Organization, transformation, and activation of the melanocortin system in Atlantic salmon

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Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2023

UNIVERSITY OF BERGEN

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Date of defense: 03.11.2023

Year: 2023

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Print: Skipnes Kommunikasjon / University of Bergen

Scientific environment

This PhD study was performed from June 2019 to June 2023 at the Marine Developmental Biology research group, a part of the Environmental and Aquaculture Biology group, at the Department of Biological Sciences, University of Bergen (UiB), Norway. The PhD study was accomplished under the supervision of Professor Jon Vidar Helvik (UiB), researcher Dr. Mariann Eilertsen (UiB), Professor Ivar Rønnestad (UiB), and researcher Dr. Ana S. Gomes (UiB).

This PhD was co-funded by the Research Council of Norway (projects LeuSense – 267626; ExcelAQUA-2.0 – 261753; NoFood2Waste – 317770; Photobiol – 254894; Lightbiotrans – 315106), the Regional Research Fund West (Greenbag – 259183), and by a four years PhD scholarship position from University of Bergen.

Acknowledgments

I would like to thank my supervisor Jon Vidar Helvik for this opportunity to do a PhD in his laboratory and scientific environment. I thank him for introducing me to the field of neurobiology, and the scientific discussions with you have been essential to understanding the overview of this project, and to see the results obtained from the lab work from a larger perspective. I would also like to thank my co-supervisor Ivar Rønnestad, whom I've had the pleasure of working with since I started as a master student, for his help and guidance on appetite control in fish and for all the constructive feedback during the writing processes.

I would also like to thank my co-supervisors Ana S. Gomes and Mariann Eilertsen, who have been essential in the work of this project. Thank you both for discussions, sharing knowledge, giving me excellent guidance in the experimental designs and laboratory work, valuable inputs in data analyses and the writing process, traveling, and being very good role models for women in science!

Thanks to the research groups of Helvik and Rønnestad. Particularly, I would like to extend my thanks to Rita Karlsen, Christine Horne, and Ann-Elise Olderbakk Jordal for taking part in samplings, sharing knowledge, and for technical assistance in the lab.

I would like to thank all former and current members of the Marine Developmental Biology group at the University of Bergen for making the days very enjoyable, for the chats and laughs during lunch, by the coffee machine and social events, and for your support through this process.

Finally, thanks to my family and friends for your support and encouragement. And especially Alex, this would never be possible without you.

Abstract

Atlantic salmon (*Salmo salar*) is a key species in Norwegian aquaculture. However, further knowledge and technology are needed to continuously develop more sustainable production, improve animal welfare, and increase the economic value of farmed fish. Owing to the high cost of food in aquaculture, a more efficient utilization of food is being developed, and feed waste is reduced. As a result, it is of interest to better understand appetite-controlling systems resulting in hunger and satiety, so that feed can be administered according to the demands of the Atlantic salmon. The melanocortin system is a key regulator of appetite and food intake in vertebrates including neurons expressing neuropeptide y (*npy*), agouti-related peptide (*agrp*), cocaine- and amphetamine-regulated transcript (*cart*), and pro-opiomelanocortin (*pomc*) that competitively interact with second-order neurons. In mammals, the hypothalamus plays a key role as an "entry point" for sensing the nutritional status of an organism, with two major neuronal circuits expressing *npy/agrp* and *cart/ pomc* that play a role in the overall stimulation or inhibition of food intake. Therefore, the main focus of this work is to gain better insight into the organization, transformation, and activation of the melanocortin system in Atlantic salmon. Specifically, by (i) mapping the topographical expression of the key neuropeptides in the melanocortin system in the Atlantic salmon brain, (ii) description of how the transition from endogenous to exogenous feeding influences genes related to food intake, and energy homeostasis in first-feeding Atlantic salmon, and (iii) investigating the effect of light regimes during development on the first-feeding Atlantic salmon, with a focus on the melanocortin neuropeptides.

To better understand the potential involvement of the melanocortin system in food intake, the topographic distribution of *npy, agrp, cart,* and *pomc* mRNA were mapped in the Atlantic salmon parr brain by chromogenic *in situ* hybridization on coronal parallel sections. After identifying the hypothalamic mRNA expression, possible intracellular coexpression of *npy/agrp* and *cart/pomc* in the tuberal hypothalamus by fluorescent *in situ* hybridization was investigated. The results showed that *npy* and *cart* were widely expressed, especially in sensory and neuroendocrine brain regions. In the

hypothalamic lateral tuberal nucleus, the putative homolog to the mammalian arcuate nucleus*, npya, agrp1, cart2b*, and *pomca* were predominantly localized in distinct neighboring neurons; but, some neurons coexpressed *cart2b/pomca*. This is the first study demonstrating coexpression of *cart2b/pomca* in the tuberal hypothalamus of a teleost. Collectively, the data suggest that the lateral tuberal nucleus is a center for appetite control in salmon, similar to that of mammals. Extrahypothalamic brain regions might also be involved in regulating food intake, including the olfactory bulb, telencephalon (and the limbic system), midbrain, and hindbrain.

To understand the involvement of the appetite controlling system during the transition from utilizing yolk to exogenous feeding in Atlantic salmon, fish were sampled during the endogenous feeding stage (1 week before pellets were introduced), and after the onset of exogenous feeding (1, 2, and 3 weeks into the first-feeding period). The results indicated that during the yolk sac utilization period, the Atlantic salmon brain was in a "satiety state" based on the transcriptomic expression of signaling pathways in the brain. Endogenous feeding fish will most likely not experience a hunger drive as the nutrients in the yolk will supply the fish with all the energy needed to support growth and development. On the other hand, the brain of an exogenous feeding Atlantic salmon seems to be in a "hunger state", indicating that the fish is constantly hungry and motivated to eat, especially during day time. Indeed, satiety systems in the salmon brain seems to not be fully developed during the first two weeks into the first-feeding period. Three weeks into the first feeding period, *npya1, pomca1,* and *a2* were the first neuropeptides of the melanocortin system that displayed a significant response to a meal, and that *pomca1* and *a2* increased in their relative expression after a meal, indicating an inhibition of appetite.

How and to what extent different light conditions during the endogenous phase affect the exogenous feeding period are not fully understood. The experiment investigating the effect of light regimes (constant light, light: dark periodicity, and constant darkness) during development on the first-feeding Atlantic salmon showed that light had no significant effect on growth, but influenced the yolk sac utilization rate. Furthermore, light conditions during embryogenesis have a significant effect on the mRNA level of

npya1, *pomca1*, and *pomca2* in the brain during the first feeding period. In general, light conditions that mimic natural light have a better stimulation of the appetite controlling system in Atlantic salmon.

Sammendrag

Atlantisk laks (*Salmo salar*) er en nøkkelart i norsk havbruk. Det trengs imidlertid mer kunnskap og teknologi for å kontinuerlig utvikle mer bærekraftig produksjon, forbedre dyrevelferden og for å øke den økonomiske verdien av oppdrettsfisk. På grunn av høye fôrkostnader utvikles det stategier for bedre fôrutnyttelse, og samtidig redusere matvinnet. Dette har vekket interesse for å øke vår forståelse av mekanismer for appetittkontroll som resulterer i sult og metthet, slik at fôringsregimet er i henhold til laksens behov. Melanokortinsystemet er en nøkkelregulator i reguleringen av appetitt og matinntak hos virveldyr som inkluderer nevroner som uttrykker nevropeptid y (*npy*), agouti-relatert peptid (*agrp*), kokain- og amfetaminregulert transkript (*cart*) og pro-opiomelanokortin (*pomc*) som kompetetivt interagerer med sekundærnevroner. Hos pattedyr spiller hypotalamus en nøkkelrolle for å registrere ernæringsstatusen til en organisme med to viktige nevronale kretsløp som uttrykker Npy/Agrp og Cart/Pomc som henholdsvis stimulerer og inhiberer matinntak. Derfor er hovedfokuset i dette arbeidet å få bedre innsikt i organisering, transformasjon og aktivering av melanokortinsystemet hos laks. Spesifikt inkluderer dette arbeidet studier for (i) kartlegging av det topografiske uttrykket av nøkkelnevropeptidene i melanokortinsystemet i hjernen, (ii) beskrivelse av hvordan overgangen fra endogen til eksogen føde påvirker gener relatert til matinntak, og energihomeostase under startfôring, og (iii) undersøke effekten av lysregimet under den embryonale utviklingen før startfôring påvirker fiskens appetittkontroll i startfôringsperioden, med en fokus på melanokortin-nevropeptidene.

For å øke forståelsen for potensiell innvirkning av melanokortinsystemet i matinntak, ble de topografiske uttrykket av *npy*, *agrp*, *cart* og *pomc* mRNA gjennom hele utstrekningen av hjernen til Atlanterhavslaks i parr stadiet kartlagt ved kromogen *in situ* hybridisering på koronale parallelle snitt. Etter identifisering av hypotalamisk mRNA-uttrykk, ble potensiell intracellulær ko-ekspressjon av *npy/agrp* og *cart/pomc* undersøkt i tuberal hypotalamus ved fluorescerende *in situ*-hybridisering. Resultatene viste at *npy* og *cart* var vidt distribuert, spesielt i sensoriske og nevroendokrine hjerneregioner. I den hypotalamiske laterale tuberalkjernen som er den antatte

homologen til pattedyrets arcuatuskjernen (ENG: arcuate nucleus), var *npya*, *agrp1*, *cart2b* og *pomca* hovedsakelig lokalisert i nabonevroner; og noen få nevroner uttrykte *cart2b/pomca*. Dette er den første studien som demonstrerer ko-ekspressjon av *cart2b/pomca* i tuberal hypotalamus til en teleost. Samlet antyder dataene at den laterale tuberalkjernen er et integeringssenter for appetittkontroll hos laks, på samme måte som hos pattedyr. Ekstrahypotalamiske hjerneregioner kan også være involvert i å regulere matinntaket, inkludert luktelappen (ENG: olfactory bulb), storhjernen (ENG: telencephalon) (limbiske systemet), mellomhjernen og bakhjernen.

For å forstå overgangen fra plommesekkutnyttelse til eksogen fôring hos laks, ble det tatt prøver av fisk under det endogene fôringsstadiet (1 uke før pellet ble introdusert), og etter starten av eksogen fôringsperiode (1, 2 og 3 uker inn i eksogen fôringsperiode). Resultatene indikerte at under endogen føde var hjernen hos laksen i en "metthetstilstand" basert på det transkriptomiske uttrykket av signalveier i hjernen i motsetning til eksogen fisk. Fisk i det endogene fôringsstadie vil mest sannsynlig ikke oppleve en sultlyst da næringsstoffene i eggeplommen vil forsyne fisken med all energien som trengs for å støtte vekst og utvikling. På den andre siden var hjernen under eksogen fødeinntak i en "sulttilstand", noe som indikerer at fisken er konstant sulten og motivert til å spise, spesielt på dagtid, men at metthetssystemet i hjernen ikke er fullt utviklet i løpet av de to første ukene inn i den eksogene fôringsperioden. Tre uker inn i fôringsperioden var *npya1*, *pomca1* og *a2* de første nevropeptidene i melanokortinsystemet som viste en signifikant respons på et måltid, og at det relative utrrykket av *pomca1* og *a2* økte etter et måltid, noe som indikerer en hemming av appetitten.

Hvordan og i hvilken grad ulike lysforhold under den endogene fasen påvirker den eksogene fôringsperioden er ikke fullstendig forstått. Eksperimentet undersøkte effekten av lysregimer (konstant lys, lys: mørke periodisitet og konstant mørke) under tidlig utvikling viste at lys ikke hadde noen signifikant effekt på veksten hos laks i startfôringsperioden, men påvirket utnyttelsesgraden av plommesekken. Videre har lys under embryogenesen en betydelig effekt på mRNA-nivået til *npya1*, *pomca1* og

pomca2 i hjernen under den starten av fôringsperioden, og lysforhold som etterligner naturlig lys gir en bedre stimulering av appetitten hos laks.

Abbreviations

Abbreviation	Description
AgRP	agouti-related peptide
AgRP/NPY ^{ARC}	AgRP/NPY-containing neurons in ARC
Akt	protein kinase B
AMPKα	AMP-activated protein kinase α
ARC	arcuate nucleus
AVP	arginin-vasopressin
α-MSH	α-melanocyte-stimulating hormone
BDNF	brain-derived neurotrophic factor
BSX	brain homeobox transcription factor
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CREB	cAMP response-element binding protein
CRF	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
DD	constant darkness
Dd	daydegrees (day °C)
FOX01	forkhead box protein O1
GABA	γ-aminobutyric acid
GHRL	ghrelin
GHSR	growth hormone secretagogue receptor
INSR	insulin receptor
ISH	*in situ* hybridization
LD	light: dark periodicity
LepR	leptin receptor
LH	lateral hypothalamic area
LL	constant light
MC3R	melanocortin 3 receptor
MC4R	melanocortin 4 receptor
MCH	melanin-concentrating hormone
mTOR	mechanistic target of rapamycin

mRNA	messenger ribonucleic acid
NAT	nucleus anterior tuberis
NLT	nucleus lateralis tuberis
NLTv	nucleus lateralis tuberis pars ventralis
NMH	nucleus magnocellularis hypothalamic
NmU	neuromedin u
NPT	nucleus posterior tuberis
NPY	neuropeptide y
NPY-R	neuropeptide y receptor
OXY	oxytocin
POMC	pro-opiomelanocortin
POMC/CART ^{ARC}	POMC/CART-containing neurons in ARC
PVN	paraventricular nucleus (also referred to as PVA or PVH)
SCN	suprachiasmatic nucleus
Ss4R	salmonid-specific fourth whole-genome duplication
TRH	thyrotropin-releasing hormone
VMN	ventromedial hypothalamic nucleus
Y1R	neuropeptide y 1 receptor

List of Publications

Paper I

Norland, S., Eilertsen, M., Rønnestad, I., Helvik, J. V., & Gomes, A. S. (2023). Mapping key neuropeptides involved in the melanocortin system in Atlantic salmon (*Salmo salar*) brain. *J Comp Neurol*, 531, 89-115. https://doi.org/10.1002/cne.25415

Paper II

Norland, S., Eilertsen, M., Gomes, A. S., Dolan, D. W. P., Rønnestad, I., Karlsen, R., & Helvik, J. V. Appetite and rhythmicity in Atlantic salmon brain transcriptome from endogenous to exogenous feeding. (Manuscript).

Paper III

Norland, S., Gomes, A. S., Rønnestad, I., Helvik, J. V., & Eilertsen, M. (2023). Light conditions during Atlantic salmon embryogenesis affect key neuropeptides in the melanocortin system during transition from endogenous to exogenous feeding. *Front. Behav. Neurosci.* 17:1162494. https://doi.org/10.3389/fnbeh.2023.1162494

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1 Introduction

1.1 Atlantic salmon as a model organism for appetite

The Atlantic salmon (*Salmo salar*) is an important aquaculture species, particularly in Norway (Iversen et al., 2020), with a production of approximately 1.55 million tons in 2022 (Fiskeridirektoratet, 2022). Consequently, huge investments are made to develop and optimize its production. According to Asche and Oglend (2016), feed is the most expensive factor used in the aquaculture of salmon. Although marine proteins have been used in fish feed for many years, the growing worldwide aquaculture industry has caused economic and environmental concerns regarding their use in fish feed (Asche & Oglend, 2016; Naylor et al., 2000). To meet these challenges, alternative sources of feed ingredients, including soybean meal, wheat, insects, and yeast, have been introduced in recent years. However, increased replacement of marine proteins with several of these novel ingredients can result in reduced food intake and growth, which might be linked to low protein digestibility, conditioning to certain food, and palatability of the diet resulting in lower appetite stimulation (Olsen et al., 2006; Torstensen et al., 2008). This can also result in increased feed waste, thus contributing to a negative environmental impact and economical losses. Thus, to have a more sustainable production, improved animal welfare, and increased economic value for the farmer, it is essential to better understand the mechanisms controlling fish hunger and satiety, so that feed can be administered according to the demands of the fish.

Despite the significant industrial importance of Atlantic salmon, the underlying mechanisms for controlling hunger and satiety are not fully understood. Wild Atlantic salmon is an anadromous species that display adjust feed intake based on several factors, that may be an adaptation to the natural food availability and seasonality. Most studies conducted on appetite control in Atlantic salmon have been performed on older stages, including parr, smolts, and post-smolts (Kalananthan et al., 2023; Kalananthan et al., 2021; Kalananthan et al., 2020a; Kalananthan et al., 2020b; Murashita et al., 2011; Murashita et al., 2009a; Tolås et al., 2021; Valen et al., 2011).

However, very little is known about appetite control in the early developmental stages, including the first-feeding period, which is a critical developmental period and may result in high mortality rates. When the yolk is exhausted the Atlantic salmon start to display a swimming (food-seeking) behavior, as well as digestive maturity (Coughlin, 1991; Dill, 1977; Sahlmann et al., 2015). At the same time, the salmon develops a photopositive tendency, such as diurnal vertical migration, in line with many teleosts that are diurnal species (Dill, 1977; Metcalfe et al., 1998; Reebs, 2002; Stefansson et al., 1990). This is opposed to nocturnal mammals, including rats and mice, that have been the subject species in most appetite studies (Volkoff, 2019). Therefore, understanding stage-specific appetite-related characteristics, including differences is key in assessing feed utilization in Atlantic salmon aquaculture.

Compared to mammals, teleost species express multiple paralogous genes due to teleost-specific 3rd round of whole-genome duplication (Glasauer & Neuhauss, 2014). In addition, the ancestor to salmonids underwent a 4th round (salmonid-specific) whole-genome duplication (Allendorf & Thorgaard, 1984; Lien et al., 2016) resulting in more copies of most genes, including genes involved in appetite control. However, the fate of duplicated appetite-related genes is not fully understood. Their functional role might be maintaining the ancestral gene function (subfunctionalization), have acquired a new function (neofunctionalization), or loss of function (nonfunctionalization) (Lynch & Conery, 2000). The additional copies may reflect increased plasticity, allowing salmon to adapt better to different environmental conditions during different life stages (Klemetsen et al., 2003). Therefore, Atlantic salmon can be used as a model organism to study comparative genomics, evolutionary processes, fates of duplicated genes, and the physiological processes associated with appetite control and energy homeostasis in salmonids.

1.2 Appetite control

1.2.1 Definition of appetite and appetite control

Appetite control is a complex process whose overall function is to regulate food intake. This complex process is influenced by hunger and satiation signaling pathways, which modulate feeding behavior, including searching for and ingesting food particles. Hunger works as a proactive signal for feeding behavior, and appetite is the motivational driver of feeding associated with an expected reward, as demonstrated by classical conditioning by Pavlov (Cannon & Washburn, 1912). On the other hand, satiety is the cumulative effect of inhibitory signals generated during feeding and counteracts hunger signals (Blundell, 1991). After a meal, a period of satiation lasts until hunger and appetite signals restart again. Appetite control is homeostatic feeding resembling a "depletion/repletion" model which refers to the purpose of eating to refill depleted energy stores in the body to support cellular metabolism and ensure the survival of the organism by initiating hunger by gradual loss of satiety signals (Berthoud, 2011; Campfield & Smith, 2003; Morley, 1987). Food intake is also controlled by sensory and hedonic inputs such as liking or wanting-driven hunger, also referred to as non-homeostatic feeding (Berthoud, 2011). A balance of steady-state conditions is maintained by neural regulators receiving inputs from central and peripheral processes (Lenard & Berthoud, 2008), including the quality and size of the meal, nutrient sensors, and available energy. All these factors contribute to the overall stimulation of hunger and satiation signaling pathways.

1.2.2 Appetite control in vertebrates

Key peripheral components of appetite control are the gustatory system, the digestive system, and adipose tissue which drive bidirectional feedback loops within the brain through neurons, hormones, and metabolites (Lenard & Berthoud, 2008) (**Figure 1**). During the hunger state, the release of ghrelin (GHRL), which is mainly synthesized in the stomach, is considered a potent orexigenic hormone resulting in an anabolic response in mammals. During a meal, satiety is conveyed by several signals,

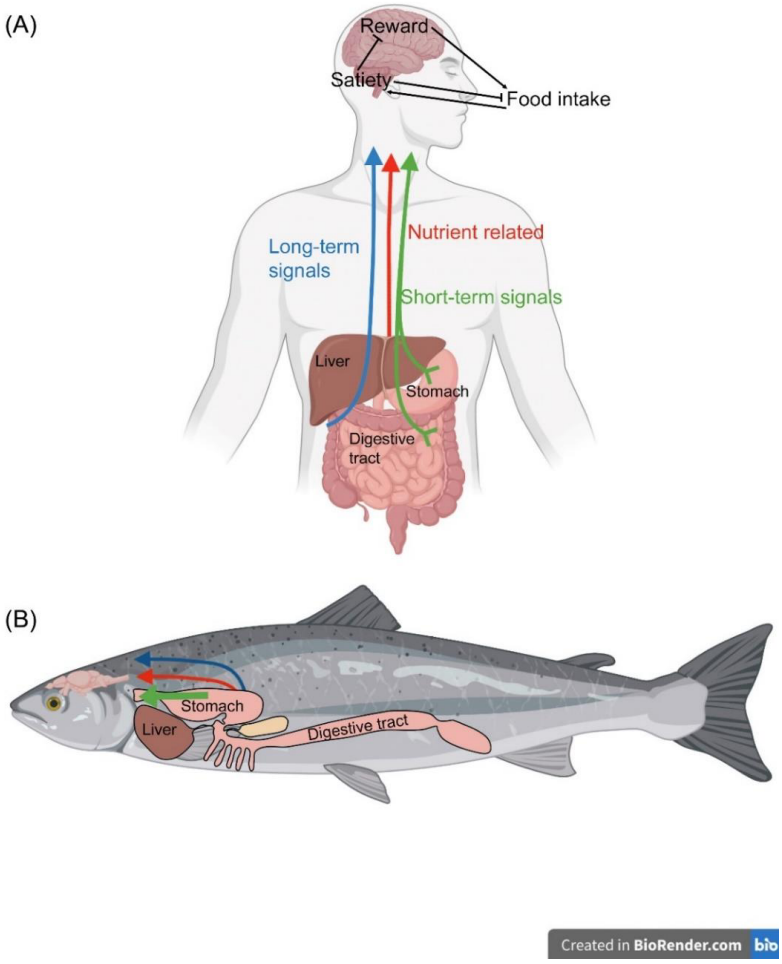

Figure 1. Integration of information for appetite control in vertebrates and key organs involved in this process. The central nervous system integrates long-term signals (represented by a blue arrow, e.g., insulin and leptin), short-term meal-related signals (represented by a green arrow, e.g., ghrelin, glucagon-like-peptide-1, CCK), and nutrient-related (represented by a red arrow, e.g., free fatty acids and glucose). In the brain, enhanced satiety inhibits food intake and reward. Food intake is stimulated when satiety signals are reduced. **(A)** Inputs in mammals. Modified from Morton et al. (2014). **(B)** Inputs in fish. The yellow color code indicates the pancreas. Modified from Rønnestad et al. (2017). Created with BioRender.com.

including nerves and hormones (Schwartz et al., 2000). Afferent nerves entering the hindbrain from the digestive tract, liver, and taste buds in the oral cavity, along with the secretion of cholecystokinin (CCK) and glucagon-like-peptide-1 from enteroendocrine cells in the intestine, and leptin mainly released from adipose tissue, stimulate the anorexigenic response and the catabolic pathway (**Figure 1A**). The satiety and hunger hormones leptin and GHRL, respectively, enter the brain and interact with their respective hypothalamic receptors activating a signal cascade (Elmquist et al., 1999; Schwartz et al., 2000). Several hypothalamic regions play a role in behavioral responses and energy homeostasis, which refers to the coordination of food intake and energy expenditure to maintain the long-term stability of stored energy (Schwartz et al., 2000). The autonomic nervous system of the caudal brain, structures of the limbic system, as well as other brain regions, are also involved in appetite control.

Brain lesions and electrical stimulations performed in mammals have proven that the hypothalamus is a major regulator of appetite almost seven decades ago (Anand & Brobeck, 1951; Elmquist et al., 1999; Morrison et al., 1958; Schwartz et al., 2000). The ventromedial hypothalamic nucleus (VMN) and hypothalamic arcuate nucleus (ARC) were identified as satiety centers, whereas the lateral hypothalamic area (LH) was termed the hunger center. These results provided the foundation for the "dual center hypothesis", in which lesions in the VMN-ARC produce hyperphagia and obesity, while LH lesions result in aphagia and weight loss (Elmquist et al., 1999). Later, the application of molecular probes identified the ARC as a key site in regulating feeding behavior (Elias et al., 1998; Hahn et al., 1998). The mammalian ARC is also the best-studied region for appetite control. The ARC contains interoceptive neurons, which are located close to the ventricle where there is a less restricted blood-brain barrier. By studying neuropeptides and receptors in the ARC, the antagonistic melanocortin system has become one of the best-characterized systems for appetite control (Cone, 2005; Nuzzaci et al., 2015).

In fish, like other vertebrates, the brain is also a key region in appetite control (Rønnestad et al., 2017; Soengas et al., 2018; Volkoff, 2016). This neuronal network

receives continuous feedback from peripheral tissues, especially the digestive tract, liver, and pancreas (Rønnestad et al., 2017) (**Figure 1B**). Selective pressure and evolutionary conservation have resulted in a conserved regulatory mechanism for appetite and food intake in the hypothalamus among vertebrates, including between mammals and fishes (Soengas et al., 2018). In fish, electrical stimulation of the inferior lobes of bluegill (*Lepomis macrochirus*) demonstrated the involvement of the hypothalamus in feeding (Demski & Knigge, 1971). In addition, track-tracing studies have shown this region to be a multisensory region, including gustatory (Rink & Wullimann, 1998). However, there are nearly 35 000 fish species that inhabit the aquatic environment (*FishBase*, 2023). While the basic mechanism underlying appetite control is conserved, the large number of fish species, habitats, food preferences, physiological factors, and intrinsic and extrinsic factors can influence this control (Volkoff, 2016).

1.2.3 Key neuropeptides in the melanocortin system

The melanocortin system is characterized by two major neuronal circuits that stimulate (orexigenic) or inhibit (anorexigenic) appetite in the hypothalamus (Cone, 2005; Elias et al., 1998; Hahn et al., 1998; Schwartz et al., 2000). Orexigenic neurons express agouti-related peptide (AgRP) and neuropeptide y (NPY), whereas anorexigenic neurons express cocaine-and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) (**Figure 2**). In mammals, both AGRP/NPY and POMC/CART neurons contain receptors to receive short-term and long-term signals from peripheral tissues and are regarded as functional units (Cone, 2005). The functional antagonism of orexigenic and anorexigenic of these first-order neurons is defined by their convergence at second-order neurons, as silencing second-order neurons promotes orexigenic stimulation (Krashes et al., 2016). Therefore, the melanocortin system constitutes of (i) neurons expressing melanocortin receptors (MCR), (ii) neurons expressing MCR agonists, such as α-melanocyte-stimulating

hormone (α-MSH) derived from POMC, and (iii) neurons expressing MCR antagonists, such as AgRP (Nuzzaci et al., 2015) (**Figure 2**).

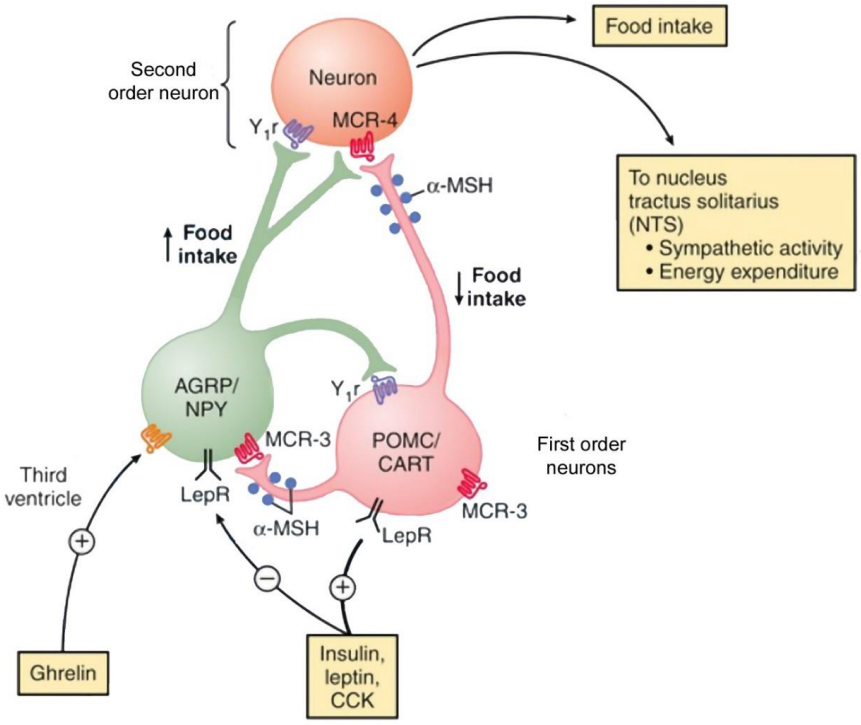

Figure 2. Schematic overview of the mammalian hypothalamic melanocortin system. Stimulation of AGRP/NPY neurons promotes food intake, while stimulation of POMC/CART neurons inhibits food intake. AGRP, agouti-related peptide; CART, cocaine-amphetamine regulated transcript; CCK, cholecystokinin; LepR, leptin receptor; NPY, neuropeptide y; MC3R, melanocortin 3 receptor; MC4R, melanocortin 4 receptor; POMC, pro-opiomelanocortin; Y1R, neuropeptide y 1 receptor; α-MSH, α-melanocyte stimulating hormone. Modified from Barsh and Schwartz (2002).

The neuropeptides *agrp, npy, cart,* and *pomc* have also been identified in the hypothalamus of several teleost species and demonstrated to respond to feeding, including goldfish *Carassius auratus* (Cerdá-Reverter et al., 2003a; Cerdá-Reverter et al., 2003b; Kah et al., 1989; Kojima et al., 2010; Matsuda et al., 2009; Volkoff & Peter, 2000), zebrafish *Danio rerio* (Forlano & Cone, 2007; Jeong et al., 2018;

Matsuda et al., 2012; Mukherjee et al., 2012; Nishio et al., 2012; Song et al., 2003; Zhang et al., 2012), and the Atlantic salmon (Kalananthan et al., 2023; Kalananthan et al., 2021; Kalananthan et al., 2020a; Kalananthan et al., 2020b; Murashita et al., 2011; Murashita et al., 2009a; Tolås et al., 2021; Valen et al., 2011).

NPY

NPY is a highly conserved neuropeptide and a potent and abundant orexigenic factor and anabolic signaling molecule in the mammalian brain (Ip et al., 2019; Larhammar et al., 2004; Loh et al., 2015). NPY increases in the ARC during fasting and it is inhibited through leptin/insulin satiety signaling in mammals, whereas upon release NPY will interact with NPY receptors in the brain (Hahn et al., 1998; Loh et al., 2015; Schwartz et al., 2000). In the teleost brain, *npy* is widely distributed (Castro et al., 1999; Garcia-Fernandez et al., 1992; Kah et al., 1989; Matsuda et al., 2012; Pickavance et al., 1992; Silverstein et al., 1998). The Npy expression was first characterized in goldfish and showed that Npy was expressed in the telencephalon and hypothalamus, indicating that this peptide is involved in various functions (including appetite and behavior) within the central nervous system of teleosts, similar to that of mammals (Kah et al., 1989; Matsuda et al., 2012). Studies have shown that Npy by immunolabelling or *npy* mRNA by *in situ* hybridization (ISH) is expressed in the hypothalamus of Atlantic salmon (Garcia-Fernandez et al., 1992), brown trout *Salmo trutta fario* (Castro et al., 1999), chinook salmon *Oncorhynchus tshawytscha* and coho salmon *Oncorhynchus kisutchand* (Silverstein et al., 1998). *npy* is involved in the control of food intake in salmon, since the brain *npy* mRNA expression differs between fed/fasted states in this species (Murashita et al., 2009a; Valen et al., 2011). Recently, *npya1, npya2,* and *npyb* were identified in Atlantic salmon, where *npya1* and *a2* are Ss4R paralogs, *npyb* is a teleost paralog (Tolås et al., 2021). Moreover, *npya1* was the most abundant paralog throughout the salmon brain. Long-term fasting (4 weeks), as opposed to short-term fasting (3-4 days), has a significant effect on hypothalamic *npya1* indicating a highly time-dependent response of *npya1* to the nutritional status in the hypothalamus (Kalananthan et al., 2023; Tolås et al., 2021). Short-term fasting had a significant effect on *npya2* in the midbrain, though non-significant changes were found in the hypothalamus (Tolås et al., 2021).

AGRP

AgRP is a natural antagonist of melanocortin-3 and -4 receptors (MC3R and MC4R) and is highly coexpressed with NPY in the mammalian ARC (Cone, 2005; Hahn et al., 1998). The activation of AgRP-neurons to promote feeding depends on AgRP and NPY released from these cells, as well as the release of GABA, which is the third inhibitory neurotransmitter found within these neurons (Cone, 2005; Tong et al., 2008). Energy deficit increases AgRP excitability, which is suppressed shortly after feeding (Mandelblat-Cerf et al., 2015). In addition to regulating food intake, AgRP also plays a role in inflammatory pain, insulin sensitivity, substrate utilization, and systemic glucose metabolism (Jais & Brüning, 2021). The first teleost studies demonstrated that *agrp* mRNA expression is restricted to the ventral region of the *nucleus lateralis tuberis* (NLT) and that fasting significantly increases hypothalamic *agrp* levels in goldfish (Cerdá-Reverter & Peter, 2003) and zebrafish (Song et al., 2003). It was later demonstrated that several fish species possess two Agouti-related peptide sequences, named *agrp1* and *agrp2* (also named *asip2b*), including salmonids, cichlids (*Cichlidae*), and cyprinids (Braasch & Postlethwait, 2011; Murashita et al., 2009a; Västermark et al., 2012; Zhang et al., 2010). While *agrp1* seems to have a conserved role in the hypothalamus to stimulate food intake (Agulleiro et al., 2014; Cerdá-Reverter & Peter, 2003; Forlano & Cone, 2007; Jeong et al., 2018; Otero-Rodino et al., 2019; Song et al., 2003), *agrp2* seems to be involved in the neuroendocrine regulation of background adaptations and stress response (Kratochwil et al., 2018; Shainer et al., 2017; Shainer et al., 2019; Zhang et al., 2010). In Atlantic salmon, Murashita et al. (2009a) identified two Agouti-like sequences, *agrp1* and *agrp2*. In the whole brain, *agrp1* mRNA levels are lower expressed after 6 days of fasting (Murashita et al., 2009a), and increasing after feeding (Valen et al., 2011). The orexigenic effect of hypothalamic *agrp1* mRNA seems to be conserved in Atlantic salmon, whereas *agrp2* does not seem to be involved in appetite control (Kalananthan et al., 2023; Kalananthan et al., 2020a; Kalananthan et al., 2020b).

CART

CART neuropeptide is widely distributed in various brain regions associated with physiological processes including memory, feeding, stress, endocrine regulation, and

drug abuse (reviewed by (Ahmadian-Moghadam et al., 2018) and (Subhedar et al., 2014)). In mammals, CART has a potent anorexigenic function in the hypothalamus, where it is coexpressed with POMC in rodents (Cone, 2005; Elias et al., 1998). Central injection of human CART fragments into goldfish inhibited food intake, which suggested that CART plays a role in appetite control in fish (Volkoff & Peter, 2000). The teleost-specific whole-genome duplication has resulted in several *cart* genes in fish that display different tissue distributions and expression levels. Two *cart* genes have been identified in goldfish (Volkoff & Peter, 2001), four in zebrafish (Akash et al., 2014), and seven in Senegalese sole (*Solea senegalensis*) (Bonacic et al., 2015). In Atlantic salmon, this system is more complex as Ss4R resulted in ten *cart* paralogs (named *cart1a, 1b1, 1b2, 2a, 2b1, 2b2, 3a1, 3a2, 3b,* and *4*) (Kalananthan et al., 2021). These *cart* paralogs have different expression patterns and their physiological roles are still far from being fully understood. Neuronal *cart2a, 2b,* and *3a* mRNA expression responded to fed/fasted state in Atlantic salmon (Kalananthan et al., 2023; Kalananthan et al., 2021).

