Expression of \textit{gpr54-chr9} in medaka (\textit{Oryzias latipes}) as revealed by fluorescent \textit{in situ} hybridization

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Marie.
Abstract
The kiss system is a well-known regulator of the brain-pituitary-gonadal (BPG) axis in vertebrate reproductive physiology, and is expressed in the brain throughout the vertebrate subphylum (except in birds). This system consists of kiss neurons excreting kiss ligands that bind the receptor GPR54. Apart from its role in control of reproduction, kiss has been found to inhibit cell migration in cancer cells. However, less is known about its other physiological functions, especially in non-mammalian species like teleosts. Teleosts make up the largest group of the extant vertebrate species, making their genome evolution and diversification important subjects for investigation of vertebrate vide physiological functions. In this study the expression pattern of one of the kiss receptor paralogs is investigated in the brain of adult medaka (*Oryzias latipes*) by a fluorescent *in situ* hybridization (FISH) assay. The work included the development of a FISH assay adapted to medaka, and generation and validation of a probe specific for the medaka kiss receptor *gpr54-chr9*. The results show expression in areas proven to be important in regulating reproduction, like in the ventral telencephalon and in the preoptic areas of the hypothalamus. Further, the FISH method was adapted to suit an embryonic model of medaka. Knockdown of the kiss system is recently found to cause abnormal embryonic development in medaka, possibly by acting on neural migration and brain formation. I established both a single – and double FISH method for use in embryos, which can potentially be used to map the expression of kiss and kiss receptors through embryogenesis in medaka.
**List of abbreviations:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>BPG</td>
<td>Brain-pituitary-gonadal</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding DNA sequence</td>
</tr>
<tr>
<td>Chr</td>
<td>Chromosome</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>Dnp</td>
<td>Dinitrophenol</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilization</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>HB</td>
<td>Habenula</td>
</tr>
<tr>
<td>Hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>HybMix</td>
<td>Hybridization mix solution</td>
</tr>
<tr>
<td>HybWash</td>
<td>Hybridization wash solution</td>
</tr>
<tr>
<td>ISH</td>
<td><em>in situ</em> hybridization</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LI</td>
<td>Lobe inferior</td>
</tr>
<tr>
<td>M13F</td>
<td>M13 forward primer</td>
</tr>
<tr>
<td>M13R</td>
<td>M13 reverse primer</td>
</tr>
<tr>
<td>MAB</td>
<td>Maleic acid buffer</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MS-222</td>
<td>Tricaine methanesulfonate</td>
</tr>
<tr>
<td>NAT</td>
<td>Nucleus anterior tuberis</td>
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<td>NFLM</td>
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</tr>
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</tr>
<tr>
<td>NVT</td>
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</tr>
<tr>
<td>OT</td>
<td>Optic tectum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PK</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>POA</td>
<td>Preoptic area</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>POm</td>
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</tr>
<tr>
<td>Pop</td>
<td>Nucleus preopticus pars parvocellularis</td>
</tr>
<tr>
<td>RT</td>
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</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate buffer</td>
</tr>
<tr>
<td>TAMRA</td>
<td>5-carboxytetramethyl-rhodamine</td>
</tr>
<tr>
<td>TE</td>
<td>Telencephalon</td>
</tr>
<tr>
<td>TSA</td>
<td>Tyrode signal amplification</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>V</td>
<td>Area ventralis telencephali</td>
</tr>
<tr>
<td>Vd</td>
<td>Area ventralis telencephali pars dorsalis</td>
</tr>
<tr>
<td>Vi</td>
<td>Area ventralis telencephali pars intermedia</td>
</tr>
<tr>
<td>VI</td>
<td>Area ventralis telencephali pars lateralis</td>
</tr>
<tr>
<td>Vp</td>
<td>Area ventralis telencephali pars posterior</td>
</tr>
<tr>
<td>Vs</td>
<td>Area ventralis telencephali pars supracommissuralis</td>
</tr>
<tr>
<td>Vv</td>
<td>Area ventralis telencephali pars ventralis</td>
</tr>
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1 Introduction

In this thesis the gene expression pattern of the kiss receptor, gpr54, was investigated in medaka (Oryzias latipes, the receptor paralog gpr54-chr9 in this species). The experiments were performed both on brains from adult medaka and on embryos, using fluorescent in situ hybridization as the main method. The information available concerning the kiss system is mostly restricted to regulation of reproductive function in the mammalian brain. I have focused my experiments mainly on the teleost brain, but also on the embryonic expression pattern – a life stage during which very little is known about the function of the kiss system.

1.1 Medaka as a model organism

Using teleosts as models instead of mammals can have several advantages (Powers, 1989). Most teleosts are oviparous, and embryonic development therefore occurs externally. The embryos are often transparent throughout most of the developmental stages, which is a huge advantage when observing the development of the brain, enabling in vivo studies. In addition, teleosts make up the largest group of vertebrates, and comprise more than half of all extant vertebrate species. They have throughout evolution adapted to a variety of marine and freshwater habitats. Their genome evolution and diversification are important subjects for the understanding of vertebrate evolution, and gives many opportunities in the study of different physiological phenomena or specific adaptations. Finally, comparative investigations between different species can reveal novel information about specific mechanisms.

Medaka (Oryzias latipes), is a small freshwater teleost fish native to Japan, Korea and China that is widely used as a model organism because of its many advantages (Kasahara et al., 2007; Kirchmaier et al., 2015). It has a short generation time, it is a diurnal species that spawns every morning provided proper environmental and social breeding conditions, and has low husbandry costs. Further, it is highly thermotolerant, as it naturally tolerates temperatures as high as 40 °C in the summer and temperatures down to 4 °C in the winter. Being a poikilothermic animal, the rate of embryonic development is highly dependent on temperature, with reduced temperatures decreasing the speed of development. The genome size of the medaka is relatively small compared to other small model fish, with a genome size estimated to be around 800 megabase pairs, this is less than half of the zebrafish (Danio rerio) genome size of approximately 1700 megabase pairs, and about one-third that of the human
genome (Naruse et al., 2004). The medaka genome is sequenced and easily accessible in the genomic database of medaka (http://utgenome.org/medaka/) and in NCBI and Ensembl. In addition, several inbred strains are established, as medaka seems remarkably tolerant to inbreeding. This is highly useful for both mutagenesis screening and genetic mapping. Among the several available inbred strains are the Hd-rR, all derived from offspring of a single stock of d-rR strain at Nagoya University, Japan in 1980 (Kinoshita et al., 2009). This inbred Hd-rR strain was utilized for the medaka genome sequencing project. The d-rR strain is characteristic by its body-color sexual dimorphism, which makes it especially suitable for experiments related to sex determination and differentiation. For the experiments performed in this thesis, fish of the d-rR strain was used.

In the following chapters, a short introduction to the anatomy of medaka embryonic development and the medaka brain is given.

1.2 Medaka embryonic development

The developmental pathway of different tissues and organs through the embryonic stages is described by Iwamatsu (2004), and consists of 39 stages, from fertilization until hatching. The time of fertilization is set to stage 1, followed by rapid synchronized cell division occurring through the following stages. The stages 10-11 of development are considered the blastula stages, where the cells begin to divide asynchronously relative to their neighboring cells. At this time the cells start to migrate, and a blastoderm is formed. Further, at the gastrula stages, stage 12-16, an involution of the blastoderm gives rise to the developing epiblast and hypoblast that will form the ectoderm and the endoderm/mesoderm respectively. A local accumulation of cells gives rise to the dorsal lip, which will develop into the embryonic shield. At the end of gastrulation the embryonic shield is visible as a thin streak. The medaka brain development begins at late gastrulation (Kage et al., 2004). In late gastrulation at stage 16 and the neurula steps at stage 17-18 the anterior part of the embryonic body thickens to generate the brain rudiment. By stage 19, the brain becomes divided into anterior-, intermediate-, and posterior brain vesicles in the early neural rod step. The anterior brain vesicle develops into the telencephalon and rostral diencephalon, including the hypothalamus. The intermediate brain vesicle develops into the caudal diencephalon, mesencephalon, and cerebellum, whereas the posterior brain vesicle develops into the medulla oblongata. See next section for description of the brain anatomy in adult medaka. At the late neural rod step, stage
formation of ventricles occurs, leading to the transformation of the neural rod into a neural tube. At the same time the heart rudiments develop in the embryo, and at stage 23 blood vessels appear in the yolk (Iwamatsu, 2004). At the next stages, 24-25, the first heartbeats appear, and the blood starts circulating in the embryonic body. During the neural tube steps, stage 23-27, the developing brain goes through a series of changes specific for teleost species (Kage et al., 2004). During brain development in teleosts, an outward bending (an eversion) of the dorsal portion of the lateral walls in the neural tube is seen, instead of an inward bending (an inversion) seen in most other vertebrates, mammals included (Nieuwenhuys, 2011). This developmental pathway gives rise to a characteristic T-shaped ventricle in transverse sections of telencephalon in teleosts, compared to the two lateral paired ventricles found in other vertebrates (Yamamoto, 2009). Further, in the late embryonic steps of brain development, stages 28-34, the main nerve fiber systems arises (Ishikawa, 1997). Gray and white matter start filling up much of the ventricular spaces. Finally, during the fry brain step, stage 35 and until hatching, the brain matures. This maturation continues to the larvae stages, where the brain matures and becomes more similar to the adult medaka brain. At the later stages, different organs and systems develop and mature towards hatching, and the embryo gradually increases in size. The larvae hatches at stage 39, when the egg envelope rips open by a combined effect of hatching enzymes and rapid body movements.

1.3 Anatomy of the medaka brain

Medaka brain atlas from Ishikawa et al. (1999) shows the anatomical features of the adult medaka brain. The brain of the medaka share the basic architecture with other vertebrate species in that it can be divided into the main parts prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). The prosencephalon consists of the telencephalon and diencephalon, the mesencephalon consists of the optic tectum and tegmentum, and the rhombencephalon consists of the cerebellum and medulla oblongata (Ishikawa et al., 1999; Yamamoto, 2009). But, despite this similar division of the main parts of the brain, the location of the different homologous brain areas are quite different between the teleosts and other vertebrate species (Nieuwenhuys, 2011) because of the teleost specific brain eversion during development described in the previous section.
When the brain is sectioned from the anterior to posterior end, the resulting transverse sections reveal many interior brain areas (Ishikawa et al., 1999; Kanda et al., 2013; Yamamoto, 2009), illustrated in Figure 2. In the most anterior sections the different areas of the telencephalon (TE) can be seen (Figure 2 A-D). In the central part, the ventral telencephalon (area ventralis telencephali, V) is located; pars dorsalis (Vd), pars ventralis (Vv), pars posterior (Vp), pars supracommissuralis (Vs), pars intermedia (Vi) and pars lateralis (Vl) respectively. More posterior, the preoptic areas of the hypothalamus emerges ventrally, including the preoptic area (POA), the nucleus preopticus pars medialis (POm) and nucleus preopticus pars parvocellularis (POp) (Figure 2 C-D). Notice that the preoptic areas is a collective term of all the three respective areas, and is not to be mistaken with the exact brain location of the POA. As the telencephalon reaches its posterior end, habenula (HB) is seen dorsally in the section (Figure 2E). In the next sections we find the optic tectum (OT) at the dorsal side, and hypothalamus at the ventral side, with the nucleus ventralis tuberis (NVT) emerging most dorsally (Figure 2 F-H). In the central regions of these sections, and dorsally in the hypothalamus, the nucleus posterioris periventricularis (NPPv) and nucleus anterior tuberis (NAT) can be found. In the last sections (Figure 2 I-J) the cerebellum is seen in the dorsal part, and lobus inferior (LI) of the hypothalamus at the ventral part. Even further back the medulla oblongata is found before we reach the spinal cord (not shown).
Figure 2. The medaka brain (transverse sections). Transverse brain sections, anterior to posterior. Dorsal side of the brain points upwards in each section on the figure, and ventral parts points downwards. A-D: the telencephalon (TE) makes up the dorsal part, while preoptic areas emerges ventrally (C and D), including preoptic area (POA), nucleus preopticus pars medialis (POm) and nucleus preopticus pars parvocellularis (POp). The area ventralis telencephali (V) is located in the central part; pars dorsalis (Vd), pars ventralis (Vv), pars posterior (Vp), pars supracommissuralis (Vs), pars intermedia (Vi) and pars lateralis (Vl) respectively. E: Telencephalon reaches its posterior end and habenula (HB) emerges dorsally. F-H: The optic tectum (OT) is seen at the dorsal side, and ventrally the nucleus ventralis tuberis (NVT) of the hypothalamus is found, while the nucleus posterioris periventricularis (NPPv) and nucleus anterior tuberis (NAT) of the hypothalamus is seen centrally. I-J: cerebellum (CE) is seen in the dorsal part, and lobus inferior (LI) of the hypothalamus at the ventral part. The hindbrain is not shown. ca, commissura anterior; ch, commissura horizontalis; CM, corpus mammillare; ct, commissura transversa; Dc, area dorsalis telencephali pars centralis; Dd, area dorsalis telencephali pars dorsalis; dDI, dorsal region DI; dDm, dorsal region Dm; DI, area dorsalis telencephali pars lateralis; DM, nucleus dorsomedialis thalami; Dm, area dorsalis telencephali pars medialis; Dp, area dorsalis telencephali pars posterior; fLM, fasciculus longitudinalis medialis; fr, fasciculus retroflexus; GR, corpus glomerulosum pars rotunda; hfb, lateral forebrain bundle; mfb, medial forebrain bundle; nII, nervus opticus; NC, nucleus corticalis; NDLI, nucleus diffuses lobi inferioris; NDLT, nucleus diffuses tori lateralis; NE, nucleus entopeduncularis; NFLM, nucleus fasciculus longitudinalis medialis; NIP, nucleus interpudendularis; NR, nucleus ruber; NRL, nucleus recessus lateralis; PGC, nucleus pregglomerulosus pars medialis commissuralis; PGM, nucleus pregglomerulosus pars medialis; pTGN, pregglomerular tertiary gustatory nucleus; TL, torus longitudinalis; TS, torus semicircularis; VM, nucleus ventromedialis thalami. Figure modified from Kanda et al. (2013).
1.4 The kiss system

The kiss system includes the kiss neurons secreting kisspeptins (ligands) and their putative kiss receptors on the innervated cells.

