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Evolutionary and Pharmacological Studies of NPY and QRFP Receptors

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Dissertation presented at Uppsala University to be publicly examined in C2, 305, Husargatan 3, BMC, Uppsala, Friday, 21 November 2014 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Adjunct professor Samuel Svensson (Linköping University).

Abstract
Xu, B. 2014. Evolutionary and Pharmacological Studies of NPY and QRFP Receptors. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1040. 59 pp. Uppsala, Sweden: Acta Universitatis Upsaliensis. ISBN 978-91-554-9059-1.

The neuropeptide Y (NPY) system consists of 3-4 peptides and 4-7 receptors in vertebrates. It has powerful effects on appetite regulation and is involved in many other biological processes including blood pressure regulation, bone formation and anxiety. This thesis describes studies of the evolution of the NPY system by comparison of several vertebrate species and structural studies of the human Y2 receptor, which reduces appetite, to identify amino acid residues involved in peptide-receptor interactions.

The NPY system was studied in zebrafish (*Danio rerio*), western clawed frog (*Xenopus tropicalis),* and sea lamprey (*Petromyzon marinus*). The receptors were cloned and functionally expressed and their pharmacological profiles were determined using the native peptides in either binding studies or a signal transduction assay. Some peptide-receptor preferences were observed, indicating functional specialization.

A receptor family closely related to the NPY receptors, called the QRFP receptors, was investigated. A QRFP receptor was cloned from amphioxus, *Branchistoma floridae*, showing that the receptor arose before the origin of the vertebrates. Evolutionary studies demonstrated that the ancestral vertebrate had as many as four QRFP receptors, only one of which remains in mammals today. This correlates with the NPY receptor family, located in the same chromosomal regions, which had seven members in the ancestral vertebrate but only 4-5 in living mammals. Some vertebrates have considerably more complex NPY and QRFP receptor systems than humans and other mammals.

Two studies investigated interactions of NPY-family peptides with the human Y2 receptor. Candidate residues, selected based on structural modeling and docking, were mutated to disrupt possible interactions with peptide ligands. The modified receptors were expressed in cultured cells and investigated by measuring binding and functional responses. Several receptor residues were found to influence peptide-receptor interactions, some of which are involved in maintaining receptor structure. In a pilot study, the kinetics of peptide-receptor interaction were found to be very slow, of the order several hours.

In conclusion, this thesis clarifies evolutionary relationships for the complex NPY and QRFP peptide-receptor systems and improves the structural models of the human NPY-family receptors, especially Y2. These results will hopefully facilitate drug design for targeting of NPY-family receptors.

Keywords: Neuropeptide Y, genome duplication, Evolution, vertebrate, Pharmacology, Modelling, Kinetics

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博学之，审问之，慎思之，明辨之，笃行之。

------《礼记·中庸》

Learn extensively, inquire thoroughly, ponder prudently, discriminate clearly and practice devotedly.

------ Doctrine of the Mean

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I   **Xu B**, Sundström G, Kuraku S, Lundell I, Larhammar D. (2012) Cloning and pharmacological characterization of the neuropeptide Y receptor Y5 in the sea lamprey, *Petromyzon marinus*. Peptides 39: (64-70).

II   **Xu B**, Lagman D, Sundstrom G, Larhammar D. Neuropeptide Y family receptors Y1 and Y2 from sea lamprey, *Petromyzon marinus*: cloning and pharmacological characterization. (Manuscript)

III Sundström G, **Xu B**, Larsson TA, Heldin J, Bergqvist CA, Fredriksson R, Conlon JM, Lundell I, Denver RJ, Larhammar D. (2012) Characterization of the neuropeptide Y system in the frog *Silurana tropicalis* (Pipidae): three peptides and six receptor subtypes. Gen Comp Endocrinol 177(3):322-31.

IV   Sundström G, Larsson TA, **Xu B**, Heldin J, Larhammar D. (2013) Interactions of zebrafish peptide YYb with the neuropeptide Y-family receptors Y4, Y7, Y8a, and Y8b. Front Neurosci 7:29.

V   **Xu B**, Lundell I, Larhammar D. Characterization of QRFP peptide and its receptor from amphioxus, *Branchiostoma floridae*. (Submitted)

VI   Larhammar D, **Xu B**, Bergqvist CA. (2014) Unexpected multiplicity of QRFP receptors in early vertebrate evolution. (Front Neurosci, in press)

VII **Xu B**, Fällmar H, Boukharta L, Pruner J, Lundell I, Mohell N, Gutiérrez-de-Terán H, Aqvist J, Larhammar D. (2013) Mutagenesis and computational modeling of human G-protein-coupled receptor Y2 for neuropeptide Y and peptide YY. Biochemistry 52: 7987-7998.

VIII **Xu B**, Varasteh Z, Orlova A, Andersson K, Larhammar D, Björkelund H. (2013) Detecting ligand interactions with G protein-coupled receptors in real-time on living cells. Biochem Biophys Res Commun 441(4):820-4.

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Contents

Abbreviations

AP	Area postrema
ARC	Arcuate nucleus
Bmax	Maximum binding capacity
BLAST	Basic Local Alignment Search Tool
cAMP	Cyclic adenosine monophosphate
CD	Circular dichroism
CNS	Central nervous system
CXCR4	CXC chemokine receptor type 4
DMH	Dorsomedial nucleus
ECL	Extracellular loop
ICL	Intracellular loop
IP3	Inositol 1,4,5 trisphosphate
GFP	Green fluorescent protein
GI	Gastrointestinal
GPCR	G protein-coupled receptor
Kd	Dissociation constant
Ki	Inhibition constant
LHA	Lateral hypothalamic area
ML	Maximum likelyhood (method)
NMR	Nuclear magnetic resonance
NJ	Neighbor joining (method)
NPY	Neuropeptide Y
NTSR1	Neurotensin receptor 1
PP	Pancreatic polypeptide
PVN	Paraventricular nucleus
PYY	Peptide YY
TM	Transmembrane region
QRFP	Pyroglutamylated RFamide peptide
RhoA	GTPase Rho kinase
RhoGEFs	RhoGTPase nucleotide exchange factors
VMH	Ventromedial nucleus
wt	Wild type
Y(n)	Neuropeptide Y receptor n
2R	Two rounds of genome duplication
3R	Third round of genome duplication

Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of receptors in vertebrates. They are involved in sensing a wide variety of signals from the extracellular environment, including light, odor molecules, neuropeptides, and lipids. The study of the biological functions, pharmacology and biochemistry of GPCRs is an important part of drug discovery and development because dysfunction of these receptors is involved in many diseases. It is estimated that about 36% of drugs are targeting GPCRs (Rask-Andersen et al., 2011).

One family of GPCRs is the neuropeptide Y (NPY) family receptors. Together with the corresponding NPY family of peptides, they have been shown to be central in the regulation of appetite and energy balance. Due to their potential in the treatment of obesity and related diseases, they are considered important drug targets. However, no effective drugs are currently available. The study of the structure of the receptors and peptides, and their interactions, might facilitate design of potential drug molecules. Aside from this, studies have also shown that the NPY system is involved in several other biological functions.

The NPY family of peptides and the corresponding GPCR family of receptors both consist of several members. Each family originated from a common ancestor in vertebrate evolution through gene and genome duplications. One interesting question is that although some pairs of receptors originated from the same ancestral gene, they have totally opposite effects on appetite regulation. To understand how their functions diverged, it is required to study their roles in different lineages. Therefore, it is necessary to identify the repertoire of peptides and receptors in a wide variety of species and to study their ligand-receptor interaction preferences and tissue expression pattern. By comparing the amino acid sequences from different species, it is also possible to identify conserved residues that might be important for the functions of the receptor.

The aim of this thesis is to study the evolution of the NPY system and the related QRFP system, as well as the ligand-receptor interactions and pharmacology of the human Y2 receptor.

Biological functions of the NPY system

The mammalian NPY system

In mammals, there are three peptides belonging to the NPY family of peptides: neuropeptide Y (NPY), peptide YY (PYY) and pancreatic peptide (PP). The preprohomones are usually 94-95 amino acid residues long and the mature peptides are all 36 amino acids long with an amidated carboxy terminus (Larhammar, 1996b). N-terminally truncated forms of NPY and PYY, NPY3-36 and PYY3-36, also exist in the circulation. These are generated by removal of the first two amino acids by dipeptidyl peptidase IV (Mentlein et al., 1993). NPY works as a neurotransmitter and is widely expressed in the nervous system, primarily in the hypothalamus, but it is also found in the basal ganglia, amygdala and nucleus accumbens. NPY is one of the most orexigenic peptides (Kalra et al., 1999). Circulating PYY (or PYY3-36) is mainly secreted from L cells in the gastrointestinal (GI) tract after food intake and the level is in proportional to the energy intake. (Field et al., 2010). PP, as its name indicates, is synthesized in the pancreatic islets by endocrine F cells (Ekblad and Sundler, 2002). Both PYY and PP function mainly as hormones to inhibit food intake.

The NPY-family peptides function through a family of receptors consisting of five functional members in mammals: Y1, Y2, Y4, Y5, Y6. See Figure 3 for their evolutionary relationships. A sixth subtype, Y3, was described based on a distinct pharmacological profile, but it has not been identified in any genome, so it does not exist as a separate gene (Chen et al., 2007). Y6 is a pseudogene (due to frame shift) in human, primates, pig and guinea pig, but it is functional in mouse and rabbit (Larhammar and Salaneck, 2004; Starbäck et al., 2000). NPY receptors belong to the rhodopsin-like G protein-coupled receptor clan (Fredriksson et al., 2003; Fredriksson et al., 2005) and they function mainly through coupling to the Gαi/o signal transduction pathway leading to inhibition of cAMP production (Figure 5). However, other pathways are also involved, such as synthesis of Inositol phosphate, inhibition of Ca^{2+} and K^+ channels, and stimulation of mitogen-activated protein kinase activity (Herzog et al., 1992; Michel et al., 1998; Misra et al., 2004).

Both the Y1 and the Y5 receptor are expressed in the hypothalamus and exert their orexigenic roles by responding to the NPY peptide synthesized in the NPY/AgRP neurons in the arcuate nucleus (ARC) (Mercer et al., 2011). Y2 is mainly expressed presynaptically as an autoreceptor to inhibit the release of NPY, thus inhibiting NPY's function through Y1 and Y5 (Chen et al., 1997; King et al., 2000). Expression of Y2 was found in ARC, paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) (Mercer et al., 2011). The ARC has a semi-permeable blood brain barrier, so PYY (or PYY3-36) from the intestine can also activate presynaptic Y2, leading to

inhibition of NPY/AgRP neurons. NPY expression levels can be affected by peripheral signals. For example, leptin and insulin can act on the NPY/AgRP neurons in the ARC by inhibiting the expression of NPY and AgRP mRNA, leading to appetite decrease. The expression levels of the orexigenic factors melanin-concentrating hormone (MCH) and orexin are negatively regulated by NPY released from the projection of NPY/AgRP neurons in the ARC (Broberger, 2005). In addition to the expression in the hypothalamus, Y2 is expressed in the area postrema (AP) of the brain stem (Dumont et al., 1996), overlapping with Y4 receptor expression (Dumont et al., 2007), where PYY and PP from the periphery can access the central nervous system. Elevated levels of PYY3-36 after food intake can activate the Y2 in ARC in the hypothalamus (Batterham et al., 2002). Thus, Y2 and Y4 in these areas respond to circulating PYY (or PYY3-36) and PP, respectively, to suppress food intake.

