Mapping the pattern of essential neuroendocrine cells related to puberty and *VA opsin* expression provides further insight in the photoreceptive regulation of the BPG axis in Atlantic salmon (*Salmo salar*)

Running head: Expression of neuroendocrine factors and *VA opsin* in Atlantic salmon

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Abstract

In Atlantic salmon (*Salmo salar*) seasonal photoperiod is shown to regulate the onset of sexual maturation, yet which brain region(s) are involved and how light information impacts the neuroendocrine system are still not fully understood in teleosts. Detailed knowledge about the photoperiodic regulation of maturation in fish is still missing. In birds, it is shown that gonadotrophin releasing hormone (GNRH) is located in the same neurons as vertebrate ancient (VA) opsin suggesting a direct photoreceptive regulation for onset of sexual maturity. This study presents a comprehensive topographic mapping of *gnrh2*, *gnrh3*, *kisspeptin 2* (*kiss2*) *gonadotrophin inhibiting hormone* (*gnih*) and *VA opsin* using *in situ* hybridization on mature Atlantic salmon brains. Neurons positive for *gnrh3* are expressed in the olfactory bulb and ventral telencephalon while *gnrh2* positive neurons are located dorsally in midbrain tegmentum. *Gnih* expressing cell bodies are present in the ventral thalamus and extend caudally to the hypothalamus with *kiss2* expressing cells appearing in a lateral position. *VA opsin* positive cells are present in the telencephalon, the rostro-dorsal ring of left habenula, the ventral thalamus and the midbrain tegmentum. The results show no similar co-location as found in birds, hypothesizing that the photoreceptive modulation of Gnrh in salmon may interact through neuronal networks. The topography analyses of the essential neuroendocrine cells related to sexual maturation in Atlantic salmon brain show that diencephalic (thalamus, hypothalamus) and midbrain (tegmentum) regions seem central for controlling sexual maturation.

Introduction

Seasonal oscillation of photoperiod is a major zeitgeber for the commence of sexual maturation in temperate fish species such as the Atlantic salmon [Migaud et al., 2010]. As in other vertebrates, sexual maturation in fish is regulated by the brain-pituitary-gonadal (BPG) axis [Weltzien et al., 2004]. Brain regions of the BPG axis include specific brain neurons that produce and release the neuroendocrine hormones: Gonadotropin releasing hormone (Gnrh), Gonadotropin inhibiting hormone (Gnih) and Kisspeptin (Kiss) that together with various neurotransmitters control the downstream synthesis and release of Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) from the pituitary [Di Yorio et al., 2019; Weltzien et al., 2004; Kitahashi et al., 2009] . In seasonal teleost breeders such as Atlantic salmon light periodicity cues are important to synchronise the onset of maturation to a specific time of year [Migaud et al., 2010]. Although the BPG axis has been mapped in many teleosts the knowledge about the light induced timing of sexual maturation is still fragmented [Irachi et al., 2021]. Photoreception and melatonin production in the pineal organ is the major system for circadian synchronizing in teleost and is also important for annual light rhythmicity [Ekström & Meissl, 1997; Falcón et al., 1987] In recent decades, understanding of the photoreceptive brain has increased dramatically by detection of nonvisual opsins in various brain regions of several vertebrate species [Davies et al., 2010; Perez et al., 2019]. This suggests direct photo-regulation of components of the BPG axis, similar to birds where the Gnrh neurons are directly photoreceptive, containing the nonvisual opsin VA opsin [García-Fernández et al., 2015]. To verify such direct photo-regulatory control in Atlantic salmon the cellular location of the central

neuroendocrine regulatory factors in the BPG axis are mapped and potential colocalization of VA opsin has been studied.

VA opsin is one of the best characterised nonvisual opsins which was first identified in Atlantic salmon [Soni & Foster, 1997]. It is part of the vertebrate opsin group consisting of visual and nonvisual opsin accompanied by pinopsins, parapinopsins and parietopsins [as reviewed in Davies et al., 2010; Shichida & Matsuyama, 2009]. In zebrafish, two VA opsin paralogues (*valopa* and *valopb*) have been identified, where one of the genes has two isoforms, *VA opsin long* and *VA opsin short* [Kojima et al. 2000; Kojima et al., 2008]. Both *valopa* and *valopb* have been found to have diurnal changes in expression in the thalamus, where *valopa* peaked in the evening and *valopb* peaked in the morning. The change in *valopb* expression was shown to be regulated by light while *valopa* was independent of light indicating that VA opsin is, at least in part, involved in deep brain light and time dependent physiology [Hang et al., 2015]. In addition, deep brain VA opsin long has been linked to maturation in goldfish as an increase of mRNA expression of various maturation related hormones was observed when injecting goldfish with VA opsin long [Choi & Choi, 2018]. Phylogenetic analyses of nonvisual opsins in teleosts have suggested that one of the VA opsin genes has been lost after the third whole genome duplication in Euteleosteomorpha. The fourth whole genome duplication in salmonids has however provided Atlantic salmon with two paralogues of VA opsin [Beaudry et al. 2017].

In most teleost species studied to date, when conditions are appropriate for sexual maturation, releasing factors such as Gnrh, Gnih and Kiss will be transmitted from the brain through hypothalamic nerve fibres to the pituitary, resulting in production of gonadotropins [Weltzien et al., 2004]. Topographic mappings of these neuroendocrine hormones are an important part of clarifying their potential roles and possible interactions with photoreceptors.

Gnrh is a key factor for sexual maturation for a wide range of animals and is predicted to have an evolutionary history dating back 650 million years ago [Kah et al., 2007]. In salmonids, *gnrh2* and *gnrh3* have been described revealing *gnrh3* cell bodies in the olfactory bulb, ventral telencephalon and preoptic area, while *gnrh2* cell bodies are located in the midbrain tegmentum [Amano et al., 1991; Bailhache et al., 1994]. In zebrafish, *gnrh2* cells is also located in the midbrain tegmentum and *gnrh3* cells are located in the olfactory bulb, ventral telencephalon and hindbrain [Steven et al., 2003]. Further, Gnrh3 has been located in the saccus vasculosus (sv) of Atlantic salmon [Chi et al., 2017], which is indicated to be the site of photoperiodic input in Masu salmon shown by opsin expression in the sv [Nakane et al., 2013].

Kisspeptins were first described to have a suppressor role in melanoma in humans [Lee et al., 1996], but was later found to play a role in sexual maturation, as loss of function in the KiSS1 receptor leads to impairment of pubertal development in human and mice [de Roux et al., 2003; Seminara et al., 2003]. Kisspeptins are also important for reproductive functions in fish where Kiss2 stimulates *lh* and *fsh* gene expressions in zebrafish. The expression pattern was located in the posterior tuberal nucleus and the periventricular hypothalamic nucleus [Kitahashi et al.,

2009]. Kiss is suggested to be regulated by photoperiod both in the Syrian hamster [Revel et al., 2006] and Atlantic salmon [Chi et al., 2017].

