates the modulatory signaling by orexic (NPY) and anorexic (CART) neuropeptides that are critical for maintaining hunger and satiety states, respectively, in adult zebrafish. This study also characterizes a common substrate of CART and NPY activity- Dorsomedial telencephalon (Dm), wherein antagonistic activities of these peptides differentially modulate the activation of this region, which can be correlated with modulation of feeding drive. Our data demonstrate that antagonistic actions of CART and NPY differentially phosphorylate NMDARs to modulate the excitability of Dm neurons. Such bistable excitability forms a neural representation of the internal energy state that can be correlated with the modulation of the feeding drive.

Although the role of CART and NPY as potent anorexic and orexic agents respectively is well established in fish species (Lin et al., 2000a, 2000b; MacDonald and Volkoff, 2009; Matsuda, 2009, 2019; Soengas et al., 2018; Volkoff et al., 2005), this is the first study which delineates the circuit-level mechanisms of peptidergic neuromodulation that result in energy state-dependent regulation of food intake in zebrafish. Here, we show that the antagonistic actions of CART and NPY converge on NMDAR function in Dm to regulate feeding drive in adult zebrafish. Consistent with our findings, multiple studies in vertebrates have evidenced the involvement of NMDARs in the regulation of homeostatic feeding. (Resch et al., 2014, Guard et al., 2009, Burns and Ritter, 1997). In rodents, NMDARs in the hindbrain are required for CCK-induced reduction in food intake under sated conditions (Wright et al., 2011). Along with CCK, NMDARs are also showed to be involved in the anorexic actions of leptin (Neyens et al., 2020) and PACAP (Resch et al., 2014).

Furthermore, NMDAR signaling has been demonstrated to be essential for the processing of hunger-related signals in rodents. NMDARs are involved in the mediation of orexia in the

lateral hypothalamus (Lee and Stanley, 2005) and are shown to be important for the regulation of AGRP neuronal activity in the establishment of starvation state (Liu et al., 2012). These independent reports indicated that NMDAR signaling in various brain regions could regulate food intake bidirectionally and mediate modulatory actions of multiple orexic and anorexic neuropeptides, which corroborate our findings.

We further characterize the role of NMDARs in regulating feeding drive in zebrafish. We show that CART signaling activates PKA and PKC to increase NMDAR dependent activation of Dm neurons. On the other hand, NPY signaling downregulates PKA activity via inhibitory GPCR (Gi) signaling and antagonizes PKC activity by activating calcineurin, which is known to dephosphorylate NR1 subunit at serine 896 where PKC phosphorylates it. (Salter et al., 2009). These data suggest that antagonistic actions of CART and NPY differentially phosphorylate NMDA receptors in Dm by regulating PKA and PKC activities. For validation, we performed direct quantification of pNR1 levels in Dm neurons by immunofluorescence studies in brain slices using phospho-NR1 antobody.The specificity of the antibody in zebrafish was tested using westen blotting in whole brain lysate. However, since only a subset of neurons in Dm shows significant changes in pNR1 levels upon peptide treatment, we resoned that western blot analysis with the bulk isolated samples might not be able to detect the differences in phosphorylation status of NR1.

The immunofluorescence results demonstrate that the CART treatment in starved fish significantly increases the phosphorylation of the NR1 subunit via activation of PKA and PKC. In contrast, treatment of NPY in sated fish significantly decreases phosphorylation of NR1 subunit of NMDARs in Dm. Alternative techniques like immunoblot analysis can be used to test the phosphorylation status of NR1 subunit. However, since only a subset of neurons in Dm shows significant changes in pNR1 levels upon peptide treatment, western blot analysis with the bulk isolated samples might not be able to detect the differences in phosphorylation status of NR1.

In summary, in this study, we find that antagonistic actions of CART and NPY differentially phosphorylate NR1 subunit of NMDARs that results in differential activation of Dm neurons, probably via modulation of NMDAR function.

Phosphorylation state-dependent modulation of NMDAR functioning has been implicated in regulating behavioural plasticity (Chatterjea et al., 2010b; Haberny and Carr, 2005; Lin et al., 2005; Shanley et al., 2001). Phosphorylation of NR1 subunit by PKA is known to enhance the calcium permeability of the receptor (Skeberdis et al., 2006), while PKC mediated phosphorylation results in an increased opening probability of the receptor (Lan et al., 2001).

Hence, PKA and PKC mediated phosphorylation of NR1 subunit results in potentiation NMDAR function (Zito and Scheuss, 2009), making the component neurons highly sensitive to excitatory cues like glutamate. Hence, we reasoned that CART-induced phosphorylation of NMDARs might result in increased excitability of Dm neurons that is manifested as increased activation of Dm under sated conditions or upon CART treatment.

In contrast, NPY mediated reduction in PKA activity has been shown to depotentiate NMDARs and reduce the excitability of neurons (Molosh et al., 2013a). Calcineurin, a phosphatase involved in NPY signaling (Fig 3.7 C-E), has been implicated in the desensitization of NMDARs. (Chen and Roche, 2007). These reports suggest that NPY signaling might lead to depotentiation of NMDARs to reduce the excitability of Dm neurons that is manifested as decreased activation of Dm under starvation or upon NPY treatment.

Considering these findings, we speculated that CART and NPY differentially potentiate NMDAR function to modulate the excitability of Dm neurons in order to regulate satiety-hunger behavioural outcomes.

To test if the excitability of Dm neurons is modulated by energy states, we performed real-time activity imaging in *ex-vivo* whole-brain preparation from the starved fish and evaluated the initial response of Dm neurons to glutamatergic cue. Next, the brain prep was treated with glucose for 30 mins to mimic satiety conditions, and the response of Dm neurons was re-evaluated. We observed that in response to glucose treatment (or satiety mimicked conditions), the Dm neurons showed significantly higher activation to glutamate stimulation while the same region remained highly inactive under initial starvation-like conditions. These data thus provide the evidence that the Dm neurons are more excitable under satiety conditions and led to the speculation that increased CART signaling via glucose-sensitive neurons in EN and the hypothalamus majorly contribute to increase the excitability of Dm neurons upon glucose treatment.

We directly tested this hypothesis by performing real-time activity imaging in *ex-vivo* telencephalon preparations from the starved fish treated with CART or vehicle. *Ex-vivo* telencephalon preps were used to cut off any neuropeptidergic inputs from the hypothalamus. We found that CART treated Dm neurons showed heightened response to glutamate, which was dependent on activation of PKA, PKC, and NMDAR.

In absence of well characterized receptor for CART, understanding detailed mechanism of CART mediated activation PKA and PKC remains a difficult task. One possibility is that CART might drive initial firing of Dm neurons that leads to NR1 phosphorylation via PKA and PKC activation. Electrophysiological techniques can be employed to test if acute

stimulation with CART peptide induces neuronal firing in Dm neurons even in presence of PKA-PKC inhibitors.

Our findings from calcium imaging and immunohistochemistry revealed that activation of PKA-PKC and NMDARs is critical for CART mediated increased activation of Dm neurons. Hence, it is possible that CART activates PKA, PKC and subsequently NMDARs via first increasing the firing of Dm neurons. However, CART induced sustained increase in excitability and hence activation of Dm is mediated by increased functioning of NMDARs via activation of PKA and PKC.

Since NPY and CART antagonistically regulate NMDAR activity at Dm, we reasoned that the decreased excitability of Dm under starved conditions might largely be attributed to increased NPY signaling. Indeed, our activity imaging data suggested that NPY treatment leads to a reduced glutamate-induced response of Dm neurons. (Fig 3.9.5)

In summary, this study demonstrates that Dm excitability is bidirectionally modulated by the antagonistic activities of CART and NPY to regulate satiety and hunger behavioural outputs, respectively. The region referred to as Dm in this study is specific to a subpopulation in dorsomedial telencephalon in zebrafish, which spans 450µm region around anterior commissure as described in Fig 3.3.1.

Dm is thought to be the homolog of the mammalian amygdala (Northcutt, 2006, Wullimann and Mueller, 2004) and plays a vital role in regulating emotional behaviours in teleosts. (von Trotha et al., 2014a). Ablation of Dm disrupts aggressive, parental behaviour and conditioned avoidance response, whereas its stimulation induces arousal, escape response in fish. (Portavella et al., 2004; Salas et al., 2006)

Since the amygdala is known to be involved in the regulation of CART and NPY mediated anxiety and stress behaviour in mammals (Giesbrecht et al., 2010; Heilig, 1995; Molosh et al., 2013b; Sajdyk et al., 2008, Rale et al., 2017; Upadhya et al., 2013), we tested if CART and NPY mediated modulation of Dm activity mediates any anxiety-like behaviours in zebrafish. We evaluated behavioural paradigms like time spent in the upper zone, the ratio of time spent in the upper Vs. the lower zone, zone transition frequency (upper to lower), total distance covered, and average velocity in a novel tank diving test to assess anxiety in zebrafish. (Assad et al., 2020; Egan et al., 2009). Results show that neither application of CART or NPY via ICV route nor changes in internal energy states evoked any anxiogenic responses in zebrafish.

(Fig 3.2.2 and unpublished data by Aditi Maduskar), validating that external application of CART, NPY at a given concentration, and three days of starvation does not induce stress or

anxiety-related response in the zebrafish and observed changes in food intake emerge from modulation of feeding drive.

To our knowledge, this is the first study that describes the novel role of Dm as a nodal structure regulating feeding in adult zebrafish, although the mammalian homolog of Dm, amygdala, has been implicated in the regulation of food intake (White and Fisher, 1969; Zhang et al., 2011). Padilla et al. have demonstrated that the amygdala receives projections from interoceptory NPY neurons where NPY acts via NPY Y1 receptors to increase food intake. Additionally, projections from dopamine receptor-positive neurons in the prefrontal cortex activate the medial basolateral amygdala to increase food intake (Land et al., 2014). The amygdala has also been involved in hindbrain satiety circuits, where PKC-δ+ neurons in the central amygdala integrate multiple anorexigenic signals to inhibit food intake (Cai et al., 2014, Carter et al., 2013). Several anorexigenic neuromodulators, when administered in the CeA, were shown to affect food intake (Beckman et al., 2009, Fekete et al., 2007, Kovács et al., 2012).

Multiple lines of evidence suggest that, in mammals, different subpopulations of neurons in the amygdala are involved in processing hunger and satiety-related inputs from interoceptory neurons in the arcuate nucleus and hindbrain. This data corroborates our findings wherein we show CART and NPY neurons in energy sensing areas of the zebrafish brain (EN and Hypothalamus) project to Dm (previous data from the lab). We show that the antagonistic activities of CART and NPY differentially modulate the activity of Dm neurons to form the neural representation of internal energy state that can be correlated with modulation of feeding drive. Hence we propose that a detailed investigation of the role of Dm in feeding behaviour using a simpler vertebrate system like zebrafish would help to uncover the function of the amygdala in the regulation of food intake in mammals.

Limitations of the study

This study describes modulatory mechanisms and characterizes neuroanatomical substrates involved in the regulation of feeding pertaining to CART and NPY mediated signaling. Although we have reported that CART and NPY act as major anorexic and orexic signals respectively in zebrafish, the involvement of other factors like orexin, ghrelin in regulating feeding behaviour cannot be ruled out.

Earlier studies in the lab have identified a specific region of dorsomedial telencephalon (Dm) that responds to changes in energy states. This study elucidates the circuit level neuropeptidergic mechanisms that modulate the activity of Dm, which can be correlated with

the internal energy state. Our study talks about the global changes in the excitability and activity of Dm based on the population level analysis, which limits our interpretations and allows us to deduce only correlations between modulation of Dm activity and changes in the feeding drive. Dm possibly has multiple populations of neurons that are functionally involved in the regulation of various functions. For example, one report suggests that Calbindin and vglut2.2 positive neurons in the subregion of the dorsomedial telencephalon, spanning the 150 µm, immediately rostral to AC are activated by acute injection of D-amphetamine and involved in the regulation of drug-seeking behaviour (von Trotha et al., 2014). Another report from Lal et al. reported that the anterior part of Dm, defined by expression of emx3 (Gantz et al.,2015), is involved in fear conditioning.

Hence it is likely that Dm has distinct populations of neurons that respond to CART and NPY and are involved in processing orexic-anorexic inputs. Genetic characterization of such populations would allow the specific activity manipulations to dissect their role in regulating food intake in adult zebrafish.

4 Chapter 4: Towards the identification of CART peptide receptor/s

Introduction

Cocaine and amphetamine-regulated transcript (CART) is a widely distributed, multifunctional neuropeptide that has established roles in neural regulation of feeding, body weight, drug addiction, nociception, stress, neuroendocrine functions, etc. (Bannon et al., 2001; Chiu et al., 2009; Lau and Herzog; Rogge et al., 2008; Subhedar et al., 2014). Despite the well-studied behavioural effects of the CART peptide, the mechanisms of CART signaling remain elusive, owing largely to the lack of a well-characterized receptor/s for the peptide.