The role of the NPY system in energy homeostasis is not just limited to the CNS. Conditional Y1 knockout in peripheral tissues of adult mice showed increased fatty acid transport and oxidation (Zhang et al., 2010). Y1 mRNA is highly expressed in human adipose tissue, and Y1 has an antilipolytic effect which can lead to increased leptin level (Serradeil-Le Gal et al., 2000). The germline knockout and CNS knockout of the Y2 gene showed different results in weight gain, suggesting also a peripheral role of Y2 in energy homeostasis (Sainsbury et al., 2002; Shi et al., 2011).

Aside from the roles in appetite regulation and energy homeostasis discussed above, the NPY system also has several other functions involving both central and peripheral actions, including development of different types of cancers (Reviewed in (Zhang et al., 2011), anxiety (Wu et al., 2011), learning and memory (Redrobe et al., 2004; Redrobe et al., 1999), blood pressure regulation (Pedrazzini, 2004), and bone formation (Shi and Baldock, 2011). The variety of biological functions of the NPY system might be caused by the multiple members of peptides and receptors and also their wide expression in both the central and peripheral nervous systems.

A large number of agonists and antagonists have been developed targeting different NPY receptors, aiming to understand the function of specific receptors, treat energy homeostasis disorders or other diseases (Yulyaningsih et al., 2011). However, due to modest effects or side effects, no effective drugs are yet available on the market targeting this system.

NPY system in other vertebrates

Functional studies of the NPY system have been carried out on species other than mammals, including amphibians (Carr et al., 2002; Crespi et al., 2004), birds (Boswell et al., 1999; Dodo et al., 2005; Saneyasu et al., 2011), reptiles (Morris and Crews, 1990), teleost fishes, (Matsuda et al., 2012; Narnaware and Peter, 2001; Yokobori et al., 2012). The NPY system showed some conserved functions with mammals, such as stimulation of food intake. Al-

though the studies above have been valuable, the information we have obtained so far has been limited and difficult to interpret without enough knowledge of the repertoire of peptides and receptors, the ligands' affinities for different receptor subtypes, and the expression patterns of the NPY peptides and receptors in those species.

Evolution of the NPY and QRFP systems

Whole genome duplications in vertebrates

Several mechanisms are involved in the generation of new genes. Gene duplication and whole genome duplication are proposed as the two main mechanisms for the generation of new genes and corresponding new functions (Long et al., 2003). Early on, it was proposed that two rounds of genome duplication (tetraploidization) took place during vertebrate evolution (Ohno 1970). Several lines of evidence from recent studies now support this idea although the exact timepoints have been adjusted relative to Ohno's initial suggestions (Dehal and Boore, 2005; Nakatani et al., 2007; Putnam et al., 2008; Sundström et al., 2008). This 2R theory, for 2 rounds of genome duplication, is now widely accepted. Early in the evolution of the teleost fish lineage a third round of whole genome duplication occurred, called 3R (Jaillon et al., 2004). The timing of the 1R, 2R and 3R events and the divergence times of the different lineages of vertebrates are shown in Figure 1 (the divergence time points are the mean values from several references summarized in www.timetree.org/). Some vertebrate lineages have gone through additional whole genome duplications, such as the frog *Xenopus laevis* which has a quite recent genome doubling compared with other frog species (Hellsten et al., 2010; Hellsten et al., 2007). In the teleost fish lineage, salmonid fishes and cyprinid fishes have independently gone through a fourth round of genome duplication after 3R (David et al., 2003; Davidson et al., 2010; Xu et al., 2014).

After a whole genome duplication, three different fates have been proposed for the redundant genes. Some gene duplicates may acquire a new function (neofunctionalization), some gene pairs may partition old functions between them (subfunctionalization), and lastly, some of the duplicated genes will become pseudogenes and eventually disappear due to nonfunctional mutations (Dehal and Boore, 2005). Pseudogenization and deletion are by far the most common fate (Lynch and Conery, 2000). Other mechanisms are also involved in the generation of new genes along with whole genome duplication, including local tandem duplications, retrotransposition as well as the duplication of chromosome segments (Long et al., 2003). However, whole genome duplications have a larger potential for the generation of new genes simply due to the large amount of duplicated genetic material.

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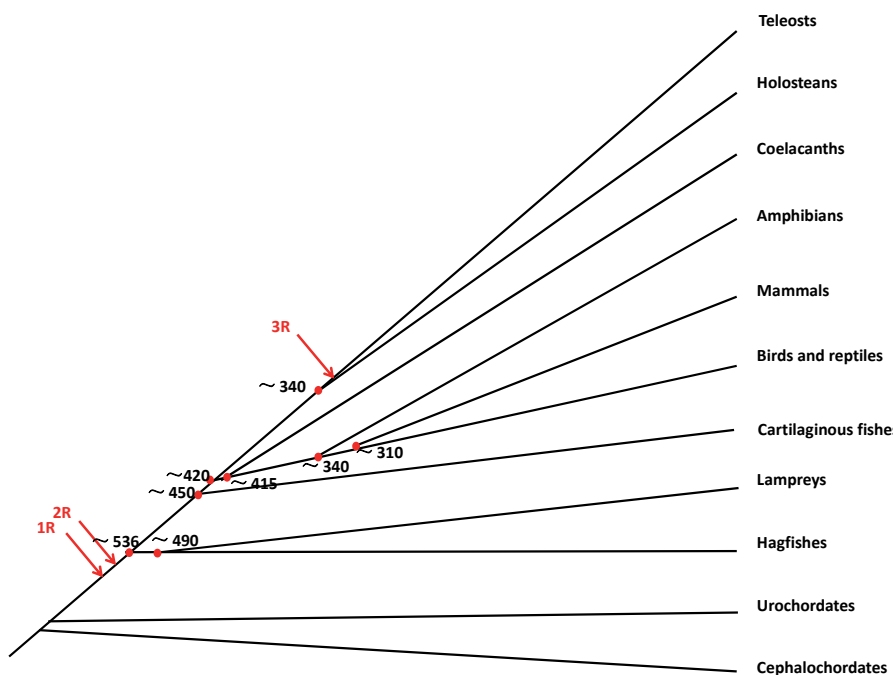
Figure 1. Evolution of vertebrates. The red arrows show the approximate timepoints of the 1R, 2R and 3R whole genome duplications in vertebrate evolution. Red dots mark approximate divergence times of different lineages.

Thus, whole genome duplication is an important evolutionary force in the generation of gene families with multiple members. The NPY family of peptides and the corresponding family of NPY receptors are examples of such gene families. The NPY peptide genes and receptor genes originated from one ancestral peptide gene and one ancestral receptor gene, respectively. Subsequently, the Y1 and Y2 have diverged considerably in sequence and structure, to the degree that their functions in food intake regulation are opposite. Hence, studies of the evolution of the NPY system will not only help us understand its physiological functions, identifying conserved amino acid residues of the peptides and receptors can also help us elucidate their structures, identify positions important for ligand-receptor interactions, and clarify their pharmacology.

Evolution of NPY peptides

NPY and PYY have been found in all vertebrates investigated, and it has been proposed that they originated from one ancestral peptide through whole genome duplication (Larhammar, 1996a; Söderberg et al., 2000; Sundström et al., 2008). The evolution of NPY family peptides is summarized in Figure 2.

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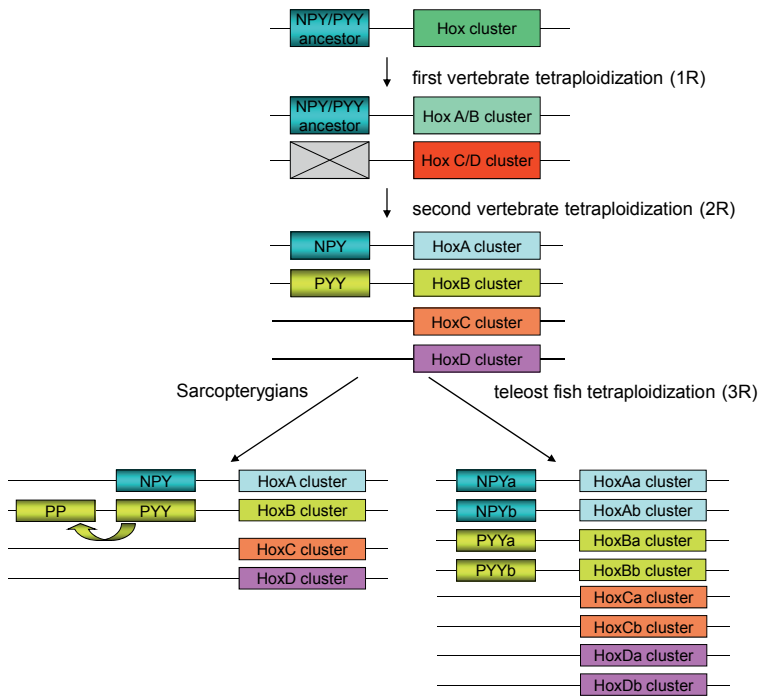
Figure 2. Evolution of the NPY family peptides. The NPY family peptides originated from one ancestral gene by genome duplication together with the Hox genes. A local duplication of PYY generated PP.

Tetrapods have three NPY-family peptides: NPY, PYY and PP (Cerdá-Reverter et al., 2000). The PP gene has been confirmed to be a local duplication of the PYY gene (Hort et al., 1995; Larhammar, 1996a). The amino acid sequences of NPY and PYY are well conserved through evolution, whereas the PP sequence is the least conserved among all the NPY-family peptides (Cerdá-Reverter et al., 2000; Larhammar, 1996a). Recently, all three peptides, including PP, have been identified in the coelacanth, *Latimeria cha-lumnae*, which diverged from the lobe-finned fish lineage before the emergence of tetrapods. This suggests that the local duplication that gave rise to PP occurred already before the origin of Sarcopterygians (lobe-finned fish) (Larhammar and Bergqvist, 2013). Additional lineage-specific duplications have given some species more than three peptide genes. For example, the African clawed frog, *X. laevis,* has two copies of each gene (Griffin et al., 1994; van Riel et al., 1993) and primates have duplicates of PYY and PP, although they are nonfunctional (Couzens et al., 2000; Hort et al., 1995).

In the teleost fish lineage four NPY peptides can be found. Duplicates of both NPY and PYY were generated in the teleost-specific whole genome duplication, 3R (Christoffels et al., 2004; Jaillon et al., 2004; Sundström et

al., 2008; Van de Peer, 2004). The two NPY duplicates, named NPYa and NPYb, have been identified from several species of teleost fishes, although NPYb has been lost in zebrafish (*Danio rerio*), and the teleost fish-specific PY peptide has now been confirmed to be the duplicate of PYY, and therefore should be named PYYb. PYYb has probably been lost in medaka (*Oryzias latipes*) (Sundström et al., 2008).

