Mechanisms underlying peptidergic modulation of feeding and feeding-associated behaviours

A Thesis Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

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Dr. Aurnab Ghose

Thesis advisor

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Abstract

Of the various neuromodulatory agents found in the central nervous system, the class of peptide modulators is of particular interest in the context of innate motivated behaviours, wherein they have been shown to modify the synaptic gain and alter intrinsic properties of neurons to initiate or extend stable behavioural states (e.g., sleep/arousal, roaming/dwelling). Feeding is an important behaviour where peptides are known to exert modulatory control. Although much is known about the neuropeptides and anatomical regions regulating feeding and associated behaviours, the underlying molecular mechanisms remain poorly understood. This thesis focuses on Cocaine-and-Amphetamine-Regulated-Transcript (CART) and Neuropeptide Y (NPY), two peptides strongly implicated in regulating feeding and associated modalities. Using a combinatorial approach, we explore the biochemical changes triggered due to CART and NPY signalling and their consequences on behaviour and neural activity in the Zebrafish (*Danio rerio*) nervous system.

Previous data from our lab used a neural activity marker, phosphorylated extracellular signal-regulated kinase (p-ERK), to identify CART peptide responsive regions. In this study, we find a strong correlation between high levels of p-ERK in the dorsomedial telencephalon (Dm) and an associated anorexic behavioural output. We thus used pharmacological intervention coupled with behavioural monitoring and neuronal activity imaging in Dm to better understand the molecular mechanisms involved in establishing CART-mediated anorexia. We found that N-methyl-D-aspartate receptor (NMDAR) activity is necessary for CART signalling, as the CART-induced increase in activity of Dm neurons and reduction in feeding drive were both disrupted by blocking NMDAR signalling. We then tested if protein kinase A (PKA) is also involved in mediating this effect. Indeed, we found that PKA activity was required for CART-induced upregulation of neural activity in the Dm and suppression of feeding drive. PKA-mediated phosphorylation of NR1 subunits is known to alter NMDAR conductance and opening probability; we, therefore, tested if CART treatment alters phospho-NR1 (p-NR1) levels. Indeed, we found higher phospho- Ser 897 levels in Dm of CART injected animals which were suppressed by inhibiting PKA activity. Additionally, results of neuronal activity imaging in *ex vivo* brain preparations from transgenic animals expressing the calcium indicator GCaMP6f under a pan-neural promoter indicate that CART neuropeptide sensitises Dm

neurons to excitatory inputs, which is correlated to CART-induced anorexia. Together these data suggest that CART action via PKA sensitises NMDA receptors via post-translational modification of receptor subunits, leading to heightened excitatory tone in Dm and a neural representation of the sated state.

In the context of feeding behaviour, the sense of smell provides information about the exact location, palatability and reward value of food. It is thus a necessary input in the decision-making process when foraging and selecting between different food sources. Previous work done in the lab demonstrated that the levels of NPY, an orexigenic peptide, in the terminal nerve, olfactory epithelium, and olfactory bulb of Zebrafish reflect the animal's nutritional status. Furthermore, it was found that heightened NPY signalling under low energy conditions is necessary and sufficient to increase attraction towards amino acids, a major food cue. In the second study, we have investigated the mechanisms underlying this nutritional state-dependent increase in olfactory sensitivity. Results from activity-based p-ERK labelling revealed that amino acid detection requires Phospholipase C (PLC) activity.

Additionally, we found that olfactory sensory neurons (OSNs) express NPY Y1 receptors and inhibiting its activity abrogates amino acid-induced OSN activation. Further testing confirmed crosstalk between odour-processing and peptide signalling pathways. We propose that this interaction of NPY signalling onto PLC-dependent odour processing allows for sensory gating at the level of OSNs. Altogether, our work has uncovered molecular pathways employed by neuropeptides to drive need-based alterations in neural activity and behaviour, which can be further explored to gain more insights into the neural computations underlying bistable behaviours.

Synopsis

All animals, be it invertebrates like nematodes or higher-order vertebrates like mammals, are equipped with neural systems that perform goal-directed behaviours essential for their survival. These behaviours are innate and neural connections involved in their execution hard-wired. Despite this, such neural networks display flexibility in their functional output. One process that confers such flexibility is neuromodulation. The anatomical connections serve as a basic framework over which various neuromodulatory substances generate a functional circuit. Therefore, the output of this network can differ depending on which neuromodulator(s) is affecting the change and at what level. Neuromodulation has been well studied in invertebrate and vertebrate systems, mainly focusing on mechanisms governing motor pattern generation. A large body of data now highlights the role of modulatory peptides in controlling transitions between distinct behavioural states. **Chapter 1** provides an overview of these studies and summarises our current understanding of peptidergic neuromodulation in the context of feeding behaviour.

Food search and consumption are crucial processes in feeding behaviour and energy homeostasis. Studies in rodents identified several neuropeptides that regulate feeding under hunger-satiety conditions and the anatomical networks they affect. However, the molecular details of peptide-induced changes that are necessary to establish these internal states (i.e hunger or satiety) and to keep sensory processing tuned to the prevailing energy conditions are still unclear. This thesis was thus designed with a two-part approach towards understanding the molecular mechanisms underlying neuropeptide signalling at both these levels in the context of feeding behaviour. **Chapter 2** focuses on Cocaine and Amphetamine Regulated Transcript (CART), a potent anorexigen in many vertebrates for which the underlying molecular signalling is still unknown. We explore the consequences of changes in CART signalling on food intake in *Danio rerio* (Zebrafish). This freshwater teleost offers a relatively simple vertebrate model system capable of performing complex behaviours. Moreover, there is appreciable conservation between neural circuit organisation and function between Zebrafish and mammals. We used a combination of techniques like behaviour assays and pharmacological interventions coupled with analysis of neural activity to address the following questions:

1. Is CART necessary for processing satiety signals and inducing anorexia in Zebrafish?
2. What effectors lie downstream of CART?

3. What are the effects of increased CART signalling on the activity of downstream neurons?

Analysis of food intake in adult fish revealed that CART is required for processing of glucose-induced satiety signals. Furthermore, exogenous CART is sufficient to induce anorexia. Together, these data demonstrate for the first time that CART functions as an anorexigenic agent in adult Zebrafish. We also provide evidence to support the involvement of NMDA receptors and Protein Kinase A in mediating these effects of CART. Additionally, analysis of dynamic activity in a telencephalic nucleus, viz. Dm, revealed that heightened CART signalling increases the sensitivity of Dm neurons to excitatory stimuli. We propose that this increased excitatory tone allows for the neural representation of satiety.

In Chapter 3, we focus on determining the molecular details that underlie starvation-induced increased olfactory sensory response to amino acids, a prominent class of food-related odours in teleost fish. Using activity-dependent labelling of olfactory sensory neurons (OSNs) and different pharmacological agents, we address the following questions:

1. How do OSNs detect a mixture of amino acids?
2. Does disrupting Neuropeptide Y signalling interfere with the detection of amino acids?
3. Is there a point of convergence for amino acid sensing pathways and NPY signalling?

Our data indicate that activation of microvillar OSNs upon exposure to amino acids requires phospholipase C (PLC). We also find that NPY signalling via the Y1R receptor is necessary for amino acid-induced OSN activation. Furthermore, the ability of exogenously delivered NPY to restore amino acid-induced activation under conditions of mimicked satiety required PLC signalling. Thus, indicating that starvation-induced elevations in NPY signalling act via Y1R and PLC pathway to enhance responses to food-cue.

In summary, this thesis provides critical insights into the molecular pathways employed by two neuropeptides, CART and NPY, in regulating feeding and related modalities. Moreover, the identification of Dm as a downstream target for processing CART-induced satiety signals provides a new entry point to further our understanding of hunger-satiety circuits.

Chapter 1: Introduction

All animals, be it invertebrates like nematodes or higher-order vertebrates like mammals, are equipped with neural systems that perform goal-directed behaviours such as feeding, mating, and sleeping. These are essential for the survival of an organism and are therefore under tight regulatory control. The circuits underlying these behaviours are characterised by the presence of certain 'interoceptory' neurons tuned to the animal's physiological state and its external environment (Fig. 1.1 A; Flavell et al., 2020; Sternson, 2013). The execution of the appropriate behaviour depends on the internal signals detected and their integration with cues from the environment. For example, neural circuits regulating arousal behaviour respond to changes in sleep pressure and circadian cues to allow the animal to switch between sleeping state (i.e. periods of inactivity with increased stimulus-response threshold) and wakeful state (i.e. periods of high activity and low stimulus-response threshold) (Adamantidis et al., 2007; Parisky et al., 2008; Taghert and Nitabach, 2012; Vosko et al., 2007). Variable response to pheromones exhibited by the nematode worm, *Caenorhabditis elegans* (Jang et al., 2012), and energy status-dependent changes in odour valence observed in *Drosophila melanogaster* (Root et al., 2011; Sengupta, 2013; Su and Wang, 2014; Vogt et al., 2021) illustrate context-dependent changes in the output of sensory circuits. It is thus evident that neural circuits of innate behaviour can show a high degree of flexibility in their functional output with a fixed set of anatomical connections. One process which allows for such behavioural flexibility is neuromodulation.

1.1 General characteristics of neuropeptide signalling

Neuromodulators are divided into two types - neuropeptides and monoamines. Unlike classical neurotransmitters, which are docked in small clear vesicles at the synaptic cleft, these are packed into dense-core vesicles in the peri-synaptic regions (Nässel, 2009; van den Pol, 2012). Neuropeptides and biogenic amines have long been studied in invertebrate systems with regards to their role in generating and extending behavioural states (Flavell et al., 2013; Jing et al., 2007; Pool and Scott, 2014; Wood et al., 2000).

Early studies on the crustacean stomatogastric (STG) system (Fig. 1.1 B) have informed much of what we know about these two groups' characteristics and neuromodulatory potential. Extensive studies by Marder and colleagues highlighted key functional features of neuromodulators: they act on neural circuits by changing the firing properties and altering

synaptic gain, leading to a variable or flexible output (Marder and Thirumalai, 2002; Swensen and Marder, 2000; Wood et al., 2000).

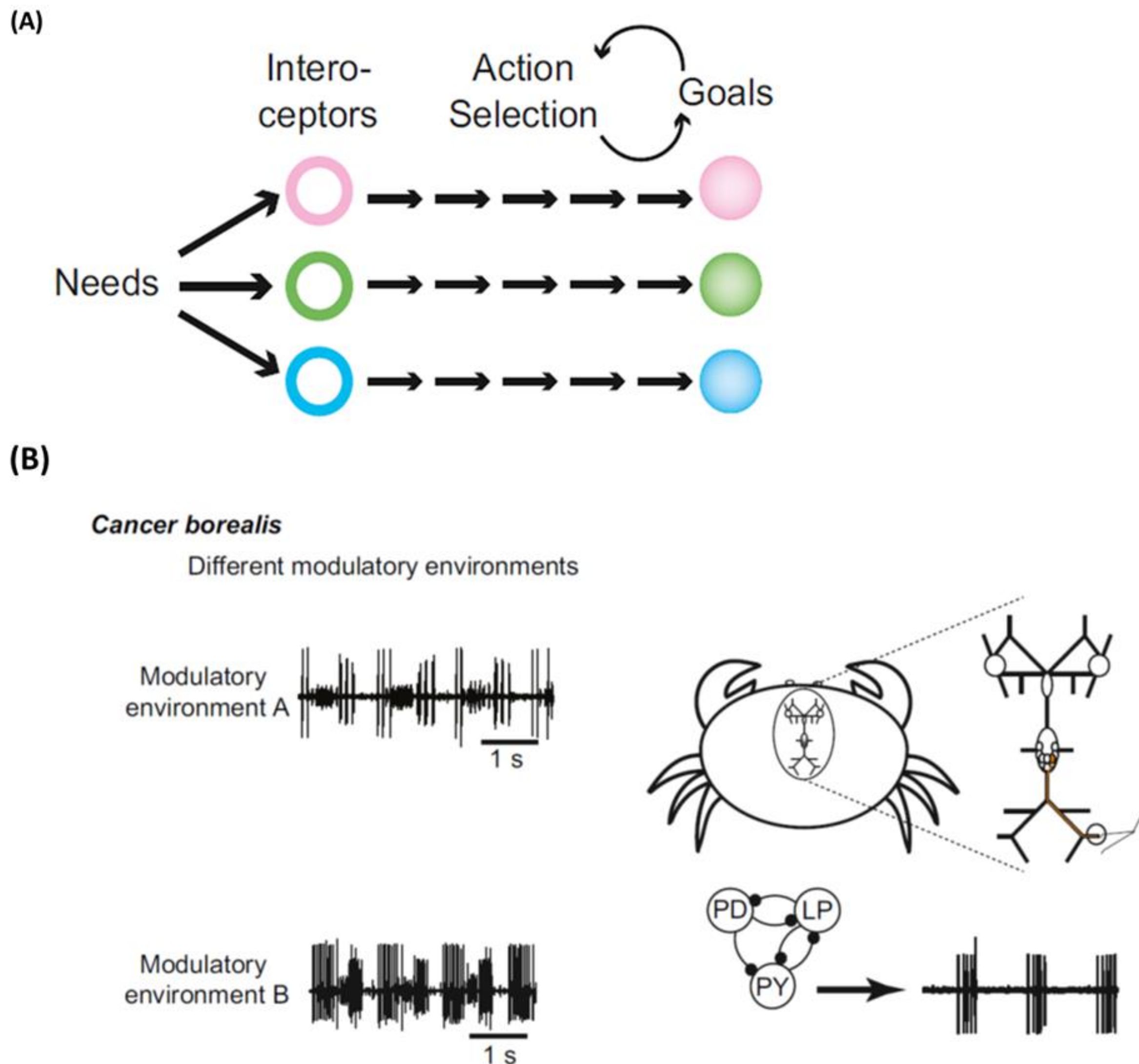


Fig. 1.1 Neuromodulation alters neural circuit output.

(A) Specialised neurons, called interoceptors, detect physiological needs and drive changes in downstream targets to execute appropriate behavioural response (reproduced from (Sternson, 2013). Extensive studies on the crustacean Somatogastric (STG) system analysed the functional consequences of changing neuromodulator environment on neural circuit output. **(B)** A change in modulatory environment was found to drastically alter the motor patterns generated by the LP, PD and PY neurons governing the gastric mill and pyloric rhythm in the STG. These studies underscored the importance of neuromodulation in mediating context-dependent changes in neural activity (Reproduced from Jékely et al., 2018).

Neuropeptides, in particular, show great versatility in their actions which may be attributed to differences in their expression patterns, presence of multiple receptors or the differences in the way they are released, i.e. as circulating neurohormones or as local modulators (Chini, 2019; Nässel, 2009). For instance, the Drosophila tachykinin (DTK) receptor, DTKR, can activate intracellular signalling in the presence of any of the six DTK peptides identified to date. In contrast, the other Drosophila tachykinin receptor, NKD, selectively responds only to DTK-6 (Nusbaum and Blitz, 2012). Another case is that of NPY mediated inhibition of hypocretin cells via its action on Y2 and Y1 receptors, which are differentially located to the pre-and post-synaptic terminals, respectively (Nusbaum and Blitz, 2012; Svensson et al., 2001; van den Pol, 2012). Studies in frog sympathetic ganglion demonstrated that neuropeptides could diffuse over tens of micrometres to reach their cognate receptors (Jan and Jan, 1982; Nässel, 2009), allowing for the recruitment of non-synaptically connected neurons into the functional connectome (Bargmann, 2012; Brezina, 2010). However, the sphere of action is thought to be limited by factors such as distance, the presence of membrane-bound peptidases, and mechanisms like desensitisation of receptors or receptor internalisation (Salio et al., 2006; van den Pol, 2012).

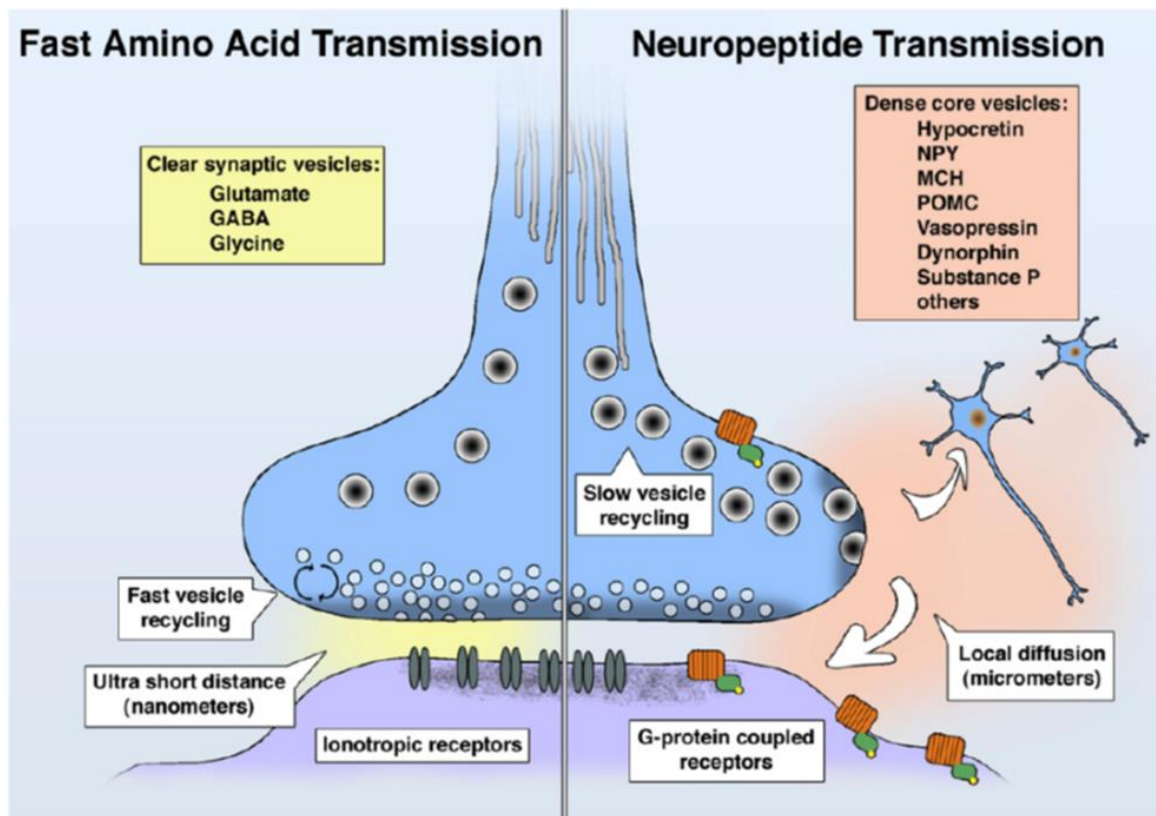


Fig. 1.2. Characteristics of neuropeptide-based transmission.

Unlike classical neurotransmitters that are stored in clear vesicles at the synaptic cleft, neuropeptides are docked and released from peri-synaptic sites (reproduced from (van den Pol, 2012). Neuropeptide release requires high frequency trains of action potential and occurs over slower time scales (~50 msec or more) than the fast release of amino acid transmitters (e.g. glutamate and GABA).

Criteria such as biological function, localisation, sequence homology, and presence of common precursors have been used to classify neuropeptides into different peptide families (Salio et al., 2006). Across the various peptide families, most neuropeptides act via G-protein coupled receptors (GPCRs) that activate various second messenger cascades to mediate more long-lasting network changes (Fig. 1.2) (Chini, 2019). Not surprisingly, therefore, this class of modulators is a major contributor to the multifunctional nature of neural circuits and has been extensively studied to better understand neural networks underlying innate behaviours.

1.2 Neuropeptide signalling affects behaviour network states

Behaviours can often be composed of discrete, long-lasting behavioural states (Flavell et al., 2013; Lee and Dan, 2012). Bistability in behavioural states has primarily been studied using invertebrate model systems. A notable example is the study of neural circuits governing locomotion states, roaming and dwelling, in *C.elegans* foraging behaviour wherein serotonin and pigment dispersing factor (PDF) were found to have parallel, antagonistic roles. The amine is necessary for initiating and maintaining the dwelling state while the peptidergic input initiates and maintains roaming (Flavell et al., 2013). In Aplysia, two distinct inputs to the feeding central pattern generator (CPG), cerebral-buccal interneuron-2 (CBI-2) and oesophagal nerve (EN), activate two competing motor responses viz. ingestive versus egestive motor patterns, respectively (Jing et al., 2007). Critical components in this circuit are the two interneurons B20 and B40 that functionally oppose each other and the modulatory CPG element B65. B65 was shown to contribute to the establishment of the egestive- state by promoting the activity of B20 and suppressing the activity and synaptic outputs of B40 (Wu et al., 2010). These pathways are thought to constitute a single composite feed-forward loop that acts in concert to promote the desired network state (Wu et al., 2010). Recent advances in optogenetic tools and the availability of better resolution microscopy techniques have facilitated the study of modulation of network states in various vertebrate systems. Like in the case of Zebrafish sleep/wake behaviour (Elbaz et al., 2012) and hunger-satiety circuits in rodents (Yang et al., 2011).

1.3 Central circuits in the establishment of hunger-satiety states and food intake regulation

Feeding is a behaviour in which neuromodulation has been quite extensively studied. It is essential for the growth and survival of the organism and requires a multitude of processes that need to be coordinated for ensuring homeostasis. Each of these processes is subject to neuropeptidergic control, from sensing of energy deficit or surplus and the establishment of a hunger or satiety state, to the detection and consumption of food (Basiri and Stuber, 2016; Ferrario et al., 2016; Sternson and Eiselt, 2017).

Based on their effect on food intake, neuropeptides are classified into orexigenic or anorexigenic peptides. The former class of peptides is upregulated under conditions of energy deprivation and increases the activity of hunger-promoting neurons to increase food

consumption while the latter is upregulated under conditions of energy surfeit and activates satiety-promoting neurons to ultimately supresses food intake. Table 1 provides examples from each group, most of which have conserved functions across the animal kingdom, from flies (Pool and Scott, 2014), teleost's (Matsuda et al., 2011) to mammals (Sternson and Eiselt, 2017). In the subsequent sections, we explore the details of how changes in nutritional status are sensed and modify the behavioural output of feeding circuits by the activity of neurons expressing orexigenic/anorexigenic neuropeptides.

Orexigenic Neuropeptides	Anorexigenic Neuropeptides
NPY	CART
AgRP	α-MSH
Orexin	GLP
MCH	CRH
Galanin	Neurotensin

Table 1. Classification of feeding regulatory neuropeptides.

1.3.1 Metabolic signals of energy status

A crucial step for forming a neural representation of energy states is sensing metabolic signals of energy sufficiency or deficit. These can be in the form of adiposity signals such as leptin or insulin and enteroendocrine hormones like CCK or ghrelin (Dietrich and Horvath, 2013; Routh, 2010; Sutton et al., 2016). Receptors for these circulating signals are expressed in hypothalamic and brainstem interoceptive neurons which then respond with a change in their neuropeptide expression profile to establish hunger or satiety state and initiate homeostatic changes. For example, an increase in leptin signalling results in the upregulation of POMC and CART mRNA and downregulation of AgRP and NPY mRNA in the arcuate nucleus (ARC) (Klok et al., 2007). Under well-fed conditions, high insulin/leptin signalling activates catabolic pathways, suppresses food intake and weight gain. In contrast, fasted conditions result in high

ghrelin levels and low insulin/leptin signalling, promoting anabolic pathways and increasing food intake and weight gain (Woods et al., 1998).