POMC

As a precursor peptide, POMC is post-translational cleaved into several peptides, including α-MSH, β-melanocyte-stimulating hormone, adrenocorticotropic hormone, and β-endorphin (Takahashi & Mizusawa, 2013). POMC regulates a wide range of functions, including feeding behavior, energy homeostasis, glucose homeostasis, lipid metabolism, skin pigmentation, and blood pressure (Cerda-Reverter et al., 2011; Jais & Brüning, 2021; Krashes et al., 2016). POMC is coexpressed with CART in the ARC inhibiting appetite through stimulation of MC4R on second-order neurons. α-melanocyte-stimulating hormone (derived from POMC peptide cleavage) is an agonist of MC4R, whereas AgRP acts as an antagonist, and will competitively interact with the MC4R on second-order neurons (Cone, 2005; Krashes et al., 2016). In fish, *pomc* was first shown to be expressed in the hypothalamus and demonstrated to inhibit food intake in goldfish (Cerdá-Reverter et al., 2003b), though the main site of expression is the pituitary (Royan et al., 2021; Segura-Noguera et al., 2000; Weltzien et al., 2003), similar to mammals. To note that teleosts lack γ-melanocyte-stimulating hormone derived from *pomc*, which is in contrast to mammals that have

three MSH peptides (Takahashi & Mizusawa, 2013). Three *pomc* paralogs have been identified in Atlantic salmon (*pomca1, pomca2,* and *pomcb*), where *pomca* and *b* are the results of teleost-specific whole-genome duplication and *a1* and *a2* are a result of the Ss4R (Murashita et al., 2011; Valen et al., 2011). *pomca1* has demonstrated a postprandial anorexigenic response (Valen et al., 2011), while *pomca1* and *pomca2* showed an orexigenic role in long-term fasting (Kalananthan et al., 2023).

1.2.4 Nutrients and the melanocortin system

Beyond short- and long-term signals that influence appetite control, the brain also respond to metabolic state or nutrient levels by nutrient sensors (Cone, 2005). Based on the glucostatic hypothesis, the initiation of a meal, in part, can be regulated through sensing blood glucose levels, and nutrient sensors influencing appetite are, therefore, an extension of this hypothesis (Cone, 2005; Mayer, 1955). In fish, the activation of nutrient sensors enhances the anorexigenic potential by decreasing the expression of Agrp and Npy, and increasing Cart and Pomc, which results in an inhibition of food intake (Delgado et al., 2017; Soengas, 2021; Soengas et al., 2018). The nutrient-sensing system involves nutrient sensors (including amino acids, free fatty acids, and glucose) and different signaling pathways, including AMP-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR), and protein kinase B (Akt) mediates transcription factors, including brain homeobox transcription factor (BSX), phosphorylated cAMP (CREB), and forkhead box protein 01 (FOX01) (Soengas, 2021). For instance, glucokinase is extensively expressed in the ependymal cells of NLT adjacent to *pomc1a* expressing cells, and potentially coexpressed with *agrp1* in rainbow trout *Oncorhynchus mykiss* (Otero-Rodino et al., 2019). Intracerebellar injection of amino acids resulted in a decrease of *npy* and *agrp* and an increase of *pomca1* mRNA in the hypothalamus of rainbow trout (Comesaña et al., 2018). The diet composition have also been shown to influence the mRNA levels of signaling pathways players and neuropeptides in the melanocortin system (Bonacic et al., 2016; Conde-Sieira et al., 2010; Conde-Sieira et al., 2015; Delgado et al., 2017; Li et al., 2019; Libran-Perez et al., 2015; Senzui et al., 2020).

1.3 Brain regions related to appetite

1.3.1 Involvement of the hypothalamus in appetite control

The ARC is a key hypothalamic region in appetite control and a major site for transducing afferent inputs, including circulating leptin and insulin, into a neural response through the high concentration of receptors in the ARC (**Figure 2**). The NLT has been suggested to be the teleostean putative homolog of the mammalian ARC (Cerdá-Reverter et al., 2003b; Lin et al., 2000). The NLT is considered a site for the integration and release of neurotransmitters to higher-order neurons linked to neuroendocrine appetite control and feeding behavior (Rønnestad et al., 2017). This is in line with the presence of melanocortin neuropeptides detected by mRNA expression methods or immunoreactivity in the NLT region of several teleost species, including goldfish (Cerdá-Reverter & Peter, 2003; Cerdá-Reverter et al., 2003b; Kah et al., 1989; Kojima et al., 2010), sea bass *Dicentrarchus labrax* (Agulleiro et al., 2014), African cichlid fish *Astatotilapia burtoni* (Hu et al., 2016; Porter et al., 2017), rainbow trout (Otero-Rodino et al., 2019), and zebrafish (Akash et al., 2014; Forlano & Cone, 2007; Jeong et al., 2018; Kawahara et al., 2018; Koch et al., 2019; Mukherjee et al., 2012; Nishio et al., 2012; Shainer et al., 2017; Shainer et al., 2019; Song et al., 2003; Wasserman-Bartov et al., 2022; Zhang et al., 2012; Zhang et al., 2010). This is further supported by the expression of neurokinin b, kisspeptin, and growth hormone-releasing hormone in teleost NLT (reviewed in (Biran et al., 2015)). In teleosts, Npy-and α-MSH-positive cell terminals (nerve fibers) are in direct contact with each other at neuronal cell bodies in the NLT and nucleus posterioris periventricularis, respectively (Kojima et al., 2010), similar to that of mammals (Menyhért et al., 2006). However, the hypothalamic coexpression in the ARC neurons of AGRP/NPY and CART/POMC in mammals is not observed in teleosts. Jeong et al. (2018) investigated the expression of *npy* and Agrp in the hypothalamus of zebrafish and observed that they were expressed in distinct neurons, and not coexpressed. Shainer et al. (2019) confirmed the absence of *npy*/Agrp coexpression in zebrafish. Thus, suggesting that the *npy*/Agrp system might not be needed to regulate appetite control in teleosts (Jeong et al., 2018). In contrast, the majority of ARC

neurons that express *Npy* also coexpress *Agrp*, and play a crucial role in appetite regulation in mammals (Morton & Schwartz, 2001; Schwartz et al., 2000).

Second-order neurons (also referred to as 'downstream sites') located within and outside the hypothalamus modulate the signals from the AgRP/NPY-containing neurons in ARC (AgRP/NPY ^{ARC}) and POMC/CART-containing neurons in ARC (POMC/CART ^{ARC}), and are important in the overall effect of ARC neurons (Waterson & Horvath, 2015). Within the hypothalamus, AgRP/NPY ^{ARC} and POMC/CART ^{ARC} project to the paraventricular nucleus (PVN), ventromedial (VM), and lateral (LH) hypothalamus (Fischer & O'Connell, 2017; Morton et al., 2006) (**Figure 3**). 2nd-order neurons are found in the PVN and contain melanocortin-4-receptors (MC4R) (**Figure 2, Figure 3**). PVN neurons synthesize a wide range of anorexigenic peptides, including corticotropin-releasing factor (CRF) (activates the sympathetic nervous system, and is the major regulator of the hypothalamo-pituitary-interrenal axis), TRH (stimulates the thyroid axis), and oxytocin (regulates uterine function) (Schwartz et al., 2000). Accordingly, synthetic activation of the MC4R-neurons in the PVN reduces food intake. On the other hand, a subset of PVN neurons (TRH- and Pituitary adenylate-cyclase-activating polypeptide (PACAP)-expressing neurons) projects to the ARC and provides excitatory input to AgRP/NPY ^{ARC} neurons to stimulate feeding (Krashes et al., 2014). As a result, MC4R on PVN neurons is both necessary and sufficient for regulating hunger (reward) and satiety through the hindbrain (Andermann & Lowell, 2017) (**Figure 3**).

The teleostean preoptic region is functionally and neurochemically associated with the hypothalamus (Porter et al., 2017), and the magnocellular preoptic nucleus has been suggested to be the teleostean homolog of the PVN (Forlano & Bass, 2011; Forlano & Cone, 2007). The preoptic region is considered the supraopto-paraventricular region (Wullimann, 2022), and similar peptide expression has been observed in fish as in mammals in this region, including those that are potentially involved in appetite control, such as CCK, corticotropin-releasing hormone (CRH), enkephalin, somatostatin, and glucagon/secretin-related genes (e.g., vasoactive intestinal peptide and vasopressin) (Herget et al., 2014). In mammals, 2nd order

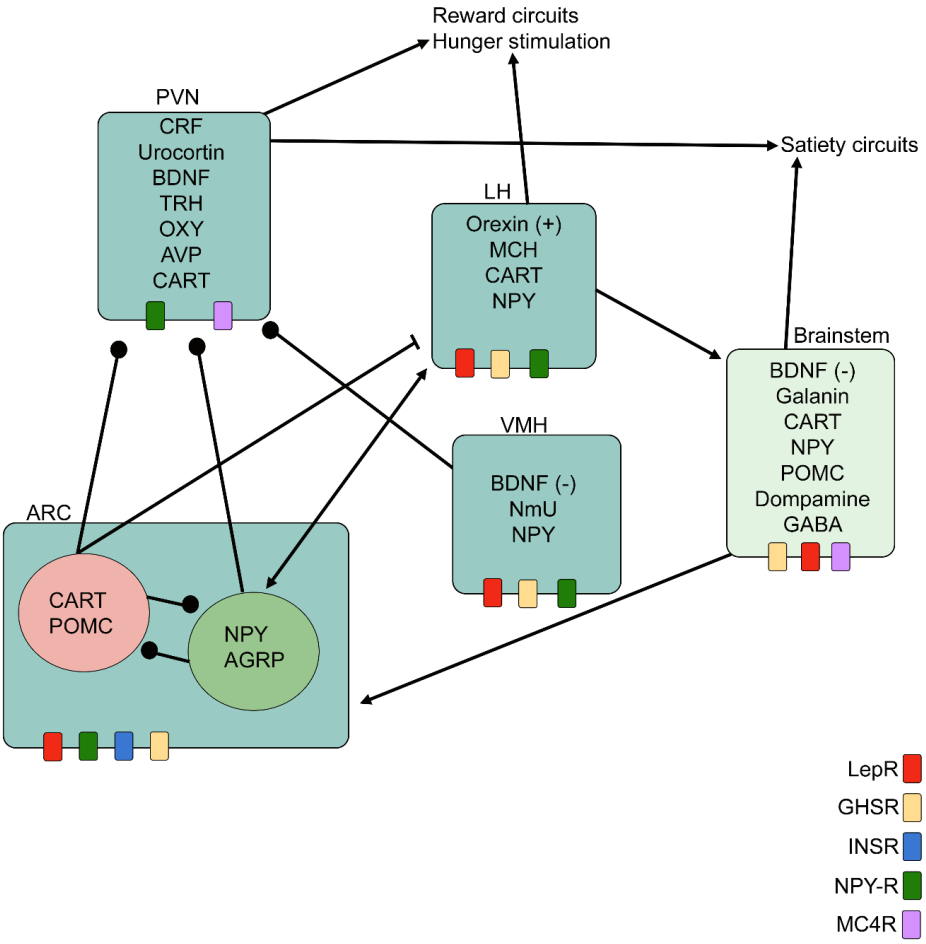

Figure 3. The architecture of hypothalamic neurocircuits and the involvement of the melanocortin system in mammals. The arcuate nucleus (ARC) contains both CART/POMC and NPY/AgRP neurons that are stimulated or inhibited by signal transduction mechanisms of various receptors. Both neurons project to adjacent hypothalamic areas, including the paraventricular nucleus (PVN) which both stimulates and inhibits food intake, lateral hypothalamus (LH) which stimulates hunger and reward circuits. The overall outflow from these regions supplies downstream brain regions that regulate satiety (including the hindbrain) as well as a reward system. CART/POMC and NPY/AgRP may also influence each other by the release of neurotransmitters. Modified from Fischer and O'Connell (2017) and (Morton et al., 2006).

neurons in the LH (also referred to as the pericornial area, PFA) integrate reward signal and is considered to be orexigenic. These neurons express melanin-concentrating hormone (MCH) and orexin/hypocretin (Schwartz et al., 2000) (**Figure 3**). In teleost, somatostatin is found in 2nd order neurons in the preoptic area of zebrafish, which project into the pituitary (Löhr et al., 2018). The hypothalamic anterior tuberal hypothalamus in fish is suggested to be the putative homolog structure to VMN in mammals (Bshary et al., 2014; Forlano & Bass, 2011).

1.3.2 Involvement of other brain regions in appetite control

An important progression in understanding appetite control was the discovery in mammals that feeding-related neuroendocrine, behavioral, and autonomic responses are organized in diffuse neural uni- and bidirectional networks to respond to changes in metabolic status (Grill & Kaplan, 2002). The neuronal architecture of the brain allows for a high degree of communication and signal integration between the regions. The degree of crosstalk and association between brain regions supports the idea that physiological functions can not be separated into specific brain regions (Pessoa et al., 2019). Different mechanisms accounting for this plasticity are 1) a large number of polymodal nerve fibers regions that integrate different stimuli, 2) a large number of different receptors expressed in a single nerve to integrate various stimulations, and 3) rapid changes in the expression of neuropeptides and their corresponding receptors in response to various stimuli (Watts et al., 2022). Feeding is a complex behavior that involves motivational, sensory, and motor circuits. For example, hunger may result in higher risk-taking behavior for successful food capture, while satiety results in inhibition of seeking out novel, possibly dangerous surroundings (Burnett et al., 2016; Comeras et al., 2019). Consequently, several brain regions are directly or indirectly involved in appetite control (Fischer & O'Connell, 2017; Krashes et al., 2016; Morton et al., 2006; Schwartz et al., 2000; Waterson & Horvath, 2015). In fish, the hypothalamus is connected to the telencephalon (both pallium and subpallium) which is involved in multisensory processing and modulates

brain activity (Folgueira et al., 2004b). In fish, the thalamus shows little interaction with the telencephalon compared to mammals, although it receives retinal input (non-olfactory cues) and is equally connected with the midbrain and the hypothalamus (Pessoa et al., 2019).

Telencephalon

The sensory perceptions of food are key factors that influence its capture and ingestion by an organism. The smell of potential food, as well as recognition of kin/predators, drive anticipation and motivation to eat (Wee et al., 2022). In the telencephalon; olfactory bulb, pallium, and subpallium are linked to the processing and transmission of sensory information, which in turn is linked to functions such as immune responses, reproduction, behavior, and appetite (Maruska et al., 2017; Rodríguez et al., 2021; Saha et al., 2015; Uezono et al., 2015; Wee et al., 2022; Ye et al., 2020). The telencephalon has afferent and efferent connections, which communicate with the preoptic region and tuberal hypothalamus (Folgueira et al., 2004a, 2004b). Therefore, the expression of neuropeptides of the melanocortin system in the telencephalon can be linked to behavioral state-dependent actions of reward and satiety signals (Devika et al., 2023; Ip et al., 2019; Senzui et al., 2020).

Midbrain

Modulation of appetite control by the mesolimbic reward system goes beyond the animal's homeostatic requirements by the melanocortin neuropeptides. For example, orexin and glutamate co-releasing neurons in the ventral tegmental area (VTA) are sensitive to peripheral signals of the energy state, including glucose, and may inhibit dopaminergic VTA-neurons (Waterson & Horvath, 2015). However, no dopamine-expressing cells were found in the midbrain of teleosts (reviewed by (Yamamoto & Vernier, 2011)). More research is needed to understand the teleostean homolog for VTA, but it is suggested to be the periventricular region of the posterior tuberculum (Maruska et al., 2017) or the posterior tuberal nucleus (Forlano & Bass, 2011).

In many fish species, visual information modulates feeding behavior, and the organism's nutritional state modulates the activity of sensory processing involved in the response to the external stimuli, including prey capture (or avoidance behavior)

(Chen et al., 2018; Corradi & Filosa, 2021; Filosa et al., 2016; Muto et al., 2017). The optic tectum, often highly developed in teleosts, plays a key role in this neuronal network as a hub for multisensory information integration, influencing feeding behavior (Heap et al., 2018; Rodríguez et al., 2021; Ye et al., 2020). The pretectal region is involved in the connection between prey detection, initiated in the retina, and the neural signaling processed in the optic tectum. Thus, pretectal and tectal neurons are stimulated by multiple stimuli as they overlap with visual function (Chen et al., 2018). Food presented to zebrafish stimulates pretecto-hypothalamic pathway neurons that project to the hypothalamic inferior lobes, possibly converting food detection into feeding motivation (Muto et al., 2017). In zebrafish, the feeding state modulates the visual perception in the optic tectum as hunger recruits prey-responsive neurons (Filosa et al., 2016). Another midbrain region is the thalamus which integrates various stimuli, which are involved in the modulation of sensory inputs (Folgueira et al., 2004a, 2004b; Mueller, 2012), and may be influenced by the melanocortin system as MC4Rs are found in this region (Cerdá-Reverter et al., 2003a).

Hindbrain

Satiation signals (including mechanical and chemical) generated during feeding are largely conveyed to the nucleus tractus solitarius (NTS) of the hindbrain through afferent fibers of the vagus nerve passing into the spinal cord from the digestive tract (Cone, 2005; Ritter et al., 1994). In mammals, POMC-expressing cells, MC4R, and hormone receptors (**Figure 3**) have been found in the hindbrain and brainstem indicating a coordination between the brainstem and the hypothalamic melanocortin system (Cone, 2005). In fish, as in mammals, hypothalamic *pomc* neurons project to the spinal cord, and the activation of *mc4r* decreases food intake (Reinoß et al., 2020), supporting that the hindbrain has a role in appetite control. Indeed, neuropeptides attributed to appetite control, including those of the melanocortin system, are expressed in the teleostean hindbrain and have contributed to the understanding of neural and whole-body physiology, including *npy* (Cerdá-Reverter et al., 2000; Porter et al., 2017), and *cart* (Akash et al., 2014; Le et al., 2016; Porter et al., 2017). It is suggested that these neuropeptides in the hindbrain may be involved

in sensory inputs from innervating nerves (e.g., from the oral cavity) or play a role in descending control from the brain stem (Akash et al., 2014; Pirone et al., 2008).

Pituitary

The hypothalamo-pituitary axis is integral in the regulation of energy expenditure through the control of basic physiological processes. In mammals and elasmobranchs, the portal system divides the hypothalamus from the adenohypophysis – compared to that of teleosts where hypothalamic neurons are in direct contact with the adenohypophysis (Cerdá-Reverter & Canosa, 2009). This direct contact, in teleosts, allows the hypothalamic neurons to terminate directly to the adenohypophysis and make close synaptic contact. Several studies in teleost have demonstrated that the preoptic region may innervate the pituitary via the hypothalamic-neurophyseal tract (Akash et al., 2014; Forlano & Cone, 2007; Herget et al., 2014; Holmquist & Ekström, 1995). The activity of the pituitary is regulated by innervated hypothalamic fibers, releasing hormones, and inhibiting hormones, which stimulate or inhibit the secretion of pituitary hormones that regulate peripheral glands (interrenal, thyroid, and gonads) (Volkoff, 2016). Common for all these pathways is to maintain homeostasis in the organism, including food intake. The pituitary consists of several cell types, including adrenocorticotropic hormone (Acth) derived from Pomc cleavage in corticotropes, growth hormone (Gh) in somatotropes, thyroid-stimulating hormone (Tsh) in thyrotropes and follicle-stimulating hormone and luteinizing hormone in gonadotrophs (Weltzien et al., 2003). For instance, characteristic behavioral traits for stressed fish are reduced feeding behavior (search and capture of food) and reduced food intake, which involves ACTH released from the pituitary (Bernier & Peter, 2001).

1.4 Transition from endogenous to exogenous feeding

While the abovementioned section describes the control of appetite in adults, much less is known in the early developmental stages of fish. This is related to how the fish acquire energy to support their development, metabolism, and growth. There is a relationship between egg size, size at hatching, the start of branchial ventilation, and

first feeding in fish (reviewed by (Osse & van den Boogaart, 1995)). Atlantic salmon are rich in yolk matter at hatching (**Figure 4A**). During the endogenous feeding period, fish have an energetically closed system where nutrients stored in the yolk sac support development, growth, and metabolism (Rønnestad et al., 1993) (**Figure 4B**). Consequently, the salmon are larger and more developed at the time of exogenous feeding compared to many marine teleost species and zebrafish, one of the most common teleost model species. When salmon begin to ingest exogenous food, their digestive system is segmented, with a stomach, midgut, hindgut, and a differentiated liver and pancreas and the system is ready to digest and absorb nutrients before the yolk is fully utilized (Sahlmann et al., 2015). On the other hand, most marine fish species have an undeveloped gut at the onset of feeding, resulting in different feeding regimes and weaning protocols in aquaculture (Gomes et al., 2014; Kamisaka & Rønnestad, 2011; Norland et al., 2022; Sahlmann et al., 2015). To secure a steady supply of nutrients when the yolk matter starts to become depleted, the fish need to ingest external feed (exogenous feeding) (**Figure 4C**). To successfully achieve the transition to exogenous feeding (**Figure 4D**), the fish have behavioral and physiological features that support this transition (Rønnestad et al., 2013; Yúfera & Darías, 2007b; Zambonino Infante et al., 2008). These features can be morphological features and integrated physiological functions for the detection, capture, ingestion, digestion, and assimilation of food (Rønnestad et al., 2013). These changes display allometric growth periods, meaning different organs and systems involved in feeding biology develop at different rates. Functional head and tail regions need to be present, including skeletal structures required for opening the mouth and capture of food particles, and skeletal muscles in the head and trunk support feeding and escape behavior (Norland et al., 2022; Osse & van den Boogaart, 1995; Yúfera & Darías, 2007b). The digestive system undergoes significant morphological, physiological, and functional changes that occur at species-specific rates prior to the onset of exogenous feeding to deal with ingested prey items (Rønnestad et al., 2013; Yúfera & Darías, 2007b; Zambonino Infante et al., 2008).

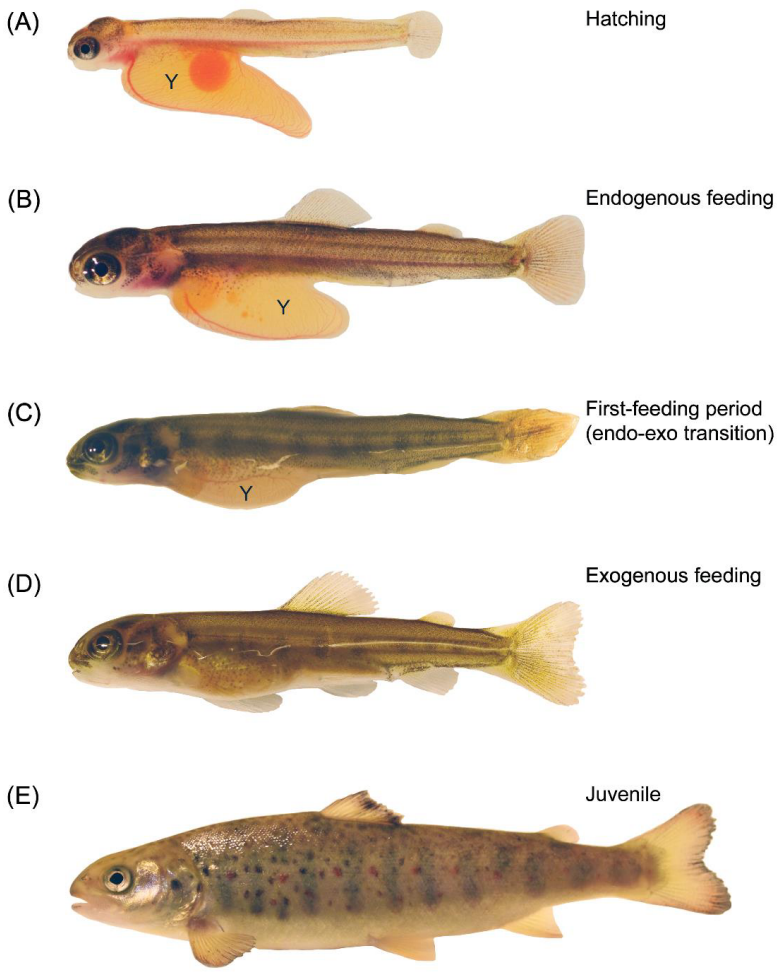

Figure 4 Atlantic salmon, *Salmo salar*, development from hatching until juvenile with corresponding outer morphology. (A) Newly hatched alevin (555 day degrees) with the presence of a large yolk sac. (B) Endogenous feeding alevin (690 day degrees) with an energetically closed system. (C) First-feeding fish (830 day degrees) before the yolk is fully internalized. (D) Exogenous feeding fry (1000 day degrees). (E) Juvenile Atlantic salmon. Abbreviation: Y; yolk sac.

1.4.1 The first feeding window

The transition from endogenous to exogenous feeding (endo-exo transition) is a critical ontogenetic period for survival during early life, where food availability and

the timing of feeding behavior influence the survival of the organism. One may speculate that endogenously feeding fish (utilization of yolk) experience no feeding stimuli; hence, their costs of acquiring nutrients and energy are minimized by the absence of foraging and ingestion of external food.

Fish adapt from endogenous to exogenous (external) nutrient sources during the first-feeding window. In this regard, if successful feeding does not occur shortly after yolk matter is fully depleted, the fish will reach the point-of-no-return. Beyond this point, even if the fish ingest feed, they may not be able to digest or assimilate food and eventually will die (Yúfera & Darías, 2007b). The switch in nutritional strategy from endogenous to exogenous feeding is accompanied by genome-wide transcription changes, and during the first-feeding period, fish can be less resistant to stress (Benini et al., 2022; Xu et al., 2021; Xu et al., 2017). Several studies have reported that growth regulation (DNA replication and cell cycle) and energy metabolism pathways (glycolysis, gluconeogenesis, and fatty acid metabolism) are significantly affected by the endo-exo transition (Guerrero-Tortolero et al., 2021; Tang et al., 2022; Xu et al., 2021). As a result, the general recommendation for aquaculture production is to present fish with feeding opportunities as soon as they achieve feeding ability (innate gut that can digest food particles or possess a developed and functional digestive system) or display active food-searching swimming behavior. Although, there can be a delay between when food is offered and when the fish larvae will actively ingest food particles (Yúfera & Darías, 2007b). Food particles may be green water and copepods before a weaning protocol with pellets is introduced or, can start directly with pellets. Therefore, offering feed to fish earlier than complete yolk sac depletion may be a strategy to ensure higher survival and quality of fish larvae (Benini et al., 2022).

After the onset of exogenous feeding, fish continue to grow and develop as they obtain juvenile and adult morphological traits (Rønnestad et al., 2017) (**Figure 4E**). Somatic growth is influenced by hypothalamic and hypophysiotropic factors, including GH, insulin-like growth factors, digestive hormones (e.g., leptin), neuropeptides (appetite-controlling neuropeptides and sex steroids), and cortisol

(Canosa & Bertucci, 2020; Kim et al., 2015). To support a high fish growth rate, a high rate of ingested and absorbed nutrients is required. In line with this, several hormones related to digestion and control of energy metabolism, including *ghrl*, *cck*, *leptin*, and *peptide yy* are upregulated around exogenous feeding that also assists to control the digestive capacity to support the metabolic demands of developing salmon (Moen et al., 2010; Sahlmann et al., 2015). However, these hormones are only a few of the factors necessary for homeostasis and energy metabolism in fish (Delgado et al., 2017; Rønnestad et al., 2017; Rønnestad et al., 2013; Soengas et al., 2018; Volkoff, 2019; Volkoff et al., 2005).

1.4.2 Development of control mechanisms for food intake and metabolic regulation

Little is known about the function of the appetite-controlling network during the first-feeding period. Ontogenetic gene expression of key peptides involved in appetite control has been investigated in some fish species, including Atlantic salmon (Moen et al., 2010; Sahlmann et al., 2015), European eel (*Anguilla angiulla*) (Benini et al., 2022; Politis et al., 2018), giant grouper (*Epinephelus lanceolatus*) (Anderson et al., 2018), Atlantic cod (*Gadus morhua*) (Le et al., 2016; Tillner et al., 2013), and Atlantic halibut (*Hippoglossus hippoglossus*) (Gomes et al., 2015; Gomes et al., 2022). The expression profile of these key peptides may underline a pre-programmed expression as well as influence digestive capacity during fish development (Rønnestad et al., 2013). Zebrafish is a common model organism for exploring molecular mechanisms. However, during vertebrate evolution, the stomach has been lost in some advanced species, including teleosts. Wrasses (*Labridae*), gobies (*Gobiidae*), scarids (*Scaridae*), and cyprinids (*Cyprinidae*, which includes zebrafish and other families) are stomach-less throughout their life cycle (Jobling, 1994; Ng et al., 2005; Norland et al., 2022; Smith et al., 2000). As a result, ingested food particles will move directly from the esophagus to the intestine. Other teleost species studied for appetite control have a neutral pH in the stomach, which means they lack

acidification (Yúfera & Darías, 2007a). These are some of the factors that influence digestion, passage rates, and the hormone and neuropeptide responses, and may be different in Atlantic salmon compared to other fish species. The responses of key factors in appetite control between fed/fasted states may differ between teleosts. Therefore, understanding species-specific and stage-specific appetite-related characteristics is key in assessing feed utilization.

In the central control of the brain, active regulation depends on the ability of the signal(s) to generate a change in neural modulations in the brain to stimulate or inhibit food intake. There are several reports of marine fish larvae that continue to ingest food even though their gut is already full (Harboe et al., 2009; Rønnestad et al., 2013). This indicates that satiety systems may not be fully developed during the first-feeding period. Other studies have shown that fish larvae develop a "circadian-like" feeding pattern that is influenced by a light: dark periodicity (LD) (Navarro-Guillén et al., 2018; Navarro-Guillen et al., 2017a; Navarro-Guillén et al., 2016). Those results indicated that fish larvae rely on visual inputs at the beginning of the first-feeding period to ingest food particles, while stimulation of reward-based appetite (other stimulations, including olfactory) may play a role in the detection of food particles after metamorphosis (Navarro-Guillén et al., 2018). The sensory properties of foods (taste, smell, and texture) are associated with animal learning after the food particle is consumed (Kulczykowska & Sánchez Vázquez, 2010). For example, if a rodent ingests a toxic food, it will develop a conditioned aversion to its flavor (and post-ingestive consequences), whereas, in a positive condition, the animal will acquire a preference for its flavor (Sclafani, 1995). It has also been demonstrated that ontogenetic expression patterns for digestive enzymes and melanocortin neuropeptides are under transcriptional control but can also be triggered by the nutritional composition of the diet (Bonacic et al., 2016; Zambonino Infante & Cahu, 2001). In mammals, the cellular mechanisms forming hypothalamic circuits are suggested to be categorized by neurogenesis, neuronal migration, cell death, axon growth, and synapse formation which continue to differentiate postnatally (Bouret, 2010; Stocker & Cawthorne, 2008). Key neurotropic agents in controlling the

development of hypothalamic circuits in mammals are leptin and long-form leptin receptors in ARC. Bouret et al. (2004a) showed that the ARC projections are immature at birth, but will innervate DMH, PVN, and LH in an age-dependent manner during the post-natal stage. As a result, young brains are thought to be relatively insensitive to metabolic cues or neurons might fail to relay signals to other brain regions (reviewed by (Coupe & Bouret, 2013)). In summary, one may speculate that the development of appetite control is partly learning signals to stimulate or inhibit food intake and partly developing neuronal circuits in the brain. However, one can not rule out that fish larvae possess a rudimentary regulatory system for energy metabolism (Rønnestad et al., 2017).

1.5 Appetite dynamics

1.5.1 Appetite control in response to a meal

In mammals, AgRP/NPY ^{ARC} are interoceptive neurons that link energy deficits with the decision to seek and consume food. During hunger, GHRL is released from the stomach, crosses the blood-brain barrier and binds to growth hormone secretagogue receptors on AgRP/NPY ^{ARC} (Willesen et al., 1999). This binding will stimulate AgRP/NPY ^{ARC} to increase their preprandial firing rate as a response to chemical stimulation (Cone, 2005; Mandelblat-Cerf et al., 2015). AgRP antagonizes the MC4R on second-order neurons and directly inhibits POMC/CART-containing neurons in ARC (POMC/CART ^{ARC}). The overall result is a stimulation of appetite. During and after a meal, peripheral satiety signals (e.g., insulin, leptin, peptide yy, and CCK) have a dual mechanism in the ARC (Cone, 2005). They inhibit the action of AgRP/NPY ^{ARC}, which results in a rapid drop in their spiking activity and an inhibition of their action (Mandelblat-Cerf et al., 2015). At the same time, satiety signals stimulate POMC/CART ^{ARC} neurons, resulting in overall decreased feeding and increased energy expenditure through the stimulation of MC3R and MC4R on AgRP/NPY ^{ARC} neurons and the 2nd-order neurons containing MC4R.

Although the mammalian melanocortin system has been extensively characterized and is critical for appetite control, new paradigms continue to emerge. The presumed orexigenic and anorexigenic definitions of ARC neurons have shown that these are flexible in coordinating appetite to maintain homeostasis. Increased feeding behavior is observed when cannabinoid receptors on POMC neurons are stimulated, resulting in a selective increase in β-endorphin but not the α-melanocyte-stimulating hormone released in the hypothalamus (Koch et al., 2015). While α-melanocyte-stimulating hormone is a direct suppressor of appetite, β-endorphin can antagonize α-melanocyte-stimulating hormone downstream signaling pathways (Mercer et al., 2013). Bilateral communication between the ARC and inter- and intrahypothalamic brain regions is important in the overall effect of ARC neurons (reviewed by (Waterson & Horvath, 2015)).

Beyond AgRP/NPY and POMC/CART, several neuropeptide pathways (ligands and receptors) and neurotransmitters have emerged with orexigenic and anorexigenic actions. Orexigenic pathways include MCH, hypocretin/orexin, galanin, and noradrenaline, while anorexigenic actions include CRH, TRH, interleukin-1B (IL-1B), urocortin, glucagon-like peptide-1 (GLP-1), oxytocin, neurotensin and serotonin (Figlewicz, 1999; Rossi & Stuber, 2018; Schwartz et al., 2000). Nutrient sensors and downstream signaling pathways also regulate feed intake, by glucose, lipids, and glucocorticoid levels (Cone, 2005). For example, dopaminergic neurons influence hedonic (reward-based) appetite control (Palmiter, 2007; Rezitis et al., 2022; Soengas et al., 2018). In contrast, rodents lacking dopamine do not eat and will eventually perish (Palmiter, 2007). A serotonergic activation stimulates POMC neurons while inhibiting AgRP/NPY, resulting in anorexigenic stimulation in mammals (Heisler et al., 2006; Rezitis et al., 2022; Xu et al., 2010). Similarly, serotonin inhibits food intake in fish (Filosa et al., 2016; Mancebo et al., 2013; Ortega et al., 2013), but in contrast to mammals, no significant effect on *npy* expression in the brain of rainbow trout was found (Mancebo et al., 2013).

1.5.2 Factors influencing appetite

Other factors beyond the neuroanatomical network influence appetite control, including temperature, stress, food availability, genetics, and metabolites (Morton et al., 2006; Rønnestad et al., 2017).

Light

Light is an important regulator of behavior and physiology, and the effects appear to be species-specific (Villamizar et al., 2011). There is a synergic effect between light, growth, and food intake in many diurnal teleosts (Berg et al., 1992; Biswas et al., 2005; Corona-Herrera et al., 2022; Fjelldal et al., 2011; Ginés et al., 2004; Hou et al., 2019; Jonassen et al., 2000; Stefansson et al., 1990; Villamizar et al., 2013; Villarreal et al., 1988). The longer days (hours of light), the larger the number of ingested food particles, which results in better feed utilization and growth (Biswas et al., 2005; Boeuf & Le Bail, 1999). The presence of light allows fish to visualize their surroundings, but light also influences their central nervous system (Peirson et al., 2009; Perez et al., 2019). As the water acts as a chromatic filter, the use of short wavelength artificial light conditions in aquaculture mimicking natural light conditions (blue and green spectrum) has positive effects on fish growth (Hou et al., 2019; Sierra-Flores et al., 2016; Takahashi et al., 2016; Villamizar et al., 2011; Villamizar et al., 2009; Villamizar et al., 2014). On the other hand, red light often caused reduced growth, low feeding activity, and poor survival. The underlying biological mechanisms are not fully understood, but it is suggested that a red light environment might act as an endocrine disruptor influencing stress coping and disturbing the circadian rhythm (Hou et al., 2019).