Both NPY and PYY have been identified in lamprey genome sequences, which suggests an early emergence of NPY and PYY peptides, before the divergence of the jawless vertebrates from the ancestor of jawed vertebrates (Montpetit et al., 2005; Söderberg et al., 1994; Wang et al., 1999). Another NPY family peptide, PMY, has also been identified in sea lamprey *Petromyzon marinus*, although the evolutionary relationship with the other two peptides has not yet been clarified (Conlon et al., 1991; Sundström et al., 2008)

Evolution of NPY receptors

In the human genome there are more than 800 genes that encode GPCRs (Fredriksson et al., 2003). The GPCRs have been divided into five major groups according to the so-called the GRAFS system, similiar to an older system which divided them into seven classes A-F (Kolakowski, 1994): Glutamate (Class C), Rhodopsin (Class A), Adhesion (Class B), Frizzled/Taste2 (Class F) and Secretin (also Class B).

Class A , which is the largest group of GPCRs, can be further subdivided into α, β, γ and δ subgroups (Fredriksson et al., 2003). The NPY receptors belong to the β subgroup together with several RFamide peptide receptors.

All vertebrate lineages have several NPY receptors that can be divided into three main clades, and it has been proposed that three corresponding ancestral NPY receptor genes, Y1-like, Y2-like and Y5-like, existed on the same chromosome before the two early vertebrate whole genome duplications (2R) (Figure 2). This ancestral triplet was generated by local duplications from single ancestral NPY receptor (Larhammar and Salaneck, 2004; Wraith et al., 2000). Subsequently, a repertoire of seven receptor genes was produced by 2R in the gnathostome ancestor (Figure 3). Based on phylogenetic and chromosomal analyses, Y1, Y4, Y6 and Y8 receptors belong to the Y1 subfamily, Y2 and Y7 to the Y2 subfamily, and Y5 forms its own clade (Larhammar and Salaneck, 2004; Larsson et al., 2009). Subsequently, through lineage-specific deletions, local duplications and teleost 3R, different repertoires of NPY receptors have arisen in different classes of vertebrates (Larhammar and Salaneck, 2004; Larsson et al., 2008; Salaneck et al., 2008). The evolution of the NPYRs in different species is depicted in Figure 3.

In our lab, we have characterized the receptors from a broad range of ver-tebrates, including chicken (*Gallus gallus*) (Bromée et al., 2006; Holmberg et al., 2002; Lundell et al., 2002), zebrafish (*Danio rerio*) (Fällmar et al., 2011; Salaneck et al., 2008), rainbow trout (*Oncorhynchus mykiss*) (Larson et al., 2003; Larsson et al., 2006; Schjolden et al., 2009), Western clawed frog (*Silurana tropicalis*) (Paper III), coelacanth (*Latimeria chalumnae*), (Larhammar and Bergqvist, 2013) and elephant shark (*Callorhinchus milii*) (Larsson et al., 2009).
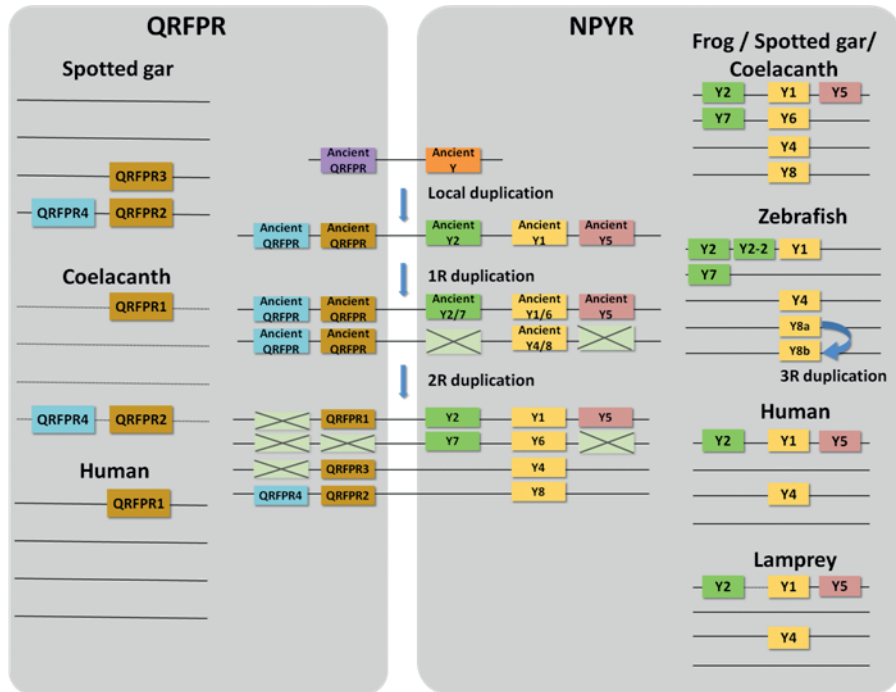
Figure 3. Evolution and repertoire of NPY family and QRFP family receptors in vertebrates. The left panel shows the repertoire of QRFPRs in different species and the right panel shows the repertoire of NPYRs in different species. The middle panel shows the expansion of QRFPRs and NPYRs by genome duplications.

Cartilaginous fishes and amphibians have the complete repertoire of seven NPY receptors ((Blomqvist et al., 1995; Larsson et al., 2008; Larsson et al., 2009; Salaneck et al., 2003), and Paper III). A recent study in our group showed that the coelacanth (*Latimeria chalumnae*) genome also contains the complete repertoire of seven NPY receptors (Larhammar and Bergqvist, 2013; Larsson et al., 2007). This also applies to the spotted gar (*Lepisosteus oculatus*) (unpublished). As for the teleost fish lineage, there have been dif-ferential losses: Y1, Y5 and Y6 are absent in the genome databases of two pufferfishes *Tetraodon nigroviridis* and *Takifugu rubripes*, as well as medaka (*Oryzias latipes*) and three-spined stickleback (*Gasterosteus aculea-*

tus), however Y1 was found in the zebrafish (*Danio rerio*) genome database, which suggests a more recent loss of Y1 in some euteleost lineages (Salaneck et al., 2008). In zebrafish, a second Y2 sequence was identified recently, named Y2-2, which was generated by a local duplication of Y2 (Fällmar et al., 2011). Y1, Y2, Y4, Y5, Y6 and Y7 have been identified from chicken, although Y8 has been lost in this lineage (Bromée et al., 2006; Holmberg et al., 2002; Lundell et al., 2002; Salaneck et al., 2000). In the mammalian lineage, Y7 was lost (Larhammar and Salaneck, 2004). In primates, pig and guinea pig Y6 is a pseudogene (Starbäck et al., 2000; Wraith et al., 2000). Among the three NPY receptor subfamilies, the Y5 subfamily has only one member in all the species studied, and it has been identified in all lineages mentioned above except teleost fishes. The ancestral Y5-like receptor gene that existed before the origin of gnathostomes and the basal tetraploidizations (Larsson et al., 2007; Larsson et al., 2009) did not have any surviving duplicates.

In papers I and II, we describe the identification and molecular cloning of the Y5 and Y1 receptors from sea lamprey (*Petromyzon marinus*). Two receptors from sea lamprey, a Y2/Y7-like, and Y4-like (Larsson et al., 2009; Salaneck et al., 2001) have been identified before, and the Y2/Y7-like receptor is also described in paper II. There is now some evidence that the lineage leading to lampreys diverged after the 2R whole genome duplications, which makes it likely that more NPY family receptor genes could be identified from the lamprey genome sequence. In this thesis, the synteny analysis supports that the previously identified Y4-like receptor should be the Y4, and the Y2/Y7-like should be the Y2. The Y1, Y2 and Y5 were cloned and their pharmacological profiles were studied using a functional assay (Paper I and II).

As mentioned above, our laboratory has previously cloned and characterized the receptors pharmacologically from a broad range of vertebrates. This thesis updates this work with the characterization of NPY receptors from sea lamprey (Paper I and II), and Western clawed frog (Paper III). Some special ligand-receptor preferences were discovered, like the mammalian PP which has a special preference for Y4 while in chicken all three ligands bind to Y4 with almost equal affinities, and in paper III, all three peptides are reported to have higher affinities for Y8 than Y5 and Y7 whereas lamprey PYY is the only potent ligand for lamprey Y2 (Paper II). Most ligands bind to the different receptor subtypes with very similar affinities which, unsurprisingly, suggests that also the expression patterns are important for their physiological functions.

The expression pattern of NPY family peptides and receptors have been studied in several species other than mammals, including chicken (Bromée et al., 2006; Holmberg et al., 2002; Lundell et al., 2002), zebrafish (Paper III), Western clawed frog (Paper II), and lamprey (Perez-Fernandez et al., 2013; Perez-Fernandez et al., 2014). The peptides and receptors in general had a

wide expression pattern. NPY and Y1 are expressed in the CNS in all species investigated, suggesting prominent and presumably evolutionary conserved roles.

Evolution of the QRFP system

There are several peptides ending with arginine-phenylalanine-amide (RFamide) in vertebrates, namely neuropeptide FF (NPFF), GnIH which also called neuropeptide VF (NPVF), pyroglutamylated RFamide peptide (QRFP) and prolactin-releasing hormone (PRLH) (Elphick and Mirabeau, 2014; Fukusumi et al., 2006; Osugi et al., 2006). The sequence similarity between these peptides is quite low, except for the C- terminal RFamide. The evolutionary relationships between these peptides are still unclear. However, it has been concluded that these peptides and their receptors already existed before the split between protostomes and deuterostomes (Elphick and Mirabeau, 2014; Jekely, 2013; Mirabeau and Joly, 2013).

In searching for an NPY receptor homolog in the genome sequence of an amphioxus, the Florida lancelet (*Branchiostoma floridae*), one receptor was identified and found to be the orthologue of the vertebrate QRFP receptor. A QRFP like peptide could also be identified in *B. floridae*. (Paper V).

The QRFP peptide (also called 26RFa or Peptide P518), one of the RFamide peptides, was identified in the frog *Pelophylax esculenta (Chartrel et al., 2003)* and mammals, including rat and human (Fukusumi et al., 2003; Jiang et al., 2003). The QRFP receptor was identified at the same time, and was found to function through the Gαq signal transduction pathway stimulating inositol 1,4,5 trisphosphate (IP3) production (Fukusumi et al., 2003; Jiang et al., 2003). Functional studies of this system have shown that QRFP is involved in appetite regulation in both mammals (Lectez et al., 2009; Primeaux, 2011; Primeaux et al., 2013; Primeaux et al., 2008; Takayasu et al., 2006) and birds (Tobari et al., 2011; Ukena et al., 2010), and is also involved in locomotor activities and the activation of the gonadotropic axis (Lectez et al., 2009; Navarro et al., 2006; Patel et al., 2008).

In paper V, we describe the identification, cloning and pharmacological characterization of the amphioxus QRFP peptide and receptor. Amphioxus like human has only one QRFP receptor while rat and mouse have two genes (Takayasu et al., 2006). In the light of paper V and whole genome duplications, the evolution of the QRFP receptor was studied in several different species of vertebrates (Paper VI).