Since the discovery of the CART peptide in 1995, several attempts have been made to identify its receptor from the brain tissue. However, radiolabelled CART failed to bind in the brain tissue specifically even though exogenous CART administration was shown to activate in discrete groups of neuronal populations in the same region (Vrang et al., 1999). The reasons for the failure could be that radiolabelling might have hampered the functioning of CART peptide, or CART receptors might be the low-affinity receptors expressed in low abundance (Vicentic et al., 2006).

The studies in the cultured cells delivered more promising results. The first evidence for receptor-mediated activity came from the experiments in primary hippocampal cultures. CART peptide was shown to inhibit voltage-dependent intracellular Ca^{2+} signaling, which was sensitive to pertussis toxin, suggesting that the CART receptor is of Gi/o type in hippocampal neurons (Yermolaieva et al., 2001). Subsequently, the Kuhar group reported a saturable binding of CART peptide in mouse pituitary cell line AtT20, which increased phospho ERK levels by activating MEK signaling. This effect was blocked by pertussis toxin, reaffirming that the CART receptor is of Gi/o type (Lakatos et al., 2005, Vicentic et al., 2005). Parallelly, receptor-like binding of CART in dissociated hypothalamic neurons, nucleus accumbens (Acb) neurons, and HepG2 cells was demonstrated (Jones and Kuhar, 2008, Keller et al., 2006), but the classical criterion for receptor binding (saturable and specific) was not fulfilled (Keller et al., 2006). The demonstration of specific and saturable binding of radiolabelled CART on rat pheochromocytoma cells PC12 marked the first step towards identifying CART receptor/s from PC12 (Maletínská et al., 2007; Nagelová et al., 2014). Following this, researchers tried to characterize the CART binding sites on PC12 cells. They found that differentiation of PC12 cells to neuronal phenotype led to three times increase in the number of binding sites in these

cells. Subsequently, Lin et al. identified pituitary adenylate-cyclase-activating polypeptide (PACAP - another neuropeptide) as a CART receptor antagonist, which could displace CART binding on PC12 cells. These in-vitro studies provided evidence for the existence of CART receptor/s and suggested that at least one of the receptors would be of Gi/o type.

In vivo study by Quintana et al. in 2013 reported that CART mRNA was one of the significantly upregulated transcripts in a GPR88 knockout in the striatum of the mouse brain. Interestingly another report showed that GPR88 was one of the significantly upregulated GPCRs in *cartpt* knockout mice (Lau et al., 2016). The same group further elucidated the role of GRP88 in energy homeostasis and demonstrated that germline knockout of GPR88 resulted in lean mice phenotype with reduced spontaneous feeding and energy expenditure. Mechanistically, the global knockout of GPR88 modulated levels of different feeding-related neuropeptides, including *cartpt* in feeding regulatory regions of the mouse brain. GPR88 null mice showed decreased expression of *cartpt* in the arcuate nucleus and upregulated levels of *cartpt* in the dorsomedial hypothalamus, which is in line with the functionally opposing feeding regulatory action of CART in these regions. These data provided indirect evidence that GPR88 might be one of the candidate receptors for CART.

Meanwhile, the most substantial evidence for identifying plausible receptors for CART was reported by Yosten et al. They showed that, in KATO III cancer cells and differentiated PC-12, GPR 160 is required for the CART mediated expression of Fos and increased phosphorylation of ERK, respectively. Moreover, it was demonstrated that CART was coimmunoprecipitated with GPR160 in these cells. In vivo, CART working through GPR160 activated ERK-CREB to induce mechano-hypersensitivity in mice spinal cord (Yosten et al., 2020). Interestingly, a recent report shows that interaction between CART and GPR160 is also required to regulate food and water intake. Immunoneutralization of GPR160 in the hindbrain by injecting GPR160 antiserum in the 4th ventricle in mice reduced food intake in the dark cycle, indicating that the receptor might regulate satiety behaviour. Furthermore, blocking GPR160 activity in the hindbrain by pre-injection of GPR160 antibody before CART administration prevented the anorexic effect of CART peptide. In agreement with the above data, GPR160 was expressed in the brain regions associated with feeding, specifically NTS, PBN, hypothalamus, amygdala, etc. (Haddock et al., 2021). These findings suggest that GPR160 is required for CART mediated signaling that regulates pain modulation and feeding, making it one of the candidate receptors for CART. These studies provided strong evidence for GPR160 as one of the putative receptors for CART; however, detailed characterization of ligand-receptor interactions to assess binding kinetics, affinity, etc., is still lacking.

Although one of the plausible receptors of CART has been identified, the CART peptide is likely to have more than one cognate receptor. CART peptide has two functional fragments- CART 55-102 and CART 62-102, showing differential potencies in various behavioural paradigms. For example, Bannon et al. found that in the acoustic startle response (ASR), CART 62–102 did not affect ASR at the same doses that CART 55–102 did, and in prepulse inhibition CART 55–102 was less potent than 62–102. CART 55-102 showed five times higher potency as an anorexic agent than CART 62-102. Based on these reports, it is predicted that CART peptide operates via more than one receptor to modulate different or even a single behaviour.

This study is focused on identification and characterization of a novel CART receptor/s from a CART responsive cell line of pituitary or adrenal origin. CART signaling is known to regulate the expression of prolactin, growth hormone in the pituitary (Baranowska et al., 2003; Kuriyama et al., 2004) and glucocorticoids in the adrenal glands (Koylu et al., 2006) and hence predicted to be involved in the regulation of stress responses (Stanley et al., 2001). Therefore, we reason that the receptor for CART in the pituitary or adrenal cell line should be different from GPR160 that is involved in the mediation of pain and feeding in rodents (Yosten et al., 2020, Haddock et al., 2021).

The aim of this study is to-

1. Identify the cell line of pituitary or adrenal origin which responds to CART.
2. Optimize ligand based receptor capture (LRC) method for isolation and identification of novel CART receptor from CART responsive cell line.

Results

4.1 Identification of CART responsive cell line

Following cell lines of pituitary or adrenal origin were selected as candidate cell lines and screened for CART induced responses.

1. Rat pituitary cell line: **GH3**
2. Mouse pituitary cell line: **AtT20**
3. Rat pheochromocytoma cell line: **PC12**

A report by Lakatos et al. suggested that AtT20 and GH3 cells upregulate pERK levels in response to Rat CART (55-102) treatment. Hence we screened for CART mediated upregulation of pERK in these cells using western blotting and immunocytochemistry. However, none of the cell lines showed increased phosphorylation of ERK across the range of CART concentrations in our experiments. (data not shown).

Alternatively, we examined the CART-induced changes in intracellular calcium as CART is shown to regulate voltage-gated calcium levels and thereby modulate intracellular calcium in primary hippocampal cultures (Yermolaieva et al., 2001).

Of the cell lines screened, we found that the rat pituitary cell line- GH3 showed consistent upregulation of intracellular calcium in response to CART (55-102) (Fig 4.1.1).

To test if the response of GH3 cells is specific to CART treatment, we treated these cells with immunoneutralized CART peptide and compared the response with active CART peptide and found that treatment with preadsorbed CART peptide showed a significantly reduced response than active CART peptide (0.1± 0.02 for IgG+CART Vs. -0.008± 0.02 for CART ab+CART) (Fig 4.1.2), suggesting that increase in intracellular calcium in GH3 cells is specific to CART treatment.

In summary, we have identified a rat pituitary cell line-GH3 which responds specifically to CART treatment via increasing intracellular calcium levels. This cell line will be further used for the isolation and characterization of a novel CART receptor.

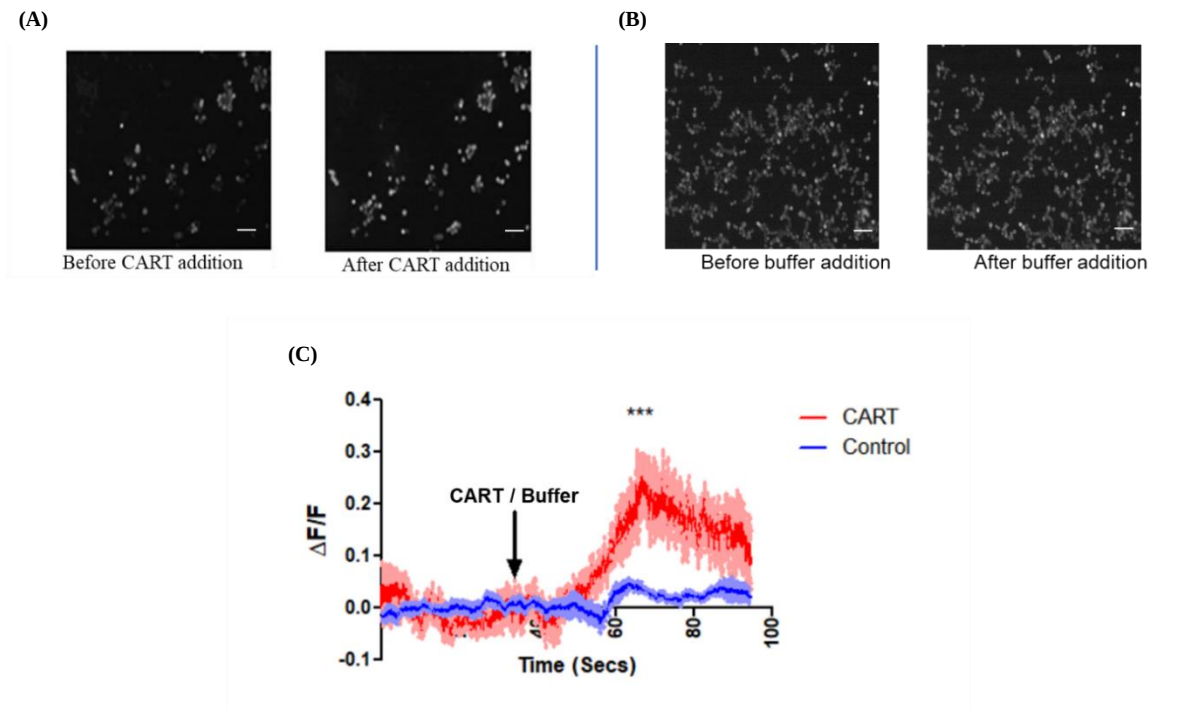


Figure 4.1.1 CART treatment mediates an increase in intracellular calcium in GH3 cells

(A-C) The calcium activity of GH3 cells increases in response to CART treatment. The traces represent the average change in fluorescence intensity (dF/F) over time in response to CART (red trace) or Vehicle treatment (blue trace). The dark line represents mean +/- SEM, with the shaded area denoting the limits for standard error. The arrow indicates the time point at which the CART / Vehicle was added.
Scale bar-10µm (C) Representative snapshots at a single frame showing calcium fluorescence before and after CART treatment (A) and before and after vehicle treatment (B). The data were analyzed using a t-test with Welch's correction. (N=6, ***p<0.001).

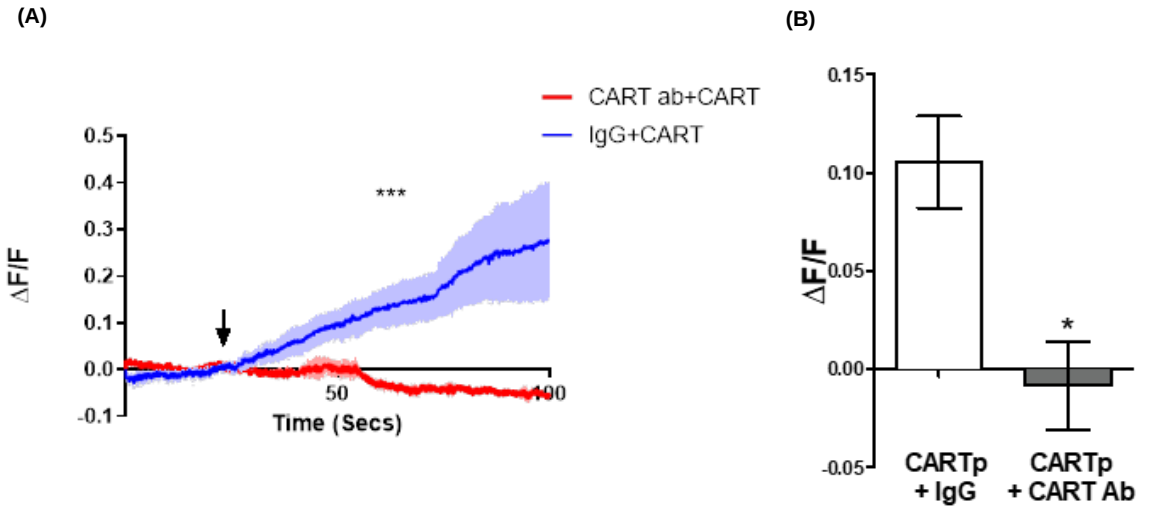


Figure 4.1.2: Preadsorption of CART peptide with CART antibody abolishes CART mediated increase in calcium activity in GH3 cells

Calcium activity of GH3 cells is significantly reduced after immunoneutralization of CART action. The traces represent the average change in fluorescence intensity (dF/F) over time in response to CARTab+CART (red trace) or IgG+CART (blue trace). The dark line represents mean +/- SEM, with the shaded area denoting the limits for standard error. The arrow indicates the time point at which the drug was added. Means were compared using t-test with Welch's correction (N=3,***P< 0.001).