Rhythmicity

Biological timekeeping is important for many organisms and, in nature, the daily solar cycles of light and dark is the most predictable environmental cue to influence the circadian rhythm. Photoperiod-dependent circadian feeding pattern is observed in rats, showing increased feeding soon after the onset of darkness (Kalra et al., 1999). It is suggested that this occurs through increased release of orexigenic factors (NPY, galanin, and β-endorphin), or indirectly by suppressing anorexigenic signals. In

humans, leptin exhibits a circadian pattern, peaking at night (Sinha et al., 1996). This is in contrast to leptin expression in rodents, where leptin is secreted in a regular, pulsatile fashion approximately every 44 min (Kalra et al., 1999).

Melatonin produced in the pineal organ is the major hormonal output of the vertebrate circadian clock mechanism. Rhythmic information is given to the organism through the secretion of melatonin into the blood and cerebrospinal fluid during darkness, controlling daily and seasonal rhythms such as locomotion, osmoregulation, food intake, reproduction, and growth (Falcon et al., 2010). Melatonin is involved in appetite inhibition, but the relationship with appetite is often contradictory, depending on the species (Kulczykowska & Sánchez Vázquez, 2010; Piccinetti et al., 2010; Pinillos et al., 2001). In zebrafish, melatonin administered via water in a dose-dependent manner reduced food intake by increasing anorexigenic *leptin* and *mc4r* and by reducing the expression of orexigenic *npy* and *ghrl*, as well as by reducing the endocannabinoid system through *cb1* (Piccinetti et al., 2010). In goldfish, intracerebroventricular injection of melatonin did not affect food intake, while intraperitoneal injections significantly reduced food intake (Pinillos et al., 2001).

The photoperiod is considered the most important synchronizer of biological rhythm; but periodic feeding can also work as an entrainment (Bolliet et al., 2001; Boulos & Terman, 1980; Deota et al., 2023; Mistlberger, 1994; Sanchez-Vazquez et al., 2019; Steindal & Whitmore, 2019). A recent study in mammals showed that periodic feeding, in contrast to continuous feeding, influenced the rhythmicity of >80% of protein-coding genes indicating a systemic signal integration of a feeding-fasting cycle (Deota et al., 2023). As previously mentioned, fish larvae are visual feeders, but will adopt a daily feeding pattern that is continuous, diurnal, or preferentially at sunrise or sunset (Rønnestad et al., 2013). In goldfish, mRNA expression profiles of clock genes and appetite-controlling genes (*npy* and *orexin*) are affected by photoperiod as well as feeding (Hoskins & Volkoff, 2012). Fish may display entrainment to the feeding regime, called food-entrained oscillators, such as increased locomotor activities before a meal, suggesting an involvement of orexigenic factors in the internal biological rhythm (Delgado et al., 2017).

Other factors affecting appetite

Photoperiod and temperature are considered the most important factors influencing food intake in fish (Volkoff et al., 2009). In general, increased temperature will increase the voluntary feed intake in fish, until the temperature goes beyond their optimal species-specific temperature range (Volkoff & Rønnestad, 2020). However, few studies have investigated whether temperature affects the endocrine factors controlling fish feeding. In Atlantic cod, it has been demonstrated that *cart* may contribute to changes in food intake at different temperatures (Kehoe & Volkoff, 2008). Other factors such as water quality (e.g., salinity, turbidity, pH, dissolved nitrogen), overcrowding, tank shape, and tank background color have also been shown to affect feeding (reviewed in Volkoff et al. (2010)). Similarly, these external factors influence growth and GH levels, but the mechanisms involved are not fully understood.

2 Objective and research questions

As Atlantic salmon is a major aquaculture species, understanding the systems that control appetite and food intake is important to optimize their feeding regimes. Many factors influence the overall hunger and satiety signals in the vertebrate brain, and the antagonistic melanocortin system has become one of the best-characterized systems in appetite control. The main objective of this work was **to gain better insight into the organization, transformation, and activation of the melanocortin system in Atlantic salmon**. More specifically, the following three major research questions were identified:

Q1: How is the topographic distribution of mRNA expression of neuropeptides of the melanocortin system in the Atlantic salmon brain?

Taking into consideration that appetite is controlled by neuronal circuits in the brain, mapping various neuroendocrine cell clusters in different brain regions is key to uncovering the contribution of the melanocortin system. In this context, and based on previous studies in salmon, **Paper I** describes the topographical mRNA expression of *npy*, *agrp*, *cart*, and *pomc* in the Atlantic salmon parr brain by chromogenic *in situ* hybridization (ISH) on coronal parallel sections across the whole brain. The presence of possible intracellular coexpression of *agrp*/*npy* and *cart*/*pomc* in the NLT, similar to that in the mammalian ARC, was evaluated by dual fluorescent ISH. Based on the topographical distribution results of key neuropeptides of the melanocortin system in **Paper I**, selected genes were also assessed in the hypothalamus of endogenous-feeding Atlantic salmon by chromogenic ISH (**Paper II**).

Q2: How does the transformation from endogenous to exogenous feeding influence appetite control and energy homeostasis in Atlantic salmon?

During the endo-exo transition of feeding, the fish undergoes irreversible changes linked to metabolism and nutritional sources. Atlantic salmon was sampled during the

endogenous (1 week before pellets were introduced) and after the onset of the exogenous feeding stage (1, 2, and 3 weeks into the first-feeding period). To better understand neuronal dynamic changes, a protocol for dissecting the brain from the skull of alevin and fry stages was developed. RNA sequencing was used to evaluate the transcriptome remodeling of the Atlantic salmon brain before (1 week before pellets were introduced) and after (2 weeks into the first-feeding period) start of exogenous feeding (**Paper II**). Exogenous feeding fish (1 and 3 weeks into the first-feeding period) were sampled before and after a meal and evaluated by quantitative polymerase chain reaction (qPCR) for the mRNA expression levels of key neuropeptides in the melanocortin system (**Paper III**).

Q3: Does light influence the activation of the melanocortin system in first-feeding Atlantic salmon?

How and to what extent different light conditions during the endogenous phase affect the exogenous feeding period are not known. Light and feeding regimes may influence the internal clock, which, in turn, affects appetite dynamics and the regulatory function of the melanocortin system. Thus, Atlantic salmon embryos and alevins were incubated under three different light conditions (constant light, light: dark periodicity, and constant darkness) during development. **Paper III** aimed to describe the light effects on the mRNA expression levels of neuropeptides *npya1, npya2, agrp1, cart2a, cart2b, cart4, pomca1,* and *pomca2* (selected based on the results from **Paper I** and Kalananthan et al. (2021)) before and after a meal during the first feeding period.

3 Discussion

The hypothalamus is a key region in the neuroendocrine system with relevance to appetite control in Atlantic salmon

The neuroendocrine system in teleosts consists of the hypothalamus, pituitary, and extrahypothalamic-hypothalamic systems, such as the preoptico-hypophysial tracts, which, collectively regulate physiology and behaviour (Cerdá-Reverter & Canosa, 2009; Holmquist & Ekström, 1995). The hypothalamus is the largest diencephalic region (Cerdá-Reverter & Canosa, 2009), and contains two cell types; neuroendocrine cells that produce and secrete neurohormones into the circulation, and non-neuroendocrine neurohormone-expressing cells (neurons) that release neurotransmitters, respectively (Clarke, 2015; Yoo et al., 2021). In line with this, mammalian and fish studies using brain lesions and electric stimulations have demonstrated that the hypothalamus is a major region for appetite control (Anand & Brobeck, 1951; Demski & Knigge, 1971; Morrison et al., 1958).

Since the discovery of leptin and the application of molecular probes, the neuroendocrine regulation of energy homeostasis has been linked to the hypothalamic arcuate nucleus (ARC), a key region for appetite control (Elmquist et al., 1999; Watts et al., 2022; Zhang et al., 1994). The ARC contains two major neuronal orexigenic and anorexigenic circuits that are targeted as “entry points” to the homeostatic feeding circuits in mammals (Cone, 2005; Elmquist et al., 1999; Morton et al., 2006; Rossi & Stuber, 2018). There is consensus that the regulatory mechanism of appetite control is evolutionarily conserved in the vertebrate hypothalamus (Soengas et al., 2018), and that the teleostean NLT is the putative homolog of the mammalian ARC (Cerdá-Reverter et al., 2003b; Lin et al., 2000; Rønnestad et al., 2017; Soengas et al., 2018; Volkoff, 2019). The NLT is considered a site for the integration and release of neurotransmitters to higher-order neurons linked to neuroendocrine appetite control and feeding behavior (Rønnestad et al., 2017). For comparative identification of hypothalamic nuclei, it is important to determine whether the mRNA and protein expression patterns are evolutionarily conserved (Biran et al., 2015). **Paper I**

demonstrated that NLTv in Atlantic salmon is a key hypothalamic region for appetite control based on the topography of *agrp1, npya, cart2b,* and *pomca* mRNA expression, determined by *in situ* hybridization.

NLTv: the key hypothalamic region for appetite control in Atlantic salmon

In mammals, ARC contains opposing orexigenic and anorexigenic signals produced by AgRP/NPY and CART/POMC, respectively (Cone, 2005; Elias et al., 1998; Hahn et al., 1998; Schwartz et al., 2000). Several studies have mapped teleostean hypothalamic nuclei to their mammalian homologs (Biran et al., 2015; Fernandes et al., 2013; Herget et al., 2014; Löhr et al., 2018). In goldfish, one of the main teleost model organisms, both NPY, orexins, galanin, CRF, β-endorphin, CCK, bombesin, tachykinins, and serotonin are involved in the neuronal regulation of food intake (Lin et al., 2000). Cerdá-Reverter et al. (2003b) were the first to demonstrate that *pomc* is involved in food intake in fish. This was achieved by investigating the dose-dependent effect of administrating a synthetic melanocortin receptor agonist ([Nle^4, D-Phe^7]-α-MSH (NDP-a-MSH)) on the goldfish food intake and by demonstrating the presence of *pomc* expression in the hypothalamus by ISH.

In line with **Paper I**, several studies have confirmed the hypothalamic expression of key neuropeptides of the melanocortin system in the teleost NLT (Agulleiro et al., 2014; Akash et al., 2014; Amano et al., 2005; Cerdá-Reverter & Peter, 2003; Forlano & Cone, 2007; Jeong et al., 2018; Kojima et al., 2010; Otero-Rodino et al., 2019; Porter et al., 2017; Sakharkar et al., 2005; Shainer et al., 2017; Song et al., 2003; Zhang et al., 2012) (**Figure 5**). **Paper I** demonstrated that *npya, agrp1, cart2b,* and *pomca* mRNA-expressing cells are located in the NLTv region of Atlantic salmon, supporting previous evidence that this region and these genes are involved in appetite control as their levels respond to fed/fasted state in Atlantic salmon (Kalananthan et al., 2023; Kalananthan et al., 2021; Kalananthan et al., 2020b; Murashita et al., 2011; Murashita et al., 2009a; Tolås et al., 2021; Valen et al., 2011). In contrast to the mammalian ARC, no *agrp1/npy* colocalization in the NLT in fish has been observed,

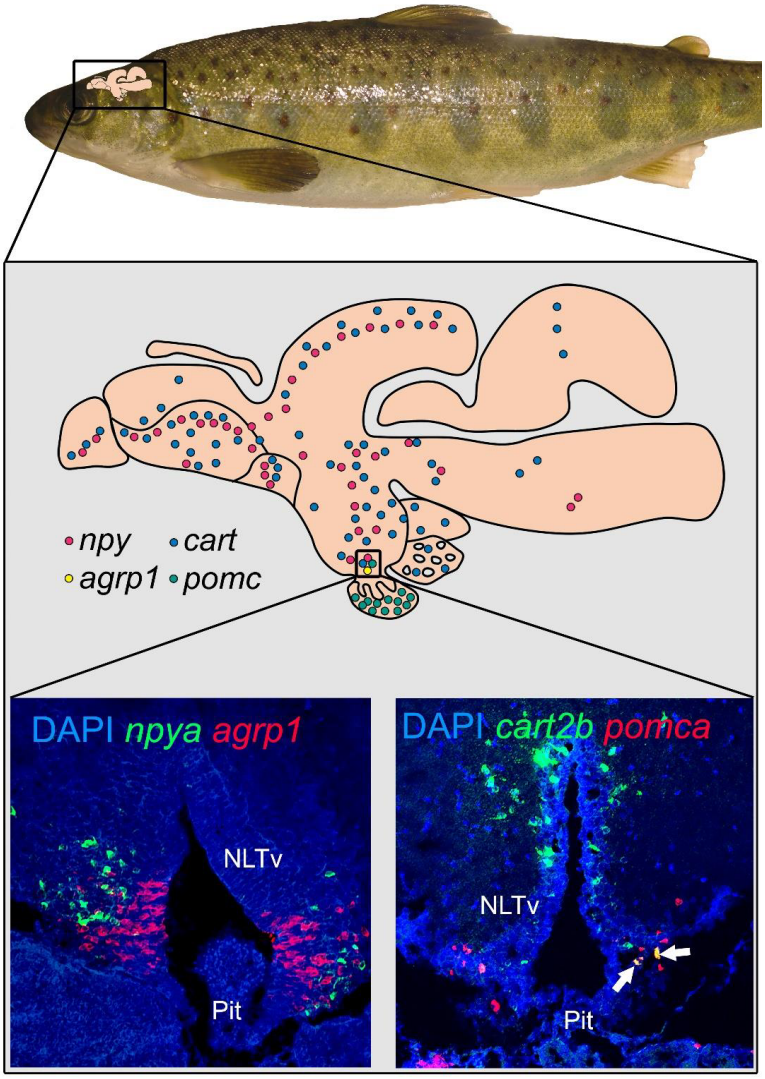

Figure 5 Top: a schematic overview of *npy* (red dots), *agrp1* (yellow dots), *cart* (blue dots), and *pomc* (green dots) mRNA expression in Atlantic salmon brain. Bottom: *npya* (TSA-green), *agrp1* (FastBlue-red), *cart2b* (TSA-green), and *pomca* (FastRed-red) mRNA are expressed in neighboring neurons of the NLTv. The absence of yellow staining indicates no coexpression between *npya* and *agrp1* mRNA. The presence of yellow staining (white arrows) indicates the coexpression of *cart2b* and *pomca* mRNA in the NLTv. Abbreviations: NLTv: ventral nucleus lateralis tuberis. Pit: pituitary. Modified from **Paper I**.

including zebrafish (Jeong et al., 2018; Shainer et al., 2019) and Atlantic salmon (**Paper I**) (**Figure 5**). These findings indicate that colocalization of *agrp1/npy* might not be needed for appetite control in teleosts. On the other hand, *cart2b* is coexpressed with *pomca* in Atlantic salmon in the NLTv (**Paper I**) (**Figure 5**). Interestingly, this coexpressing is in line with rodents, but not humans (Elias et al., 1998; Menyhert et al., 2007). Elias et al. (1998) demonstrated that >90 % of CART-expressing neurons in the ARC also expressed POMC mRNA in rodents. Menyhert et al. (2007) showed that CART is colocalized with AGRP and NPY in the human hypothalamus, but not with α-MSH, and questioned whether CART colocalization with orexigenic neuropeptides associates CART with an orexigenic role in the human brain. Indeed, double labelling immunofluorescence has shown that Npy and Cart are also colocalized in the tuberal area of catfish *Clarias batrachus* (Singru et al., 2008), and enhanced feeding behaviour has been observed when Npy and Cart are co-injected intracerebroventricularly in goldfish (Volkoff & Peter, 2000). Moreover, *cart2b* is upregulated during long-term fasting in Atlantic salmon (Kalananthan et al., 2023). Thus, to summarize, the hypothalamic NLT in Atlantic salmon is a key region for appetite control that contains neighbouring neurons expressing *npya, agrp1, cart2b,* and *pomca* mRNA, and a few *cart2b/pomca* coexpressing cells (**Paper I**).

Motivational, sensory, and motor circuits in several brain regions are directly or indirectly involved in appetite control and feeding behavior. As a consequence, several regions in the brain play a role in appetite control (Andermann & Lowell, 2017; Fischer & O'Connell, 2017; Krashes et al., 2016; Morton et al., 2006; Schwartz et al., 2000; Waterson & Horvath, 2015; Watts et al., 2022). Although the hypothalamic NLT is a key region in appetite in Atlantic salmon, other brain regions are engaged in this process, either directly or indirectly, through the melanocortin system (**Paper I**) (**Figure 5**). In fish, the hypothalamus is connected to the telencephalon (both pallium and subpallium), which is involved in multisensory processing and modulates brain activity, including reproduction, behavior, and appetite (Akash et al., 2014; Comesaña et al., 2018; Folgueira et al., 2004b; Saha et al., 2015; Singru et al., 2008; Subhedar et al., 2011; Uezono et al., 2015). The wide distribution of *npy* and *cart* in the telencephalon suggests that these neuropeptides

may be involved in sensory processing or hedonic regulation of appetite in Atlantic salmon (**Paper I**). Recently, a study in zebrafish demonstrated that dorsal telencephalic *npy* and *cart* signaling requires modulation of glutamate to generate flexibility in feeding behavior (Devika et al., 2023). Visual information modulates feeding behavior, as well as fed/fasted state influences response to external stimuli (prey capture or avoidance) (Chen et al., 2018; Corradi & Filosa, 2021; Filosa et al., 2016; Muto et al., 2017). Atlantic salmon have *npy* and *cart*-expressing neurons in the optic tectum, pretectal region, and thalamus that may be involved in (visual) signal integration and feeding status that stimulate hunger or satiety (**Paper I**). *npy* and *cart* may also interact with several neurotransmitters, including dopamine and serotonin, which may influence physiological processes, including appetite and reproduction (Filosa et al., 2016; Mancebo et al., 2013; Palmiter, 2007; Rezitis et al., 2022; Saha et al., 2015). Saha et al. (2015) showed that dopamine neurons innervate Npy-producing neurons and that superfusion with dopamine receptor agonists increased Npy-immunoreactivity. Serotonin functions as an anorexigenic factor in mammals (Heisler et al., 2006; Rezitis et al., 2022; Xu et al., 2010) and fish (Filosa et al., 2016; Mancebo et al., 2013; Ortega et al., 2013).

The ontogeny of melanocortin system in Atlantic salmon

While the main neuropeptides of the melanocortin system have been investigated in several adult fish species (Rønnestad et al., 2017; Soengas et al., 2018; Volkoff, 2016), much less is known about the ontogeny of these neuropeptides and their involvement in appetite control during fish development. It has been shown that key elements of appetite control, including *cck*, *ghrl*, *leptin*, *npy, agrp, cart,* and *pomc*, are expressed early in development as their mRNA levels are found in newly hatched Atlantic salmon (Moen et al., 2010; Sahlmann et al., 2015), giant grouper (Anderson et al., 2018), and zebrafish (Jeong et al., 2018; Mukherjee et al., 2012; Song et al., 2003; Zhang et al., 2012) or around the first-feeding period as seen in Atlantic cod (Kortner et al., 2011; Le et al., 2016; Tillner et al., 2013), Atlantic halibut (Gomes et al., 2022; Rojas-García & Rønnestad, 2002), Senegalese sole (Bonacic et al., 2016;

Navarro-Guillen et al., 2017a; Navarro-Guillen et al., 2017b), gilthead sea-bream (Koch et al., 2019), and European eel (Benini et al., 2022; Politis et al., 2018). To better understand the endo-exo transition in Atlantic salmon, fish were collected before and after the onset of feeding, and the brains were analyzed using RNA sequencing (**Paper II**). **Paper II** showed that the neuronal mRNA expression of key elements of appetite control, including key neuropeptides of the melanocortin system (*npya, agrp1, cart,* and *pomca)* are present in the brain of endogenous feeding Atlantic salmon. The early expression of these neuropeptides suggests a hard-wired activation of systems necessary for adequate functionality during the first-feeding period.

Hypothalamic mRNA levels of *agrp1* and *pomca2* have indicated the orexigenic and anorexigenic roles of these neuropeptides, respectively, in short-term fasted Atlantic salmon post-smolts (Kalananthan et al., 2020b). The topographical mRNA expression of melanocortin neuropeptides in the hypothalamus was investigated by ISH in endogenous feeding Atlantic salmon (**Paper II**). The results showed that *agrp1* and *pomca* were expressed in hypothalamic NLT, while *npya* and *cart2b* were absent in the NLT, but observed dorsally towards NAT (**Paper II**). This differs from older stages (parr) when both *npya, agrp1, cart2b*, and *pomca* are expressed in neighboring cells of the NLTv (**Paper I**). In rodents, studies on neuronal development have demonstrated that *Pomc*-positive cells are widely distributed in the immature hypothalamus, but many of these neurons will adapt to a non-POMC fate in adults (Padilla et al., 2010). Padilla et al. (2010) also demonstrated that some *Pomc*-positive cells in the embryonic rodent brain will differentiate into NPY neurons and suggested that this gradual transition is part of the hypothalamic maturation process throughout gestation. In other words, *Pomc*-expressing progenitor cells give rise to antagonistic neuronal populations that have opposing roles in appetite control (Coupe & Bouret, 2013). When the Atlantic salmon NLT will subsequently differentiate into *npya, agrp1, cart2b*, and *pomca* expressing cells, and if it is a similar process as in rodents, is unknown. However, it might take place around the first-feeding period, if following the mammalian model, since it has been shown that hypothalamic projections will form in an age-specific manner postnatally in mammals (Bouret,

2010; Bouret et al., 2004a; Coupe & Bouret, 2013). Particularly if one considers that the first feeding period in oviparous salmon mimics that of viviparous teleosts and mammals when oral food intake occurs (Iida et al., 2019).

The early expression of neuropeptides in the melanocortin system before the onset of exogenous feeding is also in line with previous studies suggesting that these neuropeptides have roles besides appetite control. *agrp1* is essential for larval growth and metabolism in zebrafish (Shainer et al., 2019; Zhang et al., 2012). In mammals, NPY and CART are abundant neuropeptides with a wide distribution in the central nervous system (Loh et al., 2015). Studies have demonstrated that *npy* and *cart* are widely distributed in the brain before the onset of exogenous feeding fish, as seen in newly hatched Atlantic cod (Le et al., 2016) and zebrafish (Mukherjee et al., 2012), suggesting an involvement of these neuropeptides in sensory brain regions. Ten *cart* paralogs have been identified in the genome of Atlantic salmon (Kalananthan et al., 2021), and those have a wide distribution in the brain (**Paper I**). The fact that some *cart* paralogs are relatively higher expressed in endogenous than in exogenous feeding Atlantic salmon, supports that *cart* also plays a functional role(s) besides appetite control during development (**Paper II**). This indicates that further studies are needed to understand the role of *cart* in salmon development.

Appetite control maturation on Atlantic salmon

When endogenous yolk nutrients are insufficient to meet the metabolic demands of a growing fish, fish need to start consuming exogenous nutrients. At the same time, to ensure a steady supply of nutrients when the yolk matter becomes depleted, the digestive system undergoes morphological, physiological, and functional changes at species-specific rates and characteristics to support this endo-exo transition (Rønnestad et al., 2013; Yúfera & Darías, 2007b; Zambonino Infante et al., 2008). When Atlantic salmon begin to ingest exogenous food, their digestive system consists of a segmented digestive tract, a differentiated liver and pancreas, and digestive enzymes and gut hormones (Moen et al., 2010; Sahlmann et al., 2015).

During the endogenous feeding period, the fish relies on the nutrients deposited within the yolk sac until it can commence exogenous feeding (Rønnestad et al., 1993). Therefore, it can be considered that fish have an energetically closed system, and the yolk supports development, growth, and metabolism. Catabolism of proteins, amino acids, and lipids stored in the yolk provides energy during development (Rønnestad et al., 1999). The yolk consumption dynamics have been described as a sigmoid curve that displays a slow absorption rate at the start, decreases faster during the late embryonic stage, and slows down at the end of the yolk sac stage (Rønnestad et al., 1992a; Rønnestad et al., 1992b; Skjærven et al., 2003). There also seems to be a selection of the nutrient-dense yolk as free amino acids are utilized first, followed by fatty acids and later amino acids derived from proteins (Kamler, 2008; Rønnestad et al., 1999). The brain of endogenous feeding Atlantic salmon can probably sense nutrient levels (glucose and essential amino acids) and this ability may differ during development or be more relevant in certain developmental windows (**Paper II**) (**Figure 6**). Nutrient sensors can activate appropriate metabolic responses that might be linked to a rudimentary system regulating overall energy expenditure and metabolism, and time-specific nutritional conditioning.

At the end of the yolk sac period, but before exogenous feeding, Atlantic salmon have relatively high expression levels of *leptin* and *cck* while having low levels of expression of their receptors (**Paper II**) (**Figure 6A**). Opposite patterns with high hormone levels and low levels of receptors were observed in exogenous feeding Atlantic salmon (**Paper II**) (**Figure 6B**). It has been demonstrated that early expression of Leptin is linked to its role in neurodevelopment in mammals, and will work as an anorexigenic factor some weeks into oral feeding (Bouret, 2010; Bouret et al., 2004a; Bouret et al., 2004b). Therefore, *leptin* may play a role in Atlantic salmon neurodevelopment, and a reduction of receptor expression causes the brain to be less sensitive to *leptin*. This will allow for a more specific response to feeding-related *leptin* secretion in the brain in exogenous-feeding fish that have utilized all the nutrients stored in the yolk sac (**Paper II**). Studies in marine larvae have shown that Cck-producing cells are present at hatching or around the first-feeding period (Kamisaka et al., 2005; Kamisaka et al., 2002; Rønnestad et al., 2007). Furthermore,

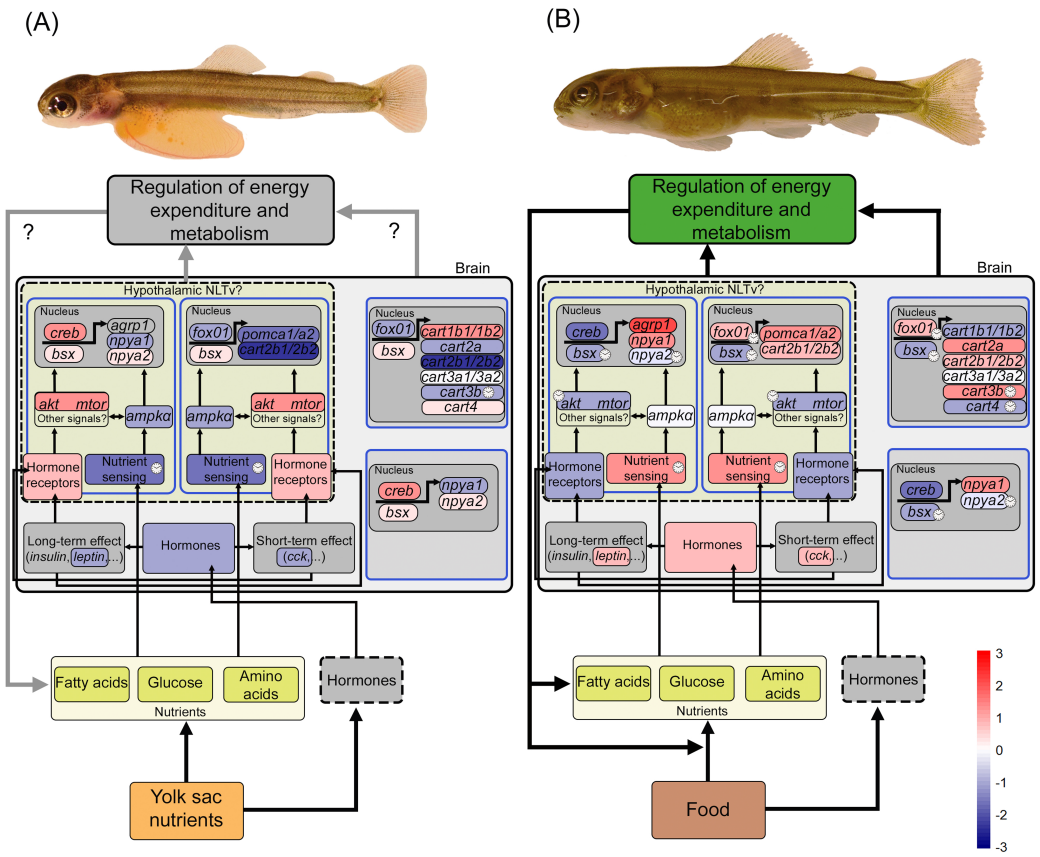

Figure 6 Schematic overview of the appetite control regulation of energy expenditure and metabolism (control the supply of nutrients) in the Atlantic salmon brain of 720 dd, during endogenous feeding (**A**) and 920 dd, during the first-feeding period (**B**). Colour codes are based on the average normalized counts in the heatmap of grouped genes related to appetite and metabolism at 720 dd and 920 dd for each age (Figure 1C in **Paper II**) and the heatmap for the expression pattern of individual appetite-related genes (Supplemental Figure 2 in **Paper II**). The clock indicates if there is a significant cyclic expression (MetaCycle, p-value < 0.05) for a gene or a gene within a group (i.e., genes that are linked to nutrient sensing) (Table 1, 2 in **Paper II**). *agrp1* was absent in the normalized count file at for 720 dd and 920 dd merged, and given no colour code at 720 dd, while at 920 dd the colour code is based on the 920 dd normalized count file. Abbreviations: agrp, agouti-related peptide; Akt, protein kinase B; AMPKα, AMP-activated protein kinase; BSX, brain homeobox transcription factor; cart, cocaine- and amphetamine-related transcript; CCK, cholecystokinin; CREB, cAMP response-element binding protein; FoxO1, forkhead box protein O1; GHRL, ghrelin; mTOR, mechanistic target of rapamycin; NLTv, lateral tuber nucleus pars ventralis; npy, neuropeptide Y; pomc, pro-opiomelanocortin. Modified from Soengas (2021) and **Paper II**.

cck does not seem to respond to fed/fasted state during the start of the first feeding period when the larvae may utilize endogenous and exogenous nutrients, in contrast to larvae that are fully dependent on exogenous nutrients (Kamisaka et al., 2013; Koven et al., 2002). Taken together, these observations suggest that the gut has a genetically pre-programmed production of *cck* that is influenced by dietary factors after the yolk is utilized in fish (Rønnestad et al., 2007). As digestive factors are present in the gut at the onset of exogenous feeding when Atlantic salmon rely on a mixed nutritional basis (endogenous and exogenous) (Sahlmann et al., 2015), it can be speculated that these factors will not respond to the feeding state until the yolk sac is fully absorbed.

The hypothalamic integration of signals includes a network of hormone receptors and a nutrient-sensing system that may alter the expression of *agrp1, npy, cart,* and *pomc* (Delgado et al., 2017; Soengas, 2021; Soengas et al., 2018). In mammals, binding of ligands to the insulin receptor activates AKT, which causes downstream mTOR activation and AMPK inhibition, and results in increased cell growth, proliferation, energy metabolism, and apoptosis inhibition (Hay, 2005; Lawlor & Alessi, 2001; Shimobayashi & Hall, 2014; Yoon, 2017). It has been demonstrated that *akt* and *mtor* are activated in fish under fed conditions and under treatments that stimulate an anabolic status (Libran-Perez et al., 2015; Soengas, 2021; Velasco et al., 2019). In endogenous feeding Atlantic salmon, higher levels of *akt/mtor* were observed compared to exogenous feeding salmon, and the opposite trend was observed for *ampk* (**Paper II**) (**Figure 6**). Therefore, higher levels of *akt/mtor* may indicate an anabolic state in the brain of endogenous feeding Atlantic salmon, and result that the fish does not experience any feeding stimuli. In contrast, the switch in nutritional strategy from endogenous to exogenous feeding is accompanied by a change towards a catabolic state in the brain of exogenous-feeding Atlantic salmon. A catabolic state in the brain can be linked to an overall orexigenic state and stimulate hunger signaling pathways(s) in the brain. As a result, first-feeding fish might be constantly hungry and motivated to eat, at least until their satiety systems are fully developed. This is in line with several reports indicating that marine fish larvae may continue to

ingest food particles even though their gut is full (Harboe et al., 2009; Rønnestad et al., 2013).

None of the melanocortin neuropeptides displayed a clear response to a meal two weeks into the first-feeding period in Atlantic salmon (**Paper II**). However, three weeks into the first-feeding period, *npya1, pomca1,* and *pomca2* displayed significant changes in mRNA expression levels in response to a meal (**Paper III**). *pomca1* and *a2* were upregulated after the meal, indicating that they are involved in satiety signaling in the brain, while the role of *npya1* in hunger or satiety signaling pathways was undetermined. This suggests that during the first-feeding period in Atlantic salmon, the brain may be insensitive to metabolic cues, or the neurons fail to send signals to other brain regions (**Paper II**). Mammalian neurodevelopment demonstrates that the brain becomes more advanced around the onset of exogenous feeding (Bouret, 2010; Bouret et al., 2004a; Coupe & Bouret, 2013; Padilla et al., 2010). There also seems to be a rhythmicity synchronization that takes place in the transition from endogenous to exogenous feeding that influences the internal clock of Atlantic salmon, since several appetite-related genes obtained a 24 h periodicity at exogenous feeding compared to endogenous feeding, probably aligning feeding to the circadian cycle (**Paper II**).

Neurons releasing npy, agrp1, cart, and pomc-derived peptides interact with their corresponding receptors on second-order neurons. agrp1 and pomc-derived peptides competitively interact with the mc4r, as silencing of second-order neurons promotes orexigenic stimulation (Krashes et al., 2016). *In silico* analyses have demonstrated that Atlantic salmon have four *mc4r* genes, named *mc4ra1, a2, b1,* and *b2,* and all the *mc4r* paralogs are expressed in the hypothalamus (Kalananthan et al., 2020a). All four receptors are present in endogenous-feeding salmon, but only *mc4ra1* and *a2* are significantly higher expressed in the brain during the transition from endogenous to exogenous feeding (**Paper II**). In rodents, MC4R is potentially involved in hypothalamic neurogenesis (Coupe & Bouret, 2013). This might be similar in salmon as *mc4r* genes are present in the brain from an early stage. *mc4r* might also play a role in the anorexigenic response by *pomca1* and *a2* peptides. It is interesting to note

that *pomca1* and *a2* mRNA levels are significantly upregulated after feeding, indicating an anorexigenic response and inhibition of food intake (**Paper III**). Overall, further studies are needed to understand the ontogeny of appetite control in teleost species.

Light conditions during early development modulate appetite and growth of exogenous feeding fry

The brain integrates all environmental inputs and controls physiological systems outputs (Johnston, 2006). The resulting changes may affect behavioral responses, growth, as well as metabolic functions. In **Paper III**, Atlantic salmon were kept under three different light conditions (DD, LD, and LL) from fertilization through the yolk sac utilization period, before they were transferred to the first feeding tanks, where they were kept under equal LD conditions throughout the first-feeding period. No significant differences in growth, i.e., standard length and myotome height, were observed between the groups. Thus, light conditions from fertilization to first feeding seem to not have a direct effect on first-feeding Atlantic salmon growth performance. RNA sequencing of whole salmon embryos and alevins has shown that transcripts of non-visual opsins, which code for light-sensing proteins with non-visual functions, are present from an early stage (255 dd) (Eilertsen et al., 2022). This early expression supports that salmon embryos and alevins can receive different light cues during endogenous feeding; however, based on **Paper III**, light conditions (DD, LD, and LL) during embryo and yolk sac stage do not influence alevin growth during the first feeding period, but effects on the overall growth performance later in life were not tested. In contrast to light conditions, temperature seems to be a major abiotic influencer on growth during embryonic development in Atlantic salmon. Atlantic salmon reared in cold water (2-5 ° C) compared to those kept at 8-10 ° C from fertilization until the eyed stage, when transferred to equal rearing conditions, demonstrated that temperature treatments during this short time period during

development were sufficient to produce changes in growth rates up until smoltification (Macqueen et al., 2008).

First-feeding Atlantic salmon kept under constant light conditions (DD and LL) during embryogenesis had less yolk than fish kept under LD at first feeding (**Paper III**). This indicated that salmon have an enhanced energy requirement during embryogenesis when kept under constant light conditions. This is in line with a previous study on Atlantic salmon reared under different light conditions (DD, LD, and LL) prior to exogenous feeding, which demonstrated increased muscle activity and use of energy in constant light conditions compared to LD (Eilertsen et al., 2022). Different light conditions during embryogenesis affected the mRNA expression profiles of neuropeptides of the melanocortin system during the first-feeding period in Atlantic salmon when they were all under the same light condition (**Paper III**). Periprandial responses were only observed in fish reared under LD from fertilization, which mimic natural light conditions. *npya1* displayed a periprandial response, but it could not be determined if *npya1* acted orexigenic or anorexigenic(**Paper III**). However, *pomca1* and *a2* displayed an anorexigenic response with significantly higher mRNA expression after feeding (**Paper III**). This indicates that the photoperiod and endogenous appetite-controlling systems for hunger and satiety are synchronized during the first-feeding period (**Papers II, III**). Furthermore, these results support the use of LD periodicity from fertilization to have a better stimulation of appetite in first-feeding fish without compromising Atlantic salmon growth (**Paper III**).