Structure of NPY family peptides and receptors

Even though the functions of the NPY system in appetite regulation have been studied extensively using both endogenous and synthesized ligands for the varous receptors, the ligand interactions with the respective receptors are still not well understood at the molecular level. This limits the potential of NPY receptors to be used as drug targets.

Structure of NPY family peptides

The first NPY-family peptide structure, the avian pancreatic polypeptide (PP), was determined in 1981 using X-ray crystallography (Blundell et al., 1981). Several 3D structures of NPY-family peptides were then determined in aqueous solution (Darbon et al., 1992; Neumoin et al., 2007; Nygaard et al., 2006). These 3D structures of the NPY family peptides revealed common structural features (Figure 4a). They are all 36 amino acids long, and contain a flexible and amidated C-terminus (residues 32-36). They share a pancreatic-polypeptide (PP) -fold structure consisting of a polyproline helix (residues 1-8) and an α-helix (residues 14-31) connected by a ß-turn (residues 9-13) (Bettio et al., 2002; Cerdá-Reverter and Larhammar, 2000; Keire et al., 2000). The C-terminal amidation has been suggested to prevent the ionization of the COOH-terminus rendering it more hydrophobic and thus better able to bind to its receptor (Beck-Sickinger and Jung, 1995; Eipper et al., 1993). Whether the amide group of NPY family peptide contributes to the binding or not is still not known.

a. b.
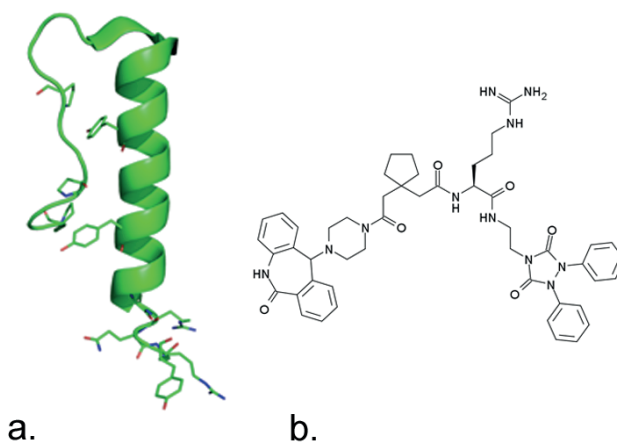
Figure 4. Ligands for human Y2. a. 3D structure of human PYY (Nygaard et al., 2006). b. Y2 specific antagonist, BIIE0246, mimicking the C-terminus of NPY/PYY (Doods et al., 1999).

An alanine scan study (systematic replacement of one residue at a time with an alanine) of NPY suggested that several residues showed differential importance in Y1 and Y2 binding, which suggested a difference in binding mode between the two receptors. This study also showed that the conserved C-terminal pentapeptide-amide, Thr32-Arg33-Gln34-Arg35-Tyr36amide, is critical for binding to both receptors (Beck-Sickinger et al., 1994). There are several modifications of the C-terminus that have special selectivity for different receptor subtypes, for example, [Pro34]-NPY is a Y1/Y5 agonist whereas [Gln34] -PP will increase the affinity for Y2 (Pedragosa-Badia et al., 2013). [Leu31, Pro34]-NPY is a Y1-selective agonist and [Ala31, Aib32]-NPY is a Y5 agonist (Lindner et al., 2008a).

The same alanine scan study also suggested that several residues in the alpha helix also contribute to the binding affinities, but mainly for Y1. For Y2, the effects seem rather modest which suggest some difference in binding mode between Y1 and Y2 (Beck-Sickinger et al., 1994). The conserved α helix (formed by residues 14-31) in these peptides has been proposed to bind to the cell membrane and thereby serve as the first step for ligand-receptor binding (Bader and Zerbe, 2005). NPY analogs with a shorter α helix have reduced affinity for the Y2, substituting the whole α helix for an arm-like linker will abolish the binding (Fournier et al., 1994; Fuhlendorff et al., 1990). This idea has been challenged since one Y1 specific agonist that was modified from NPY28-36, with almost no alpha-helix, still has high potency (15 times less than the full length NPY) (Zwanziger et al., 2009). It suggests that the orientation of the C-terminus of the peptide, rather than the alpha helix, is critical for peptide-receptor interactions.

Unlike the Y2, the receptor Y1 and Y5 are quite sensitive to N- terminal truncation of the peptides; both NPY3-36 and PYY3-36 are Y2-specific agonists (Gue et al., 1996). One study has shown the nuclear magnetic resonance (NMR) structural differences between PYY, [Pro34]-PYY and PYY3-36 (Keire et al., 2000). According to this study, the change to Pro34 affected the C-terminus structure, and PYY3-36 affected the structure of the PP-fold.

A large number of ligands, both peptide agonists and antagonists, have been developed for different NPY receptor subtypes (Brothers and Wahlestedt, 2010). Although those ligands have shown some limitations as potential drugs, they can still help us understand the peptide-receptor interactions. Many antagonists were also developed targeting different receptor subtypes. One of these is the Y2 specific antagonist BIIE0246 that was used in paper VII, which was designed to structurally mimic the C-terminus of the NPY/PYY (Figure 4b.).

Considering the fact that the modification of the C-terminus of the NPY/PYY/PP peptides will affect the binding affinities and selectivities, as well as the fact that NPY/PYY have conserved C-terminus, studying the interaction of the conserved C-terminus Thr32-Arg33-Gln34-Arg35-

Tyr36amide with different receptors might serve as a first step to determined the binding modes of the full length NPY family peptides.

Structure and pharmacology of GPCRs

GPCRs are a group of membrane receptors that react to a variety of extracellular signals including light, odors, lipids and peptides, then undergo conformational changes from an inactive to an active state to activate the following components, including G-proteins, beta-arrestins, GPCR kinases, to exert their biological functions (Ritter and Hall, 2009).

All GPCRs share some common features of their structure and signaling mechanisms. The common structural features of the GPCRs are that they all form a seven transmembrane (TM) bundle, with alpha helices embedded counterclockwise in the cell membrane (when viewed from outside), connected by extracellular loops (ECLs) and intracellular loops (ICLs). The extracellular parts, involving the N-terminus, ECLs and the extracellular part of TM region, is responsible for the ligand binding, and the intracellular parts, including C-terminus, ICLs and introcellular part of TM region is responsible for interaction with G proteins, beta-arrestins or GPCR kinases (Ritter and Hall, 2009). Across different families of GPCRs, the most conserved feature in GPCRs in the extracellular part is a disulfide bridge between ECL 1 and ECL 2, which connects the TM3 and TM5 and has been found to be critical for the GPCR structure (Ahuja and Smith, 2009).

The transmembrane (TM) regions are the most conserved structural features of GPCRs. While the extracellular part of the GPCRs is responsible for the ligand binding, the TM regions contain several conserved motifs that are important for the activation process, including the DRY/REH motif (TM3), WxP motif (TM6) and NPxxY motif (TM7) (Ahuja and Smith, 2009). One of the generic numbering systems for GPCRs is the Ballesteros Weinstein System (Ballesteros and Palczewski, 2001), which uses the most conserved residues in each TM region as reference number 50, plus the number of the TM region, to name the residues and compare the residues across different GPCRs. For example, one of the most conserved residue in TM5 is a Pro, hence it is named as Pro5.50. The preceding residue is named X5.49, and the subsquent residue is X5.51.

The GPCRs normally have relatively low expression levels and due to their flexible structures, they are diffuculit to be crystalized (Ahuja and Smith, 2009). Before 2007, only the rhodopsin 3D structure was available, a milestone in the understanding of GPCR structures (Palczewski et al., 2000). Thanks to technical advancements more and more crystal structures of Rhodopsin family/class A GPCRs have become available (Katritch et al., 2013), including adenosine A_{2A} receptor and rat neurotensin receptor 1 (NTSR1), which were used for the homology modeling of the human Y2 receptor. Recently, four receptors belonging to other classes have also been

crystallized, including two that belong to th Adhesion receptor family/class B receptors (Hollenstein et al., 2013; Siu et al., 2013), two receptors that belong to the Glutamate receptor family/class C (Dore et al., 2014; Wu et al., 2014), as well as one of the Frizzled receptors (Wang et al., 2013). Most of them were crystallized with antagonists or inverse agonists, so most of the structures were in an inactive conformation. Until today, four crystal structures have been determined by crystallization with agonists in an active or active-like conformation, including human β2 adrenergic receptor, bovine rhodopsin, rat neurotensin receptor, and the human adenosine A_{2A} receptor (Venkatakrishnan et al., 2013). This information is central to increase our understanding of the ligand binding and activation process of GPCRs. Furthermore, the availability of an increasing number of crystal structures also helps in the design of small molecule drugs targeting these receptors, and as templates to model other GPCRs (Rodriguez and Gutierrez-de-Teran, 2013).

The GPCR crystal structures reveal that GPCRs can be quite different in their extracellular part (Katritch et al., 2012). Even within the class A, the long ECL2 shows some divergent features, whereas ECL1 and ECL3 have less differences due to their short length (Katritch et al., 2012; Venkatakrishnan et al., 2013). The extracellular part of the TM regions also show structural diversity to a larger extent. Unsurprisingly, the binding pockets of the crystallized structures are quite different from each other, affected both by the diversity of side chains of the residues around the pocket and the difference in TM conformations (Katritch et al., 2012). This is due to the fact that different subfamilies of GPCR bind to molecules with very different properties, including size, shape and electrostatic profile. For example, the opioid receptors, CXCR4 and NTSR1 structures showed larger binding cavities than the GPCRs that bind to smaller molecules (Manglik et al., 2012; Thompson et al., 2012; White et al., 2012; Wu et al., 2010). Those structural differences as well as the identities should be considered for selecting the template for homology modeling.

GPCRs transfer extracellular signals across the plasma membrane to intracellular effectors via heterotrimeric G proteins. Although the extracellular regions of GPCRs are quite different between different families of receptors, or even within the same family of receptors, the intracellular part that is involved in signaling is quite conserved. Heterotrimeric G proteins consist of three protein subunits, α, β and γ. The Gα proteins can be subdivided into four major families, Gαs, Gαi/o, Gαq/11, and Gα12/13.
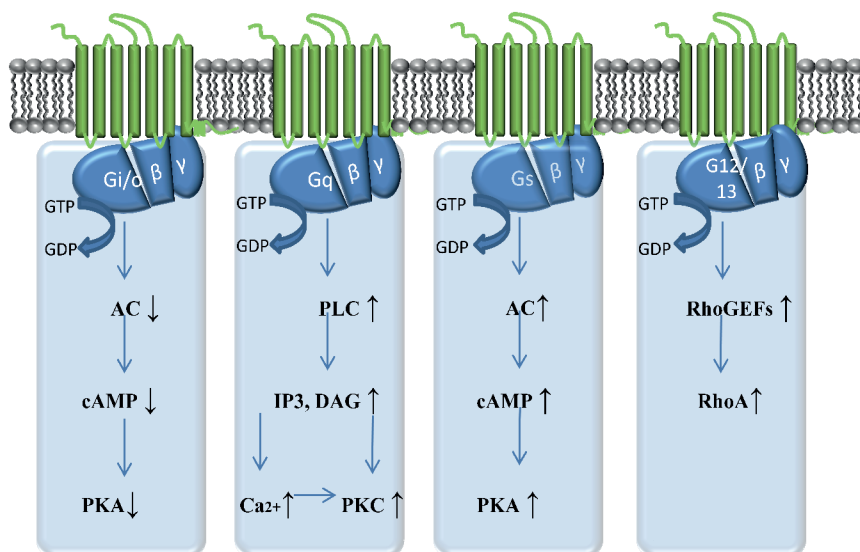
Figure 5. The classical signaling pathways of GPCRs. The different pathways depend on different interacting Gα proteins, Gαi/o, Gαq/11, Gαs and Gα12/13.