![Figure 3. Kiss neuron and kiss receptor.](image)

Figure 3. Kiss neuron and kiss receptor. The Kiss neuron excretes ligands, called kisspeptins. Kisspeptins binds kiss receptors on innervated cells, thereby activating the receptor.

The kisspeptins are proteins expressed by the kiss gene. The resulting polypeptide is processed into bioactive peptides of variable length, in mammals that is either a 54, 14, 13 or 10 -amino acid peptide, all including a well conserved 10 amino acid sequence called kisspeptin-10 (Oakley et al., 2009; West et al., 1998). These kisspeptins are related to the RF-amide superfamily of neuropeptides, which possess an Arginine (R) and a phenylalanine (F) motif at their C-terminus, -Arg-Phe-NH2. The kiss receptors, called GPR54, are of the G-protein-coupled receptor superfamily and belong to the rhodopsin family, characterized by seven transmembrane domains (Oakley et al., 2009). Screens for additional ligands that bind the kiss receptor have revealed several candidates, but with varied binding abilities (Clements et al., 2001).

1.4.1 Evolutional relationship of the kiss system genes

Several different homologs of the kiss system genes are found throughout the vertebrate subphylum. Phylogenetic and synteny analyses made it possible to reveal kiss/kiss receptor classifications, and gave information about the evolution of these gene families. In 2012 a proposed overview of this relationship was published (Pasquier et al., 2012b), where the evolution of both gene families could be traced back to the early steps of vertebrate history. The different homologs of kiss system genes found among vertebrates are a result of gene duplication events. Gene duplications have had a major impact on genome evolution, shaping
the vertebrate genomes, with whole genome duplications working as a dominant force (Lu et al., 2012). When replicates of a gene emerge, it gives room for rapid evolution of one of the two forms, which may give rise to new gene function. Whole genome duplication could therefore increase the frequency of genome evolution drastically. The 2R hypothesis suggests that the relatively large and complex vertebrate genome seen today is a result of two ancient, whole genome duplication events arisen in the ancestral vertebrate (Dehal and Boore, 2005). These two events led to a fourfold replication of the total genome, which can still be detected in the structure of the genomes of today’s vertebrates, since several gene families still present up to four variants of each gene. In the teleost lineage, an additional third round of whole genome duplication (3R) has occurred. The teleost-specific genome duplication event resulted in up to eight paralogous gene variants of the genes (Meyer and Van de Peer, 2005). Based on synteny analysis, Pasquier et al. (2012b) found that four kiss paralogs and four kiss receptor paralogs were likely generated via these two rounds of genome duplication in the early vertebrates. This was followed by multiple independent gene loss events of both Kiss and Gpr54 through evolution, leading to a reduction of the kiss system gene homologs to the numbers found in living species today. However, it seems that all replicates for kiss and kiss receptor genes resulting from this teleost-specific genome duplication was lost, shown in genomic synteny maps for the areas around kiss genes, Kiss, and kiss receptor genes, Gpr54 (Pasquier et al., 2012b). The massive gene loss events of the kiss genes have resulted in only two homologous forms of Kiss present throughout the vertebrate subphylum in the species today (Lee et al., 2009). In all the mammalian species investigated (except the mammalian platypus; Ornithorhynchus anatinus), only one paralogous version of the gene is found: Kiss1, while fish species have two paraloguos variants: kis1 and kis2. The non-mammalian kis1 gene was found to be the ortholog of the mammalian variant, whereas the kis2 was found to be a novel form. Birds on the other hand, have lost all their kiss genes through evolution (Pasquier et al., 2014). Regarding the kiss receptor, all four homologous forms are still found in the vertebrate subphylum, but most species possess fewer (Pasquier et al., 2012a). These four homologs are sorted in separate clades; Kissr-1 clade, Kissr-2 clade, Kissr-3 clade and Kissr-4 clade. Only one paralogous variant of the kiss receptor gene is known in mammals (except the mammalian platypus (Ornithorhynchus anatinus)), Gpr54-1. The mammalian Gpr54-1 gene is found among the Kissr-1 clade. Fishes show great variation between species when it comes to the number of kiss receptor paralogs, with two species, the coelacanth (Latimeria chalumnae) and the spotted gar (Lepisosteus oculatus), having representatives in every clade. At least one of the four different fish receptor paralogs is found to be present in
all fish species investigated so far, and is found among the Kissr-2 clade. The medaka and zebrafish species both have two kiss receptor gene paralogs, found in the Kissr-2 and Kissr-3 clades. Regarding these to teleost species, no mammalian ortholog (Kissr-1 clade) is found. As for the ligand, birds have lost all kiss receptor genes through evolution (Pasquier et al., 2014).

There are major disagreements concerning the kiss receptor nomenclature in the literature, especially in the medaka (see Table 1). Throughout this thesis the general receptor name in use will be GPR54, and in medaka the two kiss receptors will be named Gpr54-chr17 and Gpr54-chr9, belonging to the suggested Kissr-2 and Kissr-3 clade respectively, following the analyses of Pasquier et al. (2012a). This nomenclature is based on the gene location in the medaka, located on chromosome 17 (gpr54-chr17) and 9 (gpr54-chr9).

Following is an overview of the different kiss receptor names in use.

**Table 1. Kiss receptor gene nomenclature.**

<table>
<thead>
<tr>
<th>Proposed future naming:</th>
<th>Gpr54-chr9</th>
<th>Gpr54-chr17</th>
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<tr>
<td>NCBI:</td>
<td>XM_004072255.1</td>
<td>XM_004079431.1</td>
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<td>Ensembl:</td>
<td>ENSORLTO0000002103.1</td>
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<tr>
<td>Classified receptor clade:</td>
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<td>Kissr-2</td>
</tr>
<tr>
<td>(Pasquier et al., 2012a)</td>
<td>Gpr54-2</td>
<td>Gpr54-1</td>
</tr>
<tr>
<td>(Lee et al., 2009)</td>
<td>Medaka-1#</td>
<td>Medaka-2#</td>
</tr>
<tr>
<td></td>
<td>Gpr54-1</td>
<td>Gpr54-2</td>
</tr>
<tr>
<td>(Kanda et al., 2013)</td>
<td>gpr54-1</td>
<td>gpr54-2</td>
</tr>
<tr>
<td>(Hodne et al., 2013)</td>
<td>gpr54-1</td>
<td>gpr54-2</td>
</tr>
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</table>

The table gives an overview of the nomenclature regarding the two kiss receptor genes in medaka. Because there seems to be some confusion and disagreements about the nomenclature of these genes we include the chromosome location of the genes in the gene names; gpr54-chr9 and gpr54-chr17 (gene location on chromosome 9 and 17, respectively).

During my thesis the receptor paralog in focus will be the gpr54-chr9. The decision to investigate this receptor, in contrast to gpr54-chr-17, was based on the temporal expression pattern found by Hodne et al. (2013), described in detail later. In short this study reveals an exceptionally early expression of this gene, which occurs in parallel with kissl expression. In addition, knockdown of each of the two genes gives the same phenotypic effect in embryo,
which might suggest a coupling of gpr54-chr9 and kiss1 in early embryogenesis, which makes investigating this paralog particularly interesting.

As explained above, several Kiss- and Gpr54 gene homologs are present throughout the vertebrate subphylum, but the genetic variability between them is sparse (Lee et al., 2009). An alignment of the kisspeptin amino acid sequences showed that the conserved core sequence of kisspeptin-10 is well preserved between species of vertebrates; just differed by a couple of amino acids. The similarity was confirmed by receptor treatment with synthetic kisspeptins resembling KISS1 and KISS2, which activated all types of GPR54, although with different affinity (Lee et al., 2009). This might reflect a high grade of conservation of the bioactive parts in the ligand/receptor coupling.

1.4.2 The kiss system’s role in the vertebrate

The kisspeptins were first discovered as metastasis suppressors, having anti-metastatic effects on human malignant melanoma cells (Lee et al., 1996), by inhibiting cell mobility and suppress cell proliferation (Hori et al., 2001). The kiss gene was originally termed metastin because of this effect. The kiss receptor, GPR54 was discovered a few years later, when cloned from rat brain (Lee et al., 1999).

It was later known that the kiss system also has a central role as a major gatekeeper of reproduction in vertebrates, after studying kiss mutations leading to hypogonadotrophic hypogonadism in humans (de Roux et al., 2003; Seminara et al., 2003), and metastin was then co-named Kiss. Hypogonadotropic hypogonadism is defined as a deficiency of the pituitary secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which results in impaired pubertal maturation and reproductive function. Mouse knockout (KO) models for both the kiss ligand and the receptor have been established and phenocopy the human disease in that both models lead to infertility (Funes et al., 2003; Lapatto et al., 2007; Seminara et al., 2003). These studies linking loss of function of kiss ligand/receptor to reproductive deficiency identified GPR54 and kisspeptins as major players in the brain-pituitary-gonadal (BPG) axis, where gonadotropin-releasing hormone GnRH neurons in the hypothalamus stimulates the gonadotrophes of the pituitary to release FSH and LH, which in turn will stimulate gametogenesis and the production of sex steroids (Clarke and Pompolo, 2005). The current model in mammals suggest that the kiss system act as regulators of the BPG-axis by kiss neurons working as primary transducers of major endogenous and environmental cues, which
then provide an integrated signal, directly or indirectly through interneurons, to the GPR54 containing GnRH neurons (Oakley et al., 2009; Popa et al., 2008). Such endogenous cues could be gonadal steroids (e.g. estradiol, progesterone and testosterone) working by a feedback mechanism, or different metabolic factors (e.g. leptin). Environmental cues influencing the system could be information related to photoperiod and season, possibly mediated by melatonin.

In the teleosts, the BPG-axis work by the same principals, with only minor differences from the mammalian BPG-axis (Weltzien et al., 2014). The influence of the kiss system in regulating the axis is less studied in teleosts than in mammals, but several studies indicate a regulatory role here as well. A study in European sea bass (*Dicentrarchus labrax*) show that stimulation by Kiss2 gave a clear positive effect on the gonadotropin synthesis and release, while Kiss1 stimulation had less effect (Felip et al., 2009). The same was found in zebrafish (Kitahashi et al., 2009), which might suggest that the Kiss2 ligand is the most potent activator for gonadotropin secretion in some fish species. However, in the medaka it is found that kiss1, but not kiss2 neurons are sensitive to steroids, and thus it is most likely Kiss1 that regulates the gonadotropin release in this species (Mitani et al., 2010). Also the location of kiss neurons in the brain suggest that Kiss1 is the variant controlling reproductive function in medaka, as it is found in the NVT area in hypothalamus, with projections to the pituitary (Hasebe et al., 2014). In contrast, zebrafish has no kiss1 neurons in this area, but kiss2 can be found here (Kanda and Oka, 2012).
Figure 4. Direct kiss system regulation of BPG-axis. The kiss system is shown to regulate the BPG axis in vertebrates (here illustrated in mammals). Kiss neuron activity is regulated by different endogenous- and environmental cues. Activation releases kiss ligands, kisspeptins, which bind kiss receptors on the GnRH neurons. Stimulation of these neurons results in secretion of GnRH, which stimulates the release of FSH and LH from the gonadotrophes. FSH and LH will in turn stimulate gametogenesis and the production of sex steroids. Note that the different cells and organs in this figure are not in proportion to each other, and is just a simplified illustration of the regulatory pathway. FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

1.4.3 Kiss system gene expression in the brain

Because of its important role in regulating the BPG axis, the kiss system’s expression patterns in the brain have been investigated in several vertebrate species. In the mammalian brain the kisspeptins are expressed by two main populations of neurons in the hypothalamus, one within the preoptic area (POA) and one within the arcuate nucleus (ARC; Franceschini and Desroziers, 2013).

In the zebrafish and medaka kiss1 and kiss2 spatial expression patterns are mapped in the brain. These expression maps reveal a different pattern of expression between these two teleost species. In the zebrafish kiss1 is only found in the habenula (HB), while kiss2 is distributed in the nucleus ventralis tuberis (NVT), the nucleus posterioris periventricularis (NPPv) and the nucleus recessus lateralis (NRL; Kitahashi et al., 2009; Servili et al., 2011). In medaka kiss1 expression is found in the habenula (HB), as well as in the nucleus ventralis tuberis (NVT) and nucleus posterioris periventricularis (NPPv), while kiss2 is found only in
nucleus recessus lateralis (NRL; Kanda et al., 2008; Kitahashi et al., 2009; Mitani et al., 2010). The medaka kiss1 neurons localized in NVT, are found to have neural projections to the pituitary (described above), but also to the preoptic areas in the hypothalamus and ventral telencephalon (Hasebe et al., 2014).

A study carried out in medaka has revealed the spatial expression pattern of the kiss receptor genes \textit{gpr54-chr17} and \textit{gpr54-chr9} in the brain of this species, done by nonradioactive, classical \textit{in situ} hybridization on brain slices (Kanda et al., 2013). The \textit{in situ} hybridization study revealed that the \textit{gpr54-chr17} was widely distributed throughout the brain at many specific areas, while the expression of \textit{gpr54-chr9} was more restricted to a couple of areas. \textit{gpr54-chr9} was mainly expressed by neurons in the prosencephalon, where the expression is restricted to large cells in the preoptic area (POA), and both large and small cells in the dorsomedial areas; nucleus preopticus pars magnocellularis (POm) and nucleus preopticus pars parvocellularis (POp). Expression is also found in several ventral parts of the telencephalon, like the area ventralis telencephali pars dorsalis (Vd), area ventralis telencephali pars supracommissuralis (Vs), area ventralis telencephali pars posterior (Vp). There was also detection of mRNA in the most posterior part of the telencephalon the habenula (HB).