Does salmon-specific whole genome duplication improve salmon plasticity?

All teleosts share at least three whole-genome duplication rounds. In addition, there was a fourth salmonid WGD (Ss4R) that occurred approximately 80 million years ago (Allendorf & Thorgaard, 1984; Lien et al., 2016) resulting in the potential nonfunctionalization, subfunctionalization, and neofunctionalization of the duplicated genes (Lynch & Conery, 2000). There is a high capacity for plasticity in response to

environmental changes in salmonids, though the underlying mechanisms are not fully understood (Klemetsen et al., 2003). The additional genome duplication in salmonids has also impacted appetite-controlling genes and added to the complexity of understanding Atlantic salmon appetite control and feed intake.

Several *npy, cart,* and *pomc* paralogous genes have been identified in Atlantic salmon (Kalananthan et al., 2021; Murashita et al., 2011; Tolås et al., 2021; Valen et al., 2011). The fates of these duplicate genes are not yet fully understood, however, one hypothesis is that the genes have maintained a role in appetite control by facilitating physiological, sensory, or periprandial responses. **Paper I** demonstrated that these neuropeptides are expressed in brain regions known to be related to feeding and energy status, including the hypothalamus, where *npya, agrp1, cart2b,* and *pomca* are expressed in the NLT. In addition, *cart2a* and *3a* are expressed in the hypothalamus. The extrahypothalamic regions, such as the telencephalon, may also play a function in the sensory regulation of appetite (Das et al., 2019; Singru et al., 2008; Subhedar et al., 2011; Ye et al., 2020). Even though *npya* and *cart2b* may have roles besides appetite control in the brain (**Paper I**), *npya, agrp1, cart2b,* and *pomca* mRNA may be differentially expressed by short- and long-term changes in feeding strategies, light conditions, or at different developmental stages. In this way, the Ss4R may have contributed to the ability of Atlantic salmon to adapt to varying environmental conditions and feeding strategies (**Papers I, II,** and **III**) (Kalananthan et al., 2023; Kalananthan et al., 2021; Kalananthan et al., 2020a; Kalananthan et al., 2020b; Moen et al., 2010; Murashita et al., 2011; Murashita et al., 2009a; Murashita et al., 2009b; Rønnestad et al., 2010; Tolås et al., 2021; Valen et al., 2011) (**Figure 7**).

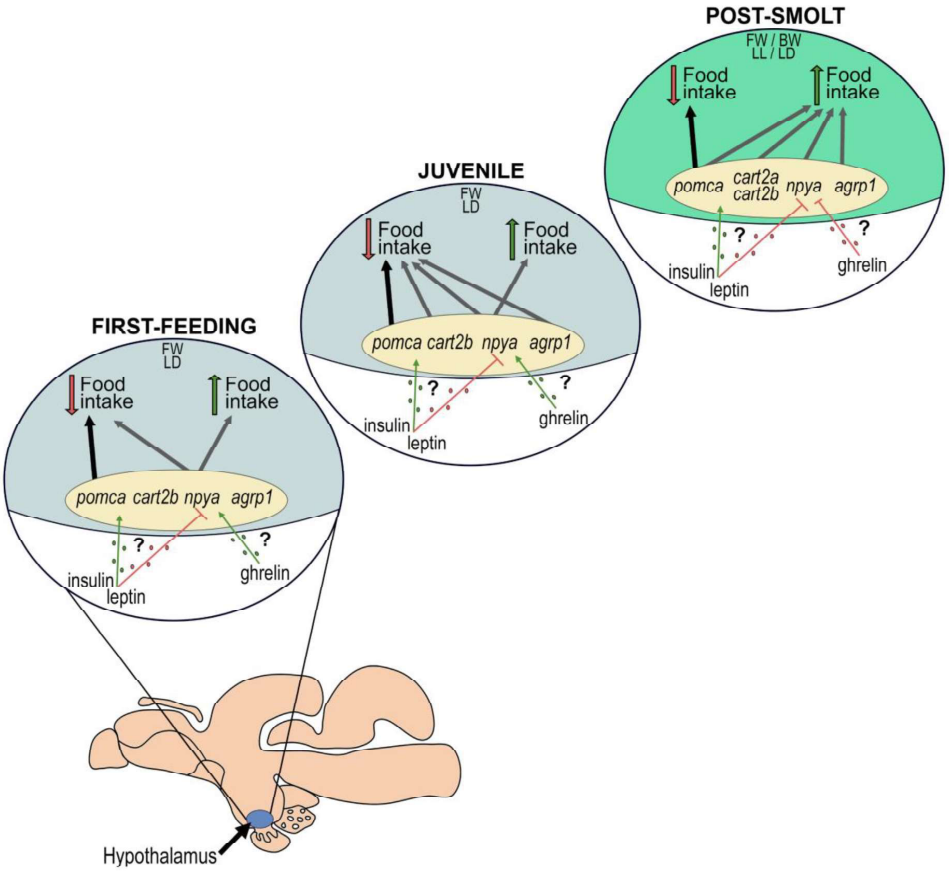

Figure 7 Prandial responses and overall role for the key neuropeptides in the melanocortin system during different developmental stages (first feeding/juvenile / post-smolt) and environmental settings for Atlantic salmon based on the literature (**Papers I, II,** and **III**) (Kalananthan et al., 2023; Kalananthan et al., 2021; Kalananthan et al., 2020a; Kalananthan et al., 2020b; Moen et al., 2010; Murashita et al., 2011; Murashita et al., 2009a; Murashita et al., 2009b; Rønnestad et al., 2010; Tolås et al., 2021; Valen et al., 2011). Dark arrows indicate a maintained anorexigenic role for *pomca* in all developmental stages. Grey arrows indicate possible plasticity of mRNA expression of neuropeptides in response to feeding status. Abbreviations: *agrp1*; agouti-related peptide 1, BW; brackish water, *cart2b*; cocaine-amphetamine regulated transcript 2b1 and 2b2, FW; freshwater, LD; light: dark periodicity, LL; constant light, *npya*; neuropeptide y a1 and a2, *pomca*; pro-opiomelanocortin a1 and a2.

Atlantic salmon *npya1* and *a2* are the results of Ss4R while *npyb* is a teleost duplicate (Tolås et al., 2021). Phylogenetic analyses showed that salmonids have two *npya* genes, while most teleost species only possess one *npya* gene. In the Atlantic salmon brain, *npya1* is more abundant than *npya2* (**Papers II** and **III**) (Tolås et al., 2021). During the first feeding period, *npya1* demonstrated a response to feeding in fish kept under LD (**Paper III**). However, the salmon were fed twice a day, and sampled before and after the first meal of the day. A significant decrease in *npya1* mRNA expression observed was observed between 3 h and 6 h after the first meal of the day, which was equivalent to 4 h and 1 h before the second meal of the day. Therefore, it was uncertain if the observed change in mRNA was a postprandial response to the first meal or a preprandial response to the second meal of the day. In addition, *npya2* did not display a significant periprandial response (**Paper III**). Interestingly, *npya2* shifted from a no significant cycling during endogenous feeding towards a 24 h periodic mRNA expression in exogenous feeding fish with a peak, acrophase, at 06:00 in the morning during the first-feeding period in Atlantic salmon kept under LD (**Paper II**). This indicates that *npya2* obtains a daily variation during the onset of exogenous feeding that may have a rhythmic function in salmon under LD. This is in contrast to goldfish displaying significant daily variations of *npy* when kept under LL with a peak before feeding, but no significant variation was observed for *npy* in LD, indicating that photoperiod affects internal daily variations (Hoskins & Volkoff, 2012). In older stages, the available data suggest that *npya* displays a bidirectional role in Atlantic salmon – anorexigenic after a meal, but with an orexigenic trend after 6 days of fasting in parr (Murashita et al., 2009a; Valen et al., 2011). Valen et al. (2011) demonstrated a significant anorexigenic response of *npya* in the brain after a meal in Atlantic salmon parr kept under 12:12 LD light condition. Recently, specific primers for *npya1* and *a2* demonstrated a region-specific decrease in the olfactory bulb of *npya1* and increased *npya2* expression in the midbrain followed by 4 days of fasting in Atlantic salmon post-smolt kept under LL (Tolås et al., 2021). No significant responses were, however, observed for either of these paralogs in the hypothalamus at this stage. In contrast, *npya1* displayed an orexigenic response in the

hypothalamus during long-term starvation in Atlantic salmon post-smolt kept under LD (Kalananthan et al., 2023).

In first feeding salmon kept under LD, there was a significant postprandial response for the paralogs *poma1* and *a2* (**Paper III**). This is in line with what was observed in older Atlantic salmon (juveniles, 44.7 ± 2.1 g) kept under LD for *pomca1*, while no response was observed for *pomca2* (Valen et al., 2011). Atlantic salmon post-smolt kept under LL displayed no significant changes for any of the *pomc* in the brain after 3-4 days of fasting (Kalananthan et al., 2020a; Kalananthan et al., 2020b), while both *pomca1* and *a2* were significantly upregulated in the hypothalamus after long-term fasting (Kalananthan et al., 2023). This suggests that *pomca* has maintained its anorexigenic role in Atlantic salmon, particularly in short-term responses to a meal and when the light conditions mimic natural light (**Paper III**) (Valen et al., 2011). However, long-term fasting may halt and counteract hunger signals to save energy during catabolic conditions (Kalananthan et al., 2023) (**Figure 7**).

4 Concluding remarks

This work has described the topographical distribution and investigated the maturation and activation of key neuropeptides of the melanocortin system in Atlantic salmon. The key conclusions are:

- The paralogs of *npy, agrp, cart,* and *pomc* analyzed were widely distributed in the brain, especially in sensory and neuroendocrine brain regions.
- The data supports that NLT is the putative homolog to the mammalian ARC with the expression of *npya, agrp1, cart2b,* and *pomca* in neighboring neurons in Atlantic salmon.
- A few neurons coexpress *cart2b* and *pomca* in the NLT. This is the first study demonstrating *cart2b/pomca* coexpression in the NLT of a teleost.
- Several of the genes potentially involved in the appetite-controlling systems and energy metabolism are present in the Atlantic salmon brain before the onset of exogenous feeding, underlining a pre-programmed expression of appetite and energy metabolism.
- During the transition from endogenous to exogenous feeding, there is a major whole-transcriptome remodeling in the brain including the expression levels of appetite-controlling genes.
- The brain of exogenous-feeding Atlantic salmon is characterized by an overall orexigenic state that may stimulate constant hunger, which includes nutrient sensors and signaling pathways, and this suggested that the fish is motivated to ingest food.
- A satiety system involving the melanocortin system seems to be functional approximately three weeks into the first-feeding period in Atlantic salmon.
- *npya1, pomca1,* and *pomca2* are the first neuropeptides of the melanocortin system that display a significant periprandial response.

- Light during embryogenesis has a significant effect on the mRNA level of *npya1, pomca1,* and *pomca2* in the brain during the first feeding period, and light conditions that mimic natural light better stimulate appetite in Atlantic salmon.

5 Future perspectives

This work has covered functional genomics (quantitative/qualitative gene expression) and developmental biology in Atlantic salmon. The use of genomic resources in Atlantic salmon research has opened up for studies on paralog-specific functions. Hence, this work provides key observations on appetite control in fish. Future studies may consider:

- Effect of light on appetite: **Paper III** demonstrated that light conditions during development have a significant effect on mRNA levels in the brain during the first-feeding window. This could be followed up by transcriptional analyses of long-term experiments (from fertilization until parr, smolt, or post-smolts) to investigate if the light effects persisted in older stages. Future studies should also investigate the role of gut hormones to understand some of the pathways in the gut-brain axis.
- Population differences: The topography, transformation of endogenous to exogenous feeding, and the periprandial activation of key neuropeptides in Atlantic salmon are based on 16 or 17 generations of domestication and selective breeding of farmed Atlantic salmon (**Papers I, II, III**). Domesticated and selective breeding of salmon is a fish that is genetically and phenotypically different from wild salmon populations (Houston & Macqueen, 2019). Differences between farmed and wild Atlantic salmon may suggest adaptations to different niches in terms of food availability and appetite control.
- Rhythmicity of genes: Light and feeding regimes may influence the internal clock, which may in turn impact appetite dynamics (**Papers II, III**). Time-restricted feeding has pleiotropic health benefits in mammals, including increased rhythmicity and improved metabolic flexibility (phases of anabolic and catabolic genes) (Deota et al., 2023). In this context, could be of interest to investigate time-restricted feeding versus continuous feeding transcriptional changes in salmon to understand the impact of feeding regimes on the biology of salmon.

- Translational studies: **Papers I, II, and III** are functional genomics (mRNA levels) studies but may not reflect the same trend as the active form of the protein of a specific gene. Future studies may combine mRNA levels and protein levels to improve our understanding of the salmonid neuroendocrine systems.

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Received: 24 June 2022 | Revised: 29 August 2022 | Accepted: 1 September 2022

DOI: 10.1002/cne.25415

RESEARCH ARTICLE

JCN RESEARCH IN SYSTEMS NEUROSCIENCE THE JOURNAL OF COMPARATIVE NEUROLOGY WILEY

Mapping key neuropeptides involved in the melanocortin system in Atlantic salmon (*Salmo salar*) brain

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Funding information
Regional Research Fund West, Grant/Award Number: 259183; Norges Forskningsråd, Grant/Award Numbers: 254894, 261753, 267626, 315106, 317770

Abstract

The melanocortin system is a key regulator of appetite and food intake in vertebrates. This system includes the neuropeptides neuropeptide y (NPY), agouti-related peptide (AGRP), cocaine- and amphetamine-regulated transcript (CART), and pro-opiomelanocortin (POMC). An important center for appetite control in mammals is the hypothalamic arcuate nucleus, with neurons that coexpress either the orexigenic NPY/AGRP or the anorexigenic CART/POMC neuropeptides. In ray-finned fishes, such a center is less characterized. The Atlantic salmon (*Salmo salar*) has multiple genes of these neuropeptides due to whole-genome duplication events. To better understand the potential involvement of the melanocortin system in appetite and food intake control, we have mapped the mRNA expression of *npy*, *agrp*, *cart*, and *pomc* in the brain of Atlantic salmon parr using in situ hybridization. After identifying hypothalamic mRNA expression, we investigated the possible intracellular coexpression of *npy/agrp* and *cart/pomc* in the tuberal hypothalamus by fluorescent in situ hybridization. The results showed that the neuropeptides were widely distributed, especially in sensory and neuroendocrine brain regions. In the hypothalamic lateral tuberal nucleus, the putative homolog to the mammalian arcuate nucleus, *npya*, *agrp1*, *cart2b*, and *pomca* were predominantly localized in distinct neurons; however, some neurons

Abbreviations: CC, cerebellar corpus; Ce, nucleus centralis lobi inferioris hypothalami; Cho, optic chiasm; Cp, commissura posterior; D, dorsal telencephalon; Dc, central zone of dorsal telencephalon; Dd, dorsal zone of dorsal telencephalon; Dl-d, dorsal part of lateral zone of dorsal telencephalon; Dl-v, ventral part of lateral zone of dorsal telencephalon; Dm, medial zone of dorsal telencephalon (dorsal pallium); DTN, dorsal tegmental nucleus; ent, nucleus entopeduncularis; EW, Edinger–Westphal nucleus; FLM, medial longitudinal fasciculus; fMth, fiber of Mauthner cell; Ggl, stratum ganglionare (cerebellum); Gran, stratum granulare (cerebellum); Hab, habenula; inf, infundibulum; Lcoer, locus coeruleus; Lih, inferior hypothalamic lobe; Mc, layer of mitral cells (bulbi olfactori); Mcba, tractus mesencephalo-cerebellaris anterior; Mfb, medial forebrain bundle; mol, stratum moleculare (cerebellum); NAT, nucleus anterior tuberis; NDlLm, medial part of the diffuse nucleus of inferior lobe; nIII, nucleus oculomotorius; NLT, nucleus lateralis tuberis; NLTa, anterior nucleus lateralis tuberis; NLTp, posterior nucleus lateralis tuberis; NLTv, ventral nucleus lateralis tuberis; NLV, nucleus lateralis valvulae; NMFL, nucleus medial longitudinal fasciculus; NMH, nucleus magnocellularis hypothalami; NPP, nucleus posterioris periventricularis; NPT, nucleus posterior tuberis; Nrl, nucleus recessi lateralis; nV, nervi trigemini; nVm, nucleus motorius nervi trigemini; OT, Optic tectum; Pit, pituitary; Ppa, preoptic area—anterior parvocellular preoptic nucleus; Ppp, preoptic area—posterior parvocellular preoptic nucleus; Psp, nucleus pretectal superficialis magnocellularis; Pt, posterior tuberculum; PTN, nucleus posterior tuberis; PVO, paraventricular organ; Retm, formatio reticularis pars medialis; Rets, formatio reticularis pars superior; RF, reticular formation; rl, recessi lateralis; Rpo, recessus preopticus; SAC, stratum album centrale (tecti mesencephali); SGC, stratum griseum centrale (tecti mesencephali); SM, stratum marginale (tecti mesencephali); SO, stratum opticum (tecti mesencephali); SOC, supraoptic/suprachiasmatic nucleus; SPV, stratum periventriculare (tecti mesencephali); Stgr, stratum granulare (bulbi olfactori); SV, saccus vasculosus; Tbc, tractus tecto-bulbaris cruciatus; Thd, dorsal thalamus; Thv, ventral thalamus; TL, Torus longitudinalis; Tlat, torus lateralis; TLw, white matter region of torus longitudinalis; Tod, tractus opticus dorsalis; TolI, tractus olfactorius lateralis; TS, Torus semicircularis; Valv, Valvula cerebelli; Vd, dorsal nucleus of ventral telencephalon; Ve4, fourth ventricle (rhombencephali); vHab, ventral habenula; Vl, lateral nucleus of ventral telencephalon; Vv, ventral nucleus of ventral telencephalon.

coexpressed *cart2b/pomca*. This is the first demonstration of coexpression of *cart2b/pomca* in the tuberal hypothalamus of a teleost. Collectively, our data suggest that the lateral tuberal nucleus is the center for appetite control in salmon, similar to that of mammals. Extrahypothalamic brain regions might also be involved in regulating food intake, including the olfactory bulb, telencephalon, midbrain, and hindbrain.

KEYWORDS
Agrp, appetite, Atlantic salmon, Cart, hypothalamus, Npy, Pomc

1 | INTRODUCTION

In fish, like in other vertebrates, appetite and food intake are controlled by endocrine signals and neuropeptides released in neural pathways in the brain (Comesaña et al., 2018; Rønnestad et al., 2017; Soengas et al., 2018; Volkoff, 2016). The neuronal network receives continuous feedback from peripheral tissues, especially the gastrointestinal tract, liver, and pancreas, where nutrients and endocrine and neuronal signals interact to regulate food intake and energy balance (Rønnestad et al., 2017). Food intake is also controlled by sensory and hedonic inputs, such as liking and wanting, that drive hunger and satiety. These inputs originate from a motivation/reward center with dopaminergic neurons (Palmiter, 2007; Soengas et al., 2018); the neurons assist in the underlying mechanism of food intake through conditioning, chemosensory stimulation from the smell of food (Rossi & Stuber, 2018), or nutrient sensing in the brain (Comesaña et al., 2018).

The melanocortin system is a key player in neuronal appetite control. In mammals, it is well characterized and comprises two major neuronal circuits in the arcuate nucleus of the hypothalamus (Elias et al., 1998; Hahn et al., 1998; Schwartz et al., 2000). These neurons are known to either stimulate (orexigenic) or inhibit (anorexigenic) appetite. Both orexigenic and anorexigenic neurons are competitively interacting with the melanocortin receptors (Nuzzaci et al., 2015). Coexpression of neuropeptide y (NPY) and agouti-regulated peptide (AGRP) increases orexigenic activity resulting in an anabolic response. In contrast, neurons coexpressing cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) act as anorexigenic, providing a catabolic response.

The key neuropeptides involved in the melanocortin system described in mammals have also been identified in teleosts (Delgado et al., 2017; Rønnestad et al., 2017; Soengas et al., 2018; Volkoff, 2016; Volkoff et al., 2005). However, teleosts typically possess multiple paralogs of these genes compared to mammals due to the additional teleost-specific whole-genome duplication, and some teleost families, like salmonids, have additional copies because of the salmonid-specific fourth round of whole-genome duplication event (Allendorf & Thorgaard, 1984; Lien et al., 2016). For most of these paralogs, their effects on appetite and food intake control remain unclear.

NPY is a potent and abundant orexigenic factor in the brain and plays a key role in energy homeostasis and food intake in mammals (Loh et al., 2015), as well as in several teleosts (Volkoff, 2016). Earlier studies on Atlantic salmon, *Salmo salar*, supported the involvement of *npy* in food intake control (Murashita et al., 2009; Valen et al., 2011). Recently, three *npy* paralogs have been identified in the Atlantic salmon, named *npya1*, *npya2*, and *npyb* (Tolås et al., 2021). Tolås et al. (2021) showed that neither of the *npy* paralogs was significantly affected by feeding status in the hypothalamus, albeit a trend of increased *npya2* mRNA expression following 4 days of fasting was observed. AGRP is also a key player in the orexigenic melanocortic pathway (Morton & Schwartz, 2001). In Atlantic salmon, Murashita et al. (2009) identified two Agouti-like sequences, named *agrp1* and *agrp2* (also named *asip2b*, see Braasch and Postlethwait (2011) and NCBI GenBank[1]). The orexigenic effect of Atlantic salmon *agrp1* seems to be in line with those reported in mammals (Kalananthan, Murashita, et al., 2020). However, *agrp2* seems to not be directly involved in appetite control in Atlantic salmon (Kalananthan, Lai, et al., 2020) but may play other functional roles, as demonstrated in the zebrafish, *Danio rerio* (Shainer et al., 2017, 2019; Zhang et al., 2010).

CART is a neuropeptide involved in several processes in the brain, including appetite control. Mammals have one *cart* gene that plays an anorexigenic role (Akash et al., 2014). However, there are 10 *cart* paralogues in Atlantic salmon with varying and differential expressions in different brain regions, and their full physiological function(s) are not fully established (Kalananthan et al., 2021). POMC is a precursor peptide that is post-translationally cleaved into several peptides with a wide range of functions, including α- and β-melanocyte-stimulating hormones, adrenocorticotropic hormone, and β-endorphin (Takahashi & Mizusawa, 2013). Three *pomc* paralogs (*pomca1*, *pomca2*, and *pomcb*) have been previously identified in Atlantic salmon (Murashita et al., 2011; Valen et al., 2011) and are primarily expressed in the pituitary and hypothalamus (Kalananthan, Lai, et al., 2020).

The topology of central neuropeptides of the melanocortin system has been mapped in the whole brain or in specific brain regions in several teleost species, including catfish *Clarias batrachus* (Gaikwad et al., 2004; Singru et al., 2008; Subhedar et al., 2011), Indian major carp *Cirrhinus cirrhosus* (Saha et al., 2015), goldfish *Carassius auratus* (Cerdá-Reverter & Peter, 2003; Cerdá-Reverter, Schiöth, et al., 2003; Kojima et al., 2010; Matsuda et al., 2009), zebrafish (Akash et al., 2014; Forlano & Cone, 2007; Jeong et al., 2018; Kaniganti et al., 2021; Koch et al., 2019; Mukherjee et al., 2012; Shainer et al., 2017, 2019), sea

bass *Dicentrarchus labrax* (Agulleiro et al., 2014; Cerdá-Reverter et al., 2000), Atlantic cod *Gadus morhua* (Le et al., 2016), and the African cichlid fish *Astatotilapia burtoni* (Porter et al., 2017). However, to our knowledge, coexpression of *npy/agrp* and *cart/pomc* in the hypothalamus has never been observed in a teleost species. In salmonids, *pomc* and *agrp* have been identified in the hypothalamus of rainbow trout *Oncorhynchus mykiss* (Otero-Rodino et al., 2019). Npy expression has been documented in the brown trout *Salmo trutta fario* brain including the dorsal and ventral telencephalon, habenula, periventricular and tuberal hypothalamus, saccus vasculosus, tectum, tegmentum, and the rhombencephalon (Castro et al., 1999). In Atlantic salmon and Gambusia affinis brain, Npy expression was found in the ventral telencephalon, tectum, tegmentum, and rhombencephalon (Garcia-Fernandez et al., 1992). However, the spatial distribution of these melanocortin neuropeptides has not been fully explored in the whole brain of salmonids. Atlantic salmon is an important aquaculture species and understanding the systems that control appetite and food intake is central to optimize their feeding regimes. Taking into consideration that appetite is controlled by neuronal circuits in the brain, mapping the various neuroendocrine cell clusters in the different brain regions is key to uncover the melanocortin system contribution.

In this study, we have described the mRNA expression of *npy*, *agrp*, *cart*, and *pomc* genes in the Atlantic salmon parr brain by in situ hybridization (ISH). Next, to identify potential key neural circuits involved in appetite control, we investigated the possible coexpression of putative anorexigenic and orexigenic neuropeptides in the Atlantic salmon tuberal hypothalamus.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The Atlantic salmon were obtained from the Industrial and Aquatic Laboratory (Bergen High Technology Center, Norway) which has all the necessary approvals for running trials on fish. Atlantic salmon were reared following the Norwegian Veterinary Authorities' standard protocols. The fish did not undergo any treatment or handling except for euthanasia; thus, special approval from the food authorities and ethics committee was deemed unnecessary according to Norwegian National legislation via the Norwegian Animal Welfare Act (LOV-2015-06-09-16-65) and Regulations on the Use of Animals in Experiments (FOR-2017-04-05- 451), as required in the European Union (Directive 2010/63/EU) for animal experiments. All fish used were euthanized with an overdose of MS-222 (MS-222™; MSD Animal Health, the Netherlands) on site, before further handling.

2.2 | Sampling

For RNA extraction and cloning, the brain and pituitary were dissected from one Atlantic salmon (weight = 900 g, standard length = 38.5 cm), stored in RNAlater (Invitrogen, Carlsbad, USA) at 4°C overnight, and then transferred to −80°C. For ISH, 18 Atlantic salmon parr (weight = 33.7 ± 3.5 g, standard length = 14.1 ± 0.5 cm) were killed with an overdose of 200 mg/L MS-222. An incision was made mid-ventral to expose the heart for whole-animal perfusion fixation with 4% paraformaldehyde (PF) in phosphate-buffered saline (PBS) pH 7.4 (4% PF). Thereafter, brains were carefully dissected out of the skull and post-fixed in 4% PF for 48 h, rinsed in 1× PBS, and immersed in 25% sucrose/25% OCT (CellPath, UK) for 24 h as described in Eilertsen et al. (2021). The brains were embedded in 100% OCT and coronal parallel cryosectioned across the entire extent of the brain at 10 μm using Leica CM 3050s cryostat (Leica Biosystems, Germany) and collected on SuperFrost Ultra Plus glasses (Menzel Glaser, Germany). Sections were dried at 65°C for 30 min and stored at −20°C until analyzed by ISH.

2.3 | RNA extraction and cDNA synthesis

Total RNA was isolated from both Atlantic salmon brain and pituitary using TRI reagent (MilliporeSigma, St. Louis, USA) following the manufacturer's instructions, and further treated with TURBO DNA-free (ThermoFisher Scientific, Indianapolis, USA). First-strand cDNA was synthesized from 1.5 μg of DNase-treated total RNA using oligo(dT)20 primer from SuperScript III First-Strand Synthesis system for RT-PCR kit (ThermoFisher Scientific).

2.4 | Molecular cloning

Primer design was done in ApE-A plasmid editor (http://biologylabs.utah.edu/jorgensen/wayned/ape/, RRID: SCR_014266). Primers and product sizes are listed in Table 1. Atlantic salmon *npya*, *npyb*, *agrp1*, *agrp2*, *pomca*, *pomcb*, *cart1b*, and *cart2b* amplification was performed with Advantage 2 PCR kit (Clontech, Mountain View, CA, USA) using Advantage SA buffer. PCR amplification was performed using a BIO-RAD C1000 Touch Thermal Cycler (Bio-Rad, Germany) with an initial step of 95°C for 3 min, and 34 cycles of 30 s denaturation at 95°C, 30 s annealing at 58–60°C, and 1 min extension at 68°C ending with a final extension at 68°C for 10 min.

cart1a, *cart3a*, *cart3b*, and *cart4* were amplified with Q5 High Fidelity 2X polymerase (New England Biolabs, Ipswich, MA, USA) using the following conditions: 98°C for 30 s; 34 cycles of 98°C for 10 s, 60°C for 20 s, 72°C for 30 s; and a final step at 72°C for 2 min.

PCR amplicons were purified from agarose gel using the MinElute Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol before being cloned into a StrataClone PCR cloning vector (Agilent Technologies, CA, USA). The products were sequenced at the University of Bergen Sequencing Facility using BigDye protocol (BigDye™ Terminator v3.1, ThermoFisher Scientific). Sequence identity was confirmed in Vector NTI software to ensure 100% sequence identity with the public available sequences.

TABLE 1 List of primers used for molecular cloning of genes involved in the melanocortin system in Atlantic salmon

Target gene	Accession number	Probe length (bp)	Primer sequence (5′–3′)
npya	*npya1* (**NM_001146681**)	359	F: GCCTGAGGACAACTTCTATC
	npya2 (XM_014178359)		R: GACACTATTACCACAACGACG
npyb	*npyb* (**XM_045697117** and XM_014184208)	423	F: GCGAGCACAGAACAGTCATTC
			R: GTGGTGTTGTGACAAACAGGC
agrp1	*agrp1* (**NM_001146677**)	612	F: GAAGCGCTTTGTTGCATCAGC
			R: GTACACCCAACGTAACATCCATC
	T3 and T7 primers for agrp1 probe		T3: CATTAACCCTCACTAAAGGGAAGAAGCGCTTTGTTGCATCAGC
			T7: TAATACGACTCACTATAGGGCTATAGGCCCCACCTCATGGA
agrp2/asip2b	*agrp2* (**NM_001146678**)	479	F: GAGCGAGAACATTCTGAGCTG
			R: GTCTAGGTCTTCTTGGGGCAG
cart1a	*cart1a* (**XM_014149393**)	482	F: CGTATAAAACCTTGGTCCAGG
			R: CATACAACATTGAGTCATCCCG
cart1b	*cart1b1* (**XM_014150559**)	618	F: CTGTATCTCCATCCCTTCTG
	cart1b2 (XM_014151634)		R: GACAACAAACCCTCCATTAC
cart2a	*cart2a* (**ENSSSAG00000015472**)	894	F: ATGGAGAGCTCTAAACTGTGGA
			R: CACAAGCACTTCAACAGAAAGAAG
cart2b	*cart2b1* (NM_001146680)	567	F: CGGGACCTTTTGGAGACGAAA
	cart2b2 (**XM_014183838**)		R: TGGGGTTTGGACAATCTCTCAG
cart3a	*cart3a1* (XM_014177116)	585	F: GAACTGCAAATTAGAGAGGGAG
	cart3a2 (**NM_001141227**)		R: TCAAGACAGTCATACATGCAG
cart3b	*cart3b* (**XM_014127320**)	389	F: CATTGGGAAGCTCTGTGAC
			R: GCTGTAAATGCTTTCTGGG
cart4	*cart4* (**XM_014141614**)	811	F: GCCTACAGCTTGTGTCAACC
			R: GACGTACTGGGAAAGTGTTCAT
pomca	*pomca1* (NM_001198575)	689	F: GTTCTGACCTCACCGCCAAA
	pomca2 (**NM_001198576**)		R: GAGCTAACTGGCTCTAAGTCCT
pomcb	*pomcb* (**NM_001128604**)	624	F: AGGTAGTCCCCAGAACCCTC
			R: CAGTACGGTTCTCCGCTTCTT

Note: Accession numbers from GenBank or Ensembl are provided for the different target genes. In bold are the genes on which the probe synthesis was based.

2.5 | Riboprobe

The cloned inserts were used to synthesize antisense and sense (control) digoxigenin (DIG)-labeled (MilliporeSigma) and fluorescein (FITC)-labeled (Roche Diagnostics, Germany) riboprobes. Synthesis was carried out as described in Thisse and Thisse (2008) using PCR products with added T3 (5′-CATTAACCCTCACTAAAGGGAA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) on the forward and reverse cloning primers for sense and anti-sense riboprobes, respectively (Table 1). The one exception was the T7 primer for *agrp1* (5′-TAATACGACTCACTATAGGGCTATAGGCCCCACCTCATGGA-3′). The RNA probes were precipitated using 4 M LiCl, 1 μg/μl tRNA (Roche Diagnostics), and 100% EtOH. When two paralogue genes shared a high sequence identity of the targeted region (> 92% identity level), one template was used in the riboprobe synthesis as the probe will label both genes. Note that for the paralogs of *pomca*, the overall sequence similarity of the target region was 81%; however, large fragments of the probe will hybridize to both genes (100% identity between 240–543 bp and 613–928 bp).

2.6 | In situ hybridization

ISH was carried out using a modified protocol from Sandbakken et al. (2012) and replacing 50% deionized formamide with 4 M urea. In summary, sections were dried at room temperature for 30 min, and then at 65°C for 30 min before being rehydrated using an ethanol series (95%–50%). Afterward, sections were permeabilized with 10 μg/ml proteinase K (MilliporeSigma), post-fixed in 4% PF, and treated with 0.25% acetic anhydride (MilliporeSigma) in 0.1 M triethanolamine (MilliporeSigma), ending with dehydration using an ethanol series (50%–100%).

TABLE 2 Summary of the mRNA expression of *npy*, *agrp*, *cart*, and *pomc* in the Atlantic salmon brain

Gene	Telencephalic regions	Diencephalic regions	Pituitary	Midbrain	Hindbrain
npya	MC, Vd, Vl	Ppa, SOC, vHab, Thv, Thd, Pt, NAT, NLTa, NLTv, NMH, Lih NLTp/NPT		SPV, SGC, EW*	FLM*, nV*
npyb	Vd, Vl	Thd		FLM*	
agrp1		NLTv, NLTp/NPT			
agrp2	Dm, Dc, Dl-v	Thv			nV*, RF*
cart1a				NFLM*, nIII*	
cart1b				NFLM*	
cart2a	Vv,	Tod, Thd, PTN		SPV, SAC, SO	
cart2b	Stgr, Vv, Vd, Vl, Dm, Dl, Dd	Ppp, NAT, NLTa, SPV, TS, Tlat, Ce, NLTv, NMH, Lih, PVO, PRN, NRL, NDILm, SV		Gran, lcoer, Rets	
cart3a	Vv, Ent	SOC, Thd, NMH/PVO		TLw, EW*	FLM*, lcoer*, nV*
cart3b		Ppp, Thd		tlat, TS	FLM*, nV*, rets*
cart4		ppp			
pomca		NLTa, NLTv, NLTp/NPT	pit		
pomcb			pit		

*Cells expressing this gene near the indicated brain region. See Section 3 more details.

The hybridization was carried out with a DIG-labeled RNA probe overnight at 65°C. After hybridization, sections were washed and treated with RNase A (0.02 mg/ml, MilliporeSigma) before being incubated with sheep polyclonal anti-DIG antibody (anti-digoxigenin-alkaline phosphatase FAB-fragment, 1:2000, cat. # 11093274910, Roche Diagnostics, RRID: AB_514497) in 1× blocking solution (MilliporeSigma) overnight at room temperature. The result was visualized using 4-Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate system (NBT/BCIP Ready-to-use tablets, MilliporeSigma). Parallel sections were Nissl stained with cresyl fast violet (Chroma-Gesellschaft, Germany). Sections were rehydrated in an ethanol series (96%–50%), dipped in staining solution (0.35% cresyl violet), differentiated in 70% ethanol, and dehydrated in 100% ethanol (2 × 5 min) ending with clearing in xylene. For all genes, sense probes were applied as a control for nonspecific staining.