Gonadotropin inhibiting hormone (Gnih) is the first peptide discovered to inhibit the release of gonadotropins in vertebrates [Tsutsui et al., 2000]. Since its discovery, GnIH and its inhibitory effect on the release of gonadotrophs has been quite clear in the avian [Tsutsui et al., 2000] and mammalian clades [Son et al., 2016; Tsutsui, 2009; Tsutsui et al., 2010]. However, the function and mechanisms of this peptide amongst teleost fish seem to vary dramatically between species and no clear role has been established [Di Yorio et al., 2019]. It has been shown that in goldfish Gnih stimulates the release of Fsh, Lh and in Sockeye salmon the release of growth hormone (Gh) [Amano et al., 2006]. Gnih is also suggested to be a positive regulator of reproductive endocrinology in Nile tilapia [Biran et al., 2014] and not an inhibitor as previous research from mammalian and avian clads suggests. A neuroanatomical study in zebrafish has located Gnih in the ventral hypothalamus by immunolabelling [Spicer et al., 2017].

Supporting evidence from other vertebrate clades suggests there is much to gain by better understanding the neuroanatomy of the neuroendocrine system and its relationship to the photoreceptor system in teleost fish. This knowledge is essential for better understanding the regulatory mechanisms controlling the photoreceptive modulation of sexual maturation in fish. Atlantic salmon is an important economical species for aquaculture where controlled light conditions are central for regulating life history transitions like sexual maturation. Early sexual maturation is a major issue in the industry and a deeper understanding of the topic is important for increasing yield, profits and animal welfare. In this study, possible relationships between deep brain photoreceptor cells and key factors for sexual maturation in the anadromous Atlantic salmon are investigated. Here, we present a thorough topographic neuroanatomical mapping of the expression pattern of *gnrh2*, *gnrh3*, *kiss2*, *gnih* and *VA opsin* in the Atlantic salmon brain. We find several regions where both expression of *VA opsin* and essential factors for sexual maturation are present, such as the telencephalon, hypothalamus and midbrain tegmentum. However, there is no cellular co-expression of *VA opsin* and maturation hormones as shown in birds.

4. Materials & methods

The protocols for perfusion fixation, total RNA isolation and *in situ* hybridization involve safety hazards posed by chemicals and reagents. See updated safety datasheet from producer for chemicals and reagents used in these protocols. The ARRIVE guidelines have been complied in this study [Percie du Sert et al., 2020].

4.1 Animals

In total 15 Atlantic salmon postsmolt mature and postsmolt immature males (500-1250g) were sampled at the Institute of Marine Research at Matre research station. The fish originated from the all-male populations recently described in [Fjelldal et al., 2020] and were from the dam 4 x sire 1 cross. The fish were created in November 2016 and were first fed in the spring 2017 under continuous light and 12C, and subsequently temperature was changed to ambient at

summer solstice 2017 and photoperiod shifted to stimulated natural on 01 October 2017. The currently used fish were kept in freshwater until sampling on 23 October 2018. At this stage, the males were either immature postsmolts or fully mature jacks (mature postsmolts) which had running milt. At sampling, the fishes were euthanized with a lethal dose of methacaine [Ackerman et al., 2005] (MSD Animal Health, The Netherlands) and fixated by cardiovascular perfusion with 4% paraformaldehyde after death. All experiments described were given ethical approval from the Norwegian Food Safety Authority and follow the local animal care guidelines [Fjelldal et al., 2020].

4.2 Molecular cloning of gnrh2, gnrh3 and kiss2

Total RNA was isolated from the brain of a mature Atlantic salmon by TRI reagent (Sigma, St. Louis, MO, USA) in accordance with the producer's protocol. Total RNA was DNase treated by using TURBO DNA-free™ Kit (ThermoScientific, Waltham, MA, USA) and cDNA was reversely transcribed by the SuperScript III kit (Invitrogen, Carlsbad, CA, USA) as recommended by the producers. *In silico* analyses of the genes were conducted using the Atlantic salmon genome (Lien et al., 2016) in BLASTP or BLASTN on NCBI (Sayers et al., 2018) and if possible the primers were placed in the UTR or in the start/stop codon to provide full-length gene sequences. Two paralogues of Gnrh2, Gnrh3, Kiss2 and Gnih was found but only *gnrh2a*, *gnrh2b*, *gnrh3b*, *kiss2a*, *gniha* and *gnihb* were cloned, however this was satisfactory for generating paralogue specific probes. Amplification was conducted with 35 cycles using Advantage 2 PCR kit (TaKaRa, Japan), with a nested approach for *gnrh2*, and PCR product were extracted from agarose gel by MinElute® Gel Extraction Kit (Qiagen®, Germany). StrataClone PCR Cloning Kit (Aglient Technologies, LA Jolla, CA, USA) was used for cloning and QIAprep® Spin Miniprep Kit (Qiagen®, Germany) for purification of the plasmids. Samples were prepared for sequencing using BigDye™ Terminator v3.1 (ThermoScientific, Waltham, MA, USA) and sequencing was conducted at the sequence facility at University of Bergen.

4.3 mRNA probe synthesis

Digoxigenin (DIG) –labelled mRNA probes were prepared following the manufacturer's instructions (Roche Diagnostics, Germany). Purified PCR product was used as template in the synthesis as described by Thisse and Thisse [Thisse and Thisse, 2008], and LiCl, EtOH and tRNA were used to precipitate the synthesized probes (Roche Diagnostics, Germany). Primers used to amplify PCR product is presented in **Table 1**. The opn4 probe is a mixture of three probes, specific for the *opn4m1a1*, *opn4x1*a and *opn4x1b1*/*opn4x1b2* generated by our previous work in [Sandbakken et al., 2012]. The *sws1* probe covers the entire ORF of the gene [Dann et al., 2004]. The VA opsin probe is specific for both paralogues. A mixture of paralogue specific probes was made for all maturation hormones, *gnrh2*, *gnrh3*, *kiss2* and *gnih*. Individual expression patterns were verified by running the paralogue specific probes in separate *in situ* hybridization reactions (data not shown). Sense probes were made and sense *in situ* hybridization reactions were conducted as verification for the associated antisense probe (data not shown).

4.4 In situ hybridization (ISH)

ISH on sections was conducted as described in [Sandbakken et al., 2012]. Briefly, brains were mounted in Tissue-Tek® O.C.T.™ Compound (Sakura®, Netherland) and sectioned using Leica CM3050 S Research Cryostat (Leica Biosystems, United States). The brain presented in the result figures is from a mature male, except from the brain in **Figure1d4**,**5** which was immature. ISH was done on five additional brains to confirm and control the expression patterns presented. Sections were rehydrated and treated with proteinase K (Sigma, St. Louis, MO, USA) to increase availability for the probe to enter the cells. The tissue was also treated with triethanolamine and acetic anhydride (Sigma, St. Louis, MO, USA) to reduce background staining. Sections were dehydrated and mRNA probe hybridization reaction was left overnight at 65^0C. All excessive probe was washed away the next day, and the tissue was treated with RNase A (Sigma, St. Louis, MO, USA) to remove partially bound probe. Sections were washed in a solution containing 2xSSC, 0.05% Triton X-100 (Sigma, St. Louis, MO, USA) and 2% Blocking reagent (Roche Diagnostics, Germany) for 3 hours. Antibody solution for DIG (anti-digoxigenin conjugated with alkaline phosphatase, Fab fragments (1:2000), Roche Diagnostics, Germany, RRID: AB_514497) was later introduced and left overnight at room temperature. Excessive antibody was thoroughly washed away, and 4-Nitro blue tetrazolim chloride solution (NBT) and BCIP® *p*-toluidine salt, X-phosphate *p*-toluidine salt (BCIP) (Roche Diagnostics, Germany) substrate was applied for visualization. Nissl staining was conducted with 0,35% Cresyl Violet (Chroma-Gesellschaft, Germany) on parallel sections.