4.2 Optimization of ligand-based receptor capture method for isolation and characterization of CART receptor from GH3 cells

Previous studies in our lab have attempted to pull down CART receptor/s using biotinylated CART peptide from membrane preparations of PC12 cells. But the affinity-based isolation of the CART receptor was unsuccessful which supported the notion that CART receptor/s are low-affinity receptors, expressed in low abundance, and hence are difficult to purify by affinity purification. Therefore, we looked for alternative approaches that can be used for successful isolation of low affinity cell surface receptors.

Ligand-based receptor capture (LRC) method has been employed to identify and isolate low affinity receptors which are expressed less numbers. This technology has been developed as an extension of cell surface capture method and allows unbiased and sensitive identification of one or several specific low affinity receptors for a given ligand under near-physiological conditions. (Frei et al., 2013). LRC technology uses a trifunctional reagent, TRICEPS, which contains three functional groups. NHS ester group attaches to the primary amines or lysines on the ligand of interest. The hydrazine group covalently attaches to the oxidized glycopeptides on the cell surface receptors, and the biotin group allows affinity purification of the ligand-receptor complex. (Fig 4.2.1A).

The LRC experiment parallelly runs on two samples- test sample wherein the ligand of our interest is coupled with TRICEPS and control sample wherein the peptide which does not show specific binding on the cell surface (e.g., glycine) is coupled to the TRICEPS.

First, preoxidized cells/ tissue are treated with TRICEPS coupled ligand. The specific interaction between the ligand and the target receptor increases the probability of covalent capture of the target receptors by the hydrazine group on TRICEPS. Successive digestion and affinity purification of TRICEPS coupled glycoproteins yield the array of purified cell surface receptor proteins which are subsequently identified using high mass accuracy mass spectroscopy. The receptor protein of our interest is identified by comparing the array of identified cell surface receptors in test and control samples wherein the target receptor protein is enriched in the test sample.

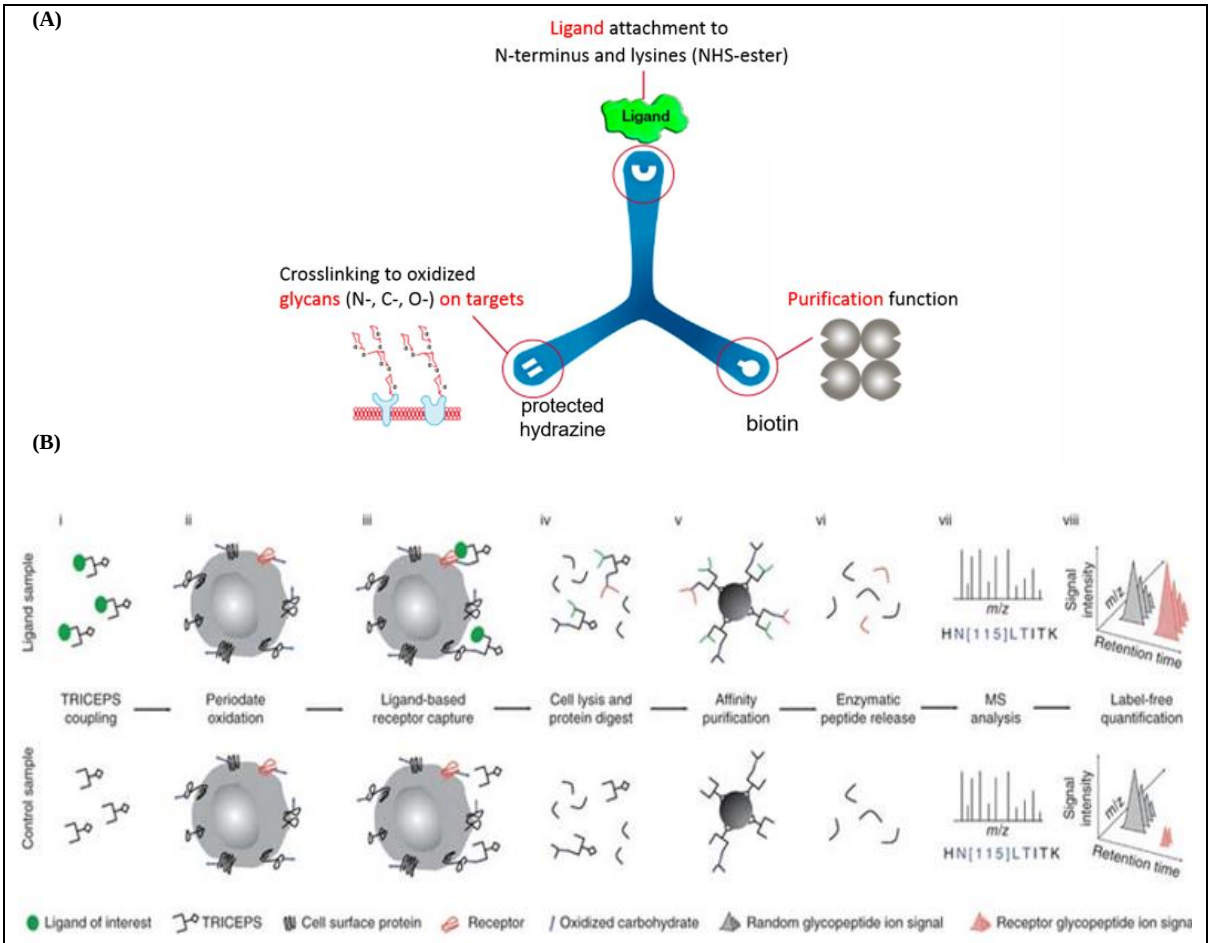


Figure 4.2.1: LRC-TRICEPS technology for successful identification of cell surface receptors

(A) TRICEPS: the trifunctional reagent consisting of three functional groups. NHS ester group for attachment to ligands, protected hydrazine group for crosslinking to oxidized glycans on the cell surface, and biotin group for purification (B) Workflow for the LRC and identification on living cells. (i) Conjugation of a purified ligand of interest to TRICEPS and quenching of an equimolar amount of TRICEPS with glycine (or coupling to a control ligand with known or no binding preferences) the control reaction. (ii) Mild periodate oxidation of target cells or tissue to introduce aldehydes into carbohydrate structures for TRICEPS capture. (iii) LRC and stochastic biotinylation of random cell surface glycoproteins according to their abundance. (iv) Cell lysis and tryptic digest of proteins. (v) Biotin-mediated affinity enrichment of captured glycopeptides on streptavidin beads. (vi) Cell surface N-glycopeptide release by PNGase F treatment, which introduces the N[115]-X-S/T motif (N[115], deamidated asparagine; X, any amino acid except proline; S/T, serine or threonine, respectively) in formerly N-glycosylated peptides. (vii) Glycopeptide identification by high mass accuracy MS and peptide filtering for the presence of the N[115]-X-S/T motif. (viii) Relative label-free quantification of formerly glycosylated cell surface peptides to identify specific LRC events (replicated from Frei et al., 2013).

In this study, we optimized the experimental conditions for isolation of CART receptor from GH3 cells using LRC technology.

First the coupling efficacy of CART with TRICEPS was tested. We coupled TRICEPS reagent with CART, transferrin (Positive control), and glycine (Negative control) and tested for coupling efficiency using a dot blot. CART-TRICEPS signal (Fig 4.2.2A middle row) on the dot blot was comparable to transferrin-TRICEPS (Fig 4.2.2A first row) which was used as a positive control. The glycine-TRICEPS (Fig 4.2.2A third row) was used as a negative control. These results indicated that CART peptide could be successfully coupled with TRICEPS. Next, we optimized the conditions for binding of TRICEPS coupled CART to GH3 cells. FACS analysis was done to assess the efficiency of binding. The fluorescence intensity analysis showed that TRICEPS coupled CART could successfully bind to cell surface receptors on GH3 cells (Fig 4.2.2B) CART treated GH3 cells (red curve) showed a significant increase in fluorescence as compared to glycine, a negative control (blue curve), and a good overlap with transferrin (Green curve), a positive control which is suggestive of efficient binding between TRICEPS-CART and GH3 cells. Note that these cells were non-oxidized, so TRICEPS itself won't bind to the surface of the cells. Therefore this assay detected only ligand-receptor interactions.

Lastly, we tested if GH3 cells could sustain the oxidation treatment used in the LRC experiment. The cells were treated with sodium metaperiodate and tested for survival rate using propidium iodide (PI) staining and subsequent FACS analysis. The FACS analysis showed that the PI fluorescence curve for oxidized cells (Green) coincided with the PI fluorescence curve for non-oxidized cells (Red), suggesting that GH3 cells could survive in oxidation treatment used in the LRC experiment (Fig 4.2.2C).

In summary, these experiments indicate that CART can be successfully coupled with TRICEPS reagent, and GH3 cells can withstand the oxidation treatment required for LRC experiments. Further, we have optimized the binding conditions and shown that TRICEPS coupled CART is able to bind to the cell surface. Our findings suggest that LRC technology can be employed to successfully isolate CART receptor/s from GH3 cells. Moreover, these experiments also provide the direct evidence for receptor-like activity in GH3 cells.

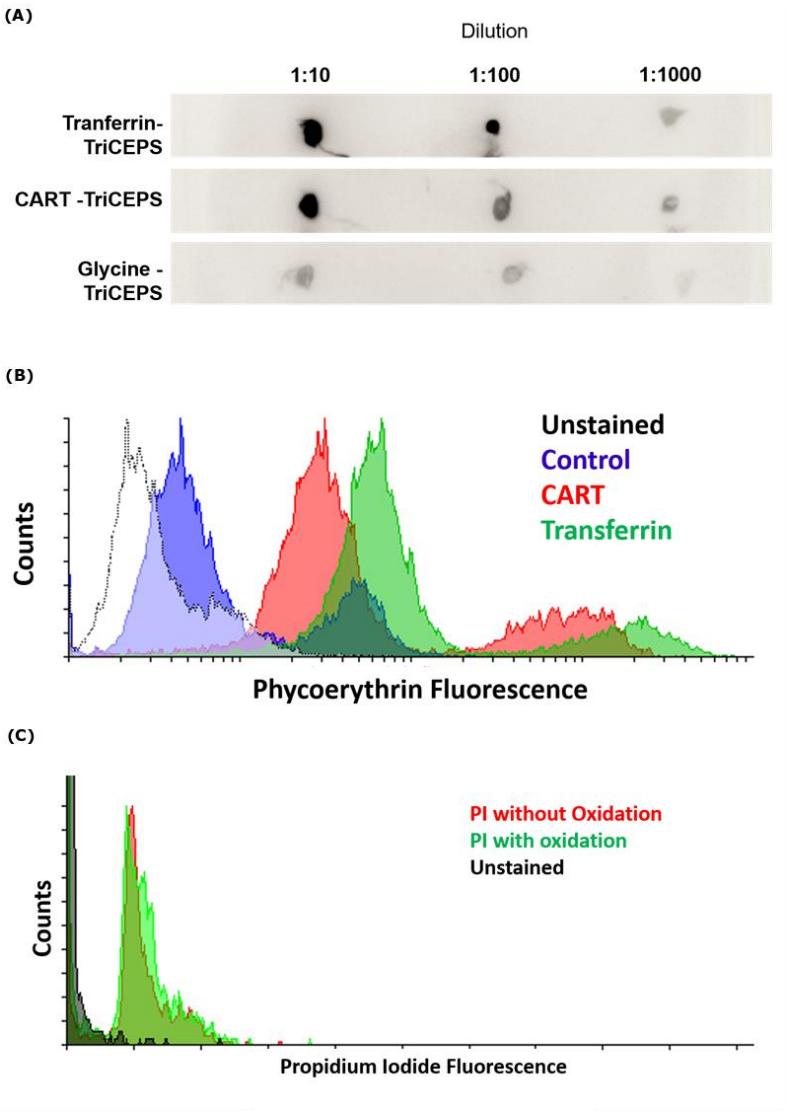


Figure 4.2.2 Optimization of ligand-based receptor capture method for isolation of CART receptor from GH3 cells

(A) CART peptide can be efficiently coupled to the TriCEPS molecule.
Dot blot to assess the coupling of a ligand with the TriCEPS molecule. The first row represents the blot for transferrin (positive control), the second row represents the blot for CART peptide, and the third row represents the blot for negative control (glycine). Each column represents different dilutions of ligand molecules.
(B) TriCEPS coupled CART peptide could successfully bind to GH3 cells. FACS analysis of GH3 cells treated with ligand coupled phycoerythrin labeled TriCEPS.Fluorescence plots for transferrin (green), CART (Red), and Glycine (Blue) treated and untreated (Black) GH3 cells are shown.
(C) GH3 cells can successfully survive the oxidation treatment. The fluorescence plots for propidium iodide treated GH3 cells with(green) and without (red) oxidation treatment and unstained cells (black) are shown.