In addition to detecting changes in circulating signals of energy availability, certain neurons in the ARC, ventromedial hypothalamus (VMH) and paraventricular nucleus (PVH) are also equipped with cellular mechanisms that enable them to respond to changes in interstitial glucose levels (Grill and Kaplan, 2002; Routh, 2010). These are categorised as glucose excited (GE) or glucose inhibited (GI) neurons based on changes in their action potential firing in response to glucose. A change in interstitial glucose, from 0.1 mM to 2.5 mM, increases action potential firing in GE neurons but is inhibitory for GI neurons (Routh, 2010). Similarly, neurons that show an increase in the frequency of action potentials as a result of increased interstitial glucose levels from 5 mM to 20 mM are termed as high glucose excited or HGE, and those that respond with a reduction in action potential frequency are termed as high glucose inhibited or HGI (Routh, 2010). The signalling mechanisms in these neurons are found to be similar to those observed in pancreatic β-cells. GE neurons express glucose transporter 2 (GLUT2), the enzyme glucokinase (GK) and utilise ATP-sensitive potassium (K_{ATP}) channels to sense glucose (Ibrahim et al., 2003; Wang et al., 2004). A subset of neurons also employs non- K_{ATP} channel-dependent mechanisms to sense glucose. Instead, these utilise sodium coupled-glucose cotransporters (SGLT) to detect changes in glucose levels. These transporters couple the influx of sugar molecules with the entry of Na^+ ions, and the resultant Na^+ flux generates an inward current in proportion to the glucose levels (O'Malley et al., 2006). While some of these components are also found in GI neurons (e.g., VMH GI expresses GLUT 2 and GLUT 4), the pathways underlying their response to glucose are quite distinct (Routh, 2010). A drop in glucose levels increases the cellular AMP: ATP ratio and activates the enzyme AMP-activated protein kinase (AMPK) (Routh, 2010; Rutter et al., 2003). Activation of AMPK is thought to gate responses of hypothalamic nuclei to peripheral hormones and is essential for the representation of energy states in vertebrate hypothalamic circuits (Dietrich and Horvath, 2013). Ghrelin induced AMPK activation and ryanodine receptor-mediated opening of internal calcium stores potentiates inputs onto $ARC^{AgRP/NPY}$ neurons to maintain persistent neural activity for several hours (Fig 1.3 A; Dietrich and Horvath, 2011). This maintains the ARC circuits in a hunger state till they are acted upon by a satiety signal such as leptin. Leptin inhibits AMPK and activates the release of β-endorphins from ARC POMC neurons leading to inhibition of the AgRP/NPY neurons and switches the circuit to a sated state (Fig. 1.3 A; Dietrich and Horvath, 2011; Yang et al., 2011). Such establishment of hunger/satiety state

results in changes in the activity of distributed downstream circuits via the action of neuropeptides and modifies food intake and energy expenditure.

1.3.2 Neuropeptidergic circuits in food intake regulation

Investigations of genetic mutants, lesion studies, and electrophysiological and optogenetic manipulation data from rodent models have revealed local and long-range ARC circuits acting together with the central melanocortin receptor systems as critical components in regulating food intake in accordance with the established energy state. As described earlier, ghrelin induces sustained activity in the $ARC^{AgRP/NPY}$ neurons. This population is shown to be necessary and sufficient for driving appetitive behaviour and increases food intake in hungry animals (Aponte et al., 2011; Cansell et al., 2012; Padilla et al., 2016). In addition to VMH and dorsomedial hypothalamus (DMH), these neurons also receive inputs from PVH neurons which increase their firing rate and promotes food intake (Krashes et al., 2014). Targeted ablation of $ARC^{AgRP/NPY}$ neurons in adult mice leads to severe weight loss and starvation (Luquet, 2005), while photo-stimulation of these neurons is sufficient to induce voracious feeding (Aponte et al., 2011). These neurons form GABA and NPY containing termini onto neighbouring NPY Y1R expressing POMC/CART neurons and inhibit their activity (Cowley et al., 2001). While inhibition of ARC^{POMC} activity is not necessary for $ARC^{AgRP/NPY}$ evoked acute feeding, pharmacological inhibition of GABA and NPY co-transmission from $ARC^{AgRP/NPY}$ termini in PVH successfully prevents rapid feeding after stimulation of $ARC^{AgRP/NPY}$ axons (Atasoy et al., 2012). The three neurotransmitters released from $ARC^{AgRP/NPY}$ neurons, viz. AgRP, NPY and GABA, act over different timescales to regulate food intake. NPY and GABA are necessary for rapid feeding and likely mediate meal to meal feeding regulation (Krashes et al., 2013). Whereas AgRP, acting on downstream melanocortin receptors, induces delayed feeding and likely contributes to the chronic starvation-induced increase in feeding (Krashes et al., 2013). In addition to PVH, the $ARC^{AgRP/NPY}$ neurons project to extra-hypothalamic sites like the parabrachial nucleus (PBN) and amygdala and provide an inhibitory tone necessary to maintain normal feeding behaviour (Padilla et al., 2016; Sohn et al., 2013; Wu et al., 2009; Zhang et al., 2019).

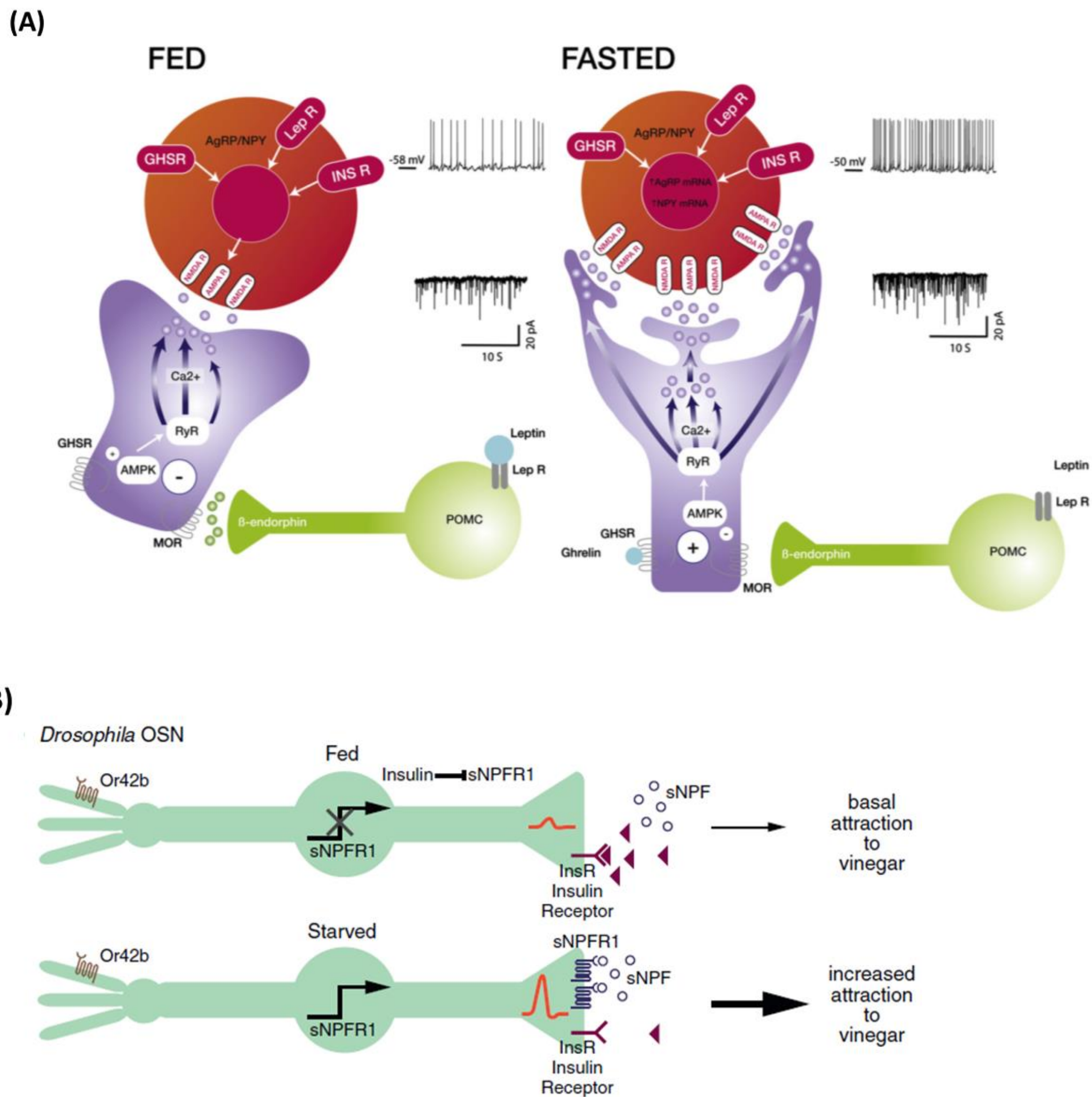


Fig. 1.3. Energy status dependent tuning of feeding circuits by neuropeptides

(A) Opposing actions of neuropeptidergic neurons help form representations of fed-fasted states in mammalian feeding circuits (Reproduced from Spanswick et al., 2012).

(B) Metabolic states alter neuropeptide signalling in olfactory circuits leading to changes in response to food related odours (Reproduced from Sengupta, 2013).

Contrary to $ARC^{AgRP/NPY}$ neurons, the $ARC^{POMC/CART}$ neurons are activated by fed conditions (i.e. high insulin/leptin signalling) and produce α-melanocyte stimulating hormone (α-MSH) and β-endorphins to inhibit $ARC^{AgRP/NPY}$ neurons (Fig. 1.3 A; Cansell et al., 2012; Spanswick et al., 2012). $ARC^{POMC/CART}$ also project to various hypothalamic (PVH and DMH) and extra-hypothalamic regions (central amygdala, CeA; nucleus tractus solitarius, NTS; bed nucleus of stria terminalis, BNST) that suppress feeding (Kwon and Jo, 2020; Sutton et al., 2016).

ARC^{POMC} neurons project to DMH, where they likely inhibit NPY release from DMH neurons to suppress feeding (Millington, 2007). Further, α-MSH released from ARC$^{POMC/CART}$ projections to PVH postsynaptically potentiate glutamatergic transmission onto PVH melanocortin receptor 4 (MC4R)-expressing neurons to mediate satiety (Fenselau et al., 2017). These PVH MC4R-neurons further project to hindbrain regions such as PBN, regulating meal termination and inducing satiety (Sutton et al., 2016). These projections are inhibited under starvation conditions by GABA and NPY co-transmission from ARC$^{AgRP/NPY}$ neurons (Atasoy et al., 2012; Dietrich and Horvath, 2013; Wu et al., 2009). ARC$^{POMC/CART}$ neurons also directly synapse onto estrogen receptor-α (ER-α) and MC4R-expressing neurons in MeA (Kwon and Jo, 2020). Activation of this pathway was recently shown to govern acute, short-term hypophagia in male and female mice (Kwon and Jo, 2020)

Together, these studies highlight the importance of peptide driven modulation of fast neurotransmitter activity in hypothalamic and extra-hypothalamic circuits for maintaining normal feeding behaviour and energy homeostasis.

1.4 Neuropeptide modulation of food-search behaviour and sensory processing

Metabolic control of olfaction

In addition to feeding regulatory circuits, metabolic factors can also affect olfactory sensitivity to facilitate homeostasis. In addition to networks that help establish an energy state, neuropeptides can form sensorimotor feedback loops to control the gain of various sensory inputs. One such feedback loop has been described for olfactory neurons in *Caenorhabditis elegans*. AWC olfactory neurons co-release glutamate and the peptide NLP-1. This peptide acts via a GPCR, NPR-11, on post-synaptic AIA interneurons. The peptidergic feedback from these neurons in the form of an insulin-related peptide (INS-1) limits activity in AWC neurons by modulating the calcium transients observed after odour removal (Chalasani et al., 2010). The role of neuropeptides in such olfactory adaptation behaviour indicates that circuit inputs can remodel sensory responses to external stimuli by integrating internal need states and sensory history.

Furthermore, responses to identical food-related stimuli are heavily influenced by the motivational state of the organism. In Drosophila, physiological responsiveness in the DM1 olfactory glomerulus and food-seeking behaviour was increased upon starvation (Root et al.,

2011). This region receives primary afferents from olfactory sensory neurons (OSNs) that express short neuropeptide F (sNPF). Using RNAi transgenic constructs for sNPF or sNPF receptor, Root and colleagues showed that sNPF signalling under starvation state alters the olfactory representation of food odours to enhance odour-driven food search (Root et al., 2011). Moreover, a satiety signal such as insulin was found to diminish DM1 glomerular responses to food by blocking sNPF mediated signalling (Fig. 1.3 B; Sengupta, 2013; Taghert and Nitabach, 2012). Energy status dependent alterations in the strength of neuropeptide signalling can thus be a way to gate sensory perception and ensure energy homeostasis.

Evidence gathered over the past decade suggests that similar strategies exist in animals with more complex nervous systems. Several essential neuropeptides known to regulate food intake (insulin, leptin, CCK, GnRH, and NPY) and their receptors are expressed in olfactory mucosa (OM), terminal nerve (TN) and olfactory bulb (OB) (Baly et al., 2007; Palouzier-Paulignan et al., 2012; Park and Eisthen, 2003; Savigner et al., 2009). OM and OB can also directly respond to changes in glucose with OSNs expressing GLUT1 and OB GLUT1, GLUT3, and GLUT4 (Palouzier-Paulignan et al., 2012) glucose transporters, indicating that olfactory system can sense changes in energy availability. Electrophysiology recordings from *ex vivo* rat olfactory epithelia revealed that leptin and insulin reduce odour-evoked activity and increase the signal to noise ratio to match olfactory function with satiety state (Savigner et al., 2009). Hunger evoked release of 2-arachidonoylglycerol (2-AG) from OSNs facilitates odour detection at lower concentrations in *Xenopus laevis* larvae (Breunig et al., 2010). In axolotls, TN derived NPY enhanced responses to a food-related odorant, L-glutamic acid, only in hungry animals (Mousley et al., 2006). Negroni et al. (2012) reported similar findings wherein the amplitude of electroolfactogram (EOG) recordings from rat OM was enhanced in fasted animals via upregulation of NPY Y1R receptors. Such changes in olfactory responses under varying metabolic status also translate to observable differences in the feeding behaviour of the animals. Indeed, fasted Zebrafish have a higher attraction to food-related odorants mediated by the upregulation of NPY, an orexigenic peptide (Kaniganti et al., 2021; Koide et al., 2009; Yokobori et al., 2012). Aimé et al. (2007) reported energy status-dependent alteration in olfactory detection of neutral odours in rats, while Seguy and Perret (2005) found that rats made anosmic by bulbectomy exhibit severe hyperphagia. Olfactory detection is a crucial input to feeding regulatory circuits in the ARC, as demonstrated by Chen et al. (2015). These studies thus support a model in which the olfactory system acts as a nutritional status sensor, and local

neuromodulation in the peripheral olfactory system modifies olfactory-driven feeding behaviour.

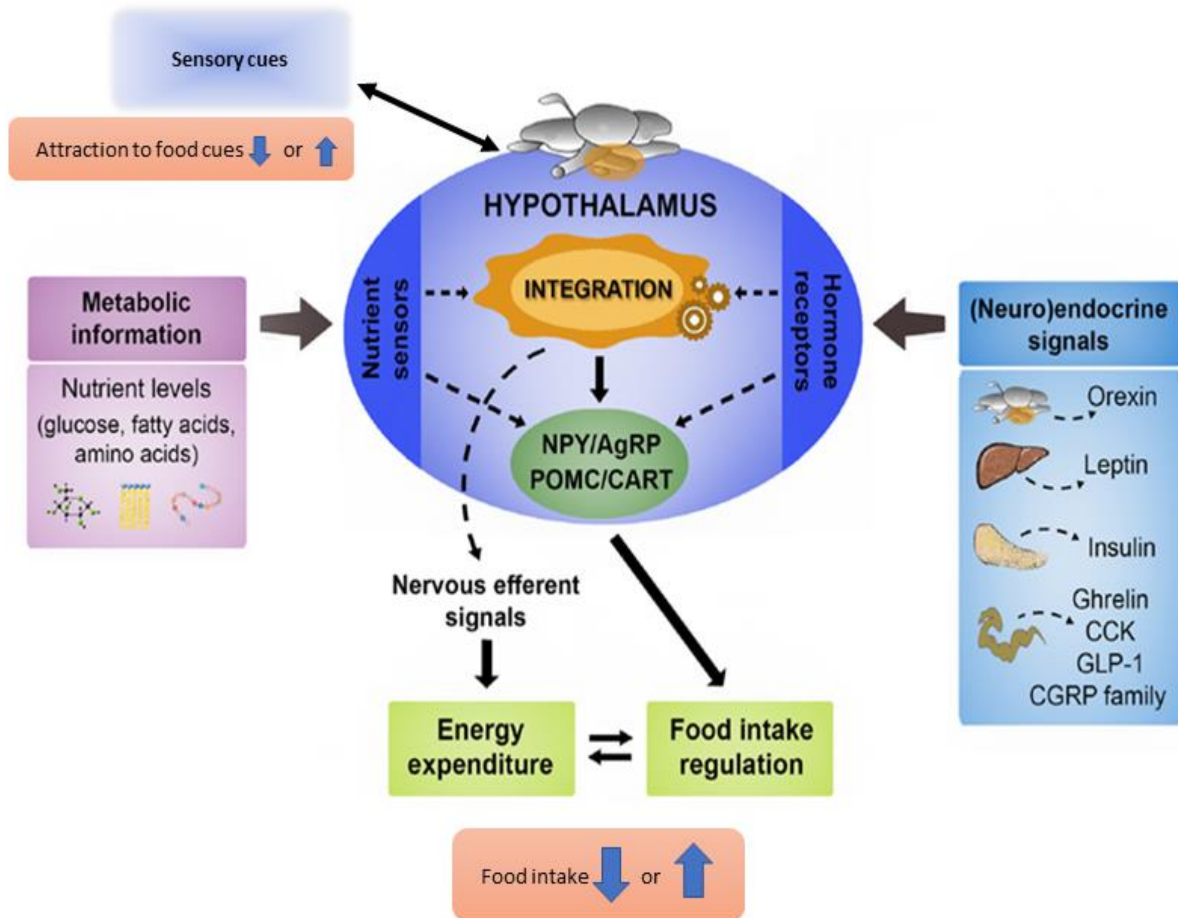


Fig. 1.4. Neuropeptides and regulation of feeding circuits.

In vertebrate feeding circuits, Arcuate AgRP/NPY expressing neurons activated in response to energy deficit signals oppose POMC/CART immunoreactive neurons that respond to signals of energy surplus. These neuropeptides together with fast amino acid transmitters, act on downstream circuits to form a neural representation of hunger-satiety states. Concomittantly, neuropeptide signalling also affects a change in processing of food-related odours in line with the established state of either hunger/satiety (Modified from Delgado et al., 2017).

1.5 Aim of the thesis

It is thus clear that the class of peptide modulators exhibits highly diverse and versatile functions in regulating feeding. Not only do they act on target neurons to establish feeding states but also modulate key sensory modalities involved in the perception of food resulting in an increase or decrease in food intake (Fig. 1.4). However, several details of peptidergic transduction remain underexplored. Using Zebrafish as a model system, this thesis explores the biochemical signalling underlying the modulatory action of two evolutionarily conserved neuropeptides, CART and NPY, in the context of food intake regulation and nutritional status dependent olfactory sensitisation, respectively. In addition to being easy to rear, Zebrafish offer a relatively small, easy-to-manipulate vertebrate nervous system. Importantly, these animals exhibit distinct and stereotyped behaviours while maintaining a fair amount of homology with tetrapod nervous systems (Bally-Cuif and Vernier, 2010; Delgado et al., 2017; Friedrich et al., 2010; Volkoff, 2016). Many of the mammalian feeding regulators have conserved expression and function in the Zebrafish brain (Ahi et al., 2019; Koide et al., 2009; Opazo et al., 2019; Polakof et al., 2008a, 2008b; Soengas et al., 2018; Yokobori et al., 2012). Comparing the neuropeptidergic signalling in such a system allows for the possibility of identifying common features or 'logic' employed by neural circuits; This would improve the translatable nature of studies carried out in model systems regarding the neuromodulatory processes.

Our lab has previously shown that the CART system in Zebrafish is sensitive to changes in energy status (Akash et al., 2014; Mukherjee et al., 2012). This peptide is a potent anorexigen in other vertebrate systems (Lau and Herzog, 2014; Singh et al., 2021; Subhedar et al., 2014) but compared to the POMC or AgRP pathways, the functional details for CART signalling are relatively unexplored. Our first study demonstrates the behavioural consequences of increased CART signalling in adult fish and outlines a mechanism underlying its effects (Chapter 2). Zebrafish gene expression data and behaviour studies indicate a conserved orexigenic role for NPY (Polakof et al., 2008a, 2008b; Yokobori et al., 2012). Moreover, NPY was also shown to increase attraction to amino acids (food cue) in food-deprived adults (Kaniganti et al., 2021; Koide et al., 2009). In the second study, we explore the biochemical signalling underlying this effect (Chapter 3). Together, our work provides key insights into the mechanistic details of CART and NPY signalling in processes critical for maintaining energy homeostasis.

Chapter 2: CART in the regulation of feeding behaviour

2.1 Introduction

Isolated and sequenced by Spies et al. in 1981and later identified by Douglas et al. as a transcript upregulated in response to cocaine and amphetamine administration in the rat brain (Douglass et al., 1995; Spiess et al., 1981), CART has since been described to play an essential role in several physiological processes. These include the regulation of various innate functions ranging from nociception, thermal regulation, motor functions, fear response, regulation of energy homeostasis as well as in mediating addiction and psychostimulant effects (Hsun Lin et al., 2005; Hubert and Kuhar, 2008; Kimmel et al., 2002; Ong and McNally, 2020; Rale et al., 2017; Vicentic and Jones, 2007). Not surprisingly, CART transcript expression is reported in several locations in the central and peripheral nervous system (Singh et al., 2021; Subhedar et al., 2014). Like most neuropeptides, the CART gene generates multiple biologically active peptides. For example, human CART mRNA produces two stable bio-active fragments, CART 42-89 and CART 49-89, while the rat transcript yields CART 55-102 and CART 62-102 after tissue-specific post-transcriptional processing (Singh et al., 2021; Thim et al., 1999; Vicentic and Jones, 2007). Within the central nervous system, gene expression studies have found CART transcripts in key regulatory loci in rodents, humans, amphibians and several fish species (Lau and Herzog, 2014; Singh et al., 2021; Zhang et al., 2012). Some areas of note include regions controlling food consumption, energy expenditure, reward and motivation such as prefrontal cortex, olfactory bulb, different hypothalamic nuclei- ARC, VMH, DMH and lateral hypothalamus (LH), amygdala, nucleus accumbens (NAc), PBN and nucleus tractus solitarius (NTS) in mammals (Singh et al., 2021). Non-mammalian vertebrate systems also show the presence of CART containing neurons in interoceptory regions such as the entopeduncular nucleus (EN) and nucleus recessus lateralis (NRL) and nucleus lateralis tuberis (NLT) in the teleost brain (Akash et al., 2014; Lau and Herzog, 2014; Nishio et al., 2012; Subhedar et al., 2014). Moreover, there is considerable evolutionary conservation between CART systems across species, pointing to its critical role as a neuromodulator (Ong and McNally, 2020; Singh et al., 2021).

Of the multiple processes influenced by the CART peptidergic system, regulation of food intake and energy homeostasis is of particular interest since polymorphisms in the CART gene

and dysregulated CART peptide levels have been correlated with disorders such as obesity, anorexia nervosa and binge eating in humans (Lau and Herzog, 2014; Singh et al., 2021; Subhedar et al., 2014).

Studies in vertebrate models have identified CART expressing neurons that respond to changes in energy status of the animal with a corresponding change in CART mRNA levels. ARC, DMH and LH neurons of rodents and non-rodent models such as rhesus monkeys show downregulation in CART mRNA following fasting, which is recovered upon refeeding (Kristensen et al., 1998; Singh et al., 2021; Subhedar et al., 2014). On the other hand, signals of satiety like the peptide hormones, leptin and cholecystokinin (CCK) upregulate CART expression in these regions and CART immunoreactive (CART-ir) projections to the vagus nerve, originating from extra-hypothalamic areas like the NTS, are thought to mediate the post-prandial effects of CCK (Vicentic and Jones, 2007). Also, central administration of CART leads to a prominent suppression of food intake and dampens the effects of orexigens like NPY (Farzi et al., 2018; Kristensen et al., 1998; Lau and Herzog, 2014; Vicentic and Jones, 2007) Centrally delivered CART has also been shown to be anorexigenic in non-mammalian systems like goldfish (Volkoff and Peter, 2001a, 2001b).