4.5 Slide scanning and image processing

Pictures were taken using the ZEISS Axio Scan.Z1 (Zeiss, Germany) and ZEN software (Zeiss, Germany) with brightfield setting and 20x magnification. Adobe Photoshop CC (San Jose, CA, USA) was used to arrange the panels, adjust brightness and contrast of the pictures and display the schematic overview of the expression patterns.

2. Results

2.1 Expression pattern for gnrh2, gnrh3, kiss2 and gnih in the Atlantic salmon

The maturation hormones studied are all suggested to have an essential role in the regulation of the onset of sexual maturity in the Atlantic salmon. Expression of all the maturation hormones studied in the mature Atlantic salmon brain reveal several maturation hormone expressing cells in the olfactory bulb, telencephalon, diencephalon and tegmentum (shown in **Fig. 1**). The topographic analyses were done in brains of both immature and mature salmon, giving similar results, here presented in a mature brain (see Supplementary figure).

Beginning in an anterior to posterior direction, the expression pattern starts with *gnrh3* in the olfactory bulb, in the mitral cells (shown in **Fig. 1a1-a5**), and this expression extends to the ventral part of the telencephalon in the region of tractus olfactorius medialis (shown in **Fig. 1b1-b5**). The *gnih* expression is detected in the diencephalon, in the ventral thalamus (thv), close to the third ventricle (shown in **Fig. 1c1-c5**). *Gnih* also appears at the same section as *kiss2* (shown in **Fig. 1d1-d5**), where *gnih* expression is located in the region of nucleus magnocellularis hypothalamic, close to the paraventricular organ (pvo) (shown in **Fig. 1d4**).

The *kiss2* positive cells are located in the same region, but in a more lateral position (shown in **Fig. 1d5**). *Kiss2* expression extends in a caudal direction, still in the region of nucleus magnocellularis hypothalamic, close to the infundibulum (inf) (shown in **Fig. 1e1-e5**). The *gnrh2* positive cells are first discovered in the midbrain tegmentum most likely in the nucleus oculomotorius (shown in **Fig. 1f1-f3**), and this expression extends caudally in the same location but in a slightly different pattern (shown in **Fig. 1e1-e5**).

2.2 Expression pattern of VA opsin in the mature Atlantic salmon brain

VA opsin expression is previously described in the diencephalon and midbrain of the Atlantic salmon for juvenile Atlantic salmon parr (freshwater stage) [Philp et al., 2000; Sandbakken et al., 2012]. Figure 2 gives an extended description of the *VA opsin* expression in the brain of a sexually mature Atlantic salmon. *VA opsin* expression is first detected in the dorsal telencephalon, as a scattered pattern in the dorsal and lateral regions (shown in **Fig. 2a1-a5**). *VA opsin* positive cells are also detected in the dorsal ring in the most rostral part of the left habenula (hab) (shown in **Fig. 2b1-b5**). The expression is detected in the transition between the most ventral part of the habenula and the dorsal thalamus (shown in **Fig. 2c1-c5**), and further extends to the dorsal thalamus (thd) (**Figure 2d1-d5**), and into the more caudal regions of dorsal thalamus (thd), where the expression pattern is altered into a different pattern (shown in **Fig. 2e1-e5**). There are some *VA opsin* positive cells in the in the dorsal tegmentum in a ventro-lateral position to the valvula (valv) (shown in **Fig. 2f1-f5**).

2.3 In situ hybridization results in the saccus vasculosus (sv)

The sv has been suggested to be the site of the photoperiodic regulation of sexual maturation [Nakane et al., 2013] and was therefore an important site of investigation in our study. **Figure 3** shows *in situ* hybridization results from the sv using *gnrh2*, *gnrh3*, *kiss2* and *gnih* as a mixture of probes (shown in **Fig. 3a1-a2**) and *sws1* mRNA probe (shown in **Fig. b1-b2**) and *opn4* mRNA probe (shown in **Fig. 3 c1-c2**) on a sexually mature Atlantic salmon brain. No expression is detected. An overview of the expression pattern described in this study is presented in Figure 4.

3. Discussion

Atlantic salmon life history transitions such as smoltification and sexual maturation are strongly linked to seasons, and photoperiod oscillation is an important cue that synchronizes these biological events to annual periods [Migaud et al., 2010]. To understand the brain organization of the neuroendocrine system involved in sexual maturation an expression analysis of *gnrh2*, *gnrh3*, *gnih* and *kiss2* along with the investigation of co-localization of deep brain *VA opsin* expressing cells have been performed in this study. An overview of the expression patterns located is presented in **Figure 4**. The neuroendocrine cells regulating onset of maturation have to some extent been described in salmonids, by characterization of the Gnrh system. In accordance with our results, expression of *gnrh3* has been detected in the ventral olfactory bulb, extending caudally to the ventral telencephalon in Atlantic salmon [Bailhache et al., 1994] and *gnrh2* positive cells is found in the midbrain tegmentum of Masu salmon [Amano et al. 1991]. However, here for the first time we show a detailed localization of central hormones in a mature

Atlantic salmon brain (shown in **Fig. 1**). Mapping the expression pattern of *gnrh2*, *gnrh3*, *kiss2* and *gnih* revealed distinct clusters of cells close to the third ventricle and the infundibulum. We find a prominent *gnih* positive cluster in the ventral thalamus that is only a single cell layer away from the third ventricle (shown in **Fig. 2c**). This is similar to immunohistochemistry findings in zebrafish adult brain [Spicer et al., 2017]. In the hypothalamus we find *gnih* expression close to the infundibulum where the paraventricular organ is located and interestingly *kiss2* positive cells are located just laterally to the *gnih* expression (shown in **Fig. 2d5**). The fact that both the peptide [Spicer et al., 2017] and mRNA from our results are found in the same region could suggest a paracrine action of Gnih cells in communication with nearby Kiss2 cells.