4.3 Discussion

Previous studies attempting to identify the CART receptor from the brain tissue reported that the labeled peptide failed to show specific binding because CART receptors are predicted to be low-affinity receptors expressed in low abundance. (Vrang et al., 1999, Vicentic et al., 2006). Hence, alternatively, we planned to identify a cell line responding to CART and subsequently use it for identifying and isolating the receptor. Cell lines allow the relatively easy scaling up so that an adequate amount of protein can be isolated for biochemical analysis. Additionally, cell lines produce robust responses, making it easier system to evaluate CART induced responses.

Using calcium imaging analysis, this study has identified a candidate CART responsive cell line- GH3, which shows a robust response to the CART treatment by upregulating intracellular calcium levels. We have also provided evidence for receptor-like activity in GH3 cells and showed direct binding of CART on GH3 cells. These data suggest that GH3 cells can potentially be used to identify and isolate the CART receptor/s.

Immuno-affinity-based techniques have been previously used to isolate many neuropeptide and hormone receptors. In affinity chromatography, the ligand is chemically coupled to beads, specifically capturing cognate receptors when membrane preparation is passed through the column. The subsequent mass spectroscopic analysis allows for the identification of the bound receptor protein. NPY Y1 /Y2 receptors (Sheikh et al., 1991), β-adrenergic receptor (Shorr et al., 1981), Insulin receptor (Fujita-yamaguchi et al., 1983), Opioid receptor (Loukas et al., 1994), Dopamine receptor (Williamson et al., 1988) have been purified in fully functional form using affinity chromatography.

Some of the limitations of affinity-based purification techniques are they can only be used to purify the receptors, which retain their ability to bind to ligands after solubilization and have high-affinity interactions.

Cell surface capture (CSC) technologies coupled with MS have been developed to overcome the inherent problems of cell surface receptors. CSC allows identifying and quantifying an array of glycoproteins expressed on the cell surface at a given point (Bausch-Fluck et al., 2012). As an extension to this technology, ligand-based receptor capture (LRC) has been developed, allowing unbiased and sensitive identification of one or several specific receptors for a given ligand under near-physiological conditions (Frei et al., 2013).

The primary advantage that LRC offers over other purification techniques is that it detects low-affinity interactions by stabilizing ligand-receptor complexes with new covalent bonds, and it works in the cellular microenvironment that enhances binding efficacy (Frei et al., 2013).
This technology has been employed to successfully identify putative receptors for Novel Adipokine CTRP3 (Li et al., 2016) and peptide Dilp8 (Garelli et al., 2015; Li et al., 2016).
Hence, we propose that LRC technology holds great potential to isolate and identify CART receptor/s from GH3 cells successfully.

In this study we have optimized the conditions for LRC mediated isolation of CART receptor/s from GH3 cells and propose that this method could be employed to isolate CART receptor from GH3 cells successfully.
Future studies will use the ligand-based receptor capture (LRC) method to isolate CART receptor/s from GH3 cells. A subsequent mass spectroscopic analysis will be carried out to identify these CART receptor/s.The identified candidate receptor protein/s will be expressed in CART non-responsive cell line-HEK 293 (Lakatos et al., 2005). CART binding and cellular responses will be assessed in the transformed HEK293 cells to validate and characterize the novel receptor/s for CART.

4.4 Materials and methods

4.4.1 Cell culture

Cell lines used-

1. PC12 cells
   Culture medium: DMEM + 5% Horse serum + 5 % FBS
   Differentiation medium: DMEM + 1% Horse serum + 50 ng/mL NGF
2. AtT20 cells (D16v-F2/ CRL-1795 from ATCC)
   Culture medium: DMEM + 10% FBS
3. GH3 cells (NCCS)
   Culture medium: Ham's F12K + 15% Horse serum + 2.5% FBS

Method-

Cells around passage numbers 10 to 35 were used for experiments. The frozen cell aliquots were thawed and cultured in respective culture medium supplemented with serum and 1X PenStrep (Gibco), 1X sodium pyruvate (Invitrogen) at 37^0C with 5% CO2. After the cells were 80-90% confluent, the media was removed, and cells were washed with DPBS. Then cells were trypsinized. For trypsinization1-2 ml of 1X trypsin-EDTA (Lonza) was added to the cells and incubated at 37^0C for 3-5 min. The detached cells were collected in a 15 ml falcon after adding 4 ml medium to the trypsinized cells and then centrifuged at 100xg for 3 min. After aspirating the media, the cell pellet was resuspended in a 1 ml complete medium. Cells were seeded in a 10 ml complete culture medium with approximately 2 X 10^5 density.

4.4.2 Calcium Imaging in cell lines

Chemicals-

- Loading buffer-124 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 24 mM $NaHCO_3$, 5 mM HEPES, 5 mM Glucose, 2 mM $MgSO_4.7H_2O$ and 0.5 mM $CaCl_2.2H_2O$, pH 7.4
- Loading solution-10µM Fluo-4 AM, 2.7 µM probenecid, and 0.1% pluronic in loading buffer.
- Drugs - 200nM CART, CART peptide preadsorbed with anti-mouse CART antibody or anti-mouse IgG (20:1 molar ratio)

Method-

8-chamber slides were precoated with a 1:1 mixture of 0.5 mg/mL Concanavalin A and 0.1 mg/mL Poly-l-lysin for 30 min., and the cells were seeded in the wells at the density of 1 X 10^4

per well and incubated overnight so that cells can adhere to the bottom of wells. The following day cells were washed with pre-warmed (37°C) loading and then loaded with Fluo-4 AM. For loading, 150 µL loading solution was added per well. Loading was carried out in the dark for ~30 min at 37°C. Cells were washed and incubated in a recording medium for 15 mins to allow complete de-esterification of the dye. Calcium activity was recorded at 10fps. Drugs (200nM CART, 2/3.CART peptide preadsorbed with anti-mouse CART antibody or anti-mouse IgG) were added manually 30 sec after the start of recording in the wells with the help of micropipette.

For preadsorption, CART antibody/ Anti Mouse IGg and CART peptide (20:1 molar ratio) were incubated overnight at 4°C.

The data was analyzed using IMAGEJ. The individual cells were segmented manually using threshold tools in IMAGEJ, and the fluorescence intensity of each cell was calculated for each time frame. F0 was obtained by calculating the average of fluorescence values before stimulant treatment. The relative change in fluorescence (dF/F0) for each cell was obtained using the formula F-F0/F0, where F stands for the fluorescence intensity at a given time.

Imaging data were analyzed and compared using a t-test with Welch's correction and plotted using the GraphPad Prism 8.0 statistical software.

4.4.3 Optimization of ligand-based receptor capture method for isolation of CART receptor/s from GH3 cells.

The experiments were performed according to the protocols in the pretest manual provided by Dual-systems biotech. https://www.dualsystems.com/manual/

1) Coupling TRICEPS with the ligand and testing the efficacy using dot blot

Coupling reaction

Method-

1µl of the TRICEPS was added to 0.4µg/µl ligand in 25mM HEPES (pH 8.2) and the mixture was incubated at 22°C under gentle agitation in a thermomixer for 90min.

Dot blot-

Chemicals -

- Blotting buffer (for 1 Litre): 3.03g Tris base 14.4g Glycine 200ml MeOH 0.1g SDS
- Washing buffer: PBS pH7.4, 0.1% Tween 20

- Blocking buffer: PBS pH 7.4, 0,5% milk powder

Method-

TRICEPS-ligand mixture was diluted in blotting buffer in the ratio 1:100 and 1:1000 and 2µL of each dilution was spotted onto the wet nitrocellulose membrane and allowed to dry. The membrane was washed 3 times with washing buffer and put in blocking buffer for 30 mins at room temperature. The membrane was incubated with Streptavidin-HRP (1:1000) and then washed three times with washing buffer for 5 mins each. Finally, the dot-blot was developed with a commercially available enhancer solution for HRP.

2) FACS: to determine the binding of TriCEPS coupled ligand on not-oxidized target cells.

Chemicals-
FACS buffer (PBS pH 6.5, 1% FBS)

Method-

25µl of TriCEPS coupled ligand was incubated on ice for 30 mins. (The incubation duration was optimized to 30 mins). 0.5 x 10^6 cells/sample were harvested by centrifugation at 350xg for 5 min and resuspended in a 200 µL FACS buffer. 3µl TriCEPS coupled ligand was added to the cells and mixture was incubated on ice in the dark. (The incubation duration was optimized to 60 mins). The cells were washed twice using 400µl FACS buffer and stained with R-Phycoerythrin by incubating for 30 min on ice in the dark. Cells were washed twice with with 400 µL FACS buffer by centrifugation at 400xg for 2min and resuspended in 200 µL FACS buffer. The sample was used for FACS analysis.

3) FACS: to determine the survival rate of the cells during oxidation.

Chemicals-
FACS buffer (PBS pH 6.5, 1% FBS)
Oxidation solution: 1.5mM of Sodium metaperiodate in FACS buffer
Live dead cell stain: Propidium Iodide

Method-

0.5 x 10^6 cells/sample were harvested by centrifugation at 350xg for 5 min and resuspended in a 100 µL oxidation solution. The mixture was incubated for 15 min on ice in the dark and cells were washed twice with FACS buffer. Propidium Iodide was added with resultant dilution factor of 1000 and FACS analysis was performed.

5 Chapter 5: Conclusions and Future directions

Feeding behaviour, one of the survival-oriented innate behaviours, is intricately controlled by well-conserved neural mechanisms. This behaviour is composed of two alternating states- hunger and satiety. The duration of each state and transition between them are predominantly regulated by the internal energy conditions of the body. Interoceptory neurons in the brain sense the metabolic requirements and accordingly release orexic/anorexic neuropeptides. These peptides play a central role in establishing stable hunger and satiety states and regulating the transition between them. Neuropeptidergic mechanisms enable neural circuits to acquire functional flexibility to tune the food intake with the body's energy demands. Studies in vertebrates have demonstrated that orexic/anorexic neuropeptides can potently regulate food intake and are critical for establishing hunger-satiety states. However, the complexity of neural circuits in higher vertebrates like mammals limits understanding cellular and molecular level mechanisms of neuropeptidergic actions regulating hunger-satiety states. Simple invertebrate systems like *Drosophila* and C *elegans* have been used to elucidate the molecular mechanisms of neuropeptides and identify neuroanatomical substrates involved in regulating distinct behavioural states- hunger and satiety (Pool and Scott, 2014; Su and Wang, 2014; Yu et al., 2016). However, the translational value of the knowledge gained from invertebrates systems is very limited as their neural systems differ substantially from vertebrates.

Zebrafish, a relatively simple non-mammalian vertebrate, serves as an excellent model system to study mechanisms of peptidergic actions regulating hunger-satiety states. The neuroanatomical substrates and the modulatory agents involved in regulating food intake are well conserved within zebrafish and higher mammals, providing the translational advantage to the general modulatory principles derived from zebrafish research.

Using zebrafish as a model system, this study elucidates molecular level modulatory mechanisms regulating feeding drive and characterizes a novel neuroanatomical region that seems to be involved in regulating huger-satiety bistable states. Here, we demonstrate that antagonistic actions of orexic neuropeptide NPY and anorexic neuropeptide CART regulate the activity of the neural circuit to establish hunger-satiety states and coordinate transition between them. This study identifies molecular candidates involved in neuromodulatory actions of CART and NPY operating *in vivo*, characterizes neuroanatomical correlates that mediate the effects of these neuropeptides, and link them to hunger-satiety behavioural output in adult zebrafish

CART is a well-conserved endogenous satiety factor in vertebrates. Previous studies in our lab found that CART expression in energy sensing regions- EN and the hypothalamus of zebrafish brain is increased in induced satiety conditions (upon ICV glucose administration), indicative of its involvement in regulating satiety state. Interrogation of CART signaling in induced satiety conditions failed to suppress feeding, demonstrating that endogenous CART signaling is essential for reducing feeding drive under satiety conditions. Moreover, we show that exogenous application of CART leads to extreme anorexia even in starved conditions. These data demonstrated that CART signaling is critical for establishing or transitioning into the satiety state in zebrafish.

The anorexic activity of CART is opposed by NPY, a well-conserved orexic agent in vertebrates. Previous studies in the lab showed that NPY is also expressed in EN and the hypothalamus, and in contrast to CART, its levels are upregulated under starved conditions. Further manipulation of endogenous NPY signaling showed that it is necessary for increasing feeding drive under starved conditions, and exogenous application of the peptide can drive orexia. This data demonstrates that NPY signaling is critical for establishing or transitioning into the hunger state in zebrafish. To sum up, these results show that CART and NPY signaling is crucial for maintaining opposing feeding states- Satiety and Hunger, respectively and orchestrating transition between them.