Effects of localised delivery, however, are somewhat confounding. Targeted delivery into the NAc inhibits feeding while delivery into certain discrete hypothalamic areas increases food intake (Abbott et al., 2001; Vicentic and Jones, 2007). Direct activation of CART neurons also results in differential effects on feeding depending on the co-transmitter repertoire of the activated cell, as seen when activating MCH-containing LH CART neurons as opposed to ARC CART neurons (Farzi et al., 2018; Ong and McNally, 2020). CART induced effects on feeding are thus complex, often involving multiple sites of action and possibly several independent appetite circuits (Ong and McNally, 2020; Singh et al., 2021; Vicentic and Jones, 2007).

Even so, very few studies have looked at the cellular details of CARTs action in regulating feeding behaviour. One challenge in the field has been the lack of a known receptor which limits even the use of classical pharmacological approaches such as the use of peptide-receptor antagonists or agonists as well as more advanced opto- or chemo-genetic approaches that directly manipulate the target neurons.

One process that is sensitive to changes in CART and in which studies have mapped some of the downstream effectors of the CART pathway is the regulation of nociceptive transmission. Studies in spinal dorsal horn neurons have revealed that CART acts upon NMDARs to

modulate nociceptive transmission (Chiu et al., 2009; Hsun Lin et al., 2005). Evidence for a similar mechanism in the processing of innate fear responses in rats was also recently provided by Rale et al. (2017). Although a host of anatomical and functional evidence supports CARTs' role in feeding behaviour, a similar understanding of the cellular pathways involved in this context is lacking.

Therefore, the present study was undertaken to explore the function of this peptide in regulating feeding and parse out the neurophysiological mechanisms involved therein using adult Zebrafish as a model system. Apart from rodents, teleosts have emerged as a popular model to study feeding regulatory circuits, offering a shorter life cycle, ability to learn behavioural protocols, and a relatively less complex vertebrate brain with conserved functions for many of the feeding-related peptides identified in mammals (Delgado et al., 2017; Volkoff, 2016). With regards to CART peptidergic systems, fasting leads to a decrease in mRNA levels of CART while refeeding, glucose administration or hyperglycaemia were found to enhance CART expression in Zebrafish, similar to observations in mammals (Akash et al., 2014; Mukherjee et al., 2012; Nishio et al., 2012; Soengas et al., 2018; Subhedar et al., 2014). The behavioural consequences of these changes and the neuroanatomical regions involved are as yet unknown. Therefore, we designed this study to explore the functional role of CART in feeding behaviour in Zebrafish and provide insights into the underlying biochemical pathways.

The approach undertaken was to address the following questions:

1. Is CART necessary for processing satiety signals and inducing anorexia in Zebrafish?
2. What effectors lie downstream of CART?
3. What are the effects of increased CART signalling on the activity of downstream neurons?

2.2 MATERIALS AND METHODS

2.2.1 Animals

Adult Zebrafish (*Danio rerio*) of both sexes were housed in a stand-alone techniplast system and maintained on 14 hr light/12 hr dark cycle. Fish were fed Ziegler feed and live artemia three times a day. For calcium activity imaging experiments, adult Tg(NeuroD: GCaMP6f) in nacre background 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. No randomisation methods were used to allocate animals to different experimental

groups. The Institutional Animal Ethics Committee approved all the procedures employed in this study.

2.2.2 Chemicals

CART peptide (55-102 a.a) (10 ng) used was provided by Thim lab and Phoenix Pharma (Cat. No. 003-62). MK801 (0.015 nmol, Sigma-Aldrich, Cat. No.77086), KT5720 (1.39 pmol, Sigma-Aldrich, Cat. No. K3761), GF109203X (1.5 pmol, Tocris, 0741), 2-Phenoxy Ethanol (1: 2000, Sigma-Aldrich, Cat. No. P1126), D -(+)- glucose monohydrate (8000 ng, glucose; Sigma-Aldrich, Cat. No. 49161), paraformaldehyde (Sigma-Aldrich, Cat. No. 158127), sucrose (Sigma-Aldrich, Cat. No. S9703), poly L- lysine (Sigma-Aldrich, Cat. No. P8920), normal goat serum (Sigma-Aldrich, Cat. No.9023) were procured from Sigma. D-AP5 (0.1 nmol, Tocris, Cat. No. 0106). Bovine serum albumin (BSA) (SRL pvt ltd, Cat. No. 83803) was purchased from Sisco Research Laboratories.

2.2.3 Intracerebroventricular (icv) injections

Fish were anesthetised in 2-phenoxy ethanol (1: 2000 in system water). The anesthetised fish were placed on a cotton bed containing anaesthesia solution to submerge the head of the fish. Icv delivery protocol was modified after (Yokogawa et al., 2007). Briefly, pharmacological agent or vehicle was delivered directly into the ventricular space using an insulin needle (Becton Dickinson Insulin Syringe U40 - 31G) attached via a catheter to a 10 µl Hamilton microsyringe. After injection, the fish were returned to their tanks and allowed to recover before proceeding for either behavioural recordings, immunohistochemistry or dynamic activity imaging. Unless otherwise indicated, all the behavioural and immunofluorescence studies were undertaken 15 mins following the injection.

2.2.4 Immunohistochemistry

Fish were icv injected with drug or vehicle and allowed to recover. After recovery, fish were anesthetised and craniotomised to expose the dorsal surface. Samples were fixed in 4% PFA overnight (O/N) at 4°C. After fixation for 12-14 hrs (or 10 hrs in case of p-NR1 staining), brain was dissected out and cryoprotected in 25% sucrose (O/N at 4°C) prior to sectioning. Serial 15-20 µm thick sections of the entire telencephalon were collected onto lysine coated glass slides and stored at -40°C till further processing. Sections were allowed to dry for 2 hrs at room temperature (RT) before proceeding to rehydration and permeabilisation with 0.5% Triton-X100 in 1X PBS (PBST) 3 times for 10 min each. Sections were then blocked using 5% BSA-PBST for 1 hr prior to addition of primary antibody and incubated overnight at 4°C. The

following primary antibodies were used in this study: anti-p-ERK 1/2 (Thr 202; Tyr 204) (1:700, Cell Signalling technologies, Cat. No. 9101), anti-p-NR1(Ser-897) (1:70 , millipore, Cat. No. ABN99).

Next day, washes were repeated and sections blocked in 5% BSA/ 5% heat inactivated goat serum (HIGS) for 1 hr. Secondary antibody [anti-rabbit Alexa-488 (1:500; Invitrogen, Cat. No. A11034) and anti-mouse Alexa-568 (1:500; Invitrogen, Cat. No. A11031)] was added and incubated at RT for 2.5 hrs in dark. Sections were washed three times for 10 min each using PBST and mounted in mounting media (N propyl gallate, 70% glycerol and 1X PBS) containing DAPI (Invitrogen, Cat. No. D1306). Sections were observed under an epifluorescence microscope (Axioimager Z1 with Axiocam HRm camera; Carl Zeiss) and representative images were acquired using Leica SP8 or LSM 780 (Carl Zeiss) laser scanning confocal microscopes. In order to ensure reliable comparisons across different groups and maintain stringency in tissue preparation and staining conditions, all brain sections were processed concurrently and imaged under identical conditions.

2.2.5 Feeding behaviour protocol

Fish were isolated from home tanks and housed singly in the experimental tank. Fig. 2.1 outlines the protocol followed for all feeding behaviour experiments. Briefly, for first three days of habituation, the experimental tanks were moved to the recording chamber and the fish were allowed to feed on coloured food pellets (~ 10-15 pellets; Taiyo) for 1 hour before returning to the housing chamber. For habituation to injection and handling stress, the fish were anesthetised and received icv saline (0.9% NaCl) injection (day 4) or a mock injection (day 5). They were allowed to recover in recording chamber for 1 hour, before returning the tanks to the housing chamber. Fish were observed on all days of the protocol for any anxious/abnormal behaviour. Fish which exhibited these signs were not used in the study. The fish were food deprived starting from day 3 (post habituation) till experimental day. On the day of the experiment, fish were anesthetised and icv injected appropriate drugs/ vehicle. Following a recovery period of 15 mins, pellets of food (~10-15) were added to the tank and the behaviour of the fish, taken one at a time, was monitored using a video recorder (Sony Handycam) for one hour. In all of the experiments animals that were food deprived for three days are referred to as ‘starved’.

2.2.6 Calcium imaging

Starved adult Tg(NeuroD:GCaMP6f) fish were injected with either saline or CART peptide (10 ng). After 15 min, the fish were anesthetised in ice-cold HEPES-based Ringer's solution [pH 7.2 containing NaCl, 134 mM; MgCl2, 1.2 mM; CaCl2, 2.1 mM; KCl, 2.9 mM; HEPES, 10 mM; Sucrose, 10 mM (for glucose-deprived samples) or Glucose, 10 mM (for samples subjected to mimicked satiety conditions)] continuously bubbled with 100% O_2 and craniotomised to dissect the telencephalon. For experiments testing neural activity under different physiological conditions, starved fish were anesthetised in ice-cold HEPES-based Ringer's solution and used for dissection of the whole brain. Intact telencephalon or whole brain was mounted in 4% low-melt agarose (in 50% Ringers) onto RC-26 GLP low profile bath chamber from Warner Inst (Cat. No. 640236). The imaging chamber was mounted onto an upright confocal microscope (Leica TCS SP8 MP) and continuously perfused with HEPES-based Ringer's solution via VC 8 perfusion system (Warner Inst, Cat. No.640186) trigger controlled using the recording software (Leica). Images were acquired in resonant bi-directional scanning mode on Leica TCS SP8 MP with 25x water-immersion objective (N.A 0.95) and binned to 512x300 pixels at 9.8 Hz using the confocal laser (488 nm) and PMT detector.

2.2.7 Data Analysis

(a) <u>IHC experiments: activity based p-ERK labelling in Dm:</u> The tissue slices were observed under Zeiss Apotome microscope (Axioimager Z1, 20x objective), and the number of p-ERK positive cells in Dm were scored manually. To avoid overestimation of cell count due to serial sections, the cell numbers were corrected using Abercrombie's method (Abercrombie, 1946)

(b) <u>Average p-NR1 intensity in Dm:</u> Images were aquired at 40x on LSM 780 (Carl Zeiss) under identical conditions. DAPI channel was used to mark out the Dm and for ROI segmentation using Image-J ROI manager. Image-J 3D manager plugin was then used for estimation of mean p-NR1 fluorescence intensity values.

(c) <u>Dynamic neural activity experiments:</u> Measurements of the population level calcium activity in Dm were performed using Image-J, where the average GCaMP6f fluorescence in Dm was calculated for each time point. F0 was obtained by taking the average of fluorescence values in first 620 frames i.e before glutamate treatment. The relative change in fluorescence ($\Delta F/F0$), i.e F-F0/F0, where F stands for the fluorescence value at a given time, and the

maximum response amplitude i.e max $\Delta F/F0$ were calculated using Microsoft Excel. The total response elicited was calculated using the AUC function in GraphPad Prism 8.

(d) Feeding behaviour assay: Videos were analysed manually at 1.5x speed and the number of biting attempts were scored. The data was recorded in bins of 15 mins and cumulative biting attempts were plotted using GraphPad Prism software.

2.2.8 Statistical Analysis

Data were tested for normality using Shapiro-Wilk test in GraphPad Prism 8. Behaviour and immunohistochemical data analyses were performed using t-test with Welch's correction or Mann-Whitney test (for single comparison) and One-Way ANOVA with Tukey's test or Two-way ANOVA with Bonferroni's post-hoc 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.

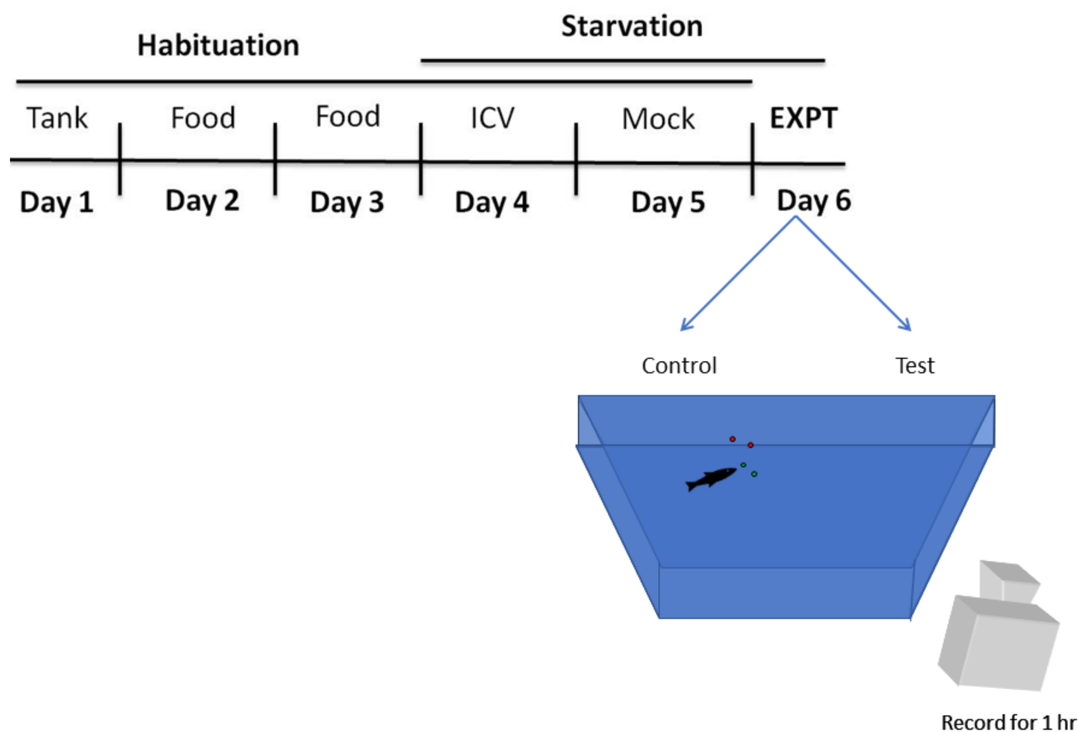


Fig. 2.1. Experimental design for food-intake assay

Adult Zebrafish (3-4 months, Indian Wild-type strain) were isolated (day 1) and habituated to pellet food (day 2-3), followed by food-deprivation and habituation to injection stress (day 4-5). On the test day, fish were injected via the intra-cerebroventricular (icv) route with vehicle or drug and the feeding behaviour was recorded for a period of 1hr.

2.3 Results and Discussion

2.3.1 CART induces anorexia in Zebrafish

Given that the CART transcript and peptide levels are upregulated in CART-ir neurons in the EN and Hypothalamus in well-fed or glucose treated Zebrafish (Akash et al., 2014; Mukherjee et al., 2012), we tested if these changes translate to an alteration in the behaviour of adult fish using the feeding assay described in the Methods (section 2.2.5). We first compared the feeding behaviour of animals in two different physiological states, i.e. starvation and satiety. For this, we icv injected starved fish with either vehicle or glucose (8000 ng). Glucose delivered centrally is known to establish a state of satiety in vertebrates, including teleosts, in addition to upregulating the CART system (Subhedar et al., 2011). Moreover, this ensures a more uniform

baseline satiety condition than recently fed fish which may exhibit variability due to differences in individual feeding tendencies. As shown in Fig. 2.2 A, control fish show a robust feeding response when presented with food pellets which is reflected in the high number of cumulative biting attempts for this group (852.016±133.3498). On the other hand, glucose treatment drastically reduces biting attempts (221.22±33.6738). To test if CART plays a role in mediating the observed glucose-induced anorexia, we disrupted the endogenous CART function by co-injecting fish with an immuno-neutralising anti-CART antibody (Thim, 1: 500) along with glucose. Neutralising endogenous CART peptide restored the feeding drive to levels comparable to controls (629.8866±79.3060 cumulative biting attempts). Thus, indicating that endogenous CART is required for the expression of glucose-induced anorexia.

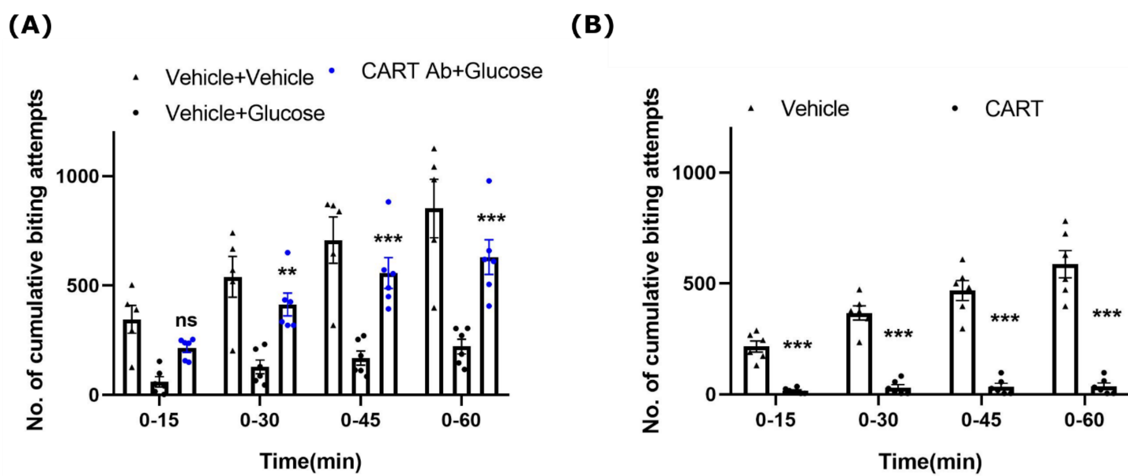


Fig. 2.2. CART is necessary and sufficient for inducing anorexia in Zebrafish.

(A) Treatment with CART antibody (1:500, 2 µl) reduces glucose (8000 ng) induced anorexia (N= 5 vehicle control animals and N=6 glucose and CART antibody + glucose treated animals; **p<0.01, ***p<0.001, ns, not significant). **(B)** Treatment with CART peptide (10 ng) induces anorexia in starved fish (N=6 animals per group; ***p<0.001). Data are represented as cumulative biting attempts in 15 min bins over a 1-hour period and are compared using Two-way ANOVA, with Bonferroni's post-hoc analysis (Error bars represent ±SEM).

To test if CART is sufficient to induce anorexia, we injected starved fish with vehicle or synthetic rat CART peptide (55-102 a.a., 10 ng) and recorded the feeding behaviour. As seen in Fig. 2.2 B, fish treated with this peptide make fewer biting attempts (36.2133±14.8895) compared to controls (586.9866±61.2811).

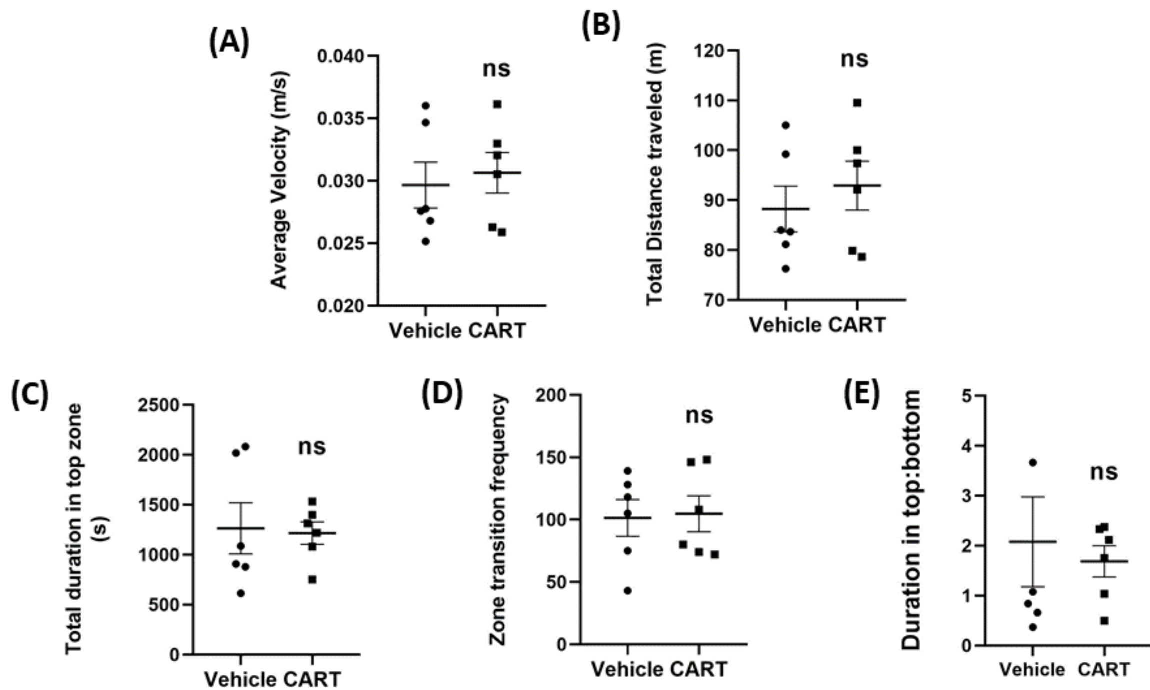


Fig. 2.3. CART treated animals do not display markers of anxiety-like behaviour

In the absence of a strong motivating factor such as food, starved animals injected with vehicle or CART peptide do not show any appreciable difference in terms of their locomotor abilities (**A** and **B**). These animals also exhibited similar exploration tendencies making fewer transitions to the bottom half of the tank and preferred to stay mostly in the top half (**C**-**E**). The data are shown as scatter plots of individual data points for each group along with the Mean±SEM and analysed using t-test with Welch's correction (N=6 animals per group; ns, not significant).

An analysis of the locomotor dynamics (Fig. 2.3. A and B) and parameters of anxiety-like behaviour such as time spent in the top half of the tank and number of transitions to the bottom (Fig. 2.3. C-E) in the absence of food revealed no significant differences in the control and peptide treated groups. Indicating that the decrease in biting attempts was not a result of anxiety or defects in locomotor systems of the fish but rather a decrease in the motivation to feed, suggesting a conserved role for CART as an anorexic agent in the vertebrate feeding circuits.

2.3.2 CART acts on the dorsomedial telencephalon

An immunohistological screen conducted in our lab using phospho-ERK (p-ERK) as a marker for recent neural activation identified a region in the Zebrafish forebrain that is activated in response to CART (unpublished lab data).

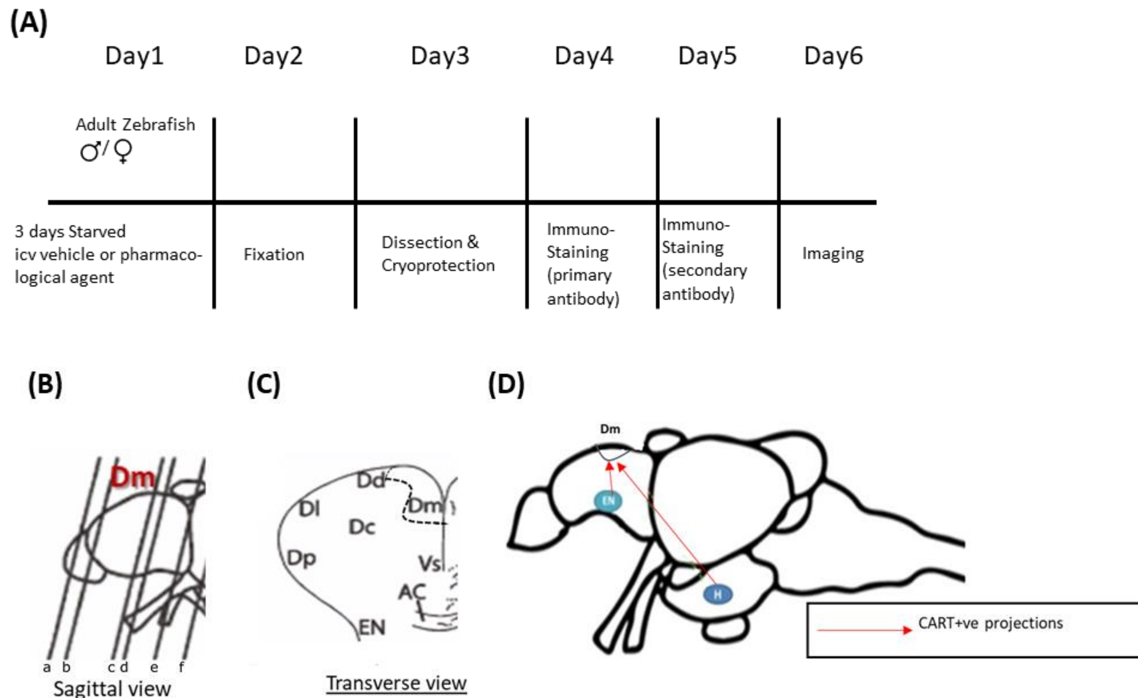


Fig. 2.4. Activity-based labelling identified dorsomedial telencephalon as a CART responsive region.

