ZEBRAFISH IN THE DRUG DEVELOPMENT PROCESS: CHARACTERIZATION OF THE ZEBRAFISH OXYTOCIN SYSTEM

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Abstract
Zebrafish (*Danio rerio*) is a vertebrate model organism. It is suited for many phases of drug development process like toxicological studies. The major advantage of using zebrafish is the possibility to conduct high-throughput screens on a whole vertebrate animal. However, there is not as much knowledge about zebrafish as there is about other model organisms. Therefore there might be differences between zebrafish and humans that affect the use of zebrafish as a model in the drug development process. The purpose of this thesis was to characterize the structure of the zebrafish oxytocin system and assess the role of oxytocin on zebrafish behaviour. In humans defects in the oxytocin system have been linked to many psychiatric disorders like autism. If the mammalian and zebrafish oxytocin systems resembled each other functionally and structurally, it would enable the use of zebrafish as a model when studying the role of oxytocin in pathophysiology of diseases and also in oxytocin system related drug development.

The structure and development of zebrafish oxytocin system was studied by staining adult zebrafish brain cryosections and larval brains with antibodies made against mammalian oxytocin. The specificity of the antibodies to recognize zebrafish oxytocin was determined by absorption and cross-reactivity controls. The role of oxytocin on zebrafish locomotion was studied by inhibiting the splicing of oxytocin messenger RNA with morpholino oligonucleotides (MOs). The MOs were used to address the relevance of the model in pharmacology, since the zebrafish oxytocin receptors have not been expressed and pharmacologically characterized.

In zebrafish oxytocin was produced in the cells of the preoptic nucleus. There were thick oxytocin fibers towards the pituitary and also thinner fibers into areas in the telencephalon, diencephalon, mesencephalon and rhombencephalon. One of the MOs was able to inhibit the production of oxytocin with a dose that did not cause morphological abnormalities. The MO reduced the locomotor activity of the fish, but the specificity of the MO has to be determined. The structure of the zebrafish oxytocin system resembles mammalian oxytocin system in terms of the location of oxytocin cells and fiber projections. Therefore zebrafish seems a suitable model organism for oxytocin research. However, the structure of the zebrafish oxytocin receptor system and the effect of oxytocin on other behavioural aspects have to be determined in order to further evaluate the applicability of zebrafish for oxytocin research.

Keywords
Zebrafish, drug development, oxytocin, immunohistochemistry, morpholino oligonucleotide

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Tiivistelmä

Seeprakala (*Danio rerio*) on selkärankaisiin kuuluva mallieläin, jota voidaan käyttää monissa lääkekehityksen eri vaiheissa kuten toksisuuskokeissa. Seeprakalan käytön merkittävin etu on mahdollisuus suorittaa laajoja tehoseulontoja selkärankaisella mallieläimellä. Seeprakaloista ei kuitenkaan ole yhtä paljon tietoa kuin yleisemmin käytettävistä mallieläimistä. Seeprakalan ja nisäkkäiden välillä voikin olla eroja, jotka vaikuttavat seeprakalan käyttöön mallieläimenä lääkekehityksessä. Tämän tutkimuksen tarkoitus oli kartoittaa seeprakalan oksitosiinijärjestelmän rakenne sekä tutkia oksitosiinin merkitystä seeprakalan käyttäytymisessä. Ihmisillä oksitosiinin toiminnan häiriintyminen on yhdistetty moniin psyykkisiin sairauksiin kuten autismiin. Jos seeprakalan ja nisäkkäiden oksitosiinijärjestelmät vastaisivat rakenteellisesti ja toiminnallisesti toisiaan, tämä mahdollistaisi seeprakalan käytön tutkittaessa oksitosiinijärjestelmän osuutta sairauksien patofysiologiassa sekä oksitosiinijärjestelmään liittyvässä lääkekehityksessä.

Seeprakalan oksitosiinijärjestelmän rakennetta ja kehitystä tutkittiin värjäämällä aikuisten seeprakalojen aivojen kryostaattileikkeitä sekä poikasten aivoja nisäkkäiden oksitosiinivasta-aineilla. Vasta-aineiden spesifisyys seeprakalan oksitosiinin tunnistamiseen tutkittiin absorptio- ja ristireaktiokokeilla. Oksitosiinin merkitystä liikeaktiivisuuteen tutkittiin estämällä oksitosiinin lähetti-RNA:n silmukointi morfolino-oligonukleotideilla. Morfolino-oligonukleotideja käytettiin, koska seeprakalan oksitosiinireseptorien ominaisuudet tunnetaan huonosti.

Seeprakaloilla oksitosiinia tuottivat preoptisen tumakkeen solut. Soluista lähti paksuja oksitosiinisäikeitä aivolisäkkeeseen sekä ohuempia säikeitä etu-, väli-, keski- ja taka-aivojen alueelle. Tutkituista morfolino-oligonukleotideista yksi esti oksitosiinin ilmentymistä annoksella, joka ei vaikuttanut kalojen morfologiaan. Kyseinen morfolino-oligonukleotidi vähensi kalojen liikeaktiivisuutta. Morfolino-oligonukleotidin spesifisyys täytyy kuitenkin vielä selvittää. Seeprakalan oksitosiinijärjestelmä rakenne muistuttaa nisäkkäiden oksitosiinijärjestelmää solujen sijainnin ja säikeiden kulun osalta. Tämän perusteella seeprakala vaikuttaa sopivalta mallilta oksitosiinitutkimukseen. Seeprakalan oksitosiinireseptori-järjestelmän rakenne ja oksitosiinin vaikutus käyttäytymisen eri osa-alueisiin tulisi kuitenkin selvittää, jotta voitaisiin paremmin arvioida seeprakalan soveltuvuutta oksitosiinijärjestelmän tutkimiseen.

Avainsanat

Seeprakala, lääkekehitys, oksitosiini, immunohistokemia, morfolino-oligonukleotidi

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LIST OF ABBREVIATIONS

ALT	alanine transaminase
BMP	bone morphogenetic protein
BNST	bed nucleus of stria terminalis
CCe	corpus cerebelli
cDNA	complementary DNA
CNS	central nervous system
cntrlMO	control morpholino oligonucleotide
CPP	conditioned place preference
CRISPR-Cas	clustered regularly interspaced short palindromic repeats-CRISPR associated
crRNA	CRISPR RNA
D	dorsal telencephalic area
DarT	*Danio rerio* teratogenicity assay
DIL	diffuse nucleus of the inferior lobe
DiV	diencephalic ventricle
Dl	lateral zone of the dorsal telencephalic area
Dm	medial zone of the dorsal telencephalic area
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
dpf	days post fertilization
EC_{50}	half maximal effective concentration
ef1α	elongation factor 1-alpha
ENU	ethylnitrosourea
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GSK3β	glycogen synthase kinase 3β
H	hypothalamus
HERG	human *ether-à-go-go-related* gene
hpf	hours post fertilization
HTS	high-throughput screening
IL-6	interleukin-6
LC	locus coeruleus
LC_{25}	lethal concentration 25%
LC_{50}	lethal concentration 50%
LD_{50}	lethal dose 50%
MAO	monoamine oxidase
MO	morpholino oligonucleotide
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NGS	normal goat serum
NOAEL	no observed adverse effect level
OB	olfactory bulb
6-OHDA	6-hydroxydopamine
ON	optic nerve

orx	orexin
oxt	oxytocin
oxtMO	oxytocin morpholino oligonucleotide
PA	preoptic area
PB	phosphate buffer
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline containing Triton x-100
PCR	polymerase chain reaction
PFA	paraformaldehyde
PM	magnocellular preoptic nucleus
PP	parvocellular preoptic nucleus
PPa	anterior parvocellular preoptic nucleus
PPI	prepulse inhibition
PPv	ventral part of the periventricular pretectal nucleus
pre-crRNA	pre-CRISPR RNA
pre-mRNA	precursor messenger RNA
PTU	phenylthiourea
R	raphe nuclei
rag1	recombination activating gene 1
RT-PCR	reverse transcription polymerase chain reaction
SAR	structure-activity-relationship
TALEN	transcription activator-like effector nuclease
TelV	telencephalic ventricles
TeO	optic tectum
TeV	tectal ventricle
TH	tyrosine hydroxylase
TILLING	targeting induced local lesions in genomes
TPp	periventricular nucleus of the posterior tuberculum
tracrRNA	trans-activating crRNA
TSA	tyramide signal amplification
V	ventral telencephalic area
VAST	vertebrate automated screening technology
Vc	central nucleus of the ventral telencephalic area
Vd	dorsal nucleus of the ventral telencephalic area
VEGF	vascular endothelial growth factor
Vl	lateral nucleus of the ventral telencephalic area
VMH	ventromedial hypothalamus
Vp	postcommissural nucleus of the ventral telencephalic area
Vs	supracommissural nucleus of the ventral telencephalic area
VT	ventral thalamus
Vv	ventral nucleus of the ventral telencephalic area
ZFN	zinc finger nuclease

1 INTRODUCTION

Zebrafish (*Danio rerio*) is a vertebrate model organism, which has traditionally been used as a model in the field of developmental biology and genetics. The use of zebrafish as a model organism has been spreading to other areas as well, such as neuroscience. Recently also the pharmaceutical industry has become interested in the potential of using zebrafish in the drug development process.

Developing a new drug is a long and expensive process and it usually takes more than ten years to get a new drug to the market (Dickson and Gagnon 2004). There are also many risks associated with the drug development process and the majority of the drug candidates do not reach the market at all. For example, the clinical approval success rate was estimated to be only 16% in the United States during 1993–2004 (DiMasi et al. 2010). Another problem related to drug development is that the cost of developing a new drug is constantly rising but at the same time there are fewer new drugs coming to market each year (Pammolli et al. 2011).

The major reasons for the failure in the drug development process are insufficient efficacy and excessive toxicity of the drug candidates (Kola 2008). Therefore the methods that are currently being used to discover new drugs, such as *in vitro* high-throughput screening (HTS), do not always provide safe and effective drug candidates. Thus, new tools for the drug development process are needed. Zebrafish may prove to be such a tool because it offers the pharmacological industry a unique possibility to combine a vertebrate model organism to HTS. With zebrafish it is also possible to conduct the screens on a whole animal. Therefore, the use of zebrafish might lead to discovery of drug candidates with high efficacy and low toxicity also in humans.

However, there is not as much knowledge about the basic biology of zebrafish as there is about other animal models traditionally used in the drug development process. Therefore, the use of zebrafish in the drug development process requires further studies about the species. The aim of this thesis was to review the use of zebrafish as a model organism and its applicability to the drug development process. In the experimental part

of the thesis the zebrafish oxytocin system was characterised. The aim was to assess the suitability of this model organism to study the role of oxytocin in the pathophysiology of diseases and in the drug development process related to the oxytocin system.

I LITERATURE REVIEW: ZEBRAFISH IN THE DRUG DEVELOPMENT PROCESS

2 ZEBRAFISH AS A MODEL ORGANISM

Zebrafish (*Danio rerio*) is a freshwater fish, which is used as a model organism in many fields of research ranging from genetics to neuroscience. Zebrafish belongs to the teleost infraclass (*Teleostei*) of the minnow family (*Cyprinidae*). The use of zebrafish as a model organism has many advantages related to its unique characteristics. The use of zebrafish as a model organism is constantly increasing.

2.1 Practical considerations

Zebrafish are easy and fast to breed: one female can produce hundreds of eggs at one mating and the fish reach maturity in 3–4 months. The average lifespan of zebrafish is 2–3 years. Zebrafish has external development and the embryos are transparent. Larvae hatch about 2–3 days after fertilization and at this stage the organogenesis is mostly complete (Kimmel et al. 1995). The larvae develop pigment but this can be prevented with phenylthiourea (PTU), an inhibitor of tyrosinase (Karlsson et al. 2001). PTU treatment may, however, have toxic and teratogenic effects. At five days post fertilization (dpf) the larvae swim freely and certain behavioural characteristics, like locomotion, can be assessed already at this stage (Colwill and Creton 2011).