Neuronal tracing studies performed previously in the lab identified a specific region of dorsal telencephalon - Dm, which is a common downstream target for CART and NPY positive neurons in EN and Hypothalamus. Based on this finding, we predicted that this region might be involved in regulating food intake. Interestingly, we find that Dm shows differential activation under fed and starved conditions or upon exogenous application of CART and NPY. Dm is highly active in fed conditions or upon ICV delivery of CART peptide, as indicated by the increased number of pERK positive neurons in these conditions. On the other hand, the same region shows reduced activation in the starved state or upon NPY delivery. These data indicate that antagonistic activities of CART and NPY bidirectionally modulate the activity of Dm according to the energy state of the body.

To identify the plausible molecular candidates involved in feeding regulatory actions of CART and NPY that lead to differential activation of Dm, we used pharmacological interventions and monitored their effects on feeding drive and Dm activity.

Our data suggest that antagonistic activities of CART and NPY activate opposing biochemical signaling pathways that converge on NMDAR function in Dm. Differential regulation of NMDAR activity by CART and NPY leads to energy state-dependent regulation of Dm activity which can be correlated with modulation of feeding drive.

CART signaling activates PKA and PKC to phosphorylate the NR1 subunit of NMDARs, resulting in increased activation of Dm and reduction in feeding drive. In contrast, NPY opposes CART action by dephosphorylating NR1 subunits of NMDARs to decrease activation of Dm neurons and increase feeding drive.NPY signaling reduces PKA activity via downregulation of cAMP levels in the Dm neurons and activates protein phosphatase calcineurin which is known to dephosphorylate NR1 subunit at serine 896, which is a target for PKC.

In summary, antagonistic actions of CART and NPY differentially phosphorylate NR1 subunit of NMDARs by regulating PKA and PKC activities in Dm neurons to regulate feeding drive.

PKA and PKC-mediated phosphorylation of the NR1 subunit is known to increase NMDAR activity and, as a result, excitability of component neurons. Hence, we hypothesized that opposing modulatory actions of CART and NPY regulate the excitability of Dm neurons which is manifested as a differential activation in alternating energy states.

We tested this hypothesis by performing real-time activity imaging to evaluate response to Dm neurons to the excitatory cue-glutamate under different nutritional conditions and upon delivery of peptides that mimic the sated or hungry state. The results indicate that CART signaling leads to the sustained increase in the excitability of Dm neurons under satiety conditions, and NPY signaling leads to decreased excitability of Dm neurons under starvation conditions. Together these results suggest that antagonistic actions of CART and NPY modulate the excitability of Dm neurons to maintain hunger and satiety bistable states. Future studies can employ electrophysiological techniques like patch-clamp to further characterize NMDAR mediated modulation of Dm excitability at the cellular level.

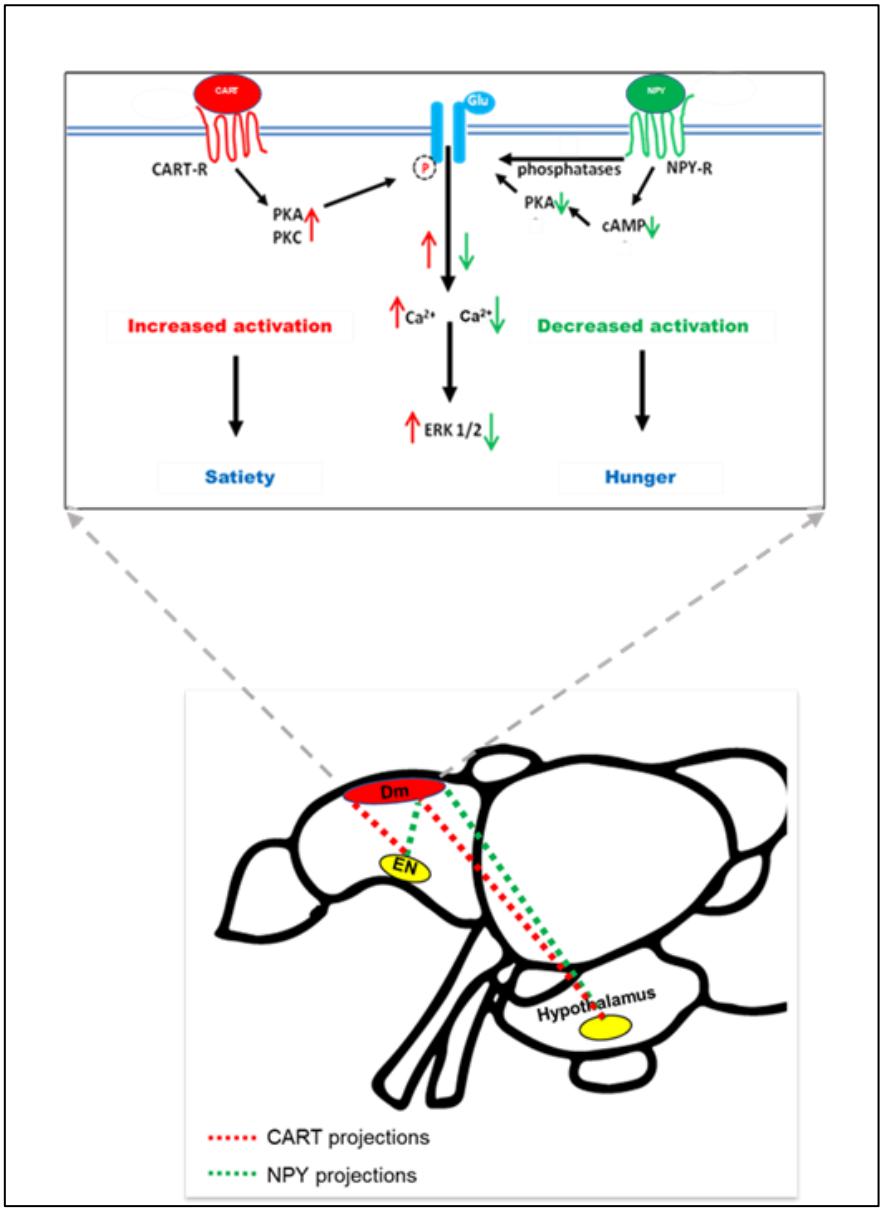
To sum up, our data suggest that the modulatory configuration of biochemical signaling allows Dm to exhibit bi-stable excitability, which forms a neural representation of the energy state of the body and can be correlated with the modulation of feeding drive in zebrafish.

Based on these data, we propose that Dm acts as an integrator of energy-status-related information, possibly relaying this processed information to motor output centers to regulate the transition between two long-lasting states of feeding behaviour - hunger and satiety.

To our knowledge, this is the first study that elucidates circuit-level mechanisms of integration of orexic-anorexic inputs regulating feeding behaviour in vertebrates. Studies in mammals have identified the energy-sensing neurons which produce orexic/ anorexic neuropeptides in response to changes in energy states (Barsh and Schwartz, 2002), but there is very little data available regarding the integration of these signals. Rodent studies suggested that orexic and anorexic activities of NPY and POMC, respectively, are integrated on melanocortin receptor 4 in the paraventricular nucleus of the hypothalamus (Aponte et al., 2011; Atasoy et al., 2012b), but the detailed molecular and circuit-level mechanism are not yet characterized. Using zebrafish as a model system, our research identifies molecular players involved in the integration of orexic and anorexic inputs by NPY and CART, respectively, and also characterizes the cellular-level effects of their antagonistic actions that form a neural representation of the energy status of the body. The general principles derived from this study can be directly applied to mammalian systems to get a better insight into mechanisms of integration of orexic-anorexic inputs

This study also characterizes the novel role of Dm as an integrator of energy status-related signals. Dm is suggested to be the fish homolog of the amygdala (Northcutt, 2006, Wullimann and Mueller, 2004). Though the role of the amygdala in the regulation of feeding is not well characterized, its involvement in the regulation of feeding has been well documented. (White and Fisher, 1969; Zhang et al., 2011). Additionally, the amygdala has also been shown to receive inputs from the interoceptory region- the arcuate nucleus of the hypothalamus (Land et al., 2014). Hence, there is a good chance that the amygdala might be involved in integrating energy status-related signals in mammals. The findings in this study can be directly extended to dissect the role of the amygdala in regulating food intake.

Schematic of the functional model



CART and NPY positive neurons in the EN and the Hypothalamus sense the energy conditions of the body and accordingly regulate the release of orexic neuropeptide (NPY) and anorexic neuropeptide (CART) over Dm neurons. Antagonistic activities of CART and NPY modulate the excitability of Dm neurons by regulating NMDAR activity. The configuration of modulatory signaling allows Dm neurons to attain bistable excitability, which can be correlated with hunger-satiety behavioral output.

Future Directions

This is the first study that demonstrates the involvement of Dm in the regulation of feeding behaviour in zebrafish. The correlation between the activity state of Dm and internal energy conditions is deduced from global changes in expression of neural activity marker pERK in Dm under different nutritional conditions and upon delivery of peptides that mimic the sated or hungry state. Population-level analysis limits our interpretations and allows us to deduce only correlations between modulation of Dm activity and changes in the feeding drive.

Future studies will be employed to identify distinct subpopulations of neurons within Dm that respond to CART and NPY and characterize their role in regulating feeding behaviour in zebrafish. Preliminary results in the lab have shown the presence of NPY-Y1 receptor containing subpopulation of neurons within Dm. Further studies will be performed to identify and characterize these NPY responding subpopulations within Dm. Since the receptor for CART, involved in its anorexic action, is not well characterized, identification of CART responsive populations within Dm remains a difficult task. Hence, we plan to evaluate the binding of labeled CART peptide in the brain slices to identify CART responsive populations in Dm and perform co-labeling studies to test if there is any overlap between modulatory targets of CART and NPY within Dm. Subsequent studies will be performed to identify the specific genetic markers expressed in CART and NPY responding populations in Dm. Specific activity manipulation of these genetically characterized populations using optogenetic and chemogenetic tools would allow us to probe their role in regulating food intake and demonstrate if Dm is causally linked with the regulation of feeding drive in zebrafish.

The modulatory mechanisms regulating long-lasting bistable states like hunger and satiety are known to induce self-sustaining feedback loops that retain the memory of a long-lasting behavioural state until the opposing modulatory input kicks in (Bhalla and Iyengar, 1999). For example- under hunger conditions, ghrelin activates the AMPK-mediated feedback loop, which persistently upregulates the presynaptic activity onto AGRP neurons which serve as a memory for hunger state and activates the hunger circuit till the satiety signals kick in. The elevated presynaptic activity onto AGRP neurons is counter-regulated by the satiety hormone leptin, which activates the satiety circuit. (Yang et al., 2011).

The involvement of PKC and ERK signaling in inducing self-sustaining feedback loops for long-term memory storage in neural systems has already been demonstrated (Bhalla and

Iyengar, 1999; Tanaka and Augustine, 2008). It would be interesting to test if CART mediated activation of kinases and ERK signaling induces self-sustaining feedback loops for maintenance of satiety state and sustained excitability of Dm neurons.

Apart from homeostatic mechanisms, the hedonic properties of food can also drive feeding even under satiety conditions. Hedonic feeding is regulated by reward circuits in the brain. CART has also been suggested to be involved in regulating reward circuits mediating feeding. (Hunter et al., 2004; Jones and Kuhar, 2006; Lau and Herzog, 2014; Rogge et al., 2008a). For example- CART signaling in the nucleus of accumbance is suggested to regulate hedonic feeding via interacting with the dopaminergic system (Lau and Herzog, 2014). However, the mechanisms remain poorly characterized. This study elucidated molecular mechanisms and characterized the brain region involved in CART-mediated regulation of homeostatic feeding. Future studies can extend these findings to explore the role of CART in the regulation of hedonic feeding in zebrafish.

Parallelly, we have undertaken work to identify and characterize novel receptor/s for CART peptide. In this study, we report the identification of a candidate CART responsive cell line- GH3. We find that GH3 shows receptor-like activity and increases intracellular calcium in response to peptide treatment indicating that it can be further used to isolate a CART receptor. We have optimized the protocol for the ligand-based receptor capture (LRC) method, which will be used for isolating the CART receptor from GH3 cells. Future studies will be employed to isolate CART receptor/s using LRC technology with subsequent identification of isolated receptor/s by mass spectroscopy.