Immunohistochemistry protocol outlined in **(A)** was followed to identify CART responsive regions in adult Zebrafish brain using phospho-ERK immunoreactivity (p-ERK-ir) as a marker for recent neural activation. This approach identified a region in the dorsomedial telencephalon (Dm; sagittal planes b to d in **(B)** and dashed outline in **(C)** as a target region for CART (D. Wakhloo and T. Kaniganti, unpublished lab data). Dm receives CART immunoreactive projections from energy sensing regions in Zebrafish brain (**D**; unpublished lab data) and was thus selected for further investigation of CART signalling in this thesis. [EN: Entopeduncular nucleus; Dm: medial zone of the dorsal telencephalic area; Dc: central zone of the dorsal telencephalic area; Dp: posterior zone of the dorsal telencephalic area; Dl: lateral zone of the dorsal telencephalic area; Dd: dorsal zone of the dorsal telencephalic area; Vs: supracommissural nucleus of the ventral telencephalic area; H: Hypothalamus; AC: Anterior commissure; icv: intracerebroventricular]

Located in the dorsomedial telencephalon, this region referred to as 'Dm' receives projections from energy-sensing regions such as EN and hypothalamus and is highly active in CART

treated fish compared to vehicle injected controls (Fig. 2.4 and Fig. 2.5). We have thus focused on this region in our analysis of the cellular mechanisms underlying CARTs anorectic actions described in the following sections.

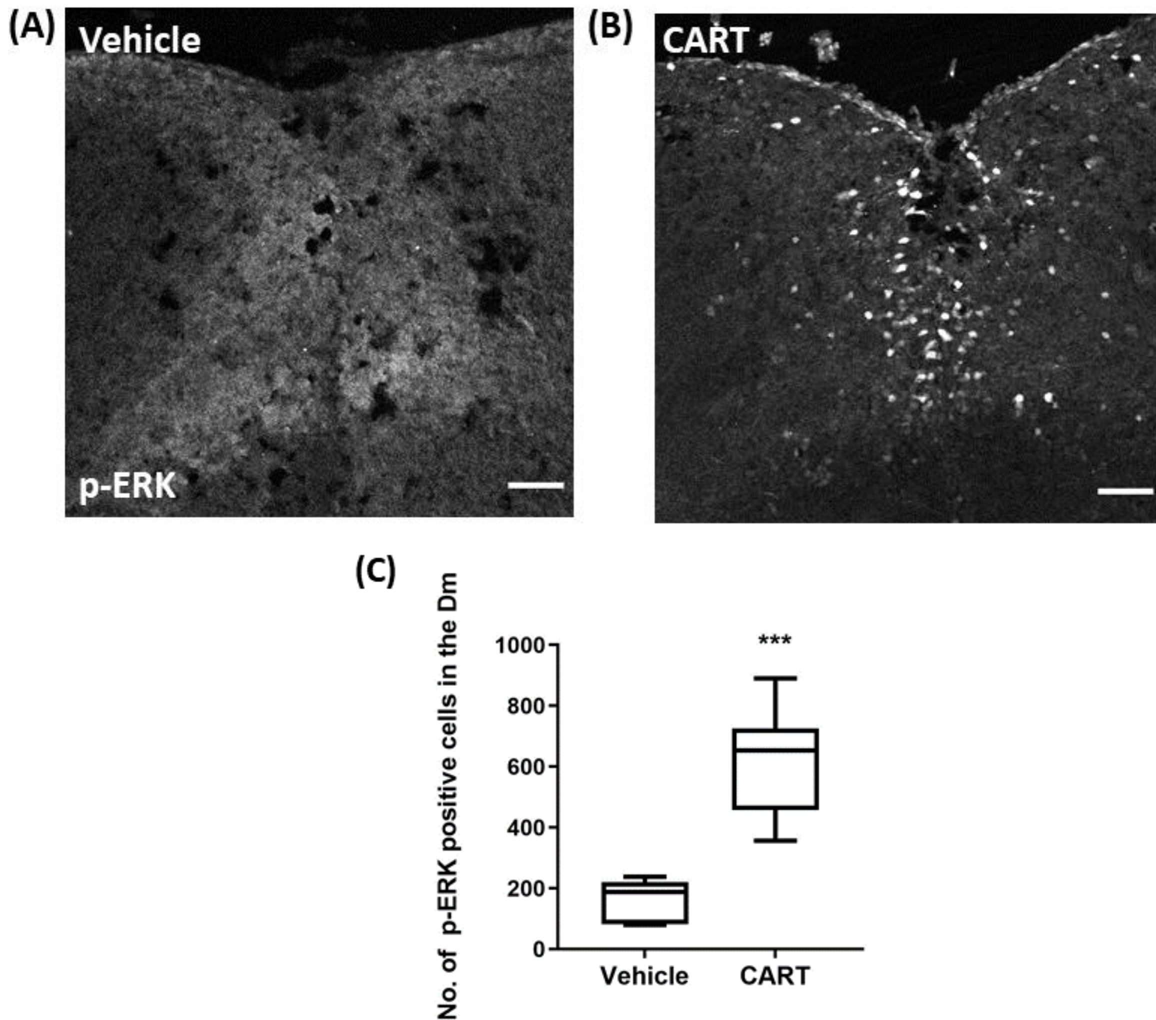


Fig. 2.5. CART treatment upregulates neural activity in Dm.

Number of p-ERK immunoreactive cells in the Dm across all 15 µm sections of the telencephalon in starved fish treated with either vehicle **(A)** or CART peptide (10 ng) **(B)**. Data are shown as mean ± SEM and means were compared using unpaired t-test with Welch's correction (N=6; ***, p< 0.001). [Scale bar 50 µm]

2.3.3 CART mediated anorexia and upregulation of neural activity in Dm requires signalling via NMDARs

Although the lack of a known CART receptor has limited our knowledge of possible molecular players recruited by CART, reports studying CART signalling in nociceptive transmission (Chiu et al., 2009; Hsun Lin et al., 2005) and in processing innate fear responses (Rale et al., 2017) suggest NMDAR as a target of CART based modulatory action. The involvement of NMDARs in regulating food intake has also been well documented by studies in various vertebrate systems such as rats and goldfish (Campos and Ritter, 2015; Guard et al., 2009; Kang et al., 2011; Liu et al., 2012). To investigate if NMDAR signalling is involved in mediating CART peptides effects on feeding behaviour in Zebrafish, we tested the effect of NMDAR blockade on CART induced anorexia. We thus co-injected MK801 (non-competitive NMDAR antagonist, 0.015 nmol) along with CART (10 ng) 15' prior to the start of the behavioural experiment. While application of the antagonist alone showed no significant difference in biting attempts (602.5746±55.3894) as compared to vehicle controls (873.0303±83.8026), treatment with MK801 and CART together abrogated CART induced anorexia (798.2±113.0729 cumulative biting attempts for inhibitor+ CART group as compared to 42.6666±19.7613 for CART alone) (Fig. 2.6 A).

To test if NMDAR antagonism also blocks the upregulation in neural activity seen in response to treatment with CART, we co-administered MK801 (0.015nmol) with CART (10 ng) in starved fish via the icv route. Treatment with the drug alone did not affect basal neural activity in the Dm (Fig. 6 C-D and Fig. 5 A and C). As seen from the representative micrographs in Fig. 2.6 B & C and the corresponding quantitation in Fig. 2.6 D, NMDAR blockade reduced CART induced neural activity in the Dm. Together these data indicate that NMDAR activity is required for processing CART induced satiety signals.

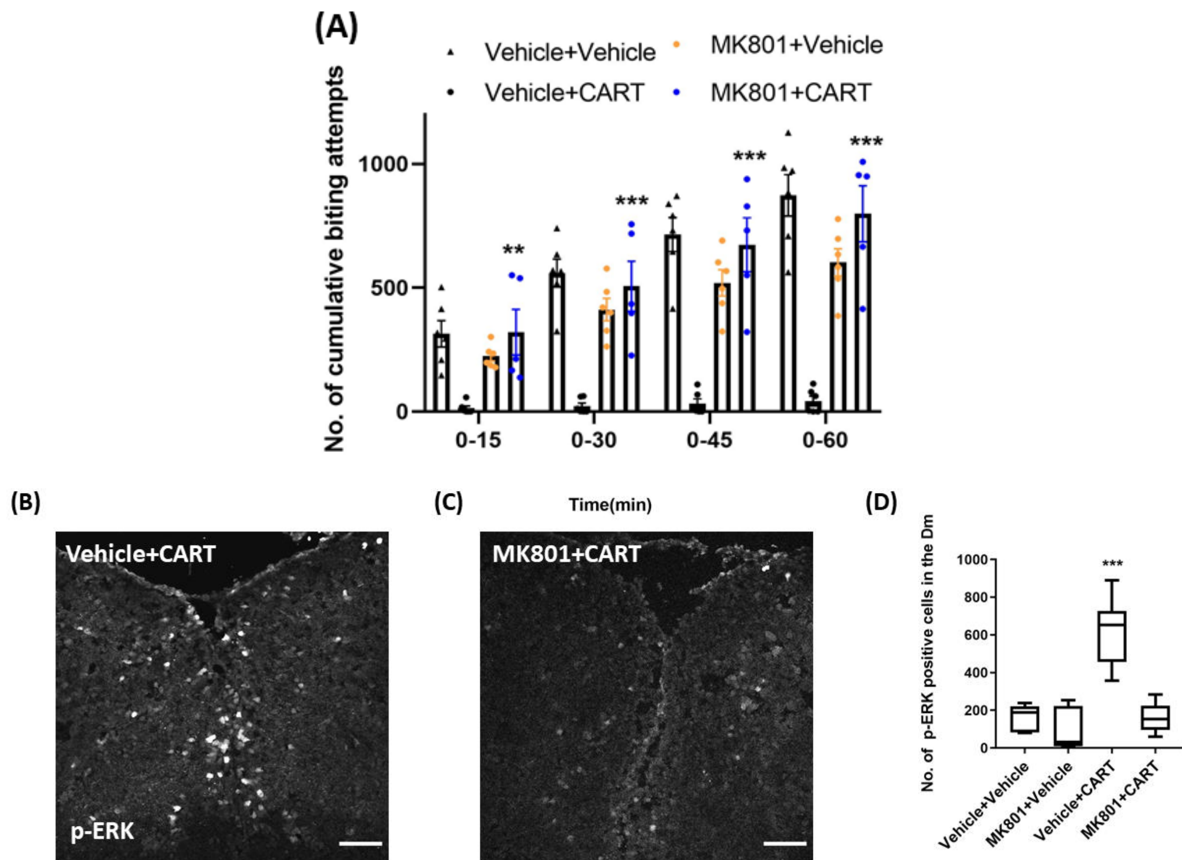


Fig. 2.6. CART induced anorexia and neural activation in Dm requires NMDAR signalling.

Treatment with non-competitive NMDAR antagonist, MK801 (0.015 nmol) abrogates CART induced anorexia **(A)**. Data are represented as cumulative biting attempts in 15 min bins over a 1-hour period and compared using Two-way ANOVA, with Bonferroni's post-hoc analysis (N=6 animals for all treatments except MK801+ CART where N=5); error bars represent ±SEM; **p<0.01, ***, p< 0.001). Representative transverse sections showing the immunoreactivity of p-ERK (p-ERK –ir) in starved fish treated with either vehicle **(B)** or MK801 **(C)** prior to CART treatment. **(D)** Quantification of the data in **(B-C)** (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. [Scale bar 50 µm]

2.3.4 CART signalling acts via Protein Kinase A (PKA)

As discussed in chapter 1, most neuropeptides act via activating G protein-coupled receptors. Although the CART receptor is as yet unidentified, studies in cell lines such as At20 (Lakatos et al., 2005) and PC12 (Lin et al., 2011) indicate that this is likely to be the case for CART as well. Indeed, a recent report by Haddock et al. proposes GPR160 as a putative CART receptor in the context of nociceptive transmission (Haddock et al., 2021). Protein kinase activation due to GPCR activation is a well-known mechanism whereby acute signals result in sustained changes in cellular function. Our data from behaviour and activity-based p-ERK labelling identified changes in NMDAR activity as an essential step in CART signalling (section 2.3.3). Numerous studies have indicated a role for protein kinases in modulating NMDAR activity (Castillo et al., 2011; MacDonald; Pitcher et al., 2008; Ron, 2004; Wang et al., 2014) and *in vitro* studies using DRG cultures have identified PKA as one of the downstream effectors of CART (Chiu et al., 2009). We, therefore, tested if PKA activity is also required for CART induced anorexia. With this aim, KT5720, a selective PKA inhibitor (0.75 ng), was co-injected along with CART (10 ng) 15' prior to the start of behavioural recording. While animals treated with the inhibitor alone showed feeding drive comparable with controls (713.92±215.6766 cumulative biting attempts for KT5720 vs 586.9866±61.2811 for vehicle), delivery of CART alone significantly reduced feeding drive over a one-hour period (30.3333±10.6134 cumulative biting attempts) (Fig. 2.7 A). This anorectic effect of CART was significantly reduced by co-injection with the selective PKA inhibitor (697.4933±105.5933) (Fig. 2.7 A).

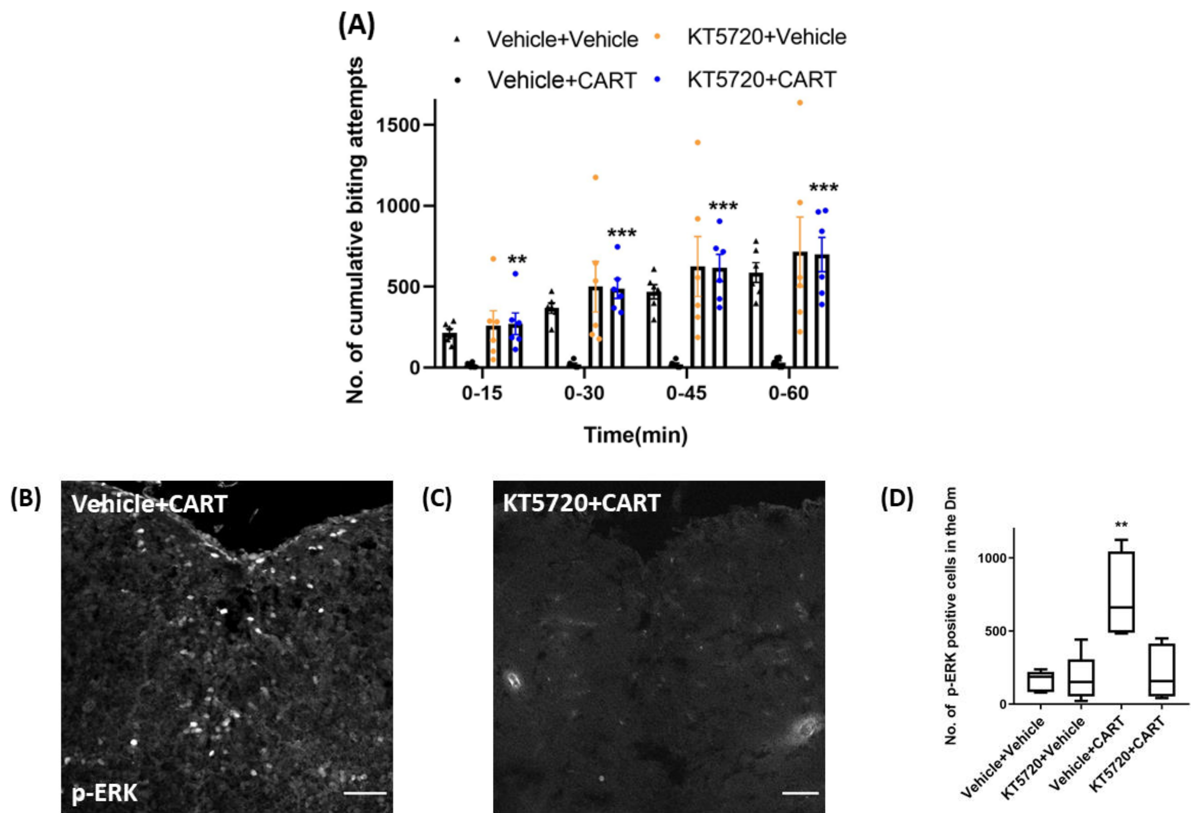


Fig. 2.7. CART induced anorexia and neural activation in Dm requires PKA activity.

Treatment with **(A)** selective PKA inhibitor, KT5720 (0.75ng) abrogates CART induced anorexia. Data are represented as cumulative biting attempts in 15 min bins over a 1-hour period and compared using Two-way ANOVA, with Bonferroni's post-hoc analysis (N=6 animals per group; error bars represent ±SEM; **p<0.01, ***, p< 0.0001). Representative transverse sections showing the immunoreactivity of p-ERK (p-ERK–ir) in starved fish treated with either vehicle **(B)** or PKA **(C)** prior to CART treatment. **(D)** Quantification of the data in **(B-C)** Data was analysed using Two-way ANOVA, with Bonferroni's post-hoc analysis (N=6 animals per group, error bars represent ±SEM; **, p<0.01).
[Scale bar 50 μm]

We also tested if the increase in the activity of Dm neurons following CART treatment requires PKA, for which we co-injected KT5720 (0.75 ng) and CART in starved animals. CART treated samples showed high activity in Dm neurons compared to samples treated with vehicle or inhibitor alone (Fig. 2.5 A and C, Fig. 2.7 C). As illustrated in the representative micrographs (Fig. 2.7 B-C) and the corresponding quantitation in Fig. 2.7 D, inhibition of PKA activity significantly blocks CART induced upregulation of Dm activity. These data, therefore, suggest

a role for the PKA pathway in mediating CARTs modulatory actions, possibly via altering NMDAR subunit phosphorylation levels.

2.3.5 CART increases the phosphorylation of NR1 subunit of the NMDA-R in Dm neurons via PKA

PKA belongs to the class of Serine/Threonine kinases that have known phosphorylation sites on NMDAR subunits. PKA mediated phosphorylation of NR1 subunit of NMDARs is well documented (Chen and Roche, 2007; MacDonald; Wang et al., 2014). Phosphorylation of receptor subunits changes the channel properties and consequently NMDAR activity and often generates changes in an animal's behaviour. Phosphorylation of NR1 subunit at the Ser 897 site has been shown to enhance NMDAR function and excitatory postsynaptic currents (EPSCs) by altering channel opening probability, Ca^{2+} permeability and receptor trafficking (MacDonald; Pitcher et al., 2008; Ron, 2004).

To assess if CART recruits PKA to alter the phosphorylation status of NMDAR NR1 subunits in the Dm, we compared levels of phospho (Ser 897)-NR1 (p-NR1), which is the target site of PKA activity. Fluorescence intensity analysis of samples from CART treated animals that were co-injected with vehicle or PKA inhibitor (KT5720, 0.75 ng) and labelled with anti-p-NR1 antibody revealed that PKA inhibition markedly reduced p-NR1 levels in the Dm when compared with brains of animals that were co-administered with vehicle (Fig. 2.8 A-C).

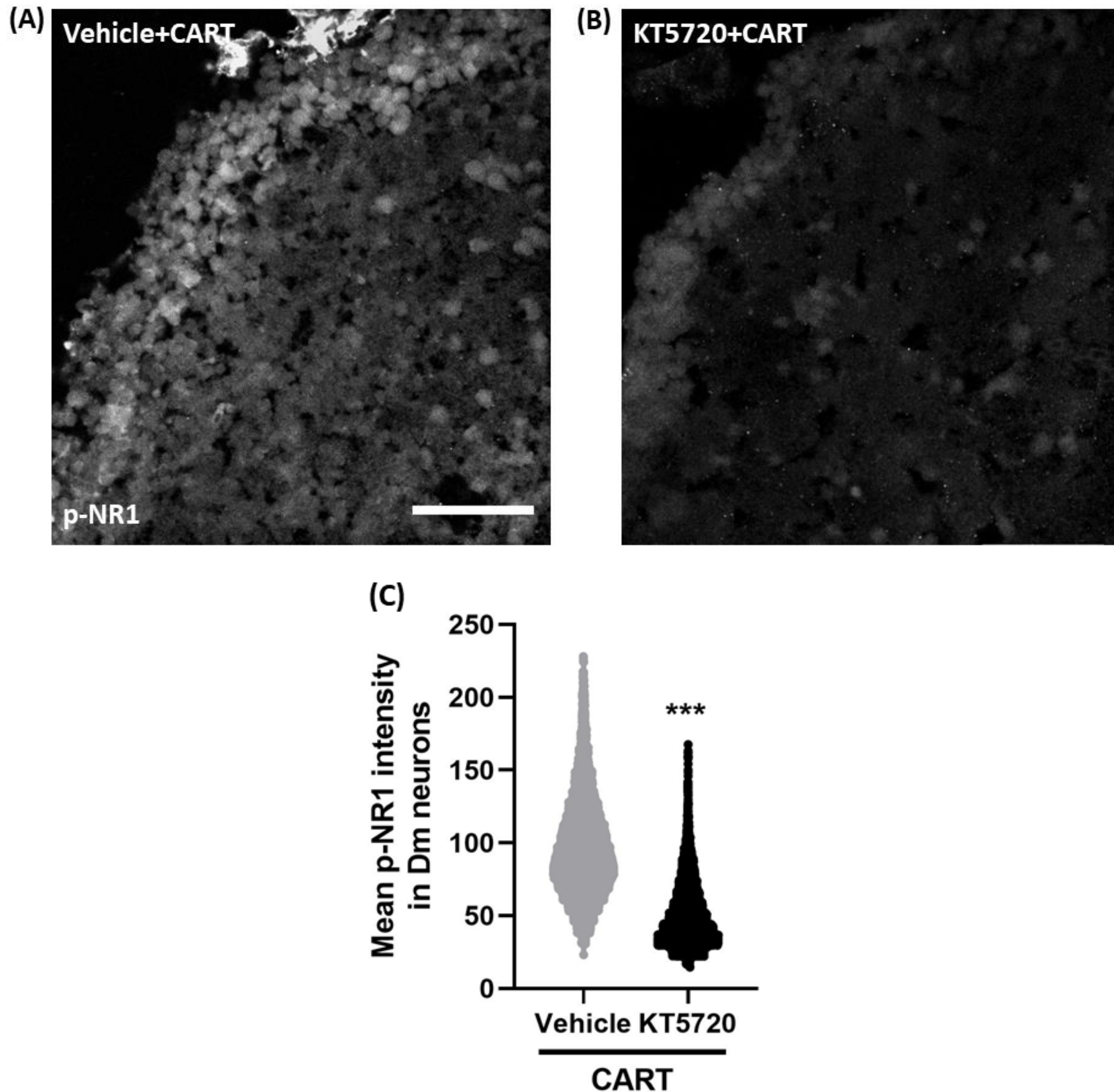


Fig. 2.8. CART treatment increases the levels of NMDAR subunit p-NR1(Ser897) in a PKA dependent manner.

Representative transverse sections showing the immunoreactivity of phospho-NR1 (Ser897) (p-NR1) in the Dm of starved fish treated with either vehicle **(A)** or KT5720 **(B)** 15 min prior to CART treatment. **(C)** Mean p-NR1 intensity in Dm neurons is higher in CART treated samples as opposed to samples treated with PKA inhibitor prior to CART injection. Data are represented as scatter plot of fluorescence intensity per neuron across all 20 µm sections of the Dm and analysed using Mann-Whitney U-test (N = 3 animals per group; ***p<0.001) [Scale bar 50 µm]

The data thus indicate that CART acts via PKA to alter phosphorylation of NMDARs in Dm neurons leading to an increase in neural activity in the Dm (Fig. 2.5 A-C and Fig. 2.8 A-C).