The current study describes *VA opsin* expression for the first time in the mature Atlantic salmon brain (shown in **Fig. 2**). In agreement with previous studies at the parr stage, *VA opsin* was detected in the rostro-dorsal ring of the left habenula [Sandbakken et al., 2012], in the transition between ventral habenula and the dorsal thalamus, and in the more caudal parts of the dorsal thalamus [Philp et al., 2000] (shown in **Fig. 2b-e**). Further, we find *VA opsin* positive cells in the telencephalon and the ventro-lateral area of the tegmentum close to the valvula in mature salmon brain. This is not described at the parr stage and may indicate that these expression patterns appear later in life. Discovering photoreceptors in the telencephalon suggests that telencephalic functions could be directly influenced by light. The *VA opsin* positive cells in the tegmentum could be considered homologous to Ediger-Westphal cells found in zebrafish, where *VA opsin* (*valopb*) is co-localized with cells expressing thyrotropin releasing hormone (trh) [Hang et al., 2014]. Trh is a highly conserved neuroendocrine hormone which classically stimulates thyroid stimulating hormone in mammals and birds [Galas et al., 2009]. The role of trh in fish has not been established and to date has contradicting results [Abbott and Volkoff, 2011; Chatterjee et al., 2001]. Nevertheless, the presence of *VA opsin* expression in this region of the Atlantic salmon brain suggest these neuroendocrine factors could be regulated by light through this receptor.

In the zebrafish brain, the Gnrh systems have been mapped by green fluorescent report lines (GFP) [Xia et al., 2014] and their localisation of the Gnrh2 and Gnrh3 cell bodies are in accordance with our findings in Atlantic salmon. The axonal projection pattern of the Gnrh2 and Gnrh3 neurons is zebrafish are extensive, covering several regions of the brain, including Gnrh2 neurons projecting to the pituitary. The projection pattern also reveals Gnrh2 and Gnrh3 have overlapping axons [Xia et al., 2014]. The extensive projection pattern suggests possible connections with other neurons and/or cell types, not necessarily in the close vicinity.

The thalamic region of the diencephalon is of high interest as *VA opsin* is prominently expressed in the caudal habenula and in the dorsal thalamus. Ventral to the profound thalamic expression of *VA opsin* we detect a cluster of *gnih* positive cells. Both cell groups are located a few cell layers away from the third ventricle and as deep brain photoreceptors are indicated to share the unique feature of contacting the cerebrospinal fluid [Fernandes et al., 2013], this may indicate a potential interaction through the third ventricle. *VA opsin* has also been detected in the

thalamus of zebrafish where *VA opsin* has been suggested to be involved in deep brain light dependent physiology [Hang et al., 2015].

In addition, even though *gnrh3* is expressed in the telencephalon, literature points out that Gnrh3 neurons extend to the hypothalamic areas in the zebrafish brain [Abraham et al., 2010], and due to the high conservativeness of these peptides, it could be similar in salmon [Kah et al., 2007]. Besides, neuronal tracing using DiI shows migration of axons from the thalamus, approximately where we locate *VA opsin* towards the pituitary, down the path where we detect *gnih* expression in the ventral thalamus and further to the pituitary [Anglade et al., 1993]. This could suggest some communication with *VA opsin* expressing neurons and *gnih* expressing neurons.

Immunolabeling of Gnrh in the quail and chicken brain show clusters in the area of supraoptic/ suprachiasmatic nucleus (SOC) [García-Fernández et al., 2015]. This seems homologous to the *gnrh3* expression pattern found in the ventral telencephalon in our study. However, the avian Gnrh is most likely Gnrh1 and not Gnrh3 as Gnrh3 is specific for teleost species [Kah et al., 1986]. There is another cluster of avian Gnrh positive immunolabeled cells in the midbrain area, seemingly homologous to the midbrain cluster of *gnrh2* cells found in our study.

Avian VA opsin immunolabelled cells were located in the SOC area, midbrain, median eminence (ME), and pars nervosa (PN) of the pituitary in the avian brain [García-Fernández et al., 2015]. *VA opsin* expression was not detected in these areas in Atlantic salmon, suggesting different topography between fish and birds. We did not find co-localisation of *VA opsin* and any of the maturation hormones in an intracellular manner. However, it is important to point out that this does not exclude the possibility of other nonvisual opsins to be co-localized with the maturation hormones studied. In addition to VA opsin, melanopsin and neuropsin have been suggested to play important roles in the sexual maturation of birds [Nakane and Yoshimura, 2014]. However, our analyses of neuropsin in a mature Atlantic salmon brain (data not shown) and the melanopsin expression in parr does not overlap with the expression of maturation hormone expressing neurons [Sandbakken et al., 2012].

The saccus vasculosus (sv) has been suggested to be the site of photoperiodic regulation of sexual maturation in fish. Several opsins were expressed in the sv of the Masu salmon such as *rh1*, *sws1*, *lws* and *opn4* [Nakane et al., 2013]. Our findings do not locate *sws1* or *opn4* in the sv of the Atlantic salmon (shown in **Fig. 3**). However, we did not include *rh1* and *lws* in our study. Expression of *gnrh3* has also been located to the sv of Atlantic salmon [Chi et al., 2017]. No similar expression pattern could be found in our study (shown in **Fig. 3**). The results of this current study supports the more classical theory of the photoperiodic regulation of sexual maturation [Weltzien et al., 2004], where the main site of photoperiodic regulation of sexual maturation is in the hypothalamus. However, the exact mechanisms are yet to be elucidated.

Photoperiodic regulation of sexual maturation is quite established in mammals, where photic input from the eyes signals through the retinohypothalamic tract to the supra-chiasmatic nucleus [Dardente et al., 2019; Hattar et al., 2002]. Melatonin production is induced in the pineal gland,

leading to activation of melatonin receptors which increases thyroid stimulating hormone (TSH). TSH increases the expression of *DIO2*, an enzyme that converts T_4 to T_3 where T_3 modulate reproductive hormones [Hanon et al., 2008; Klosen et al., 2013]. T_3 modulate GNRH either through GNIH or through an interaction of GNIH and KiSS2 [Klosen et al., 2013]. A similar pathway is also suggested for birds, however with a significant difference in photoreception. In birds the photic input is directly in the deep brain and not through the eyes as for mammals [García-Fernández et al., 2015; Hazlerigg and Loudon, 2008]. In Atlantic salmon, expression of *tshβb* and *dio2b* is increased by longer days, indicating a common feature of photoperiodic regulation of seasonality in vertebrates [Irachi et al., 2021]. Interestingly, *dio2* expressing cells in the thalamus that are regulated by photoperiod [Lorgen et al., 2015], seem to be in the same region as the *VA opsin* expression described in this study.

Conclusion

This study provides a thorough topographic mapping of neurosecretory cells related to sexual maturation and *VA opsin* photoreceptors in the Atlantic salmon brain. The location of the essential neuroendocrine cells is in the diencephalic (thalamus, hypothalamus) and midbrain (tegmentum) regions indicate that this area is central for regulation of sexual maturation in Atlantic salmon. Earlier studies in birds have shown that GNRH neurosecretory cells in the brain are VA opsin positive, suggesting a direct photoreceptive modulation on onset of sexual maturation. This study in Atlantic salmon indicate that fish do not have such direct intra cellular VA opsin photoreceptive modulation of Gnrh but may interact through neuronal networks. The topographic map generated by this study provides a strong resource for further studies investigating these maturation pathways in salmonids.