### 1.4.4 Additional roles of the kiss system

\textit{Kiss} and \textit{Gpr54} expression are found not only in the brain, but also in other tissues. \textit{Kiss} is reported in several human organs, like the testis, pancreas, liver and small intestine (Oakley et al., 2009), while \textit{Gpr54} is reported in the spinal cord and pancreas. In mice kiss receptor are found in additional tissues, like in the heart, the aorta and in the kidneys (Sawyer et al., 2011). Further, both genes are found to be expressed in the placenta (Mead et al., 2007) with a relatively stable high expression level of the receptor, and a changing \textit{Kiss} level throughout the pregnancy. This widespread expression pattern might indicate additional roles of the kiss system other than tumor suppression and reproductive control.

There are several studies today indicating additional roles of the kiss system, which might reflect a diversity of intracellular signaling pathways triggered by GPR54 receptor activation, with many mechanisms yet to be revealed (Castaño et al., 2009). Thus, in total the kiss system shows a diverse aspect of functions in the adult vertebrate, but less is known about its expression and function during postnatal and pubertal development, and in embryogenesis.
and early development. Information regarding this is now beginning to be revealed (Sandvik et al., 2014).

1.4.5 The kiss system during vertebrate development

Kiss system during development in mammals

Very little is known about the kiss system during mammalian embryogenesis. There might be several reasons for this. Mammals often have an extended time of gestation compared to other vertebrates, and are in addition viviparous vertebrates, which is a huge disadvantage when it comes to studying embryonic development in vivo. In addition it is known that Kiss and Gpr54 knockout (KO) mice are infertile (Lapatto et al., 2007). KO animals therefore have to be generated by crossing of heterozygous parents, thus enabling maternal mRNA to be transferred from the mother to the embryo in the early developmental stages. Despite this, a couple of studies of the developing kiss system have recently been performed in mammals, mainly on mice and rats.

In the rodent species, there is a tendency of early onset of Kiss and Gpr54 expression in the brain. The early expression of Kiss1 is mainly restricted to the ARC area, and is detected already prenatally in both mice (Fiorini and Jasoni, 2010) and rats (Desroziers et al., 2012). At the same embryonic stage Gpr54-1 expression was detected in POA in mice (Fiorini and Jasoni, 2010). This offset corresponds to the time that the first GnRH neurons reach their target, and an effect of KISS1 from ARC acting on these early GnRH neurons through the kiss receptor is suggested. Kiss1 expression in the POA is not detectable until postnatal stages in both mice (Semaan et al., 2010), and rats (Navarro et al., 2004), and in both species the expression increases until puberty is reached (Franceschini and Desroziers, 2013; Navarro et al., 2004). It is clear that rodents show an early, prenatally expression of Kiss1 and Gpr54-1 in ARC, that might indicate a functional kiss system already at these current stages, but the function of this developmental kiss system gene expression is still unclear. To examine the early effect of the kiss ligand on GnRH neurons further, Fiorini and Jasoni (2010) performed a study on embryonic POA brain tissue from mice ex vivo, where KISS1 was shown to stimulate both the number and length of the GnRH neurons.

Also during human development, kiss and kiss receptor has been reported (Guimiot et al., 2012). Immunostaining of brain sections from human fetuses revealed presence of both
proteins, KISS1 and GPR54-1 in the hypothalamus at the second trimester of development (15th week of gestation), and the protein level increased until the third trimester measurements (30-31th week of gestation), before a decrease at the end of gestation. However, nothing is known about the function of the kiss system in these early human developmental stages.

**Kiss system during development in teleosts**

Only a few studies are performed that investigate the physiological regulation and function of the kiss system during early teleost development, and they are mainly performed by using zebrafish or medaka as a model.

*kiss1* and *kiss2* expression in teleost juveniles was reported in both the gonads and the brain of developing sea bass (Felip et al., 2009). Another study done on the cobia (*Rachycentron canadum*) reported expression of *gpr54-1* at larvae 24 hours post hatching (Mohamed et al., 2007).

The expression profile of both *kiss1* and *kiss2* has been investigated in zebrafish embryonic development (Kitahashi et al., 2009; Zhao et al., 2014), and expression was detected in the brain already at the first stage investigated, at 24 hours post fertilization (30 somite stage), which equals the developmental stage 28 in medaka embryos. Earlier stages were excluded in these studies. The expression increased by each day in the time interval 1-7 days post fertilization, before a marked increase was seen at 30 days post fertilization, with this level remaining high during the adult stages. This expression pattern resembles those found in rodents, described in the previous chapter.

A recent study in zebrafish has investigated the effectiveness of the kiss ligands on GnRH neuron development during embryogenesis (Zhao et al., 2014). They found that both Kiss1 and Kiss2 had a stimulatory effect on GnRH3 neuron proliferation located in the peripheral nervous system, while only Kiss1 ligand stimulation had an impact on the GnRH3 in central nervous system. Kiss1 had a stimulatory effect on the number of hypothalamic GnRH3 neurons developing during embryogenesis, but an increase resulting from high stimulation was transient as it did not extend into adulthood. These findings are comparable to the study done on mice brain tissue (Fiorini and Jasoni, 2010), previously described, where KISS1 stimulated both growth and development of GnRH1 neurons. Also seen in this zebrafish study
was a stimulatory effect of Kiss1 ligands on the number of synaptic contact points of the GnRH neurons (Zhao et al., 2014).

In 2013 a novel study working on medaka embryogenesis was published (Hodne et al., 2013). Both an expression analysis of the kiss system genes through embryogenesis, and a knockdown study of the respective genes were performed from the earliest stages. The expression analyses were performed on both the kiss ligand genes: \textit{kiss1} and \textit{kiss2}, and both the receptor genes: \textit{gpr54-chr9} and \textit{gpr54-chr17} at specific stages of development, from right after fertilization and until hatching. The study revealed that both the kiss ligand genes and receptor genes were expressed during the embryonic development, but with different expression profiles. The two kiss ligands, in addition to one of the receptors (\textit{gpr54-chr9}) were already expressed right after fertilization. This indicates a maternal provision of mRNA for these three genes (\textit{kiss1}, \textit{kiss2} and \textit{gpr54-chr9}). The zygotic expressions of genes are expected to appear after gastrulation, around stage 15 of development. After this time point all the four kiss system genes appear to be expressed in this species. Regarding the expression pattern of the two kiss genes, both showed a rapid increase in expression level at the switch between maternally and zygotic kiss expression, with a peak of expression level around stage 19. The rapid increase in kiss expression coincides with the time right after neurulation offset. The kiss receptor paralog \textit{gpr54-chr9} was proven to be expressed through the whole study at relatively high expression levels, with a peak at the earliest stages, before it declined and stabilized around the offset of zygotic expression. The other receptor paralog was not detectable before the zygotic transcription offset, but from then on the expression levels increased throughout the study.

A series of knockdowns of the kiss system genes in medaka were then performed (Hodne et al., 2013), to investigate the gene functions during embryogenesis, and to reveal any functional differences of the maternally provided genes. Knockdown of maternally provided \textit{kiss1} or \textit{gpr54-chr9} resulted in developmental arrest right before gastrulation (stage 11-12). At these specific stages the embryonic cells normally start to divide asynchronously, and cell migration begins. Therefore it seems that the function of the maternally provided kiss system transcripts is closely linked to the initial embryonic cell migration, which is vital for further embryogenesis beyond this step. Further, partial knockdowns were performed, leading to embryo development past the gastrulation. This gave rise to embryos suffering from reduced eye and brain development. \textit{kiss2} knockdown of any kind did not result in any increased mortality or morphological effects like seen in the \textit{kiss1} and \textit{gpr54-chr9} knockdowns. \textit{Gpr54-}
chr17 knockdown showed no abnormal phenotype until the transitional stage between maternal and zygotic provision of transcripts. This correlates with the pattern found in the expression analyses. At this point the developmental pathway was clearly affected and the embryos died before stage 17. During partial knockdown the development proceeded beyond this but the effect on the development of the cranial and brain area was severe, and it is therefore likely that the zygotic gene expression of this gene is vital for these developmental events.

A recent KO study performed in zebrafish (Tang et al., 2015) showed some rather contradictory results to the study of Hodne et al. (2013). In the zebrafish study it was clear that knocking out either of the kiss system genes did not give any drastic morphological effect, as the offspring develops normally into adulthood in the KO lines. But a notable difference between these studies (apart from different species studied) is that Hodne et al. (2013) performed a knockdown of the kiss system genes, preventing translation of the current transcripts, while Tang et al. (2015) performed a KO of the respective genes, interrupting the current gene and rendering it inoperative. KO of genes could activate compensatory mechanisms, which may not be the case in knockdown studies. This means that KO could lead to kiss system gene compensation by other neuropeptide -or neurotransmitter/receptor systems.

When comparing the results found in medaka and zebrafish, it is clearly a lot of functional differences in the role of the kiss system in early development. Regarding the kiss gene expression profiles (described above), variation is found between the two species, with the zebrafish expression patterns more resembling those found in rodents. The medaka expression level is lower and more stable in comparison, when comparing the stages in the same interval of development. The early embryonic expression found in medaka is not comparable with zebrafish because of lack of data in zebrafish. Also the many differences revealed when comparing these two teleost species emphasize the importance of looking at more than one model in studies aimed to reveal gene function, and the diversity seen suggests that the kiss system has a varied potential in influencing embryogenesis.

In summary, the studies performed in teleost species show that, like in mammals, the kiss system genes are expressed at early stages where they exert some sort of function in the developing embryo. In medaka, studies indicate that the kiss system might exert some function in the initial embryotic cell migration at early stages, and is essential for proper brain
development. In zebrafish there are studies indicating an early influence of Kiss1 ligands on the developing GnRH neurons, probably as a way of shaping the reproductive pathway.

1.5 **In situ hybridization**

*In situ* hybridization (ISH) is a method that allows specific nucleic acid sequences in the cytoplasm to be detected in morphologically preserved cells, tissue samples or whole embryos (Thisse and Thisse, 2008). An antisense probe, complimentary to the endogenous RNA of interest are used to reveal the spatial expression pattern (Gasnier et al., 2013). Radiolabeled probes were first used to detect expression before *in situ* procedures using nonradioactive, hapten-conjugated probes became available. Both are classical ISH methods, where probe detection is achieved by chromogenic substrates, visible in the light microscope or in a fluorescent microscope. Hapten-conjugated probe detection by chromogenic substrates is proven to be a sensitive detection method, giving a clear response if expression is present, even at small levels. A clear limitation of this technique is that it lacks any three-dimensional visualization possibilities, and multiple-color ISH detection is difficult (Lauter et al., 2011). These techniques are now gradually being replaced by the use of fluorescent labeled probes (Fluorescent ISH, FISH), a method that overcomes these limitations when combined with confocal microscopy imaging. This gives great resolution that enable visualization of specific RNAs from the whole tissue and down to a single cell (Gasnier et al., 2013). FISH is a less sensitive than classical ISH, but the sensitivity greatly increases when it is combined with a signal amplification step by the use of peroxidases in combination with fluorescent tyramide substrates in tyramide signal amplification FISH (TSA FISH).

TSA FISH also utilizes hapten-conjugated probes, specific for the cytoplasmic RNA of interest (Gasnier et al., 2013). When synthesizing a hapten-conjugated probe the sequence of choice should preferably be more than 1 kb in size, and match a unique sequence in the genome to enable highly specific hybridization. The synthesized antisense probe is then set to hybridize to the complementary cytosolic RNA in the sample of choice. The hapten conjugate on the hybridized probe is then recognized by specific antibodies coupled to a peroxidase (Gasnier et al., 2013). Peroxidase is important for the TSA step of the method, by promoting precipitation of tyramide components at the adjacent area. The peroxidases work by catalyzing the conversion of tyramide molecules into highly reactive free radicals in the presence of hydrogen peroxides (Krieg and Halbhuber, 2010). The tyramide free radicals bind
covalently to adjacent proteins. In the tyramide components, tyramides are linked to fluorophores so that precipitation of tyramide components will lead to aggregation of fluorescent molecules, thereby increasing the resulting fluorescent signal. In this way the TSA will amplify the expression signal so that even small levels of expression, undetectable by regular FISH, could be revealed.

Figure 5. Mechanisms of fluorescent in situ hybridization with tyramide signal amplification; TSA FISH. Designed probes hybridizes to the complementary target mRNA. Peroxidases are brought in by antibodies specific to the probe marker (here showed as DIG). Tyramide activation by the peroxidases. Aggregation of activated tyramides at the area of target mRNA. Ab, antibody; DIG, digoxigenin; HRP, horseradish peroxidase; T; tyramides. Figure modified from PerkinElmer webpage: In situ hybridization with TSA; http://www.perkinelmer.com/resources/technicalresources/applicationsupportknowledgebase/tsa/ish.xhtml
1.6 Aims of the study

In this study, I have investigated the spatial gene expression of the kiss receptor, \textit{gpr54-chr9}, in medaka. The work was separated into three defined aims:

The first aim of the study was to set up a fluorescent in situ hybridization (FISH) assay with a functional antisense probe for the medaka kiss receptor \textit{gpr54-chr9}. The FISH protocol was originally optimized for zebrafish, so this included setting up an assay in our lab that worked in medaka. The first aim also included validation of the specific probe for \textit{gpr54-chr9} by comparing the labelling with previously published expression maps for \textit{gpr54-chr9} in adult medaka brain.

The second aim of the study was to optimize the FISH method to work in embryonic medaka, using the validated probe. This was to be followed up by a spatial-temporal expression analysis of \textit{gpr54-chr9} expression pattern through medaka embryogenesis, to map the exact location of the expression during early development at different embryonic stages.