The external development and transparency make it easy to follow the development of the embryos and larvae. Observations can be made in real-time, on a living organism. The external development also enables easy manipulation, for example genetic manipulation with morpholino oligonucleotides (MOs). The MOs inhibit messenger

RNA (mRNA) translation to appropriate protein (Nasevicius and Ekker 2000). There are many genetic manipulation methods available for zebrafish that further reinforces the usefulness of zebrafish as a model organism. The genetic methods are described in chapter 3.1.1.

The cost of zebrafish maintenance is low. Zebrafish are small (adults about 3–4 cm long) and many individuals can be housed in a same tank. Tanks can be stored in racks so large amounts of zebrafish can be fitted into a small area. There are many commercially available aquarium systems for zebrafish, which facilitate the zebrafish maintenance. Unlike with many other model organisms the zebrafish husbandry conditions have not been fully standardized. The small size of zebrafish, especially larvae, has also its disadvantages. For example larval brains can be dissected but this usually requires fixation. Also getting enough tissue for certain assays may be problematic. This problem can, however, be overcome in some instances by pooling several individuals into one sample.

In zebrafish research many different wild type lines are being used. There is not much knowledge about the specific characteristics of these lines but it is known that different lines have differences in gene expression, neurochemical levels and behaviour (Dlugos and Rabin 2003; Pan et al. 2012). Thus, studies conducted with different lines might give different results. An inbred zebrafish strain with a homogenous genetic background would help in getting more reliable and reproducible results. However, zebrafish inbreeding has proven to be difficult, leading to high mortality of the embryos and a biased sex ratio (Shinya and Sakai 2011). Therefore, only a few inbred zebrafish strains, such as SJD and C32, are available (Streisinger et al. 1981; Johnson et al. 1996). On the contrary many transgenic and mutant zebrafish, such as the transparent *casper* mutant, are available (Ju et al. 1999; Burns et al. 2005; White et al. 2008).

2.2 Similarity to humans

The overall organ morphology and physiology of zebrafish resembles that of mammals. Zebrafish have nearly all the main organ systems that mammals have, including the

cardiovascular system, nervous system and gastrointestinal system. The zebrafish heart has two chambers, an atrium and a ventricle (Lieschke and Currie 2007). Circulation is closed and it consists of arteries and veins. There is also a separate lymphatic system (Küchler et al. 2006). The gastrointestinal system includes the alimentary tract, liver, gall bladder and pancreas (Lieschke and Currie 2007). The zebrafish immune system also resembles that of mammals: zebrafish have both adaptive and innate immunity (Trede et al. 2004). Being a marine organism, the zebrafish naturally lack a mammalian pulmonary system. Additionally, several other organs, such as the prostate, are not found in zebrafish.

The zebrafish central nervous system (CNS) has all the main areas of the mammalian CNS and many signalling molecules and neurotransmitters, such as dopamine and serotonin, are the same (Kaslin and Panula 2001; Guo 2009). The blood brain barrier of zebrafish resembles, both functionally and molecularly, that of mammals (Jeong et al. 2008). There are, however, also differences between zebrafish and mammalian CNS. Zebrafish have for example only one form of monoamine oxidase (MAO) (Setini et al. 2005). The structure of the mammalian and the teleostean telencephalon differs greatly. The structural differences are caused by different development: the mammalian telencephalon develops through evagination and the teleostean through eversion (Wullimann and Mueller 2004). Many corresponding regions have been identified but some mammalian neuroanatomical homologues of the zebrafish telencephalon are still unresolved.

The sequence of the zebrafish genome has been resolved (Howe et al. 2013). Zebrafish is estimated to have over 25000 protein-coding genes while humans are estimated to have 20000–21000 protein-coding genes (Clamp et al. 2007; Collins et al. 2012). Approximately 70% of the human genes have a zebrafish orthologue (Howe et al. 2013). Furthermore, some of the human genes that do not have a clear zebrafish orthologue might have a similarly functioning protein even though they are not recognized as orthologues. This is supported by the fact that for example human *interleukin-6 (IL-6)* gene does not have a zebrafish orthologue but there is one for *IL-6 receptor* gene. The amino acid sequence and function of many proteins is highly

conserved between zebrafish and humans (Golling et al. 2002; Ruuskanen et al. 2005; Renier et al. 2007). Thus the results of zebrafish studies may be applicable also to humans.

The zebrafish genome is duplicated (Amores et al. 1998). In teleosts many of the duplicated genes are silenced but some of them have complementary expression patterns and functions (Brunet et al. 2006). Zebrafish has for example two genes coding for tyrosine hydroxylase, *th1* and *th2*, which are expressed at different times and areas (Chen et al. 2009). The genome duplication has to be considered in the zebrafish research. For example antibodies might recognize both or just one of the protein isoforms. Genome duplication may also offer an advantage when studying the roles of different genes: for example if a knockout of a certain gene is lethal in mice, it might not be lethal in zebrafish when only the other duplicate gene is knocked out (Panula et al. 2010).

The resemblance of zebrafish to humans is not as high as that of mammalian models like rodents, but it is much higher compared to invertebrate models like *Drosophila*. At present there is not as much knowledge about the biology of zebrafish compared to animal models that have been used more extensively. However, the knowledge about zebrafish, and its resemblance to humans, is increasing rapidly. Furthermore, based on the knowledge acquired so far, the conservation between zebrafish and human biology seems high.

3 ZEBRAFISH IN DRUG DISCOVERY AND TOXICOLOGY

In zebrafish the main advantages of both invertebrate and vertebrate models are combined. The zebrafish is a vertebrate and therefore it shares some of the complexity of the mammalian models. Zebrafish can be used to model many human diseases such as cancer, cardiovascular diseases, blood disorders and neurological disorders such as Parkinson's disease and Alzheimer's disease (Wang et al. 1998; Donovan et al. 2000; Langenau et al. 2003; Langheinrich et al. 2003; Bretaud et al. 2004; Paquet et al. 2009).

The function of many proteins is conserved between zebrafish and humans and also many compounds have a similar pharmacological effect in zebrafish as they have in mammals (Golling et al. 2002; Milan et al. 2003; Langheinrich et al. 2003; Ruuskanen et al. 2005; Irons et al. 2013). On the other hand zebrafish also shares some of the simplicity of invertebrate models. Zebrafish is, for example, suited for HTS and the genetic manipulation is easy.

With zebrafish it is possible to conduct high-throughput screens on a whole organism. An advantage of using zebrafish instead of other vertebrate models is the possibility to screen high numbers of individuals in a cost-effective manner (Chan et al. 2002). Especially zebrafish embryos and larvae are well suited for HTS. They are small and fit easily in multi-well plates and survive until 6 dpf without additional nutrition. For example ten zebrafish embryos can be placed in a single well of a standard 96-well plate in a high-throughput screen (Kokel et al. 2010). Another advantage of using zebrafish is the ease of drug administration: compounds can be added to the water surrounding the fish and they are absorbed into the fish through skin and gills (Peterson et al. 2000). Zebrafish embryos and larvae also tolerate many solvents and carriers, like dimethyl sulfoxide (DMSO), which can be so used to dissolve compounds with low water solubility (Maes et al. 2012). Furthermore, the amount of a compound required for zebrafish HTS is low (Chan et al. 2002). These characteristics make zebrafish an advantageous model organism for the drug development process, especially for the drug discovery phase and the toxicity studies.

3.1 Drug discovery

Genetic screening in zebrafish can be used to study the function of different genes and their role in disease processes (Haffter et al. 1996; Donovan et al. 2000). New drug targets can be discovered based on the identified phenotype-gene interactions. The ease of genetic manipulation makes zebrafish a good model for genetic screening and target validation (Haffter et al. 1996; Donovan et al. 2000; Ito et al. 2010). The applicability to HTS makes zebrafish an advantageous model also for chemical screening, which can be used to identify new hit molecules (Peterson et al. 2004). Zebrafish is well suited for

structure-activity-relationship (SAR) studies in the lead optimization phase (Hao et al. 2010).

An advantage of using zebrafish instead of cell-based assays in these processes is the possibility to conduct high-throughput screens on a whole animal: the effect of a compound on the entire organism can be assessed. With zebrafish it is possible to analyse many parameters, such as complex physiology and behaviour, that cannot be analysed using *in vitro* assays. Whole-animal screening could also enable simultaneous assessment of both efficacy and toxicity. This would help in early identification of hits with the desired combination of high *in vivo* efficacy and low toxicity. Use of whole animals also enables the detection of compounds working as prodrugs and compounds with toxic metabolites.

3.1.1 Genetic screening

There are many methods that can be used to screen gene function in zebrafish. The methods can be divided into forward and reverse genetics. In forward genetics random mutations are introduced to the zebrafish genome. Then the individuals are screened for a wanted phenotype and the genome is characterised to find the mutated gene. Since the mutations generated in forward genetics are random, no prior knowledge about the role of the gene in different biological processes is needed. Therefore, this approach may lead to the discovery of new disease pathways and drug targets.

Random mutations can be generated into zebrafish genome by exposing fish to ethylnitrosourea (ENU) (Mullins et al. 1994). ENU is an alkylating agent that produces mainly point mutations. Chemical mutagenesis with ENU is suited for large-scale genetic screens: in the studies by Driever and others (1996) and Haffter and others (1996) over 6000 mutations were isolated and over 1500 of them characterised. ENU screens have led to the identification of many zebrafish mutants that resemble phenotypically and genetically human diseases. For example, an *yquem* mutant has a porphyria phenotype that is caused by a mutation in the same gene that leads to hepatoerythropoietic porphyria in humans (Romana et al. 1991; Wang et al. 1998).

Because of the genetic and phenotypic similarities, zebrafish mutants could be useful disease models. ENU mutation screens have also lead to the discovery of new human disease genes. For example, a *weissherbst* mutant found in an ENU screen has a hypochromic anaemia phenotype that is caused by a mutation in *ferroportin1* gene (Donovan et al. 2000). Mutations in the *ferroportin1* gene were later linked to hereditary hemochromatosis in humans (Njajou et al. 2001).

Irradiation and insertional mutagenesis with retroviruses can also be used to introduce random mutations into zebrafish genome (Chakrabarti et al. 1983; Lin et al. 1994; Gaiano et al. 1996). Insertional mutagenesis is suitable for large-scale screening and the cloning of the genes is easier than in the ENU mutagenesis (Amsterdam et al. 1999). However, the frequency of mutagenesis in insertional mutagenesis is lower compared to ENU mutagenesis (Allende et al. 1996).

A problem related to forward genetic approach is the possibility that the phenotype caused by a mutation is so subtle that it is not detected in the screens. Because the zebrafish genome is duplicated, the mutation of just one of the duplicated genes might not lead to a detectable phenotype. The function of the mutated gene might also be replaced by another gene product in the same biological pathway.

Contrary to the forward genetic approach, in reverse genetics the gene of interest is first mutated or silenced and then the effect on the phenotype is assessed. The zebrafish genome has been fully sequenced (Howe et al. 2013), which is useful for the identification of candidate genes. There are many different reverse genetic methods that can be used to study gene function in zebrafish.

Targeting induced local lesions in genomes (TILLING) is a reverse genetic method in which the DNA of a large, usually ENU, mutagenized zebrafish population is isolated and screened to discover mutations in the gene of interest (Moens et al. 2008). After a mutation is identified by a high-throughput screening method, the line carrying this mutation is recovered and the phenotype analysed. For example mutations in *recombination activating gene 1* (*rag1*), which is required for V(D)J recombination in

lymphocytes, has been identified by TILLING in zebrafish (Schatz et al. 1989; Wienholds et al. 2002). With TILLING it is possible to detect many different kinds of mutations like premature stop and missense mutations, which might for instance have different viabilities (Wienholds et al. 2002). Another advantage of using TILLING is the possibility to collect allelic series of the random mutations. An allelic series includes the different alleles of the same gene that often lead to different phenotypes when mutated.

In addition to random mutagenesis, targeted mutagenesis and gene knockdown methods are available for zebrafish. Artificial restriction enzymes called zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated (Cas) system are used for targeted mutagenesis (Meng et al. 2008; Sander et al. 2011; Hwang et al. 2013). MOs are used for targeted gene knockdown (Nasevicius and Ekker 2000).