References

- Aguilar, A.J., Conde-Sieira, M., López-Patiño, M.A., Míguez, J.M., and Soengas, J.L. (2011). In vitro leptin treatment of rainbow trout hypothalamus and hindbrain affects glucosensing and gene expression of neuropeptides involved in food intake regulation. Peptides *32*, 232–240.
- Akash, G., Kaniganti, T., Tiwari, N.K., Subhedar, N.K., and Ghose, A. (2014). Differential distribution and energy status-dependent regulation of the four CART neuropeptide genes in the zebrafish brain. J. Comp. Neurol. *522*, 2266–2285.
- Andermann, M.L., and Lowell, B.B. (2017). Review Toward a Wiring Diagram Understanding of Appetite Control. Neuron *95*, 757–778.
- Aponte, Y., Atasoy, D., and Sternson, S.M. (2011). AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat. Neurosci. *14*, 351–355.
- Assad, N., Luz, W.L., Santos-Silva, M., Carvalho, T., Moraes, S., Picanço-Diniz, D.L.W., Bahia, C.P., Oliveira Batista, E. de J., da Conceição Passos, A., Oliveira, K.R.H.M., et al. (2020). Acute Restraint Stress Evokes Anxiety-Like Behavior Mediated by Telencephalic Inactivation and GabAergic Dysfunction in Zebrafish Brains. Sci. Rep. *10*, 1–8.
- Atasoy, D., Nicholas Betley, J., Su, H.H., and Sternson, S.M. (2012). Deconstruction of a neural circuit for hunger. Nature *488*, 172–177.
- Bannon, A.W., Seda, J., Carmouche, M., Francis, J.M., Jarosinski, M.A., and Douglass, J. (2001). Multiple behavioral effects of cocaine- and amphetamineregulated transcript (CART) peptides in mice: CART 42-89 and CART 49-89 differ in potency and activity. J. Pharmacol. Exp. Ther. *299*, 1021–1026.
- Baranowska, B., Wolińska-Witort, E., Chmielowska, M., Martyńska, L., and Baranowska-Bik, A. (2003). Direct effects of cocaine-amphetamine-regulated transcript (CART) on pituitary hormone release in pituitary cell culture. Neuro Endocrinol. Lett. *24*, 224–226.
- Bargmann, C.I. (2012). Beyond the connectome: How neuromodulators shape neural circuits. BioEssays *34*, 458–465.
- Barsh, G.S., and Schwartz, M.W. (2002). Genetic approaches to studying energy balance: Perception and integration. Nat. Rev. Genet. *3*, 589–600.
- Bausch-Fluck, D., Hofmann, A., and Wollscheid, B. (2012). Cell surface capturing technologies for the surfaceome discovery of hepatocytes. Methods Mol. Biol. *909*, 1–16.
- Beckman, T.R., Shi, Q., Levine, A.S., and Billington, C.J. (2009). Amygdalar opioids modulate hypothalamic melanocortin-induced anorexia. Physiol. Behav. *96*, 568–573.
- Ben Arous, J., Laffont, S., and Chatenay, D. (2009). Molecular and sensory basis of a food related two-state behavior in C. elegans. PLoS One *4*, 1–8.
- Bhalla, U.S., and Iyengar, R. (1999). Emergent properties of networks of biological signaling

pathways. Science *283*, 381–387.

- Blevins, J.E., and Baskin, D.G. (2009). Hypothalamic-brainstem circuits controlling eating. Front. Eat. Weight Regul. *63*, 133–140.
- Burns, G.A., and Ritter, R.C. (1997). The Non-Competitive NMDA Antagonist MK-801 Increases Food Intake in Rats. Pharmacol. Biochem. Behav. *56*, 145–149.
- Bucher, D., Haspel, G., Golowasch, J., and Nadim, F. (2015). Central Pattern Generators. In ELS, (Wiley), pp. 1–12.
- Cai, H., Haubensak, W., Anthony, T.E., and Anderson, D.J. (2014). Central amygdala PKC-δ+ neurons mediate the influence of multiple anorexigenic signals. Nat. Neurosci. *17*, 1240–1248.
- Carter, M.E., Soden, M.E., Zweifel, L.S., and Palmiter, R.D. (2013). Genetic identification of a neural circuit that suppresses appetite. Nature *503*, 111–114.
- Chatterjea, D., Hamid, E., Leonard, J.P., and Alford, S. (2010). Phosphorylation-state-dependent regulation of NMDA receptor short-term plasticity modifies hippocampal dendritic Ca2+ transients. J. Neurophysiol. *104*, 2203–2213.
- Chen, B.S., and Roche, K.W. (2007). Regulation of NMDA receptors by phosphorylation. Neuropharmacology *53*, 362–368.
- Chen, B.S., Braud, S., Badger, J.D., Isaac, J.T.R., and Roche, K.W. (2006). Regulation of NR1/NR2C N-methyl-D-aspartate (NMDA) receptors by phosphorylation. J. Biol. Chem. *281*, 16583–16590.
- Chiu, H.Y., Lin, H.H., and Lai, C.C. (2009). Potentiation of spinal NMDA-mediated nociception by cocaine- and amphetamine-regulated transcript peptide via PKA and PKC signaling pathways in rats. Regul. Pept. *158*, 77–85.
- Choudhary, A.G., Somalwar, A.R., Sagarkar, S., Rale, A., Sakharkar, A., Subhedar, N.K., and Kokare, D.M. (2018). CART neurons in the lateral hypothalamus communicate with the nucleus accumbens shell via glutamatergic neurons in paraventricular thalamic nucleus to modulate reward behavior. Brain Struct. Funct. *223*, 1313–1328.
- Cowley, M. a, Smart, J.L., Rubinstein, M., Cerdán, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature *411*, 480–484.
- Cowley, M.A., Smith, R.G., Diano, S., Tscho, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L., et al. (2003). The Distribution and Mechanism of Action of Ghrelin in the CNS Demonstrates a Novel Hypothalamic Circuit Regulating Energy Homeostasis. *37*, 649–661.
- D'Agostino, G., Lyons, D.J., Cristiano, C., Burke, L.K., Madara, J.C., Campbell, J.N., Garcia, A.P., Land, B.B., Lowell, B.B., Dileone, R.J., et al. (2016). Appetite controlled by a cholecystokinin nucleus of the solitary tract to hypothalamus neurocircuit. Elife *5*.

- Delgado, M.J., Cerdá-Reverter, J.M., and Soengas, J.L. (2017). Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: Involvement in the control of food intake. Front. Neurosci. *11*.
- Douglass, J., and Daoud, S. (1996). Characterization of the human cDNA and genomic DNA encoding CART: A cocaine-and amphetamine-regulated transcript. Gene *169*, 241–245.
- Douglass, J., McKinzie, A.A., and Couceyro, P. (1995). PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. J. Neurosci. *15*, 2471–2481.
- Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., et al. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. *205*, 38–44.
- Eriksson, A., Raczkowska, M., Navawongse, R., Choudhury, D., Stewart, J.C., Tang, Y.L., Wang, Z., and Claridge-Chang, A. (2017). Neuromodulatory circuit effects on Drosophila feeding behaviour and metabolism. Sci. Rep. *7*, 1–12.
- Fan, W., Ellacott, K.L.J., Halatchev, I.G., Takahashi, K., Yu, P., and Cone, R.D. (2004). Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. Nat. Neurosci. *7*, 335–336.
- Farzi, A., Lau, J., Ip, C.K., Qi, Y., Shi, Y.C., Zhang, L., Tasan, R., Sperk, G., and Herzog, H. (2018). Arcuate nucleus and lateral hypothalamic cart neurons in the mouse brain exert opposing effects on energy expenditure. Elife *7*, 1–27.
- Fekete, É.M., Bagi, É.E., Tóth, K., and Lénárd, L. (2007). Neuromedin C microinjected into the amygdala inhibits feeding. Brain Res. Bull. *71*, 386–392.
- Flavell, S.W., Pokala, N., Macosko, E.Z., Albrecht, D.R., Larsch, J., and Bargmann, C.I. (2013). Serotonin and the neuropeptide PDF initiate and extend opposing behavioral states in C. Elegans. Cell *154*, 1023–1035.
- Förster, D., Arnold-Ammer, I., Laurell, E., Barker, A.J., Fernandes, A.M., Finger-Baier, K., Filosa, A., Helmbrecht, T.O., Kölsch, Y., Kühn, E., et al. (2017). Genetic targeting and anatomical registration of neuronal populations in the zebrafish brain with a new set of BAC transgenic tools. Sci. Rep. *7*, 5230.
- Frei, A.P., Moest, H., Novy, K., and Wollscheid, B. (2013). Ligand-based receptor identification on living cells and tissues using TRICEPS. Nat. Protoc. *8*, 1321–1336.
- Friedrich, R.W., Jacobson, G.A., and Zhu, P. (2010). Circuit neuroscience in zebrafish. Curr. Biol. *20*, R371-81.
- Fu, W., Nelson, T.S., Santos, D.F., Doolen, S., Gutierrez, J.J.P., Ye, N., Zhou, J., and Taylor, B. (2019). An NPY Y1 receptor antagonist unmasks latent sensitization and reveals the contribution of Protein Kinase A and EPAC to chronic inflammatory pain.

- Fujita-yamaguchi, Y., Choi, S., Sakamotos, Y., and Itakura, K. (1983). Purification of Insulin Receptor with Full Binding Activity *. *258*, 5045–5049.
- Garelli, A., Heredia, F., Casimiro, A.P., Macedo, A., Nunes, C., Garcez, M., Dias, A.R.M., Volonte, Y.A., Uhlmann, T., Caparros, E., et al. (2015). Dilp8 requires the neuronal relaxin receptor Lgr3 to couple growth to developmental timing. Nat. Commun. *6*, 1–14.
- Gaykema, R.P., Newmyer, B.A., Ottolini, M., Raje, V., Warthen, D.M., Lambeth, P.S., Niccum, M., Yao, T., Huang, Y., Schulman, I.G., et al. (2017). Activation of murine pre-proglucagon-producing neurons reduces food intake and body weight. J. Clin. Invest. *127*, 1031–1045.
- Giesbrecht, C.J., Mackay, J.P., Silveira, H.B., Urban, J.H., and Colmers, W.F. (2010). Countervailing Modulation of Ih by Neuropeptide Y and Corticotrophin-Releasing Factor in Basolateral Amygdala As a Possible Mechanism for Their Effects on Stress-Related Behaviors. J. Neurosci. *30*, 16970–16982.
- Gründemann, J., Bitterman, Y., Lu, T., Krabbe, S., Grewe, B.F., Schnitzer, M.J., and Lüthi, A. (2019). Amygdala ensembles encode behavioral states. Science (80-.). *364*.
- Guard, D.B., Swartz, T.D., Ritter, R.C., Burns, G.A., and Covasa, M. (2009). NMDA NR2 receptors participate in CCK-induced reduction of food intake and hindbrain neuronal activation. Brain Res. *1266*, 37–44.
- Haberny, S.L., and Carr, K.D. (2005). Food restriction increases NMDA receptor-mediated calcium-calmodulin kinase II and NMDA receptor/extracellular signal-regulated kinase 1/2-mediated cyclic amp response element-binding protein phosphorylation in nucleus accumbens upon D-1 dopamine receptor. Neuroscience *132*, 1035–1043.
- Haddock, C.J., Almeida-Pereira, G., Stein, L.M., Hayes, M.R., Kolar, G.R., Samson, W.K., and Yosten, G.L.C. (2021). Signaling in rat brainstem via Gpr160 is required for the anorexigenic and antidipsogenic actions of cocaine- and amphetamine-regulated transcript peptide. Am. J. Physiol. Regul. Integr. Comp. Physiol. *320*, R236–R249.
- Harris-Warrick, R.M., and Marder, E. (1991). Modulation of neural networks for behavior. Annu. Rev. Neurosci. *14*, 39–57.
- Harris-Warrick, R.M. (2011). Neuromodulation and flexibility in Central Pattern Generator networks. Current Opinion in Neurobiology *21*, 685–692
- Haskell-Luevano, C., and Monck, E.K. (2001). Agouti-related protein functions as an inverse agonist at a constitutively active brain melanocortin-4 receptor. Regul. Pept. *99*, 1–7.
- Heilig, M. (1995). Antisense inhibition of neuropeptide Y (NPY)-Y1 receptor expression blocks the anxiolytic-like action of NPY in amygdala and paradoxically increases feeding. Regul. Pept. *59*, 201–205.
- Huang, L.Y.M. (1993). Sustained potentiation of NMDA receptor-mediated glutamate

responses through activation of protein kinase-c by a muopioid by chen,l., huang,l.y.m. Scientist *7*, 15.