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Statement of Ethics

The ARRIVE guidelines have been complied in this study [Percie du Sert et al., 2020]. All experiments described were given ethical approval from the Norwegian Food Safety Authority with Norwegian research permit number 8521 [Fjelldal et al., 2020] and follow the local animal care guidelines.

Conflict of Interest Statement

The authors of the manuscript have no conflicts of interest to declare.

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Author Contributions

Christine Horne performed cloning, sectioning, *in situ* hybridization and Nissl staining. Christine Horne did the data analyses, made the figures (picture panels and the schematic drawing) and wrote the manuscript. Jon Vidar Helvik and Mariann Eilertsen. created the foundation of the research, designed the experiment. Jon Vidar Helvik performed sampling of the fishes, data analyses and has done major revision of the manuscript. Mitchell Stewart Fleming contributed with scientific input regarding onset of sexual maturation and revisions of the manuscript. Per Gunnar Fjelldal provided the Atlantic salmon, lead the sampling of fishes, and revised the manuscript. Mariann Eilertsen did the bioinformatic analyses, cloning, sampling, data analyses and contributed to writing and major revisions of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

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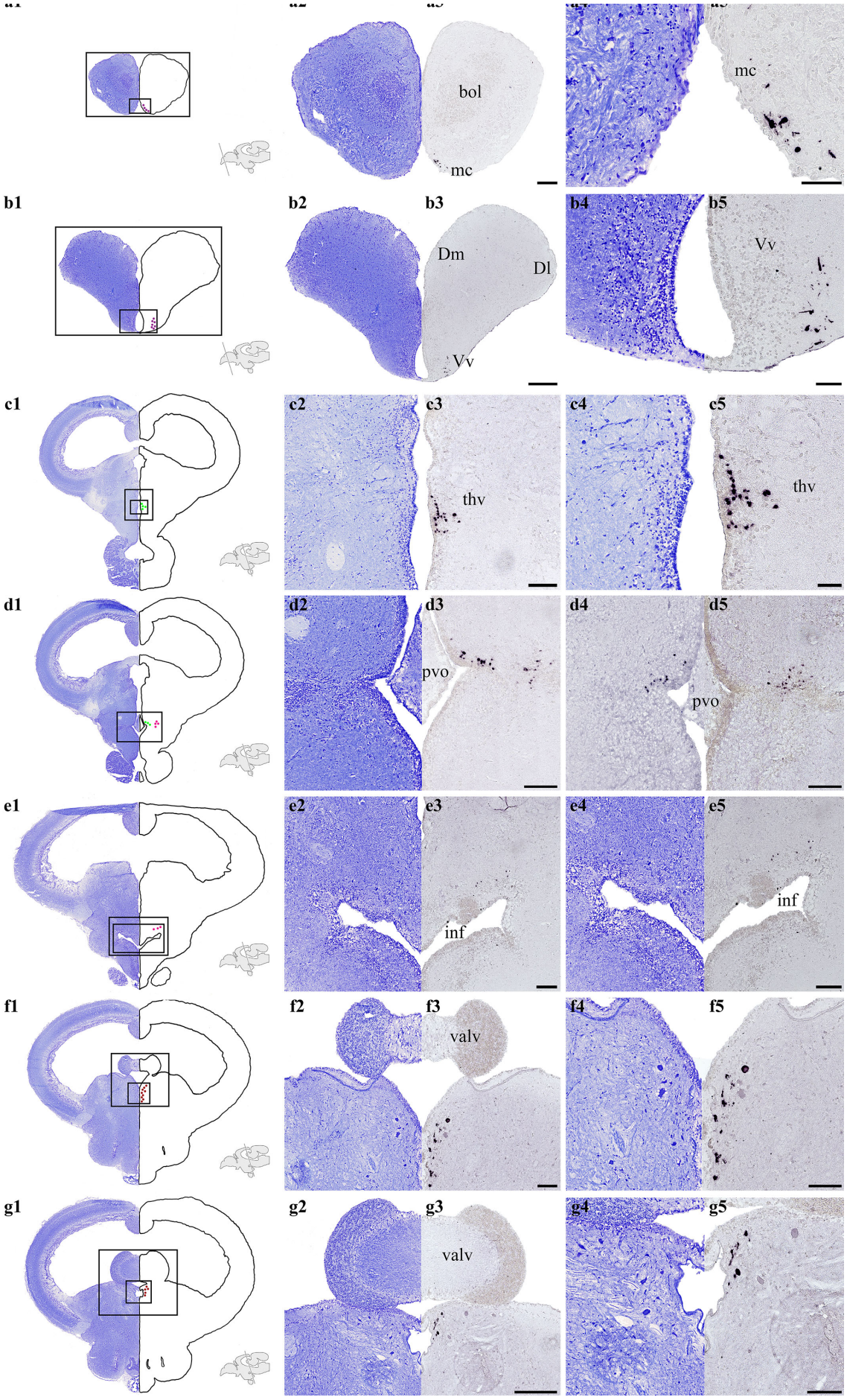
Table 1. Primers (Sigma-Aldrich Merck, Germany)

Primer name	Sequence (5′→3′)
Gnrh3F	GCGTTGGTAGCTCAGGTCAC
Gnrh3R	CAGTTCTGTCCATTTCCAAC
Gnrh2F	ATGGTGAGTGTGGCTAGAC
Gnrh2R	TTGGTCCAATGTTGTGGTTTATC
Gnrh2R	ATGTGCATGATGTTACATCTG
Kiss2.aF	CACTTGCTTTTGACAGAATG
Kiss2.aR	CCATGTAATGACAATAAGGA
GnihF	ACGGAGTMTCGAGGTGGATG
GnihR	GCCTAAATGGCACTGTATCTG
GnihR	AAGGGTTCAGGTTAAGGTTAGG


bol
mc
mc
b1
b2
b3
b4
b5
Dm
Dl
Vv
Vv
c1
c2
c3
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c5
thv
thv
d1
d2
d3
d4
d5
pvo
pvo
e1
e2
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e5
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f2
f3
f4
f5
valv
g1
g2
g3
g4
g5
valv