2.3.6 CART mediated sensitisation of Dm (performed in collaboration with Devika Bodas)

Dm receives glutamatergic projections from EN, a putative interoceptory region (unpublished lab data) and changes in excitatory tone in Dm are important for CART-mediated satiety signalling since the disruption of NDAR signalling abolished CART induced anorexia and neural activity (Fig. 2.6). Furthermore, the increase in phosphorylation of NR1 subunit of NMDARs seen in Dm neurons upon CART treatment (Fig. 2.8) could alter gating properties, calcium permeability of NMDAR as well as enhance NMDAR-mediated EPSCs (Pitcher et al., 2008; Ron, 2004). Such changes would ultimately sensitise and enhance the response of these neurons to glutamatergic inputs.

In order to test this hypothesis, we used transgenic Tg(NeuroD: GCaMP6f) fish because this transgenic line has a strong expression of the calcium indicator in the Dm (Fig. 2.9 A and B).

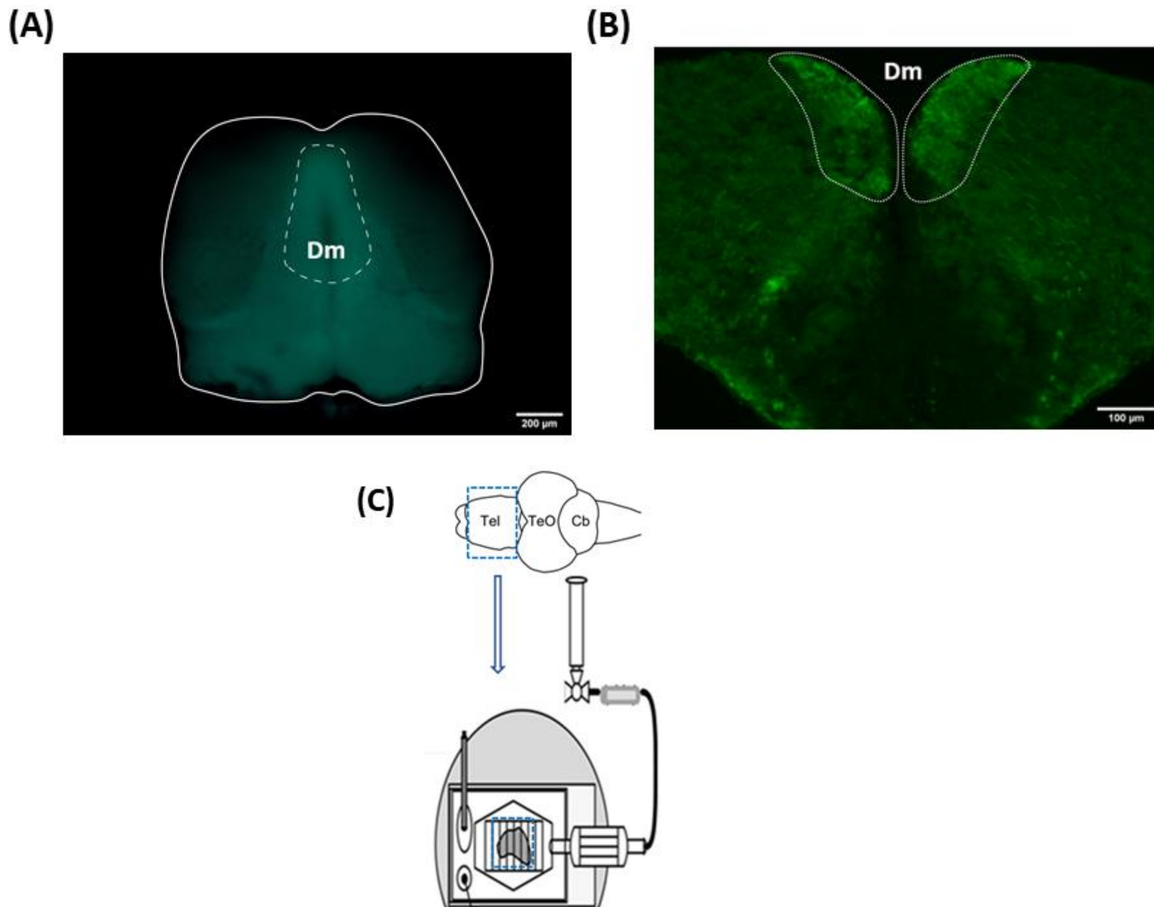


Fig. 2.9. GCaMP6f expression pattern in transgenic (NeuroD: GCaMP6f) adult fish and *ex vivo* telencephalon imaging set up.

Stereo microscope image showing GCaMP6f expression in adult Tg(NeuroD:GCaMP6f) fish craniotomised to expose the telencephalon (positioned with its rostral end towards the top of the image; white continuous line marks the telencephalon and dashed lines mark the Dm region) **(A)**. Transverse section (300 µm thick) **(B)** with a strong expression of the calcium indicator in the Dm (marked with white dotted line). **(C)** Schematic of the perfusion chamber mounted with intact telecephalon (marked with coloured dotted line) used for *ex vivo* neural activity imaging. [Scale bar 200 µm in (**A**) and 100 µm in (**B**)]

Starved Tg(NeuroD: GCaMP6f) animals were injected with either vehicle or CART and then used to make *ex vivo* preparations of intact telencephalons. Fig. 2.9 C shows the experimental

set-up, and the glutamate stimulation protocol is depicted in Fig. 2.10 A (discussed in detail in section 2.2.6) along with snapshots of neural activity in the Dm of vehicle (Fig. 2.10 B) or CART treated (Fig. 2.10 C) animals.

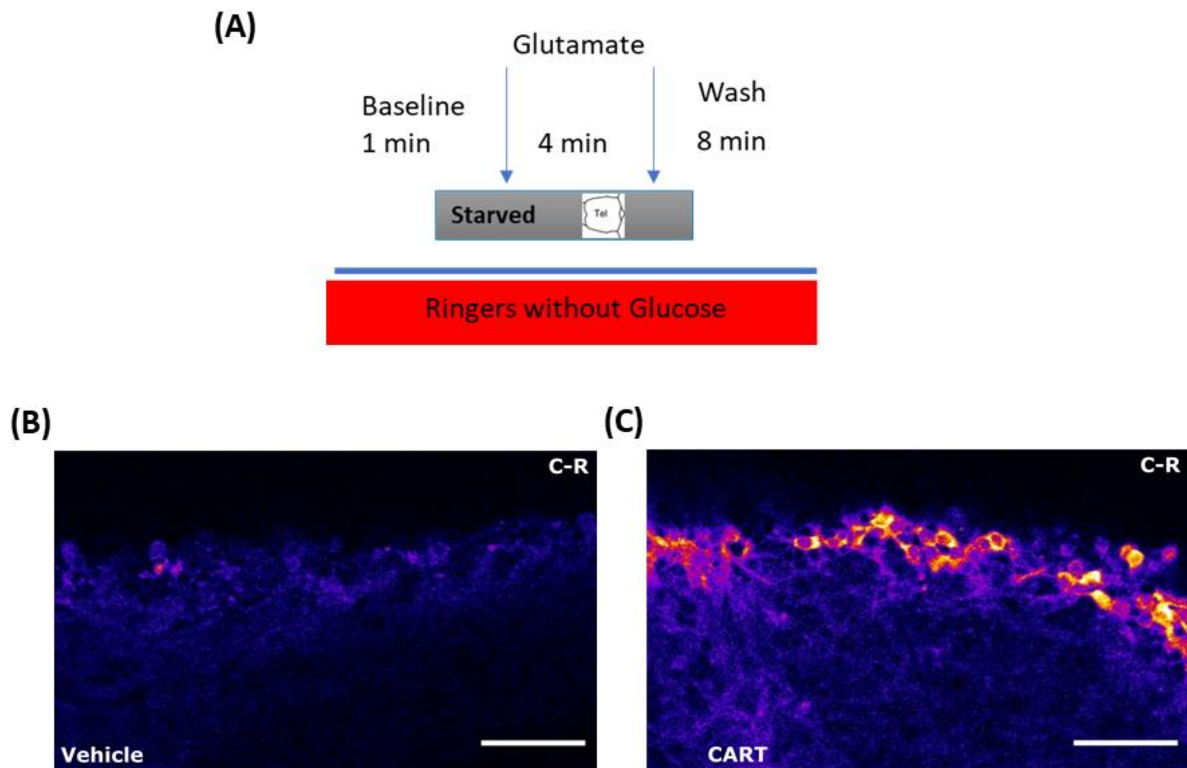


Fig. 2.10. Stimulation paradigm and example snapshots of glutamate stimulated neural activity in Dm.

Telencephalons from starved animals injected with vehicle or CART were dissected and mounted in a chamber perfused with Ringers-Sucrose (10 mM) buffer and stimulated with glutamate (75 µM) as per the protocol in **(A)**. Example snapshots of neural activity in Dm of vehicle **(B)** and CART **(C)** injected samples taken at the same time points post stimulation with glutamate (75 µM). Images are pseudo-coloured with warmer shades corresponding to higher activity. The ventricle is towards the top of the image and C and R indicate caudal and rostral ends respectively [Scale bar 50 µm].

When stimulated with glutamate (75 µM), Dm of CART treated animals showed heightened neural activity, both in terms of the maximum amplitude as well as the overall response (as indicated by an increase in the total area under the curve; AUC) (Fig. 2.11 A-C).

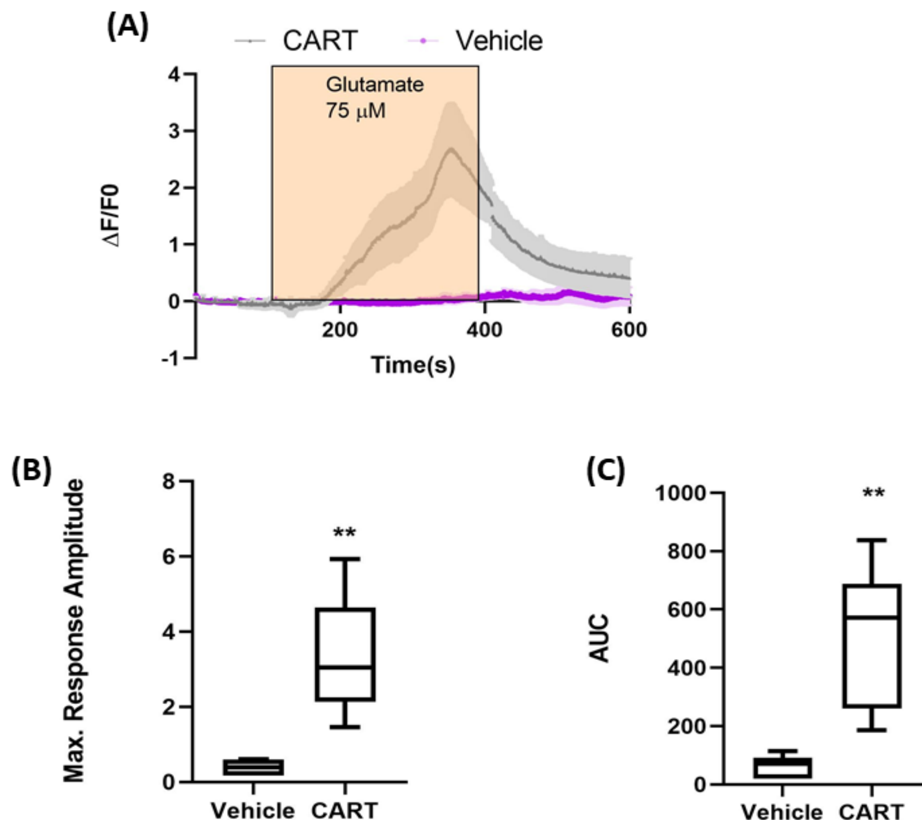


Fig. 2.11. CART treatment heightens response to excitatory stimulation.

Traces represent relative change in fluorescence intensity (ΔF/F0) 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 coloured rectangular box indicates the duration for which glutamate was presented. **(A)** Dm neurons of CART treated animals (grey trace) show increased response as compared to controls (purple trace). **(B)** The peak response amplitude [max (ΔF/F0)] as well as **(C)** total response [Area Under Curve (AUC)] in Dm neurons of CART treated animals is higher than controls. The data is plotted as box-and-whisker (Tukey) and was analysed using t-test with Welch's correction. (N=6 telencephalons per group; **, $p<0.01$).

Furthermore, fish co-treated with AP5, a competitive NMDAR antagonist, together with CART, failed to show such an enhanced response to glutamate (Fig. 2.12 A-C). Thereby confirming the contribution of NMDAR based signalling in processing CART induced modulatory changes.

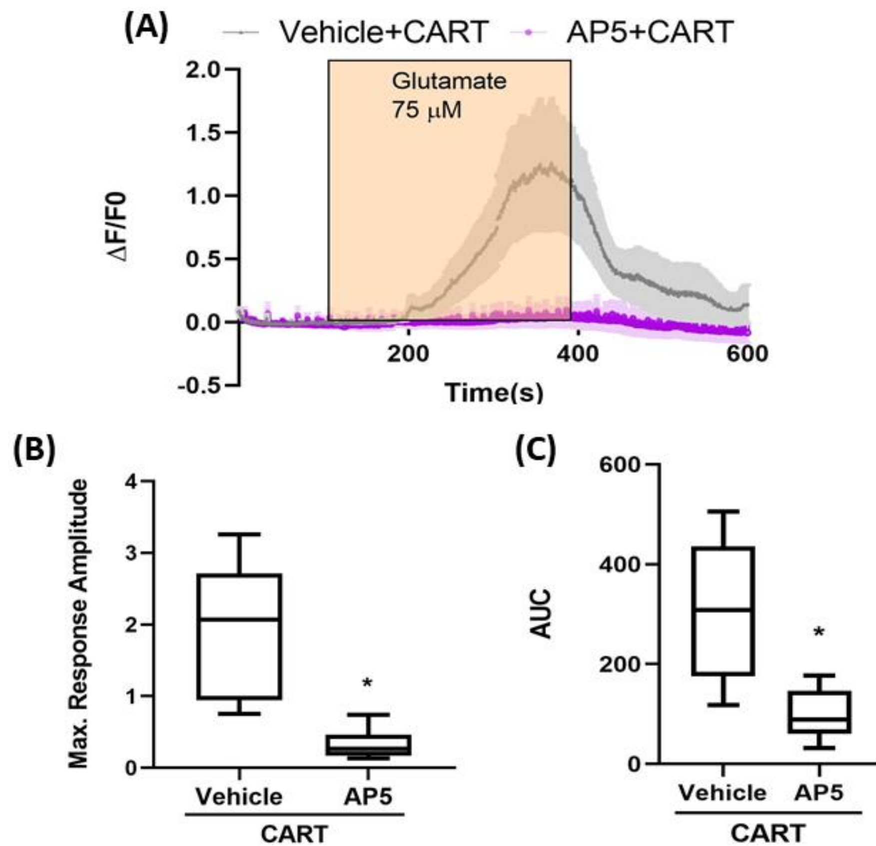


Fig. 2.12. CART based sensitisation of Dm neurons requires signalling via NMDAR.

Traces represent relative change in fluorescence intensity (ΔF/F0) 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 coloured rectangular box indicates the duration for which glutamate was presented. **(A)** Dm neurons of CART treated animals (grey trace) show increased response as compared to brains which were treated with AP5+CART (purple trace). **(B)** The max (ΔF/F0) and **(C)** AUC for Dm neurons of CART treated animals is higher than brains from animals treated with AP5+CART. The data is plotted as box-and whisker (Tukey) and was analysed using t-test with Welch's correction (N= 5 control and N=6 AP5+CART telencephalons; *, $p < 0.05$)

To determine if this change in the response profile of Dm neurons requires protein kinases known to target NMDAR subunits, we co-administered selective inhibitors for the protein kinases PKA and PKC, i.e. KT5720 and GF109206X, respectively, along with CART. Inhibitors for both kinases were used since PKA is necessary for CART induced anorexia, neural activation in Dm, and increased p-NR1 (Ser897) levels (Fig. 2.7 and Fig. 2.8) and PKC was found to have a similar role in a parallel study in our lab (D. Bodas, unpublished data).

Interestingly, blocking PKA and PKC activity prior to CART injection led to a stark decrease in the maximum amplitude and total response (indicated by AUC) of Dm neurons (Fig. 2.13 A-C). Together these data suggest that CART action via PKA (and PKC) sensitises NMDA receptors, possibly via post-translational modification of NR1 subunits, leading to ERK activation that acts as a neural representation of the sated state.

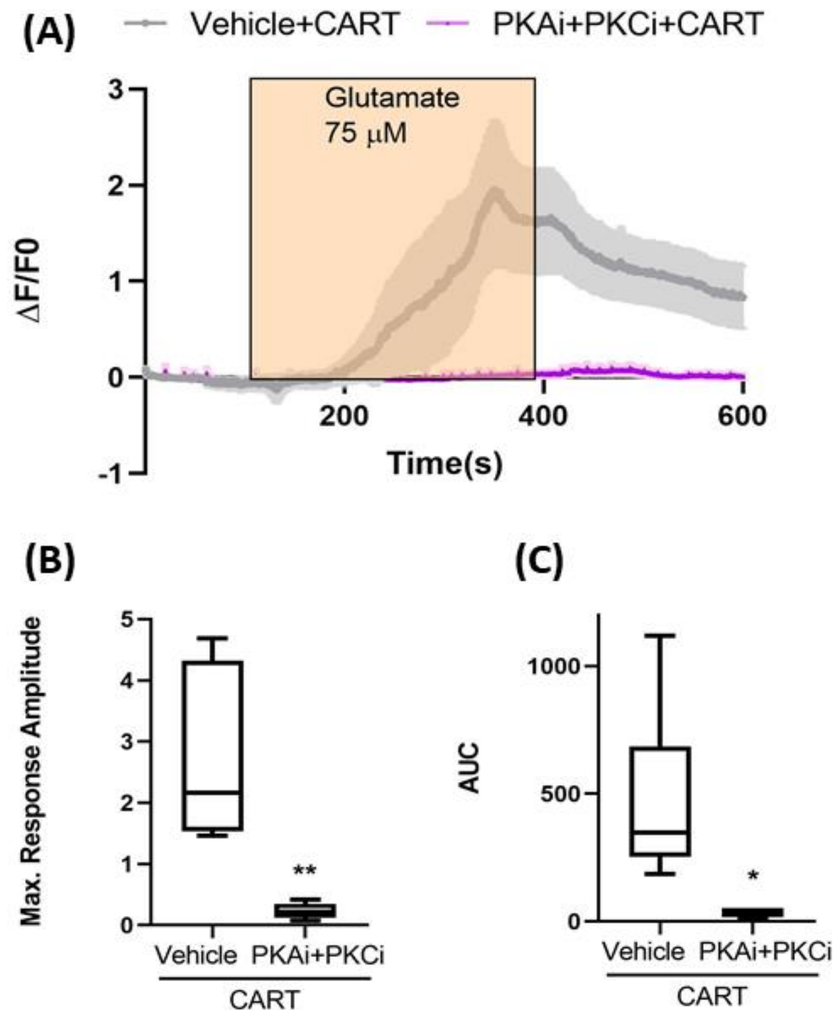


Fig. 2.13. CART based sensitisation of Dm neurons requires protein kinase activity.

Traces represent relative change in fluorescence intensity ($\Delta F/F0$) 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 coloured rectangular box indicates the duration for which glutamate was presented. **(A)** CART treated (grey trace) Dm neurons show increased response as compared to brains which were treated with PKA and PKC inhibitors + CART (purple trace). **(B)** The max ($\Delta F/F0$) and **(C)** AUC for CART treated Dm neurons is higher than brains which were treated with PKA and PKC inhibitors + CART. The data is plotted as box-and whisker (Tukey) and was analysed using t- test with Welch's correction. (N=6 telencephalons per treatment; *, $p<0.05$, **, $p<0.01$). [PKAi: KT5720, selective PKA inhibitor; PKCi: GF109206X, selective PKC inhibitor]

2.3.7 The excitability of Dm is tuned to changes in energy status (performed in collaboration with Devika Bodas)

The earlier experiments, while useful in dissecting out the pathway for CART induced modulation of neural activity, do not fully recapitulate a physiologically relevant scenario as they are performed in the absence of midbrain and hindbrain inputs. Prior studies have demonstrated that CART levels in EN and hypothalamus increase upon glucose treatment (Mukherjee et al., 2012). Furthermore, these glucose-responsive regions send CART immunoreactive projections to the Dm (Fig. 2.14 A and unpublished lab data). We thus used an *ex vivo* whole-brain preparation which keeps these interoceptive inputs intact thereby allowing us to monitor activity in the Dm from the same tissue under conditions that increase endogenous CART levels. Fig. 2.14 shows the experimental set-up (B) and stimulation protocol (C) (described in detail in the Methods section 2.2.6).

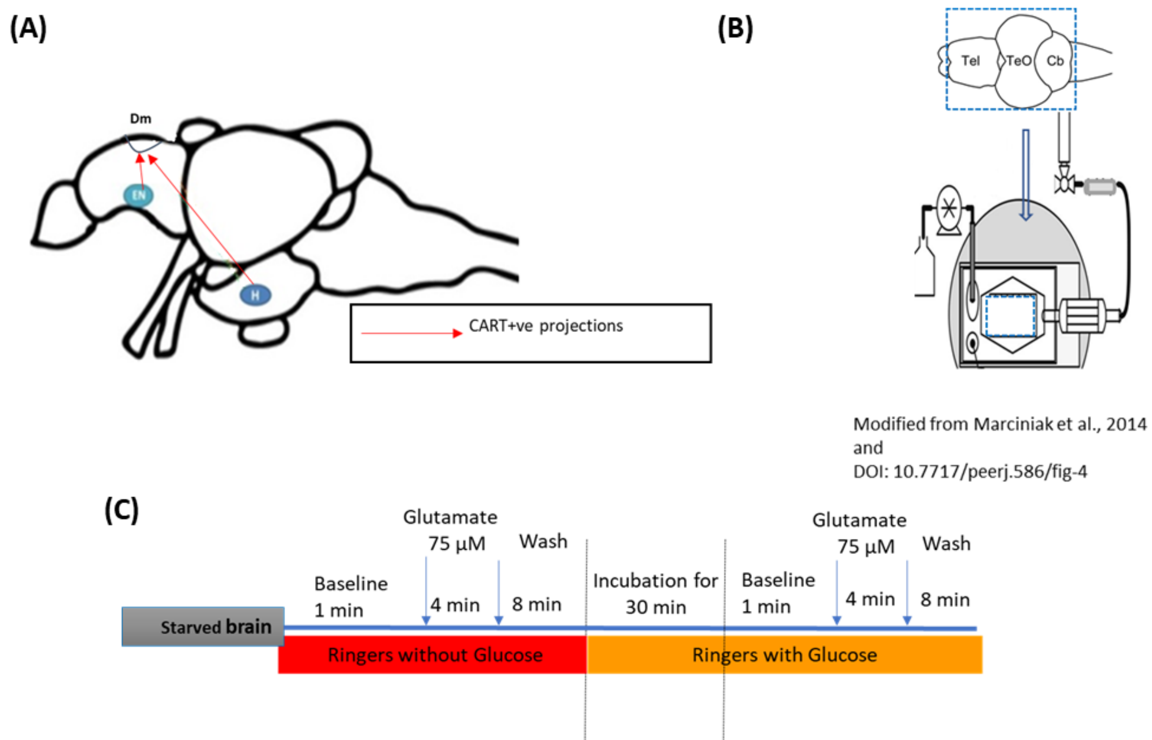


Fig. 2.14. Schematic for whole-brain ex vivo imaging set up and modified stimulation paradigm.

(A) Schematic of the whole brain showing CART-ir projections onto the Dm from interoceptory regions. Whole brain dissected from starved animals is mounted in a chamber perfused with Ringers-Sucrose (10 mM) buffer or Ringers-Glucose (10 mM) buffer **(B)** according to the glutamate stimulation paradigm shown in **(C)**.