- Hunter, R.G., Philpot, K., Vicentic, A., Dominguez, G., Hubert, G.W., and Kuhar, M.J. (2004). CART in feeding and obesity. Trends Endocrinol. Metab. *15*, 454–459.
- Inagaki, H.K., Panse, K.M., and Anderson, D.J. (2014). Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in Drosophila. Neuron *84*, 806–820.
- Jékely, G., Melzer, S., Beets, I., Kadow, I.C.G., Koene, J., Haddad, S., and Holden-Dye, L. (2018). The long and the short of it -A perspective on peptidergic regulation of circuits and behaviour. J. Exp. Biol. *221*.
- Jeong, I., Kim, E., Kim, S., Kim, H.K., Lee, D.W., Seong, J.Y., and Park, H.C. (2018). mRNA expression and metabolic regulation of npy and agrp1/2 in the zebrafish brain. Neurosci. Lett. *668*, 73–79.
- Jin, I., Kandel, E.R., and Hawkins, R.D. (2011). Whereas short-term facilitation is presynaptic, intermediate-term facilitation involves both presynaptic and postsynaptic protein kinases and protein synthesis. Learn. Mem. *18*, 96–102.
- Johannsen, D. (1933). Review of Purposive Behavior in Animals and Men. J. Abnorm. Soc. Psychol. *28*, 222–223.
- Johnson, A.W. (2013). Eating beyond metabolic need: How environmental cues influence feeding behavior. Trends Neurosci. *36*, 101–109.
- Jones, D.C., and Kuhar, M.J. (2006). Cocaine-Amphetamine-Regulated Transcript Expression in the Rat Nucleus Accumbens Is Regulated by Adenylyl Cyclase and the Cyclic Adenosine 5J-Monophosphate / Protein Kinase A Second Messenger System. J. Pharmacol. Exp. Ther. *317*, 454–461.
- Jones, D.C., and Kuhar, M.J. (2008). CART receptor binding in primary cell cultures of the rat nucleus accumbens. Synapse *62*, 122–127.
- Kaniganti, T., Deogade, A., Maduskar, A., Mukherjee, A., Guru, A., Subhedar, N., and Ghose, A. Sensitivity of olfactory sensory neurons to food cues is tuned to nutritional states by Neuropeptide Y signaling. J. Neurochem. *n/a*.
- Katsurada, K., Maejima, Y., Nakata, M., Kodaira, M., Suyama, S., Iwasaki, Y., Kario, K., and Yada, T. (2014). Endogenous GLP-1 acts on paraventricular nucleus to suppress feeding: Projection from nucleus tractus solitarius and activation of corticotropin-releasing hormone, nesfatin-1 and oxytocin neurons. Biochem. Biophys. Res. Commun. *451*, 276–281.
- Kawakami, K., Abe, G., Asada, T., Asakawa, K., Fukuda, R., Ito, A., Lal, P., Mouri, N., Muto, A., Suster, M.L., et al. (2010). zTrap: zebrafish gene trap and enhancer trap database. BMC Dev. Biol. *10*, 105.

- Keller, P.A., Compan, V., Bockaert, J., Giacobino, J.P., Charnay, Y., Bouras, C., and Assimacopoulos-Jeannet, F. (2006). Characterization and localization of cocaine- and amphetamine-regulated transcript (CART) binding sites. Peptides *27*, 1328–1334.
- Klein, M., Camardo, J., and Kandel, E.R. (1982). Serotonin modulates a specific potassium current in the sensory neurons that show presynaptic facilitation in Aplysia. Proc. Natl. Acad. Sci. *79*, 5713–5717.
- Kmiec, Z. (2006). Central regulation of food intake in ageing. J. Physiol. Pharmacol. *57*, 7–16.
- Kohno, D., and Yada, T. (2012). Arcuate NPY neurons sense and integrate peripheral metabolic signals to control feeding. Neuropeptides *46*, 315–319.
- Kovács, A., László, K., Gálosi, R., Tóth, K., Ollmann, T., Péczely, L., and Lénárd, L. (2012). Microinjection of RFRP-1 in the central nucleus of amygdala decreases food intake in the rat. Brain Res. Bull. *88*, 589–595.
- Koylu, E.O., Balkan, B., Kuhar, M.J., and Pogun, S. (2006). Cocaine and amphetamine regulated transcript (CART) and the stress response. Peptides *27*, 1956–1969.
- Krashes, M.J., Shah, B.P., Madara, J.C., Olson, D.P., Strochlic, D.E., Garfield, A.S., Vong, L., Pei, H., Watabe-Uchida, M., Uchida, N., et al. (2014). An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. Nature *507*, 238–242.
- Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., et al. (1998). Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature *393*, 72–76.
- Kuriyama, G., Takekoshi, S., Tojo, K., Nakai, Y., Kuhar, M.J., and Osamura, R.Y. (2004). Cocaine- and amphetamine-regulated transcript peptide in the rat anterior pituitary gland is localized in gonadotrophs and suppresses prolactin secretion. Endocrinology *145*, 2542–2550.
- Lakatos, A., Prinster, S., Vicentic, A., Hall, R.A., and Kuhar, M.J. (2005). Cocaine- and amphetamine-regulated transcript (CART) peptide activates the extracellular signal-regulated kinase (ERK) pathway in AtT20 cells via putative G-protein coupled receptors. Neurosci. Lett. *384*, 198–202.
- Lan, J.Y., Skeberdis, V.A., Jover, T., Grooms, S.Y., Lin, Y., Araneda, R.C., Zheng, X., Bennett, M. V, and Zukin, R.S. (2001). Protein kinase C modulates NMDA receptor trafficking and gating. Nat. Neurosci. *4*, 382–390.
- Land, B.B., Narayanan, N.S., Liu, R.J., Gianessi, C.A., Brayton, C.E., M Grimaldi, D., Sarhan, M., Guarnieri, D.J., Deisseroth, K., Aghajanian, G.K., et al. (2014). Medial prefrontal D1 dopamine neurons control food intake. Nat. Neurosci. *17*, 248–253.
- Larsen, P.J., Vrang, N., Petersen, P.C., and Kristensen, P. (2000). Chronic intracerebroventricular administration of recombinant CART(42-89) peptide inhibits food intake and causes weight loss in lean and obese Zucker (fa/fa) rats. Obes. Res. *8*, 590–596.

- Lau, J., and Herzog, H. (2014). CART in the regulation of appetite and energy homeostasis. Front. Neurosci. *8*.
- Lau, C.G., Takeuchi, K., Rodenas-Ruano, A., Takayasu, Y., Murphy, J., Bennett, M.V.L., and Zukin, R.S. (2009). Regulation of NMDA receptor Ca2+ signalling and synaptic plasticity. Biochem. Soc. Trans. *37*, 1369–1374.
- Lau, J., Shi, Y.-C., and Herzog, H. (2016). Temperature dependence of the control of energy homeostasis requires CART signaling. Neuropeptides *59*, 97–109.
- Lee, D.A., and Blackshaw, S. (2014). Feed Your Head: Neurodevelopmental Control of Feeding and Metabolism. Annu. Rev. Physiol. *76*, 197–223.
- Lee, S.W., and Stanley, B.G. (2005). NMDA receptors mediate feeding elicited by neuropeptide Y in the lateral and perifornical hypothalamus. Brain Res. *1063*, 1–8.
- Li, Y., Ozment, T., Wright, G.L., and Peterson, J.M. (2016). Identification of putative receptors for the novel adipokine CTRP3 using ligand-receptor capture technology. PLoS One *11*, 1–18.
- Lima, S.L., and Dill, L.M. (1990). Behavioral decisions made under the risk of predation: a review and prospectus. Can. J. Zool. *68*, 619–640.
- Lin, H.H., Chiu, H.Y., and Lai, C.C. (2005). Potentiation of spinal N-methyl-D-aspartate-mediated nociceptive transmission by cocaine-regulated and amphetamine-regulated transcript peptide in rats. Neuroreport *16*, 253–257.
- Lin, X., Volkoff, H., Narnaware, Y., Bernier, N.J., Peyon, P., and Peter, R.E. (2000a). Brain regulation of feeding behavior and food intake in fish. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. *126*, 415–434.
- Lin, Y., Hall, R.A., and Kuhar, M.J. (2011). CART peptide stimulation of G protein-mediated signaling in differentiated PC12 Cells: Identification of PACAP 6-38 as a CART receptor antagonist. Neuropeptides *45*, 351–358.
- Liu, T., Kong, D., Shah, B.P., Ye, C., Koda, S., Saunders, A., Ding, J.B., Yang, Z., Sabatini, B.L., and Lowell, B.B. (2012). Fasting Activation of AgRP Neurons Requires NMDA Receptors and Involves Spinogenesis and Increased Excitatory Tone. Neuron *73*, 511–522.
- Loh, K., Herzog, H., and Shi, Y.C. (2015). Regulation of energy homeostasis by the NPY system. Trends Endocrinol. Metab. *26*, 125–135.
- Loukas, S., Mercouris, M., and Panetsos, F. (1994). Purification to homogeneity of an active opioid receptor from rat brain by affinity chromatography. *91*, 4574–4578.
- MacDonald, E., and Volkoff, H. (2009). Neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) and cholecystokinin (CCK) in winter skate (Raja ocellata): cDNA cloning, tissue distribution and mRNA expression responses to fasting. Gen. Comp. Endocrinol. *161*, 252–261.
- Maletínská, L., Maixnerová, J., Matyšková, R., Haugvicová, R., Šloncová, E., Elbert, T.,

Slaninová, J., and Železná, B. (2007). Cocaine- and amphetamine-regulated transcript (CART) peptide specific binding in pheochromocytoma cells PC12. Eur. J. Pharmacol. *559*, 109–114.

- Marder, E. (2012). Neuromodulation of Neuronal Circuits: Back to the Future. Neuron *76*, 1–11.
- Marder, E., and Bucher, D. (2007). Understanding Circuit Dynamics Using the Stomatogastric Nervous System of Lobsters and Crabs. Annu. Rev. Physiol. *69*, 291–316.
- Marder, E., and Thirumalai, V. (2002). Cellular, synaptic and network effects of neuromodulation. Neural Networks *15*, 479–493.
- Matsuda, K. (2009). Recent Advances in the Regulation of Feeding Behavior by Neuropeptides in Fish. *250*, 241–250.
- Matsuda, K. (2019). Recent Advances in the Regulation of Feeding Behavior by Neuropeptides in Fish.
- Mobbs, D., Trimmer, P.C., Blumstein, D.T., and Dayan, P. (2018). Foraging for foundations in decision neuroscience: insights from ethology. Nat. Rev. Neurosci. *19*, 419–427.
- Molosh, A.I., Sajdyk, T.J., Truitt, W.A., Zhu, W., Oxford, G.S., and Shekhar, A. (2013). NPY Y1 Receptors Differentially Modulate GABAA and NMDA Receptors via Divergent Signal-Transduction Pathways to Reduce Excitability of Amygdala Neurons. Neuropsychopharmacology *38*, 1352–1364.
- Morton, G.J., Cummings, D.E., Baskin, D.G., Barsh, G.S., and Schwartz, M.W. (2006). Central nervous system control of food intake and body weight. Nature *443*, 289–295.
- Mu, Y., Narayan, S., Mensh, B.D., and Ahrens, M.B. (2020). Brain-wide, scale-wide physiology underlying behavioral flexibility in zebrafish. Curr. Opin. Neurobiol. *64*, 151–160.
- Mukherjee, A., Subhedar, N.K., and Ghose, A. (2012). Ontogeny of the cocaine- and amphetamine-regulated transcript (CART) neuropeptide system in the brain of zebrafish, Danio rerio. J. Comp. Neurol. *520*, 770–797.
- Nadim, F., and Bucher, D. (2014). Neuromodulation of neurons and synapses. Curr. Opin. Neurobiol. *29*, 48–56.
- Nagelová, V., Pirník, Z., Železná, B., and Maletínská, L. (2014). CART (cocaine- and amphetamine-regulated transcript) peptide specific binding sites in PC12 cells have characteristics of CART peptide receptors. Brain Res. *1547*, 16–24.
- Narnaware, Y.K., and Peter, R.E. (2001). Neuropeptide Y stimulates food consumption through multiple receptors in goldfish. Physiol. Behav. *74*, 185–190.
- Neyens, D., Zhao, H., Huston, N.J., Wayman, G.A., Ritter, R.C., and Appleyard, S.M. (2020). Leptin sensitizes NTS neurons to vagal input by increasing postsynaptic NMDA receptor currents. J. Neurosci. *40*, 7054–7064.
- Northcutt, R.G. (2006). Connections of the lateral and medial divisions of the goldfish

telencephalic pallium. J. Comp. Neurol. *494*, 903–943.