The third aim was to establish two working labelling approaches, to develop a double-FISH assay in the medaka embryo.
2 Materials and methods

All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.1 Experimental animals

2.1.1 Medaka

Fish of the wildtype medaka (Oryzias latipes) dr-R strain were maintained at the University of Oslo aquarium facilities on a 14:10-hour light/dark cycle at 27-28 °C. They were fed live nauplii larvae of brine shrimp (Artemia franciscana, Argent Laboratories, WA, USA) and dry feed (Special Diets Services, UK) 3-5 times per day. The experimental procedures and housing were approved by the Norwegian Animal Research Authority.

2.2 Preparation of samples for in situ hybridization

2.2.1 Embryos

Medaka fertilized eggs were harvested for the embryo expression analyses. Right after fertilization, female fish with eggs attached to their abdomen were netted and their eggs were harvested and transferred to a dish with E3-medium (Appendix IV). The eggs were stored at 28 °C in an incubator until the right embryonic stage was reached (Iwamatsu, 2004) (Table 2).
Table 2. Embryonic stages in focus.

<table>
<thead>
<tr>
<th>Embryonic stage</th>
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<td>&gt; 9dpf</td>
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hpf, hours post fertilization; dpf, days post fertilization.

When the embryos stored in the incubator reached the preferred age, they were fixed in 4 % paraformaldehyde, PFA (Electron microscopy sciences, PA, USA) in PBST (0.1 % Tween in PBS; Fisher Scientific), and stored over night at 4 °C with rotation.

After fixation, the eggs were dechorinated under a binocular using fine tweezers, and the released embryo was transferred to PBST solution. During dechorination the embryonic stage was confirmed by observation in the binocular.

To prepare the samples for storage, dechorinated embryos were dehydrated by adding an increased percentage of alcohol solution (methanol) to the embryonic samples, 25, 50, 75, and 2× 100 %, each step with 10 min incubation time. After dehydration the embryos were stored at -20 °C until usage.

2.2.2 Adult brains

Fish were collected for in situ hybridization experiments on adult brains. Male adult fish at approximately same size and with an age span of 3-4 month, all assumed to be sexually matured based on their age, were netted and transferred to a separate tank. All fish appeared healthy. The fish were anesthetized with buffered MS-222 solution (approximately 1 g/L MS-222 and 1 g/L NaHCO₃). Full anesthetization was ascertained before any procedures took
place, by observing behavioral changes (loss of equilibrium) and lack of reaction when pinching the tailfin.

Fully anesthetized fish were transferred to a paper bed soaked in water under a binocular kept in the fume hood, for perfusion with fixation solution. The beating heart was located, and the thorax was opened using a scalpel so that the heart was exposed. A glass capillary needle, coupled to a 2 mL syringe by a narrow plastic hose was used to inject 4 % PFA in PBST into the heart. The PFA solution was slowly injected into the beating heart to exchange the total blood volume of the fish with the fixation agent. The perfusion proceeded after the heart stopped, until the heart turned white and the gills lost its color. The skull roof of the perfused fish was opened to allow easier entry of the fixation solution into the brain, and the fish was transferred to a tube containing fixation agent of 4 % PFA in PBST, and incubated over night at 4 °C with rotation.

After fixation the perfused brain was carefully dissected out of the fish. First, the fixated fish were thoroughly washed with PBS to get rid of the PFA, and kept under the ventilating hood. The brain dissection was carried out under a binocular with tissue submerged in PBS. The brain was transferred to a tube containing PBST and stored at 4 °C until dehydration.

Perfused brains were dehydrated by adding an increased percentage of alcohol solution (methanol), 25, 50, 75 and 2× 100 %, each step with 10 min incubation time. After dehydration the brains were stored at -20 °C until usage.

### 2.3 Probe preparation for Fluorescent in situ hybridization

#### 2.3.1 Cloning of plasmids

The probe used in this work was synthesized from the medaka kiss receptor sequence gpr54-chr9 with RNA polymerases. This probe was complementary (antisense) to the mRNA of interest. To be able to synthesize this probe, the sequence was cloned into an expression vector, between specific promoters for the RNA polymerases.

Because there are some confusion and disagreements about the nomenclature for the genes coding for kiss receptors in medaka (see Introduction, and Table 1), we chose to name the two
medaka receptors after the chromosome locations of the genes, *gpr54-chr9* and *gpr54-chr17* (gene location on chromosome 9 and 17, respectively). The coding sequence of the medaka kiss receptor gene, *gpr54-chr9*, was found in GeneBank at NCBI (XM_004072255.1). This is identical to the receptor named Kiss1rb on Ensembl (ENSORLT00000002103), medaka GPR54-2 in Pasquier et al. (2012a), Medaka #1 and *GPR54-1* in Lee et al. (2009) and *gpr54-1* in both Hodne et al. (2013) and Kanda et al. (2013).

A vector (pUC57) containing the coding sequence of the receptor gene was ordered from GeneScript (Figure 6 and Appendix I). The received plasmid was delivered as dried material and was dissolved in nuclease free water (Invitrogen) and stored at -20 °C.

**Figure 6.** *pUC57 plasmid with inserted sequence*. The ordered plasmid pUC57 (GeneScript) included the insert of the kiss receptor gene *gpr54-chr9* from NCBI’s GeneBank (XM_004072255.1). The two gene-specific primers (F and R) binding within the inserted gene sequence were: *gpr54-ol-49-F* and *gpr54-ol-1066-R*. Figure modified from pUC57 protocol (GeneScript).
To amplify the sequence that we wanted to use for probe synthesis, a PCR with primers specific for a part of the gpr54-chr9 sequence was performed with 5 ng/µL of the pUC57 plasmid as template. The gene-specific primers for gpr54-chr9 were designed in the program Primer3, and ordered from Thermo Scientific. The primer sequences were (F, forward; R, reverse primers):

F: Gpr54-ol-49-F (5’- TGC AAC CTT TCC CTG GAG AT -3’)

R: Gpr54-ol-1066-R (5’- CTT CTC CTT TGG GTG GTT GC -3’)

The PCR was performed with Platinum Taq DNA Polymerase according to the manufacturers protocol (Invitrogen) and ran on GeneAmp PCR system 2700 (Applied Biosystems, USA). The following PCR program was applied: 94 °C for 2 min (initial denaturation), and then 35 cycles of 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing), and 72 °C for 80 s (extension), followed by a prolonged extension at 72 °C for 2 min at the end of the last cycle.

The size of the PCR product was analyzed by agarose gel electrophoresis. Five µL of the PCR product was loaded on a 1 % agarose gel and the migration of the bands was compared to the 1 kb+ ladder from Invitrogen. The PCR product which gave the expected length of 1017 bp (the number of base pairs between the selected primers (R ÷ F = 1066 bp ÷ 49 bp)) was used in the following procedures.

### 2.3.2 Sequence ligation

The PCR product was ligated into a pCRII vector (Invitrogen) which contained T7 and SP6 RNA polymerase promoters on each side of the insertion site (Figure 7). A ligation mix was made according to the manufacturer’s protocol, with 1 µL PCR product added to the ligation reaction.
2.3.3 Transformation

The plasmid resulting from the ligation reaction was used for heat shock transformation of *E. coli* bacteria following the manufacturer’s protocol (One Shot TOP10 Chemically Competent E.coli, Invitrogen), to mass produce the ligated vector. One hundred µL of the transformed bacteria was plated out on LB plates containing ampicillin (100 µg/mL) and X-gal (50 µg/mL; Promega), for screening of transformants. An ampicillin resistant gene present in the pCRII vector ensures the growth of only transformed bacteria. The X-gal is added to enable blue/white screening. The multiple cloning site of the plasmid is positioned inside the LacZα.
gene sequence of the plasmid, thus inactivating LacZα upon insertion. Thereby, the bacteria that have been transformed with a plasmid containing an insert will turn out white since the insertion leads to a dysfunctional β-galactosidase. The bacteria that have been transformed with an empty plasmid turn out blue, since the X-gal produces a distinctive blue color dye when cleaved by a functional β-galactosidase enzyme.

Five colonies of successful transformants from each plate were grown in LB-medium and the plasmids were purified with a Midiprep kit according to the protocol of the manufacturer (Wizard Plus Midiprep DNA purification system kit, Promega). The concentration of the plasmid solution was measured by NanoDrop, ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA), and the purified plasmids were sent for sequencing at Eurofin Genomics (Ebersberg, Germany). The sequenced plasmid was found to have the correct sequence inserted in the plasmid, as the sequence was comparable to the NCBI gene bank sequence (see aligning in Appendix II). In addition, sequenced cDNA from the fish used in the experiments were aligned with the sequence ordered from GeneScript, and were proven to be similar (not shown). One plasmid with the gpr54-chr9 sequence inserted was chosen and used in subsequent steps.

2.3.4 Probe linearization and amplification

To linearize and amplify the sequence before producing the probe, a PCR was performed with 10 ng/µL of the plasmid from Midiprep as a template using M13F and M13R primers (Invitrogen), flanking the two RNA polymerases (SP6 and T7) in the pCRII vector, see Figure 7. The PCR was performed with Platinum Taq DNA Polymerase according to the manufacturers protocol (Invitrogen), and ran on GeneAmp PCR system 2700. The following PCR program was applied: 94 °C for 2 min (initial denaturation), and then 35 cycles of 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 1 min and 50 s (extension), followed by a prolonged extension at 72 °C for 2 min at the end of the last cycle.

The size of the PCR product was analyzed by agarose gel electrophoresis. Four µL of the PCR product was loaded on a 1 % agarose gel and the migration of the bands was compared to the 1 kb+ ladder from Invitrogen. The PCR product which gave the expected length of 1258 bp (length of insert (1017 bp) + length from M13R to insert site (130 bp) + length from insert site to M13F (111 bp) = 1258 bp) was used in the following procedures.
2.3.5 Probe synthesis

Synthesis of the receptor probe was performed by in vitro RNA transcription with the PCR product as template and SP6 or T7 polymerases from Promega. Antisense RNA transcript was made by transcription by T7 RNA polymerase, giving a probe sequence of 1215 bp (length of linearized PCR product (1258 bp) ÷ distance from M13F to T7 promoter (43 bp) = 1215 bp). Hapten-conjugated UTP’s: DIG or FITC was added into the transcription mixture and incorporated into the RNA probe by transcription. A transcription mix was made according to the manufacturer’s protocol, with a total volume of 40 µL: 4 µL PCR product, 8 µL transcription buffer, 4 µL RNA labelling mixture (DIG/FITC, Roche Life Science) including NTPs, 1 µL RNAsin plus (Roche Life Science), 4 µL DTT (100 mM; Roche Life Science), 4 µL RNA polymerase T7 (20 u/µL; Promega) and nuclease free water until total volume was reached. The reaction was incubated at 37 °C for two hours. This resulted in single stranded copies of labelled RNA sequences in the form of antisense probes, that hybridizes with target mRNA upon in situ hybridization.

As a negative control during the experiments, the sense probe was also generated by following the same protocol, except that sense probe was made with SP6 RNA polymerase instead.

2.3.6 Probe purification

Purification of the synthesized probes was done with the Nucleospin RNA clean-up kit (Macherey-Nagel) and performed according to the protocol given by the manufacturer, with the exception of the elution step which was performed directly in deionized formamide. In short, a specific buffer that binds the sample RNA to a nucleoSpin column was added to the sample. The bound RNA were then washed and dried for 3 min, before the RNA was eluted by nuclease free water into a tube containing deionized formamide. The probes were stored at -20 °C.
2.4 Control experiments

2.4.1 Probe validation

Because \textit{in situ} hybridization of \textit{gpr54-chr9} has not been previously performed in embryonic medaka, the probe was first used to map the expression of \textit{gpr54-chr9} in adult brain from male medaka fish, to compare the expression pattern with already published data (Kanda et al., 2013).

2.4.2 Positive controls

A positive control was included in each \textit{in situ} hybridization experiment on embryo to ensure the success of each run. The positive control was a probe that hybridizes to the mRNA of the CXC motif chemokine receptor 4a (\textit{cxcr4a}), which is seen to give a characteristic expression pattern in 1.5 dpf medaka embryos.

2.4.3 Negative controls

To ensure that the probe did not bind to unspecific mRNAs in the samples, negative controls were included in the experiments by running \textit{in situ} on samples added sense probes. The sense probe is identical to the target mRNA, and should therefore not hybridize to the target mRNA. Sense controls were run on both brain and embryo samples.

Samples added no probe at all, and samples added probe, but no tyramide components (signal amplificator and carrier of fluorophore dye, described later) were also used as negative controls during the experiments, and were included in each \textit{in situ} run.
2.5 Expression analyses with fluorescent in situ hybridization: single-, double- or triple- FISH

Fluorescent in situ hybridization by tyramide signal amplification was used as a method to reveal the expression pattern of the gpr54-chr9 gene (see Introduction). In cells expressing this kiss receptor the synthesized probe will hybridize to the target mRNA, enabling targeted detection. This method also enables multi-labeling of the sample by adding more than one probe. Single- or double FISH can be performed by adding one or two different RNA probes, respectively. For each additional probe added, the total time of the FISH method increases with one day, so that the total method last for 3 or 4 days. The number of possible probes was limited to the available tyramides, which in our case was two: FITC and TAMRA.

The method was optimized from a protocol received from our collaborators in the group of Philippe Vernier at the Institute of Neurobiology Alfred Fessard, CNRS Gif-sur-Yvette, France, working on in situ hybridization on zebrafish, to fit our medaka model. The detailed optimized protocol is found in Appendix VI.

In situ hybridization was run on insituPro VSi robot (Intavis Bioanalytical Instruments, Germany), controlled by the InsituPro VSi operation software. During the in situ the tissue samples were placed in a gridded well, and the well was placed in one of the chambers on a plate in the in situ robot. Several embryos or brains can be placed in the same well; the number depends on the amount of fluid added. It was crucial for the result that all the tissue samples were embedded in fluid at all time.