2.7 | Double labeling fluorescence ISH

To investigate the coexpression of *npy* and *agrp* or *cart* and *pomc* in the tuberal hypothalamus, fluorescence double labeling ISH was done as described in Eilertsen et al. (2018), and replacing 50% deionized formamide with 4 M urea. DIG-labeled riboprobes were incubated with sheep polyclonal anti-DIG antibody (anti-digoxigenin-alkaline phosphatase FAB-fragment, 1:2000, cat. # 11093274910, Roche Diagnostics, RRID: AB_514497) and detected with either Fast Red tablet (Roche Diagnostics) dissolved in 0.1 M Tris-HCl pH 8.2 and 0.1% Tween-20 or with 100 mg/ml Fast Blue BB salt (MilliporeSigma) and 100 mg/ml naphthol AS-MX phosphate (MilliporeSigma) in 0.1 M Tris-HCl pH 8.2, 50 mM $MgCl_2$, 0.1 M NaCl, and 0.1% Tween-20 (MilliporeSigma). A 2% blocking solution (MilliporeSigma) in 2× saline-sodium citrate buffer was used for blocking the sections, followed by the visualization of FITC-labeled riboprobes using sheep polyclonal anti-FITC (anti-fluorescein conjugated with horseradish peroxidase, Fab fragments, cat. # 1142636910, Roche, RRID: AB_840257) and TSA™ Fluorescein (Akoya Biosciences Marlborough, USA) according to the manufacture's protocol. Sections were mounted with ProLong Glass antifade medium with NucBlue (Invitrogen).

2.8 | Microscopy

Whole sections were scanned at 20×/0.8 objective with ZEISS Axio Scan.Z1 Slide scanner (Zeiss, Germany, RRID: SCR_020927) and ZEN software (Zeiss). The setting for NBT/BCIP was in TL brightfield (BF) using Hitachi HV-F202SCL. Fluorescent sections were scanned with DAPI, AF488 (TSA staining for *npya* and *cart2b*), AF568 (FastRed for *pomca*), and AF647 (FastBlue for *agrp1*), and using the Hamamatsu Orca Flash imaging device.

Confocal images were acquired by a laser-scanning confocal microscope (Olympus FV3000, Olympus, Japan, RRID: SCR_017015) with 10× and 60× silicon-immersion oil objective (UPLSAPO 40XS, Olympus), using DAPI, AF488 (TSA staining for *npya* and *cart2b*), AF568 (FastRed for *pomca*), and AF647 (FastBlue for *agrp1*). Image stacks from each channel were imported into Fiji (https://fiji.sc/, RRID: SCR_002285; Schindelin et al., 2012) to create z-projections based on maximum intensity.

Figures were prepared using Adobe Photoshop (version 22.1.1, Adobe Systems, San Jose, CA, RRID: SCR_014199). The background

was removed, and brightness and contrast were adjusted if necessary. The rainbow trout, *O. mykiss*, was used for reference and nomenclature of the brain regions in this study (Folgueira et al., 2004a, 2004b; Meek & Nieuwenhuys, 2014).

3 | RESULTS

To map the expression of the neuropeptides involved in the melanocortin system in Atlantic salmon parr brain, *npy*, *agrp*, *cart*, and *pomc* mRNA were examined across the entire rostrocaudal extent of the brain by ISH in coronal parallel cryosections. A summary of the results is shown in Table 2.

3.1 | neuropeptide y (npy)

3.1.1 | npya

npya was widely distributed throughout the brain (Figure 1). In the olfactory bulb, *npya* expression was found lateroventrally in the mitral cell layer (mc, Figure 1b(1–2)). A high density of *npya* is further seen in the lateral nucleus of the ventral telencephalon (Vl). Medial scattered neuronal clusters in the dorsal nucleus of ventral telencephalon (Vd, Figure 1c(1–2)), and scattered cells in the posterior dorsal telencephalon (Figure 1(d1)) also expressed *npya*. Cells expressing *npya* were identified in the preoptic region, including ventral to the *recessus preopticus* (rpo, Figure 1(d2)), and supraoptic/suprachiasmatic nucleus (SOC, Figure 1e(1–2)). *npya* was expressed in the ventrolateral habenula (vHab) and thalamic regions including the ventral (Thv) and dorsal thalamus (Thd), and the posterior tuberculum (Pt, Figure 1f(1–2) and Figure 1g(1–3)).

Hypothalamic *npya* expression was observed at the pituitary stalk in ventral nucleus lateralis tuberis (NLTv), nucleus anterior tuberis (NAT), and nucleus magnocellularis hypothalami (NMH, Figure 1g(1–3), (h3), and (i3)). In the optic tectum, *npya* was expressed in the periventricular layer (SPV) and in a few cells in the griseum layer (SGV, Figure 1(h2)). Positive *npya* expression was found in a cluster of dorsal tegmentum nucleus (DTN), potentially near the Edinger–Westphal nucleus (EW, Figure 1j(1–3)). Two rhombencephalic *npya* expressions were found, one in a small cell cluster located lateral to the fasciculus longitudinalis medialis (FLM, Figure 1k(1–2)), and a larger cluster located ventral to the Ve4 close to the trigeminal nerve (nV, Figure 1l(1–2)).

3.1.2 | npyb

npyb was detected in the telencephalon and diencephalon brain regions (Figure 2). In the telencephalon, *npyb* mRNA expression was found in the dorsal nucleus of the ventral telencephalon (Vd) toward the telencephalic ventricle in the mid telencephalon (rostral-caudal direction) about 600 μm (Figure 2a(1–2)). Light staining for *npyb* was found in a few cells of the lateral nucleus of ventral telencephalon (Vl, Figure 2a(1–3)). Ventral to the optic tectum, one *npyb* expressing cell cluster was observed in the ventral thalamus region (Thv) (Figure 2b(1–3)) toward the hypothalamic NMH near the nucleus posterioris periventricularis (NPPv, Figure 2(b3)). A cell cluster expressing *npyb* was observed just ventral to the cerebellar valvula adjacent to the medial longitudinal fasciculus (FLM, Figure 2c(1–3)).

3.2 | agouti-related peptide 1 (agrp1)

Analysis by ISH of *agrp1* showed labeled neurons in the hypothalamic NLT, including the ventral NLT (NLTv, Figure 3). *agrp1*-expressing neurons located at the pituitary stalk were situated medially toward the infundibulum as infundibular cerebrospinal-fluid contacting cells, and a few neurons laterally from cerebrospinal fluid, connecting the posterior pituitary and caudal hypothalamus. *agrp1* mRNA expression was not detected in any other brain region.

3.3 | agouti-related peptide 2 (agrp2)

agrp2 was mainly expressed in the telencephalon, but a scattered expression was also found in the diencephalon and rhombencephalon (Figure 4). In the telencephalon, neurons expressing *agrp2* demonstrated a specific pattern from the medial region (Dm) and central region (Dc) toward the ventral part of the lateral zone (Dl-v) of the dorsal telencephalon (Figure 4a(1–3)). A few *agpr2*-positive neurons were located in the ventral thalamus (Thv, Figure 4b(1–3)). In the rhombencephalon, *agrp2* mRNA expression was identified in cells lateroventral to the rhombencephalic ventricle in small nuclei near large nuclei of the nucleus motorius nervi trigemini (nV, Figure 4c(1–3)) and laterally to the FLM in the reticular formation (RF, Figure 4d(1–3)).

3.4 | cocaine- and amphetamine-regulated transcript (cart)

The *cart* paralogs mRNA distributions in the brain of Atlantic salmon parr were analyzed by ISH using seven distinct RNA probes (Table 1). The results are presented in a rostrocaudal direction from the most abundant, *cart2b*, to the lowest abundant *cart* paralogs (*cart4*, *1a*, and *1b*).

3.4.1 | cart2b

cart2b was the most abundant *cart* paralog and showed a wide distribution in several brain regions (Figure 5), being continuously observed from the olfactory bulb to the thalamus. In the olfactory bulb, numerous cells expressing *cart2b* were found in the granular cell layer (strg, Figure 5b(1–2)). *cart2b*-positive cells were found in the subpallium in the dorsal nucleus of ventral telencephalon (Vd) near the telencephalic ventricle (Figure 5c(1–3)). In the lateral telencephalon, a cluster of cells expressing *cart2b* was observed in the lateral nucleus of the ventral telencephalon (Vl). Scattered cells expressing *cart2b* were observed

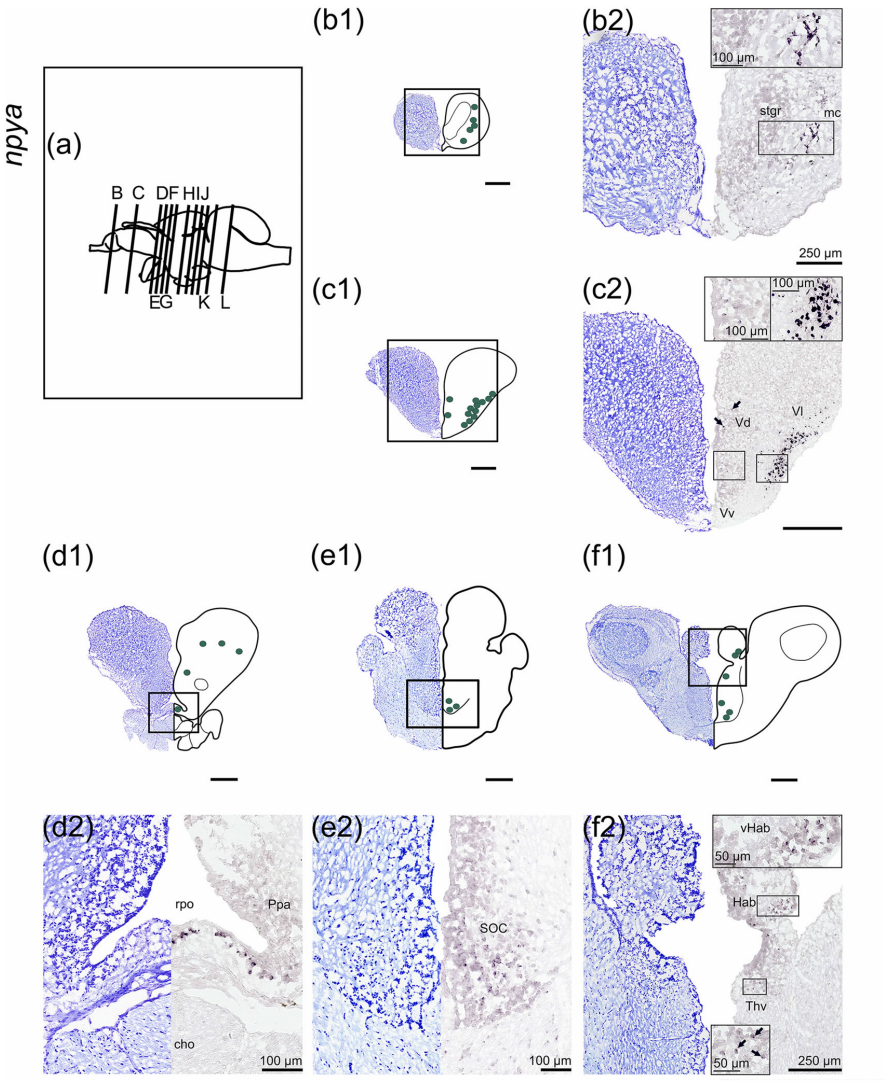

FIGURE 1 *npya* mRNA expression in Atlantic salmon parr brain. (a) Schematic representation of the brain indicating the position of each transverse section. (b1–l1) Nissl-staining compared to schematic drawing illustrating *npya* expression by green dots. (b2–l2, g3–j3) Nissl-staining and corresponding *npya* expression along with neuroanatomical structures. (b) *npya* expression in the mc of the olfactory bulb. (c) *npya* expression in the Vd, and Vl of the telencephalon. (d) *npya* expression in dorsal telencephalon and preoptic area—ppa. (e) *npya* expression in the SOC. (f) *npya* expression in the vHab and Thv. (g) *npya* expression in the SPV, Thv, Pt, NAT, and NLTv. (h) *npya* expression in the SPV, SGC, and NMH. (h) *npya* expression in the Thd and NMH. (j) *npya* expression in the dorsal tegmentum near EW. (k) *npya* expression near FLM. (l) *npya* expression near nV. Abbreviations; Cho, optic chiasm; D, dorsal telencephalon; EW, Edinger–Westphal nucleus; FLM, fasciculus longitudinalis medialis; Hab, habenula; Lih, lobus inferior hypothalami; mc, layer of mitral cells; NAT, nucleus anterior tuberis; NLT, nucleus lateralis tuberis; NLTv, ventral nucleus lateralis tuberis; NMH, nucleus magnocellularis hypothalami; nV, nervus trigemini; Ppa, preoptic area—anterior parvocellular preoptic nucleus; Pt, posterior tuberculum; Rpo, recessus preopticus; SGC, stratum griseum centrale; SM, stratum marginale; SOC, supraoptic/suprachiasmatic nucleus; SPV, stratum periventriculare of the optic tectum; stgr, stratum granulare (bulbi olfactory); Thd, dorsal thalamus; Thv, ventral thalamus; Ts, torus semicircularis; Valv, valvula cerebelli; Vd, dorsal nucleus of ventral telencephalon; vHab, ventral habenula; Vl, lateral nucleus of ventral telencephalon; Vv, ventral nucleus of ventral telencephalon. Scale bar (if no other indication) = 500 µm

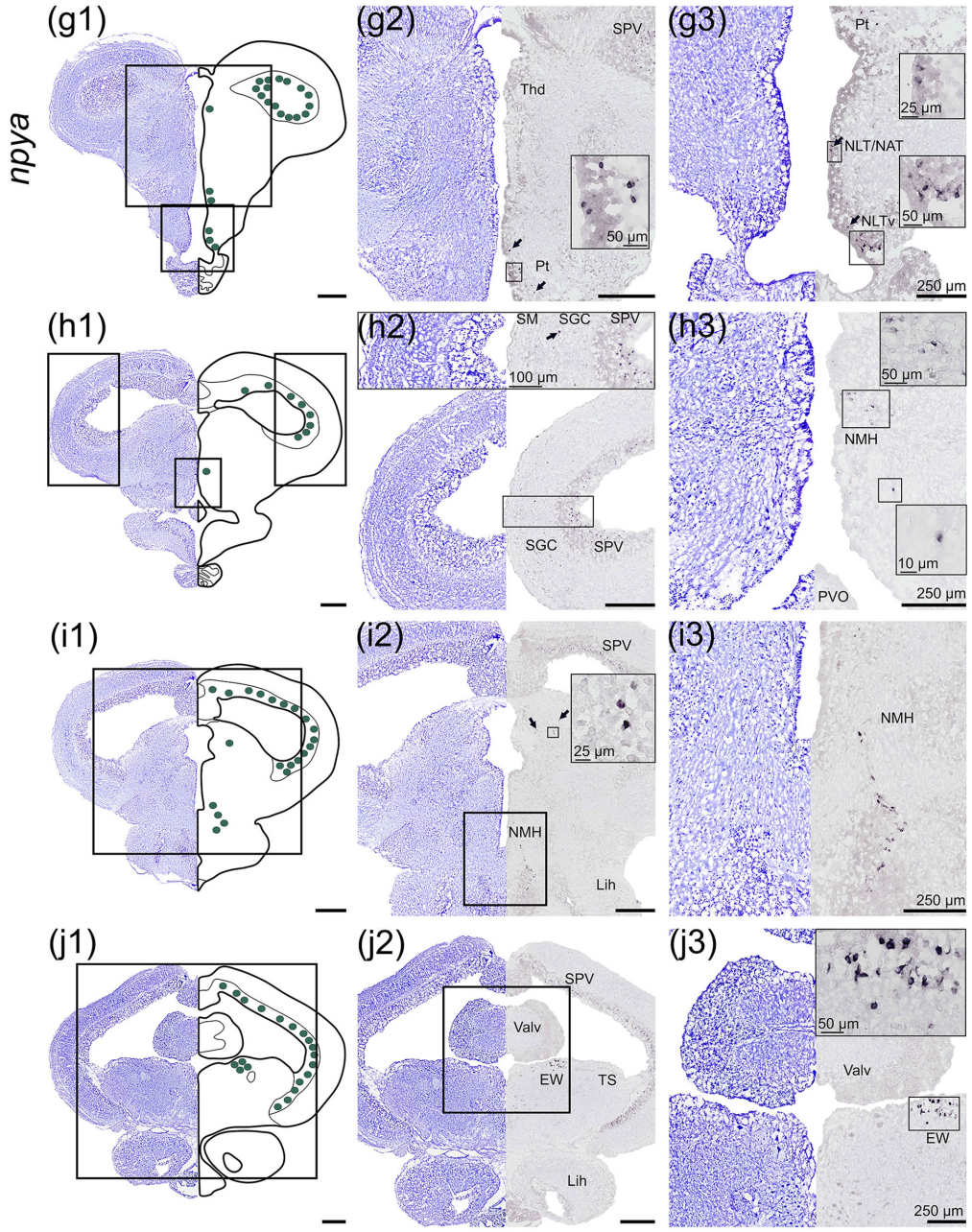

FIGURE 1 Continued

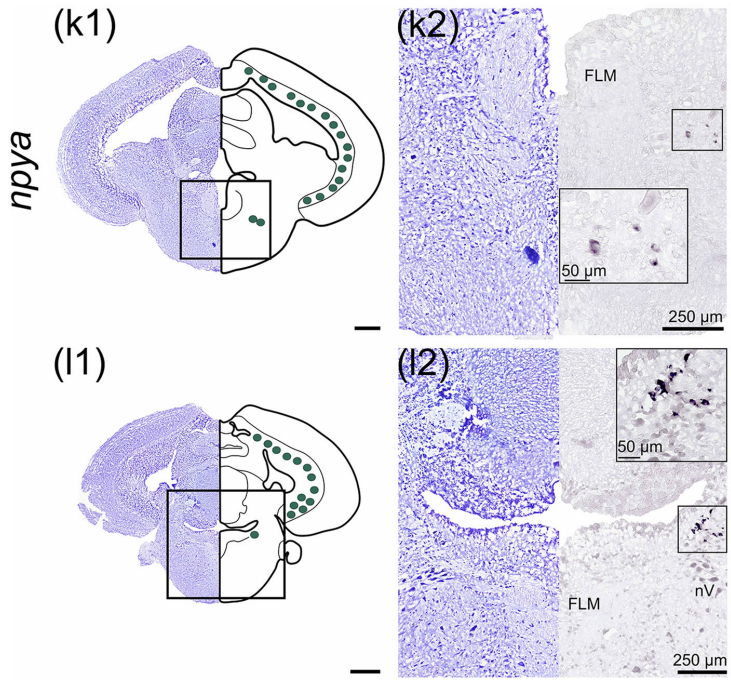

FIGURE 1 Continued

in the ventral nucleus of ventral telencephalon (Vv), lateral (Dl-d and Dl-v), medial (Dm), and dorsal (Dd) zone of the dorsal telencephalon (Figure 5(c3)). The *cart2b* mRNA expression from strg and Vd could be followed continuously to the periventricular preoptic region, as shown in nucleus posterior periventricularis (Ppp, Figure 5d(1–3)). *cart2b* was detected from the optic tectum in the periventricular layer (SPV) toward the stratum album centrale (SAC) border (Figure 5(e2)), and from the dorsal-most region adjacent to torus longitudinalis until the base of the optic tectum near torus semicircularis (Figure 5(e1), (f1), (g1), (h1), and (i1)). In the midbrain, *cart2b* was present in the dorsal thalamus (Thd), posterior tuberculum (Pt) toward the diencephalic ventricle, and in the hypothalamus (Figure 5e,h).

cart2b mRNA was abundant in the hypothalamus, in a dorsoventral direction from the NAT toward the ventral NLT (NLTv, Figure 5(e3)). Scattered cells expressing *cart2b* were observed laterally in *torus lateralis* (tlat) and *lobus inferior hypothalami* (Lih) toward the cerebrospinal fluid (Figure 5(f3)). A cluster of cells expressing abundantly *cart2b* were also observed in the paraventricular organ (PVO), nucleus posterior tuberis (PTN) as well as in NMH (Figure 5g(1–3)). From NAT, *cart2b* expression continued to be detected dorsolateral to the infundibulum, into nucleus recessi lateralis (NRL) and medial part of the diffuse nucleus of Lih (NDILm, Figure 5(h1) and (h3)). Scattered cells expressing *cart2b* were observed in *saccus vasculosus* (SV, Figure 5(h3)).

Ventral to the valvula in dorsal tegmentum, scattered neurons expressing *cart2b* were observed in torus semicircularis (Ts) toward nucleus lateralis valvulae (nlv, Figure 5(g2) and (h2)). In the rhombencephalon, *cart2b* was observed in stratum ganglionare (ggl) of corpus cerebelli (Figure 5i(1–2)) and ventrolateral to the fourth ventricle near locus coeruleus (lcoer) and formatio reticularis pars superior (rets, Figure 5(i1) and (i3)).

3.4.2 | cart3a

cart3a mRNA expression was identified in the telencephalon, midbrain, and rhombencephalon (Figure 6). In the telencephalon, neurons expressing *cart3a* were identified in the ventral nucleus of ventral telencephalon (Vv, Figure 6b(1–b2)), in the ventrolateral telencephalon in nucleus entopeduncularis (ent), and within SOC of the preoptic region (Figure 6c(1–3)). The *cart3a* in the optic tectum was expressed in the less densely populated neurons in the torus longitudinalis (TL) toward the white matter of the torus longitudinalis (TLw, Figure 6(d1), (d2), (e1), and (e2)). *cart3a* expression was observed in scattered cells in the dorsal thalamus (Figure 6(d3)), and in the hypothalamus dorsal to the paraventricular organ (PVO) in PTN and NMH (Figure 6(e3)). *cart3a* expression was also found in the dorsal mesencephalic tegmentum (DTN)—possibly near the EW (Figure 6f(1–3)), dorsomedial to fasciculi longitudinalis medialis (FLM, Figure 6g(1–2)), and scattered neurons laterally to the rhombencephalic ventricle near lcoer (Figure 6(g3)). In rostral rhombencephalon, *cart3a* mRNA was

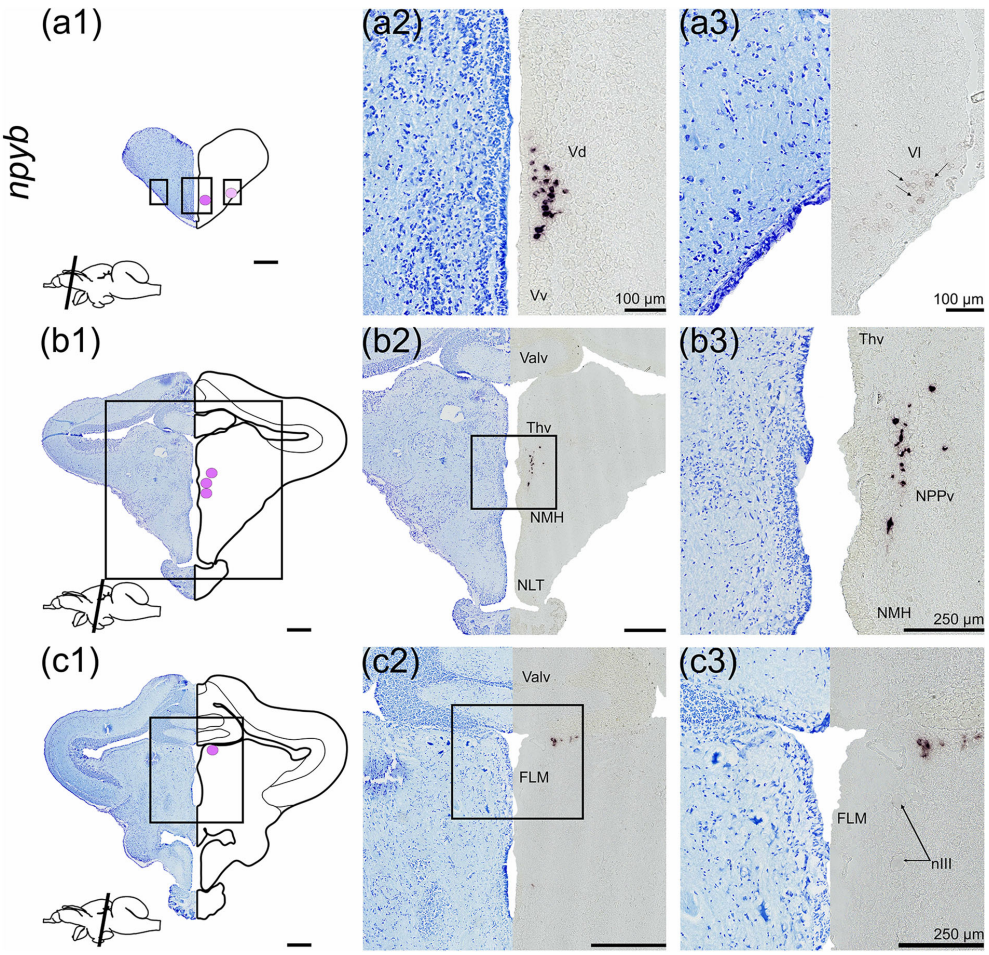

FIGURE 2 *npyb* mRNA expression in Atlantic salmon parr brain from rostral (a) to caudal (c) brain regions. (a1–c1) Nissl-staining and schematic drawing illustrating *npyb* expression by purple dots. Lower left corner represents a schematic drawing of the salmon brain indicating the position of the section (a2–c2, a3–c3). Nissl-staining and corresponding *npyb* expression with neuroanatomical structures. (a) *npyb* expression in the Vd, Vv and Vl parts of ventral telencephalon. (b) *npyb* expression near the Thv and NPPv. (c) *npyb* expression near the FLM and nIII. Abbreviations: FLM, fasciculus longitudinalis medialis; nIII, nervus oculomotorius; NMH, nucleus magnocellularis hypothalami; NPPv, nucleus posterioris periventricularis; Thv, ventral thalamus; Valv, valvula cerebelli; Vd, dorsal nucleus of ventral telencephalon; Vl, lateral nucleus of ventral telencephalon; Vv, ventral nucleus of ventral telencephalon. Scale bar (if no other indication) = 500 µm

found ventral of nervus trigeminus (nV) in nervus motorius nervi trigemini (nVm, Figure 6h(1–3)).

3.4.3 | cart3b

The localization of *cart3b* by ISH demonstrated its presence in the preoptic region, thalamus, tegmentum, and rhombencephalon (Figure 7). *cart3b* mRNA expression was detected in the caudal preoptic region, specifically in the posterior part of the parvocellular preoptic nucleus (Ppp, Figure 7b(1–2)). Ventral to the optic tectum in the diencephalon, *cart3b* probe labeled a neuronal line from the dorsal thalamus (Thd) to the ventrolateral direction of the hypothalamic *torus lateralis* (tlat, Figure 7c(1–3)). Ventral to the valvula, *cart3b* was expressed in scattered neurons of the central and ventral torus semicircularis (TS, Figure 7d(1–3)). The *cart3b* probe also hybridized scattered neurons located ventrolateral to the FLM (Figure 7e(1–3)), neurons near nervus trigemini (nV, Figure 7f(1–3)), and neurons in the formatio reticularis pars superior (rets) as well as dorsal cells to rets (Figure 7g(1–3)).

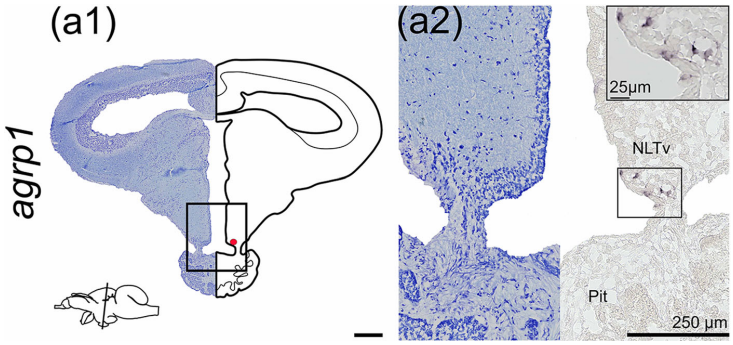

FIGURE 3 *agrp1* mRNA expression in Atlantic salmon parr brain. (a1) Nissl-staining at equivalent level to the schematic drawing illustrating *agrp1* expression by red dot. Lower left corner represents a schematic brain indicating the position of the section. (a2) Nissl-staining and corresponding *agrp1* expression in the ventral nucleus lateralis tuberis (NLTv) at the pituitary stalk. Abbreviation: Pit, pituitary. Scale bar (if no other indication) = 500 µm

3.4.4 | cart2a

cart2a-expressing cells were identified in the telencephalon, optic tectum, thalamus, and hypothalamus (Figure 8). One *cart2a* neuronal cluster was present in the ventral nucleus of the ventral telencephalon (Vv) toward the telencephalic ventricle (Figure 8b(1–2)). Scattered *cart2a*-positive cells were detected in tractus opticus pars distalis (tod, Figure 8c(1–2)). In the optic tectum, scattered cells expressing *cart2a* were observed in stratum marginale (SM), stratum opticum (SO, Figure 8(c3)), in the album layer (SAC), and evenly distributed in the periventricular layer (SPV) toward the SAC (Figure 8d(1–2) and (e1)). In the midbrain, one *cart2a* cell cluster was observed in the dorsal thalamus (Thd, Figure 8d(1–3)). In the hypothalamus, a cluster of cells expressing *cart2a* was identified in the PTN (Figure 8e(1–3)).

3.4.5 | *cart4*, *1b*, and *1a*

cart4, *1b*, and *1a* were expressed in distinct brain regions from the rostral to the caudal direction (Figure 9). *cart4* was only expressed in the most rostral area of the diencephalon, in the posterior parvocellular preoptic nucleus (Ppp, Figure 9b(1–2)). In the dorsomedial mesencephalic tegmentum ventral to the valvula near the nucleus medial longitudinal fasciculus (NMFL), *cart1a* (Figure 9c(1–3)) and *cart1b* (Figure 9d(1–3)) mRNA expression were identified. *cart1b* was only observed in one cell cluster, while the *cart1a* probe identified two separate clusters of neurons adjacent and medial to the FLM and oculomotor nucleus (NIII, Figure 9e(1–3)).

3.5 | pro-opiomelanocortin (pomc)

In the Atlantic salmon parr brain, *pomca*-expressing cells were detected in the pituitary (adenohypophysis), and in the NLTv of the hypothalamus (Figure 10a(1–2)). The hypothalamic *pomca*-expressing cells were located medially toward the infundibulum. *pomcb* was strongly expressed in the adenohypophysis of the pituitary (Figure 10b(1–2)), and was not observed in the NLT, or in any other brain regions.

3.6 | Hypothalamic expression of melanocortin system neuropeptides

To determine whether the Atlantic salmon tuberal hypothalamus coexpress *npya/agrp1* and/or *cart2b/pomca*, double labeling fluorescent ISH was used. The results show that neurons expressing *npya* did not coexpress *agpr1* (Figure 11). In the anterior NLT (NLTa) of the rostral tuberal hypothalamus, few neurons expressed *npya* mRNA, but no *agrp1* expression was found in this region (Figure 11a). Toward the NLTv at the pituitary stalk, both *npya* and *agrp1* were abundantly expressed in neighboring neurons (Figure 11b(1–4)). *npya* and *agrp1* were still present in neighboring neurons of the ventral NLT (NLTv) bordering the nucleus posterior tuberis (NPT) in the caudal tuberal hypothalamus (Figure 11c), but their expression decreased, particularly for *agrp1*, in comparison to the NLTv at the pituitary stalk.

cart2b/pomca coexpression was observed in the NLTa and NLTv of the tuberal hypothalamus (Figure 11d,e). However, *cart2b* and *pomca* were mainly expressed in distinct neurons of the tuberal hypothalamus. The *cart2b*-positive neurons were gradually located dorsally toward the NAT, while *pomca* expression remained ventrally in the NLT (Figure 11e,f). Thus, no *cart2b/pomca* coexpression was found in the caudal tuberal hypothalamus.