Briefly, the samples were first maintained in a buffer supplemented with sucrose (to maintain glucose-deprived/ starvation-like conditions) and then imaged again 30 minutes after changing to a buffer containing physiologically normal levels of glucose (10 mM) (to induce satiety-like conditions). Remarkably, we found that, while in starvation-like conditions, Dm neurons failed to respond to excitatory stimulation, mimicking satiety conditions drastically enhanced glutamate-induced activity in the Dm of the same sample, both in terms of maximum response amplitude as well as the total response (indicated by AUC) (Fig. 2.15 A red and grey traces and B, C). Importantly, brains subjected to the same protocol without switching to a glucose-containing buffer did not alter their response profile after the 30 min of 'mock' incubation (Fig 2.15 A violet and yellow traces and B, C). Hence, indicating that the observed changes were likely due to glucose-induced upregulation in CART and other endogenous satiety signals rather than a result of repeated glutamate stimulation.

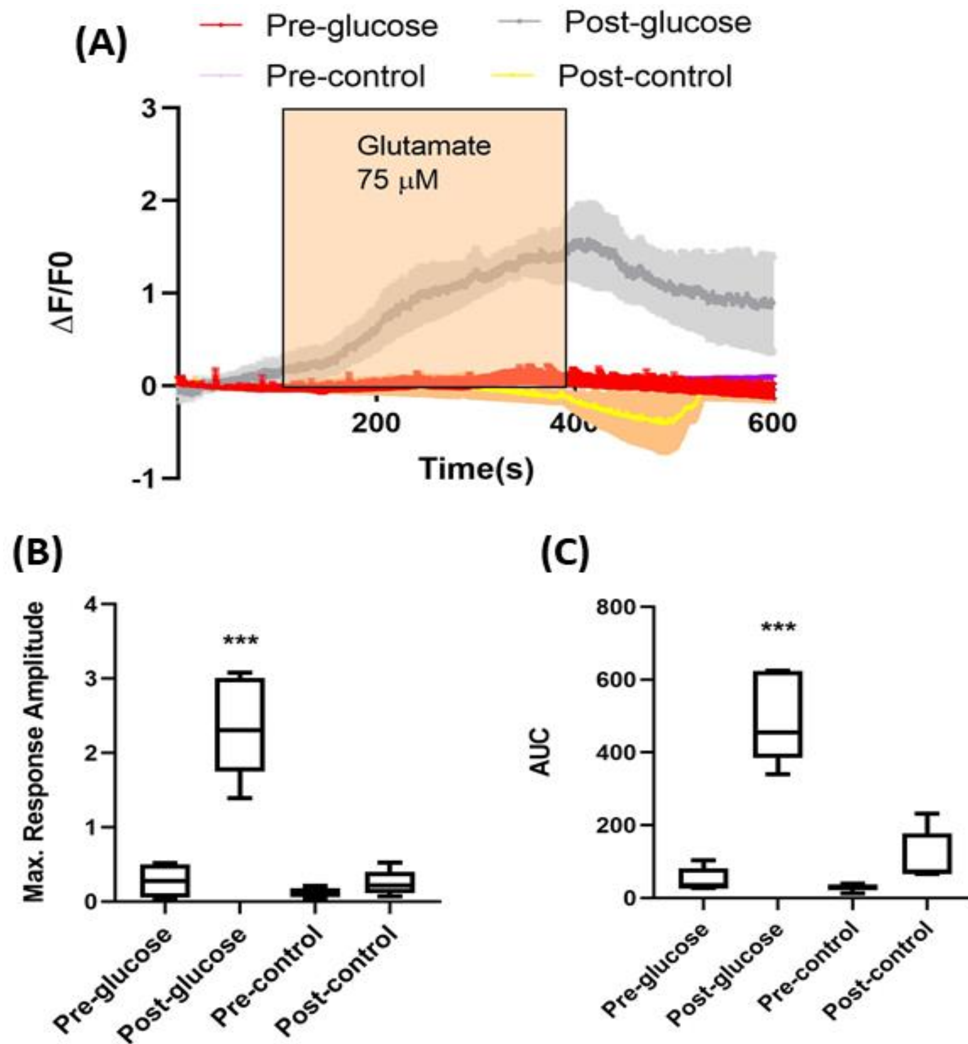


Fig. 2.15. A change in available energy conditions alters the response of Dm neurons to excitatory transmission in a manner similar to CART treatment.

Traces represent relative change in fluorescence intensity (ΔF/F0) 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 coloured rectangular box indicates the duration for which glutamate was presented. **(A)** Dm neurons of whole- brains samples show increased response post 30 min incubation with glucose (grey trace) as compared to pre-glucose treatment (red trace). Control brains were maintained in sucrose supplemented buffer for the entire duration of the experiment and did not show significant difference in response to glutamate (purple and yellow traces denote response to glutamate before and after 30 min interval between the recordings, respectively) **(B)** The max (ΔF/F0) and **(C)** AUC for Dm neurons post-glucose treatment is higher than pre-glucose treatment. The data is plotted as box-and whisker (Tukey) and was analysed using One-Way ANOVA with Tukey's multiple comparisons test (N=5 brains each for glucose and sucrose controls; ***, $p<0.001$).

These data, therefore, suggest that activity in the Dm is tuned to changes in energy status and that Dm could play a role in processing signals from interoceptive regions relevant to maintaining energy homeostasis.

2.4 Summary and future prospects

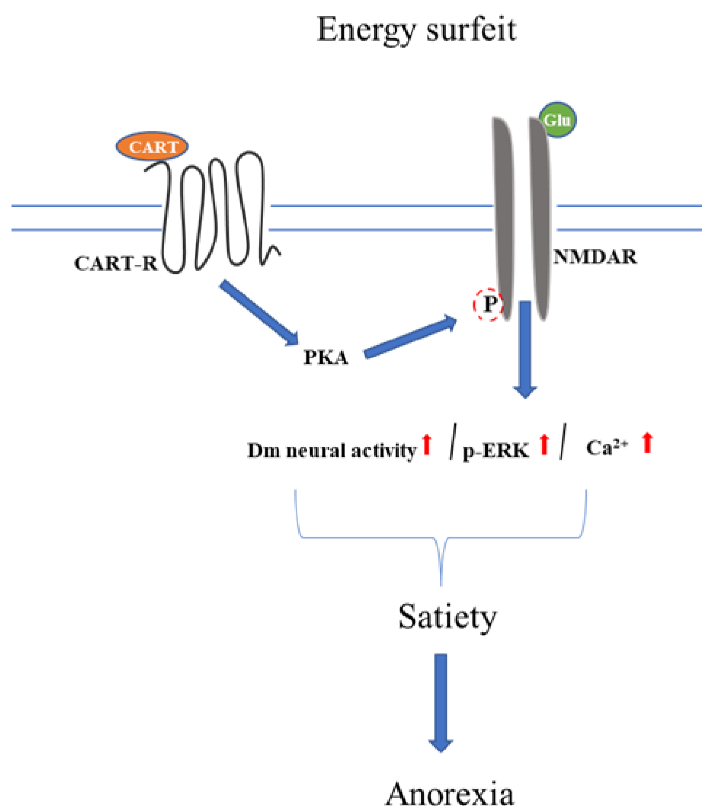


Fig. 2.16. Summary model.

Enhanced CART signalling under conditions of energy surfeit leads to increased phosphorylation of NR1 subunit of NMDARs in the Dm. This leads to heightened activity and potentiates excitatory transmission in this region resulting in a correlated anorectic behavioural output.

In conclusion, we have shown for the first time that CART regulates satiety in Zebrafish and propose that CART mediated increase in phosphorylation of NMDARs underlies the neural activity changes and suppression of feeding drive in Zebrafish (Fig. 2.16). We found that CART is not only sufficient to induce anorexia but is also required for processing satiety cues such as those mediated by glucose since fish treated with CART antibody recovered from

glucose-induced suppression of food intake. With the identity of a CART receptor still unknown and in the absence of suitable antagonists, we used a central infusion of an immuno-neutralising antibody to disrupt endogenous CART signalling in glucose-administered, food-deprived animals. Previous studies have used similar approaches to establish the endogenous function of this peptide in rodent models (Kristensen et al., 1998; Singh et al., 2021). By combining behavioural and histological analysis and dynamic neural activity imaging along with pharmacological interventions, we showed that CART acts by modulating the activity of the medial zone of dorsal telencephalon through a mechanism that requires signalling via NMDARs. Studies in rodents have previously reported the involvement of NMDA receptors in regulating food intake via the action of different orexic or anorexic factors (Burns and Ritter, 1997; Resch et al., 2014). Under satiety conditions, NMDARs in the hindbrain are required for CCK induced reduction of food intake (Wright et al., 2011). Apart from CCK, leptin (Neyens et al., 2020) and PACAP (Resch et al., 2014) mediated suppression of food intake also requires NMDARs. Our study adds to these reports by identifying NMDAR-dependent CART-induced sensitisation as another mechanism of inducing anorexia and further highlights these receptors as an important target for neuromodulators that regulate feeding circuits. NMDARs are heteromers of at least one NR1 subunit and NR2 or NR3 subunits depending on anatomical location and developmental time (MacDonald; Ron, 2004). Phosphorylation of its subunits is known to drastically alter the strength of NMDAR signalling. PKA belongs to the family of Serine/Threonine kinases, and its activity is known to enhance NMDAR currents and excitatory postsynaptic currents (EPSCs) by altering channel opening probability, Ca^{2+} permeability and receptor trafficking. It does so by phosphorylating the C-terminus of NR1 subunit specifically at Ser 897 residue (MacDonald; Pitcher et al., 2008; Ron, 2004). Phosphorylation of the NR1 subunit at this site thus leads to increased functioning of NMDARs that results in an increased sensitivity of neurons to excitatory cues like glutamate. A parallel study in our lab found that PKC, another serine/threonine kinase with known sites on NMDAR subunits, is also involved in CART mediated anorexia. As a change in phosphorylation status of receptor subunits is a key mechanism of regulating NMDAR function, we reasoned that CART via activation of PKA and PKC could increase the excitability of Dm neurons by influencing NMDAR function.

Indeed, our experiments monitoring neural activity dynamics in *ex vivo* brain samples indicate that CART peptide sensitises this region to excitatory stimuli. Analysis of various parameters of calcium activity dynamics such as a relative change in fluorescence ($\Delta F/F0$), maximal response intensity (max $\Delta F/F0$), and the total response (AUC) revealed that CART treated Dm

neurons show increased activity in response to glutamate, which is dependent on the activation of PKA, PKC and NMDARs. Our experiments were performed using two different strategies to monitor changes in Dm activity; in the first set of experiments, we used an *ex vivo* telencephalon preparation which allowed us to monitor activity changes elicited by the exogenously delivered peptide in the absence of conflicting interoceptory influences from the hypothalamus. However, such an approach does not allow for monitoring Dm activity immediately before and after altering the physiological state. Hence, in the next set of experiments, we used an *ex vivo* whole brain preparation wherein we could monitor differential activation of the Dm under conditions that mimic hunger and satiety states in the same brain. Moreover, since the interoceptory projections to Dm are still intact in this prep, the observed increase in the activity of Dm neurons likely reflects responses due to glucose-induced increase in endogenous CART (and other satiety signals). Data from the control set, which was not treated with glucose and did not show an appreciable change in response to excitatory stimulation, indicates that the observed changes in activity were indeed due to alterations in energy state and not due to repeated treatments with glutamate. These observations are in keeping with previous reports wherein CART has been shown to potentiate effects of glutamate on heart-rate and blood pressure as well as glutamatergic neurotransmission in nociceptive transmission and fear response circuits (Chiu et al., 2009; Hsun Lin et al., 2005; Rale et al., 2017; Subhedar et al., 2014). However, we cannot determine if the observed correlation in potentiation of neural activity in Dm directly affects feeding behaviour.

Immunohistochemistry and dynamic neural activity data together identify Dm as a downstream target of CART. With prominent neuropeptidergic projections from EN and Hypothalamus (unpublished lab data), Dm, a homologue of the mammalian amygdala, seems well-positioned to integrate and execute satiety related signals. This is consistent with current literature about the mammalian amygdala, given that the last decade or so has seen an increase in the number of reports highlighting the importance of amygdalar neural circuits in regulating the motivation to feed (Sweeney and Yang, 2017; Zhang et al., 2011). MeA MC4R-expressing neurons suppress feeding in rodents (Liu et al., 2013) and activation of ARC^{POMC} projections to MC4R- and ER-α-expressing MeA neurons induces a short-term reduction in liquid food intake (Kwon and Jo, 2020). Further, optogenetic activation of NPY Y1R receptor-expressing MeA neurons also suppresses feeding, indicating that the MeA is an important downstream target of ARC interoceptive circuits (Padilla et al., 2016). PKC-δ positive neurons in CeA are activated by satiety signals and inhibit feeding while serotonin receptor expressing CeA^{Htr2a} neurons

promote feeding through long-range projections to PBN (Cai et al., 2014; Douglass et al., 2017). Two neurochemically distinct populations with opposing effects on food intake were also recently identified in the BLA (Kim et al., 2017).

Based on these reports, it seems likely that such functionally distinct microcircuits could be present within the Dm. Recent neurocytochemistry data also indicate functionally distinct subdivisions within the Dm (Ganz et al., 2014; Lal et al., 2018; Porter and Mueller, 2020). Our study compares population dynamics within Dm and is also limited by the lack of neurochemical identification of CART responsive Dm neurons. Better temporal resolution and careful segmentation of neural activity data will be needed to identify any functional neural assemblies within the Dm. It should also be noted that the differential neural activity post-CART treatment that we observed – both, as marked by an increase p-ERK-ir and by monitoring calcium dynamics – was restricted mainly to the pre-and supra- commissural extent of the Dm. This anatomical expanse seems to match with the recently identified molecular subdivision of the Dm region referred to as the 'DM'- a derivative of the pallial amygdala, characterised by Porter and Mueller (2020). This region is marked by mostly vglut2a positive neurons, significant emx3 and CB1 receptor expression, and lacks parvalbumin-positive fibres and neurons. Though a complete characterisation of the genetic markers and neurotransmitter repertoire of the relevant sub-populations in the DM is beyond the scope of this study, nonetheless we present a new node in the feeding circuits that can be explored further to better understand how forebrain structures contribute to the regulation of feeding and the role that peptidergic neuromodulators have therein.

Chapter 3: Mechanism of energy status dependent modulation of olfaction by NPY

3.1 Energy state-based modulation of olfaction

Changes in the neuromodulator milieu in response to physiological changes are essential not only for the representation of a need-state but also for driving changes within sensory circuits. Olfaction is a key sensory modality that supplies information necessary for behaviours such as navigation, predator avoidance, mating, and foraging, all of which are crucial for the survival of an organism (Gerlach and Wullimann, 2021; Kermen et al., 2013, 2019; Koide et al., 2009; Wakisaka et al., 2017; Whitlock, 2006). In the context of feeding behaviour, the sense of smell provides information about the exact location, palatability, and reward value of food and is a necessary input in the decision-making process when selecting between different food sources. Metabolic context-dependent modulation of olfaction has been observed in various phyla across the animal kingdom. In 2006, Getchell et al. showed that leptin alters food-search behaviour in mice by regulating olfactory sensitivity (Getchell et al., 2006), while Aimé and colleagues reported fasting-induced increases in olfactory acuity in rats (Aimé et al., 2007). In the nematode *C. elegans*, starvation induces attraction towards CO_2, an otherwise aversive cue (Rengarajan et al., 2019). This study also established a role for the biogenic amines, dopamine and octopamine, in regulating the valence of CO_2 responses. A similar metabolic state-dependent differential response to the same odour has recently been characterised in *Drosophila* (Vogt et al., 2021).

Additionally, presynaptic facilitation at the first olfactory synapse, mediated by the neuropeptide sNPF, enhanced responses to food odours in *D. melanogaster* (Root et al., 2011) under conditions of starvation. Another study in the same model system reported decreased sensitivity to various odours in flies supplied with a high-fat diet (Jung et al., 2018). These studies highlight the crucial role that neuromodulatory agents play in tuning olfactory responses to match physiological needs. Much of the focus on understanding the cellular mechanisms underlying such modulatory effects has been limited to studying processes at the olfactory bulb level or at the first olfactory synapse. How neuromodulators interact with odour transduction pathways to alter activity at the level of olfactory sensory neurons (OSNs) warrants further study.

3.2 Neuromodulation in the Zebrafish olfactory system

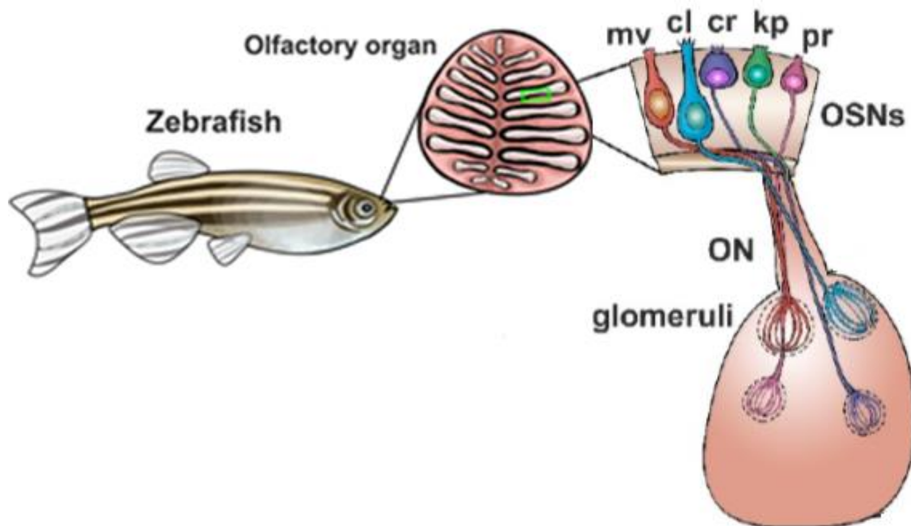


Fig. 3.1. Zebrafish olfactory system.

Schematic of the Zebrafish olfactory system depicting the different types of olfactory sensory neurons in the olfactory organ along with their projections to distinct glomerular territories in the olfactory bulb via the olfactory nerve. [mv: microvillar; cl: ciliated; cr: crypt; kp: kappe; pr: pear; OSN: olfactory sensory neurons; ON: olfactory nerve]. Adapted from (Calvo-Ochoa and Byrd-Jacobs, 2019).

Despite some evolutionary differences, teleost and tetrapod olfactory systems share several architectural and functional features. Electron microscopy, immunohistochemistry and receptor expression analysis have revealed that the Zebrafish olfactory system is organised along the same principles as that of higher vertebrate systems (Calvo-Ochoa and Byrd-Jacobs, 2019; Sato, 2005; Villamayor et al., 2021; Weth et al., 1996). It is composed of a pair of olfactory organs and olfactory bulbs (OB) connected via the terminal nerve (TN), which carries the projections of olfactory sensory neurons (OSNs). The olfactory organ, a cup-shaped structure in the nasal cavity, is composed of OSNs and support cells arranged in several lamellae that converge onto the medial raphae, giving it a characteristic 'rosette' shape. To date, five types of OSNs have been described- microvillar, ciliated, crypt, kappe and pear (Fig. 3.1) (Ahuja et al., 2015, 2018; Calvo-Ochoa and Byrd-Jacobs, 2019). The different OSN types are arranged in a pseudo-stratified manner and are identified based on differences in morphological characteristics, location and molecular markers (Table 2). These morphological and molecular

differences translate to differences in the odour classes detected as well as the behaviours affected by each of the OSN types (Calvo-Ochoa and Byrd-Jacobs, 2019; DeMaria et al., 2013; Saraiva and Korsching, 2007; Villamayor et al., 2021). For instance, the basally located $G_{\alpha/olf}$ positive ciliated OSNs preferentially detect bile salts (Friedrich and Korsching, 1998; Sato, 2005) while the Vomeronasal type-2 receptor (V2R)-expressing microvillar populations, located in the intermediate layers, are activated by amino acids (DeMaria et al., 2013; Lipschitz, 2002; Sato, 2005). The conservation of the 'one neuron-one receptor' strategy and odour transduction machinery amongst the Zebrafish and mammalian olfactory systems makes the former an attractive model to study general principles of odour detection and context-dependent neuromodulation of olfaction.

Zebrafish also show changes in olfactory behaviour due to altered energy conditions. Teleost fish have a high dietary requirement for proteins and amino acids, thus, represent a major class of food-related odours (Soengas et al., 2018). Food-deprived Zebrafish exposed to a mixture of amino acids on one side and a neutral odour on the other show a marked preference for the baited side (Kaniganti et al., 2021; Koide et al., 2009). This preference is suppressed due to disruptions in NPY signalling by the administration of a NPY Y1R receptor antagonist in the cerebral ventricle (Kaniganti et al., 2021). Additionally, compared to recently-fed and glucose injected fish, food-deprived animals show an increase in NPY levels in the OE, TN and OB (Kaniganti et al., 2021). Exogenous application of NPY over OM of fasted rats enhanced the amplitude of odour-evoked EOG recordings (Negroni et al., 2012). This presents an interesting possibility that NPY acting directly on OE-OB neurons alters olfactory responses to better match physiological needs. The cellular mechanisms underlying this fasting-induced NPY-mediated enhancement of amino acid detection, however, are not well-defined. Koide et al. (2009) have previously shown the involvement of V2R expressing OSNs in detecting amino acids in Zebrafish. Unlike classical odorant signalling, transduction via V2Rs involves PLC activation, and Diacylglycerol (DAG) mediated opening of transient receptor potential ion channels (TRPC2). This study was therefore designed with the aim to (1) test if amino acid detection in Zebrafish utilises a PLC dependent pathway, (2) test if amino acid-induced OSN activation is sensitive to NPY signalling and if so, (3) to test for a crosstalk between NPY signalling and amino acid sensing pathways.

OSN type	Shape; Location	Marker; Receptor	Odor class; Function
Microvillar	Short dendrite with apical microvilli; intermediate layers	TRPC2; VR-type	Amino acid; Response to food cue
Ciliated	Long slender dendrite with apical cilia; basal	$G_{\alpha/olf}$; OR; TAAR	Bile salts; Determine water quality
Crypt	Large spherical soma & presence of both cilia and microvilli; apical	ORA4	Schrekstoff, Pheromones; Kin recognition and predator response
Kappe	Kappe (‘cap’) shaped; apical	NA	Pheromones; Kin recognition
Pear	Pear shaped; apical	A2c	Adenosine & adenine nucleotides; Response to food cue

Table 2. Types and distinctive features of the main olfactory sensory neurons found in Zebrafish olfactory system.

3.3 Materials and methods

3.3.1 Animals

Animals were housed and fed as described in Chapter 2 section 2.2.1. In addition to wildtype animals, this study also used Tg(TRPC$^{24.5k}$:gap-Venus) obtained from the National BioResource Project, Riken Center of Brain Science, Japan.

3.3.2 Chemicals

Synthetic human neuropeptide Y (NPY, Sigma-Aldrich, Cat. No. N5017), small molecule NPY Y1 receptor inhibitor BIBP-3226 (BIBP, Sigma-Aldrich, Cat. No. B174), PLC inhibitor U73122 hydrate (U73122; Sigma-Aldrich Cat. No. U6756), dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cat. No. D8418), L- lysine (Sigma-Aldrich, Cat. No. L5626), L- histidine monohydrochloride monohydrate (Sigma-Aldrich, Cat. No. H8125), L- tryptophan (Sigma-Aldrich, Cat. No. T0254), L- cysteine hydrochloride (Sigma-Aldrich, Cat. No. C1276), L- alanine (Sigma-Aldrich, Cat. No. A7627), L- phenylalanine (Sigma-Aldrich, Cat. No. P2126), L- valine (Sigma-Aldrich, Cat. No. V0500) and L- methionine (Sigma-Aldrich, Cat. No. M9625), Levamisole (Sigma-Aldrich, Cat. No. L9756) were purchased from Sigma Aldrich). Blocking solution (Roche Applied Sciences, Cat. No. 11096176001) and BM purple solution (Roche Applied Sciences, Cat. No. 11442074001) were from Roche while Dextran sulfate (HiMedia, Cat. No. MB146) was purchased from HiMedia.