Before in situ hybridization the prepared samples, embryos or brains, were rehydrated by gradually decreasing the alcohol percentage of the incubation solution (ethanol), 100, 75, 50 and 25 %, with 10 min incubation time per step. In situ hybridization was performed on rehydrated embryo samples in the embryo expression analyses, and performed on rehydrated brain samples from male adult medaka fish during the probe validation analyses.

The samples were washed in PBST, before they were treated with proteinase K. The added proteinase K enzyme will digest the tissue by the cleavage of specific peptide bonds, so that the reagents used in the experiment will more easily penetrate the tissue. The type of treatment was dependent of the sample type (embryo or adult brain), and in the case of embryo samples it was dependent of the embryonic stage. Different proteinase K treatments
were tested, based on two different protocols received from other labs (Table 3). Proteinase K treatment was terminated by incubation in 20 mM glycine, before the samples were washed in PBST.

Subsequently, the samples were prehybridized with Hybridization mix (HybMix; see Appendix III) for 4 hours at 65 °C. The HybMix was replaced with a solution of the probe of interest diluted in HybMix solution. This dilution had to be optimized for each probe and differed between brain and embryo samples. The probes were denatured by 90 °C heat shock (10 min) before addition to generate single stranded RNA that easily could hybridize with the complementary target mRNA. When multi-labelling of the sample was done, all the probes were added at once. The added probe/probes were left to hybridize to the target mRNA overnight (minimum 15 hours) at 65 °C.

In the second day of *in situ* the samples were added several prepared buffers, one at the time, to rinse the samples so that excess probe was washed out. All buffers were preheated to 65 °C. First, the samples were added a Hybridization wash solution (HybWash; see Appendix III), secondly they were added an increasing amount of 2× SSC (25, 50, 75, and 100 %) diluted in HybWash, before the solution was replaced by 0.2× SSC (100 %). The temperature was lowered to RT, and the samples were washed in PBST. Endogenous peroxidases in the

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**Table 3. Guideline of PK treatment of the samples.**

<table>
<thead>
<tr>
<th>Embryonic stage</th>
<th>[PK] treatment</th>
<th>Time of PK treatment (min)</th>
<th>Treatment temperature</th>
<th>Protocol’s origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;18</td>
<td>None</td>
<td>0</td>
<td>-</td>
<td>A and B</td>
</tr>
<tr>
<td>18-19</td>
<td>25 µg/mL</td>
<td>5</td>
<td>RT</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>1-2.5 µg/mL</td>
<td>20</td>
<td>37°C</td>
<td>B</td>
</tr>
<tr>
<td>26</td>
<td>25 µg/mL</td>
<td>10</td>
<td>RT</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>2.5-5 µg/mL</td>
<td>20</td>
<td>37°C</td>
<td>B</td>
</tr>
<tr>
<td>30</td>
<td>25 µg/mL</td>
<td>30</td>
<td>RT</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>10 µg/mL</td>
<td>20</td>
<td>37°C</td>
<td>B</td>
</tr>
<tr>
<td>33</td>
<td>25 µg/mL</td>
<td>40</td>
<td>RT</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>10 µg/mL</td>
<td>20</td>
<td>37°C</td>
<td>B</td>
</tr>
<tr>
<td>35</td>
<td>25 µg/mL</td>
<td>40</td>
<td>RT</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>20 µg/mL</td>
<td>20</td>
<td>37°C</td>
<td>B</td>
</tr>
<tr>
<td>36</td>
<td>25 µg/mL</td>
<td>40</td>
<td>RT</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>40 µg/mL</td>
<td>20</td>
<td>37°C</td>
<td>B</td>
</tr>
<tr>
<td>37-39</td>
<td>25 µg/mL</td>
<td>40</td>
<td>RT</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>60 µg/mL</td>
<td>20</td>
<td>37°C</td>
<td>B</td>
</tr>
<tr>
<td><strong>Whole adult brain</strong></td>
<td>25 µg/mL</td>
<td>60</td>
<td>RT</td>
<td>A</td>
</tr>
</tbody>
</table>

A guideline of the PK treatment in embryos and brain samples. The treatments were recommended by collaborators from two different groups A and B. Both groups works with medaka as a model organism. A=The group of Jean-Stéphane Joly, CNRS Gif-sur-Yvette, France. B=The group of Kataaki Okubo, University of Tokyo, Japan. PK treatment, Proteinase K treatment.
samples were inactivated by adding 2 % H$_2$O$_2$ (in PBST) with 30 min incubation time at RT. This prevents the activity of naturally occurring peroxidases in further steps. Then the samples underwent a thorough PBST wash, before 2 hours of blocking using 1:10 blocking reagent (Roche Life Science) in maleic acid buffer (MAB; see Appendix III). Blocking was performed to prevent unspecific binding. After blocking, the antibody specific to the probe used was diluted in blocking buffer and added to the samples (1:150 anti-DIG-peroxidase (anti-DIG-POD; Roche Life Science) or 1:200 anti-FITC-POD (Roche Life Science) in blocking buffer; blocking reagent + MAB). The antibodies used are linked to POD, which is important for highly specific tyramide activation and fluorophore aggregation in the upcoming steps. During multi-labelling, one specific antibody is added per day to enable detection of each probe - one after the other. The added antibody was incubated overnight at 4 °C with rotation.

At the third day of in situ, excess antibodies were washed out of the samples by PBST before the tyramide substrate of choice was added (tyramide-fluorophore component; 1:100 tyr-TAMRA or 1:200 tyr-FITC, together with 0.003 % H$_2$O$_2$ in PBST) and incubated, see Appendix V for tyramide synthesis. The incubation times of the different tyramide substrates depended on the type of fluorophore coupled to the tyramide (tyr-TAMRA, 45 min or tyr-FITC, 30 min). In the resulting process, the POD on the antibody will activate the tyramides, causing the tyramide components to precipitate, and thereby aggregating fluorophores at the place of antibody-POD binding, which is the area expressing the gene of interest. Areas with no antibody binding will not have activated tyramides. The excess tyramides were washed out with PBST, before a bleaching reagent was added in the form of 2 % H$_2$O$_2$ for 1 hour to inactivate the added PODs. After POD inactivation the samples were thoroughly rinsed with PBST in several steps, and in the case of single FISH staining the method was finished at this step. In the case of double FISH the method continued, by repeating the blocking step, before a second antibody incubation overnight. First the blocking buffer was prepared; 1:10 blocking reagent in MAB buffer, and added to the samples with 1 hour incubation time. After blocking, the antibody specific to the second probe was diluted in blocking buffer and added to the samples manually (1:150 anti-DIG-POD or 1:200 anti-FITC-POD in blocking buffer; blocking reagent + MAB). The added antibody was incubated overnight at 4 °C with rotation.

The fourth day of FISH was only required for double FISH, and consisted of adding a second tyramide substrate to label the areas of antibody binding from the day before. After a wash in PBST to remove excess antibodies, the samples were added the tyramide substrate of choice -
which had to be different from the first tyramide added (tyramide-fluorophore component; 1:100 tyr-TAMRA or 1:200 tyr-FITC, together with 0.003 % H₂O₂ in PBST). The added tyramides were incubated for 45 or 30 min, depending on the type of fluorophore coupled to the tyramide, tyr-TAMRA or tyr-FITC respectively. The excess tyramides were washed out with PBST, before a bleaching reagent was added in the form of 2 % H₂O₂ for 1 hour to inactivate the added PODs. The hydrogen peroxide was washed out by PBST, and after this PBST rinsing the sample labeled with double FISH is ready.

Following *in situ* hybridization, embryo and brain samples could be stored at 4 °C for shorter periods of time (couple of days) before proceeding with the experiments.

### 2.6 Sample preparation for confocal microscopy

#### 2.6.1 Brain sectioning

Following *in situ* hybridization on whole brain samples, the brains were sectioned into slices before any subsequent analyses. This was done using a vibratome (Campden instruments LTD, UK). The brains were first rinsed in PBS, before excess fluid was removed by fine paper, and then embedded in 3 % agarose. Cubes of gel each containing a brain were cut out of the gel with a scalpel. The brain cube was then glued in a vertical position (olfactory bulbs facing up) onto a plate in a chamber, and once the glue had dried this chamber was filled with PBST until the gel cube was completely covered by the fluid. One hundred and fifty μm thick transverse sections were made. Each brain section was transferred to a tube containing PBST by a fine paint brush.

#### 2.6.2 DAPI staining

4’’,6-diamidino-2-phenylindole (DAPI) stain (5 ng/mL) was added to each sample (embryos or brain sections), before incubation in the dark (using aluminum foil). The incubation time for the embryos was 4 hours, while the incubation time for brain sections was 20 min. The DAPI will stain the cell DNA, and thus label the cell nuclei. The samples were washed repeatedly in PBST afterwards to remove excess DAPI, and stored in PBST at 4 °C until imaging with confocal microscopy.
2.6.3 Mounting of confocal microscopy samples

Before confocal microscopy imaging the samples were mounted on slides. For embryos, a well was made of donut-shaped stickers added in a stack. An embryo was placed in the middle, and the fluid was removed by a filter paper, before drops of glycerol-based Vectashield (Vector Laboratories, California, USA) was added to the sample. Vectashield gives an ideal refractive index in the microscope, and preserves the samples by preventing drying and inhibit photo-bleaching of the fluorochromes. The orientation of the embryo was optimized by gently moving the embryo with tweezers under a binocular, before a coverslip was added to seal the samples. The DAPI-stained brain sections were mounted on a glass slide under a binocular in the right anatomical order, forebrain to brainstem. The excess fluid was removed by a filter paper, and Vectashield was added to the specimen before a coverslip was added.

2.7 Confocal microscopy

The slides were analyzed by confocal microscopy (Zeiss LSM 710, Germany) with proper lasers (argon) depending of the kind of fluorescent labels used in the experiment. The settings were adjusted on the computer (Zen 2009 software). The 10× (air) objective was used to get an overview of the sample, and the 20× (air), 25× (water) and 40× (oil or water) objectives was used to achieve more detailed images. In the brain sections, the pattern of expression in each area of the brain was compared with published expression maps of kiss receptor gpr54-chr9 in the medaka brain (Kanda et al., 2013). In addition, the medaka brain atlas published by Ishikawa et al. (1999) was used in this process for correctly orientation in the brain. Z-stacks of confocal images were made, enabling three-dimensional expression patterns to be revealed. Expression was observed as fluorescent color (FITC/TAMRA) aggregating around a cell nucleus colored by DAPI. The quantity of color around each nucleus indicates the expression level of that cell, with low expression having a dotted appearance around the nuclei.
Table 4. Confocal microscopy argon lasers.

<table>
<thead>
<tr>
<th>Type of staining</th>
<th>Laser λ, nm</th>
<th>Visible color added to laser</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>405</td>
<td>Gray</td>
</tr>
<tr>
<td>FITC</td>
<td>488</td>
<td>Green</td>
</tr>
<tr>
<td>TAMRA</td>
<td>561</td>
<td>Red</td>
</tr>
</tbody>
</table>

Information of the different lasers tuned on to reveal the different types of staining, DAPI and tyramides: FITC/TAMRA.

2.8 Confocal image analyses

The Z-stacks of images from the confocal microscope were analyzed in the ImageJ-based program Fiji (http://fiji.sc/Fiji). The stacks of images taken of the expression in adult brain were sorted on the computer, and similar brain areas were aligned to compare the expression patterns. Similar areas from the positive and negative control samples were included in this comparison. The stacks of images taken of the embryonic expression were sorted on the computer by the embryonic stage, and the expression pattern in each stage was noted, and compared with the embryonic control samples.

The images shown in Results, were representatives for the average expression pattern seen. Images were made by fusing Z-stacks, fusing the stacked images together to a single picture. Further, by splitting and fusing the different colors used, it is possible to look at both each color (DAPI/FITC/TAMRA) on its own, or together. The light settings and contrast were optimized in each picture, before composites were assembled using PowerPoint.
3 Results

3.1 Fluorescent in situ hybridization method adaptation

The fluorescent in situ hybridization (FISH) method was adapted from zebrafish to medaka (embryo and adult brain) by repeated trials. Different variables were tested in these trials, and the different outcomes were assessed.

Through these trials it was found that the in situ robot had an optimal volume of 200 µL per sample well for all solutions, and working with volumes different from this was not successful. Two hundred µL was enough to cover the samples, smaller volumes increased the risk of drying of the samples, while increased volumes (300 µL tested) resulted in a decreased precision of the robot, as it failed to keep the volume distributions constant. Using this sample well volume, it was found that the optimal sample size was 1-2 brains per well, or 1-15 embryos. In addition, it was clear that the standard wells used for the brain samples had a mesh that were too coarse for the earliest embryonic stages (mesh size 100 µm), damaging the tissue during fluid exchange. This improved when using wells with a finer mesh (mesh size 55 µm). It was further found that the robot worked best with a low number of wells. The robot goes through a series of cleaning steps between every well to avoid contamination. These steps are time-consuming, giving that each additional well increases the incubation time in that step. In addition, increased number of wells sometimes led to inaccurate volume exchange distributed by the robot. The ideal number of wells was found to be less than 15 when working on the current protocol.

The proteinase K treatments tested was based on values obtained from protocols received from two separate groups (see Materials and methods, Table 3); the group of Jean-Stéphane Joly, CNRS Gif-sur-Yvette, France, and the group of Kataaki Okubo, University of Tokyo, Japan. Whole brain proteinase K treatment was tested with the given concentration, but different incubation times (the stated 60 min, and 45 min). Whole brains were found to tolerate a one hour long proteinase K treatment of 25 µg/mL at room temperature, without substantial tissue damage. This optimal proteinase K setting was similar to the one in Joly’s protocol. Medaka embryos were found to be much more fragile, and most of the settings in the received protocols were found to be too harsh. But through a series of trials it was found that Okubo’s settings worked well when the reaction temperature was reduced. In this way,
embryos of different stages were added various concentrations of proteinase K according to protocol, with younger stages receiving a more diluted enzyme. The incubation time was 20 minutes for all stages, but the reaction was carried out at room temperature, not at 37° C.