4 | DISCUSSION

In this study, ISH was utilized to map the spatial distribution of *npy*, *agrp*, *cart*, and *pomc* in the Atlantic salmon parr brain (summarized in Table 2). The topology of these neuropeptides, particularly in the lateral tuberal nucleus (NLT), supports that the hypothalamic nucleus is

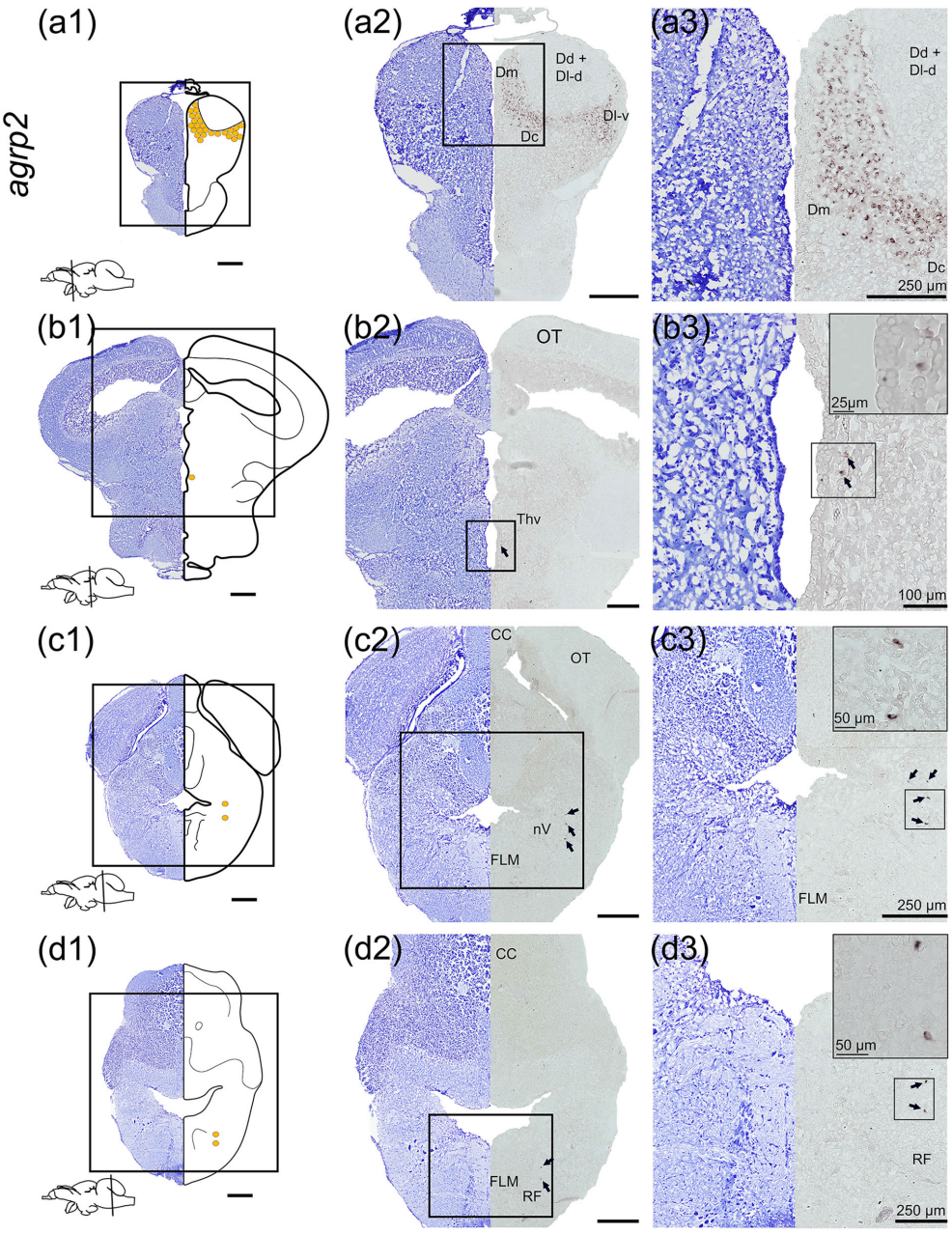

FIGURE 4 *agrp2* mRNA expression in Atlantic salmon parr brain. (a1–d1) Nissl-staining compared to schematic drawing illustrating *agrp2* expression by yellow dots. Lower left corner represents a schematic brain indicating the position of the section. (a2–d2, a3–d3) Nissl-staining and corresponding *agrp2* expression with neuron anatomical structures. (a) *agrp2* expression in the Dm, Dc, and Dl-v parts of the telencephalon. (b) *agrp2* expression in the Thv. (c) *agrp2* expression near the FLM and (d) the RF. Abbreviations: Dc, central zone of dorsal telencephalon; Dd, dorsal part of dorsal telencephalon; Dl-d, dorsal part of lateral zone of dorsal telencephalon; Dl-v, ventral part of lateral zone of dorsal telencephalon; Dm, medial zone of dorsal telencephalon; FLM, fasciculus longitudinalis medialis; OT, optic tectum; RF, reticular formation; Thv, ventral thalamus. Scale bar (if no other indication) = 500 µm

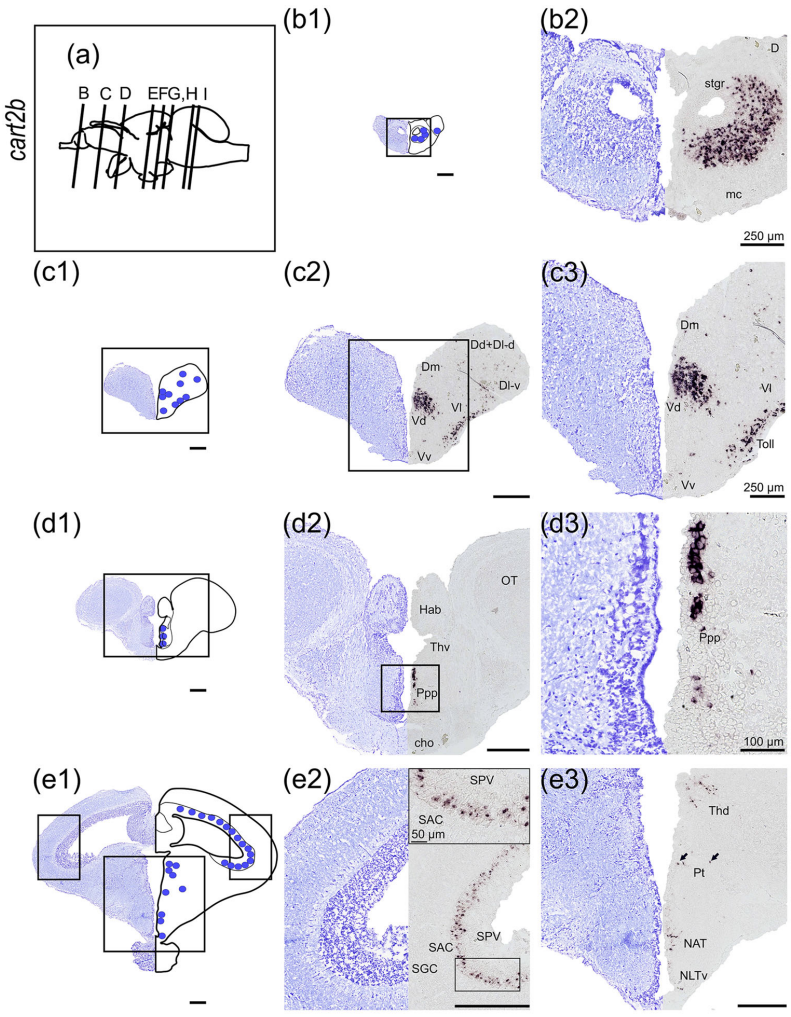

FIGURE 5 *cart2b* mRNA expression neurons in Atlantic salmon parr brain. (a) Schematic representation of the brain indicating the position of each transverse section. (b1–I1) Nissl-staining compared to schematic drawing illustrating *cart2b* expression by blue dots. (b2-i2, c3-i3) Nissl-staining and corresponding *cart2b* expression along with neuroanatomical structures. (b) *cart2b* expression in the olfactory bulb stgr. (c) *cart2b* expression in the Dm, Dd, and Dl zones of dorsal telencephalon as well as the Vd, Vl and Vv nucleus of ventral telencephalon. (d) *cart2b* expression in the preoptic region—Ppp. (e) *cart2b* expression in the SPV, Thd, Pt, NAT, and NLTv. (f) *cart2b* expression in the SPV, Thd, NMH, NAT, tlat, and Lih. (g) *cart2b* expression in the SPV, scattered neurons in the Ts, large cluster near NMH and PVO, scattered neurons in NAT, nrl, Ce, tlat, and Lih. (h) *cart2b* expression in the SPV, and dorsal midbrain tegmentum toward Ts and the nlv, in the NDILm and nrl, and SV. (i) *cart2b* expression in the SPV, gran of the cerebellum, and in the lcoer and rets. Abbreviations: Ce, nucleus centralis lobi inferioris hypothalamic; Cho, optic chiasm; D, dorsal telencephalon; Dd, dorsal zone of dorsal telencephalon; Dl-d, dorsal part of lateral zone of dorsal telencephalon; Dl-v, ventral part of lateral zone of dorsal telencephalon; Dm, medial zone of dorsal telencephalon; Ggl, stratum ganglionare—cerebelli; Gran, stratum granulare—cerebelli; Hab, habenula; Lcoer, locus coeruleus; Lih, lobus inferior hypothalami; Mcba, tractus mesencephalo-cerebellaris anterior; Mol, stratum moleculare—cerebelli; NAT, nucleus anterior tuberis; NDILm, medial part of the diffuse nucleus of inferior lobe; NLT, nucleus lateralis tuberis; NLTv, ventral nucleus lateralis tuberis; NLV, nucleus lateralis valvulae; NMH, nucleus magnocellularis hypothalami; NRL, nucleus recessi lateralis; OT, optic tectum; Ppp, posterior parvocellular preoptic nucleus; Pt, posterior tuberculum; PVO, paraventricular organ; Rets, formatio reticularis pars superior; SAC, stratum album centrale; SPV, stratum periventriculare of the optic tectum; Stgr, stratum granulare—bulbi olfactory; SV, saccus vasculosus; Thd, dorsal thalamus; Thv, ventral thalamus; Tlat, torus lateralis; Toll, tractus olfactorius lateralis; Ts, torus semicircularis; Valv, Valvula cerebelli; Vd, dorsal nucleus of ventral telencephalon; Vl, lateral nucleus of ventral telencephalon; Vv, ventral nucleus of ventral telencephalon; Scale bar (if no other indication) = 500 µm

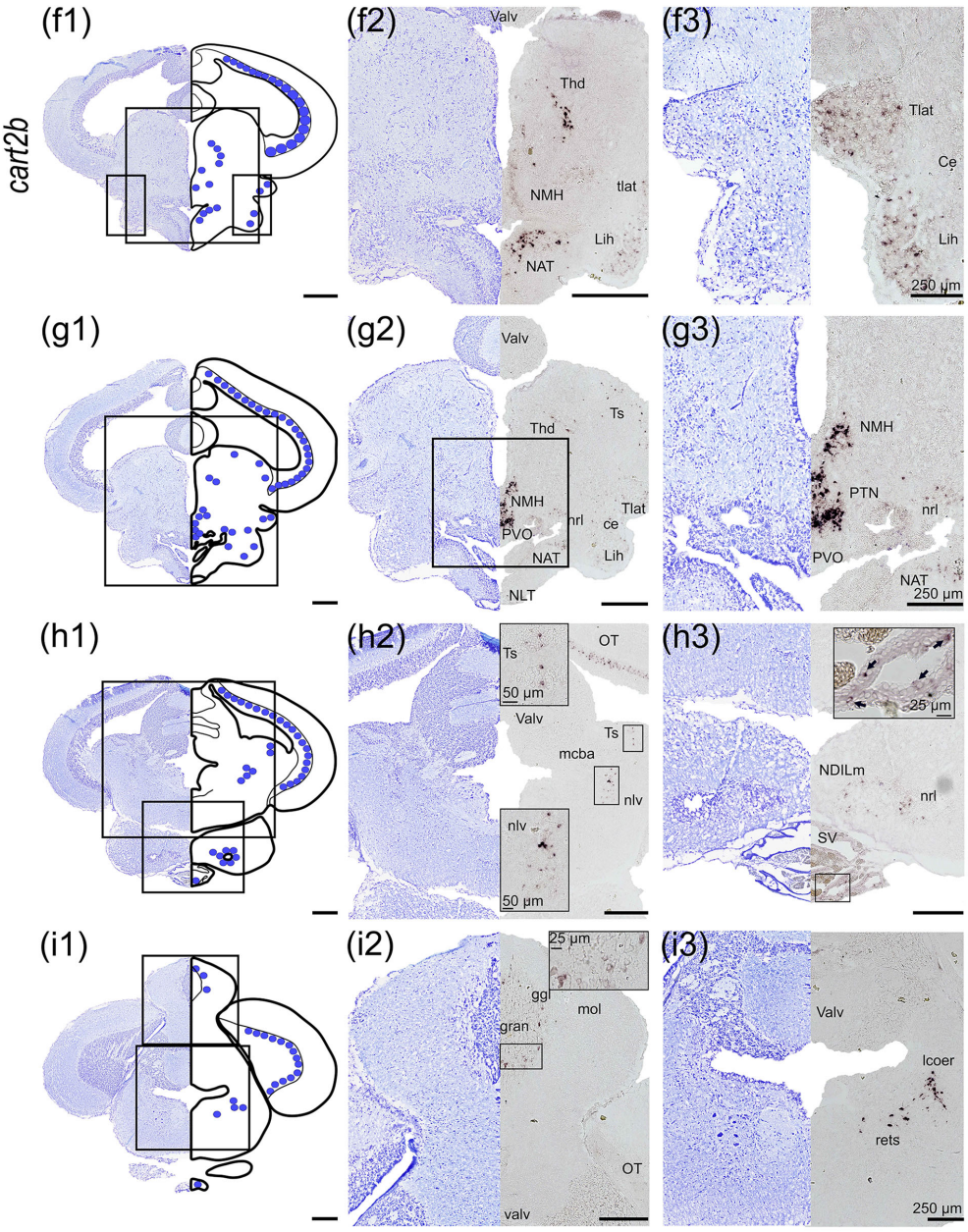

FIGURE 5 Continued

associated with appetite and food intake regulation. We also demonstrated the presence of *cart2b/pomca* coexpression in the anterior and ventral NLT.

As a result of the salmonid-specific fourth whole-genome duplication event (Allendorf & Thorgaard, 1984; Lien et al., 2016), several paralogs of *npy*, *cart*, and *pomc* have been identified in Atlantic salmon (Kalananthan et al., 2021; Murashita et al., 2011; Tolås et al., 2021; Valen et al., 2011). Although the fate of the duplicated genes of key players in the melanocortin system are not yet fully understood, one hypothesis is that some genes will still play a role in appetite control

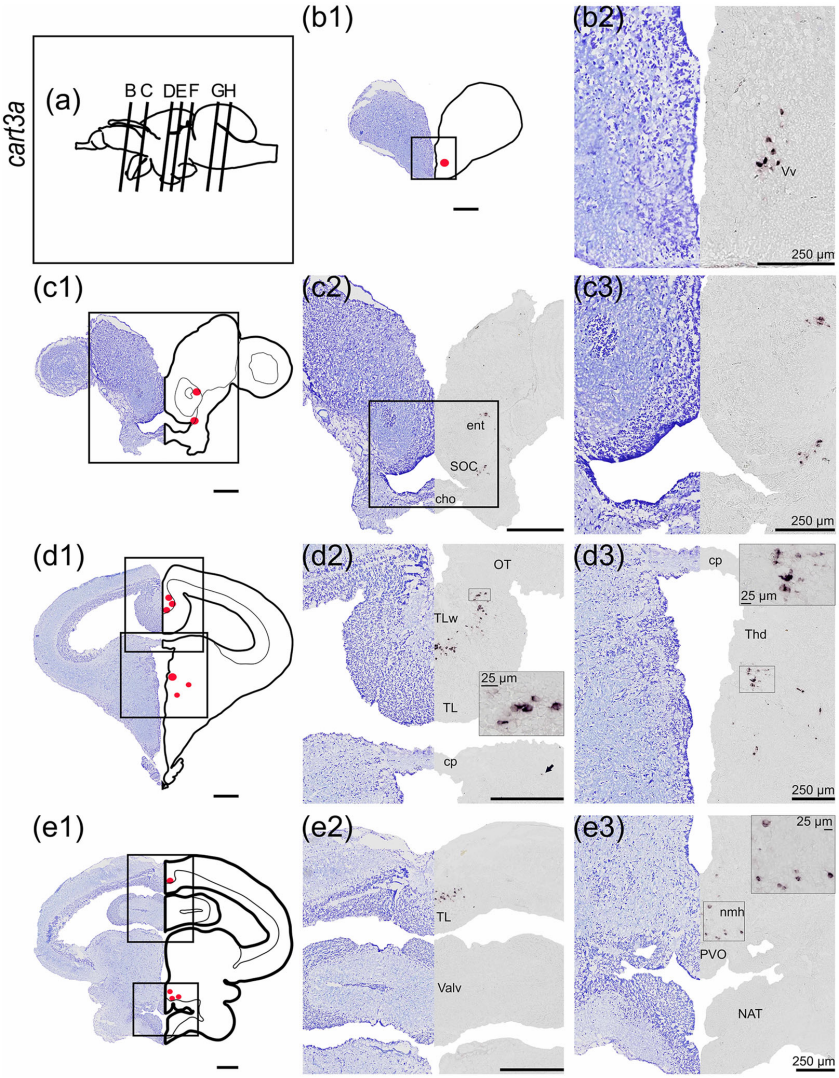

FIGURE 6 *cart3a* mRNA expression in Atlantic salmon parr brain. (a) Schematic representation of the brain indicating the position of each transverse section. (b1–h1) Nissl-staining compared to schematic drawing illustrating *cart3a* expression by red dots. (b2–h2, c3–h3) Nissl-staining and corresponding *cart3a* expression along with neuroanatomical structures. (b) *cart3a* expression in the Vv. (c) *cart3a* expression in the ent and in the SOC. (d) *cart3a* expression in the TLw of the optic tectum and in the Thd. (e) *cart3a* expression in the TL, and in the NMH and PVO. (f) *cart3a* expression in the TL and dorsal tegmentum near EW. (g) *cart3a* expression near the FLM and lcoer. (h) *cart3a* expression near the nV and nVm. Abbreviations: Cho, optic chiasm; cp, commissura posterior; Ent, nucleus entopeduncularis; EW, Edinger–Westphal nucleus; FLM, fasciculus longitudinalis medialis; fMth, fiber of Mauthner cell; Lcoer, locus coeruleus; NAT, nucleus anterior tuberis; NMH, nucleus magnocellularis hypothalami; nV, nervi trigemini; nVm, nucleus motorius nervi trigemini; OT, optic tectum; PVO, paraventricular organ; SOC, supraoptic/suprachiasmatic nucleus; Thd, dorsal thalamus; TL, torus longitudinalis; TLw, white matter of the torus longitudinalis; Valv, valvula cerebelli; Vv, ventral nucleus of ventral telencephalon. Scale bar (if no other indication) = 500 µm

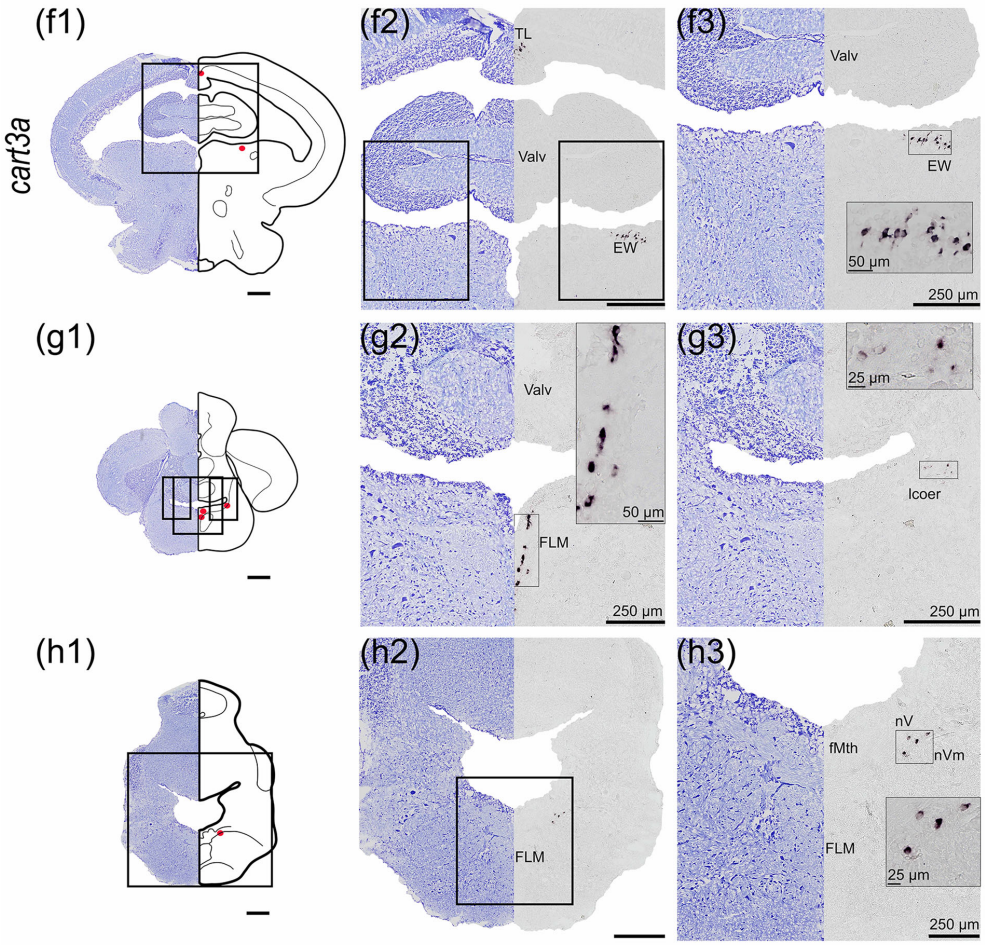

FIGURE 6 Continued

by facilitating physiological, sensory, or periprandial responses. Indeed, our results demonstrate that these neuropeptides are expressed in the salmon brain regions known to be related to feeding and energy status. These regions include the hypothalamus, known to be related to regulation of vital homeostatic feeding control in both fish and mammals, as well as the olfactory bulb, telencephalon, optic tectum, and secondary gustatory nucleus, which are linked to feeding (Demski & Knigge, 1971; Volkoff et al., 2005). Indeed, we found *npy*, *agrp2*, and *cart* in the olfactory bulb, telencephalon, and optic tectum (Figures 1–9). Several of these brain regions are indirectly linked to chemical stimulation of appetite, either through inputs from sensory organs (olfaction and taste) or by hedonic (nonhomeostatic) regulation (Arikawa et al., 2020; Rossi & Stuber, 2018; Volkoff, 2019).

4.1 | Hypothalamic expression of melanocortin system neuropeptides

The hypothalamic neuropeptides *npya*, *agrp1*, *cart2b*, and *pomca* are involved in appetite control as their expression levels responded to a fed/fasted state in Atlantic salmon (Kalananthan et al., 2021; Kalananthan, Murashita, et al., 2020; Murashita et al., 2011, 2009; Tolås et al., 2021; Valen et al., 2011). Here, we show the presence of these neuropeptides in the NLT region of the Atlantic salmon parr, the putative homolog to the mammalian arcuate nucleus ((Cerdá-Reverter & Peter, 2003; Cerdá-Reverter, Ringholm, et al., 2003) and reviewed in Biran et al. (2015)), supporting previous evidence that this region and these genes are involved in appetite control. This can be further supported

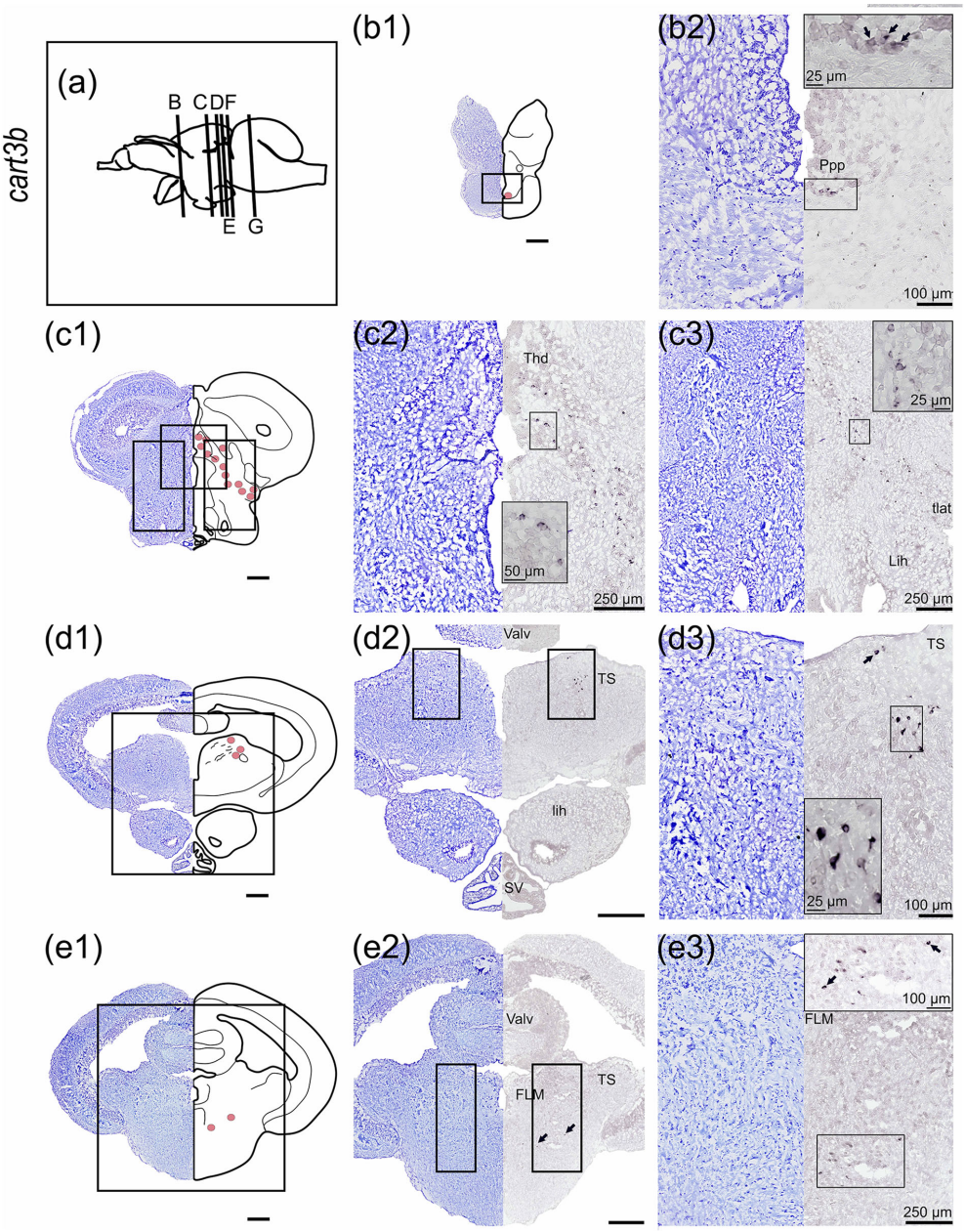

FIGURE 7 *cart3b* mRNA expression in Atlantic salmon parr brain. (a) Schematic representation of the brain indicating the position of each transverse section. (b1–g1) Nissl-staining compared to schematic drawing illustrating *cart3b* expression by pink dots. (b2–g2, c3–g3) Nissl-staining and corresponding *cart3b* expression along with neuroanatomical structures. (b) *cart3b* expression in the preoptic region—Ppp. (c) *cart3b* expression in a ventrolateral direction from the Thd toward tlat. (d) *cart3b* expression in dorsal tegmentum near Ts. (e) *cart3b* expression ventral to the FLM. (f) *cart3b* expression near nV. (g) *cart3b* expression ventral to the FLM near the rets. Abbreviations: FLM, fasciculus longitudinalis medialis; fMth, fiber of Mauthner cell; Lih, lobus inferior hypothalami; nV, nervi trigemini; ppp, posterior parvocellular preoptic nucleus; Rets, formatio reticularis pars superior; Tbc, tractus tecto-bulbaris cruciatus; Thd, dorsal thalamus; Tlat, torus lateralis; Ts, torus semicircularis; Valv, valvula cerebelli; Ve4, fourth ventricle (rhombencephali). Scale bar (if no other indication) = 500 µm

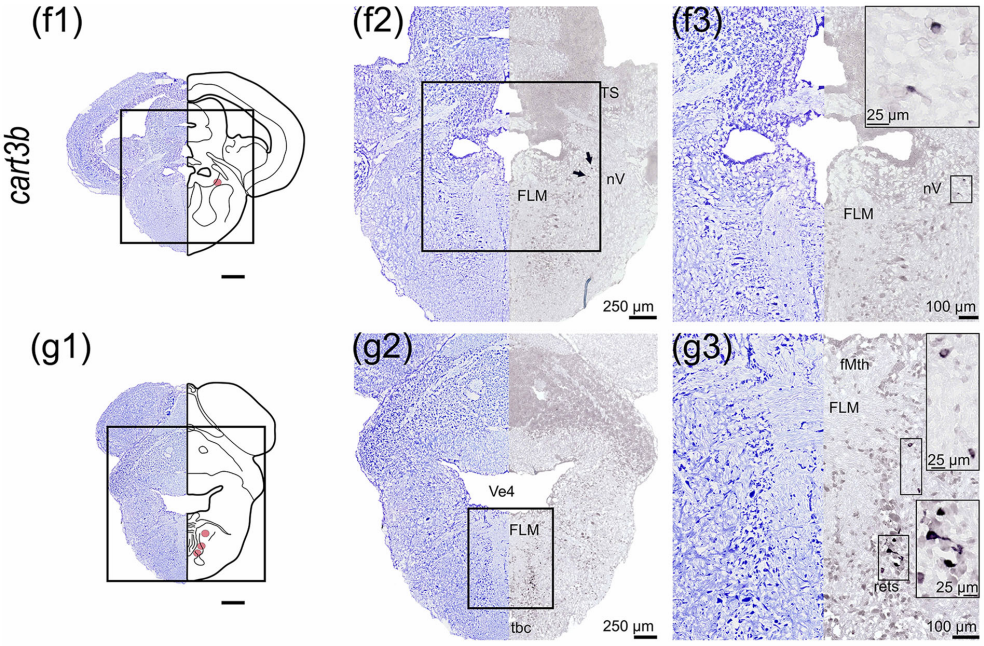

FIGURE 7 Continued

by the presence of a few neurons coexpressing *cart2b/pomca*, and the expression of *agrp1* and *npya* in neighboring cells in the NLT (Figure 11). There is evidence that the homeostatic control of appetite by the melanocortin system involves the stimulation of hypothalamic arcuate nucleus first-order orexigenic and anorexigenic neurons, which then project to second-order hypothalamic neurons which in turn project to autonomic centers in the hindbrain (Morton et al., 2006; Schwartz et al., 2000). The resulting neuronal net output stimulates anabolic or catabolic pathways. Previous studies mapping the neuroanatomical distribution of melanocortin circuits in teleosts have hypothesized possible coexpressions (Delgado et al., 2017; Porter et al., 2017; Soengas et al., 2018); however, this has never been demonstrated. Thus, to our knowledge, this is the first evidence of coexpression between *cart2b/pomca* in the NLT region of a teleost species. In agreement with the findings of Jeong et al. (2018) in the hypothalamus of zebrafish, no coexpression of *npy/agrp1* was observed in Atlantic salmon NLT. Therefore, as previously suggested, coexpression of *npy/agrp1* might not be required for the action of these neuropeptides in appetite control of teleost fishes (Jeong et al., 2018). As a contrast, at least 90% of the neurons in the mammalian arcuate nucleus that express *Npy* or *Cart* also express *Agrp* or *Pomc*, respectively, and play a crucial role in a homeostatic regulation of appetite (Elias et al., 1998; Hahn et al., 1998; Schwartz et al., 2000). The limited number of neurons coexpressing *cart2b/pomca* in the tuberal hypothalamus of Atlantic salmon, and that there was no coexpression of *npya/agrp1* suggest that coexpression might not be required for homeostatic feeding control in Atlantic salmon and other teleost species.

The presence of *npy* in the NLT region seems to be conserved throughout evolution since it has been observed in several teleost species including sea bass (Cerdá-Reverter et al., 2000), goldfish (Kojima et al., 2010), Atlantic cod (Le et al., 2016), and African cichlid fish (Porter et al., 2017). The NLT region is considered a site for integrating and releasing neurotransmitters to higher-order neurons linked to neuroendocrine appetite control and feeding behavior (Rønnestad et al., 2017). In Atlantic salmon, *agrp1* was exclusively detected in the hypothalamic NLT (Figure 3). The NLT *agrp1* expression is in line with observations for other species like goldfish (Cerdá-Reverter & Peter, 2003), zebrafish (Forlano & Cone, 2007; Koch et al., 2019; Shainer et al., 2017), sea bass (Agulleiro et al., 2014), African cichlid fish (Porter et al., 2017), and rainbow trout (Otero-Rodino et al., 2019). Indeed, *agrp1* function has been associated with appetite control in Atlantic salmon (Kalananthan, Murashita, et al., 2020; Murashita et al., 2009). Furthermore, in zebrafish, it has been shown that *agrp*-neurons are hypophysiotropic, projecting from the NLT to the pituitary (Zhang et al., 2012). The high degree of similarity in the NLT *agrp1* population among fish suggests that the involvement of this region in controlling appetite is well conserved. The hypothalamic nuclei expressing *cart2b* mRNA (Figure 5e,h) in Atlantic salmon are consistent with previous studies of hypothalamic *cart* expression (Akash et al., 2014; Porter et al., 2017). Additionally, salmon hypothalamic *cart2b* expression has been shown to respond to a fed/fasted state (Kalananthan et al., 2021). Atlantic salmon hypothalamic neurons also expressed *cart3a* in the NMH area. Indeed, it has been shown that *cart3a* expression is upregulated in the hypothalamus after 3 days of fasting, indicating a

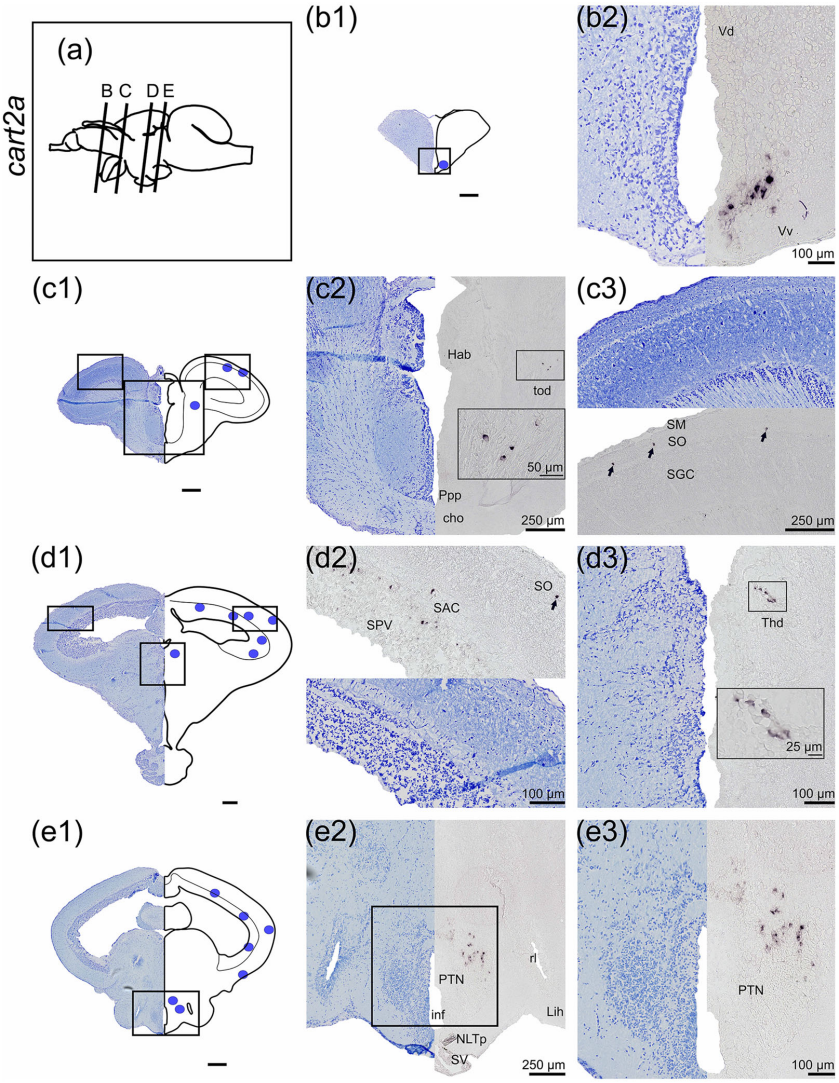

FIGURE 8 *cart2a* mRNA expression in Atlantic salmon parr brain. (a) Schematic representation of the brain indicating the position of each transverse section. (b1–e1) Nissl-staining compared to schematic drawing illustrating *cart2a* expression by blue dots. (b2-e2, c3-e3) Nissl-staining and corresponding *cart2a* expression along with neuroanatomical structures. (b) *cart2a* expression in Vv of telencephalon. (c) *cart2a* expression in tod and SO. (d) *cart2a* expression in SPV, SAC, and SO of the optic tectum and ventrally in the Thd. (e) *cart2a* expression in the hypothalamus near the PTN. Abbreviations: Cho, optic chiasm; Hab, habenula; inf, infundibulum; Lih, lobus inferior hypothalamic; NLTp, posterior nucleus lateralis tuberis; Ppp, posterior parvocellular preoptic nucleus; PTN, nucleus posterior tuberis; Rl, recessi lateralis; SAC, stratum album centrale (tecti mesencephali); SM, stratum marginale (tecti mesencephali); SO, stratum opticum (tecti mesencephali); SPV, stratum periventriculare of the optic tectum; SV, saccus vasculosus; Thd, dorsal thalamus; Tod, tractus opticus dorsalis; Vd, dorsal nucleus of ventral telencephalon; Vv, ventral nucleus of ventral telencephalon. Scale bar (if no other indication) = 500 µm

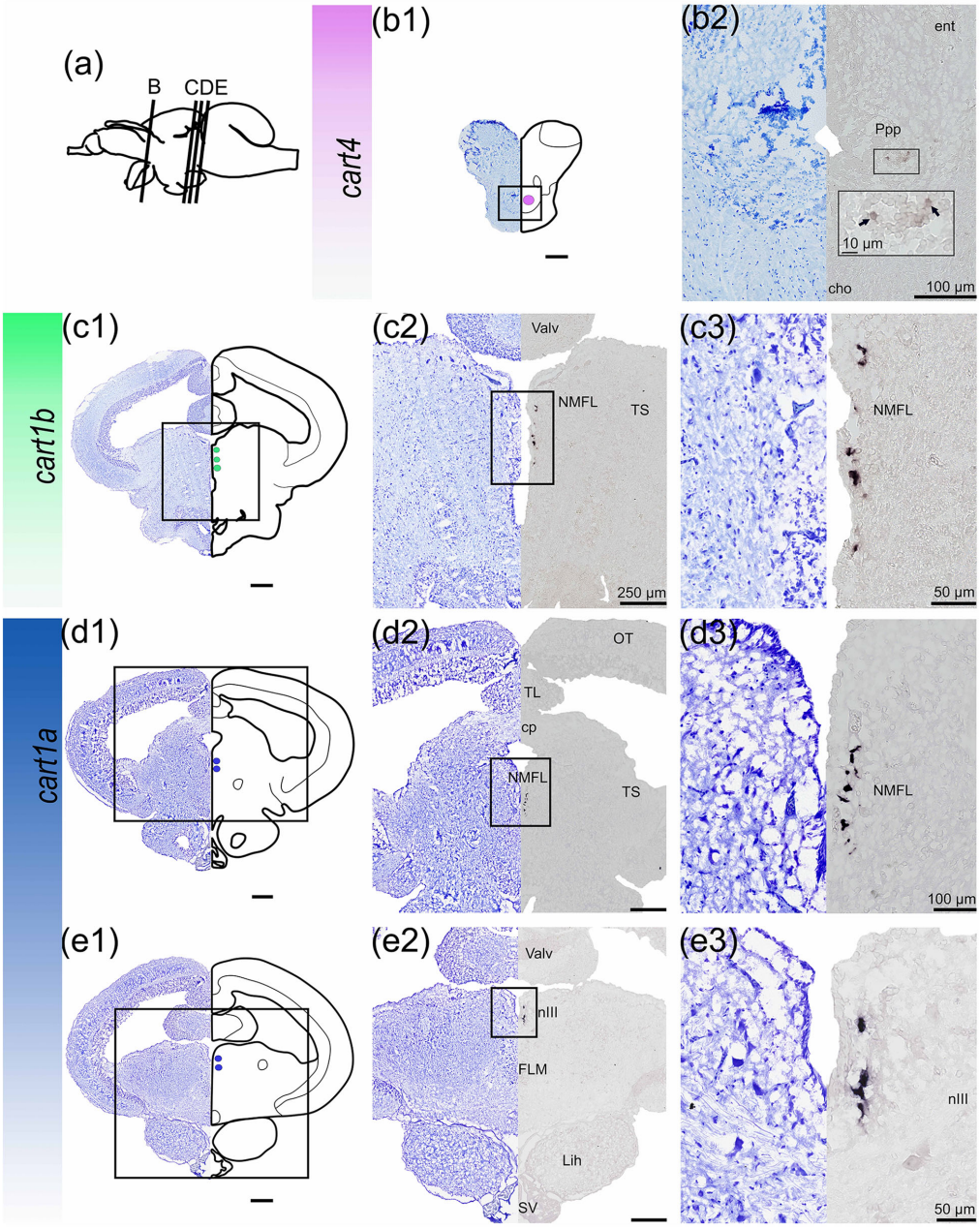

FIGURE 9 *cart4*, *1b*, and *1a* mRNA expression in Atlantic salmon parr brain. (a) schematic representation of the brain indicating the position of each transverse section. (b1–e1) Nissl-staining compared to schematic drawing illustrating *cart4* expression by pink dot, *cart1b* by green dots, and *cart1a* by blue dots. (b2–e2, c3–e3) Nissl-staining and corresponding *cart4*, *1b*, and *1a* expression along with neuroanatomical structures. (b) *cart4* expression the preoptic region—Ppp. (c) *cart1b* expression near the NMFL. (d) *cart1a* expression near the NMFL. (e) *cart2a* expression near the nIII. Abbreviations: Cho, optic chiasm; Cp, commissura posterior; Ent, nucleus entopeduncularis; FLM, medial longitudinal fasciculus; Lih, inferior hypothalamic lobe; nIII, Nucleus oculomotorius; NMFL, nucleus medial longitudinal fasciculus; OT, optic tectum; Ppp, posterior parvocellular preoptic nucleus; SV, saccus vasculosus; TL, torus longitudinalis; Ts, torus semicircularis; Valv, valvula cerebelli. Scale bar (if no other indication) = 500 µm