ZFNs are engineered restriction enzymes, which consist of a DNA cleavage domain and a zinc finger DNA binding domain (Kim et al. 1996). The cleavage domain contains a non-sequence specific restriction enzyme, such as the type II restriction enzyme FokI (Smith et al. 1999). The zinc finger domain contains sequence specific zinc finger motifs and it can be modified to target a wanted sequence. The ZFNs induce double-strand breaks in the target DNA, which can be repaired by non-homologous end joining or homologous recombination (Bibikova et al. 2001, 2002). Non-homologous end joining is prone to errors and it usually leads to small insertions or deletions at the site of the double-strand break, which disrupts the function of the target gene (Bibikova et al. 2002). With homologous recombination mediated repair it is possible to use an exogenous donor template to insert a DNA sequence into the break site (Urnov et al. 2005; Moehle et al. 2007).

In zebrafish ZFNs have been used to generate targeted mutations in many genes such as *dopamine transporter* and *kdra*, a zebrafish orthologue of the vascular endothelial growth factor-2 receptor (Meng et al. 2008; Foley et al. 2009; Ben et al. 2011). The ZFN mRNAs are microinjected into zebrafish embryos and induced mutations can be

transmitted through the germline (Doyon et al. 2008; Meng et al. 2008). In zebrafish the ZFN induced mutations have been caused mainly by induced errors in the non-homologous end joining (Meng et al. 2008; Foley et al. 2009). Homologous recombination mediated repair after ZFN treatment has not been reported for zebrafish. The main problem related to ZFNs is the insufficient specificity of the zinc finger motifs, which leads to off-target cleavage. A method that produces more specific ZFNs, oligomerized pool engineering, has been developed and it has been used successfully also in zebrafish (Maeder et al. 2008; Foley et al. 2009). However, the specificity of the ZFNs remains to be a problem.

Like ZFNs, TALENs are engineered restriction enzymes that can be used for targeted mutagenesis in zebrafish (Miller et al. 2011; Cade et al. 2012). They consist of a transcription activator-like effector domain and a cleavage domain. The effector domain contains repeat units with different repeats binding to different DNA bases (Boch et al. 2009). By combining these repeats it is possible to target the construct to a certain gene sequence. Also TALENs work by introducing double strand breaks into the DNA and their gene modification efficiency is similar to ZFNs (Miller et al. 2011).

TALEN induced mutations can be transmitted through the germ line in zebrafish (Cade et al. 2012). In zebrafish the reported mutations are caused mainly by insertions and deletions but TALEN-mediated targeted knock-in of DNA fragments by homologous recombination has also been reported (Huang et al. 2011; Sander et al. 2011; Zu et al. 2013). If the targeted knock-in approach proves to be effective, it will allow the introduction of specific mutations, like point mutations, into zebrafish genome and also the production of conditional knockouts. This would be extremely useful for the use of zebrafish as a disease model. TALENs have usually higher target specificity and less off-target cleavage than ZFNs, also in zebrafish (Hockemeyer et al. 2011; Huang et al. 2011; Li et al. 2011; Cade et al. 2012). TALENs are also easier to engineer and faster to produce than ZFNs. There are even high-throughput methods available for TALEN assembly (Briggs et al. 2012; Reyon et al. 2012).

The CRISPR-Cas method was developed very recently for targeted mutagenesis (Jinek et al. 2012; Cong et al. 2013). It is based on an adaptive immunity mechanism of prokaryotes, in which foreign nucleic acids, like plasmids, are detected and silenced by small RNAs (Wiedenheft et al. 2012). There are three types of CRISPR-Cas systems of which type II is used for gene targeting (Jinek et al. 2012). In type II fragments of foreign nucleic acids are incorporated into CRISPR loci and transcribed into pre-CRISPR RNAs (pre-crRNAs) (Wiedenheft et al. 2012). Trans-activating crRNAs (tracrRNAs) recognize the pre-crRNAs by their sequence and form a complex, which is cleaved by RNase III to mature CRISPR RNAs (crRNAs). The mature crRNA then forms complexes with Cas nucleases, which recognize and cleave the foreign nucleic acid. The foreign nucleic acids are recognized by their complementary base pairing with crRNAs.

In targeted mutagenesis with CRISPR-Cas a guide RNA, which consists of crRNA and tracrRNA, is designed and used to program the Cas9 endonuclease to cleave the genomic sequence of interest (Jinek et al. 2012). This method has been used successfully also in zebrafish (Hwang et al. 2013). The efficiency of the CRISPR-Cas system in zebrafish is similar to that of ZFNs and TALENs. In comparison to ZFNs and TALENs, the guide RNAs are easier to engineer and assemble. However, a disadvantage of this method is that there are limitations in the target sequence whereas with TALENs it is possibly to target nearly any given sequence. Further studies are needed to assess the amount of off-target cleavage and germline transmission.

With MOs it is possible to study the function of a particular gene in zebrafish (Nasevicius and Ekker 2000). The MOs are nucleic acid analogues that are designed to transiently knock down gene function (Corey and Abrams 2001). This can be achieved by blocking the translation initiation or the splicing of the RNA with MOs (Nasevicius and Ekker 2000; Draper et al. 2001). The MOs bind to complementary sequence in the nucleic acid that they are targeted against (Ekker 2000; Corey and Abrams 2001). The structure of MOs resembles RNA but in the MOs the sugar moieties are replaced by morpholino rings and the anionic phosphates are replaced by non-ionic phosphorodiamidates. Thus, in contrast to nucleic acids the MOs are non-ionic. These

modifications protect the MOs from degradation by catabolic enzymes and the immune system.

Depending on the purpose of the study, the MOs can be microinjected directly into zebrafish embryos or into the yolk sac (Nasevicius and Ekker 2000; Amack and Yost 2004). The MOs can also be delivered by electroporation into adult fish (Thummel et al. 2006). The knockdown effect of MOs is transient and the duration of the effect varies between different MOs. Usually the effect lasts for several days but the duration of each MO has to be determined individually. There are also MOs that can be activated by light, which enables spatial and temporal control of the gene knockdown (Shestopalov et al. 2007). MOs might have off-target effects: they may inhibit other genes than the ones they were designed to knockdown. Therefore, the use of MOs requires careful controls and rescue experiments to distinguish possible off-target effects from the specific ones.

The use of artificial restriction enzymes and MOs is rather laborious and therefore they are not well suited for large-scale genetic screens. They can, however, be used to screen promising candidate genes. They are also useful in target validation. For example the target through which thalidomide exerts its teratogenic activity was verified in zebrafish using MOs (Ito et al. 2010). An advantage of using the artificial restriction enzymes rather than MOs is that with artificial restriction enzymes the gene is knocked out permanently. This enables the role of a gene to be studied also later in the development. However MOs are easier to engineer than the artificial restriction enzymes.

3.1.2 Phenotype screening

After the function of a gene is disrupted by genetic methods, the phenotype needs to be analysed to assess the gene function. The gene function can be analysed for example based on changes in the morphology or physiology. Because zebrafish embryos and young larvae are transparent, it is possible to assess the morphology visually. For example in the study by Haffter and others (1996) mutated fish were scored by visual inspection of the phenotypes. Mutations that do not cause clear, visible morphological

abnormalities can be detected using molecular markers. Immunohistochemistry and *in situ* hybridization can be used to detect differences in RNA and protein levels. They are however time-consuming methods and usually require sample fixation and dissection. Therefore they are not well suited for HTS.

Fluorescence can be used to facilitate the detection of changes in the morphology and physiology. Transgenic zebrafish, which have a green fluorescent protein (GFP) linked to the gene or promoter of interest, can be produced. There are, for example, transgenic zebrafish in which the GFP is linked to tissue specific promoters such as muscle-specific creatine kinase or in which only certain cell populations, like dopamine neurons, are labelled (Ju et al. 1999; Xi et al. 2011). Also fluorescent molecules can be used to assess the phenotype. For example fluorescent lipids have been used to screen the effect of mutations on lipid metabolism (Farber et al. 2001). A quenched fluorescent moiety was attached to a phospholipid cleavage site. Cleaving of this modified phospholipid by phospholipase A_2 led to detectable fluorescence in fish with normal lipid metabolism. Thus, mutations affecting lipid metabolism could be screened based on reduced fluorescence. The *fat free* mutant, with defects in bile synthesis, was discovered in this screen based on diminished fluorescence. The mutant was morphologically normal and therefore the mutation could not have been detected in a screen based solely on morphology.

There are high-throughput methods for the phenotype screening of zebrafish embryos and larvae. For example, vertebrate automated screening technology (VAST) is a high-throughput method that can be used for genetic and pharmaceutical screens (Pardo-Martin et al. 2010). It is an automated method for zebrafish larvae that can be used to study for example organ development and function. With VAST it is possibly to perform optical manipulations like localized activation of fluorescent reporters. VAST consists of fully automated cycles in which a larva is extracted from a multiwell plate into a capillary within the imaging and manipulation system, moved into a wanted orientation based on an automated image-processing algorithm, imaged and finally removed. The cycle lasts about 20 seconds per well.

In order for the image-based screening methods to be high-throughput, the analysis of the acquired images needs to be automated. For this purpose an assay combining automated imaging with image analysis based on artificial intelligence has been developed (Vogt et al. 2009). In the assay, fluorescent transgenic zebrafish embryos are imaged with a high-content reader in a multi-well plate. The orientation of the embryos does not need to be adjusted for the imaging. The images are analysed with cognition network technology.

Behavioural phenotyping, in which the gene function is analysed through its effect on zebrafish behaviour, can also be used to assess zebrafish phenotypes. Many different aspects of zebrafish behaviour can be analysed. The motility of zebrafish embryos and larvae can be easily analysed (Granato et al. 1996). For example touch response and opto-kinetic response has been assessed to identify genes related to muscle formation, neuronal development and visual-behaviour responses (Granato et al. 1996; Neuhauss et al. 1999). More complex behaviours, such as social behaviour, anxiety and learning, can also be studied (Peitsaro et al. 2003; Bilotta et al. 2005; Bencan et al. 2009; Braida et al. 2012). For example anxiety can be studied with novel tank test (Bencan et al. 2009). When zebrafish is placed into a new tank, it stays at the bottom of the tank. Over time the fish starts to explore upper levels of the tank. Anxiolytic drugs, like diazepam, reduce the time that the fish spends in the bottom of the tank.

Conditioned place preference (CPP) and prepulse inhibition (PPI) can be studied in zebrafish: weak prepulses attenuate the acoustic startle response of zebrafish larvae in a similar manner to mammalian PPI and for example cocaine induces CPP in zebrafish (Darland and Dowling 2001; Burgess and Granato 2007). CPP and PPI can be used to identify genes and pathways related to many psychiatric disorders such as schizophrenia and addiction. When ENU-mutagenized zebrafish were screened based on their cocaine induced place preference, *dumbfish*, *jumpy* and *goody-two-shoes* mutants were discovered to have an abnormally low response to cocaine (Darland and Dowling 2001). The results of further assays suggested that they had mutations in genes related to dopaminergic signalling.

Different aspects of behaviour, such as learning, anxiety and social behaviour, can be studied with high-throughput methods (Blaser and Gerlai 2006; Gerlai et al. 2009; Pather and Gerlai 2009). For example Pather and Gerlai (2009) developed a high-throughput learning assay for adult zebrafish. In this method a zebrafish is presented with an animated image of a zebrafish shoal on a computer screen. The zebrafish is a shoaling fish species so the fish tries to stay close to the image of the shoal. Two computer screens are placed on opposite sides of the tank. The image is shown first on one screen for 20 seconds, turned off for 90 seconds and then shown on the other screen for 20 seconds. This cycle is repeated for several times and the movement of the fish is tracked. In the beginning of the test zebrafish stays close to the screen that last showed the image. During the experiment it spends more and more time in the proximity of the screen where the image will be shown next. If the image is shown randomly at the screens, the behaviour of the fish does not change: the fish stays close to the screen that last showed the image. There is also a protocol available for zebrafish CPP with rather high throughput (Mathur et al. 2011). The procedure is based on a single exposure and it takes about two days to complete.