After an agonist bound to a GPCR, the conformation will be changed to an active state which in turn will lead to the activation of the different Gα proteins. Depending on the subtype, activation of the Gα proteins will lead to different signaling pathways (Figure 5) (summarized in (Ritter and Hall, 2009)). The Gαi/o and Gαs proteins can affect the activity of adenylate cyclase by inhibiting (Gαi/o) or stimulating (Gαs) the synthesis of cyclic adenosine monophosphate (cAMP). Activation of the Gαq/11 family will lead to an intracellular Ca^{2+} mobilization from the endoplasmatic reticulum (ER) and activation of protein kinase C (PKC), by generation of inositol 1,4,5-triphosphate. The signaling pathways activated by Gα12/13 are much less studied, but seem to mainly act on the RhoGTPase nucleotide exchange factors (RhoGEFs) to activate the GTPase Rho kinase (RhoA).

Structure of NPY receptors

It is already known that NPY and PYY as well as PP bind to their receptors with their amidated C-terminus (Beck-Sickinger et al., 1994; Larhammar, 1996b; Rose et al., 1995), but the exact interaction points are still not very clear. No crystal structure is available for this family of receptors right now. Another way to study their structures is to use computational methods to predict the structure by homology modeling and verify it with mutagenesis and pharmacological studies.

The four human NPY receptors belong to three subfamilies, Y1 (Y1 and Y4), Y2 and Y5. The shared sequence identities among Y1, Y2 and Y5 are rather low, about 30% (Larhammar and Salaneck, 2004), but they have simi-

lar ligand binding profiles. They bind to NPY and PYY with high affinities, but low affinities for PP. In contrast Y4 has higher sequence identity to Y1 than the other two receptors but a very different ligand preference, with a high affinity for PP (summarized in (Berglund and Donald, 2005)). So there should presumably be some residues that are conserved between different receptor subtypes that participate in the interactions with the NPY family peptides in a similar way. Also some other interaction points should contribute to the selectivity as the receptors do have a different peptide preferences or have different tolerance for different peptide modifications.

Among all the NPY receptors, the human Y1 (hY1) structure is the most well studied using mutagenesis since it is a potential drug target to treat obesity and hypertension. Mutants have been generated for 68 positions (Berglund and Donald, 2005; Lindner et al., 2008b). In earlier mutagenesis studies, several positions were identified to be important for ligand binding. For example, Tyr100 (2.64), Phe286 (6.58) and His298 (7.31) (Bromée et al., 2006; Sautel et al., 1995). Four acidic amino acids in the extracellular parts of Y1, Asp104 (2.68), Asp194 (4.68), Asp200 (5.27), and Asp287 (6.59), were also reported to be critical for peptide binding (Sjödin et al., 2006; Walker et al., 1994). Some other positions were also identified to be important for cell surface expression, receptor internalization or antagonist binding, as reviewed in (Berglund et al., 2003). A few models for Y1 were generated using computer modeling to interpret the mutagenesis result, based on certain assumptions, using as template the low resolution bacteriorhodopsin structure and later on the crystallized bovine rhodopsin structure (Du et al., 1997; Sautel et al., 1995; Sjödin et al., 2006; Walker et al., 1994). With new crystallized receptors available, these Y1 models likely need to be re-evaluated.

In human Y2 (hY2), only a few positions have been studied. Gln135 (3.37), Leu227 (5.46) and Leu284 (6.51) in hY2 were replaced with corresponding positions in chicken Y2, which reduced binding of the antagonist BIIE0246 but still retained peptide binding (Berglund et al., 2002). The hY2 positions, corresponding to Tyr2.64, Phe6.58 and His7.31 in hY1, are less important for peptide binding: Tyr2.64 and His 7.31 still interact with peptides, but the interactions might be different compared with hY1, and Val 6.58 does not affect ligand binding at all in hY2 (Akerberg et al., 2010). Another three positions, Gly2.68, Leu4.60, Gln6.55, were also selected based on their importance for ligand binding in Y1 (Fallmar et al., 2011). However, it turns out that the mutants for Gly2.68, Leu4.60 showed modest effects on ligand binding, while Ala mutantation of Gln6.55 surprisingly increased some affinity. The residue was further studied in paper VII in this thesis. Three other residues were also evaluated, including Glu5.24, Asp6.61 and Asp6.59, among which Glu 5.24 and Asp6.59 were found to be important for binding (Merten et al., 2007).
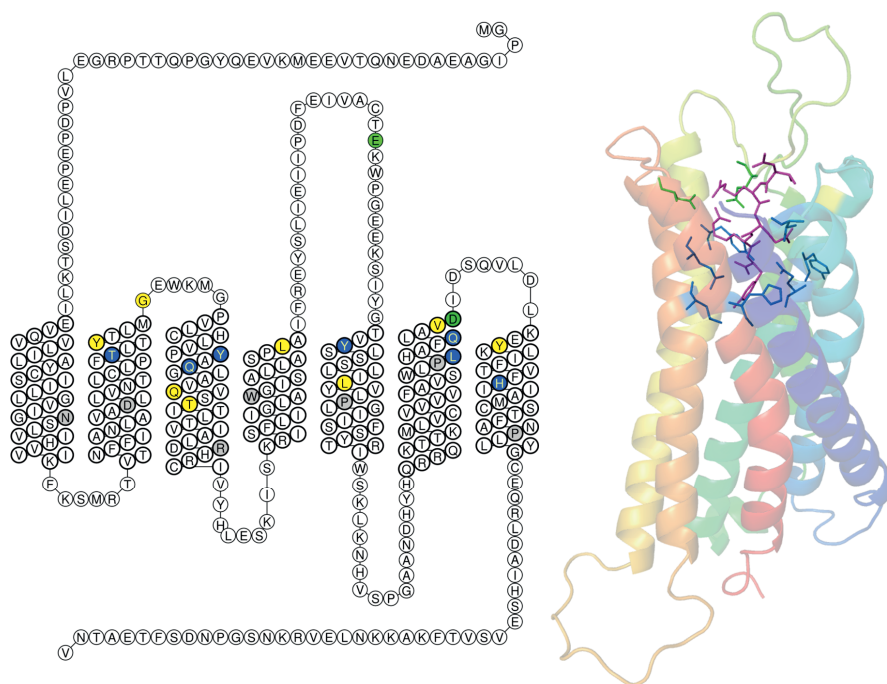
Figure 6. Snake plot of human Y2 (a) and the Y2 model, based on the NTSR1 structure, with docked Thr32-Arg33-Gln34-Arg35-Tyr36amide (magenta). Residues marked yellow have been studied previously by our research group (Akerberg et al., 2010; Fällmar et al., 2011). Residues marked blue (Thr2.61, Tyr3.30, Gln3.32, Tyr5.38, Leu6.51, Gln6.55 and His7.39) have been studied as described in Paper VII. The two residues marked green were shown to be important for ligand binding in a previous study (Merten et al., 2007).

For the Y4 receptor, only two studies have been published until now (Merten et al., 2007; Pedragosa-Badia et al., 2014) involving Gln3.32, His7.39, Glu5.27, Asp6.59, Tyr2.64, Asp2.68, Asn6.55, Asn7.32, and Phe7.35. Most of them were shown to be involved in the peptide binding. Gln3.32 in Y4, in contrast, had no effect on peptide binding which is different from Y1 and Y2 where Gln3.32 can affect peptide binding and receptor structure (Du et al., 1997; Sjödin et al., 2006) (Paper VII). In Y5, three acidic residues, Glu5.27, Gul6.62 and Asp6.59, were studied, whereas the Glu6.62 had no effect on ligand interaction (Merten et al., 2007).

Asp6.59 is conserved in all four receptors, and the data from mutagenesis of receptors and peptide modifications suggest that this residue in Y1/Y4 interact with Arg35 in NPY whereas in Y2/Y5 receptors, it interacts with Arg33 (Merten et al., 2007; Pedragosa-Badia et al., 2013). This proposed

binding mode is not convincing for two reasons. From an evolutionary point of view, the multiple members of peptides and receptors are both originated from single ancestral gene each, making it unlikely that they diverged to form such a huge difference in peptide-receptor interactions. Secondly, the pharmacological data that were obtained by these authors to support their hypothesis has two limitations. Firstly, they used a functional assay, which is an indirect way to measure the ligand-receptor interactions; and secondly, even their results are not in agreement with this hypothesis. For example, the Arg33 to Ala mutant of NPY reduced more potency for Y5 than for Y4, and the Arg35 to Ala mutant of NPY reduced more potency for Y2/Y5 than for Y1/Y4, which is opposite to their hypothesis.

Based on the mutagenesis and pharmacological studies, two homology models were generated for hY2 using the human β2 adrenergic receptor and the human adenosine A_{2A} receptor structures as templates, respectively (Akerberg et al., 2010; Fallmar et al., 2011). In paper VII, we desribe the continuation of previous studies from our research group. The A_{2A}-based model was docked with the conserved C-terminal dipeptide to study the binding mode of the peptide to the receptor. During the study, the antagonist bound neurotensin receptor type 1 (NTSR1) has been crystallized which is the first peptide-bound structure available (White et al., 2012). Although the TM regions showed moderate identity (28%) when compared with hY2, it might be a better template for modeling of hY2 and for the peptide docking. Thus, a new model was generated based on NTSR1 structure to verify the mutagenesis data and as a guidance for the future study.

In the Y2 mutagenesis study (Paper VII), both binding assays (including saturation and competition assays) and a functional assay were used. Another way to study the ligand-receptor profiles is the time-resolved binding kinetic study, which can provide not only the affinity but also the association and dissociation rates. The dissociation rate reflects how long the drug molecules can form complex with the receptor which is relevant for a potential drug effect (Copeland et al., 2006). In one binding kinetic study (Dautzenberg and Neysari, 2005), PYY showed an irreversible binding profile for the Y2 but not for the Y1 and Y5 receptors. This interesting phenomenon led to the study described in paper III, in which the binding kinetics of PYY to Y2 was studied using the LigandTracer technique in living cells. As a proof of concept, firstly, we also wanted to explore the possibility of adopting the LigandTracer technique for GPCR research in general to improve the understanding of GPCRs in an *in vivo* situation. Secondly, we wanted to study the binding kinetics of PYY to Y2 *in vivo*, which might give us insight into the pharmacology of NPY family peptides and receptors.