Fig. 1: Expression pattern for *gnrh2*, *gnrh3*, *kiss2* and *gnih* in the brain of a sexually mature (immature for the sections in **d4**,**5**) Atlantic salmon. **a**-**g3**,**5** (and **d4**): *in situ* hybridization. **a**-**g1**,**2**,**4** (except from **d4**): Nissl staining to provide an overview of the anatomical structures of the brain. **a1** - **g1**: An Illustration of the section, with first half is Nissl stained, and second is an illustration indicating the position of the expression pattern coloured purple for *gnrh3*, light green for *gnih*, magenta for *kiss2* and red for *gnrh2*. Also including the lateral view of the brain with a line indicating the section plane in the right bottom corner. Squares represent the frame of **a**-**g2**,**3** and **4**,**5**. **a**-**g2**,**3**: provides a lighter zoom, and **a**-**g4**,**5** provides a greater zoom. **a1**-**a5**: The *gnrh3* expression first appears in the olfactory bulb (bol), perhaps in the mitral cells (mc). **b1**-**b5**: Expression extends to the ventral telencephalon (Vv) medial to the region of tractus olfactorius medialis. **c1**-**c5**: *Gnih* expression appears in the diencephalon in the ventral thalamus (thv), close to the third ventricle. **d1**-**d5**: Expression pattern of *gnih* and *kiss2*. **d3**: Expression of both *gnih* and *kiss2* appears on the same section, where *gnih* positive cell cluster is in the most medial position and the *kiss2* positive cells are in the more lateral cluster. **d4**: Cluster of *gnih* positive cells on a separate section, using a gene specific mRNA probe. The *gnih* expression is located in the region of nucleus magnocellularis hypothalami, close to the paraventricular organ (pvo). **d5**: Cluster of *kiss2* cells on a separate section, using separate mRNA probe. The *kiss2* expression is in the lateral to the region of nucleus magnocellularis hypothalami. **e1**-**e5**: *Kiss2* positive cells extent caudally in the region of nucleus magnocellularis hypothalami, close to the infundibulum (inf). **f1**-**f5**: The *gnrh2* expression first appears in the midbrain tegmentum most likely in the nucleus oculomotorius. **g1**-**g5**: Expression for *gnrh2* extends caudally in the same location in a slightly different pattern. Scale bars: **a2**-**a3, b2**-**b3, g2**-**g3**: 500 μm, **a4**-**a5**, **c2**-**c3**, **d2**-**d3**, **d4**-**d5**, **e2**-**e3**, **e4**-**e5**, **f2**-**f3**, **f4**-**f5**, **g4**-**g5**: 200 μm, **b4**-**b5**, **c4**-**c5**: 100 μm.

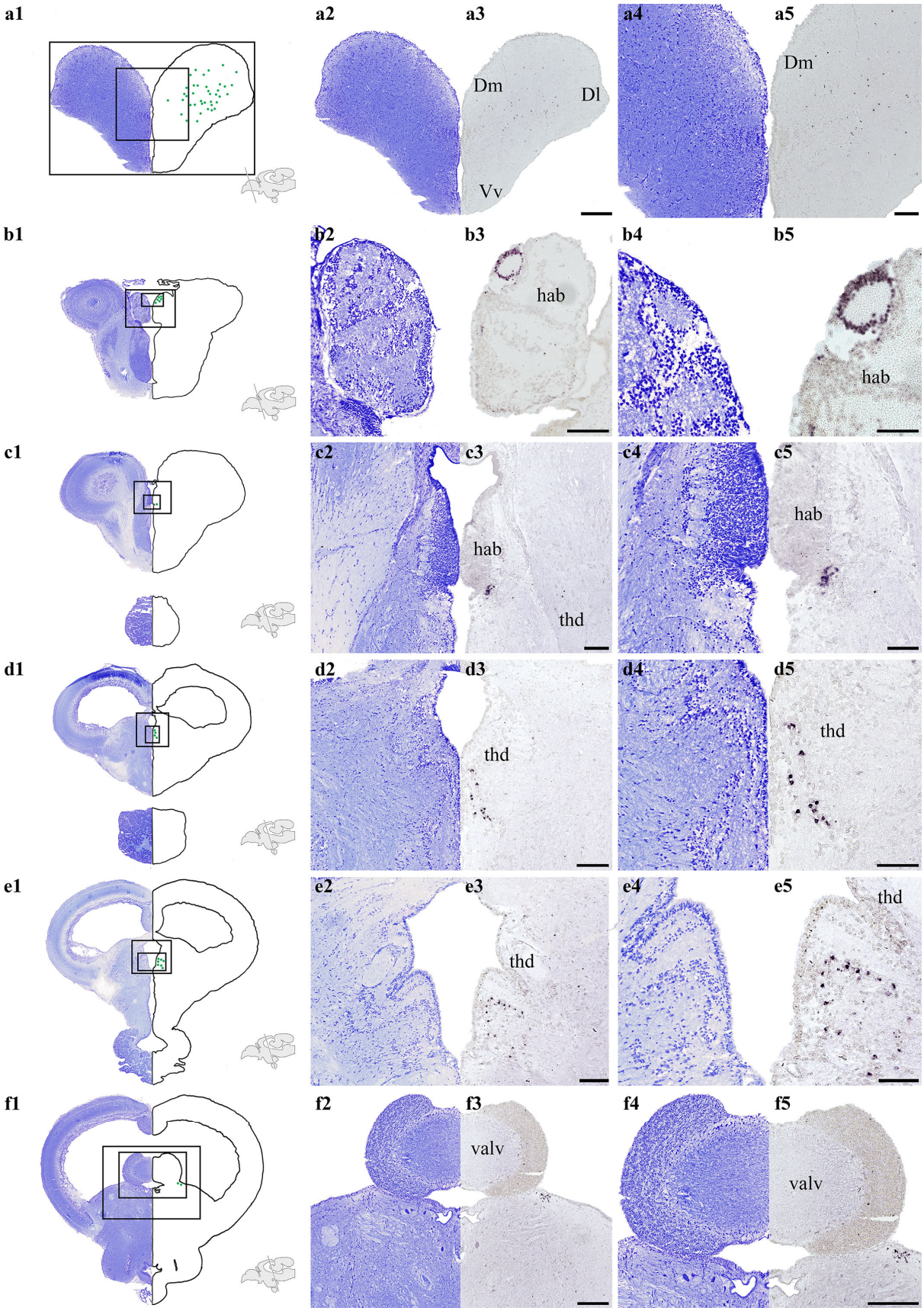


Fig. 2: Expression pattern for *VA opsin* in the brain of a sexually mature Atlantic salmon. **a-f3,5**: *in situ* hybridization. **a-f1,2,4**: Nissl staining to provide an overview of the anatomical

structures of the brain. **a1**, **b1**, **c1**, **d1**, **e1**, **f1**: An overview and illustration of the section, where the first half is Nissl stained, and the second half is an illustration indicating the position of the expression pattern coloured in dark green. Also including the lateral view of the brain with a line indicating the section plane in the right bottom corner. The squares represent the frame of **a-f2**,**3** and **4**,**5**. **a-f2**,**3**: provides a lighter zoom, and **a-f4**,**5** provides a greater zoom. **a1-a5**: Expression is first present in the dorsal telencephalon, in the in the dorsolateral pallium (Dl) and in the dorsomedial pallium (Dm) as a scattered pattern. **b1-b5**: *VA opsin* positive cells are detected in the dorsal ring in the most rostral part of the left habenula (hab). **c1-c5**: The expression pattern is also detected in the transition between the most ventral part of the caudal habenula (hab) and the dorsal thalamus (thd). **d1-d5**: The expression extends to the dorsal thalamus (thd) near the third ventricle, and **e1-e5**: continues to the more caudal regions of the dorsal thalamus (thd), evolving into a different pattern. **f1-f5**: *VA ops*in positive cells are also discovered in dorsal tegmentum ventro-lateral to the valvula (valv) pointed out by arrows. Scale bars: **a2-a3**: 500 μm, **a4-a5, b2-b3**, **c2-c3**, **d2-d3**, **e2-e3**, **f2-f3**, **f4-f5**: 200 μm, **b4-b5**, **c4-c5**, **d4-d5**, **e4-e5**:100 μm.