3.3.3 Intracerebroventricular (icv) and intranasal delivery

Prior to icv delivery, fish were anesthetized following immersion in 2-phenoxyethanol (Sigma-Aldrich, 1: 2000). All pharmacological agents were dissolved in 1X Phosphate buffered saline or in DMSO depending on solubility. The final concentration of DMSO did not exceed 0.1% for BIBP and 0.5% for U73122 treatment. Icv delivery protocol was as described in section 2.2.3. All immunofluorescence studies were undertaken within 30 minutes (mins) following injection. The following doses were used for icv delivery of glucose (8000 ng); BIBP (100 pmol); U73122 (1 µM) and NPY peptide (1 pmol).

Intranasal delivery of BIBP-3226 (66.67pmol) or vehicle was carried out by superfusing both the OE with 5 µl of the solution over the nares of anesthetized fish for 30 secs using a Hamilton microsyringe. The mouth and gills were covered with a small piece of tissue paper to prevent the solution from spreading. Following the procedure, fish were returned to the tank after 1 minute and allowed to recover from anesthesia before proceeding for odorant stimulation assay.

3.3.4 Amino acid stimulation of the OE for evaluation of OSN activation

Post 30 mins icv delivery of pharmacological agents, fish were transferred into the habitat tank containing 1 litre aquarium water. A mixture of 8 amino acids (Ala, Cys, His, Lys, Met, Phe, Trp, and Val; to a final concentration of 0.8 mM each in distilled water or solvent was introduced into these tanks using a syringe. Following 5-7 min of exposure, the fish were immediately anesthetized in ice-cold aquarium water and processed for p-ERK immunofluorescence.

3.3.5 Immunofluorescence

Adult fish were anesthetized, craniotomised and fixed for 14-16 hours in 3% paraformaldehyde at 4ºC. The olfactory epithelia were dissected out and transferred to 25% sucrose solution for cryoprotection, embedded in OCT compound (Tissue-Tek, Sakura), sectioned in the transverse plane at 20 µm thickness using a cryotome (CM1950; Leica) and mounted on poly-L-lysine coated glass slides. The slides containing the sections were stored at -40ºC until they were processed for immunofluorescence. The frozen sections were rehydrated and permeabilized using 0.5% Triton-X in phosphate buffer saline (PBST). The sections were treated with 5% BSA in PBST for 1 hour at room temperature (RT) and then incubated with the primary antibody overnight (O/N) at 4 ºC. The sections were washed using 0.5% PBST and blocked with 5 % heat inactivated goat serum (HIGS) in PBST for 1 hour before incubation with desired secondary antibody for 3 hours at RT. Sections were then washed and mounted with mounting media (70% glycerol, 0.5% N-propyl gallate in 1 M Tris, pH 8.0, DAPI). The following primary antibodies were used in this study- monoclonal anti-phosphorylated ERK 1/2 (1:500; Abcam, Cat. No. ab50011) and polyclonal anti-TrpC2 (1:50; Abcam, Cat. No. ab69101), anti-NPY (1:5000; Sigma-Aldrich, N9528), anti-GFP (1:1000; Abcam, Cat. No. Ab13970). Anti-rabbit Alexa-488 (1:500; Invitrogen, Cat. No. A11034), anti-chick 488 (1:500; Invitrogen, Cat. No. Ab13970) and anti-mouse Alexa-568 (1:500; Invitrogen, Cat. No. A11031) were used as secondary antibodies. Sections were observed using an upright epifluorescence microscope (Axioimager.Z1 with Axiocam HRm camera; Carl Zeiss) and imaged using laser scanning confocal microscopes (Leica SP8 or Zeiss LSM 780).

3.3.6 NPY Y1 Receptor RNA in situ hybridization

Adult fish were starved for three days and anesthetized followed by the removal of skull and immersion in 4% paraformaldehyde (for 14-16 hours at 4ºC), olfactory rosettes were dissected out, washed in phosphate buffered saline (PBS), and stored in 100% methanol O/N at -20°C.

Digoxigenin labeled sense and antisense riboprobes were transcribed in vitro using Zebrafish NPY Y1R TOPO pcDNA 3.1 vector template (received from Larhammar lab) and the following primer pair: Forward: 5'-GACACCAACTCATCCTGCACC-3'; Reverse: 5'-TGGGTACAGTTCATCGCCAC-3' by following the manufacturer's instructions (Roche Applied Sciences). Resulting probes were then column purified according to the manufacturer's instructions (BioRad Micro Bio-Spin). Samples were stepwise rehydrated to PTW (phosphate buffered saline, 0.1% Tween-20 pH 7.3) and permeabilized in 2% hydrogen peroxide for 35 min at RT. Samples were further permeabilized by a 40-min proteinase K treatment and post-fixed for 20 min in 4% paraformaldehyde at RT. Prehybridization, probe hybridization and washes were performed as described in Akash et al. (Akash et al., 2014) except for the addition of 5% dextran sulfate to the hybridization buffer. The olfactory rosettes were embedded in 4% low melting point agarose blocks and sectioned (30 µm) using a Vibratome (VT 1200; Leica). The resulting sections were collected in 1X PBS and washed thrice in PTW. These were then blocked in 1% blocking solution (Roche Applied Sciences) for 10 min at RT and incubated overnight at 4°C with sheep anti-DIG alkaline phosphatase conjugated antibody (1:2000; Roche Applied Sciences). Sections were washed thrice with PTW buffer and twice with coloration buffer (100 mM Tris-HCl, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20, 2mM Levamisole). DIG-labeled probes were detected using pre-warmed BM purple solution (Roche). The reaction was continuously monitored at 37°C for 30 min to 2 h or overnight at 4°C depending on the strength of the signal. The reaction was stopped by washing three times with PTW buffer. The sections were then mounted in 70% glycerol and were either processed for imaging or stored at 4°C. DIC photomicrographs were acquired using Axioimager Z1 (Carl Zeiss).

3.4 Results and Discussion

3.4.1 Amino acid-responsive OSNs express TRPC2 ion channel and require PLC signalling for their activation

Previous studies on the Zebrafish olfactory system indicated that the TRPC2-expressing population of OSNs mediate the detection of amino acid odorants (Koide et al., 2009; Sato, 2005). Using activity-dependent labelling of OSNs, we confirmed that amino acid-responsive OSNs are immunoreactive to TRPC2 (Fig. 3.2).

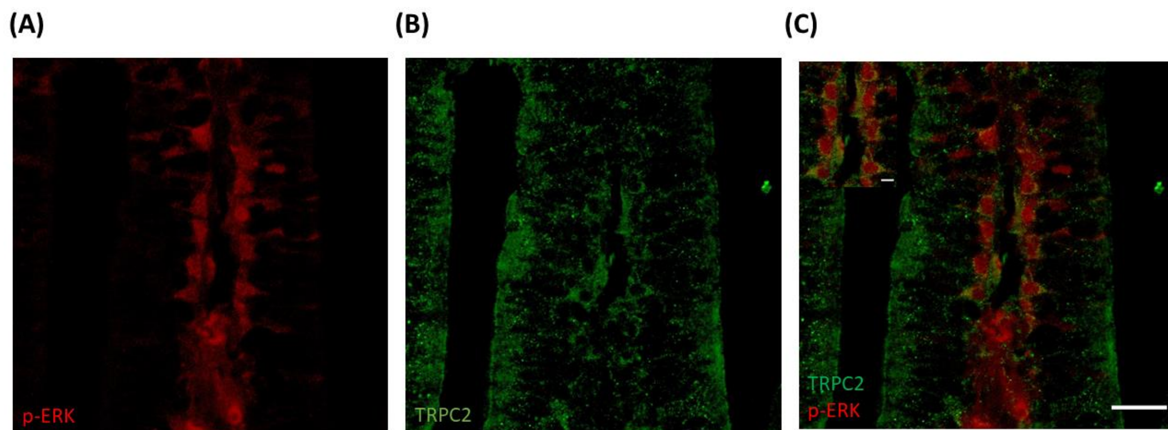


Fig. 3.2. Amino Acid activated neurons express TRPC2 ion channels.

Starved fish exposed to a mixture of amino acids show activity dependent p-ERK labelling **(A)** in TRPC2 immunoreactive cells **(B)**. Merged image **(C).** [Scale bar: 15 µm, inset: 5 µm]

In Zebrafish, microvillar OSNs expressing TRPC2 are also known to express vomeronasal type receptors, with the latter implicated in mediating amino acid detection (DeMaria et al., 2013; Sato, 2005). This suggests the possibility that amino acid sensing pathways in fish could be similar to the Phospholipase C (PLC)-dependent odorant sensing mechanisms reported in amphibians (Sansone et al., 2014) and rodents (Lucas et al., 2003; Szebenyi et al., 2014). We thus used U-17322, a selective inhibitor of PLC activity and tested its effect on amino acid-induced OSN activation. Using phospho-ERK (p-ERK) as a marker for recent neural activity, we compared the number of OSNs activated in response to exposure to a mixture of amino acids in vehicle (Fig. 3.3 A) and inhibitor (Fig. 3.3 B) injected starved animals. We found that disrupting PLC signalling leads to a pronounced reduction in the number of activated OSNs (Fig. 3.3 C; N=12 olfactory rosettes per group). Together these data indicate that the TRPC2-expressing microvillar OSNs employ PLC dependent cascade for detecting amino acid cues.

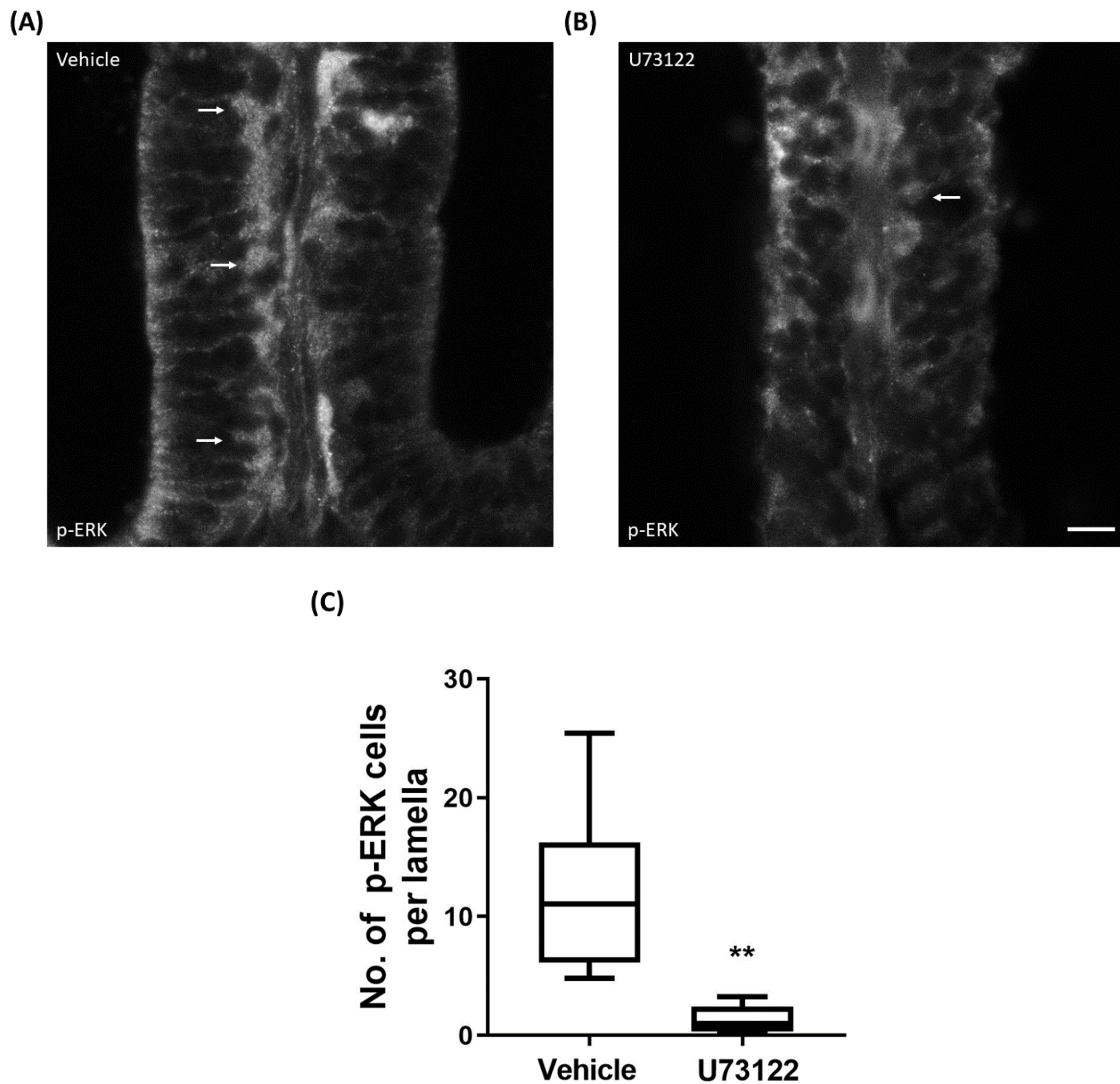
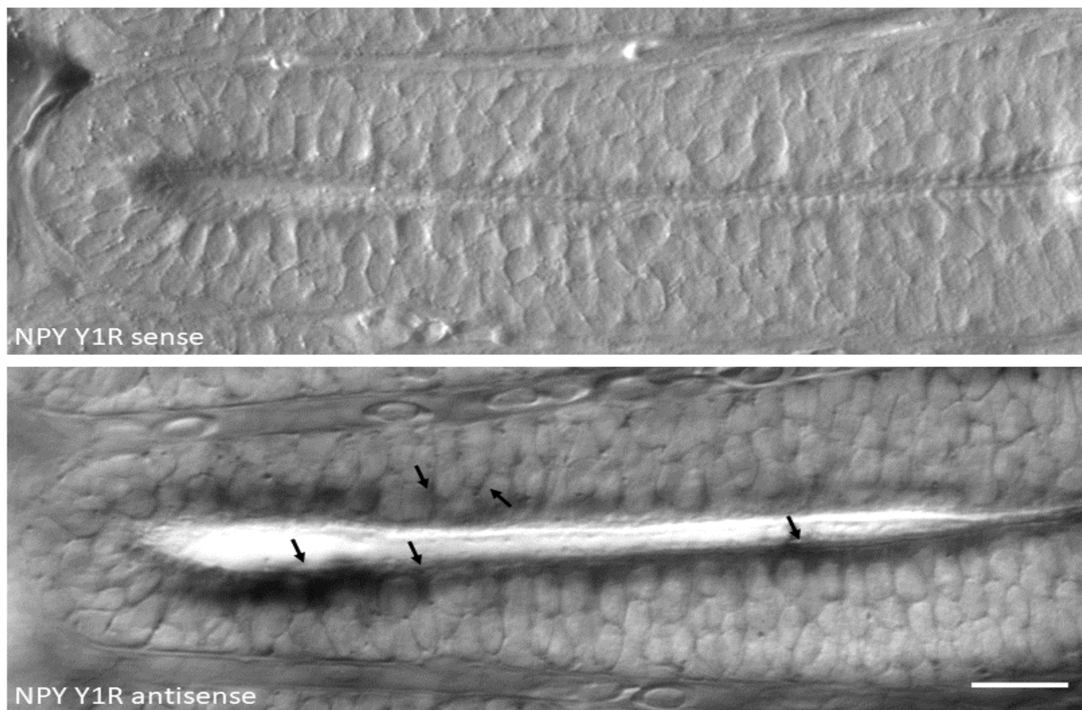


Fig. 3.3. Amino acid stimulated OSN activation requires PLC activity.

Inhibition of PLC activity drastically reduces the number of OSNs activated in response to amino acids. Representative micrographs showing p-ERK immunoreactive (p-ERK-ir) cells in the olfactory epithelium (OE) in response to exposure to amino acids mixture for vehicle **(A)** and U73122 (PLC inhibitor, 1 µM) **(B)** injected animals. Quantitation of the number of p-ERK-ir cells in the OE across all 20 µm sections of each olfactory rosette in response to amino acids for vehicle and inhibitor. Data are shown as mean ± SEM and means were compared using unpaired t-test with Welch's correction (N= 12 OE per group; **, $p<0.01$).[Scale bar: 15 µm]

3.4.2 NPY signalling is required for OSN activation in response to amino acid exposure

Previous data from our lab has shown that food-deprived animals have a higher behavioural attraction towards amino acids. This was found to be abolished by disrupting NPY signalling via the central administration of BIBP 3226 (BIBP), a selective NPY Y1R antagonist (Kaniganti et al., 2021). To determine if this could be due to the disruption of NPY action at the level of OSNs, we first tested if the Zebrafish OSNs express NPY Y1 receptor (NPY Y1R). *In situ* hybridisation revealed NPY Y1R mRNA in cells with short projections located in intermediate layers of the olfactory lamellae (Fig. 3.4 A), indicating a microvillar OSN identity.



(B)

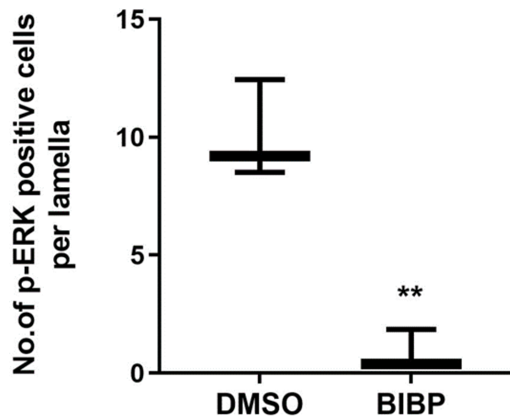


Fig. 3.4. NPY signalling is necessary for amino acid-induced OSN activation.

OSNs in the adult Zebrafish OE express NPY Y1R mRNA (A). Selective inhibition of this receptor via BIBP 3226 (BIBP, 100 pmol) results in a loss of amino acid stimulated OSN activity (B). Quantitation of the number of p-ERK-ir cells in the OE across all 20 µm sections of each olfactory rosette in response to amino acids for vehicle and inhibitor. Data are shown as mean ± SEM and means were compared using unpaired t-test with Welch's correction (N= 6 OE per group; **, $p<0.01$). [Scale bar: 15 µm]

To determine the mechanism underlying the increased sensitivity to amino acids, we first tested the effect of blocking NPY signalling on the amino acid-induced activation of OSNs. We found that exposure to amino acids increases the number of activated OSNs in starved fish. However, fish treated with BIBP, prior to exposure to amino acids, failed to show a significant OSN response (Fig. 3.4 B). This indicates that amino acid-induced OSN activation requires NPY signalling via the Y1R receptor.

3.4.3 NPY acts via PLC to induce OSN activation

Based on our observations that OSN activation by amino acids is affected by disruptions in PLC activity as well as NPY signalling, we tested if NPY-induced modulation of OSN function requires the PLC pathway. To test this, we first reduced the endogenous NPY signalling in starved animals by the icv injection of glucose, a satiety mimic that reduces NPY levels in the olfactory system (Kaniganti et al., 2021). We then exposed these fish to a mixture of amino acids. As expected, glucose-treated fish showed significantly fewer p-ERK immunoreactive (p-ERK-ir) OSNs (Fig. 3.5 A, D).

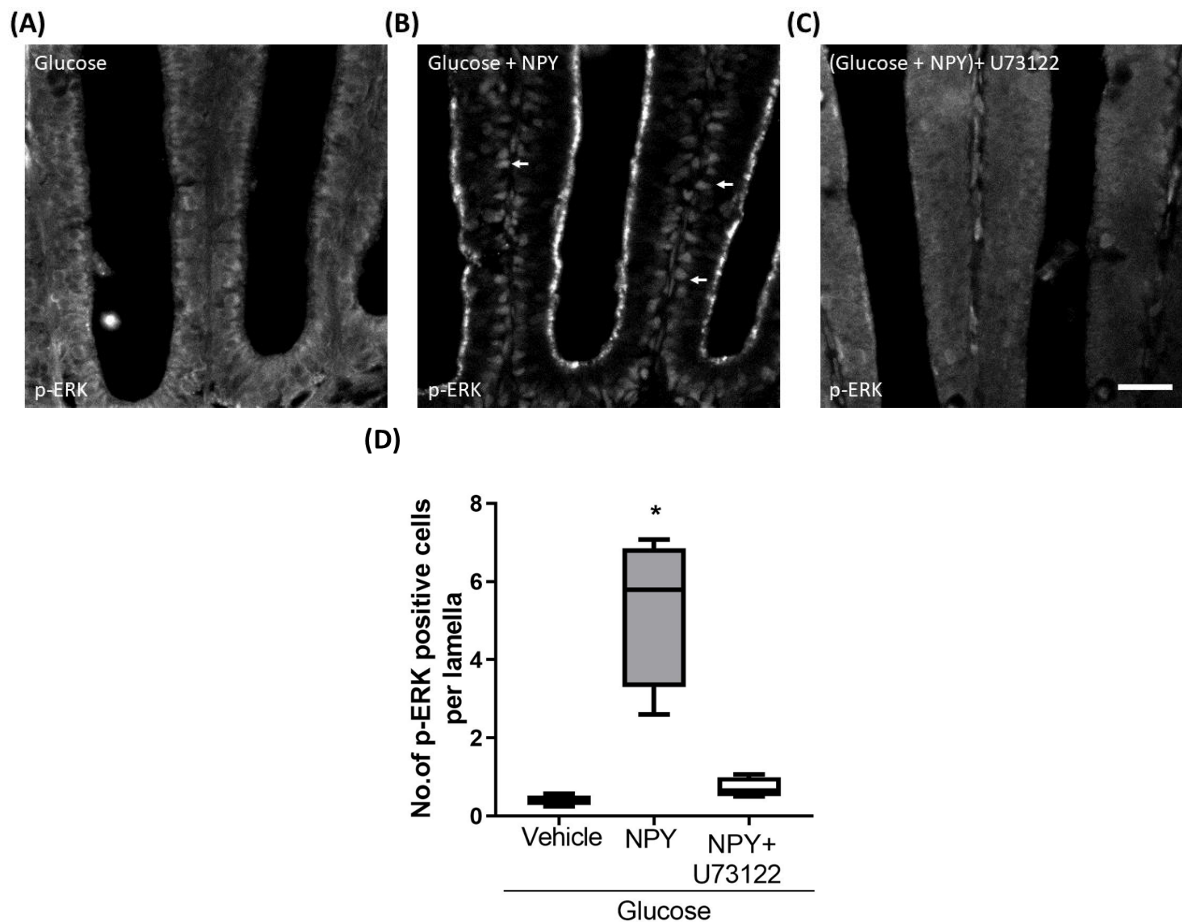


Fig. 3.5. NPY signaling and amino acid detection converge at the level of PLC.

Representative transverse sections showing p-ERK-ir OSNs in starved fish injected with glucose and **(A)** vehicle **(B)** NPY or **(C)** NPY+ U73122. Under conditions of mimicked satiety, NPY injection restores amino acid induced OSN activation as compared with vehicle injected animals (A, B and D). However, co-injection of **(C)** PLC inhibitor, U73122 (1 µM), along with NPY suppresses OSN activation back to control levels. Quantification of the data in **A-C (D)**. Data are represented as mean±SEM and compared using t-test with Welch's correction (N= 6 vehicle, N= 8 for NPY and NPY+ U73122 OE per group; *, $p < 0.05$). [Scale bar: 15 µm]

We then reintroduced NPY by co-injecting synthetic NPY peptide along with glucose. Restoring NPY levels in the background of reduced endogenous NPY-signalling was sufficient to increase the number of amino acid-activated OSNs to levels comparable with starved controls (Fig. 3.5 B, D). Interestingly, blocking PLC activity before injecting glucose+NPY attenuated amino acid-induced OSN activation (Fig. 3.5 C, D). These data suggest that NPY

signalling converges onto PLC signalling, allowing heightened OSN activation in response to amino acid stimulation.