Aims

There are two main aims in this thesis. One is to identify, clone and express NPY receptors and related receptors from species representing diverse vertebrate lineages, and to study their pharmacological profiles. The second aim is to use homology modelling, docking, mutagenesis and pharmacological studies to investigate how NPY family peptides bind to their receptors.

Papers I and II

To identify NPY receptors in the sea lamprey, *Petromyzon marinus* and study their pharmacological profiles using lamprey endogenous NPY-family peptides: NPY, PYY and PMY.

Paper III

To study the neuropeptide Y system in the frog *Silurana tropicalis*, including the identification, pharmacological study and expression patterns of NPY family peptides and receptors.

Paper IV

To investigate the binding profiles of the zebrafish PYYb binding to four of the receptors, Y4, Y7, Y8a and Y8b, to compare the PYYb binding to the other two endogenous peptides, NPY and PYYa, and to investigate the expression pattern of the zebrafish NPY system.

Papers V and VI

To characterize the QRFP peptide from amphioxus, *Branchiostoma floridae,* to investigate its pharmacological profile, and to study the evolution of vertebrate QRFP receptors in the light of these findings.

Paper VII

To investigate how NPY family peptides bind to hY2. The approach was to use homology modeling and docking of the conserved C-terminus of NPY peptides to identify residues likely to be involved in peptide binding, and to investigate these by mutagenesis and pharmacological studies.

Paper VIII

To study the time-resolved binding kinetics of PYY for the hY2 in living cells.

Materials and methods

The detailed description of the experimental procedures of different methods can be found in the papers or manuscripts included in this thesis. The methods used are summarized as below.

Sequence identification and analysis

Sequence identification

In papers I and II, putative lamprey NPY receptor sequences were sought in the genome assembly PMAR3 of the sea lamprey (*Petromyzon marinus*) available from http://genome.wustl.edu/ as well as in the genome assembly LetJap 1.0 of the Artic lamprey *Lethenteron camtschaticum* available from http://www.ncbi.nih.gov/. Sequences were identified by TBLASTN or BLAST searches using NPY receptor sequences from different vertebrates as queries.

In paper III, NPY receptor family and peptide family sequences were sought by BLAST searches in the *Silurana (Xenopus) tropicalis* genome available in the Ensembl database (www.ensembl.org), using human and chicken sequences as queries.

In paper V, a putative amphioxus QRFPR sequence was sought by TBLAST searches in the *Branchiostoma floridae* genome assembly version 2 available in the NCBI Genome database. The human QRFPR sequence was used as a query.

Phylogenetic and synteny analysis

Sequences were aligned using clustalX version 1.81 (Paper III) and version 2.012 (Papers I and II) with the following settings: Gonnet weight matrix, gap opening penalty 10.0 and gap extension penalty 0.20. In papers V and VI, sequences were aligned using Jalview version 2.8 using the MUSCLE web align tool with standard settings.

Neighbor-joining (NJ) trees were constructed using clustalX version 1.81 (Paper III) and version 2.012 (Paper I, II, V and VI) (Larkin et al., 2007). A boostrap analysis of 1000 iterations was used for branch support. Phylogenetic Maximum Likelihood (PhyML) trees were built using the PhyML 3.0

algorithm available at http://www.atgc-montpellier.fr/phyml, (Guindon et al., 2010) (Paper II, III, V and VII) using a bootstrap test of 100 iterations.

For papers II and VI, a synteny analysis was performed to further confirm the identities of the receptors by analyzing their chromosomal locations.

Tissue expression of cloned peptide and receptor genes

For papers III and IV, quantitative Real-time PCR was used to measure the mRNA levels of different peptides and receptors in tissue panels from zebrafish (*Danio* rerio) and the Western clawed frog *Silurana tropicalis*. Total RNA was extracted using the Trizol Reagent (Invitrogen, Sweden) according to instructions. cDNA was synthesized with MLV reverse transcriptase and random hexamers according to the manufacturer's protocols (Invitrogen, Sweden). Real-time PCR reactions were performed in a MyIQ thermal cycler (Bio-Rad Laboratories, Sweden). The cycles (Ct) values were derived from MyIQ software V1.04 (Bio-Rad laboratories, Sweden) and transformed using the delta Ct method (Livak and Schmittgen, 2001). The expression levels of different genes in different tissues were normalized with housekeeping genes.

Homology modeling and docking

In paper VII, firstly, the fragment of the conserved C- terminal, CH3C(O)-R35-Y36amide, was docked to the previously built Y2 model based on the human A_{2A} receptor (hA_{2A}) (Fällmar et al., 2011), using the GOLD version 4.0 software. Based on the docking mode of the dipeptide, and previous mutagenesis data, the human NPY peptide was docked to the Y2 model. Several residues were selected for mutagenesis to test whether they are involved in the peptide binding as suggested by the docking mode.

A new Y2 model was generated by MODELER using NTSR1 crystal structure (PDB entry 4GRV) as a template to evaluate the mutagenesis data. The automated docking of the NPY/PYY C-terminal pentapeptide, CH3C(O)-Thr32-Arg33-Gln34-Arg35-Tyr36NH2, by the Induced Fit Docking protocol (Sherman et al., 2006), and followed by Molecular dynamics (MD) which is implemented in the GPCR-Modsim web server (Gutierrez-de-Teran et al., 2013).

Molecular cloning and mutagenesis

For papers I and II, the cloning primers were designed based on the identified sequences, and PCR products were amplified using GC-rich PCR sys-

tem, DNTPPack reagent (Roche), PCR products were inserted into pcDNA3.1/CT-GFP-TOPO® vector (Invitrogen). For paper III, PCR primers were designed based on the sequences of different frog receptors identified from the genome of *S. tropicalis* in the Ensembl database (www.ensembl.org). PCR products were inserted into a pcDNA3 vector. For paper V, the coding region of the *Branchiostoma floridae* QRFP receptor was synthesized by GeneScript® after codon usage optimization and inserted into a pcDNA3.1/CT-GFP expression vector.

To generate different Y2 mutants for paper IV, the QuikChange II site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's protocol. Briefly, the mutations were introduced through a PCR with primers specifically designed for each mutation. A pcDNA-DEST47 vector (Invitrogen) inserted with the wild type human Y2 (wt hY2) receptor coding region was used as parental template (Fällmar et al., 2011). After PCR, the methylated parental template was digested with DpnI enzyme and only the PCR amplified plasmids with mutation will be transformed to the *E. coli*. The residues used for mutagenesis were named using Ballesteros and Weinstein system (Three letter abbreviation for residues and one letter abbreviation for the mutants (Ballesteros and Weinstein, 1995).

Upon sequence confirmation, plasmids were purified from *E.coli* by PureLink HiPure Plasmid DNA purification kits (Invitrogen).

Transfection

To check the receptor expression or prepare the receptors for pharmacological studies, the receptor plasmids were transfected into HEK293 cells using lipofectamine 2000 (Invitrogen) according to product instructions. For papers I, II, and VII, the receptors were co-transfected with chimeric Gα-protein Gqi4 with the same procedure. For paper V, a stable transfection was performed for the QRFP receptor by using growth medium containing the Geneticin as selecting medium.

For papers I, II, III, V, and VII, the cloned receptors or the mutants were tagged with GFP at the C-terminus of the coding region. After transfection of the cells growing on coverslips, the receptor expression was visualized by GFP expression using florescence or confocal microscopy.

Ligands

Radioligand ^{125}I-porcine PYY (pPYY) with a specific activity of 2200 Ci/mmol (PerkinElmer) was used as a tracer ligand in all binding assays. Different ligands were used in competition assays for different papers: zebrafish PYYb for paper IV, frog NPY, PYY and PP for paper III, as well as

human PYY3-36 and Y2 specific non-peptide antagonist BIIE0246 for paper VII. The lamprey NPY, PYY and PMY (Paper I, II), amphioxus QRFP, human QRFP 43RFa, human QRFP 26RFa (Paper V), and human PYY3-36 (VII) were used for the functional Inositol phosphate (IP) assays.

Pharmacological studies

In this thesis, three different methods were used to determine the pharmacological profiles of ligands and receptors, including binding assays (Saturation and competition assays) and functional Inositol phosphate (IP) assay. A kinetic binding study was performed for the hY2.

Binding assays

For papers III, IV and VII, saturation assays were performed to determine the Kd values of the radioligand ^{125}I-pPYY for different receptors and mutants. Competition assays were performed to determine the Ki values of competing ligands for the different receptors. Saturation assays were performed using 12 different concentrations of radioligand but constant concentration of receptors and competing ligands, the concentration of which should be 1000 times higher than concentration of the radioligand. Human PYY was used to determine nonspecific binding. Competition assays were performed using a constant concentration of radioligand and receptors together with 12 different concentrations of competition ligands. To determine the Bmax values in fmol/mg protein of the wt hY2 and its mutants, the total protein concentration of cell homogenate was determined using the Bio-Rad Protein Assay.

Inositol phosphate assay

For papers I, II and VII, Inositol phosphate (IP) assays were used as functional assays to study the potencies of endogenous lamprey ligands for the lamprey NPY receptors or human PYY3-36 for the corresponding receptors. The Gαi signal transduction pathway for NPY receptors, which leads to the inhibition of cAMP, was changed to the Gαq pathway, which leads to Inositol phosphate production after stimulation with agonists (Kostenis, 2002). This was done by transfecting HEK293 cells with receptor and chimeric G protein Gαqi4, which has the last four amino acid from Gαi. The following day, the myo-[2-^{3}H]-inositol was loaded to the cells and after another 24 hours, the cells were stimulated with a serial dilution of the peptides. The generated inositol phosphate was isolated by ion exchange chromatography on AG 1-X8 resin column, then the radioactivity was measured with a liquid scintillation counter.

Ligand binding kinetics

For paper VIII, the LigandTracer technique was used to measure the binding kinetics of ^{125}I-pPYY for the hY2. Briefly, the stable or transient transfected hY2 expression cells were seeded in a small area of the petri dish, then the radioligand was added to the dish before measurement using LigandTracer (Grey) instrument which detect the radioactivity of 125I-labeled ligands. After a few hours' measurement of the association, the radioligand was changed to fresh culture medium to measure the dissociation of the radioligand from the cells. For detailed procedures see paper VIII.

Data analysis

The Kd and Ki values of the saturation and competition assays (Paper III, IV and VII), and EC50 values from the IP assays (Paper I, II, V and VII) were calculated using nonlinear regression curve-fitting in the Prism software version 4.0 or 5.0 (Graphpad). Scatchard plots were generated for saturation assays and Hill-slope values were calculated for competition assays as well. One-way ANOVA followed by Dunnett's multiple comparison test (Paper V and VII), or Tukey-Kramer Multiple Comparison test (Paper I, II, III and IV), were performed using the pKd, pKi or pEC50 values (-log values for Kd, Ki or EC50).

Results and discussion

Paper I and II

In paper I, we reported the identification and cloning of Y5 from sea lamprey, *Petromyzon marinus* (Pma). In paper II, two other lamprey NPY receptors, Y1 and Y2, were identified and cloned for pharmacological study, the identities of which were based on the synteny analysis.