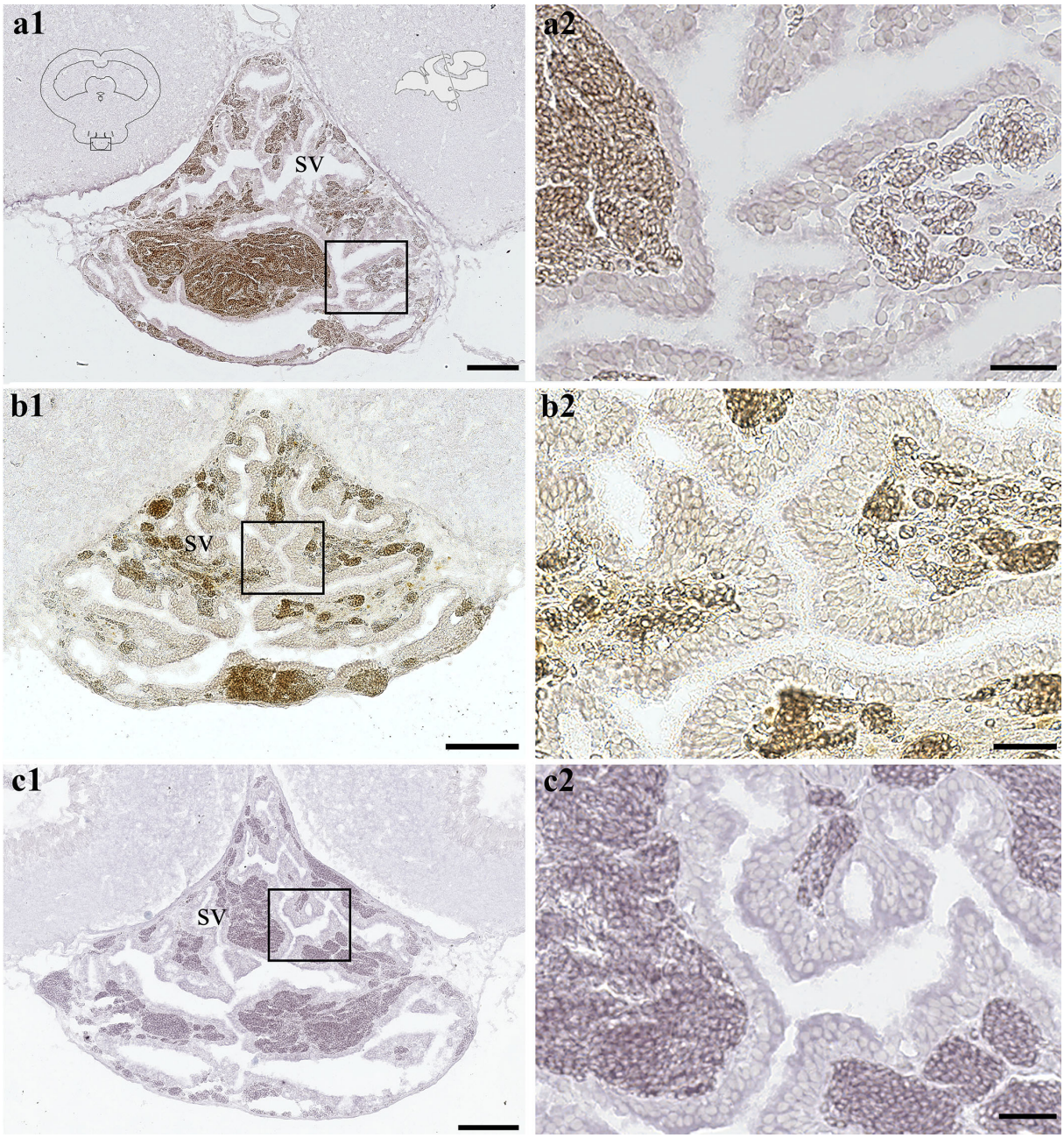


Fig. 3: *In situ* hybridization on sv sections from a sexually mature Atlantic salmon. Probes used are **a1**-**a2**: *gnrh2*, *gnrh3*, *kiss2* and *gnih*, **b1**-**b2**: *sws1* and **c1**-**c2**: *opn4*. **a1** includes an illustration of the section with a square representing the view **a**-**c1** (left) and a lateral view of the section plane (right). Squares in **a**-**c1** represent the view in **a2**-**c2**. No signal is detected. Scale bars: **a**-**c1**: 200 µm. **a**-**c2**: 50 µm.