- Ollmann, M.M., Lamoreux, M.L., Wilson, B.D., and Barsh, G.S. (1998). Interaction of Agouti protein with the melanocortin 1 receptor in vitro and in vivo. Genes Dev. *12*, 316–330.
- Opazo, R., Plaza-Parrochia, F., Cardoso Dos Santos, G.R., Carneiro, G.R.A., Sardela, V.F., Romero, J., and Valladares, L. (2019). Fasting Upregulates npy, agrp, and ghsr without Increasing Ghrelin Levels in Zebrafish (Danio rerio) Larvae. Front. Physiol. *10*, 1–13.
- Otero-Rodiño, C., Rocha, A., Álvarez-Otero, R., Ceinos, R.M., López-Patiño, M.A., Míguez, J.M., Cerdá-Reverter, J.M., and Soengas, J.L. (2018). Glucosensing capacity of rainbow trout telencephalon. J. Neuroendocrinol. *30*, 0–2.
- Padilla, S.L., Qiu, J., Soden, M.E., Sanz, E., Nestor, C.C., Barker, F.D., Quintana, A., Zweifel, L.S., Rønnekleiv, O.K., Kelly, M.J., et al. (2016). Agouti-related peptide neural circuits mediate adaptive behaviors in the starved state. Nat. Neurosci. *19*, 734–741.
- van den Pol, A.N. (2012). Neuropeptide Transmission in Brain Circuits. Neuron *76*, 98–115.
- Pool, A.H., and Scott, K. (2014). Feeding regulation in Drosophila. Curr. Opin. Neurobiol. *29*, 57–63.
- Portavella, M., Torres, B., Salas, C., and Papini, M.R. (2004). Lesions of the medial pallium, but not of the lateral pallium, disrupt spaced-trial avoidance learning in goldfish (Carassius auratus). Neurosci. Lett. *362*, 75–78.
- Quintana, A., Sanz, E., Wang, W., Storey, G.P., Güler, A.D., Wanat, M.J., Roller, B.A., Torre, A. La, Amieux, P.S., Stanley, G., et al. (2013). HHS Public Access. *15*, 1547–1555.
- Rale, A., Shendye, N., Bodas, D.S., Subhedar, N., and Ghose, A. (2017). CART neuropeptide modulates the extended amygdalar CeA-vBNST circuit to gate expression of innate fear. Psychoneuroendocrinology *85*, 69–77.
- Randlett, O., Wee, C.L., Naumann, E.A., Nnaemeka, O., Schoppik, D., Fitzgerald, J.E., Portugues, R., Lacoste, A.M.B., Riegler, C., Engert, F., et al. (2015). Whole-brain activity mapping onto a zebrafish brain atlas. Nat. Methods *12*, 1039–1046.
- Resch, J.M., Maunze, B., Phillips, K.A., and Choi, S. (2014). Inhibition of food intake by PACAP in the hypothalamic ventromedial nuclei is mediated by NMDA receptors. Physiol. Behav. *133*, 230–235.
- Rogge, G., Jones, D., Hubert, G.W., Lin, Y., and Kuhar, M.J. (2008). CART peptides: regulators of body weight, reward and other functions. Nat. Rev. Neurosci. *9*, 747–758.
- Sainsbury, A., and Zhang, L. (2010). Role of the arcuate nucleus of the hypothalamus in regulation of body weight during energy deficit. Mol. Cell. Endocrinol. *316*, 109–119.
- Sajdyk, T.J., Johnson, P.L., Leitermann, R.J., Fitz, S.D., Dietrich, A., Morin, M., Gehlert, D.R., Urban, J.H., and Shekhar, A. (2008). Neuropeptide Y in the Amygdala Induces Long-Term Resilience to Stress-Induced Reductions in Social Responses But Not Hypothalamic-Adrenal-

Pituitary Axis Activity or Hyperthermia. J. Neurosci. *28*, 893–903.

- Salas, C., Broglio, C., Durán, E., Gómez, A., Ocaña, F.M., Jiménez-moya, F., and Rodríguez, F. (2006). Vertebrate Brain Evolution. Zebrafish *3*, 157–171.
- Salter, M.W., Dong, Y., and Kalia, L. V (2009). of NMDA 7 Regulation Receptors by Kinases and Phosphatases. 123–148.
- Shanley, L.J., Irving, A.J., and Harvey, J. (2001). Leptin enhances NMDA receptor function and modulates hippocampal synaptic plasticity. J. Neurosci. *21*, 1–6.
- Sheikh, S.P., Hansen, A.P., and Williams, J.A. (1991). Solubilization and affinity purification of the Y2 receptor for neuropeptide Y and peptide YY from rabbit kidney. J. Biol. Chem. *266*, 23959–23966.
- Shorr, R.G.L., Lefkowitz, R.J., and Caronf, M.G. (1981). Purification of the /? -Adrenergic Receptor. *256*, 5820–5826.
- Singh, A., de Araujo, A.M., Krieger, J.P., Vergara, M., Ip, C.K., and de Lartigue, G. (2021). Demystifying functional role of cocaine- and amphetamine-related transcript (CART) peptide in control of energy homeostasis: A twenty-five year expedition. Peptides *140*, 170534.
- Skeberdis, V.A., Chevaleyre, V., Lau, C.G., Goldberg, J.H., Pettit, D.L., Suadicani, S.O., Lin, Y., Bennett, M.V.L., Yuste, R., Castillo, P.E., et al. (2006). Protein kinase A regulates calcium permeability of NMDA receptors. Nat. Neurosci. *9*, 501–510.
- Smedh, U., Scott, K.A., and Moran, T.H. (2015). Fourth ventricular CART peptide induces c-fos in the area postrema and nucleus of the solitary tract via a CRF-receptor dependent mechanism. Neurosci. Lett. *609*, 124–128.
- Soengas, J.L., Cerdá-Reverter, J.M., and Delgado, M.J. (2018). Central regulation of food intake in fish: An evolutionary perspective. J. Mol. Endocrinol. *60*, R171–R199.
- Sohn, J.W., Elmquist, J.K., and Williams, K.W. (2013). Neuronal circuits that regulate feeding behavior and metabolism. Trends Neurosci. *36*, 504–512.
- Spiess, J., Villarreal, J., and Vale, W. (1981). Isolation and Sequence Analysis of a Somatostatin-like Polypeptide from Ovine Hypothalamus. Biochemistry *20*, 1982–1988.
- Stanley, B.G., and Leibowitz, S.F. (1984). Neuropeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. Life Sci. *35*, 2635–2642.
- Stanley, S.A., Small, C.J., Murphy, K.G., Rayes, E., Abbott, C.R., Seal, L.J., Morgan, D.G.A., Sunter, D., Dakin, C.L., Kim, M.S., et al. (2001). Actions of cocaine- and amphetamine-regulated transcript (CART) peptide on regulation of appetite and hypothalamo–pituitary axes in vitro and in vivo in male rats. Brain Res. *893*, 186–194.
- Sternson, S.M., Nicholas Betley, J., and Cao, Z.F.H. (2013). Neural circuits and motivational processes for hunger. Curr. Opin. Neurobiol. *23*, 353–360.
- Su, C.Y., and Wang, J.W. (2014). Modulation of neural circuits: How stimulus context shapes

innate behavior in Drosophila. Curr. Opin. Neurobiol. *29*, 9–16.

- Subhedar, N., Barsagade, V.G., Singru, P.S., Thim, L., and Clausen, J.T. (2011). Cocaine- and amphetamine-regulated transcript peptide (CART) in the telencephalon of the catfish, Clarias gariepinus: Distribution and response to fasting, 2-deoxy-D-glucose, glucose, insulin, and leptin treatments. J. Comp. Neurol. *519*, 1281–1300.
- Subhedar, N.K., Nakhate, K.T., Upadhya, M.A., and Kokare, D.M. (2014). CART in the brain of vertebrates: Circuits, functions and evolution. Peptides *54*, 108–130.
- Sutton, A.K., and Krashes, M.J. (2020). Integrating Hunger with Rival Motivations. Trends Endocrinol. Metab. *31*, 495–507.
- Takahashi, K. a, and Cone, R.D. (2005). Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. Endocrinology *146*, 1043–1047.
- Tanaka, K., and Augustine, G.J. (2008). A positive feedback signal transduction loop determines timing of cerebellar long-term depression. Neuron *59*, 608–620.
- Tatemoto, K., Carlquist, M., and Mutt, V. (1982). Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature *296*, 659–660.
- von Trotha, J.W., Vernier, P., and Bally-Cuif, L. (2014b). Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. Eur. J. Neurosci. *40*, 3302–3315.
- Upadhya, M.A., Kokare, D.M., and Subhedar, N.K. (2013). Cocaine- and amphetamine-regulated transcript peptide (CART) in the central nucleus of amygdala potentiates behavioral and hormonal responses of the rat exposed to its predator. Behav. Brain Res. *243*, 129–137.
- Vergoni, A.V., and Bertolini, A. (2000). Role of melanocortins in the central control of feeding. Eur. J. Pharmacol. *405*, 25–32.
- Vicentic, A., Lakatos, A., and Kuhar, M.J. (2005). CART (cocaine- and amphetamine-regulated transcript) peptide receptors: Specific binding in AtT20 cells. Eur. J. Pharmacol. *528*, 188–189.
- Vicentic, A., Lakatos, A., and Jones, D. (2006). The CART receptors: Background and recent advances. Peptides *27*, 1934–1937.
- Volkoff, H., and Peter, R.E. (2000). Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, Carassius auratus: Actions on neuropeptide Y- and orexin A-induced feeding. Brain Res. *887*, 125–133.
- Volkoff, H., Canosa, L.F., Unniappan, S., Cerdá-Reverter, J.M., Bernier, N.J., Kelly, S.P., and Peter, R.E. (2005). Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. *142*, 3–19.
- Vrang, N., Larsen, P.J., Kristensen, P., and Cart, C.Ž. (1999). Recombinant CART peptide induces c-Fos expression in central areas involved in control of feeding behaviour. 499–509.
- Warrick, R.M.H. (1991). Modulation of neural networks for behavior. 39–57.

- White, N.M., and Fisher, A.E. (1969). Relationship between amygdala and hypothalamus in the control of eating behavior. Physiol. Behav. *4*, 199–205.
- Williamson, R.A., Worrall, S., Chazot, L., and Strange, P.G. (1988). Purification of brain D2 dopamine receptor. *7*, 4129–4133.
- Wright, J., Campos, C., Herzog, T., Covasa, M., Czaja, K., and Ritter, R.C. (2011). Reduction of food intake by cholecystokinin requires activation of hindbrain NMDA-type glutamate receptors. Am. J. Physiol. - Regul. Integr. Comp. Physiol. *301*, 448–455.
- Wu, Q., Zheng, R., Srisai, D., Mcknight, G.S., and Palmiter, R.D. (2013). NR2B subunit of the NMDA glutamate receptor regulates appetite in the parabrachial nucleus. *110*, 14765–14770.
- Wullimann, M.F., and Mueller, T. (2004). Erratum: Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior (Journal of Comparative Neurology (2004) 475 (143-162)). J. Comp. Neurol. *478*, 427–428.
- Wyart, C., and Del Bene, F. (2011). Let there be light: zebrafish neurobiology and the optogenetic revolution. Rev. Neurosci. *22*, 121–130.
- Yang, Y., Atasoy, D., Su, H.H., and Sternson, S.M. (2011). Hunger states switch a flip-flop memory circuit via a synaptic AMPK-dependent positive feedback loop. Cell *146*, 992–1003.
- Yermolaieva, O., Chen, J., Couceyro, P.R., and Hoshi, T. (2001). Cocaine- and amphetamine-regulated transcript peptide modulation of voltage-gated Ca2+ signaling in hippocampal neurons. J. Neurosci. *21*, 7474–7480.
- Yokobori, E., Azuma, M., Nishiguchi, R., Kang, K.S., Kamijo, M., Uchiyama, M., and Matsuda, K. (2012). Neuropeptide Y Stimulates Food Intake in the Zebrafish, Danio rerio. J. Neuroendocrinol. *24*, 766–773.
- Yosten, G.L.C., Harada, C.M., Haddock, C., Giancotti, L.A., Kolar, G.R., Patel, R., Guo, C., Chen, Z., Zhang, J., Doyle, T.M., et al. (2020). GPR160 de-orphanization reveals critical roles in neuropathic pain in rodents. J. Clin. Invest. *130*, 2587–2592.
- Yu, Y., Huang, R., Ye, J., Zhang, V., Wu, C., and Cheng, G. (2016). Regulation of starvation-induced hyperactivity by insulin and glucagon signaling in adult Drosophila. 1–19.
- Zhang, Q., Li, H., and Guo, F. (2011). Amygdala, an important regulator for food intake. Front. Biol. China *6*, 82–85.
- Zheng, H., Patterson, L.M., and Berthoud, H.R. (2002). CART in the dorsal vagal complex: Sources of immunoreactivity and effects on Fos expression and food intake. Brain Res. *957*, 298–310.
- Zigman, J.M., Elmquist, J.K., Z, D.E.J.M., and Israel, B. (2014). Minireview : From Anorexia to Obesity — The Yin and Yang of Body Weight Control Leptin : A Molecular Signal of. *144*, 3749–3756.
- Zito, K., and Scheuss, V. (2009). NMDA Receptor Function and Physiological Modulation. In:

Encyclopedia of Neuroscience. Elsevier 276–283.

- Zou, X., Lin, Q., and Willis, W.D. (2002). Role of protein kinase A in phosphorylation of NMDA receptor 1 subunits in dorsal horn and spinothalamic tract neurons after intradermal injection of capsaicin in rats. Neuroscience *115*, 775–786.