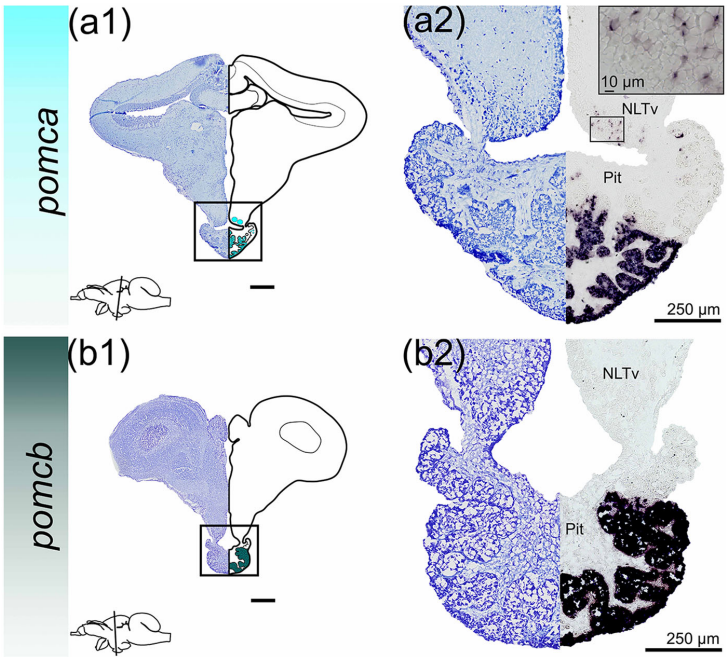

FIGURE 10 *pomc* mRNA expression in Atlantic salmon parr brain. (a1–b1) Nissl-staining compared to schematic drawing illustrating *pomca* expression by light blue dots, and *pomcb* expression by green dots. Lower left corner represents a schematic brain indicating the position of the section. (a2–b2) Nissl-staining and corresponding *pomca* and *pomcb* expression with neuroanatomical structures. (a) *pomca* expression in the NLTv and adenohypophysis of the Pit. (b) *pomcb* expression in the adenohypophysis of the Pit. Abbreviations: NLTv, ventral nucleus lateralis tuberis. Pit, pituitary. Scale bar (if no other indication) = 500 μm

potential role in modulating appetite control (Kalananthan et al., 2021). In zebrafish, *cart2a* (previously named *cart2*) presence in the NRL indicated a role in mediating energy homeostasis (Akash et al., 2014). Thus, the expression of *cart2a* in the salmon PTN near the infundibulum supports the observations that *cart2a* might modulate food intake in salmon (Kalananthan et al., 2021). In Atlantic salmon, *cart2* (*cart2a* and *2b*) seems to be the only *cart* gene with similar potential proteolytic sites as its mammalian homolog, based on their sequence alignment (Kalananthan et al., 2021). POMC is a key regulator in the melanocortin system that is post-transcriptionally cleaved into α- and β-melanocyte-stimulating hormones, adrenocorticotropic hormone, and β-endorphin (Takahashi & Mizusawa, 2013). Here, *pomca* was expressed in the NLT area in the brain of Atlantic salmon parr. This result is in line with previous studies of *pomc* or α-melanocyte-stimulating hormones in goldfish (Cerdá-Reverter, Schiöth, et al., 2003; Forlano & Cone, 2007; Porter et al., 2017), barfin flounder *Verasper moseri* (Amano et al., 2005), zebrafish (Zhang et al., 2012), African cichlid fish (Porter et al., 2017), and rainbow trout (Otero-Rodino et al., 2019). These observations together with the findings of Kalananthan, Murashita, et al. (2020) suggest the involvement of *pomca* in Atlantic salmon appetite control.

4.2 | Expression of melanocortin system neuropeptides in other brain regions

The widespread distribution of *npy* and *cart* in the brain of Atlantic salmon parr indicates various potential functional roles in the central nervous system. The neuropeptides *npya* and *cart2b* were the most abundant, as previously demonstrated (Kalananthan et al., 2021; Tolås et al., 2021). Additionally, *cart2b* expression resembled that of *npy* in brain areas associated with sensory processing, such as its presence in the olfactory bulb, which is known to be linked with processing chemosensory information, immune responses, and reproduction (Ye et al., 2020). This expression is consistent with other studies in teleosts for *npy* (Cerdá-Reverter et al., 2000; Gaikwad et al., 2004; Kaniganti et al., 2021; Pirone et al., 2008; Porter et al., 2017) and *cart* or *cart2b* (Akash et al., 2014; Bonacic et al., 2015; Kalananthan et al., 2021; Le et al., 2016). Interestingly, fasted zebrafish express higher levels of *npy* in the olfactory bulb compared to those fed (Kaniganti et al., 2021), while in Atlantic salmon fasting decreased *npya1* and increased *cart2b* mRNA levels in the olfactory bulb (Kalananthan et al., 2021; Tolås et al., 2021). In fact, *npy* has been suggested to serve as a neurotransmitter, while *cart* is involved in modulating the activity that

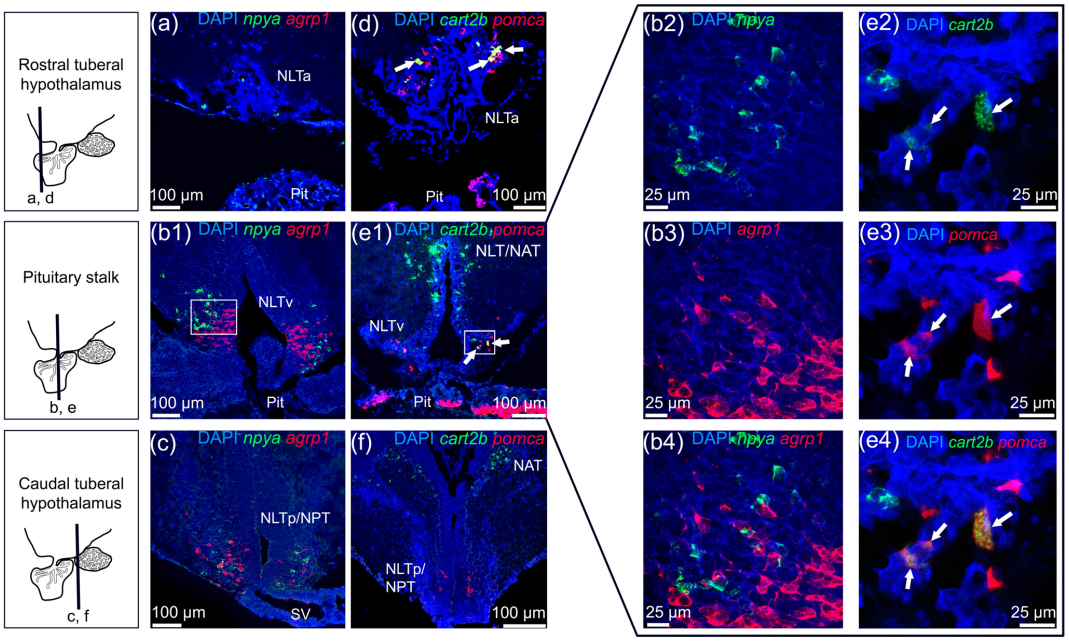

FIGURE 11 *npya*, *agrp1*, *cart2b*, and *pomca* mRNA expression in Atlantic salmon tuberal hypothalamus. Left side: schematic representation of the tuberal hypothalamus indicating the position of each transverse section. (a–c) *npya* (TSA-green) and *agrp1* (FastBlue-red) expression. (d–f) *cart2b* (TSA-green) and *pomca* (FastRed-red) expression. (a) *npya* expression in the NLTa of the rostral tuberal hypothalamus. (b1) *npya* and *agrp1* expression in the NLTv at the pituitary stalk. (b2–b4) *npya* and *agrp1* expression in neighboring neurons in the NLTv. The absence of yellow staining indicates no coexpression between *npya* and *agrp1* mRNA. (c) *npya* and *agrp1* expression in the NLTp/NPT of the caudal tuberal hypothalamus. (d) *cart2b* and *pomca* expression in the NLTa of the rostral tuberal hypothalamus. The presence of yellow staining (white arrows) indicates coexpression of *cart2b* and *pomca* mRNA. (e) *cart2b* and *pomca* expression in the NLTv of the tuberal hypothalamus. (e2–e4) The presence of yellow staining (white arrows) indicates coexpression of *cart2b* and *pomca* mRNA in the NLT. (f) *cart2b* expression in NAT, and *pomca* expression in the NLTp/NPT. Sections were mounted with ProLong Glass antifade medium with NucBlue (Invitrogen). Abbreviations: NAT, nucleus anterior tuberis; NLTa, anterior nucleus lateralis tuberis; NLTp, posterior nucleus lateralis tuberis; NLTv, ventral nucleus lateralis tuberis; NPT, nucleus posterior tuberis; Pit, pituitary; SV: saccus vasculosus

can affect chemosensory processing and food-seeking behavior (Akash et al., 2014; Gaikwad et al., 2004; Kalananthan et al., 2021; Kaniganti et al., 2021; Singru et al., 2008; Tolås et al., 2021). Taken together, these two peptides (*npya* and *cart2b*) may work together or independently in processing and transmitting olfactory sensory information in Atlantic salmon.

npy is expressed in the telencephalon of all teleost species investigated to date (Castro et al., 1999; Cerdá-Reverter et al., 2000; Gaikwad et al., 2004; Le et al., 2016; Pirone et al., 2008; Porter et al., 2017; Saha et al., 2015; Singru et al., 2008; Tolås et al., 2021). In the ventral telencephalon, *npya* was abundantly expressed (Figure 1c), while *npyb* was much less abundant (Figure 2a). Further, *cart2a*, *2b*, and *3a* were also expressed in the ventral telencephalon (Figures 5c, 6b, and 8b). Telencephalon plays a role in sensory input processing connected to various functions such as reproduction (Saha et al., 2015; Uezono et al., 2015), behavior (Comesaña et al., 2018), and appetite control (Ye et al., 2020). Anatomically, the telencephalon has afferent and efferent connections with many brain regions, including the olfactory bulb, preoptic region, and tuberal hypothalamus (Folgueira et al., 2004a, 2004b). Telencephalic *cart* expression is linked with sensory-motor function, while *npy* expression in the telencephalon has been linked to olfactory sensory processing (Singru et al., 2008), suggesting that *npy* might be involved in the hedonic control of food intake in this brain region. Interestingly, zebrafish *cart2* (Akash et al., 2014) and catfish *cart* (Subhedar et al., 2011) decrease in the entopeduncular nucleus during starvation, while fasting had no impact on Atlantic salmon *npy* and *cart* transcripts in the telencephalon (Kalananthan et al., 2021; Tolås et al., 2021). The species-specific *cart* responses indicate that more research is needed to understand the role of telencephalic *cart* in appetite control in teleost species. *agrp2* was strongly expressed in the dorsal telencephalon in Atlantic salmon parr (Figure 4a), which is in line with previous findings in salmon (Kalananthan, Lai, et al., 2020). Opposite to that found in zebrafish, no *agpr2* expression was observed in the pineal cells of Atlantic salmon pa (Shainer et al., 2017, 2019; Zhang et al., 2010). Zebrafish *agrp2* has been found in novel, uncharacterized, nonphotosensitive pineal cells,

in addition to a few neurons in the preoptic region that project to the adenohypophysis, indicating that this neuropeptide is linked to the hypophysiotropic stress axis in zebrafish (Shainer et al., 2017). As suggested in zebrafish, *agrp2* in salmon might have a functional role in the spatial navigation network or a stress response via cortisol and the medial and lateral zones of the dorsal telencephalic serotonergic system (Rodríguez et al., 2021; Silva et al., 2015).

The preoptic region, located rostral to the hypothalamus, is functionally and neurochemically associated with the hypothalamus—including reproduction and sensory processing (Porter et al., 2017). In fact, the preoptic region functions as a key region for downstream signaling as the neurons from the preoptic region may be connected to several brain regions (Folgueira et al., 2004b), and innervate the pituitary via the hypothalamic-neurohypophyseal tract (Akash et al., 2014; Forlano & Cone, 2007; Herget et al., 2014). These signals include serotonergic and corticotropin-releasing factor systems that can affect food intake (Ortega et al., 2013). Preoptic expression of Atlantic salmon *npya* was observed in several subregions, including the SOC (Figure 1e), as it has previously been shown for other teleost species (Cerdá-Reverter et al., 2000; Jeong et al., 2018; Le et al., 2016; Perez Sirkin et al., 2013; Pirone et al., 2008; Porter et al., 2017). Moreover, *cart2b*, *3a*, *3b*, and *4* were expressed in the preoptic region (Figures 4d, 5c, 6b, and 8a), similar to that reported for other teleosts (Akash et al., 2014; Le et al., 2016; Mukherjee et al., 2012; Porter et al., 2017), suggesting that *cart*, like *npya*, might be involved as preoptic neuroendocrine regulators. *npya* was expressed ventrally in the left and right habenula (Figure 1f), which is homologous to the mammalian lateral habenula (Amo et al., 2010) where NPY modulates excitatory transmissions (Cheon et al., 2019). Moreover, lateral habenular NPY might be indirectly linked to the hedonic regulation of appetite in primates (reviewed by Rezitis et al. (2022)). The lateral habenula is indeed a central node connecting rostral and caudal brain regions; afferent connections originate from the nucleus entopeduncularis (ENT, homologous to the globus pallidus in primates), and efferent connections to the median raphe nucleus in the ventral tegmentum (Hikosaka et al., 2008; Turner et al., 2016). Furthermore, *agrp2*, *cart2a*, *2b*, *3a*, *3b*, and *npya* were expressed in the thalamus (Figures 1g, 4b, 6e,d, 7c, and 8d), suggesting an involvement of these neuropeptides in the modulation of sensory inputs to the telencephalon (Folgueira et al., 2004a, 2004b; Singru et al., 2007). In the midbrain, *npyb* was expressed exclusively near NPPv as well as in proximity to NIII (Figure 2b), with no expression observed ventrally toward the hypothalamus. This suggests that *npyb* might not have a direct role in appetite control, which agrees with previous studies on Atlantic salmon (Tolås et al., 2021), tiger puffer *Takifugu rubripes* (Kamijo et al., 2011), and Nile tilapia *Oreochromis niloticus* (Yan et al., 2017).

In zebrafish, visual information is essential to modulate feeding behavior (Muto et al., 2017), and feeding state modulates the activity of sensory processing involved in fine-tuning the response to external stimuli, such as prey capture or avoidance behavior (Corradi & Filosa, 2021). Atlantic salmon *npya* expression in the SPV and scattered cells in SGV of the optic tectum (Figure 1g,l) was in line with previous studies in teleost species (Cerdá-Reverter et al., 2000; Das et al., 2019; Porter et al., 2017). Together, this indicates that *npya* may have a role in both signaling feeding status and visual perception, as suggested previously for Atlantic salmon (Tolås et al., 2021) and zebrafish (Filosa et al., 2016), and also supported by the high expression of *npy* in the salmon eye (Murashita et al., 2009). In the optic tectum, *cart2b* expressed in the SPV (Figure 5e,a,i) was similar to that of *npya*, while *cart2a* and *3a* were expressed in the distal layers of SPV and torus longitudinalis, respectively, (Figures 6d–f and 8d,e) suggesting a role in integrating visual information for the later (Filosa et al., 2016).

In mammals, the EW plays a vital role in the integration and modulation of sympathetic outflow affecting stress and energy homeostasis through orexigenic and anorexigenic projections from the hypothalamic arcuate nucleus and paraventricular nucleus (Cano et al., 2021). In contrast, the EW in teleosts is rarely connected to appetite but it is described to be photosensitive in zebrafish (Hang et al., 2014). In this study, *npya* and *cart3a* were found near EW (Figures 1j(1–3), 6f(1–3)), which is in line with previous studies for *cart* in teleosts, including catfish (Singru et al., 2007) and zebrafish (Akash et al., 2014), and this indicates that further studies are needed to better understand this region. Laminated TS receives inputs from the lateral line and visual system (Pirone et al., 2008), suggesting that *cart2b* and *3b* might be involved in the processing of both visual and lateral stimuli. In the rhombencephalon, *npya*, *b*, *agrp2*, *cart1a*, *3a*, and *3b* were observed proximally to the FLM and nV. The *npy* expression observed here is similar to previous findings in Atlantic salmon and *Gambusia affinis* by NPY-immunoreactivity (Garcia-Fernandez et al., 1992). The expression of these neuropeptides near the nV indicates a possible involvement in food intake and sensory inputs from the oral cavity (Pirone et al., 2008). The expression of *cart1a*, *3a*, and *3b* in the rhombencephalic region suggests that these neurons may innervate the spinal cord and, thus, these neuropeptides may play a role in descending control from the brain stem, as speculated for *cart* in zebrafish (Akash et al., 2014).

The main site for *pomc* expression was the adenohypophysis, in line with previous observations in Atlantic salmon by qPCR (Kalananthan, Lai, et al., 2020; Kalananthan, Murashita, et al., 2020) and other teleost species (Amano et al., 2005; Forlano & Cone, 2007; Otero-Rodino et al., 2019; Zhang et al., 2012). Downstream signaling from the adrenocorticotropic hormone, one peptide produced from *pomc*, is the hypothalamus-pituitary-interrenal axis affecting food intake through glucocorticoid production. Interestingly, starved zebrafish have been shown to have lower cortisol levels than fed fish (Filosa et al., 2016). Downstream signaling from the melanocyte-stimulating hormones includes physiological color change mechanisms and stress response (Segura-Noguera et al., 2000) that indirectly affect food intake. Mapping hypophysiotropic neurons in the hypothalamus by immunocytochemical studies has shown that α-melanocyte-stimulating hormone fibers project from NLT down to the pituitary in zebrafish (Zhang et al., 2012), but not in barfin flounder (Amano et al., 2005). The contradictory effects of *pomca* observed in previous studies might be explained by the end-product of the post-translational cleavage of *pomc*. While α-melanocyte-stimulating hormone has been shown to be a direct suppressor of appetite, β-endorphin can antagonize the α-melanocyte-stimulating hormone downstream signaling pathways

directly (Mercer et al., 2013). Thus, more research is needed to investigate the relationship between *pomc* and appetite and energy balance in vertebrates.

5 | CONCLUSION

This study shows that the Atlantic salmon neuropeptides *npy*, *cart*, *pomc*, and *npy* are expressed in brain regions known to be related to feeding and energy status. This includes the hypothalamus, supporting the hypothesis that the melanocortin system and the NLT region of the hypothalamus are involved in the control of appetite in Atlantic salmon and that this function is conserved across vertebrates. In the Atlantic salmon hypothalamus, a distinct neuronal *npya*, *agrp1*, *cart2b*, and *pomca* expression was found, as well as a few neurons coexpressing *cart2b/pomca*. To what extent does this hypothalamic coexpression affect the physiological regulation of food intake compared to the distinct expression of these neuropeptides in Atlantic salmon is a question that needs further investigation. In addition, our data suggest that several of the neuropeptides investigated might be involved in the control of food intake and energy homeostasis through transmission and processing of sensory signals. This is based on their mRNA expression profile in the olfactory bulb, telencephalon, midbrain, and hindbrain.

AUTHOR CONTRIBUTIONS

Conceptualization: Ivar Rønnestad and Jon Vidar Helvik. *Sampling*: Sissel Norland, Mariann Eilertsen, Jon Vidar Helvik and Ana S. Gomes. *Methodology*, *investigation*, *and analysis*: Sissel Norland, Mariann Eilertsen, Jon Vidar Helvik, and Ana S. Gomes. *Writing-original draft and review and editing*: Sissel Norland, Ivar Rønnestad, Mariann Eilertsen, Jon Vidar Helvik, and Ana S. Gomes.

ACKNOWLEDGMENTS

The authors thank Christine Horne and Mitchell Fleming for assistance during sampling, Tharmini Kalananthan for the donation of *cart2a* plasmid, Rita Karlsen for technical assistance in the lab, and Elsa Denker for assistance with the confocal microscope. The study was funded by the Research Council of Norway (projects LeuSense - 267626; ExcelAQUA-2.0 – 261753; NoFood2Waste - 317770; Photobiol - 254894; Lightbiotrans - 315106) and the Regional Research Fund West (Greenbag - 259183).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this paper are primarily presented within the scope of this publication. Additional materials are available upon reasonable request to the corresponding author.

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ENDNOTE

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How to cite this article: Norland, S., Eilertsen, M., Rønnestad, I., Helvik, J. V., & Gomes, A. S. (2023). Mapping key neuropeptides involved in the melanocortin system in Atlantic salmon (*Salmo salar*) brain. *Journal of Comparative Neurology*, *531*, 89–115. https://doi.org/10.1002/cne.25415

frontiers | Frontiers in Behavioral Neuroscience

TYPE Original Research
PUBLISHED 21 April 2023
DOI 10.3389/fnbeh.2023.1162494

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

RECEIVED 09 February 2023
ACCEPTED 30 March 2023
PUBLISHED 21 April 2023

CITATION
Norland S, Gomes AS, Rønnestad I, Helvik JV and Eilertsen M (2023) Light conditions during Atlantic salmon embryogenesis affect key neuropeptides in the melanocortin system during transition from endogenous to exogenous feeding.
Front. Behav. Neurosci. 17:1162494.
doi: 10.3389/fnbeh.2023.1162494

Light conditions during Atlantic salmon embryogenesis affect key neuropeptides in the melanocortin system during transition from endogenous to exogenous feeding

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During the first feeding period, fish will adapt to exogenous feeding as their endogenous source of nutrients is depleted. This requires the development of a functional physiological system to control active search for food, appetite, and food intake. The Atlantic salmon (*Salmo salar*) melanocortin system, a key player in appetite control, includes neuronal circuits expressing neuropeptide y (*npya*), agouti-related peptide (*agrp1*), cocaine- and amphetamine-regulated transcript (*cart*), and proopiomelanocortin (*pomca*). Little is known about the ontogeny and function of the melanocortin system during early developmental stages. Atlantic salmon [0–730 day degrees (dd)] were reared under three different light conditions (DD, continuous darkness; LD, 14:10 Light: Dark; LL, continuous light) before the light was switched to LD and the fish fed twice a day. We examined the effects of different light conditions (DD_{LD}, LD_{LD}, and LL_{LD}) on salmon growth, yolk utilization, and periprandial responses of the neuropeptides *npya1, npya2, agrp1, cart2a, cart2b, cart4, pomca1,* and *pomca2*. Fish were collected 1 week (alevins, 830 dd, still containing yolk sac) and 3 weeks (fry, 991 dd, yolk sac fully consumed) into the first feeding period and sampled before (−1 h) and after (0.5, 1.5, 3, and 6 h) the first meal of the day. Atlantic salmon reared under DD_{LD}, LD_{LD}, and LL_{LD} had similar standard lengths and myotome heights at the onset of first feeding. However, salmon kept under a constant light condition during endogenous feeding (DD_{LD} and LL_{LD}) had less yolk at first feeding. At 830 dd none of the neuropeptides analyzed displayed a periprandial response. But 2 weeks later, and with no yolk remaining, significant periprandial changes were observed for *npya1, pomca1,* and *pomca2*, but only in the LD_{LD} fish. This suggests that these key neuropeptides serve an important role in controlling feeding once Atlantic salmon need to rely entirely on active search and ingestion of exogenous

food. Moreover, light conditions during early development did not affect the size of salmon at first feeding but did affect the mRNA levels of *npya1, pomca1,* and *pomca2* in the brain indicating that mimicking natural light conditions (LD_{LD}) better stimulates appetite control.

KEYWORDS

***agrp1*, appetite, Atlantic salmon, *cart*, *npy*, photoperiod, *pomc*, yolk**

1. Introduction

Atlantic salmon (*Salmo salar*) is the most successful aquaculture species in Norway and has a considerable economic impact (Iversen et al., 2020). In nature, the life-history transition of Atlantic salmon is closely linked to photoperiod with spawning in rivers taking place in the autumn, hatching in the spring, and the migratory smolt phase occurring in the late spring of the following years (Metcalfe and Thorpe, 1990). In aquaculture production, Atlantic salmon are typically reared in indoor freshwater facilities until they reach the end of smoltification when they are transferred to open net pens in seawater for the outgrowth phase. Under intensive rearing conditions, artificial light, and temperature are actively used to extend the day length and optimize production in indoor facilities compared to the natural environment (Villarreal et al., 1988; Stefansson et al., 1990; Berg et al., 1992; Handeland and Stefansson, 2001; Fjelldal et al., 2011).

Light is an important cue for the regulation of behavior, physiological functions, and rhythms in most vertebrates, including teleosts. In addition to retinal photoreception, which allows fish to visualize their surroundings, a high abundance of extraretinal photoreceptors is found in the central nervous system (Peirson et al., 2009; Perez et al., 2019). This is in line with the activation of specific brain regions following dark-to-light stimulation, such as the habenula, suprachiasmatic nucleus, thalamus, and hypothalamus in Atlantic salmon (Eilertsen et al., 2021). In the pineal organ, light affects melatonin production involved in the regulation of the circadian rhythm (Philp et al., 2000; Perez et al., 2019). The light-sensitive hypothalamus is also a key region for neuroendocrine systems such as reproduction and appetite (Philp et al., 2000; Sandbakken et al., 2012; Delgado et al., 2017; Perez et al., 2019). Indeed, several studies have shown that diurnal fish species have better growth under constant light (LL) (Villarreal et al., 1988; Stefansson et al., 1990; Berg et al., 1992; Jonassen et al., 2000; Ginés et al., 2004; Biswas et al., 2005; Fjelldal et al., 2011; Villamizar et al., 2013; Hou et al., 2019), likely because of the synergetic effect between feed intake and light. The photoperiod is considered the most important synchronizer of biological rhythms; but periodic feeding can also act as entrainment (Boulos and Terman, 1980; Mistlberger, 1994; Bolliet et al., 2001; Sanchez-Vazquez et al., 2019; Steindal and Whitmore, 2019).

Feed intake is also controlled by internal signals, in which several hormones and nerve signals are integrated and controlled centrally in the brain and affect the overall energy homeostasis and growth. When a salmon embryo and alevin feed on the yolk, it has an energetically closed system in which the nutrients in the yolk fully support the development, growth, and metabolism of the organism. The utilization rate of the yolk is limited by hydrolytic enzyme activities and the surface area of the syncytium layer (Skjærven et al., 2003; Kamler, 2008). To secure a steady supply of nutrients, salmon alevins initiate exogenous feeding while there is still some yolk matter left. The onset of exogenous feeding and regulation of appetite are critical for survival and are influenced by factors such as motivation, and prey type, as well as morphological features and integrated physiological functions for the detection, capture, ingestion, digestion, and assimilation of food particles (Rønnestad et al., 2013). During the first-feeding period, little is known about the dynamics of the appetite-controlling network or how the presence of endogenous yolk might influence it. Ontogenetic gene expression of some key peptides involved in appetite control has been investigated in some fish species, including Atlantic salmon (Moen et al., 2010), European eel (*Anguilla anguilla*) (Politis et al., 2018), giant grouper (*Epinephelus lanceolatus*) (Anderson et al., 2018), Atlantic cod (*Gadus morhua*) (Le et al., 2016), and Atlantic halibut (*Hippoglossus hippoglossus*) (Gomes et al., 2015, 2022). In first-feeding marine teleosts, several observations have shown that larvae continue to ingest food even though the gut is already full (Harboe et al., 2009; Rønnestad et al., 2013). This indicates that their satiety (anorexigenic) systems may not be fully developed in the first-feeding stages.

The melanocortin system is among the most studied and best-characterized networks for appetite control in vertebrates. This system is characterized by two major hypothalamic neuronal circuits known to stimulate (orexigenic) or inhibit (anorexigenic) appetite (Schwartz et al., 2000). Key neuropeptides of the melanocortin pathway include neuropeptide y (*npy*), agouti-related peptide (*agrp1*), cocaine- and amphetamine-regulated transcript (*cart*), and proopiomelanocortin (*pomc*) that are shown to respond to fed/fasted state in salmon (Murashita et al., 2009a, 2011; Valen et al., 2011; Kalananthan et al., 2020b, 2021, 2023; Tolås et al., 2021). Because of whole-genome duplication events, Atlantic salmon possess multiple paralogs for most genes (Allendorf and Thorgaard, 1984; Lien et al., 2016). The topological distribution of *npya*, *agrp1*, *cart2b,* and *pomca* has recently been mapped in Atlantic salmon demonstrating that these neuropeptides are expressed in the tuberal hypothalamus, the putative homolog of the mammalian arcuate nucleus, indicating that this is a key region for appetite regulation in teleosts (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003; Norland et al., 2023).

How, and to what extent different light conditions during the endogenous phase affect the exogenous feeding period is not fully understood. Light and feeding regimes may influence the internal clock, which may in turn impact the appetite dynamics. Therefore,

eggs and alevins kept under constant conditions lack zeitgeber, in contrast to individuals kept under a light-dark periodicity that mimics natural light conditions. How this affects the regulatory function of the melanocortin system in first-feeding alevins is not known. This study aimed to describe the effects of three different light conditions (LL, LD, and DD) during development on the mRNA expression of neuropeptides *npya1, npya2, agrp1, cart2a, cart2b, cart4, pomca1,* and *pomca2* before and after a meal during the first feeding period in Atlantic salmon, as well as its effects on the fish growth. Based on the results we propose that the activation of key neuropeptides in the brain is linked to the depletion of the yolk content.

2. Materials and methods

2.1. Ethical statement

All animal treatments were performed according to the national animal welfare legislation and complied with the ARRIVE guidelines (Percie du Sert et al., 2020). As fish did not undergo handling except euthanasia, no special approval was required according to Norwegian National legislation via the Norwegian Animal Welfare Act (LOV-2015-06-09-16-65) and Regulations on the Use of Animals in Experiments (FOR-2017-04-05-451), given by the EU (Directive 2010/63/EU) for animal experiments. All fish were euthanized with metacaine (MS-222^{TM}; MSD Animal Health, Netherlands) on-site, before further handling. The trials were conducted at an approved laboratory facility by the Norwegian Food Safety Authority (VSID 2135) at Bergen High Technology Center (University of Bergen, Bergen, Norway). All personnel involved in the experiment had undergone training (FELASA-C) approved by the Norwegian Food Safety Authority, which is mandatory for running experiments involving animals included in the Animal Welfare Act.

2.2. Experimental design

Two sibling groups of Atlantic salmon eggs and sperm were obtained from Mowi (Tveitevågen, Askøy, Norway) and transferred to a wet lab at the Bergen High Technology Center where eggs were fertilized. The fertilized eggs were randomly divided into three light conditions: continuous darkness (DD), light-dark photoperiod (14:10 LD, 0.1 W/m^2), and continuous light (LL, 0.1 W/m^2) and incubated in racks in egg incubator chambers (45 × 45 × 45 cm, 500 eggs/racks) at 5.7 ± 0.5°C (2 replicate chambers × 3 light conditions, 6 tanks in total; **Figure 1A**). Salmon development was estimated based on the number of day-degrees (dd) from fertilization. One week before the start of exogenous feeding (730 dd), the fish in each light treatment were transferred into feeding tanks (Ø = 60 cm, 3 replicates × 3 tanks, 9 tanks in total). In the feeding tanks, the light was set at 14:10 LD for all tanks at an intensity of 1.0 W/m^2 until the end of the experiment. The temperature was increased from 5.7 ± 0.5°C to 11.5 ± 0.3°C at 730 dd. Fish were fed from 762 dd with a commercial diet (EWOS Micro starter diet, 0.6 mm) twice a day (09:00–09:30 and 16:00–20:00). The amount of feed per day was 4% of the total biomass per tank (total fish wet weight) + 25% excess feed. Of this, 25% was given in the morning and 75% in the afternoon. At 21:30 the day before sampling, the excess feed from the afternoon meal was manually removed from the tanks. The DD_{LD}, LD_{LD}, and LL_{LD} refer to the light treatment applied during the endogenous, and subscript letters refer to the exogenous feeding stages.

2.3. Sampling

To evaluate the effect of light on the fish growth, yolk utilization, and mRNA profile of appetite-controlling genes, fish samples were collected 1 and 3 weeks after the onset of exogenous feeding—830 dd (5 days after exogenous feeding started and some yolk sac remaining) and 991 dd (19 days after feed were introduced and fish yolk sac fully consumed) (**Figure 1B**). Three fish per tank (3 fish × 9 tanks) per sampling point [1 h before feeding (BF) and 0.5, 1.5, 3, and 6 h after feeding (AF) the first meal of the day (**Figure 1C**)] were collected and sacrificed with a lethal dose of MS-222 (200 mg/L). The fish were photographed (NIKON D7500) to measure standard length (SL), myotome height (MH), and yolk sac height, length, and surface area. The head was cut and transferred to RNAlater (Invitrogen, Carlsbad, CA, USA), kept at 4°C overnight, and stored at −80°C. In total, 270 fish were collected in the two sampling days.