Biological pathways can be highly complex and therefore finding a direct drug target based on phenotype-gene interaction is not always straightforward. In addition, there is not as much knowledge about zebrafish biology as there is about the biology of other vertebrate models, which might complicate the interpretation of the results even more. Furthermore, not all the behavioural traits that can be studied in other model organisms can be studied in zebrafish because suitable assays have not yet been developed. To be able to develop new behavioural assays that are valid and reliable, more information about specific characteristics of zebrafish behaviour is needed.

3.1.3 Chemical screening

Chemical screening in zebrafish can be used to identify new hits and also to assess gene function (Peterson et al. 2000; Tran et al. 2007). Chemical libraries that contain small molecule compounds can be screened in wild type or transgenic zebrafish to identify compounds that alter their phenotype. The phenotypes can be assessed with similar

methods that are described in chapter 3.1.2. Since chemical screens are conducted on a whole animal, no prior knowledge of a target is necessary for these screens. Therefore zebrafish chemical screens can also be used to discover compounds with therapeutic potential for diseases without known targets.

Transgenic zebrafish are extremely useful for high-throughput chemical screening. Tran and others (2007) used transgenic zebrafish with fluorescent blood vessels to identify drugs that affect angiogenesis. They screened the LOPAC1280 compound library and identified three hits with dose-dependent anti-angiogenic activity. Two of the hits were compounds with known anti-angiogenic effect. One hit was, however, a new anti-angiogenic compound and it proved to be anti-angiogenic also in human endothelial cells. Another example of the use of fluorescence is a study by Burns and others (2005). They used transgenic zebrafish embryos that express GFP in the myocardium to screen the effect of drugs on heart rate. They found that drugs that prolong the QT interval in humans, like astemizole and amiodarone, decrease the heart rate in zebrafish.

Another approach is to screen the ability of different compounds to suppress or enhance disease phenotypes achieved with pharmacological or genetic manipulation. For example, the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) destroys dopaminergic neurons also in zebrafish and can be used as a zebrafish model of Parkinson's disease (Bretaud et al. 2004). MPTP-treated zebrafish can then be used to study neuroprotective effect of compounds. Using this method an anti-cancer agent SU4312 was found to protect zebrafish against MTPT-induced neurotoxicity (Cui et al. 2013).

A study by Peterson and others (2004) is an example of combining genetic manipulation to phenotype suppressor screen: a new class of compounds that affect angiogenesis was found in a screen with a zebrafish *gridlock* mutant. G*ridlock* mutants have a mutation in a *hey2* gene that causes a deformed aorta and prevents circulation to the trunk. In order to identify compounds that suppress the *gridlock* phenotype, embryos were incubated with compounds of a small-molecule library. Two structurally related compounds were found to rescue the aorta formation through activating the vascular endothelial growth

factor (VEGF) pathway. These compounds also promoted tubule formation in human endothelial cells.

The disease phenotype approach and fluorescent markers can also be combined in zebrafish. For example transgenic zebrafish expressing fluorescent labelled, mutant human tau-P301L protein in its neurons has been created (Paquet et al. 2009). This mutation of tau protein has been linked to frontotemporal dementia in humans (Hutton et al. 1998). The transgenic zebrafish have many pathological features of tauopathies, like tau hyperphosphorylation, tangle formation and neurodegeneration (Paquet et al. 2009). Glycogen synthase kinase 3β (GSK3β) phosphorylates tau and is therefore considered as a therapeutic target for tauopathies (Mazanetz and Fischer 2007). Inhibitors of GSK3β were found to reduce the hyperphosphorylation of tau in the transgenic zebrafish (Paquet et al. 2009).

Behaviour based chemical screening in zebrafish is also an effective approach. Rihel and others (2010) screened small molecule compounds with an automated HTS assay to study their effect on the rest-wake behaviour. The assay was conducted with zebrafish larvae and many behavioural parameters, such as the number of rest bouts, were analysed. Over 5000 compounds were screened and over 500 of them modified the behaviour. Each compound was given a behavioural fingerprint based on the individual combination of altered parameters. The compounds were then clustered into groups based on their fingerprints. Analysis of these clusters revealed that compounds with at least one shared target had similar behavioural phenotypes. Furthermore, in almost every case the compounds from the same structural or therapeutic class had a highly similar behavioural pattern. The detected behavioural effects were also similar to the effects these drugs have in humans. Rihel and others identified also new molecules and pathways that regulate rest-wake cycle at least in zebrafish. For example two structurally related podocarpatrien-3-ones had a specific, rest latency increasing effect.

Kokel at al. (2010) conducted a similar, behaviour-based chemical screen with zebrafish embryos. A high-intensity light stimulus evokes a stereotypic series of motor behaviour in zebrafish embryos called the photomotor response. The effect of 14000 compounds

on different features of the photomotor response was analysed. There were compounds with both known and unknown targets. Each compound was given a barcode representing the individual effect of the compound on the photomotor response. After clustering the compounds based on their barcodes, it was discovered that compounds with the same mechanism of action had similar effects on the behavioural parameters. They were also able to discover new compounds with potential therapeutic effect like novel acetylcholinesterase inhibitors. However, some of the known neuroactive compounds did not alter behaviour in this screen. It is not known whether this was due to problems in the screening assay, such as inadequate dosage, or differences between zebrafish and humans.

The results of Rihel and others (2010) and Kokel and others (2010) indicate that combining HTS chemical screens with behavioural phenotyping could be a useful method in identifying new drugs. Based on their results behavioural phenotyping may also help in resolving the mechanism of action, which is a common problem related to phenotype based chemical screening: The compounds with the same molecular target have a similar behavioural phenotype. Therefore the behavioural fingerprint, or barcode, of a compound can be compared with the behavioural profiles of known pathways to identify its target.

The identified hits usually require optimization to become lead compounds with wanted characteristics. In SAR studies the structure of the hit compound is modified in order to improve its efficacy, specificity and pharmacokinetic properties. When the SAR studies are conducted on zebrafish, it is possible to assess the effect of the structural modifications on all the properties simultaneously. Zebrafish have for example been successfully used in lead optimization of a compound called dorsomorphin (Hao et al. 2010). Dorsomorphin was discovered as a hit in a zebrafish chemical screen (Yu et al. 2008). It inhibits bone morphogenetic protein (BMP) signalling. However dorsomorphin had severe off-target effects through inhibition of VEGF signalling (Hao et al. 2010). Therefore a SAR study was conducted using zebrafish embryos and dorsomorphin analogues with improved specificity were identified.

3.2 Toxicology

Zebrafish is emerging as a promising animal model for toxicology studies. The same advantages that make zebrafish a good model for drug discovery also support its use in toxicology: zebrafish is a vertebrate model that is suited for large-scale, whole animal screening. Although zebrafish cannot replace mammalian models in toxicity studies, it offers a cost-effective alternative that could be used to assess toxicity early in the drug development process. Because zebrafish is suited for HTS, toxicity profiles of many compounds could be screened effectively. Zebrafish could also be used later in the discovery pipeline, for example to study the toxicity of discovered lead compounds. Toxicity could even be assessed simultaneously with efficacy due to the use of whole animals.

Many cellular pathways and metabolic enzymes that are related to toxicity, such as apoptosis related p53 pathway and many phase I and phase II metabolic enzymes, are conserved between zebrafish and mammals (Cheng et al. 1997; Thisse et al. 2000; Langheinrich et al. 2002; Bresolin et al. 2005; Jones et al. 2010). There are, however, also species-specific differences in human and zebrafish biology, which might lead to differences in toxicity. For example, differences in the metabolism might lead to production of toxic metabolites in just one of the species.

At present there is not enough knowledge about the differences between zebrafish and humans to know how well zebrafish results predict human toxicity. Therefore further studies using compounds with known toxicity profiles are needed to fully assess the usefulness of zebrafish as a toxicity model. However, based on the studies so far the correlation between zebrafish and mammalian toxicity seems high. Many compounds have similar toxicological effects in zebrafish as they have in mammals (Parng et al. 2002; Milan et al. 2003; Berghmans et al. 2008; Brannen et al. 2010). There is also a strong correlation between the dose that leads to 50% lethality (LD_{50}) in mammals and the concentration that leads to 50% lethality (LC_{50}) in zebrafish of compounds from different chemical classes (Parng et al. 2002; Ali et al. 2011). Toxicity related drug-drug

interactions, such as observed with erythromycin and cisapride, has also been reproduced in zebrafish (Milan et al. 2003).

3.2.1 Mutagenicity and cytotoxicity

There are methods to assess mutagenicity and cytotoxicity in zebrafish. One way to detect mutagenesis is to use transgenic zebrafish embryos carrying a shuttle vector plasmid (Amanuma et al. 2000). The plasmid contains genes related to antibiotic resistance. After zebrafish embryos are exposed to a test compound the plasmid is isolated and transformed into bacteria. Mutagenesis can be recognized based on changes in the antibiotic resistances of the bacteria. Cytotoxicity can be assessed in zebrafish for example with acridine orange staining and there are also commercial kits for cell death detection (Parng et al. 2002; Li et al. 2012).

Many compounds that are cytotoxic in rodent models, like neomycin and taxon, are also cytotoxic in zebrafish assays (Parng et al. 2007; Li et al. 2012). The zebrafish assays might be more sensitive to detect mutagenicity and cytotoxicity than the traditionally used cell-based assays, like Ames test and MTT assay: in zebrafish mutagenicity and cytotoxicity could be assessed in the presence of whole, functional physiological systems, such as DNA repair mechanisms (Zon and Peterson 2005). For example, many compounds with a known apoptosis-inducing effect were identified as apoptotic also in a zebrafish assay but not in the MTT assay (Li et al. 2012). Therefore, zebrafish mutagenicity and cytotoxicity assays could be used to detect cytotoxic or mutagenic compounds earlier in the drug development.

3.2.2 Teratogenicity and carcinogenicity

Teratogenicity of many compounds, like ethanol and thalidomide, correlates between zebrafish and mammals (Reimers et al. 2004; Brannen et al. 2010; Ito et al. 2010). There are assays to screen teratogenicity of compounds using zebrafish embryos (Nagel 2002; Brannen et al. 2010). One of them is called a *Danio rerio* teratogenicity (DarT) assay (Nagel 2002). In the DarT assay the teratogenic potential is assessed based on the

ratio of the LC_{50} value and the half maximal effective concentration (EC_{50}) of malformations. The DarT assay predicted the teratogenicity correctly in 88% of the test compounds. It also identified teratogens that require metabolic activation. In another zebrafish assay, teratogenicity is assessed based on the ratio of the concentration resulting in 25% lethality (LC_{25}) and no observed adverse effect level (NOAEL) (Brannen et al. 2010). The assay predicted teratogenic potential correctly in 87% of the test compounds that represented different teratogenic potencies, pharmacological targets and structural classes.

The use of zebrafish in carcinogenicity studies has been limited although many mechanisms related to cancer, such as oncogenes and tumour suppressor genes, are conserved between zebrafish and mammals (Khodaei et al. 1999; Deltour et al. 2001; Spitsbergen and Kent 2003). Also carcinogen-induced alterations in DNA methylation are similar in mammals and zebrafish (Mirbahai et al. 2011). The carcinogenicity of some compounds is conserved but there are species-specific differences (Khudoley 1984; Spitsbergen et al. 2000a, 2000b). There are for example differences in the types of carcinogen-induced neoplasms between zebrafish and other species. The carcinogenic potential of compounds also varies depending on the age of the zebrafish used. While there are many zebrafish cancer models available (Langenau et al. 2003; Yang et al. 2004), proper zebrafish carcinogenicity assays are still to be developed.

3.2.3 Organ toxicity

Organ toxicity has for long been a major reason for abandoning promising drug candidates in the drug development process and withdrawing drugs from the market (Shah 2006). Organ toxicity may be missed in preclinical and even clinical studies due to its rare occurrence (Dykens and Will 2007). The zebrafish is emerging as a useful model for organ toxicity studies because its organ morphology and function are similar to humans. Furthermore it is suited for large-scale screens and could therefore reveal even rarely observed toxic effects. Cardiotoxicity, hepatotoxicity and neurotoxicity are the most studied types of zebrafish organ toxicity.