Phylogenetic analysis of the three cloned NPY receptors showed that one of them shared high identity with Y5 receptors from other species. It also contains a large intracellular loop 3 (IL3) like in other Y5s. Thus we confirmed this receptor to be the Y5. For the other two receptors, their identities cannot be identified by the phylogenetic analysis due to low statistical support. Therefore, synteny analysis was used to confirm their identities by identifying the TMA16 and MAP9 genes, which are located in the same chromosome region as the Y1, Y5 and Y2 genes in human and spotted gar. In the Arctic lamprey (*Lethenteron camtschaticum,* Lca), the Y1 and Y2 genes are also located on the same genomic scaffold. We used this *L.camtschaticum* Y1 sequence in order to design primers to clone the Y1 from the *P. marinus*, since only a partial sequence could be identified in the current genome assembly. We were able to identify the Y2 gene in sea lamprey since it is located in the vicinity of the MAP9 gene, like in the human and spotted gar genomes. However, the orientation of the lamprey Y2 has been inverted, likely due to a local rearrangement. Previously, our lab identified a Y1-like receptor in the sea lamprey (Salaneck et al., 2001). this has now been confirmed to be the sea lamprey Y4 due to its location in the same region as MARCH8 gene, as in the human and spotted gar genomes.

The Y5 receptors in general have a IL3 and shorter carboxy terminus compared with other NPY receptor subtypes. The lamprey Y5 has an even longer IL3 than other Y5 sequences, including an Asp-rich region, a His- and Gln-rich region, and an Ala-rich region. The *P. marinus* Y1 has 20 amino acid insertion in the long IL3, and longer C-terminus with a 24 aa insertion with Gly repeats. The *P. marinus* Y2 has a longer N-terminus and C-terminus.

All three cloned receptors, Y1, Y2 and Y5 could be expressed on the cell membrane, whereas Y5 showed high expression in the cytoplasm. The pharmacological characteristics of these three receptors were studied using the Inositol phosphate functional assay with three peptides: river lamprey,

Lampetra fluviatilis (Lfl) NPY, Pma PYY and Pma PMY. One additional peptide was used for the Y5, porcine PYY (pPYY). The results showed that all four ligands, Lfl NPY (14.3 nM), PmaPYY (18.6 nM), PmaPMY (48.1 nM), pPYY (44.4 nM), bind to Y5 with similar affinities in the nanomolar range. For *P. marinus* Y1, the potencies of the three peptides, NPY (3.1 nM), PYY (4.4 nM) and PMY (2.3 nM), are also quite similar to each other. Only PmaPYY (2.0 mM) had a similar potency for the Y2 receptor.

In conclusion, four NPY receptors have been identified in lamprey lineage, the same subtypes as found in human.

Paper III

In paper III, the NPY families of peptides and receptors were studied in the Western clawed frog, *Silurana (Xenopus) tropicalis*. Three NPY-family peptides (NPY, PYY and PP) were identified using the previously determined tetraploid frog *Xenopus laevis* peptide sequences which differ at only a few positions from the *S. tropicalis* peptides. Six receptors, Y1, Y2, Y4, Y5, Y7 and Y8, were identified in *S. tropicalis* using the human and chicken receptor sequences as queries.

Thus, the ancestor of the amphibian lineage had the full repertoire of NPY-family receptors as the gnathostome ancestor ((Larsson et al., 2008; Larsson et al., 2009) and Paper III).

In *S. tropicalis*, the Y1 and Y5 genes have the same head to head orientation as in human and chicken, although the distance between Y1 and Y5 is longer in *S. tropicalis* (86 kb), than in human (20 kb) and chicken (18 kb). No introns were found in Y5 while the Y1 has one intron as in other vertebrates. The receptor gene identified as Y2 in the *S. tropicalis* genome database turned out to be Y7 based on our analysis.

The three identified peptides were synthesized and all six identified receptors were cloned. However, only Y5, Y7 and Y8 showed high enough specific binding for the binding assay. As we describe in the paper, although we have tried several other frog cell lines, not enough specific binding could be obtained for Y1, Y2 and Y4 to perform pharmacological studies. The binding data showed that PYY had higher affinity for all three receptors (0.042-0.34 nM) than the other peptides. NPY bound to Y5 with much lower affinity compared with mammals and chicken.

The mRNA expression levels of NPY-family peptides and receptors were investigated through quantitative PCR using a panel of 18 tissues from male and ovaries from female *S. tropicalis*. All peptides and receptors showed broad expression in different tissues, but only relatively low level of Y2 mRNA was detected in the ovary. As for the neuronal tissues, only NPY and Y1 mRNA could be detected.

Paper IV

In zebrafish, the NPY system consists of three peptides, NPYa, PYYa and PYYb, and seven receptors, including Y1, Y2, Y2-2, Y4, Y7, Y8a, and Y8b (the two last-mentioned previously called Yc and Yb, respectively). The Y5 receptor, which has been cloned from all other non-teleost species studied, has not been found in zebrafish and other teleost. This suggests that Y5 has been lost from the teleost lineage. Aside from the Y1 receptor, which was discovered more recently, all other receptors have been cloned and studied with regard to their pharmacological profiles using binding assays. Among these, Y2 and Y2-2 have been studied using all three peptides (Fällmar et al., 2011; Fredriksson et al., 2006) whereas only the peptides NPYa and PYYa were used in the studies of Y4, Y7, Y8a, Y8b (Berglund et al., 2000; Fredriksson et al., 2004; Lundell et al., 1997; Starbäck et al., 1999).

In this paper, we determined the affinities of PYYb for the remaining four receptors, Y4, Y7, Y8a and Y8b. Thus the binding profile of three peptides for all six cloned receptors was completed.

In summary, the PYYa displays lower affinity (3 nM) for Y2 than NPY (0.17 nM) and PYYB (0.066 nM). Taking together the results from the other NPY peptides and receptors in zebrafish, all three peptides have higher affinity for Y4 (0.028-0.034 nM) than for the other five receptors. The strongest peptide-receptor selectivity was PYYb for Y2, as compared to NPY and PYYa. These affinity differences may be helpful to elucidate specific details of peptide-receptor interactions.

We also investigated the level of mRNA expression in different organs using qPCR. All peptides and receptors have higher expression in heart, kidney, and brain.

These quantitative aspects of pharmacological studies and mRNA distribution may serve as a basis for physiological studies of the NPY system in zebrafish.

Paper V and VI

There are several RFa peptides, including neuropeptide FF (NPFF), GnIH, PRLH and QRFP/26RFa, that bind to a group of closely related GPCRs, including the QRFP receptor. This group of GPCRs is more closely related to the NPY receptor family than to other GPCRs. In order to explore the origins of the RFa and RYa peptides and their GPCRs during the vertebrate evolution, we have searched the genome database for the amphioxus, *Branchiostoma floridae*, which might help to sort out the ancestral repertoires of QRFP-related receptors.

In paper V, we describe searches in the genome sequence of the amphioxus *B. floridae*. Initially this was done in order to search for NPY-family

receptors. Although no NPY receptors could be identified from the amphioxus genome database, several related receptors were found. Among these, two putative QRFP receptor orthologues were identified using the human QRFP receptor as the query sequence. The two candidate receptor sequences have high nucleotide identity to each other (94%, 65 differences). We cloned one these and characterized its pharmacological profile. Four ligands were used for studies of the cloned QRFP, namely amphioxus QRFP and human 43RFa, 26RFa, and 26RF (lacking the amide group). In the signal transduction assay, the amphioxus QRFP had the highest potency (0.28 nM). Also the human 26RFa and 43RFa gave quite high potencies (1.9 nM and 5.1 nM, respectively) whereas 26RF had a potency of more than 1 uM, which suggest that the amide group is essential for the ligand-receptor interactions. The identification of a QRFP receptor from amphioxus, together with the fact that the ancestral PRLH receptor was found to have duplicated in the 2R events (Kuraku and Kuratani, 2011), suggests an early existence of QRFP receptors before the early vertebrate tetraploidizations (2R).

In paper VI, we investigated the evolution of QRFP receptors in light of the 2R tetraploidizations. We identified three QRFPRs in spotted gar (*Lepisosteus oculatus*), coelacanth (*Latimeria chalumnae*) and zebrafish (*Danio rerio*). Two receptors were found other species' genome sequences, including Western clawed frog (*Silurana (Xenopus) tropicalis*), Chinese softshelled turtle (*Pelodiscus sinensis*), Japanese eel (*Anguilla japonica*) and the cave fish *Astyanax mexicanus*. Two receptors genes were also found in the Carolina anole lizard (*Anolis carolinensis*) and American alligator (*Alligator mississippiensis*) genomes. However, one of the two gene sequences was incomplete in both species and they were excluded from the phylogenetic analysis. Only one QRFPR sequence was found in mammals and birds, except rat and mouse that have two QRFPR genes, which are likely the result of a lineage-specific duplication in rodents (Takayasu et al., 2006).

Our synteny analysis and phylogenetic analysis demonstrated that the ancestral QRFP receptor type 1 (QRFPR1) gene was located on the same region as the NPY receptor Y2-Y1-Y5 gene triplet. Subsequently, four subtypes were generated due to a combination of local duplication and the 2R whole genome duplications, concomitantly with the ancestral NPY receptor genes. Due to differential gene losses, different subtypes were preserved in the different lineages. Although four subtypes of QRFPR were identified in vertebrates, no single species investigated contains all four sybtypes.

Paper VII

NPY-family peptides and receptors are involved in several biological functions. Y1 and Y2 have attracted special interest as they have opposite roles in appetite regulation.

Previously, we built a human Y2 (hY2) structural model based on the adenosine A_{2A} receptor crystal structure. In this study, the conserved C-terminal dipeptide fragment of NPY/PYY was docked into this model. The possible interaction points were identified based on this peptide-receptor complex. Several residues seemed to be potentially involved in peptide binding, including Thr2.61, Tyr 3.30, Gln3.32, Tyr5.38, Leu6.51, and His7.39, as well as the previously studied residues Tyr2.64, Gln6.55, Asp6.59 and Tyr7.31 (Merten et al., 2007). Asp6.59 seemed to form a salt bridge with Arg35 in NPY based on this model, which disagrees with the previous hypothesis that the NPY Arg33 interact with the hY2 receptor. Residues Gln3.32 and His7.39 also seem to have a role in connecting the TM3 and TM7 to maintain the stability of the receptor. To verify the importance of the newly identified residues in peptide binding, mutants of those residues were made to disrupt the possible interactions with the peptide, mainly by substituting the original residue to Ala or Leu. In total, including signal and double mutants, 17 mutants were made for the above six residues and two mutants were also made for Gln6.55 to further investigate its role in ligand interaction.

The membrane expression of all the mutants as well as wt hY2 were visualized with GFP, which was added as a tag at the C-terminus. All the mutants could be expressed on the cell membrane, except the hydrophobic substitutions of Gln 3.32 and His7.39. The Leu substitution of Gln3.32 and His7.39 and Ala substitution of His7.39 totally abolished membrane expression, which suggests that Gln3.32 and His7.39 are involved in receptor stability. The Ala substitution of Gln3.32 showed very low membrane expression which made subsequent pharmacological studies impossible. The Leu substitution of Gln6.55 abolished GFP expression which also suggests a role in receptor structure

During the course of this study, the first crystal structure of the peptide binding receptor, neurotensin receptor type 1 (NTSR1), was published (White et al., 2012). The NTSR1 amino acid sequence is approximately 26% identical in the TM region compared with hY2. A new structural hY2 model based on NTSR1 was therefore developed. The pharmacological data in this paper (summarized in Table 1), suggest that Tyr3.30 may have less important roles in ligand binding while other residues seem to be involved in ligand binding or maintain the structural stability as predicted by the A_{2A}-based model. The NTSR1-based model agrees with these findings.