2.4. Growth and yolk utilization

Assessment of standard length (SL), myotome height (MH), and yolk sac height, length, and surface area were performed using ImageJ (NIH, Bethesda, MD, USA,[1] RRID:SCR_003070). The specific growth rate (SGR) was calculated using the average SL and MH for each light condition group using Equation 1, where *g1* is the average value at 830 dd and *g2 is* the average value at 991 dd and Δt is the elapsed days between 830 and 991 dd (14 days) (Crane et al., 2020). Yolk length (Ysl) and height (Ysh) were measured to estimate the yolk sac volume using Equation 2 (Rønnestad et al., 1992).

$$\text{Equation 1 : } SGR = 100 \times \left(\left(\frac{g2}{g1}\right)^{\frac{1}{\Delta t}} - 1\right)$$

$$\text{Equation 2: } Yolk\ sac\ volume = \frac{\pi}{6} \times Ysl \times Ysh^2$$

2.5. RNA isolation and cDNA synthesis

Atlantic salmon heads were thawed on ice, and the brains were dissected out of the skull and transferred into new RNAlater. Total RNA was extracted using TRI-reagent (SigmaMillipore, MO, USA) according to the manufacturer's protocol. RNA quantity, purity, and integrity were analyzed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA;

1 https://imagej.net/

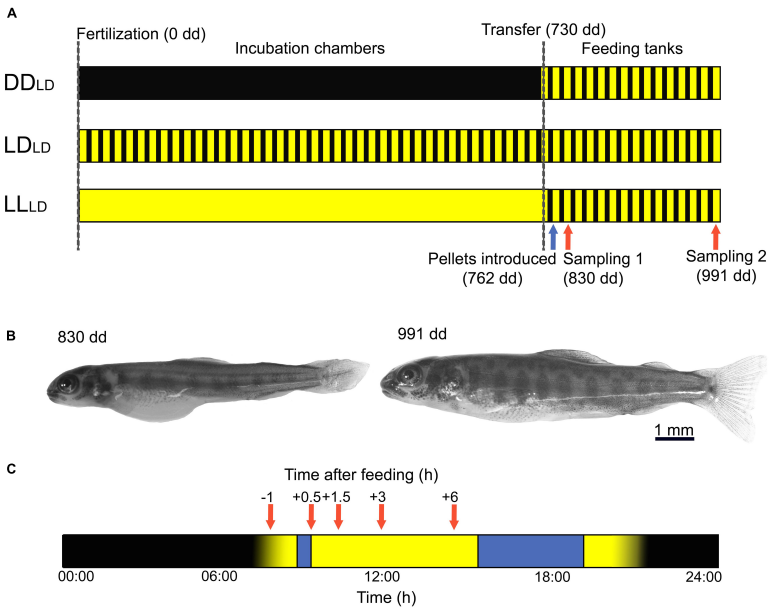

FIGURE 1
Experimental design and sampling of first feeding Atlantic salmon. **(A)** Atlantic salmon were reared under three different light conditions: constant darkness (DD), 14 h light-10 h dark (LD), and constant light (LL) from fertilization (dashed line, 0 dd) until 1 week before the onset of exogenous feeding when alevins were transferred to feeding tanks (dashed line, 730 dd). In the feeding tanks, the light was set to 14:10 LD for all tanks, and pellets were first introduced at 762 dd (blue arrow). Fish were sampled at 1 (830 dd) and 3 weeks (991 dd) after the onset of exogenous feeding (red arrows). **(B)** First-feeding Atlantic salmon alevin with yolk sac at 830 dd; and Atlantic salmon fry with clear cryptic (spotted) coloration at 991 dd. **(C)** The timeline of the sampling days is shown in hours, from 00:00 to 24:00. The light was switched on at 08:00. Fish were fed twice a day (blue box) and sampled (red arrows) before (−1 h) and after (0.5, 1.5, 3, and 6 h) the first meal of the day (9:00–9:30). Blue box: Feeding period. Red arrows: sampling time points. Yellow: Lights on. Black: Lights off. dd, day-degrees.

RRID:SCR_016517) and a 2100 Bioanalyzer (Agilent Technologies, CA, USA; RRID:SCR_018043) with a RNA 6000 Nano Kit (Agilent). DNase treatment and cDNA synthesis were carried out using Quantitect Reverse Transcription kit (cat. # 205313, Qiagen, Germany) according to the manufacturer's protocol.

2.6. Quantitative RT-PCR

The mRNA expression of *npya1, npya2, agrp1, cart2a, cart2b, cart4, pomca1,* and *pomca2* were analyzed in Atlantic salmon whole brains. The genes in this study were chosen based on their importance in appetite control and response to feeding in Atlantic salmon parr and post-smolt (Murashita et al., 2009a, 2011; Valen et al., 2011; Kalananthan et al., 2020a,b, 2021, 2023; Tolås et al., 2021) and their topological distribution in the parr brain (Norland et al., 2023). Primers for the selected neuropeptides were based on previous studies in Atlantic salmon (Kalananthan et al., 2020a, 2021; Tolås et al., 2021; **Supplementary Table 1**). The primer pairs efficiency was determined using a standard curve dilution series (10-fold) generated using each target gene cloned into the pCR4-TOPO vector (Thermo Fisher Scientific). Each assay had an efficiency between 95–103% and R^2 between 0.996–0.999. qPCR assays were performed using 10 µl of iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.4 µM of each forward and reverse primer, 8 µl cDNA template (between 12.5–50 ng), and Ultrapure water (Biochrom, Berlin, Germany) to a total volume of 20 µl reaction. Assays were run on a Bio-Rad CFX96^{TM} Real-Time System (RRID:SCR_018064) under the following cycling conditions: 95°C for 30 s; 40 cycles of 95°C for 5 s, and 60°C for 25 s. Melting curve analysis was performed over a range of 65–95°C (increment of 0.5°C for 2 s). Three controls were included in each plate (no template, minus reverse transcription control and between-plate control). The copy number for each gene was calculated based on the specific standard curve using Equation 3. The copy number was normalized using the ng of total RNA used for the assay (copy number/ng of total RNA) and further normalized to the copy number of the reference gene ribosomal protein s20 (*rps20*).

$$\text{Equation 3 : } copy\ number = 10^{\frac{Cq - intercept}{slope}}$$

2.7. Statistical analysis

Data exploration was conducted to identify outliers and evaluate normality. A generalized linear mixed model (GLMM) with gamma distribution (log-link function) was used to model SL

and MH as a function of light L and age A (Equation 4), the yolk sac surface area and yolk sac volume as a function of L at age 830 dd [no yolk was left at age 991 dd (Equation 5)], and the target gene relative expression as a function of light L, feeding F and age A (Equation 6). Tanks were added as random intercepts to account for possible tank effects.

$$\text{Equation 4 : } n(B_{i,L,A,t}) = b_{0,t} + \beta_1 L_{i,t} + \beta_2 A_{i,t} + \varepsilon_i$$

$$\text{Equation 5 : } n(Y_{i,L,t}) = b_{0,t} + \beta_1 L_{i,t} + \varepsilon_i$$

With B as the biometry parameters SL or MH in fish i at age A 830 dd or 991 dd and light condition L, and Y as the yolk sac area or volume at age 830 dd; $b_{0,t} \sim N(0, \sigma_I)$ as random intercepts for tank t to represent potential differences between tanks (**Supplementary Figure 1**); $\beta_1 L_{i,t}$ as fixed effect of light (factor) and $\beta_2 A_i$ as fixed effect of fish age (factor).

$$\text{Equation 6 : } n(E_{i,L,F,A,t}) = b_{0,t} + \beta_1 A_{i,t} + \beta_2 L_{i,t} + \beta_3 F_{i,t} + \beta_{4,F_{i,t}} L_{i,t} + \beta_{5,F_{i,t}} A_{i,t} + \varepsilon_i$$

With E as the target gene expression by fish i at age A 830 dd or 991 dd and light condition L; $b_{0,t} \sim N(0, \sigma_I)$ as random intercepts for tank t to represent potential differences between tanks (**Supplementary Figure 2**). In addition to $\beta_1 A_{i,t}$ as fixed effect of age (factor), $\beta_2 L_{i,t}$ as a fixed effect of light (factor), and $\beta_3 F_{i,t}$ as fixed effect of feeding time (factor), interaction terms between feeding time and light $\beta_{4,F_{i,t}} L_{i,t}$ and between feeding time and age $\beta_{5,F_{i,t}} A_{i,t}$ were also included. The interactions were added to account for the potential dependency of the effect of light and feeding and between age and feeding. The error distribution was assumed to be Gamma-distributed ε_i $Gamma(\lambda)$ with log-link.

Backward selection was applied based on the Akaike information criterion. The models were evaluated by the residual distributions. *Post-hoc* tests with Tukey approximation were used to analyze pairwise differences and contrasts between groups (light, feeding, and age). Statistical significance was set at $p < 0.05$. All data are presented as mean ± 95% confidence interval. Data exploration and statistical analyses were performed in RStudio (RRID:SCR_000432)[2] R.4.1.1 with the packages *glmmTMB* (Brooks et al., 2017) to fit the model in Equations 4, 5 and 6, *DHARMa* (Hartig, 2022) was used to validate the model fit, (simulated) residual distribution and to evaluate residual distributions, and *emmeans* (Lenth et al., 2018) for *post-hoc* testing.

3. Results

3.1. Effect of light during development on growth

The Atlantic salmon grew significantly ($p < 0.0001$) in SL and MH between the first (830 dd) and second sampling (991 dd) for all light conditions (**Figure 2**; **Table 1**). No significant differences caused by light conditions during development were observed for SL or MH in first-feeding salmon at 830 dd or 991 dd. There were no observable differences for the mean specific growth rate (SGR) between 830 dd and 991 dd for SL or MH between light conditions (**Table 2**).

3.2. Effect of light on yolk utilization

One week into the first feeding (830 dd), the LD_{LD} group had a significantly larger yolk sac surface area ($p = 0.034$) and volume ($p = 0.049$) than the LL_{LD} fish group (**Figure 3**; **Table 1**). A similar trend was found between LD_{LD} and DD_{LD} albeit non-significant. The yolk sac was fully consumed between 1 and 3 weeks after starting exogenous feeding (830 dd and 991 dd) for all light condition groups.

3.3. Light effect on the mRNA expression of appetite-controlling neuropeptides

All target genes were expressed in the brain 1 week into the first feeding period in Atlantic salmon when the alevins (830 dd) still had yolk sac (**Figure 4**; **Supplementary Figure 3**). Light affected the mRNA expression of *pomca2* in fish kept in LD_{LD}, which had a significantly ($p < 0.01$) lower expression in unfed fish (1 h before a meal) compared to the LL_{LD} and DD_{LD} fish (**Figure 4**; **Supplementary Table 2**). At 0.5 h after feeding the mRNA expression of *pomca2* in fish kept in LD_{LD} was significantly lower compared to the LL_{LD} fish group (**Figure 4**; **Supplementary Table 2**). No significant differences were found between DD_{LD} and LL_{LD} groups in the *pomca2* mRNA expression at 830 or 991 dd (**Figure 4**). No significant changes were found for *npya1, npya2, agrp1, cart2a, cart2b, cart4*, or *pomca1* mRNA expression between the light conditions.

3.4. mRNA expression of appetite-controlling neuropeptides in response to a meal

The overall expression of central appetite-related neuropeptides in response to a meal was analyzed 1 h before feeding (BF), and 0.5, 1.5, 3, and 6 h after feeding (AF) in the brain of alevins and fry. For alevins (at 830 dd), no effect of feeding was observed on the mRNA expression of the target genes in any of the light groups (**Figure 4**; **Supplementary Table 2**). In the fry (991 dd), the relative expression of *npya1, pomca1,* and *pomca2* in response to a meal were significantly ($p < 0.05$) affected in the LD_{LD} group. In the LL_{LD} and DD_{LD} groups, the mRNA levels of these genes remained unchanged over time (BF and AF). For the LD_{LD} fry, *npya1* remained unchanged between 1 h BF until 3 h AF, before significantly dropping between 3 and 6 h AF ($p = 0.015$) (**Figure 4**; **Supplementary Table 2**). *pomca1* in LD_{LD} increased after feeding until 1.5 h AF ($p = 0.014$) and remained significantly higher until 6 h AF than the unfed fry (BF) ($p < 0.05$) (**Figure 4**; **Supplementary Table 2**). *pomca2* showed a similar response to *pomca1* in the LD_{LD} fry, increasing

2 http://www.rstudio.com/

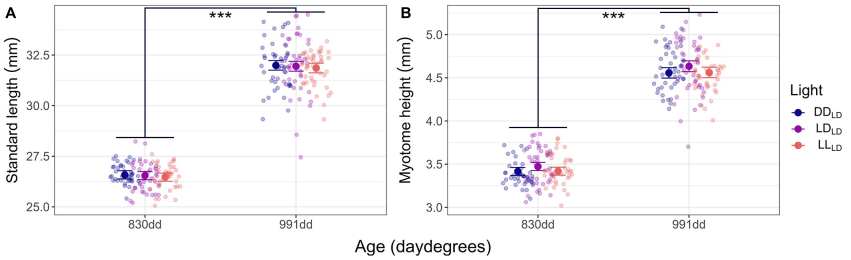

FIGURE 2
Model predicted values and raw data of growth in first feeding Atlantic salmon at 830 dd and 991 dd. **(A)** Standard length (mm). **(B)** Myotome height (mm). The larger dots and lines indicate model-predicted means and 95% confidence intervals, respectively, for each age. Transparent dots represent individual raw data points. Per light condition group at 830 dd $n = 41$ for DD_{LD} and LD_{LD}, and $n = 42$ for LL_{LD}. Per light condition group at 991 dd $n = 44$ for DD_{LD} and LL_{LD}, and $n = 42$ for LD_{LD}. Asterisks show the significant level (*** indicates $p < 0.001$).

TABLE 1 Morphometric data for first feeding Atlantic salmon at 830- and 991-day degrees (dd) (1 and 3 weeks after offered feed for the first time).

Age	830dd			991dd		
Light regime	DD_{LD}	LD_{LD}	LL_{LD}	DD_{LD}	LD_{LD}	LL_{LD}
SL (mm)	26.6 ± 0.71	26.5 ± 0.71	26.5 ± 0.6	31.9 ± 1.15	32.0 ± 1.49	31.8 ± 0.84
MH (mm)	3.39 ± 0.14	3.48 ± 0.22	3.44 ± 0.18	4.59 ± 0.24	4.63 ± 0.33	4.53 ± 0.19
Ysa (mm^2)	19.9 ± 2.68	20.7 ± 2.66	19.2 ± 2.65	–	–	–
Ysv (mm^3)	41.3 ± 9.80	45.4 ± 9.11	40.6 ± 8.20	–	–	–

Larvae were reared under continuous light (LL), 14 h light-10 h darkness (LD), and continuous darkness (DD) until 730 dd, when all fish were transferred to an LD regime. The data are presented as mean ± sd. SL, standard length; MH, myotome height; Ysa, yolk sac surface area; Ysv, yolk sac volume.

significantly ($p < 0.05$) in fed fry (0.5–6 h AF) compared to unfed (Figure 4; Supplementary Table 2). No significant changes were found for *npya2, agrp1, cart2a, cart2b,* and *cart4* mRNA expression.

4. Discussion

This study investigated the effects of three different light conditions during Atlantic salmon development on growth, yolk utilization, and mRNA expression levels of key neuropeptides in the melanocortin system during the transition from endogenous to exogenous feeding. Results showed differences in terms of yolk consumption and activation of the mRNA transcription of the neuropeptides in the brain between light conditions. Additionally, the periprandial mRNA expression for some of the neuropeptides differed between the two stages (830 dd versus 991 dd) and between light conditions.

TABLE 2 Morphometric changes in first-feeding Atlantic salmon from 830 to 991-day degrees (dd) (14 days in between samplings).

Light regime	DD_{LD}	LD_{LD}	LL_{LD}
SL SGR (% day^{-1})	1.31	1.36	1.31
MH SGR (% day^{-1})	2.19	2.06	1.99

Larvae were reared under continuous light (LL), 14:10 L:D, and continuous darkness (DD) until 730 dd, when all fish were transferred to an LD (14:10) regime. SGR, specific growth rate; SL, standard length; MH, myotome height.

4.1. Effect of light on growth

In this study, Atlantic salmon kept under different light conditions during the yolk sac utilization period and equal LD conditions during the first-feeding period (830 dd or 991 dd), showed no significant differences on growth, i.e., SL or MH (Figure 2; Table 1). Thus, light conditions before first feeding seem to not directly affect salmon growth performance during their early development. In contrast, if the light conditions used during development were continued through the exogenous feeding, growth differences would be expected. Several studies have shown that diurnal fish species, including Atlantic salmon, have stronger growth after the onset of exogenous feeding when reared under long days or continuous light (LL) (Villarreal et al., 1988; Stefansson et al., 1990; Berg et al., 1992). However, fast-growing fish kept under LL often display a higher proportion of malformations, such as overinflated swim bladders and cranial deformities (Villamizar et al., 2009, 2014), however, no deformities were observed in the fish sampled in our study. RNA sequencing of whole salmon embryos and alevins has shown that non-visual opsins, which are light-sensing proteins with non-visual functions, are present from an early stage (255 dd) (Eilertsen et al., 2022b). At older stages (parr) these non-visual opsins are expressed in the central nervous system including the pineal organ and deep brain (Philp et al., 2000; Peirson et al., 2009; Sandbakken et al., 2012; Perez et al., 2019). The early expression of non-visual opsins in salmon (Eilertsen et al., 2022b) supports that salmon eggs and alevins can receive different light cues during endogenous feeding; however, based on the results of this study, this does not influence alevin growth during the first feeding period.

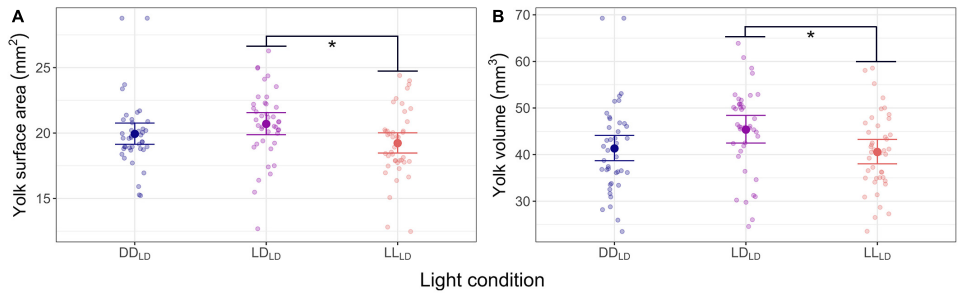

FIGURE 3
Model predicted values and raw data of yolk sac in first feeding Atlantic salmon at 830 dd for the three light conditions (DD_{LD}, LD_{LD}, and LL_{LD}). **(A)** Yolk sac surface area (mm^2). **(B)** Yolk sac volume (mm^3). The larger dots and lines indicate model-predicted means and 95% confidence intervals, respectively, for each light condition group. Transparent dots represent individual raw data points. Per light condition groups n = 41, for DD_{LD} and LD_{LD}, and n = 42 for LL_{LD}. Asterisks show the significant level (* indicates $p < 0.05$).

Most fish are visual feeders around first feeding, which means that they detect and ingest food particles that they can see (Rønnestad et al., 2013; Nikolaou and Meyer, 2015; Eilertsen et al., 2022a). First-feeding Atlantic salmon use ram feeding during the first days of exogenous feeding but gradually change towards suction-feeding, which is a more effective method to catch food supporting their growth (Coughlin, 1991). Here, after 3 weeks of exogenous feeding, no differences in growth were observed between the light condition groups. Our results suggest that the first-feeding Atlantic salmon may maintain equal growth from alevins to fry independent of previous light conditions during development. This is in line with a previous study demonstrating that salmon growth can be leveled out by an increase in food consumption regardless of the light conditions they are exposed to during early life (Villarreal et al., 1988). This indicates the ability of salmon to allocate resources toward somatic growth when the fish is in a surplus of energy.

4.2. Utilization of yolk reserves

In this study, salmon reared in constant light conditions (DD_{LD} or LL_{LD}) had a faster yolk sac utilization rate during endogenous feeding compared to LD_{LD}, which mimics natural conditions. Fish larvae reared under long days and high light intensity seem to have greater metabolism and yolk sac utilization compared to fish kept on short days or low light intensity (Downing and Litvak, 2002; Finn et al., 2002; Finn and Rønnestad, 2003). For example, haddock (*Melanogrammus aeglefinus*) larvae reared under high-intensity light (18 $\mu mol\ s^{-1}\ m^{-2}$) had significantly reduced yolk sac area, which was associated with increased larval activity during light, elevated embryonic metabolism, and growth resulting in increased consumption of endogenous energy reserves (Downing and Litvak, 2002). Moreover, Atlantic cod and turbot (*Scophthalmus maximus*) metabolism is significantly affected by light at an early age (Finn et al., 2002; Finn and Rønnestad, 2003). Our results showed that Atlantic salmon reared under DD_{LD} and LL_{LD} have enhanced energy requirements during endogenous feeding demonstrated by a smaller yolk sac at 830 dd. This is in line with a transcriptomic study on Atlantic salmon reared under different photoperiods (LD, DD, and LL) prior to exogenous feeding, which demonstrated an increased muscle activity and use of energy in constant light conditions compared to LD (Eilertsen et al., 2022b). As in Atlantic salmon, European sea bass (*Dicentrarchus labrax*) absorbed the yolk faster in LL, but slower in DD (Villamizar et al., 2009) contrasting to what was observed here for Atlantic salmon. Previous studies have found that photoperiod manipulation induces an enhanced stress response in fish (Leonardi and Klempau, 2003; Villamizar et al., 2014; Corona-Herrera et al., 2022), but not in all fish species (Biswas et al., 2004). However, how stress may have affected metabolism and yolk utilization in the present study could not be determined.

4.3. Effect of age and light on the mRNA expression of appetite-controlling neuropeptides

Despite the significant industrial importance of Atlantic salmon, little is known about when systems controlling appetite and energy homeostasis are fully established. This study demonstrated that key neuropeptides of the melanocortin system are expressed in the Atlantic salmon brain at first feeding. This is in line with a previous study showing that *npya1, agrp1, cart2b,* and *pomca1* are expressed in the head region of newly hatched Atlantic salmon [named *npy, agrp, cart,* and *pomca1* in Moen et al. (2010)]. The fact that the genes encoding key neuropeptides involved in appetite control are expressed before the first-feeding window is most likely linked with a hardwired activation of systems required for sufficient functionality during the first-feeding period. This also supports the hypothesis that these neuropeptides have roles besides appetite control, as demonstrated by *agpr1* which is required for normal growth in zebrafish (*Danio rerio*) larvae (Zhang et al., 2012).

During ontogeny, the expression level of neuropeptides usually increases as a result of an increased number of neurons, as demonstrated in the development of Atlantic

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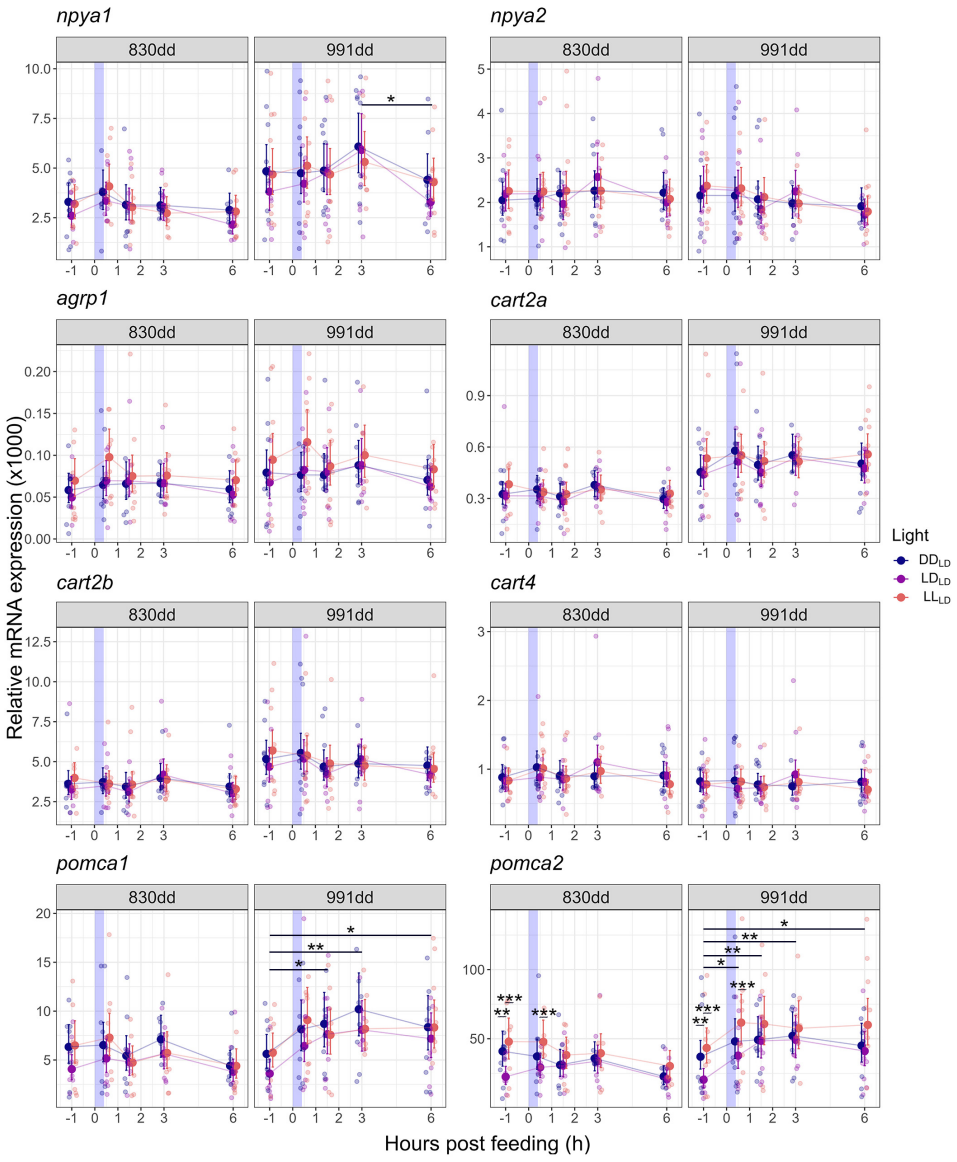

FIGURE 4
Model predicted values and raw data of relative mRNA expression of *npya1*, *npya2*, *agrp1*, *cart2a*, *cart2b*, *cart4*, *pomca1*, and *pomca2* in Atlantic salmon whole brain 1 week (alevins, 830 dd) and 3 weeks (fry, 991 dd) into exogenous feeding in response to a meal and light condition during development. Larger dots and lines indicate model-predicted means and 95% confidence intervals, respectively, for each sampling point and light condition. Smaller, transparent dots represent individual raw data points. Asterisks show the significant level (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$). Please refer to **Supplementary Table 3** for the number of individuals.

salmon (Moen et al., 2010), Atlantic cod (Le et al., 2016), and zebrafish (Mukherjee et al., 2012). In the present study, *pomca2* was lower expressed in the LD_{LD}, which approximates natural light conditions, compared to the continuous light conditions (DD_{LD}, and LL_{LD}) before a meal was given (**Figure 4**). This result may not be solely a response to appetite, given that *pomca2* is a precursor peptide that is cleaved into several peptides with varied functions (Takahashi and Mizusawa, 2013). Lower *pomca2* leads to reduced mRNA translated in peptide synthesis. As a result, the difference in *pomca2* 1 h BF may influence endogenous changes, such as plasma

cortisol which overall influences the food intake. The differences in *pomca2* expression at 1 h BF might be a result of a light-dependent modulation of the "metabolic set-point" of the hypothalamus. The set-point is an important factor in the hypothalamic response to the adrenocorticotropic hormone, a peptide derived from *pomc* cleavage (Henry et al., 2010; Walton et al., 2011). Precisely how the metabolic set-point is established is not fully understood but might involve feedback from photoperiod, melatonin, leptin, and short-term satiation signals (Morgan and Mercer, 2001; Walton et al., 2011). Extraretinal photoreceptors affect endocrine rhythms, i.e., melatonin, glucocorticoids, and thyroid hormone cycles (Perez et al., 2019). Several studies have shown that thyroid hormones play a role in appetite regulation (Kong et al., 2004; Walton et al., 2011; Deal and Volkoff, 2020; Wasserman-Bartov et al., 2022). As a result, light may indirectly affect the metabolic set-point.

4.4. Light conditions during embryogenesis influence the mRNA expression of appetite-controlling neuropeptides in response to a meal

Several reports have suggested that *agrp1, cart2b,* and *pomca1* have anorexigenic roles in Atlantic salmon parr (Murashita et al., 2009a, 2011; Valen et al., 2011). In contrast, *npya* seemed to serve an anorexigenic effect after a meal, but it also had an orexigenic role after 6 days of fasting (Murashita et al., 2009a; Valen et al., 2011). In Atlantic salmon post-smolt, fasting influences the hypothalamic mRNA levels of *agrp1, pomca2, cart2b, cart2a, npya1,* and *npya2* (Kalananthan et al., 2020b, 2021, 2023; Tolås et al., 2021). Fasting significantly increased the hypothalamic *agrp1*, and the expression was negatively correlated with stomach filling, indicating an orexigenic role in post-smolts (Kalananthan et al., 2020b). In the same study, hypothalamic *pomca2* expression was positively correlated with stomach filling (though non-significant), indicating an anorexigenic role. Hypothalamic and midbrain *npya2*; and hypothalamic, midbrain, and olfactory bulb *cart2b* mRNA increased during 3–4 days of fasting indicating orexigenic effects by these neuropeptides (Kalananthan et al., 2021; Tolås et al., 2021). During long-term (4 weeks) fasting, hypothalamic *npya1, cart2a,* and *cart2b* displayed an orexigenic role (Kalananthan et al., 2023).

Different light conditions during endogenous feeding have affected the mRNA expression profiles of key appetite-controlling neuropeptides in response to a meal during the first-feeding period. However, periprandial alterations in *npya1, pomca1,* and *pomca2* mRNA levels were only found in 991 dd fish reared under LD_{LD}, which emulates natural light conditions (**Figure 4**). The LD-regime is an environmental cue used to entrain endogenous rhythms and, thus, fish kept in LD exhibit greater rhythmic amplitudes (Isorna et al., 2017). In our study, only LD_{LD} had a significant periprandial response, suggesting that photoperiod and endogenous appetite may be synchronized during development. The expression of *npya1* significantly decreased between 3 h AF and 6 h AF, indicating either a post-prandial response to the morning meal, or a pre-prandial response to the second meal of the day. This result is in line with the increased whole-brain *npya* expression in the hours after feeding in Atlantic salmon parr maintained under 12:12 LD (Valen et al., 2011). However, whole-brain *npya* expression after 6 days of fasting in Atlantic salmon parr indicated an orexigenic role (albeit non-significant) (Murashita et al., 2009a). Spatial distribution analysis have demonstrated that *npya* mRNA is expressed in the lateral tuberal hypothalamus neighboring *agrp1*-positive cells, indicating an important hypothalamic region in appetite control in salmon (Norland et al., 2023). Hypothalamic *npya1* increased during long-term fasting indicating an orexigenic effect under a natural simulated photoperiod, while *npya2* displayed an orexigenic trend after 4 days of fasting, albeit non-significant, under continuous light (Tolås et al., 2021; Kalananthan et al., 2023). In salmon, *npya* is also expressed in several brain regions, including the telencephalon, optic tectum, and thalamus (Norland et al., 2023). Thus, *npya* is present in brain regions linked with sensory inputs driving behavioral modulations and food intake (Filosa et al., 2016; Chen et al., 2018; Wee et al., 2019, 2022; Corradi and Filosa, 2021; Norland et al., 2023). In this study, the *npya1* expression was higher than that of *npya2* during the first-feeding period, which is in line with older stages (Tolås et al., 2021). The duplication of *npya* is a result of the salmon id-specific genome duplication (Tolås et al., 2021). Thus, the periprandial responses of *npya1* and *a2* might be a stage-specific subfunctionalization, reflecting possible neural plasticity in Atlantic salmon allowing the fish to adapt to different environmental conditions during different life stages.

In the current study, *pomca1* and *a2* showed a clear anorexigenic effect in the LD_{LD} group, with significantly higher mRNA expression after feeding at 991 dd (**Figure 4**). The main sites of *pomca* expression are the adenohypophysis and basal hypothalamus (Norland et al., 2023). Increased whole-brain *pomca1* expression was observed after feeding in Atlantic salmon parr kept under 12:12 LD, but no change in *pomca2* expression was observed between fasted and fed states (Valen et al., 2011). Atlantic salmon post-smolt kept under a natural simulated photoperiod had elevated expression of hypothalamic *pomca1* and *a2* after 6 weeks of fasting, indicating an orexigenic effect (Kalananthan et al., 2023).

4.5. Behavioral aspects and transition to exogenous feeding

To be successful, alevins and fry need to detect, capture, ingest, digest, and assimilate food items for exogenous feeding (Rønnestad et al., 2013). The feeding state of fish modulates the activity of sensory processing involved in fine-tuning the response to external stimuli, such as prey capture or avoidance behavior (Corradi and Filosa, 2021). Thus, we speculate that the first-feeding salmon need a motivator (i.e., hunger or desire to eat) that will influence their behavior and locomotor performance during food capture. An example is that increased swimming activity will decrease the time to capture food. This behavioral plasticity highlights the role of appetite in fish larvae. In this study, we demonstrated that the mRNA expression of key neuropeptides in the melanocortin system responds to a meal in Atlantic

salmon fry, but not in alevins. This may indicate that hunger and thus feeding behavior are not fully stimulated, and that ingestion of prey provides no or a weak signal for satiety in alevins. The fact that these neuropeptides show periprandial changes in expression in fry suggests that the feedback loops and neuronal control used to stimulate feeding behavior, ingestion, and digestion of exogenous nutrients are functional once the yolk is consumed.

The regulation of feeding behavior includes responses from peripheral tissues and central regulation in the brain. Compared to marine fish larvae, Atlantic salmon alevins have a differentiated gastric digestive system and are already able to digest formulated feed pellets from the onset of exogenous feeding, which simplifies the transition between endogenous and exogenous nutrient sources (Sahlmann et al., 2015). This is supported by the upregulation of ghrelin [mainly expressed in the stomach (Murashita et al., 2009b)], trypsin (secreted from the pancreas), cholecystokinin, leptin, and peptide yy around the first-feeding period to support the demands for developing salmon (Moen et al., 2010; Sahlmann et al., 2015). The peptides derived from the gut interact with the hypothalamic receptors on neurons expressing key neuropeptides of the melanocortin system. As a result, the salmon have a digestive system ready to digest food particles at the onset of first-feeding period, but before *npya1, pomca1,* and *a2* display periprandial responses in the brain. In general, the gene expression levels of the central melanocortin system in the brain reflect the metabolic state of an organism (Cone, 2005). However, studies have shown that the young brains of vertebrates are believed to be relatively insensitive to metabolic cues or that hypothalamic neurons fail to relay signals to other brain regions [reviewed by Coupe and Bouret (2013)].

Our results indicate that the first-feeding period is key in the development of rhythmic gene expression. However, the expression of key neuropeptides of the melanocortin system in first-feeding salmon was less in accordance with what is observed in older stages (Kalananthan et al., 2021, 2023; Tolås et al., 2021). This indicates an underdeveloped regulatory system different from that of older stages (Kalananthan et al., 2023), that requires time to became fully functional, as suggested for Senegalese sole (*Solea senegalensis*) (Bonacic et al., 2016; Navarro-Guillén et al., 2018) and Atlantic halibut (Gomes et al., 2015).

5. Conclusion

Our results indicate that the neuropeptides involved in the melanocortin system become actively involved in controlling feeding in Atlantic salmon when fry becomes fully dependent on exogenous feeding for energy supply. The relative expression of *npya1, pomca1,* and *pomca2* displayed a periprandial response at 991 dd when the yolk had been completely consumed, indicating that the melanocortin system plays a role in the regulation of appetite in Atlantic salmon fry and onward. Interestingly, light conditions during the development and endogenous feeding period significantly affect the periprandial mRNA levels of key appetite-controlling neuropeptides in the brain during the first-feeding window, but only for larvae reared under the LD_{LD} regime from fertilization which resembles light conditions in nature. Atlantic salmon kept under constant light conditions (DD_{LD} or LL_{LD}) during development and endogenous feeding period did not display the periprandial response of any of the key neuropeptides investigated. Thus, even though growth was not affected, we postulate that fish kept under constant conditions lack a zeitgeber, in contrast to fish kept under a light-dark periodicity from fertilization. This emphasizes that the first feeding period is a key developmental stage for the development of a rhythmic expression of several genes related to appetite control.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because the fish did not undergo handling except euthanasia. Thus, no special approval was required according to Norwegian National legislation via the Norwegian Animal Welfare Act (LOV-2015-06-09-16-65) and Regulations on the Use of Animals in Experiments (FOR-2017-04-05-451), given by the EU (Directive 2010/63/EU) for animal experiments.

Author contributions

ME, JH, IR, AG, and SN planned and prepared the study. ME, JH, SN, and AG conducted the experiment. SN and AG performed the sampling, lab work, and statistical analyses. All authors contributed to the interpretation of the data, writing of the manuscript, read, and approved the final version.

Funding

This study was funded by the Research Council of Norway (projects LeuSense–267626; ExcelAQUA-2.0—309368; NoFood2Waste–317770; Photobiol–254894; and Lightbiotrans–315106) and the Regional Research Fund West (Greenbag–259183).

Acknowledgments

We thank C. Horne and R. Karlsen for assistance during sampling and Dr. A.-E. O. Jordal for technical assistance in the lab at UIB. We also thank Dr. F. Zimmermann at the Institute of Marine Research for inputs on the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2023.1162494/full#supplementary-material

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Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

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ISBN: 9788230842966 (print)
9788230849149 (PDF)