The zebrafish is well suited for cardiotoxicity studies. The zebrafish heart starts to beat by 24 hours post fertilization (hpf) and the cardiovascular system is functional around 48 hpf (Kimmel et al. 1995). The morphology and function of the cardiovascular system can be easily assessed visually or using transgenic zebrafish (Langheinrich et al. 2003; Burns et al. 2005). The electrocardiogram of adult zebrafish can also be recorded (Milan et al. 2006). The zebrafish orthologue of the human *ether-à-go-go-related* gene (HERG) is highly conserved (Langheinrich et al. 2003). HERG is related to QT interval prolongation, which is a common and severe type of drug-induced cardiotoxicity: HERG encodes a potassium ion channel, which is involved in the repolarization phase of the cardiac action potential (Taglialatela et al. 1998). Most of the studied drugs that induce QT prolongation in humans, such as haloperidol, also increase the QT interval in adult zebrafish and cause bradycardia and atrioventricular block in larval zebrafish (Langheinrich et al. 2003; Milan et al. 2003, 2006).

Zebrafish can be used for hepatotoxicity studies. The zebrafish liver organogenesis is ready by 72 hpf (Pack et al. 1996). The overall structure of the zebrafish liver is similar to mammals but there are also differences, like the lack of portal lobules. Zebrafish has orthologues to many CYP enzymes, like CYP3A4, and drug-induced enzyme inhibition and induction can be represented in zebrafish (Bresolin et al. 2005; Li et al. 2009; Jones et al. 2010). Hepatotoxicity can be assessed based on histopathology but some forms of liver damage, such as apoptosis, can be assessed visually without dissection (Zhang et al. 2003). Therefore zebrafish could be used to assess hepatotoxicity even with high-throughput. Serum levels of enzymes related to liver function, such as alanine transaminase (ALT), have been characterized in zebrafish (Murtha et al. 2003), and they could be used to assess hepatotoxicity.

Many compounds, such as paracetamol, induce similar hepatotoxicity in zebrafish and mammals (Braunbeck et al. 1990; Zhang et al. 2003; North et al. 2010). Furthermore, a zebrafish hepatotoxicity assay identified 84% of the tested drugs correctly as hepatotoxic and nonhepatotoxic (Jones et al. 2009; Hill et al. 2012). Troglitazone, which was withdrawn from the market in 2000 due to hepatotoxicity, was identified as hepatotoxic in a zebrafish assay (Fung et al. 2001; Jones et al. 2009; Hill et al. 2012).

Thus, zebrafish appears to be a promising hepatotoxicity model. Nevertheless, the liver function of zebrafish, especially larvae, still requires further characterization in order to validate the use of zebrafish as a hepatotoxicity model.

Zebrafish is an advantageous model for neurotoxicity studies. Neurotoxicity on specific neuron classes can be examined using immunohistochemistry and *in situ* hybridization (Parng et al. 2007). Because zebrafish embryos and larvae are transparent, neurons could be examined visually using transgenic zebrafish. Neurotoxicity can also be assessed based on behaviour (Bretaud et al. 2004). Glial fibrillary acidic protein (GFAP) can be used as a marker of neurotoxicity and its structure and function is highly conserved in zebrafish (O'Callaghan 1991; Nielsen and Jørgensen 2003). Therefore GFAP could be used to assess neurotoxicity also in zebrafish. The use of zebrafish in neurotoxicity studies is further supported by the fact that neurotoxicity profiles of many compounds are similar in mammals and zebrafish. For example 6-hydroxydopamine (6-OHDA) and MPTP destroy dopaminergic neurons, ethanol damages motoneurons, acrylamide causes demyelination and taxol induces neuron apoptosis in zebrafish (Anichtchik et al. 2004; Bretaud et al. 2004; Parng et al. 2007).

3.3 Future directions

Zebrafish will not replace other models like cell-based assays and mammalian models in the drug development process: cell-based assays are important for the early phases of the drug development process due to their high throughput and reduction of animal use whereas mammalian models are important later in the process due to their high similarity to human physiology. The unique properties of zebrafish, however, make it a compromise between cells and mammalian models. Therefore the use of zebrafish as a complementary model would benefit the drug development process.

The use of zebrafish along with cell-based assays would have many advantages. By using zebrafish the effect of a gene or a compound on whole animal can be assessed. Therefore the results obtained with zebrafish will probably be more relevant to humans than the results obtained with cell-based assays. For example compared to cell-based

assays, the lead compounds discovered with zebrafish assays would more likely be effective and non-toxic also in humans. Whereas cell-based assays usually rely on known, druggable targets, zebrafish could also be used to model diseases with no validated targets. Therefore zebrafish genetic or chemical screening might lead to discovery of new targets and effective drugs for the disease of interest. Use of zebrafish would also enable the discovery of compounds with more than one target because of the whole-animal approach.

In comparison to mammalian models zebrafish offers an opportunity to study the efficacy and safety of many compounds quickly and cost-effectively. If the efficacy and toxicity of compounds were screened in zebrafish prior to mammalian models, it would reduce the number of mammals needed and lower the time and costs. In the future zebrafish could be used between cell-based assays and mammalian models. For example, the efficacy and toxicity of hits discovered in *in vitro* HTS could then be assessed in zebrafish. Also the SAR studies could be conducted in zebrafish to optimize the efficacy and toxicity simultaneously. Then the acquired lead compounds could be assessed in mammalian models. Screening first with cell-based assays might, however, result in hits with efficacy only in *in vivo* models being missed. This could be avoided by using zebrafish, especially zebrafish embryos, as the first screening tool. This would require further development of validated, high-throughput zebrafish assays.

The main question concerning the use of zebrafish in the drug development process in the future is how well zebrafish represents human physiology and can the results obtained using zebrafish be translated to humans. Many aspects of zebrafish biology are similar to humans and for example many genes and physiological processes are highly conserved between zebrafish and humans (Cheng et al. 1997; Njajou et al. 2001; Paquet et al. 2009; Jones et al. 2010). Also the efficacy and toxicity profiles of many drugs and chemicals are similar in zebrafish and humans (Peterson et al. 2004; Burns et al. 2005; Milan et al. 2006; Parng et al. 2007; Tran et al. 2007; Brannen et al. 2010; Irons et al. 2013).

However, not all of the compounds that are effective or toxic in humans are identified in zebrafish assays (Milan et al. 2003; Jones et al. 2009; Kokel et al. 2010). The observed differences may be due to problems in the assay design. For example, many of the false negative results in zebrafish assays were due to lack of absorption from the water surrounding the fish: when the compounds were injected, their effect in zebrafish was similar the effect observed in humans (Milan et al. 2003; Jones et al. 2009). Another factor in the assay design that can affect the results is the dose: the dose may be too low and thus ineffective or it may be too high and toxic. These features have to be considered when developing zebrafish assays for drug development.

The observed differences in the efficacy and toxicity may also be due to differences between zebrafish and human biology, such as differences in the metabolism or in receptor subtypes. The overall predictability of zebrafish results to human is difficult to assess because of the limited number of studies conducted so far. There may also be publication bias towards publishing only results with similarity to human results. Furthermore, the relevance of the zebrafish assays to humans has to be considered: does the assay really recapitulate complex human functions. For example how well does zebrafish shoaling behaviour represent social behaviour of humans? Future studies about the similarity of zebrafish and humans and the predictability of the results are needed to assess for which research areas and drug development stages zebrafish would be most advantageous.

4 CONCLUSIONS

Zebrafish is a vertebrate model organism that is suited for large-scale whole animal screening. The overall organ morphology and physiology of zebrafish is similar to mammals and many distinct molecular pathways and genes are highly conserved between zebrafish and humans. In addition, the efficacy and toxicity profiles of many compounds are similar in zebrafish and humans. These characteristics make zebrafish an ideal model for the drug development process, especially for drug discovery and toxicology studies. In the drug development process zebrafish can be regarded as a

compromise between cell-based and mammalian models and therefore in the future zebrafish will likely be used to complement the existing models. However, more research is needed to determine the overall predictability of zebrafish results to human efficacy and toxicity and to further assess the applicability of zebrafish to the drug development process.

II EXPERIMENTAL PART: CHARACTERIZATION OF THE ZEBRAFISH OXYTOCIN SYSTEM

5 OXYTOCIN

Oxytocin is a neuropeptide, which consists of nine amino acids (Du Vigneaud et al. 1953). In mammals oxytocin is produced in the magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus and also in the smaller parvocellular neurons of the paraventricular nucleus (Swaab et al. 1975; Buijs et al. 1978; Sawchenko and Swanson 1982). The majority of oxytocin fibers are directed towards the neurohypophysis where oxytocin is released into the circulation. Besides this hypothalamo-neurohypophyseal tract, oxytocin fibers are found in many other brain areas such as the amygdala and hippocampus (Buijs 1978; Knobloch et al. 2012). Oxytocin receptors are also found in several brain areas, for example in the lateral septal nucleus (Loup et al. 1991).

Oxytocin is best known for its effect on uterine contraction and milk ejection (Dale 1906; Schafer and Mackenzie 1911). Besides these peripheral effects, oxytocin also affects many aspects of social behaviour like pair bonding and social memory (Cho et al. 1999; Savaskan et al. 2008). Oxytocin affects also non-social behaviour. For example, it attenuates memory and reduces anxiety and stress (Kovács et al. 1978; Neumann et al. 2000; Ring et al. 2006). The effect of oxytocin on behaviour is regulated by a central release of oxytocin, either through axonal or dendritic release (Ludwig and Leng 2006; Knobloch et al. 2012). Deficits in the oxytocin system have been linked to many psychiatric disorders such as autism, schizophrenia and depression (Beckmann et

al. 1985; Scantamburlo et al. 2007; Wermter et al. 2010). It is therefore no surprise that the role of oxytocin in the pathophysiology of diseases and the therapeutic potential of affecting the oxytocin system are being studied extensively (Meyer-Lindenberg et al. 2011).

Zebrafish is emerging as a potential vertebrate model for human disease modelling and drug discovery. Because the use of zebrafish as a model organism has many advantages, it might be a valuable tool for oxytocin research. The oxytocin system has been highly conserved in the vertebrate evolution and zebrafish oxytocin shows a high degree of sequence similarity to mammalian oxytocin (Acher et al. 1997; Unger and Glasgow 2003). The amino acid sequences of zebrafish oxytocin and mammalian oxytocin differ by two amino acids. Oxytocin has also been shown to affect similar functions in teleost fish as it does in humans, such as social behaviour (Thompson and Walton 2004). These facts reinforce the use of zebrafish in the oxytocin research. However, there is only little knowledge in the literature about the structure and function of the zebrafish oxytocin system.

The aim of the experimental part of this thesis was to determine the structure of the zebrafish central oxytocin system and to study the role of oxytocin on zebrafish behaviour by manipulating oxytocin expression. This was expected to be a better approach than oxytocin receptor ligands, because zebrafish oxytocin receptors have not been expressed and pharmacologically characterized yet. If the zebrafish oxytocin system was structurally and functionally similar to that of humans, it would enable the use of zebrafish as a model in oxytocin research.

6 MATERIALS AND METHODS

6.1 Animals

Adult and larval wild-type zebrafish of the Turku strain (Kaslin and Panula 2001; Sundvik et al. 2011) were used in the experiments. Fish were kept under a 14/10-hour

light-dark cycle at 28,5 °C. Zebrafish embryos and larvae were raised in E3 medium (5 mM NaCl, 0,17 mM KCl, 0,44 mM CaCl, 0,33 mM $MgSO_4$ in H_2O). Breeding and feeding were done according to Westerfield (2000).

6.2 Immunohistochemistry

The structure of the zebrafish oxytocin system was determined by staining adult zebrafish brain cryosections with mammalian oxytocin antibodies. The development of the oxytocin system was studied by staining 1–14 dpf larval zebrafish with the mammalian oxytocin antibodies.

6.2.1 Cryosections

Adult wild type zebrafish were euthanized by immersion in water containing an overdose of tricaine methane sulfonate (0,4 mg/ml, Sigma). The brains were dissected on ice and fixed in 4% paraformaldehyde (PFA) in 0,1 M phosphate buffer (PB, recipe in Appendix 1) overnight at 4 °C. The brains were washed three times with phosphate-buffered saline (PBS, recipe in Appendix 1), transferred to 30% sucrose in PBS and kept at 4 °C until they sank. The brains were frozen in Shandon M-1 Embedding Matrix (Thermo Scientific) on dry ice and sectioned with a cryostat to 16–20 µm sagittal and horizontal sections. Sections were collected on SuperFrost Plus –slides and stored in -20 °C until further use.