When running *in situ* hybridization on whole brains, an optimal probe concentration of 10 µL *gpr54-chr9* probe per 200 µL total well volume was established after several trials. Decreased concentrations gave no signal or a reduced signal, and increased concentrations gave greater background staining. Because I did not achieve any specific *gpr54-chr9* labeling in the embryonic samples, no optimal embryonic probe concentration was established.

To decrease background staining of the samples, additional washing steps was included after the antibody incubation and after tyramide-compound activation. The peroxidase inactivation step by hydrogen peroxide (2 %) after tyramide-compound incubation was increased from 30 min to 60 min to decrease the background staining and to avoid cross-reactivity of the two different labelings when running double FISH. Effective peroxidase inactivation is crucial between the two labeling procedures to avoid false positive signal overlap of the two probes.

These adjustments to the original protocol were found to give a clearer expression signal, and/or increasing the background staining, improving the detectability of the FISH method.

### 3.2 Expression analyses by fluorescent *in situ* hybridization

Gene expression in the samples was explored under the confocal microscope by scanning through the sample. Expressing cells were seen as spots of fluorescent color surrounding a DAPI-colored cell nucleus. Some of the cells were marked brightly by fluorescence, in addition to an unusually strong DAPI color. These cells were evenly spread out through the brain, some of them oriented in chain structures, and they looked different from the expressing cells revealed by the antisense probe. Such cells were counted as auto-fluorescence, and excluded from the analyses.
3.2.1 Spatial expression of gpr54-chr9 in adult medaka brain – probe validation

The gpr54-chr9 expression pattern in adult medaka brains were revealed by looking through the sectioned in situ labeled brains, from anterior to posterior. After adapting the method, the pattern of gene expression of gpr54-chr9 was found consistently in the adult brains tested (approximately 15 brains), also between different FISH runs. This made the expression patterns presented in the following paragraphs good representatives for the average expression pattern seen in the experiments. Further, the expression found showed bilateral symmetry around the midline in every area of expression. In addition, every in situ run included negative controls (described in next paragraph), giving basis for comparison of the expression pattern resulting from the gpr54-chr9 probe to negative control samples.

When mapping the spatial expression pattern of gpr54-chr9 in adult brain, expression was revealed at distinctive brain areas. The most anterior area found to express gpr54-chr9 was area ventralis telencephali pars dorsalis (Vd) of the telencephalon (Figure 8). This area is found central in the section. The expression appeared as many TAMRA-colored red spots clustering around the DAPI nuclei. Further, the expressing cells were dense in certain areas, localized along the midline. In the same brain section expression was also found in a more ventral part of the telencephalon, close to ventralis telencephali pars lateralis (Vl), lateral on either side of the midline (Figure 9). The number of labeled cells was lower compared to what was found in Vd, but these cells were large and had a spherical shape, and in addition, the cells were found to cluster together in a distinctive manner.
Figure 8. *gpr54-chr9* expression in Vd. Whole brain *in situ* with *gpr54-chr9* probe, transverse view. A: An illustration of the brain section, with the area seen in the confocal pictures marked with a red square. B: Confocal Z-stack (24 µm) showing *gpr54-chr9* (red) and DAPI (grey). A lot of expression is seen in the central area, marked with arrows. The image is a merged image of DAPI (C) and *gpr54-chr9* (D). Images taken with 40× oil objective. Vd, ventralis telencephali pars dorsalis.
Figure 9. *gpr54-chr9* expression in ventral telencephalon, lateral of the midline. Whole brain in situ with *gpr54-chr9* probe, transverse view. A: An illustration of the brain section, with the area seen in the confocal pictures marked with a red square. B: Confocal Z-stack (17 µm) showing *gpr54-chr9* (red) and DAPI (grey). Area with clustered expressing cells marked with arrows, close to VI area. The image is a merged image of DAPI (C) and *gpr54-chr9* (D). Images taken with 40× oil objective. VI, ventralis telencephali pars lateralis.

In the posterior section bright spots of expression were found in the preoptic area (POA) of the hypothalamus, with expression seen scattered over the entire area (Figure 10). The expressing cells appeared large, with a slightly spherical shape, and many expressing cells were present. In the more central parts of the same brain area expression was found in the ventralis telencephali pars supracommissuralis (Vs; Figure 11) of telencephalon. The expression was dense in this area as well, but the cells appeared smaller in comparison.
Figure 10. *gpr54-chr9* expression in POA. Whole brain *in situ* with *gpr54-chr9* probe, transverse view. A: An illustration of the brain section, with the area seen in the confocal pictures marked with a red square. B: Confocal Z-stack (40 µm) showing *gpr54-chr9* (red) and DAPI (grey). Arrows marks some of the several expressing cells. Large cells were expressing the current gene in this area. The image is a merged image of DAPI (C) and *gpr54-chr9* (D). Images taken with 40× oil objective. POA, preoptic area.
Further, the most posterior *gpr54-chr9* expressive areas was found in the nucleus preopticus pars parvocellularis (POp) area of the hypothalamus (Figure 12). The expression was seen as large cells forming cell clusters, evenly distributed over the entire area. The expression seen in POp covered a big area of the ventral part of the section, and proceeded upward to more central regions. The expression preceded to the nucleus preopticus pars magnocellularis (POm) and ventralis telencephali pars posterior (Vp) areas (Figure 13) in the border between the hypothalamus and the telencephalon. Labeled cells were found with high density. Also here, some of the expressing cells appeared large in size, but most of the cells were normal in size and shape. Several of the expressing cells formed cell clusters in this area.
Figure 12. *gpr54-chr9* expression in POp. Whole brain *in situ* with *gpr54-chr9* probe, transverse view. A: An illustration of the brain section, with the area seen in the confocal pictures marked with a red square. B: Confocal Z-stack (22 µm) showing *gpr54-chr9* (red) and DAPI (grey). Areas with large, clustering cells showing expression are marked with arrows. Chain of auto-fluorescent cells marked by a triangle. The image is a merged image of DAPI (C) and *gpr54-chr9* (D). Images taken with 40x oil objective. POp, nucleus preopticus pars parvocellularis.
Figure 13. *gpr54-chr9* expression in POM and Vp. Whole brain in situ with *gpr54-chr9* probe, transverse view. A: An illustration of the brain section, with the area seen in the confocal pictures marked with a red square. B: Confocal Z-stack (24 µm) showing *gpr54-chr9* (red) and DAPI (grey). Areas with clustered expressing cells marked with arrows. The expression appear dense in these areas. Auto-fluorescent cells marked by triangles. The image is a merged image of DAPI (C) and *gpr54-chr9* (D). Images taken with 40× oil objective. POM, nucleus preopticus pars magnocellularis; Vp, ventralis telencephali pars posterior.

More posterior sections where all screened for *gpr54-chr9* expression, but no labelled cells were revealed. Special attention was given to the habenula, because previous reports have found expression in this area (see Introduction). However, no clear labelling of cells was found in this area in my experiments (Figure 14). The habenula often showed some evenly scattered coloring, seen on Figure 14, but not clear enough to conclude with any specific labeling in this area.
Figure 14. No gpr54-chr9 expression in the habenula. Whole brain in situ with gpr54-chr9 probe, transverse view. A: An illustration of the brain section, with the area seen in the confocal pictures marked with a red square. B: Confocal Z-stack (15 µm) showing possible gpr54-chr9 expression (red) and DAPI (grey). No clear cell expression was found. Auto-fluorescent cell marked by a triangle. The image is a merged image of DAPI (C) and gpr54-chr9 (D). Images taken with 40× oil objective. HB, habenula.
3.2.2 Negative controls

Three wells containing negative control samples were included in all FISH experiments. These were experiments on adult brain or embryos with either no probe added, no tyramides added, or using sense probes of the \textit{gpr54-chr9}. When examining at the negative controls under the confocal microscope, settings were kept constant to the values used during the expression analyses. The samples added no probe (not shown) showed only auto-fluorescent cells, by some bright tyramide colored cells, also brightly lighting up on the DAPI coloring. The samples added no tyramides (not shown) appeared dark on the TAMRA/FITC, but had similar, bright DAPI nuclei showing up on the images as auto-fluorescent cells. When turning up the fluorescent light, the same cells lit up in TAMRA/FITC. In the sense probe testing, a sense probe concentration similar to the optimal antisense concentration was used. All brain sections in the sense tests were examined for labeling, and no expression similar to those seen for the antisense probe was found in any areas. Figure 15 and Figure 16 show two examples of sense experiments. The first image (Figure 15) shows the preoptic area (POA), the same area as analyzed with the antisense probe in Figure 10. The sense probe gave no specific labelling of cells in the POA like seen in the antisense experiments, but some auto-fluorescent cells were seen lightened up bright on both TAMRA and DAPI. The second image (Figure 16) shows the Vs in the telencephalon, which is the same area pictured for the antisense probe in Figure 11. Only auto-fluorescent cells were seen here, as the structures labelled with TAMRA was also strongly labeled with DAPI. No specific labelling was seen in the Vs.
Figure 15. No expression of \textit{gpr54-chr9} SENSE probe in POA. Whole brain \textit{in situ} with \textit{gpr54-chr9} SENSE probe as negative control, transverse view. A: An illustration of the brain section, with the area seen in the confocal pictures marked with a red square. B: Confocal Z-stack (20 µm) showing any \textit{gpr54-chr9} SENSE expression (red) and DAPI (grey). No expressing cells were found. Auto-fluorescent cells marked by triangles. The image is a merged image of DAPI (C) and \textit{gpr54-chr9} SENSE (D). Images taken with 40× oil objective. POA, preoptic area.
3.2.3 Spatial expression in medaka embryos

Potential expression in the *in situ* labelled embryo samples were inspected under the confocal microscope. In addition to the embryos labeled with *gpr54-chr9* and the negative controls (described previously), a well of positive controls were included in each run.

The FISH method was established in embryos using the positive control, *cxcr4a* probe, at a concentration of 8 µl per 200 µl total well volume in 1.5 day old embryos. *cxcr4a* expressing cells were found in the same areas as previously found by members of my group (unpublished), giving rise to a characteristic expression pattern along the outer edge of the
developing retina, following the shape of the eye cavity from an anterior point and further posterior to each eye, in the outer area of the brain (Figure 17). The expression was seen as bright red spots, colored by TAMRA, each surrounding a nucleus. The edge effect was large, seen as strong labeling of the surface of the embryo.

Despite many trials variating several different parameters in the in situ protocol, the gpr54-chr9 probe gave no specific labeling in embryos at any stage.

Figure 17. cxcr4a expression in embryo 1.5 dpf. Whole embryo in situ with cxcr4a probe, labelled with TAMRA. A: DAPI stained overview image of the embryo, with the area seen in the B-D confocal pictures marked with a red square. B: Confocal Z-stack (22 µm) showing cxcr4a (red) and DAPI (grey). Some of the expressing cells are marked by arrows. Distinctive expression pattern along the eye cavities. The image is a merged image of DAPI (C) and cxcr4a (D). Images taken with 40× oil objective.
3.2.4 Double fluorescent in situ hybridization establishment

To enable double FISH experiments, two separate labelling approaches have to be established. Two different hapten-conjugates are needed, one for each probe, and in addition two tyramide components have to be established. In this way the two specific probes tested for could be labelled separately. Probes labelled with different hapten-conjugates was established for the positive control, *cxcr4a*, with two different working tyramide compounds, tyr-TAMRA (red) and tyr-FITC (green). Expression was seen in both *cxcr4a*-TAMRA labeled embryos and *cxcr4a*-FITC labeled embryos, see Figure 17 and Figure 18, respectively. The expression pattern shown by the two different labeling approaches were similar, a bilateral expression pattern with labelled cells showing along the outer edge of the developing retina, following the shape of the eye cavity from an anterior point and all the way back posterior to each of the eyes in the outer area of the brain. The *cxcr4a* expressing cells appeared approximately similar in every successive run, with almost same expression level and pattern regardless of the tyramide component used.

![Figure 18. cxcr4a expression in embryo 32 hpf.](image)

*Figure 18. cxcr4a expression in embryo 32 hpf.* Whole embryo in situ with *cxcr4a* probe, labelled with FITC. The image, is a confocal Z-stack (30 µm) merging *cxcr4a* (green) and DAPI (grey). Some of the expressing cells are marked by arrows. Distinctive expression pattern along the eye cavities and posterior to the eyes at the edge of the developing brain. Confocal microscopy imaging with 20× air objective.
4 Discussion

In this study, a fluorescent in situ hybridization (FISH) assay in whole brains from medaka has been set up. In addition, antisense probes hybridizing to the mRNA encoding the Kiss receptor gpr54-chr9 gene has been successfully made, and the spatial expression pattern in the adult brain has been mapped. Two different tyramide labels were established in embryos with a positive control probe. However, as I did not see any labeling with the gpr54-chr9 probe in embryos, the expression pattern of gpr54-chr9 through embryogenesis is still unknown.

4.1 Spatial expression pattern of gpr54-chr9 in adult brain

After adapting the method, gpr54-chr9 antisense probe was found to work in whole brains from adult medaka, giving a spatial expression pattern with many similar aspects to the expression map found in the literature (see Introduction). Through systematic trials, it was found that gpr54-chr9 expression is present in the ventral telencephalon in the anterior part of the brain (in Vd, lateral-ventral telencephalon, Vs, and Vp; see Results) and in the ventral hypothalamus in the posterior part of the prosencephalon (in POA, Pop and POm; see Results). This corresponds with the expression pattern reported in medaka by Kanda et al. (2013), except in habenula were no clear expression was detected in the current work (see Figure 14, and discussion later in this chapter). This indicates that the adapted protocol is working, and that the gpr54-chr9 probe is valid.