Table1. The binding affinities of hPYY3-36 and BEEI0246 for wt hY2 and its mutants.

Mutant	hPYY3-36 (Ki/wt hY2 Ki)	BIIE0246 (Ki/wt hY2 Ki)
T2.61A	101	0.3
Y3.30A	5.8	0.5
Y3.30L	1.8	0.2
Y3.30L+Y5.38L	38.2	1.3
Y3.30L+L6.51A	13.9	0.7
Q3.32A	-	-
Q3.32E`	56.9	6.2
Q3.32H	141	0.2
Q3.32L	-	-
Q3.32H+H7.39Q	-	-
Y5.38A	37.4	0.7
Y5.38L	11.4	0.3
Y5.38L+L6.51A	85.9	0.5
L6.51A	28.9	0.4
Q6.55L	-	-
Q6.55N	1.4	7.8
H7.39A	-	-
H7.39L	-	-
H7.39Q	9.2	5.2

Paper VIII

There are several methods to identify the pharmacological characters of ligands to their receptors, including ligand-receptor binding assays and functional assays. In paper VIII, as a pilot study, we measured the binding kinetics of the radioligand ^{125}I-pPYY for hY2 receptor using the LigandTracer (Ridgeview Instruments AB, Sweden). There are a few methods to determine the association and dissociation rates of GPCRs, for example the surface plasmon resonance (SPR) technique (Navratilova and Hopkins, 2011). However, as of yet no study has been done using living cells. As a proof of concept, we investigated the possibility of using the LigandTracer technique to determine the association and dissociation rates of GPCRs. The gastrin-releasing peptide receptor (GRPR) was also included in this study, however, it will not be discussed further in this thesis.

It has been shown that the hY2 shows quite a low dissociation rate for h PYY (Dautzenberg and Neysari, 2005), but those assays were performed *in vitro*, using the homogenate of cells expressing the GPCRs which might not reflect the real environment for *in vivo* ligand receptor interactions.

The interactions of ^{125}I-pPYY with hY2 expressed in HEK293 cells was monitored for 5 hours and then ^{125}I-pPYY was removed and monitored for another 15 hours to measure the dissociation of the ^{125}I-pPYY from the receptors. Based on the results of the curve fit analysis, both the association rate constant K_{on} (K_a in paper) ($5.0*10^5$ M-1s-1) and the dissociation rate constant K_{off} (K_d in paper) ($5.1*10^{-5}$s-1) were determined. The dissociation constant Kd (K_D in paper) was also determined accordingly, Kd=K_{off}/K_{on}=$1.02*10^{-10}$ M.

In order to determine how long is required to reach the equilibrium, we measured three cell lines, transient transfected HEK 293 cells, stable transfected HEK 293 cells and wild type HEK 293 cells. Of these, transient transfection showed the highest signal, the stable transfection showed a much lower expression level. And they both take about 10 hours to reach the equilibrium.

There is a difference in the Kd value calculated from this paper compared with the value presented in paper VII. This discrepancy might due to the following reasons. Firstly, the five hour incubation period *in vivo* might not be long enough to reach equilibrium. Secondly, the specific signal is rather low due to the low expression level of the Y2 receptors. Finally, the difference in the molecular environment *in vivo* compared with the *in vitro* assays might contribute to differences in pharmacological profiles between the two conditions.

Concluding remarks and future perspectives

In this thesis, I have studied the evolution of NPY receptors (Paper I, II, III, IV) and QRFP receptors (Paper V, VI) in different species as well as the peptide-receptor interactions of the NPY family peptides and receptors, with focus on the human Y2 receptor (VII, VIII).

In papers I and II, we have identified and cloned three NPY family receptors, Y1, Y2 and Y5 from the sea lamprey, *Petromyzon marinus*. Together with the previously cloned receptor which has been confirmed to be Y4 in this thesis, in total four functional receptors have been found in the lamprey lineages. The functional assay results show that all three lamprey NPY-family peptides have similar potencies for Y1 and Y5. However, only lamprey PYY is a potent agonist for Y2. The expression patterns of the Y5 and Y4 receptors have been studied before, and the Y1 and Y2 expression patterns will be studied to complete this story.

In paper III, three peptides, NPY, PYY and PP, and six receptors, Y1, Y2, Y4, Y5, Y7 and Y8 were identified in the Western clawed frog *Silurana (Xenopus) tropicalis*. The peptides had higher expression in skin, blood and small intestine and only NPY was present in the brain. All receptor mRNAs had similar expression profiles with high expression in skin, blood, muscle and heart. Among the six receptors, Y5, Y7 and Y8 could be characterized by binding studies using the three frog peptides. The other three receptors, Y1, Y2 and Y4, which showed low expression levels based on the radioligand binding assay, may need to be studied through functional assays, since there is a possibility that they bind poorly to the radioligand ^{125}I-pPYY.

In paper IV, we investigated the PYYb binding affinities for the four zebrafish receptors, Y4, Y7, Y8a and Y8b and compared with the other two endogenous peptides, NPY and PYYa. All peptides and receptors have high expression in heart, kidney, and brain. The expression pattern and pharmacological profiles may be used as a basis for functional studies of the NPY system in zebrafish.

In order to study the evolution of the QRFP systems, in paper VI, three QRFP receptors were identified in zebrafish (*Danio rerio*), coelacanth (*Latimeria chalumnae*), and spotted gar (*Lepisosteus oculatus*), representing four different subtypes that were generated by the 2R genome duplications early in vertebrate evolution. Mammals and birds have only one QRFP receptor, with a few exceptions. In paper V, a QRFP receptor was identified and cloned from the amphioxus, *Branchiostoma floridae* (Bfl). The amphi-

oxus QRFP peptide showed good potency for this receptor. The expression pattern of the QRFP peptide and its corresponding receptor is also on our wish list for future study. The highly conserved human QRFP peptides also have good potencies for this receptor, even though the amphioxus receptor and the human QRFP receptor only share 44% identity, so the interaction points of the two receptors must be highly conserved. Using similar methods as in paper VII, by performing homology modeling and docking for the two receptors and comparing the QRFP receptor sequences from different species, it should be possible to identify important residues for QRFP binding. Thus, we might be able to explain how the binding selectivity arose between different RFamide peptides and receptors, although the peptides all end with a conserved RFamide.

Pharmacological profiles have also been determined for most of the NPY receptors mentioned above and the Bfl QRFP receptor, providing a starting point to study their biological functions in different species and to interpret the results from the functional studies. Furthermore, this work on the evolution of these receptors as well as their peptides will also contribute to understanding how the peptides bind to their receptors in the NPY and QRFP systems.

In paper VII, to understand how the NPY peptides interact with their receptors, we have used homology modeling and docking of the conserved C-terminus of NPY family peptides to identify residues that are important for peptide binding. Mutagenesis and pharmacology studies will be needed to confirm the importance of these residues. Several residues have been confirmed to interact with the C-terminus of NPY family peptides The ongoing project is investigating more residues involved in peptide binding. In the future, with more crystal GPCR structures available, especially of receptors closely related to the NPY receptors, for example, the orexin-2 receptor, we could build a more accurate Y2 model.

In paper VIII, we demonstrate how PYY interacts with hY2 in real-time on living cells using the LigandTracer technique. We found that the radio-labeled porcine PYY binds to and also dissociates very slowly from hY2 indicated by the K_{on} and K_{off} rates. One interesting question that remains is whether the truncated PYY, PYY3-36, which is a common analogue of PYY in the circulation, also shows a similar profile. The functional implications of this low off rate binding would also be interesting to study in the future.

Acknowledgements

I would like to express my gratitude to the many people who have contributed to this thesis.

First, I would like to thank my supervisor, Professor **Dan Larhammar**, for accepting me as a PhD student. It is a real honor to be a PhD student of you - an enthusiastic scientist, a caring person, a friend. Thank you for your trust, guidance, patience (especially in correcting my writing :)) and encouragement during these years.

To my co-supervisors: **Ingrid Lundell,** thank you for taking care of me from the very beginning after I joined the lab. Thank you for introducing me to the pharmacological methods, for all the discussions and all your kind help. **Nina Mohell**, thank you for your endless knowledge of pharmacology. Every time when we met to discuss our results, I learned a lot. **Johan Åqvist,** thank you for the collaboration and all your efforts for the Y2 modeling project.

To my former and current colleagues in DanLab: A large thank you to **Christina Bergqvist**, **Görel Sundström**, **Helena Fällmar, Jasna Pruner** and **David Lagman,** for your excellent work on different projects included in this thesis. I will also never forget all the help I received from **Daniel Ocampo Daza, Jenny Widmark**, **Xesús M. Abalo**, **Kateryna Shebanits**, **Minas Al-Bagdadi, Xiao Zhang** and **Helen Haines.** Thanks all of you for the fun times we have had in DanLab. I feel so lucky to have worked and to be friends with you guys!

Many thanks to all the collaborators outside DanLab, especially to **Hugo Gutierrez de Teran** and **Lars Boukharta**, for your excellent work on building our Y2 model, and the very fruitful discussions on our data. **Hanna Björkelund**, thank you for our collaboration on the LigandTracer project.

The project students that I have worked with, especially **Al Rashid, Björn Edlund, Roja Saffari, Judith Bezgovsek, Rebecca Faresjö, Tong Zhang** and **Sofia Kaneberg,** thank you for your contributions. I learnt a lot from your questions and our discussions!

I also would like to thank my previous project supervisors in Sweden, Professor **Elena Jazin** and Professor **Kjell Malmlöf.** Thanks for accepting me as your project student, I have learnt a lot from working with you.

Thanks to all the nice people in **A2:2 corridor** and in **Department of neuroscience**, for being friends, helping me in different ways and creating a nice atmosphere here.

All the **Chinese friends** who are still in or have already left Sweden. You make my life here much easier and more fun!

My family in China. Thanks to my parents, **Qingshen Xu** (许庆申) and **Guifen Niu** (牛桂芬), and my siblings, **Li Xu** (许立), **Fang Xu** (许芳) and their families, for your love, and supports in my life! My relatives, especially my aunt **Xuemin Zhang** (张学敏), my cousins **Zhe Xu** (许哲) and **Xin Xu** (许新), thanks for your enlightening suggestions and encouragement.

My small family in Sweden. My dear wife, **Haisha Ma** (马海莎), thanks for your supports these years and thank you for always feeding me very well. My daughter **An-ti Linnea Xu** (许安缇) and my son **Jing-lang Nils Xu** (许境朗), the two biggest achievements during my PhD study, thank you both for all the laughs and happiness you brought me.

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Editor: The Dean of the Faculty of Medicine

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