The specimens were washed with PBS containing 0,1% Triton x-100 (PBS-T) for five minutes and blocked with 3% normal goat serum (NGS) in 0,3% PBS-T for 60 minutes to prevent unspecific binding of the antibodies. The primary antibodies were diluted in 1% NGS in 0,3% PBS-T. The specimens were incubated with the primary antibody for 22–24 hours at 4 °C. After the incubation the specimens were washed with 0,1% PBS-T (3 x 10 min) and incubated with Alexa Fluor secondary antibody (Invitrogen) in 1% NGS for 60 minutes. The secondary antibody was chosen according to the species in which the primary antibody was produced. The specimens were washed again with 0,1% PBS-T (3 x 10 min) and mounted on 50% glycerol in PBS.

The specimens were examined with a Leica TCS SP 2 confocal microscope. For Alexa Fluor 488 the excitation wavelength used was 488 nm and the emission was collected from 500 nm to 550 nm. For Alexa Fluor 568 the excitation wavelength used was 561 nm and the emission was collected from 600 nm to 650 nm. Acquired image stacks were transformed to maximum projection images using Leica software. The image stacks and the maximum projection images were analysed.

6.2.2 Whole mounts

Larvae older than 4 dpf were stained as brain whole mounts. The larvae were euthanized on ice and fixed in 1,5 ml 4% PFA in PB overnight at 4 °C. PFA was replaced with 1,5 ml PB (pH 7,4) and incubated for three hours at 4 °C. The brains were dissected under a preparation microscope and put into baskets in a 24-well plate containing PBS. The plate was kept on ice. The specimens were washed with 0,3% PBS-T (3 x 60 min) and incubated with preincubation solution (1% DMSO and 4% NGS in 0,3% PBS-T) overnight at 4 °C. The specimens were incubated with the primary antibody for 20–24 hours 4 °C and washed with 0,3% PBS-T (10 min + 3 x 30 min). They were incubated with Alexa Fluor secondary antibody for 20–24 hours at 4 °C. Primary and secondary antibody solutions were made in 2% NGS in 0,3% PBS-T. The specimens were washed in 0,3% PBS-T (10 min), in PBS (3 x 30 min) and in 50% glycerol in PBS (2 x 60 min). The specimens were then infiltrated in 80% glycerol in PBS overnight at 4 °C and mounted ventral side up in 80% glycerol in PBS.

Larvae younger than 4 dpf were stained as larval whole mounts and they were raised in 0,003% PTU in E3 to prevent pigment formation. The staining procedure was the same as for the brain whole mounts. Yolk sac and lower jaw were removed before mounting.

The brain and larval whole mounts were examined with a Leica TCS SP 2 confocal microscope. The excitation wavelengths used were the same as described for cryosections. Acquired image stacks were transformed to maximum projection images using Leica software. The image stacks and the maximum projection images were analysed.

6.2.3 Characterization of the oxytocin antibodies

The sensitivity and specificity of two mammalian oxytocin antibodies to detect zebrafish oxytocin was studied with immunohistochemistry. The immunostaining was conducted on cryostat sections of adult zebrafish brains according to the protocol described above. The primary antibodies tested were polyclonal rabbit anti-oxytocin and monoclonal mouse anti-oxytocin clone 4G11 (Table 1). The efficiency of the staining protocol was ensured by using anti-tyrosine hydroxylase (anti-TH) antibody (Table 1) as a positive control and the omission of the primary antibody as a negative control. Dilutions 1:100, 1:1000, 1:5000 and 1:10 000 of both oxytocin antibodies were tested. The optimal dilution for both antibodies was 1:1000 and that dilution was used in further experiments. For the anti-TH antibody dilution 1:1000 was used based on previous studies (Chen et al. 2009; Sallinen et al. 2009). Alexa Fluor 488 Goat Anti-Rabbit IgG (Invitrogen A11034, diluted 1:1000) was used to detect rabbit anti-oxytocin and Alexa Fluor 488 Goat Anti-Mouse IgG (Invitrogen A11001, diluted 1:1000) to detect mouse anti-oxytocin and anti-TH. They were also used in the negative controls.

Table 1. Primary antibodies

Antigen	Immunogen	Host	Company/ Catalog number
Oxytocin	Synthetic oxytocin (Sigma) conjugated to thyroglobulin	Rabbit	Millipore/ AB911
Oxytocin	Oxytocin conjugated to thyroglobulin	Mouse	Millipore/ MAB5296
Tyrosine hydroxylase	Tyrosine hydroxylase purified from rat PC12 cells	Mouse	Immunostar/ 22941
Orexin-A	Synthetic peptide corresponding to the C-terminal portion of the bovine Orexin-A peptide	Rabbit	Chemicon/ AB3704

The specificity of the antibodies was studied with absorption and cross-reactivity controls. In the absorption control oxytocin antibody was incubated with oxytocin peptide (Peninsula Laboratories 8152) and in cross-reactivity control with arginine-vasopressin peptide (Peninsula Laboratories 8103) for 24 hours at 4 °C. Three different

peptide dilutions (1 µM, 10µM, and 100 µM) were used. After the incubation immunostaining was conducted with the antibody-peptide-solutions on adult zebrafish brain cryosections according to the protocol described above.

6.2.4 Double staining

Double staining with rabbit anti-oxytocin and mouse anti-TH antibodies and with mouse anti-oxytocin and rabbit anti-orexin A antibodies were conducted to study the colocalization and correlative distribution of oxytocin and other neurotransmitters. Adult zebrafish brain cryosections were incubated simultaneously with both primary antibodies and simultaneously with highly cross-adsorbed Alexa Fluor 488 and 568 secondary antibodies (Invitrogen). The rabbit anti-oxytocin was detected with Alexa Fluor 488 goat anti-rabbit IgG (A11034, diluted 1:1000), the mouse anti-oxytocin with Alexa Fluor 488 goat anti-mouse IgG (A11029, diluted 1:1000), anti-orexin A with Alexa Fluor 568 goat anti-rabbit IgG (A11036, diluted 1:1000) and anti-TH with Alexa Fluor 568 goat anti-mouse IgG (A11031, diluted 1:1000). Otherwise the protocol for double staining was the same as described above for the cryostat sections. To study possible colocalization overlay images acquired with Leica confocal microscope were analysed plane by plane.

6.3 Inhibition of oxytocin splicing with morpholino oligonucleotides

Two morpholino oligonucleotides, oxytocin MO1 (oxtMO1) and oxytocin MO2 (oxtMO2), were used to inhibit the splicing of zebrafish oxytocin precursor mRNA (pre-mRNA) (Table 2). OxtMO1 was targeted at the splicing site between intron one and exon two of the zebrafish oxytocin pre-mRNA (Figure 1). OxtMO2 was targeted between intron two and exon three based on a study by Gutnick and others (2011) (Figure 1). Standard control MO (Table 2, cntrlMO) injected larvae and uninjected larvae were used as controls.

Table 2: The sequences of the morpholino oligonucleotides (MOs) used in the study. The sequence of oxtMO2 was designed by Gutnick and others (2011).

MOs	Sequence (5'-3')	Company
Zebrafish oxytocin 1 (oxtMO1)	ACA TTA CTG TGG AGG AAG AGA CGT A	GeneTools
Zebrafish oxytocin 2 (oxtMO2)	CAC TGC AGA TGG TAA GGG AAA CCT A	GeneTools
Standard control-MO (cntrlMO)	CCT CTT ACC TCA GTT ACA ATT TAT A	GeneTools

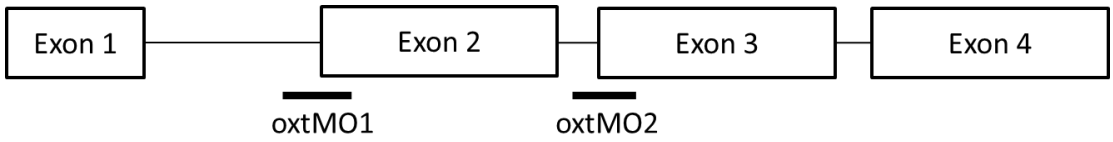

Figure 1. The target sites of oxytocin morpholino oligonucleotide 1 (oxtMO1) and oxytocin morpholino oligonucleotide 2 (oxtMO2) in the zebrafish precursor messenger-RNA.

Different amounts of the oxytocin MOs ranging from 0,5 ng to 8 ng/embryo were tested to determine the optimal dose of the MOs. The dose of the cnrtlMO was 6,8 ng/embryo. The MOs were diluted to right concentrations with sterile water and 0,1% of Phenol red solution (Sigma). 4 nl of the MO-solution was microinjected into the yolk sack of zebrafish embryo at one to four cell stage. The MOs were labelled with 3'-fluorescein and the succession of the MO delivery was assessed six hours after the injection with a fluorescent microscope. The development of the embryos was followed daily.

The efficacy of the MOs was assessed using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry. If the MOs inhibited the splicing of oxytocin pre-mRNA, that would be detected in the RT-PCR as a band shifting to another mass or as a decrease in the intensity of the oxytocin mRNA band. In the immunohistochemistry it would be detected as weaker intensity of the oxytocin staining and as a decrease in the number of oxytocin producing cells compared to the control groups.

6.3.1 Reverse transcription polymerase chain reaction

At 2 dpf 35 larvae per group were pooled and euthanized on ice for the RT-PCR. The RNA was extracted using the RNeasy Mini Kit (Qiagen). The extraction was performed according to the protocol given by the manufacturer. The amount and quality of the isolated RNA was analysed spectrophotometrically with an Eppendorf BioPhotometer. The extracted RNA was turned into complementary DNA (cDNA) using the SuperScript III reverse transcriptase kit (Invitrogen). The amount of RNA needed for the reaction varied between experiments because of differences in the RNA yield. The amount of RNA was calculated so that it was between 10 pg – 5 µg and it was the same between groups to ensure the comparability of the results. RNA was mixed with 2 µl of 50 ng/µl random primers (Roche), 1 µl of 10 mM deoxyribonucleotide triphosphate (dNTPs) mix (Fermentas) and sterile water to a total volume of 11 µl. The mixture was heated for five minutes at 65 °C and incubated on ice for five minutes. 4 µl of 5X First Strand buffer (Invitrogen), 1 µl of 0,1 M DTT (Invitrogen) and 1 µl of SuperScript III reverse transcriptase (Invitrogen) was added. The samples were incubated at room temperature for five minutes and 60 minutes at 50 °C. The reaction was inactivated by heating for 15 minutes at 70 °C after which the samples were incubated on ice for five minutes.

The cDNA was amplified using polymerase chain reaction (PCR). For the oxytocin samples 8 µl of the cDNA template was added to a mixture containing 2,5 µl of 10X optimized DyNazyme buffer (ThermoScientific), 0,5 µl of 10 mM dNTPs (Fermentas), 1 µl of 10 µM zebrafish oxytocin forward primer, 1 µl of 10 µM zebrafish oxytocin reverse primer, 0,5 µl of DyNazyme II DNA polymerase (ThermoScientific) and 11,5 µl of sterile water. For the control samples 3 µl of the cDNA template was added to a mixture containing 2,5 µl of 10X optimized DyNazyme buffer, 0,5 µl of 10 mM dNTPs, 1 µl of 10 µM zebrafish elongation factor 1-alpha forward primer, 1 µl of 10 µM zebrafish elongation factor 1-alpha reverse primer, 0,5 µl of DyNazyme II DNA polymerase and 16,5 µl of sterile water. In the negative control cDNA was replaced with sterile water. The sequences of the primers used are presented in table 3.