During my experiments, a distinctive difference was observed between the cell appearance between the ventral telencephalon and the ventral hypothalamus gpr54-chr9 expressing cells (see Results), with the latter showing cell expression in large, slightly spherical shaped cells clustering together, while in the former the expressing cells appear smaller and more normal shaped, but also showing clustering tendencies. These findings correlates with the findings made by Kanda et al. (2013), stating that gpr54-chr9 is mainly expressed by large neurons in the POA, and both large and small cells in the dorsomedial areas; POm and POp. The hypothalamic POA are known to have GnRH1 expressing neurons (Moriya et al., 2013), which is the form of GnRH found to be hypophysiotropic and thus control gonad development and maturation in most teleosts (Oka, 2009). Most vertebrate species
investigated, shows co-localization of kiss receptor genes and GnRH1 (Parhar et al., 2004), emphasizing the kiss system’s direct regulatory role during reproductive control. Some exceptions to this is found in certain teleost species, including the European sea bass (Escobar et al., 2013) and medaka (Kanda et al., 2013), where no co-localization could be observed. However, kiss receptor expression (including medaka \textit{gpr54-chr9}) was found in close proximity to \textit{gnrhl} in both studies, leaving a possibility of indirect Kiss regulation of GnRH neuron activity through interneurons. It is found that Kiss1 neurons in the NVT area of medaka sends projections to the POA, (Hasebe et al., 2014), which further indicates that Kiss1 in the NVT can control the GnRH neurons in the POA. The cell clusters of relatively large, distinctive cells, found around the POA in my experiments are therefore possibly interneurons, linking the kiss system to GnRH1 neurons in the reproductive axis. The smaller, expressing cells that were found in my experiments, mainly in the ventral telencephalon areas, looked different from the cells described above. My suggestion is that these cells exert some other physiological function in the animal, either somehow linked to reproduction or reproductive behavior, or having some other role in the animal. It is well known that many of the areas of \textit{gpr54-chr9} expression, like the POA and the ventral telencephalon, are important in regulating several homeostatic events, like regulating reproduction and reproductive behaviors in teleost species (Satou et al., 1984).

In the areas I found to express \textit{gpr54-chr9}, there were a tendency of many cells showing expression, especially in the preoptic areas, but the expression level in each cell appeared to be low. Too low levels of expression could be an explanation for the lack of expression in habenula: since the expression level already was low in the other areas showing expression, reduced levels in this area could lead to the fluorescent emission falling below the detection limit. In fact, in my analyzes indicate some labelling in the habenula, but it was too weak and diffuse to make any firm conclusion. If low expression level is the problem, small adjustments to the method to increase the expression level and reduce the background could be a breakthrough in the detection of habenula \textit{gpr54-chr9} labelling. In the expression map published by Kanda et al. (2013), the habenula expression signal appears a bit weak compared to the other expressed areas. In this study the expression pattern is revealed by classical \textit{in situ} hybridization, which proves to be a more sensitive method compared to FISH (see Introduction). Further, the lack of \textit{gpr54-chr9} expression in my experiments, compared to the findings of Kanda et al. (2013) might be a result of fish specific differences between the two aquarium facilities. Different handling of the fish could affect the kiss system expression.
level, as Kiss1 localized in the habenula is found to be affected by fear in zebrafish (Ogawa et al., 2014). Habenula Gpr54 is not restricted to medaka, as expression is reported in several other vertebrate species, in both mammals (rodent species: mice and rats; Herbison et al., 2010; Lee et al., 1999) and other teleosts (zebrafish and European sea bass respectively; Escobar et al., 2013; Ogawa et al., 2012). The habenula expression in these two teleost species has proven to be dense. As habenula Gpr54 expression appears to be a vertebrate wide phenomenon, the lack of detection during my study might be a cause of too low sensitivity of the method. To find out if expression was present in the habenula of our medaka, a qPCR could have been performed on dissected habenula tissue. This is a much more sensitive method, which probably could have detected any low-level gpr54-chr9 expression present, but I did not have the time to do so.

The expression pattern found in medaka in my experiments, and by Kanda et al. (2013) shows a different spatial expression pattern in the brain compared to what is found for the receptor ortholog in zebrafish (Ogawa et al., 2012) and in the European sea bass (Escobar et al., 2013). Zebrafish expression of this gene is found mainly in the habenula, where the expression is most abundant, and in the POp in the ventral hypothalamus. However, expression is also found in far more posterior areas, like nucleus fasciculus longitudinalis medialis (NFLM) and nucleus posterioris periventricularis (NPPv) of the mesencephalon, and additional low expression in the cerebellum of rhombencephalon. The European sea bass has an expression pattern more similar to medaka, but still showing some differences. Here, expression is found restricted to the areas of the ventral telencephalon, the preoptic areas and habenula, like in medaka, and additional expression found in a few cells in the mediobasal hypothalamus of the mesencephalon and dense expression showing in the pituitary. All the three teleost species have some expression in the preoptic areas common, in addition to the habenula expression. The many differences in the expression patterns between medaka and zebrafish is not surprising, as the kiss1 and kiss2 expression patterns between the two species is shown to differ notably, and also showing physiological differences in regulating the reproductive axis (see Introduction). Mammals lack this current receptor ortholog (see Introduction), but have another homolog in a different receptor clade, GPR54-1, showing wide distribution of expression in many areas throughout the brain, differing from both the teleosts’ expression patterns (Herbison et al., 2010). But what is similar for all vertebrate species investigated is that the kiss receptor gene is expressed in, or proximate to, the GnRH neurons that control the reproductive axis.
From the findings discussed in the above paragraphs, it is clear that the expression pattern of gpr54-chr9 found in my experiments correlates well with the available expression map in medaka. The expression pattern differs somewhat from what is found in other vertebrates, also in other teleosts, which might indicate that the receptor has intraspecies functional differences.

4.2 In situ hybridization in embryonic medaka

Seen from the expression of cxcr4a, which I used as positive control, the in situ hybridization method worked in embryos as well. However, no labeling was found with the gpr54-chr9 probe in embryos. It is known from the qPCR studies performed by Hodne et al. (2013) that gpr54-chr9 expression is present in all stages of embryonic medaka. Either the use of gpr54-chr9 probe by FISH needs further modifications for the use on embryos, or the expression level is below the detection limit of this method.

Because of Gpr54-chr9’s central role in the brain of adult medaka, and its predicted role in the developing brain seen from the knockdown studies performed by Hodne et al. (2013), a suggestion will be that expression is seen in the developing brain. The gene knockdown and partial knockdown (Hodne et al., 2013) resulted in developmental arrest, possibly as a result of affected neuron migration, and morphological defects of the developing brain and eyes region occurring, respectively (see Introduction). Therefore, during the search for embryonic expression of gpr54-chr9, my main area of focus was the developing brain, but the rest of the developing embryo was also carefully investigated. In addition, gpr54-chr9 might show expression in close proximity to GnRH1 neurons in embryos, similar to what is seen in the adult brain. The expression of gnrh1 has been mapped through embryonic development in medaka (Okubo et al., 2006; Parhar et al., 1998). Two gnrh1 expressing neuronal clusters develop. The first cluster emerges at 2 dpf bilaterally around the nasal area and the dorsal telencephalon. As the development reach 4 dpf, the neural population has migrated posterior into the forebrain through the ventral telencephalon to the POA, and when reaching 20 dpf the neurons have developed extended projections to the pituitary. The other cluster emerges first at 4 dpf bilaterally in the anterior telencephalon, developing through a more medial route, reaching the ventral telencephalon at 10 dpf. If the gpr54-chr9 probe starts working in embryo, then expression might be seen in proximity to these migratory routes.
Despite this lack of \textit{gpr54-chr9} labelling, the FISH method was successfully adapted to embryonic expression analyses with the \textit{cxcr4a} probe. Both tyramide colorings, TAMRA and FITC were proven to work in embryos for this positive control. Expression was distinctive around 1.5 dpf embryos, giving a characteristic labelling pattern in the brain and eye region. Bright expressing cells of specific labeling were found along the outer edge of the developing retina, following the shape of the eye cavity from an anterior point and further posterior to each eye, in the outer area of the brain. Cxcr4 are receptors working as important components in cell migration, among others in development (Wu et al., 2010). Teleost species have two paralogous variants of the gene; \textit{cxcr4a} and \textit{cxcr4b} (Sasado et al., 2008). The receptor ligand is Sdf1, which is considered an attractant for the cells containing the Cxcr4 receptor, thereby promoting cell migration. Because of the expected role of Gpr54-chr9 in neural migration and brain- and eye development, it could be interesting to explore the expression pattern of the \textit{gpr54-chr9} and \textit{cxcr4a} in combination in a double FISH assay, searching for any potential co-expression of the two receptor genes. Especially since \textit{cxcr4a} was shown to be expressed in both the area of the developing brain and eye in 1.5 dpf embryos during my experiments. The establishment of two working labelling approaches in this thesis enables double FISH, and could be used for investigating this relationship, once the \textit{gpr54-chr9} probe is working in embryo.

4.3 Further improvement of the method

Further improvement of the method is needed to set up a working protocol using the \textit{gpr54-chr9} probe in medaka embryos. Improvements affecting the rate of sensitivity of the method to reach above the detection limit, in addition to reducing the background staining is essential. First, the optimal probe concentration has to be found, needing more probe concentrations to be tested in embryos, to reveal the optimal dilution. Further, different probe hybridization temperatures could also be tested to find the ideal binding temperature (Yilmaz and Noguera, 2004). The receptor probe is quite long (1215 bp), making it harder to reach the target transcript within the sample tissue and thus complicating the hybridization reaction. By decreasing the hybridization temperature slightly (from 65°C) the rate of hybridization will increase because of less energetic reagents, but a decrease in specificity is expected to follow (Thisse and Thisse, 2008). Even so, this increased hybridization could prove sufficient to reach an expression above the limit of detection. Another improvement worth trying is to change the viscosity of the probe mixture to increase the rate of probe reaching the target.
transcripts. This was achieved by Lauter et al. (2011) by adding an viscosity-increasing polymer (5% dextran sulfate was used in this study) to the reaction buffer upon hybridization. Increased viscosity will increase the ability for the probe to reach the target of hybridization, giving a higher local concentration of the probe, resulting in a higher level of hybridization by significantly enhancing the signal intensity. Further, the POD inactivation efficiency after tyramide binding could be increased further to improve the expression to background level. This could be achieved by increasing the time of hydrogen peroxide treatment further, or it could be achieved by inactivation by another reagent; glycine-HCl (100 mM, pH 2.0), which is proven by Lauter et al. (2011) to give a more effective inactivation. A couple of test-runs using this inactivation procedure were performed, but there was no time for systematic testing. In the few runs tested, no significant effect was observed. Proper POD-inactivation could be even more crucial when working with double FISH, where complete inactivation is critical for good results. Improper inactivation could lead to false positive co-expression of the two probes tested (Gasnier et al., 2013). In addition, it is possible to include an additional amplification step, like stated in the protocol by Gasnier et al. (2013), for detection of low level expression. In this double signal amplification step, biotinyl-tyramide complexes are added to precipitate at the area of antibody-POD. A new round of -POD is added that specifically binds this complex, before the second tyramide complex is added coupled to fluorophores. The added tyramide-fluorophore complex is activated by the second POD, and precipitates around the POD-bound biotinyl-tyramides. By including this step an intensification of the fluorescent signal compared to original TSA FISH might be achieved, which could lead to increased signal of gpr54-chr9 in brain samples, or detection in embryo. Further, a shortened version of the probe could be made. Making a new, shorter version of the probe could be a turning point toward detecting gpr54-chr9 expressing cells in embryo. A shorter probe will more easily reach the targeted cytosol of the expressing cells, and might make detection possible. A downside is that by shortening the length of the probe, its specificity decreases (Thisse and Thisse, 2008).

At last, it could have been wise to start the expression analysis in medaka embryo with a classical ISH to get an indication of the area of expression, since no expression map is yet published. In this way an indication of the area of expression is given, and screening of the different embryonic stages could be simpler, which overall could make the probe detection during expression analyses by FISH easier (Gasnier et al., 2013).
Unfortunately, the time I had available for adaptive testing was limited, rendering an undetectable gpr54-chr9 probe expression in embryo samples. Further, it could be that the embryonic expression level of pgr54-chr9 is too sparse, making it hard to adapt the settings to increase the stained expression pattern above detection limit, and thereby making the overall mRNA detection in embryo difficult by the FISH method.

4.4 Summary

The object of this thesis was divided in three defined aims

First, I adapted a fluorescent in situ hybridization assay, originally optimized for zebrafish, to work in a medaka model of adult brain samples. This was a time-consuming process, with a lot of trouble-shooting, but eventually the adaptions contributed to getting the expression level above detection limit. A functional antisense gpr54-chr9 probe was established, and the expression pattern in the brain verified previously published expression maps in the medaka brain, thereby rendering the probe valid. With this in mind, the first aim of my study has been fulfilled.

Second, the fluorescent in situ method was adapted to work in an embryonic medaka model. This was not achieved for the validated gpr54-chr9 probe, but for the positive control probe of cxcr4a. Either the gpr54-chr9 expression is too sparse to be detected by FISH, or this probe needs further probe-specific adaptions to work in embryos. This entails that the spatial-temporal expression pattern of the gpr54-chr9 gene remains unknown for the time being, leaving this second aim just partly fulfilled.

Last, I have through these experiments established two separate labelling approaches, working in both brain and embryonic samples. In this way it is now possible to do double-FISH assays, exploring gene expression of two genes in parallel, if two working probes are available. Thereby, the third aim of the study is fulfilled.