3.4.4 Disrupting NPY signalling in the peripheral olfactory system blocks activation of amino acid-responsive OSNs

OE, TN and OB all show energy state-dependent expression of NPY (Kaniganti et al., 2021). Immunohistochemistry analysis of TRPC2-ir and NPY-ir OSNs revealed that both populations occupy spatially distinct regions in the OE (Fig. 3.6 A-C). This suggests that NPY likely acts in a paracrine mode to affect amino acid detection. In the past several years, there has been an increase in evidence to support such peripheral modulation of olfaction (Lucero, 2013).

In an effort to discern the contribution of local NPY signalling in the olfactory organ from central influences, we perfused BIBP over the nares of starved fish before exposure to a mixture of amino acids. This mode of delivery of BIBP has previously been shown to limit the site of action up to the OE-OB without affecting higher neural centres (Kaniganti et al., 2021). We found that blocking Y1R receptor signalling in the olfactory epithelium leads to a significant drop in the number of OSNs activated in response to amino acids (Fig. 3.6 D). Thus, NPY signalling acting in paracrine fashion at the level of the OSNs is required for heightened amino acid sensing in food-deprived animals.

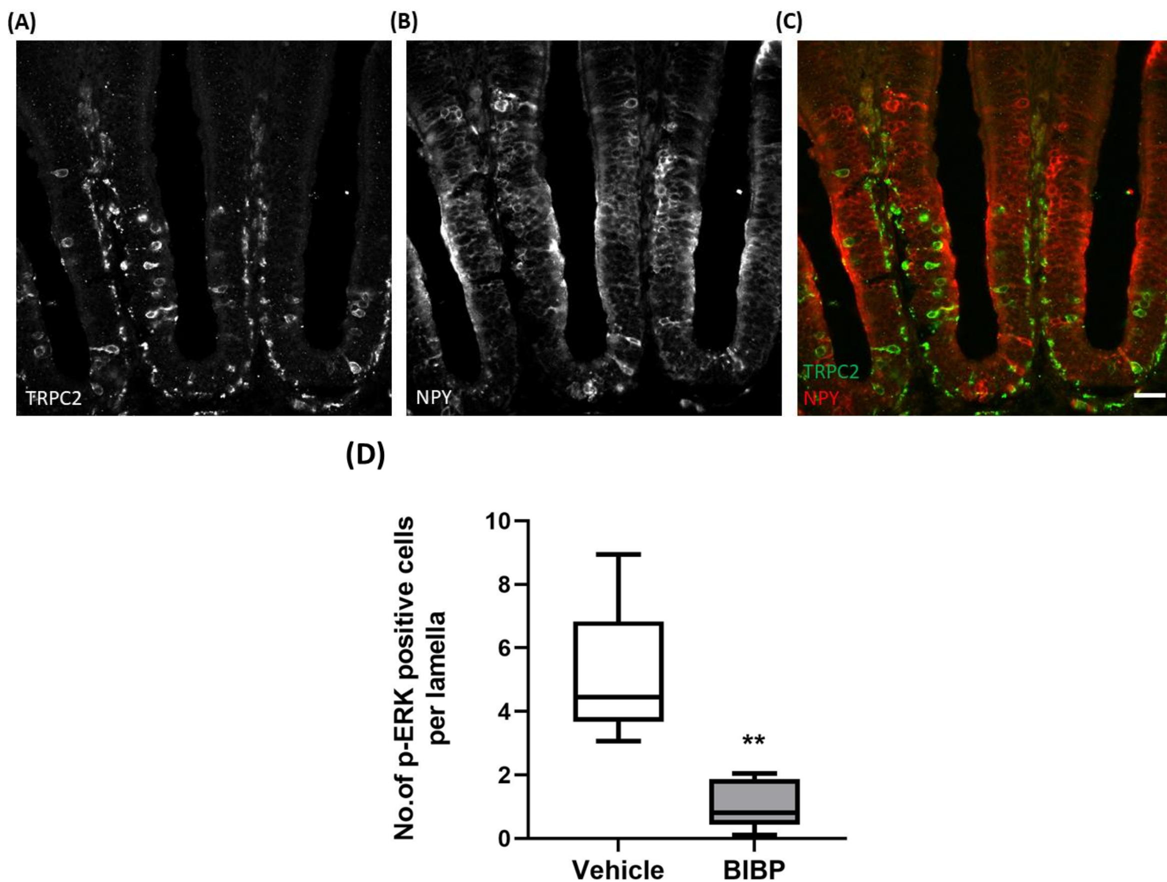


Fig. 3.6. NPY signalling in olfactory mucosa contributes to detection of amino acids.

Lack of overlap **(C)** between TRPC2-ir OSNs **(A)** and NPY-ir OSNs **(B)** suggests a paracrine mode of action for NPY in the OE. Disrupting NPY signalling in the OE by local perfusion of BIBP 3226 (66.67 pmol) over the nares reduces the number of OSNs **(D)** activated by exposure to amino acids. Data are represented as mean ± SEM and compared using t-test with Welch's correction (N= 10 OE per group; **, p< 0.01) [Scale bar: 15 μm]

3.5 Summary

Increased response to food cues under energy deprivation conditions is an important factor in ensuring robust food-search behaviour necessary to re-establish energy homeostasis. Amino acids are a major food cue for teleost fish, and food-deprived adult Zebrafish display increased attraction to amino acids, contingent on the presence of functional NPY signalling in these animals (Kaniganti et al., 2021; Koide et al., 2009). By combining activity-dependent p-ERK labelling with pharmacological interventions, our study identifies the odour transduction mechanisms underlying NPY-based modulation of amino acid detection in adult Zebrafish. We found that TRPC2-containing microvillar OSNs are activated upon exposure to amino acids

via PLC. This observation is further supported by dynamic calcium activity measurements, which revealed functional conservation between Zebrafish OlfCc1 and its orthologous mammalian V2R-receptors in amino acid-induced activation of OSNs (DeMaria et al., 2013). Our results also corroborate previous reports which identified PLC as a key mediator of olfactory transduction for pheromones and amino acids in other systems like mice (Lucas et al., 2003; Szebenyi et al., 2014; Zhang et al., 2010) and amphibians (Sansone et al., 2014).

Furthermore, inhibition of the Y1R receptor via nasal route suggests that paracrine action of NPY via Y1R receptor facilitates amino acid-induced OSN activation in starved animals. This approach limits the action of the inhibitor to the OE-OB level, and more experiments are needed to differentiate the contribution of NPY signalling exclusively in the OSNs. We found that NPY based modulation of OSN responses to amino acids is also dependent on PLC activity. Evidence suggests that, under specific contexts, signalling through NPY receptors can activate PLC via its $G_{\beta\gamma}$ subunits (Selbie et al., 1995, 1997). It remains to be seen if the convergence of NPY signalling and PLC in the amino acid detection pathway that we report here employs a similar mechanism.

Our study adds to existing reports for NPY signalling in modulating odour preferences and food intake under starvation conditions (Kaniganti et al., 2021; Yokobori et al., 2012). Studies in rat OM (Negroni et al., 2012) and *Drosophila* olfactory systems have demonstrated the ability of NPY and its orthologs to modulate olfactory responses and odour-driven food search in accordance with energy conditions (Ko et al., 2015; Root et al., 2011). Although modulation at the level of the first olfactory synapse and in cultured OSNs has been documented before, modulation of OSN activity *in vivo* and resulting behavioural implications are only just being described. A recent study in *Drosophila* by Lee et al. (2017) revealed the role of NPF (fly orthologue of Neuropeptide Y) and its receptor NPFR in modulating the responses of OSNs by altering the localisation of a particular class of odourant receptors.

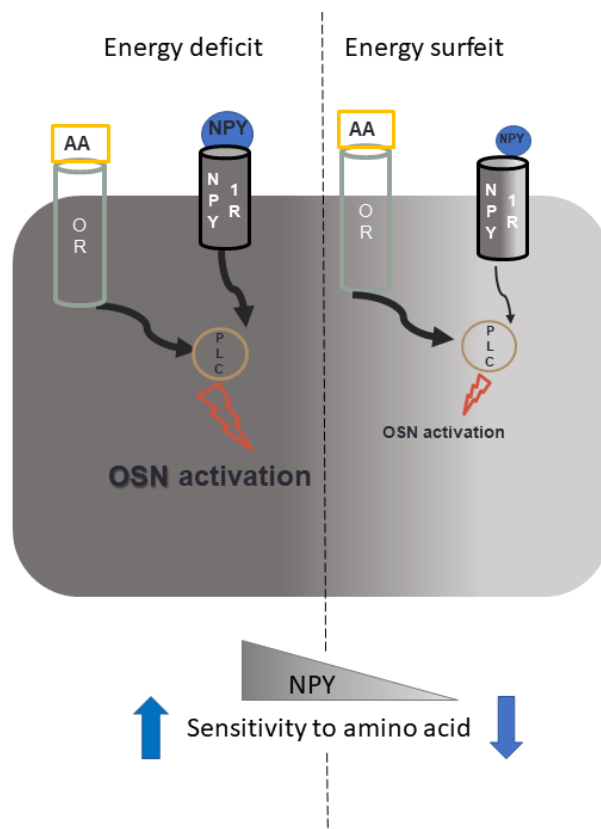


Fig. 3.7. NPY mediated tuning of olfactory response to amino acids.

NPY signalling via Y1R converges onto PLC-mediated amino acid detection to enhance microvillar OSN activation under conditions of energy deficit. Alleviating the energy deficit reduces NPY levels to baseline which in turn lowers the sensitivity to this class of food-related odours. [AA: amino acids; OR: odour receptor]

Together these data highlight a conserved neuromodulatory blueprint wherein physiological states, represented here by the levels of NPY, alter the strength of odour signalling to enhance the most relevant environmental stimuli (viz. food-related odours), thereby ensuring correct behavioural output (Fig. 3.7).

Chapter 4: Conclusions and Future directions

An intricate and well-coordinated set of processes are involved in sensing energy availability and modifying feeding behaviour to achieve homeostasis via the action of various neuromodulatory agents. Chapter 1 briefly described the role of neuropeptidergic modulation in regulating behavioural state transitions and altering sensory processing across multiple phyla. From an overview of the vertebrate feeding circuits, it is apparent that while the neuropeptidergic populations in the ARC and their neuroanatomical targets are well-characterised, the biochemical pathways employed to alter food intake are in some cases still unclear. Chapter 2 we demonstrated a conserved anorexigenic role for CART in the Zebrafish system and parsed out the underlying biochemical signalling. Using a combinatorial approach, we showed that CART-mediated anorexia requires NMDAR signalling and PKA activity. Immunohistochemistry data also revealed PKA mediated upregulation of p-NR1 levels in Dm, a downstream target of CART signalling. Results from dynamic activity imaging indicate that CART, via PKA and PKC, modifies the strength of glutamatergic transmission via NMDARs in Dm neurons.

Moreover, we find a similar sensitisation of Dm neurons in conditions that induce satiety and increase endogenous CART levels. These changes in neural activity in Dm are strongly correlated with an anorectic behavioural output. Such neuropeptide-mediated alteration of fast neurotransmitter activity is a feature of many feeding circuits, including those in the ARC, PVH, and MeA (Chapter 1). Dm is a homolog of the pallial amygdala and receives interoceptive inputs from EN and Hypothalamus. Thus, Dm is likely to drive CART-mediated anorexia however further studies are needed to confirm a direct role. It is presently technically challenging to monitor neural activity in freely behaving adult fish. One way to overcome this drawback would be to adopt protocols that measure food intake in larval or early juvenile stages (Jordi et al., 2018; Shimada et al., 2012). More detailed molecular characterisation is required to identify CART responsive microcircuits within the Dm, which would facilitate the use of targeted opto-or-chemogenetic strategies and utilise this model system to its full potential. Nevertheless, our study presents Dm as a new node in Zebrafish feeding circuits that can be explored further to gain insights into the regulation of feeding behaviour by neuropeptides.

Another aspect that requires further investigation is testing similarities in energy-sensing mechanisms that regulate CART expression between Zebrafish and other vertebrate systems. Such knowledge would be essential to develop models of metabolic disorder and their applications in drug discovery. As discussed in chapter 1, studies in vertebrate interoceptive neurons have identified a p-AMPK based feedback loop that maintains a metabolic memory of fasted state. Inhibition of AMPK also abrogates starvation-induced increase in food intake in rodents (Kim et al., 2004). Based on these reports, we tested if AMPK activity is involved in starvation induced orexia in Zebrafish. Indeed, we found that food-deprived animals injected with an AMPK inhibitor, Compound C (0.263 µg), show a stark reduction in food intake (328.25±62.4722 cumulative biting attempts) compared to vehicle injected controls (633.8571±112.053 cumulative biting attempts) (Fig. 4.1). AMPK activity is therefore necessary for maintaining an increased feeding drive under conditions of energy-deprivation.

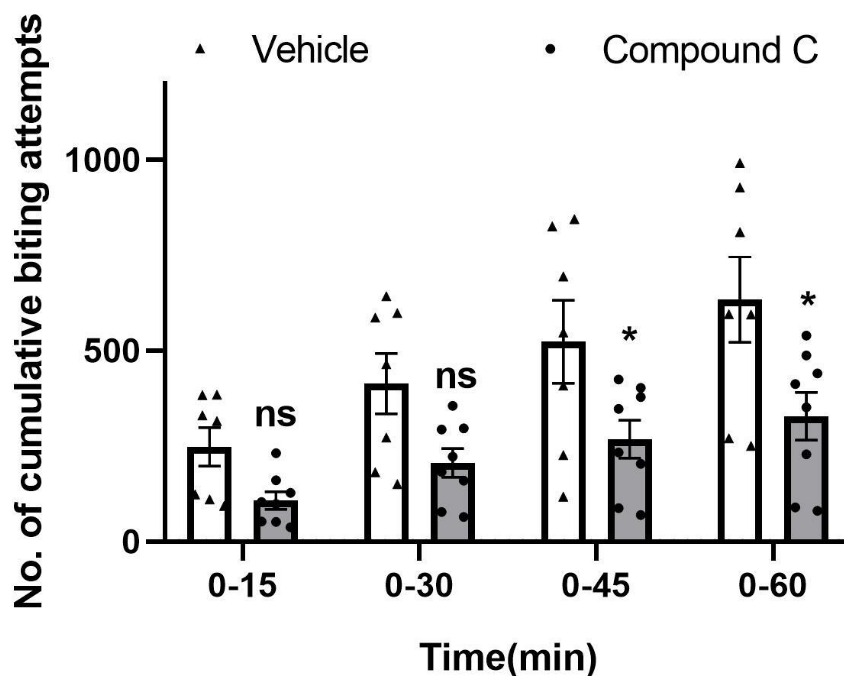


Fig. 4.1. Inhibition of AMPK activity reduces feeding drive in starved animals.

Treatment with AMPK inhibitor, Compound C, (0.263 µg) reduces food intake in starved animals (N=7 animals for control and N=8 animals for Compound C injected group). Data are represented as cumulative biting attempts in 15 min bins over a 1-hour period and are compared using Two-way ANOVA, with Bonferroni's post-hoc analysis (error bars represent mean±SEM; ns, not significant, *p<0.05).

It would be interesting to see if the CART-ir interoceptive neurons in the EN and Hypothalamus respond to such energy-deprivation conditions with an upregulation of p-AMPK which then downregulates CART expression. However, due to the lack of a suitable p-AMPK antibody that cross-reacts with the Zebrafish protein, we are currently unable to directly test this.

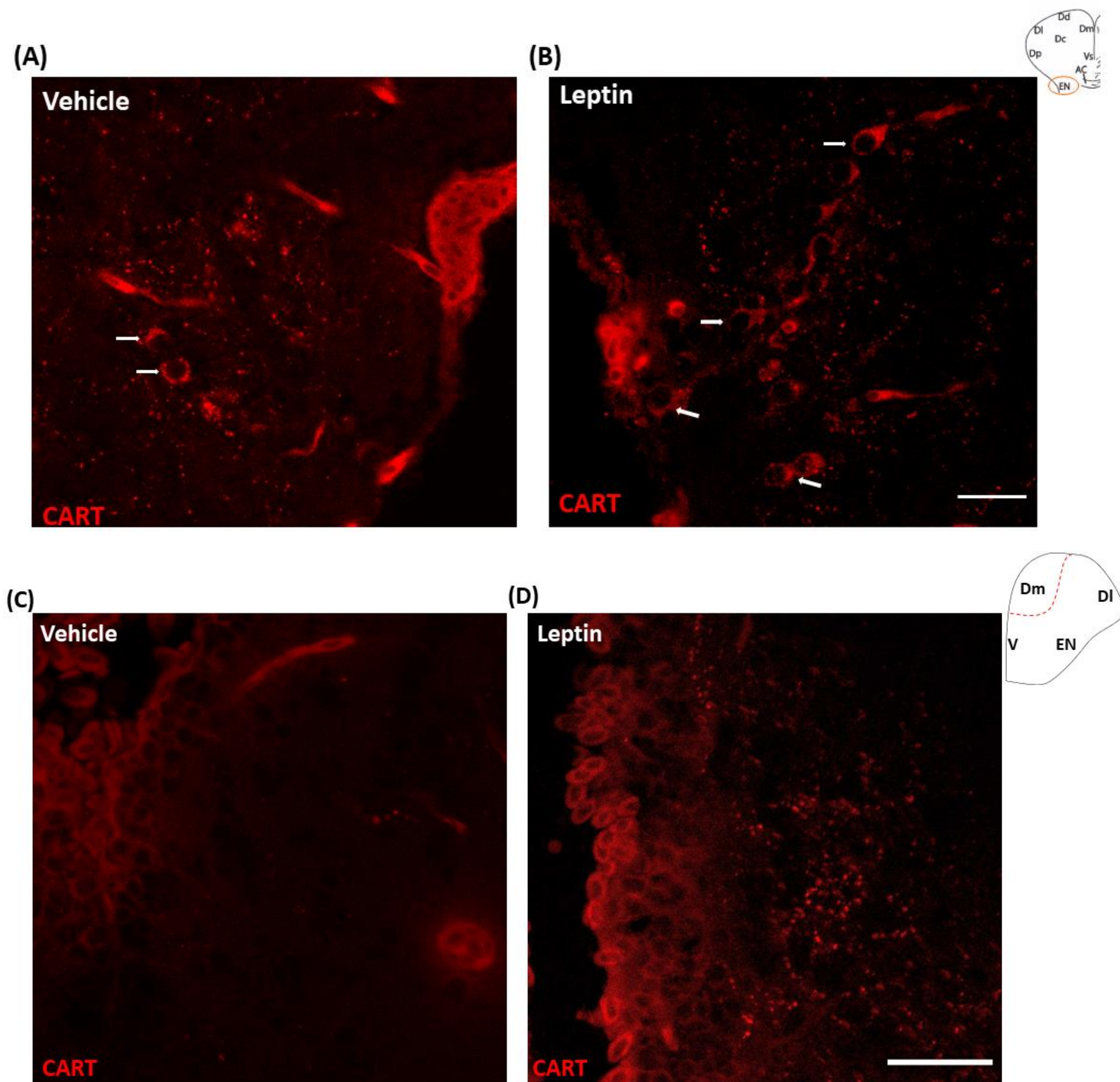


Fig. 4.2 CART expression in EN and Dm is sensitive to changes in leptin.

Representative transverse sections showing CART positive neurons in EN (top) and fibres in Dm (bottom) of starved fish treated with either vehicle **(A, C)** or Leptin (0.01µg) **(B,D)** respectively. [Scale bar 30 µm]

Conversely, we can also test the response of CART-ir interoceptive neurons in the EN and Hypothalamus to satiety signals. As a study in that direction, we analysed CART

immunoreactivity in the EN and Dm upon central administration of leptin (0.01µg) (Fig. 4.2). Preliminary results suggest that CART expression in neurons and fibres increases in response to leptin administration, similar to reports in mammals (Klok et al., 2007). Further testing will be needed to confirm these observations and determine the overlap between the energy-sensing and homeostasis mechanisms in Zebrafish and mammalian systems. (Materials and Methods for this section can be found in the appendix at the end)

In chapter 3, we outlined a mechanism by which NPY, an orexigenic neuropeptide, modulates odour transduction pathways to tune olfactory responses to a food-related odour class viz. amino acids. We found that amino acids activate TRPC2-expressing OSNs in a PLC-dependent manner. These OSNs also express NPY Y1R receptors but don't express NPY. Further, heightened OSN activation under energy deprivation requires signalling via Y1R. An increase in NPY signalling within the olfactory mucosa could thus facilitate sensory gating by modulating odour transduction pathways. Indeed, we find crosstalk between NPY signalling and amino acid detection pathways at the level of PLC. However, the mechanistic details of co-incident PLC activation by NPY and the odour transduction machinery are still unresolved. We delivered NPY Y1R inhibitor via two routes: icv and nasal. Both approaches abolished amino acid-induced OSN activation, indicating that in addition to central influences, NPY signalling locally at the OE-OB level is necessary for heightened odour response in starved animals. Further studies that disrupt Y1R signalling exclusively in microvillar OSNs would help discern the role of neuromodulatory influences at the level of OSNs themselves.

The detection of nucleotides involves a distinct group of OSNs, the A2c receptor-expressing pear OSNs (Wakisaka et al., 2017). It would also be interesting to test if and how NPY signalling affects the detection of this group of food-related odours as it likely involves different transduction machinery. Furthermore, future studies aimed at determining the glucose-sensing capabilities of Zebrafish OM and how that governs the local expression of neuromodulators would aid in understanding the role of sensory neurons in maintaining energy homeostasis. This is particularly interesting considering that patients with metabolic disorders present with compromised olfactory acuity (Palouzier-Paulignan et al., 2012). A better understanding of the role of olfactory sensory neurons in energy homeostasis and the sensory neuroendocrine processes involved present an attractive possibility to translate these findings to develop strategies that aid in the detection and treatment of metabolic disorders.

Taken together this thesis uncovered molecular pathways employed by neuropeptides that drive need-based alterations in neural activity and behaviour, which can be further explored to gain insights into the neural computations underlying bistable behaviours.

Appendix

Chemicals

Synthetic mouse Leptin (Sigma-Aldrich, Cat. No. L3772) and dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cat. No. D8418) were purchased from Sigma-Aldrich. Compound C was provided by Dr. Ullas's lab (TIFR).

Intracerebroventricular (icv) and feeding behaviour assay

Prior to icv delivery, fish were anesthetized following immersion in 2-phenoxyethanol (Sigma-Aldrich, 1: 2000). All pharmacological agents were dissolved in 1X Phosphate buffered saline or in DMSO depending on solubility. The final concentration of DMSO did not exceed 0.01% for Compund C treatment. Icv delivery and feeding behaviour protocol was as described in chapter 2 (section 2.2.3).

Immunofluorescence

Adult fish were anesthetized, craniotomised and fixed overnight in 4% paraformaldehyde at 4°C. The brain was dissected out and transferred to 25% sucrose solution for cryoprotection, embedded in OCT compound (Jung), sectioned in the transverse plane at 15 μm thickness using a cryostat (CM1950; Leica) and mounted on poly-L-lysine coated glass slides. The slides containing the sections were stored at -40°C until they were processed for immunofluorescence. The frozen sections were rehydrated and permeabilized using 0.5% Triton-X in phosphate buffer saline (PBST). The sections were treated with 5% BSA in PBST for 1 hour at room temperature (RT) and then incubated with the primary antibody overnight at 4 ^{o}C. The sections were washed using 0.5% PBST and blocked with 5 % BSA in PBST for 1 hour before incubation with desired secondary antibody for 3 hours at RT. Sections were then washed and mounted with mounting media (70% glycerol, 0.5% N-propyl gallate in 1 M Tris, pH 8.0, DAPI). The following antibodies were used in this study- Anti-CART (55-102 aa) provided by Thim lab (1:2000). Anti-mouse Alexa-568 (1:500; Invitrogen) was used as secondary antibody. Sections were observed using an upright epifluorescence microscope (Axioimager.Z1 with Axiocam HRm camera; Carl Zeiss) and imaged using an inverted laser scanning confocal microscope (LSM 780; Carl Zeiss).

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Publications

Sensitivity of olfactory sensory neurons to food cues is tuned to nutritional states by Neuropeptide Y signaling

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