Table 3. The sequences of zebrafish oxytocin (oxt) and zebrafish elongation factor 1-alpha (ef1α) primers used in the reverse transcription polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession No.
oxt	TCG GTG TCA GCC TTG GTG AA	GCG GCT CCT CCT GAG ATG AT	NM_178291.2
ef1α	GTT GCC TTC GTC CCA ATT TC	AGC AAA GCG ACC AAG AGG A	NM_131263.1

The PCR program consisted of initial denaturation (94 °C, two minutes), 35 cycles of denaturation (94 °C, 30 seconds), annealing (57 °C, 30 seconds) and polymerization (72 °C, 70 seconds) and final extension (72 °C, seven minutes). The PCR products were analysed with gel electrophoresis. The samples were mixed with 3 µl of 10X DNA Loading Dye (ThermoScientific) and run in 40 ml of 1% agarose gel in TBE buffer (0,1 M Tris, 0,1 M boric acid, 2 mM EDTA) containing 3 µl of Sybr Safe DNA gel stain (Invitrogen). GeneRuler 100 bp DNA ladder (Fermentas) was used to aid in the assessment of the band sizes.

6.3.2 Cell number quantification

Immunohistochemistry was conducted with 5 dpf and 7 dpf larvae according to the staining protocol described above for the brain whole mounts. The acquired image stacks were imported to Fiji (an Open Source imaging software: Schindelin et al. 2012) and the number of oxytocin immunoreactive neurons was counted manually. Results were analysed in SPSS 15.0 with one-way ANOVA and Tukey's *post hoc* test. The significance limit was $p<0,05$.

6.4 Locomotor activity test

The locomotor activity of 5 dpf larvae was studied for groups injected with 2,5 ng oxtMO2, 4 ng oxtMO2 and 6,8 ng cntrlMO. Uninjected larvae were also used as controls. 12 larvae per group were placed individually into wells in a 48-well plate containing 1 ml of E3 medium. The experiment was conducted three times with different larvae so the total number of animals per group was 36. The movement of

larvae was tracked for 15 minutes using a video camera connected to Ethovision 3.1 software as described earlier (Sallinen et al. 2009). The sample rate used was five samples per second. If the fish was detected in less than 90% of the frames of the track file, the track was excluded from the analysis. Tracks containing reflection artefacts were also excluded. After the exclusion the number of larvae per group was 20 in 2,5 ng oxtMO2, 15 in 4 ng oxtMO2, 29 in cntrlMO and 24 in uninjected group. Parameters analysed were total distance moved, mean turn angle, mean meander and mean angular velocity. Results were analysed in SPSS 15.0 with one-way ANOVA followed by Tukey's *post hoc* test. The significance limit was $p<0,05$.

7 RESULTS

7.1 Specificity of the oxytocin antibodies

Both oxytocin antibodies were able to recognize zebrafish oxytocin. The mouse anti-oxytocin antibody proved to be more specific than the rabbit anti-oxytocin antibody. In the absorption control of the mouse anti-oxytocin antibody incubation with 1 μM of oxytocin was enough to block the staining (Figure 2A). Incubation with vasopressin in the cross-reactivity control did not affect the staining even at a concentration of 100 μM. In the absorption control of the rabbit anti-oxytocin antibody the staining was only blocked by 100 μM of oxytocin (Figure 2B). Incubation with vasopressin weakened the intensity of the staining. This indicates that the rabbit anti-oxytocin antibody recognizes also vasopressin, in addition to oxytocin. Because of the higher specificity the mouse anti-oxytocin antibody was used in further studies.

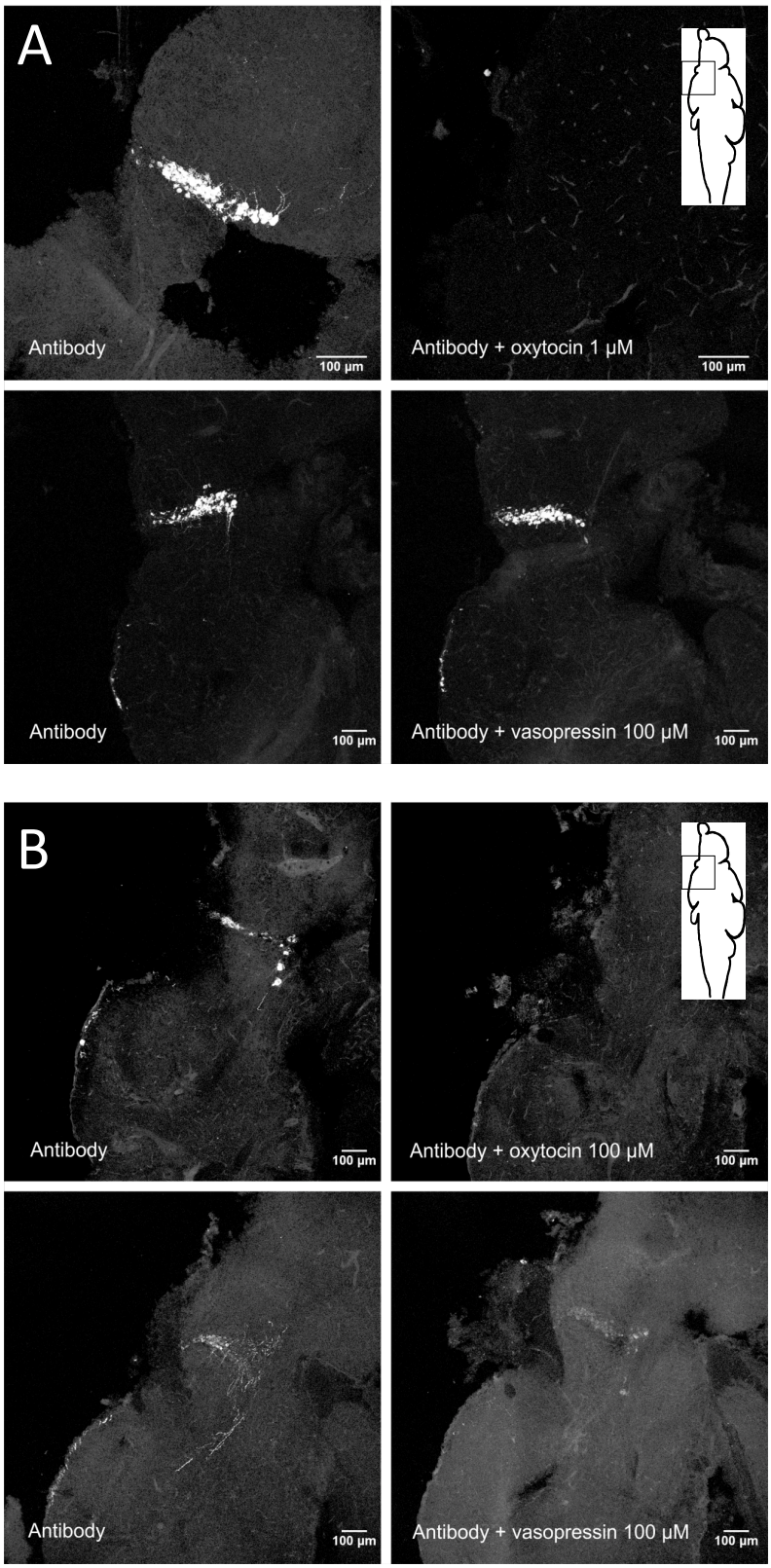

Figure 2. Absorption and cross-reactivity controls of the mouse anti-oxytocin antibody (A) and rabbit anti-oxytocin antibody (B). Sagittal cryosections of adult zebrafish brains were stained with the mouse and rabbit anti-oxytocin antibodies. Both antibodies were

able to recognize zebrafish oxytocin. In the figures A and B the upper row represents the results of the absorption control in which the antibodies were incubated with oxytocin prior to the staining. In the figures A and B the lower row represents the results of the cross-reactivity control in which the antibodies were incubated with vasopressin prior to the staining. With mouse oxytocin antibody the staining was blocked with 1 μM of oxytocin and not affected with 100 μM of vasopressin as seen in figure A. With the rabbit oxytocin antibody 100 μM of oxytocin was required to block the staining and the staining was affected by vasopressin as seen in figure B.

7.2 Oxytocin immunoreactive neurons and fibers

The zebrafish oxytocin immunoreactive neurons were located in the preoptic nucleus in the preoptic area (Figure 3). More dorsally situated larger oxytocin neurons comprised the magnocellular preoptic nucleus (PM) and more ventrally situated smaller neurons the parvocellular preoptic nucleus (PP) (Figure 3D and 3E). The neurons were arranged into two cell clusters that were situated on both sides of the diencephalic ventricle (DiV) (Figure 3B and 3C). Oxytocin neurons were not found in any other brain region besides the preoptic area.

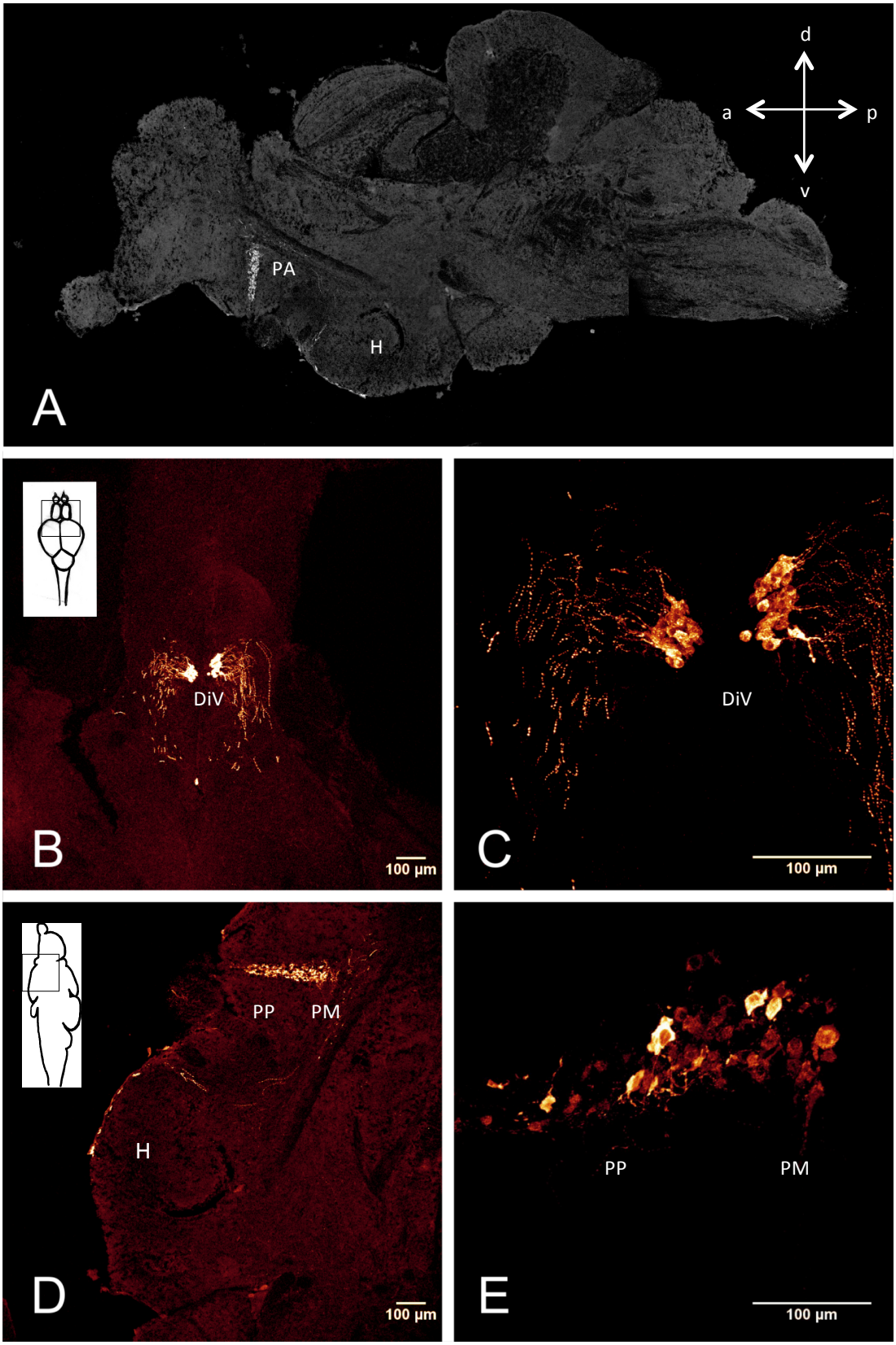

Figure 3. Oxytocin immunoreactive neurons in the zebrafish brain. Cryosections of adult zebrafish brains were stained with mouse anti-oxytocin antibody and examined

with a confocal microscope. The results are represented as maximum projection images. Figure A is a sagittal section of the whole zebrafish brain from near the midline. Figure B is a horizontal section of the preoptic area and figure C is a higher magnification of the area. Figure D is a sagittal section of the preoptic area and figure E is a higher magnification of the area. Oxytocin immunoreactive neurons were located in preoptic area (PA) as shown in figure A. There were prominent oxytocin fibers in the hypothalamus (H) toward the pituitary (Figures A and D). Two clusters of oxytocin cells were found on both sides of the diencephalic ventricle (DiV) as shown in figures B and C. Oxytocin neurons were found in both magnocellular (PM) and parvocellular (PP) preoptic nuclei (Figures D and E). Abbreviations: DiV=diencephalic ventricle, H=hypothalamus, PA=preoptic area, PM=magnocellular preoptic nucleus, PP=parvocellular preoptic nucleus.