4.5 Future experiments

With my experiments ended, several doors open for future experiments. For instance, the FISH method using the gpr54-chr9 probe could be used on adult medaka brain from the transgene line of gnrh1:Gfp, available at our aquarium facility at the University of Oslo. This
future experiment can possibly confirm indirect kiss system regulation of GnRH1 neurons through Gpr54-chr9 containing interneurons, like found by Kanda et al. (2013), or if any gpr54-chr9 is expressed directly on the GnRH1 neurons, like seen in most species throughout the vertebrate subphylum (see Introduction). Further, if gpr54-chr9 containing interneurons are present, then a characterization of these cells could be performed by combining FISH using the gpr54-chr9 probe with immunohistochemistry using antibodies for various neuron-specific proteins looking for potential co-localization and thereby characterize the gpr54-chr9 cells.

In addition, if the gpr54-chr9 probe was set to work in embryos, several interesting experiments could be set up besides finding the spatial expression pattern throughout embryogenesis. Like described previously, a double FISH assay combining the gpr54-chr9 probe with the cxcr4a probe could reveal a possible co-localization of the two receptor genes during neural migration in the developing brain. Further, FISH using the gpr54-chr9 probe could be ran on embryos of the gnrh1:Gfp transgenic line, investigating if expression is found in GnRH neurons in embryo, or if the expression is similar to the situation found in adults. This could in addition render information about the kiss system’s regulation of GnRH1 neurons during development, and might reveal if it regulates the development or the migration of the current neurons.

Another thing worth looking into is if any changes to the gpr54-chr9 expression occur in embryos when the level of Kiss is manipulated, ether by Kiss ligand stimulation or by knockdown experiments using morpholinos (splice site blocking of specific transcripts by antisense oligos, like performed by Hodne et al. (2013)).
References


Appendices:

APPENDIX I: Plasmide pUC57 insert sequence
APPENDIX II: Sequencing results of pCRII insert
APPENDIX III: Buffer recipes
APPENDIX IV: Medium recipes
APPENDIX V: Tyramide-component synthesis
APPENDIX VI: Fluorescent *in situ* hybridization protocol
Appendix I: Plasmid pUC57 insert sequence

Kiss receptor gpr54-chr.9:

**SP6:** Sequence (5’→3’)

```
CATGATTACGCAAGCTATTTAGTGACACTATAAGAATACTCAAGCTATGC
ATCAAGCT
```

**CDS:** Sequence (5’→3’)

```
ATGTCTGCAGAACCGGCGACCATTGGGAGTCCGAACCTGTTGCTGCTCGTGC
AACCTTTCCCTGGAGATCCCAACGCACCACGCAGCTGGTCGACGCCTGGTGG
GTGCCCACTTTCTCTGCCATCAATGCTGTGCTGCTGTGCTGGTGGGAACTCGC
TGCTGTGCACTATGTGTACGAGATACAGACAGATGAAGACTGTCACCAAT
TTCTACATAGTCAAATCTGGCTACTACTGACATCTTGTCTGGGTGCTGCTGCG
TTCCCTCCACCGCACTCTGTAATTCCCTCTGCCAGCTGGATCTTTTGGGAGTT
CATGTGCCGTCGTCAATTATCTACAACAGCTGACTGCGCAGGCCACTTG
CATCACACCTGCTCTGCAATAGGCGGTGGGACAGCTATGACGAGTTATCC
TCTGCAGTCTGCTGGCAACCCGACACTTGGCTGTGGCTGTGCTGCTGCG
GCCATCTGGATAAGCTCCTTGCTTCTGTCCATTCTCCGTGCTGCTGCTGAG
CTCTAGAGGAGGATCTGTTGGCTGCCAAGATTTACTGCGCGAGGT
CTTCCCCTCTGGTTTTGTCACAGAGCCCTATCATTCAAACCTTTCGCCC
ATCTAAGCTCTCCTCCCCCTCTGACATGCGCTGCTGTGCTGCTGCTGACT
AGCGCATCGGCAACCAACTGATCCATCCAGGCCGCAAGGCTCATGTTCT
CTGAGGCTGTGCTGACGGGTTGGCCTCCACTGCTTCTAACTG
AAAAGATTTGGGGCCACTGCTTGTCCTACTGCAACTCCTCCATCAACCCACT
GGTTTAGCTCTGCTACTGGCGAACAACCTTCAAGGCTCTTTCTAAACATCGCTT
CCAGCCCTTTCTCTGCTGGCGCCAGGGAAGAGATGCTGGGATTCA
AGACAGAGGAGAGCAGAGTACGCAAAACCCACACTCAAGGAGAGGCTGAG
CTGCAATCTCCTTCATCTGAGTCTCCTAA
```

**T7:** Sequence (5’→3’)

```
CATCTAGAGGCGCCCAATTTCGGCCCTATAGTGAGTCGTATTCAATTCA
```
Appendix II: Sequencing results

Sequencing was done by Eurofin Genomics. The sequencing results were plotted into the GeneDoc computer program. Alignment of the sequence from Ensemble (KissRb_Ens), the sequence from NCBI, genebank (XM_0040722), the plasmid sequenced by SP6 primer (6a_12 SP6) and the plasmid sequenced by T7 primer, complimented and reversed (6a_12_T7cr). The two lower sequences are the sequenced plasmid, showing that I have the correct sequence inserted in the plasmid, as the sequence is comparable to the NCBI gene bank sequence.
Appendix III: Buffer recipes

**Hybridization mix:**

- Deionized formamide: 25 mL
- SSC 20×: 12.5 mL
- Tween 10%: 500 µL
- Citric acid 1M: 770 µL
- Heparin (50 mg/mL stock): 15 µL
- Torula/yeast RNAt (10 mg/mL stock): 400 µL
- Nuclease free water: Complete total volume to 50 mL

  - Store in the freezer at -20°C

**Hybridization wash:**

- Formamide: 25 mL
- SSC 20×: 12.5 mL
- Tween 10%: 500 µL
- Citric acid 1M: 770 µL
- Nuclease free water: Complete total volume to 50 mL

  - Store in the freezer at -20°C, or at RT overnight
**MAB**

- Maleic acid 250 mM 200 mL
- NaCl 5M 15 mL
- Tween 10% 5 mL
- Nuclease free water Complete total volume to 500 mL

- Adjust pH with NaOH pellets until pH=7.5. Store at RT.
Appendix IV: Medium recipes

**E3-medium**

NaCl 5.0 mM
KCl 0.17 mM
CaCl$_2$ 0.33 mM
MgSO$_4$ 0.33 mM
RO water

Methylene Blue

- Store in the fridge, or for shorter periods at incubating temperature
Appendix V: Tyramide-component synthesis

TAMRA and FITC tyramide synthesis was prepared in the lab, by the protocol Fluorescein-tyramide synthesis (provided by L. Davidson):
http://www.xenbase.org/other/static/methods/FISH.jsp

TAMRA or FITC tyramide synthesis

1. Make a 10 mg/ml stock of the NHS ester (NHS-Rhodamine or NHS-fluorescein ester; Pierce) in dimethyl formamide (DMF).

2. Make DMF-TEA solution: 1 ml DMF added 10 ul TEA (triethylamine).

3. Make tyramide solution: 10 mg tyramide in 1 ml DMF-TEA.

Mix 2.5 ml NHS ester stock in DMF with 822 μl tyramide solution and incubate in dark at room temp for 2 hours. Add 21 ml 100 % ethanol, store in dark at -20°C.
Appendix VI: Fluorescent *in situ* hybridization protocol

**Embryos / Conditions:** Starting point = dehydrated embryos stored in methanol.

**Preparations:** Prepare *in situ* program. Make necessary calibrations of the machine. Check available probes, synthesize new probes if empty.

### Day 1

**Single – and double FISH**

**Rehydration**

Rehydrate the embryos through an ethanol/PBST series (100%, 75%, 50%, 25% ethanol mixed with PBST) 5 minutes per wash.

☐ Put embryos or brains in appropriate well (with grid underneath) and set well in appropriate basket in 100% ethanol. Use appropriate amount of liquid (so that all samples are covered with liquid). Start probrame:

<table>
<thead>
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<th>RT</th>
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<tr>
<td>Incubate</td>
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<tr>
<td>Incubate</td>
<td>00:10 h:m, ETOH 25 % x 2</td>
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<tr>
<td>Incubate</td>
<td>00:05 h:m, PBST WASH x 2</td>
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<tr>
<td>Incubate</td>
<td>00:15 h:m, PBST WASH x 2</td>
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<td>Pause……..</td>
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**Proteinase K treatment**

Digest with Proteinase K manually according to the sample type; embryo stage/brain.

☐ At ended enzyme incubation, start the programe at the glycine step.

<table>
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<tr>
<td>Incubate</td>
<td>00:20 h:m, PBSt x 2</td>
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</tbody>
</table>

**Hybridation:**

☐ Prepare Hybridization mix (HybMix) and Hybridization wash (HybWash, needed the next day).

☐ Prehybridize embryos in HybMix, 4 hrs at 65°C.

☐ Prepare HybMix+probe solutions (concentration is probe specific)

☐ Heat probes 10 min 90°C (denaturation; heating block). Put directly on ice afterwards.

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>ON 65°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubate</td>
<td>04:00 h:m, HybMix</td>
</tr>
<tr>
<td>Incubate</td>
<td>15:00 h:m, Probe solution; step overnight</td>
</tr>
</tbody>
</table>
Day 2

Wash

☐ Prepare buffers and heat the buffers to 65 °C for 1 hour.

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>ON 65°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubate</td>
<td>00:15 h:m, 100%Hyb 2x</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:20 h:m, 75%Hyb/25% SSC 2X</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:20 h:m, 50%Hyb/50% SSC 2X</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:20 h:m, 25%Hyb/75% SSC 2X</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:20 h:m, SSC 2X</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:20 h:m, SSC 0.2X x 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wait</td>
<td>00:30 h:m - to cool down</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:05 h:m, PBSt x 5</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:20 h:m, PBSt x 2</td>
</tr>
</tbody>
</table>

Endogenous peroxidase inactivation

☐ Prepare H2O2 2% (diluted in PBST)

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubate</td>
<td>00:30 h:m, H2O2 2%</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:06 h:m, PBSt x 5</td>
</tr>
<tr>
<td>Incubate</td>
<td>00:20 h:m, PBSt x 2</td>
</tr>
</tbody>
</table>

Blocking

☐ Prepare blocking Buffer: 1/10 Blocking in MAB

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubate</td>
<td>02:00 h:m, blocking</td>
</tr>
<tr>
<td>Pause……..</td>
<td></td>
</tr>
</tbody>
</table>

Antibody incubation

☐ Add Antibody manually: 1/150 Anti-DIG POD or anti-FITC 1/200 in blocking buffer + MAB; 200 µL per well. Incubation at 4 °C overnight.
**Day 3**

**Wash**

- **Wash samples:**
  
  | SetTemp | Incubate | Wash samples: | Temp | H:m | PBST x
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>00:06 h:m</td>
<td>PBST x 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>00:20 h:m</td>
<td>PBST x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tyramide**

- Prepare 0,003% H₂O₂ (in PBST)
- Prepare Tyramide-TAMRA (1/100 in PBST) + 0,003% H₂O₂ (1/10), 45 min incubation at RT or Tyramide-FITC (1/200 in PBST) + 0,003% H₂O₂ (1/10), 30 min incubation at RT

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>Incubate</th>
<th>Tyramide-TAMRA</th>
<th>RT</th>
<th>00:45 h:m</th>
<th>Tyr-TAMTA</th>
<th>….or….</th>
<th>00:30 h:m</th>
<th>Tyr-FITC</th>
</tr>
</thead>
</table>

- **Wash in PBSt**

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>Incubate</th>
<th>Wash in PBSt</th>
<th>RT</th>
<th>00:06 h:m</th>
<th>PBSt x 5</th>
</tr>
</thead>
</table>

**Ab peroxidase inactivation**

- Prepare H₂O₂ 2% (diluted in PBST)

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>Incubate</th>
<th>Ab peroxidase inactivation</th>
<th>RT</th>
<th>01:00 h:m</th>
<th>H₂O₂ 2%</th>
<th>x 5</th>
<th>x 2</th>
</tr>
</thead>
</table>

- Single FISH, method ended!

**Double FISH:**

**Blocking**

- Prepare blocking Buffer: 1/10 Blocking in MAB

<table>
<thead>
<tr>
<th>SetTemp</th>
<th>Incubate</th>
<th>Blocking</th>
<th>RT</th>
<th>01:00 h:m</th>
<th>blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pause….</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Antibody incubation**

- Add second antibody manually: 1/150 Anti-DIG POD or anti-FITC 1/200 in blocking buffer + MAB; 200 µL per well. Incubation at 4 °C overnight.
Day 4

**Wash**

- **Wash samples:**
  - SetTemp: RT
  - Incubate 00:05 h:m, PBST x 5
  - Incubate 00:20 h:m, PBST x 2

**Tyramide**

- Prepare 0.003% H\(_2\)O\(_2\) (in PBST)
- Prepare second tyramide, Tyramide-TAMRA (1/100 in PBST) +0.003% H\(_2\)O\(_2\) (1/10), 45 min incubation at RT or Tyramide-FITC (1/200 in PBST) +0.003% H\(_2\)O\(_2\) (1/10), 30 min incubation at RT

  - SetTemp: RT
  - Incubate 00:45 h:m, tyr-TAMTA
  - …or
  - Incubate 00:30 h:m, tyr-FITC

- **Wash in PBSt**
  - SetTemp: RT
  - Incubate 00:06 h:m, PBSt x 5

**Ab peroxidase inactivation**

- Prepare H\(_2\)O\(_2\) 2% (diluted in PBST)

  - SetTemp: RT
  - Incubate 00:30 h:m, H\(_2\)O\(_2\) 2%
  - Incubate 00:05 h:m, PBSt x 5
  - Incubate 00:20 h:m, PBSt x 2

- double FISH, method ended!