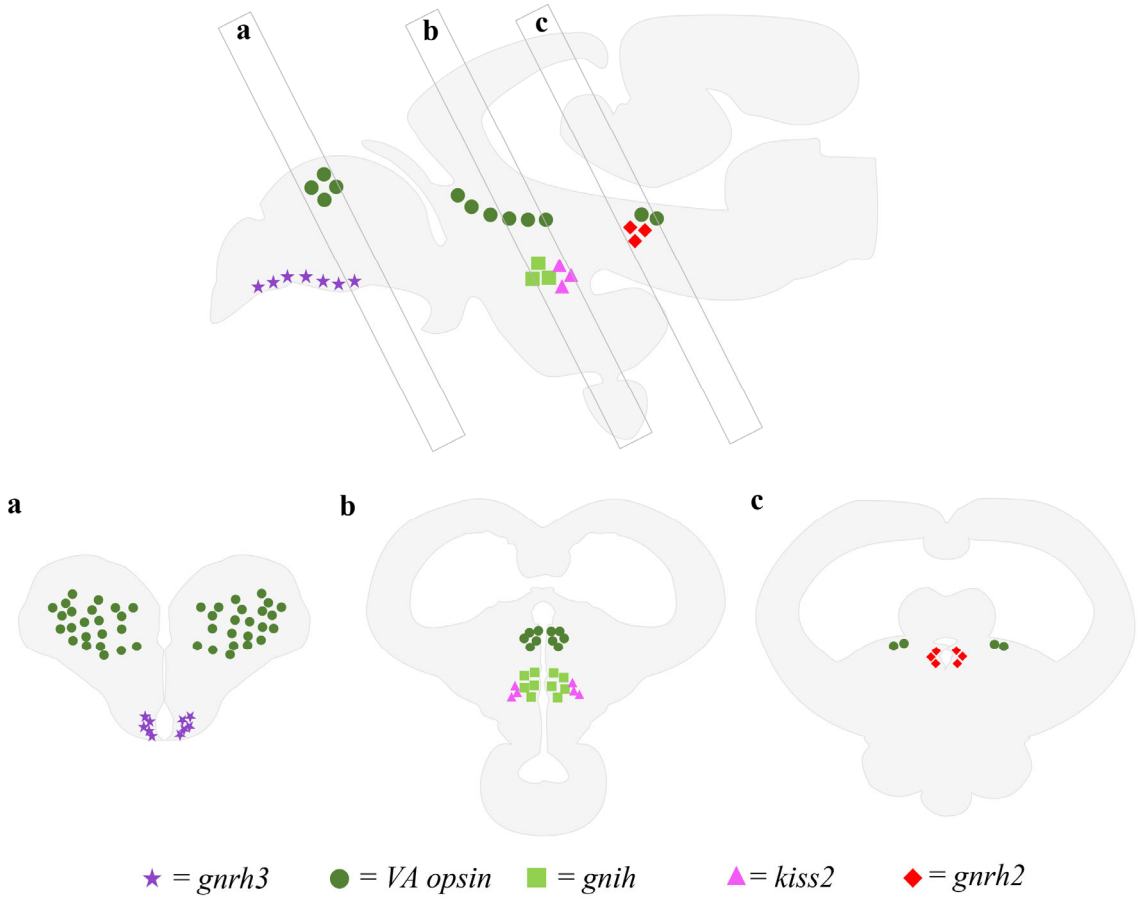
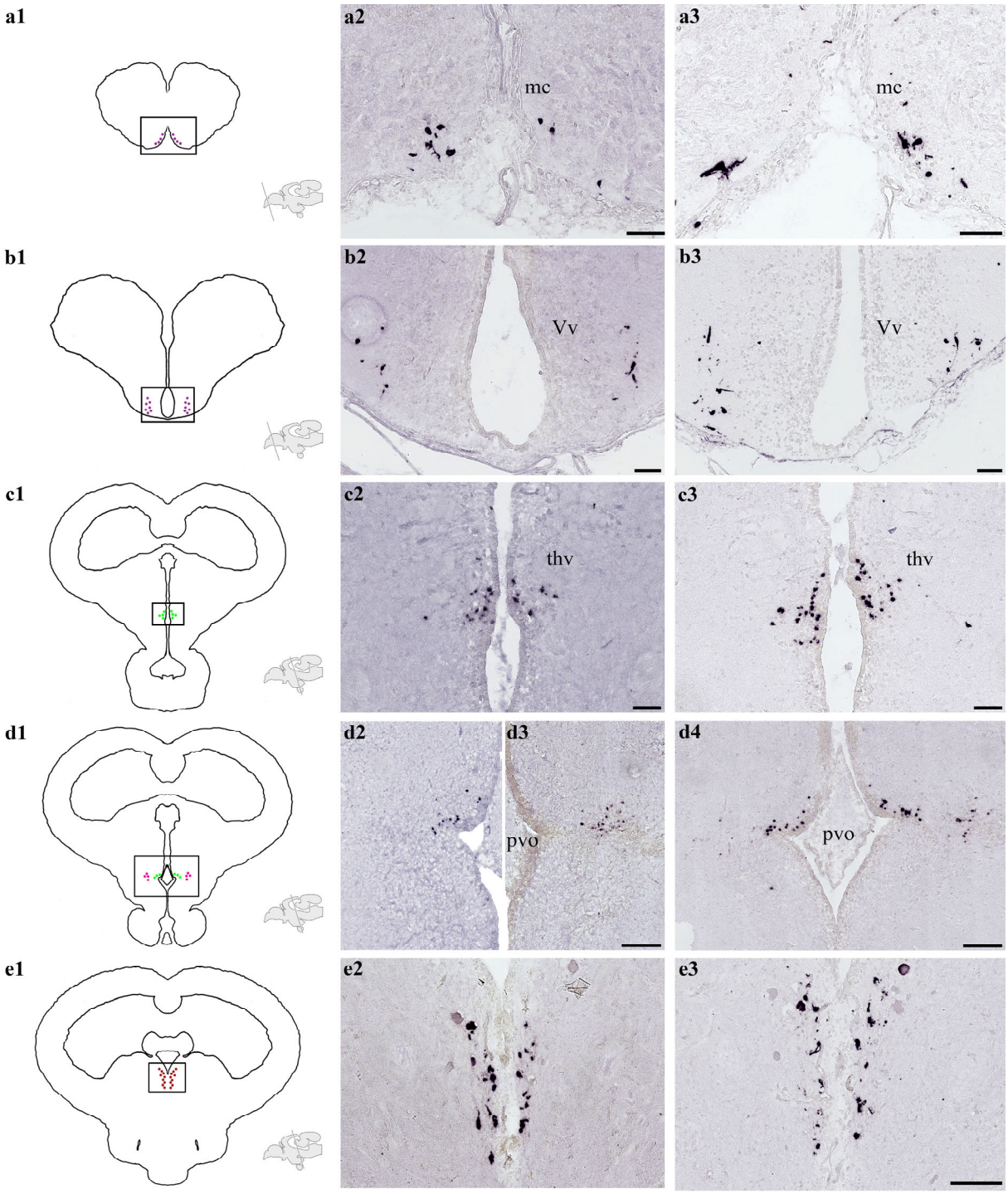


Fig. 4: Overview of expression pattern for *gnrh3*, *VA opsin*, *gnih*, *kiss2* and *gnrh2* in the Atlantic salmon brain shown in this study. Upper left corner shows a sagittal illustration with outlines **a**, **b** and **c** illustrating an array of transversal sections of the telencephalon, hypothalamic areas of the diencephalon, and tegmentum area of the midbrain, respectively.



Supplementary fig.: Comparison of gene expression for *gnrh2*, *gnrh3*, *kiss2* and *gnih* in immature and mature Atlantic salmon. **a-e1**: An illustration of the section showing the expression patterns in colour-codes: purple: *gnrh3*, light green: *gnih*, magenta: *kiss2*, red: *gnrh2*, also including a lateral view of the brain with the section plane in the right bottom corner. **a-e2**: Brain sections from an immature male Atlantic salmon. **a-e3**: Brain sections from a mature male Atlantic salmon. **a1-a3**: Expression pattern of *gnrh3* in the olfactory bulb, perhaps in the mitral cells, similar for both immature and mature salmon. **b1**-**b3**: *gnrh3* expression further extends to the area of tractus olfactorius medialis in the telencephalon, also similar for both immature and mature salmon. **c1-c3**: The *gnih* expression in the diencephalon, in ventral thalamus, close to the third ventricle for both immature and mature salmon. **d1-d4**: Expression pattern for *gnih* and *kiss2*. **d2**: *gnih* expression close to the pvo using a gene specific probe. **d3**: The *kiss2* expression in the midbrain lateral to the paraventricular organ (pvo) using a gene specific probe. **d4**: *gnih* expression and *kiss2* expression on the same section, using a mixed probe. The *gnih* expression is located closest to the pvo, and *kiss2* is located in a more lateral position in relation to the pvo in a symmetrical manner. The expression pattern is similar for both immature and mature salmon. **c1-c3**: Expression pattern of *gnrh2* in the midbrain tegmentum perhaps in the nucleus oculomotorius, also similar for both immature and mature salmon. Scale bars: **a2**, **a3**, **b2**, **b3**, **c2**, **c3**, **d2**, **e3**: 100 um. **d3**, **e2**, **f2**, **f3**: 200 um.