Oxytocin immunoreactive fibers were found in all major brain areas: telencephalon, diencephalon, mesencephalon and rhombencephalon (Figure 4A). In the ventral part of the telencephalon fibers projected though the anterior parvocellular preoptic nucleus (PPa) towards the dorsal and ventral nucleus of ventral telencephalic area (Vd and Vv) (Figure 4B). In the dorsal part of the telencephalon fibers were detected in the medial zone of the dorsal telencephalic area (Dm) (Figure 4C).

There were widespread oxytocin fiber projections in the diencephalon (Figure 4E). The most prominent fibers projected to the pituitary. These thick fibers travelled from the preoptic area to the pituitary along the ventral surface of the hypothalamus (Figure 3D). Thinner fibers projected through dorsal and medial parts of the diencephalon (Figure 4E). In the dorsal part the fibers ascended to the thalamus and the pretectum. Some of the fibers turned caudally and ran along the tectal ventricle towards the locus coeruleus (LC). Others continued to the optic tectum (TeO) (Figure 4D). In the medial part the fibers projected through the posterior tuberculum towards the rhombencephalon. Fibers were detected also in the ventral rhombencephalon but the projection could not be traced back to the preoptic area.

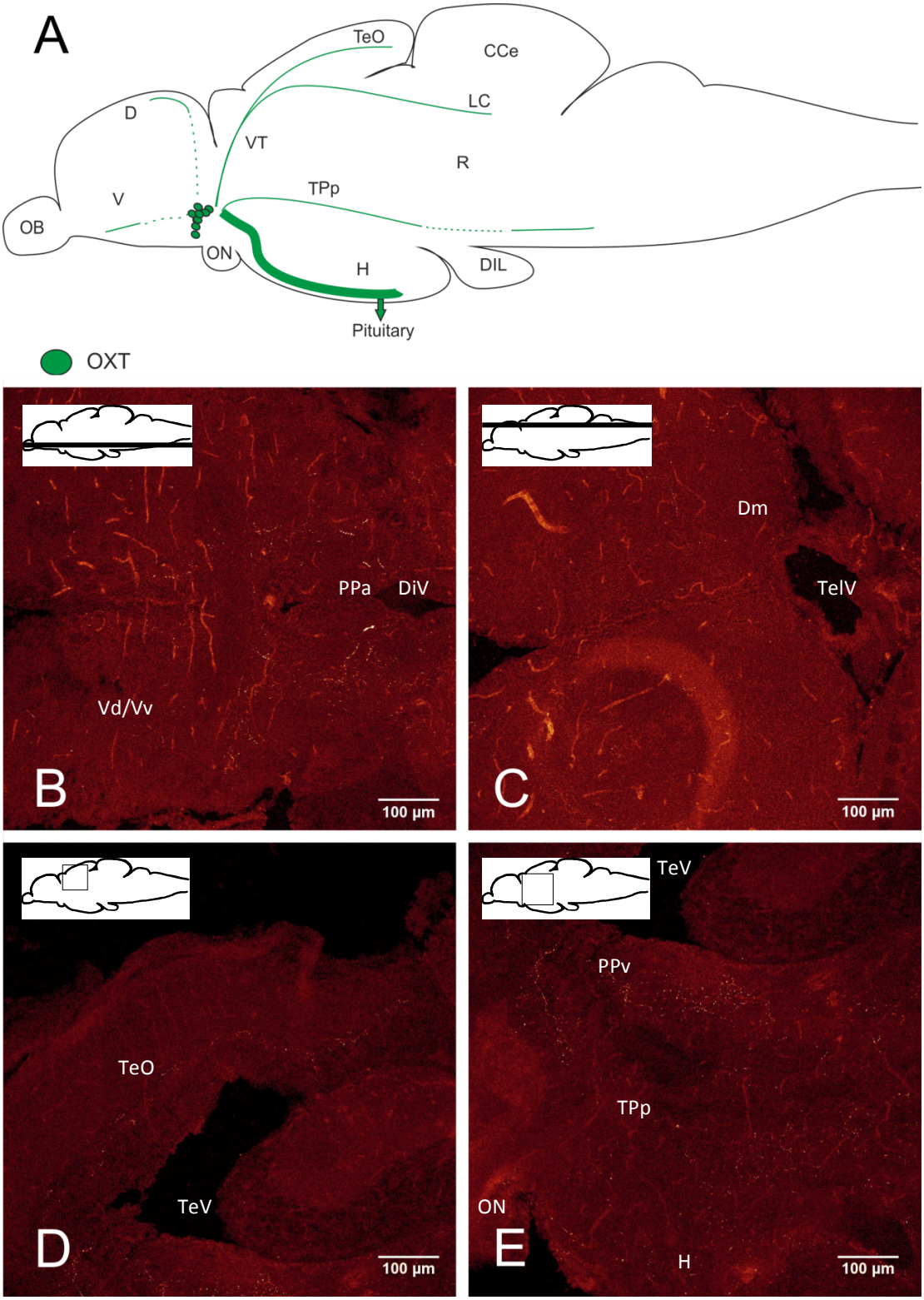

Figure 4. Fiber projections of the zebrafish oxytocin system. Cryosections of adult zebrafish brains were stained with mouse anti-oxytocin antibody and examined with a confocal microscope. The results are represented as maximum projection images. Oxytocin (oxt) fiber projections of the zebrafish central nervous system are summarized

in figure A (anterior to the left, dorsal up). Some of the fibers could not be traced back to the preoptic area and the dashed lines represent possible projection patterns of these fibers. Figure B shows the projections in the more ventral telencephalic area and figure C in the more dorsal telencephalic area. Fiber projections in the optic tectum (TeO) are shown in figure D, which is an image of a sagittal section near the midline. Fiber projections in the diencephalon are shown in figure E, which is an image of a sagittal section near the midline. Abbreviations: CCe=corpus cerebelli, D=dorsal telencephalic area, DIL=diffuse nucleus of the inferior lobe, DiV=diencephalic ventricle, Dm= medial zone of the dorsal telencephalic area, H=hypothalamus, LC=locus coeruleus, OB=olfactory bulb, ON=optic nerve, PPa= anterior parvocellular preoptic nucleus, PPv=ventral part of the periventricular pretectal nucleus, R=raphe nuclei, TelV=telencephalic ventricles, TeO=optic tectum, TeV=tectal ventricle, TPp= periventricular nucleus of posterior tuberculum, V=ventral telencephalic area, Vd/Vv= dorsal and ventral nucleus of the ventral telencephalic area, VT=ventral thalamus.

7.3 Development of the oxytocin system

No oxytocin was detected in 1–3 dpf larval whole mounts but oxytocin could be detected in 3 dpf sagittal cryosections (Figure 5). In the 3 dpf cryosections both the oxytocin producing cells and the projections to the pituitary were visible. Using zebrafish brain whole mounts oxytocin could be first detected at 5–6 dpf. At this stage the staining was strongest in the pituitary, although weak staining could also be seen in the preoptic area (Figure 5). The cells in the preoptic area became more visible after 7 dpf and the number of oxytocin immunoreactive neurons could be calculated more reliably from larvae older than 6 dpf. At 10–14 dpf the projections from the preoptic area to the pituitary became visible. No other projections were detected.

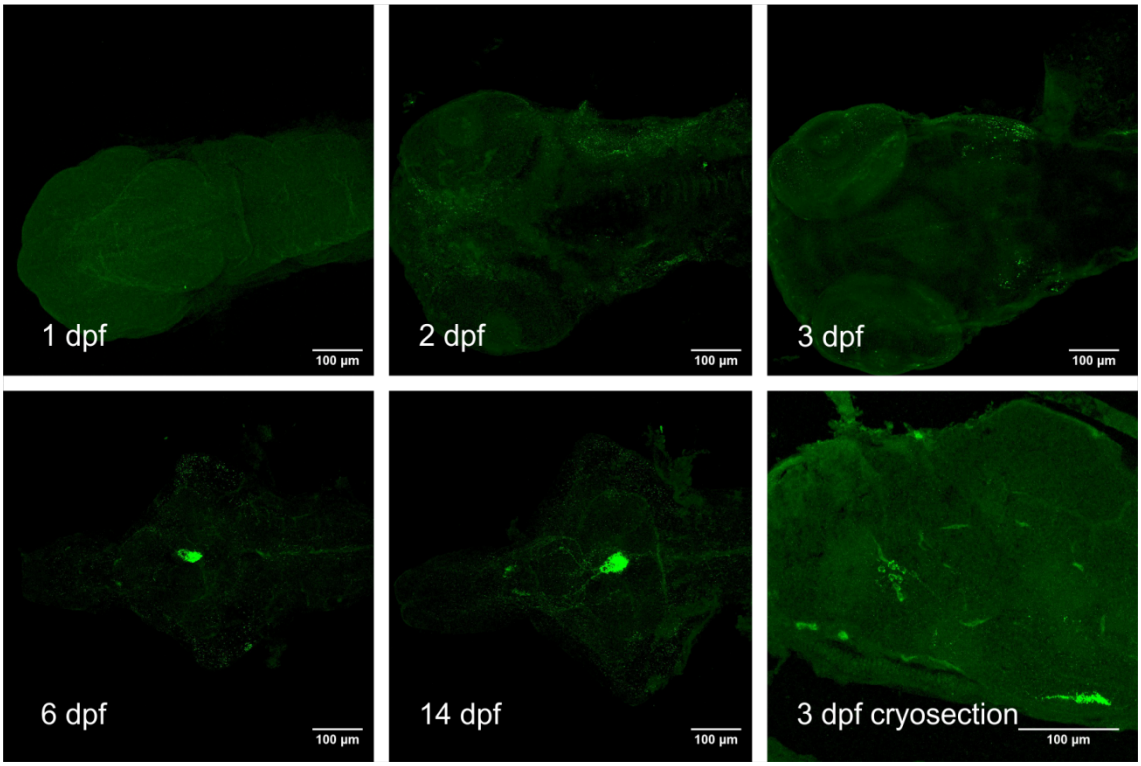

Figure 5. The development of the zebrafish oxytocin system. The development of the oxytocin system was studied by staining larval whole mounts (1–3 dpf), 3 dpf sagittal cryosections and brain whole mounts (over 4 dpf) with mouse anti-oxytocin antibody. In the whole mounts oxytocin staining was detected at 5–6 dpf while no specific oxytocin staining occurred during 1–3 dpf. At 6–14 dpf oxytocin staining was most prominent in the pituitary but oxytocin immunoreactive neurons in the preoptic area and projections to the pituitary could also be detected. Oxytocin immunoreactive neurons and oxytocin staining in the pituitary were detected in the 3 dpf sagittal cryosection. In the figures 1–14 dpf anterior is to the left and in the figure of the 3 dpf cryosection dorsal is up and anterior to the left.

7.4 Colocalization

No colocalization of anti-oxytocin and anti-TH immunoreactivities was detected in the zebrafish brain (Figure 6). The TH positive cells were situated ventrally compared to the oxytocin immunoreactive cells. There was also no colocalization in the fiber projections although the fibers were situated close to each other and projected through the same tract.

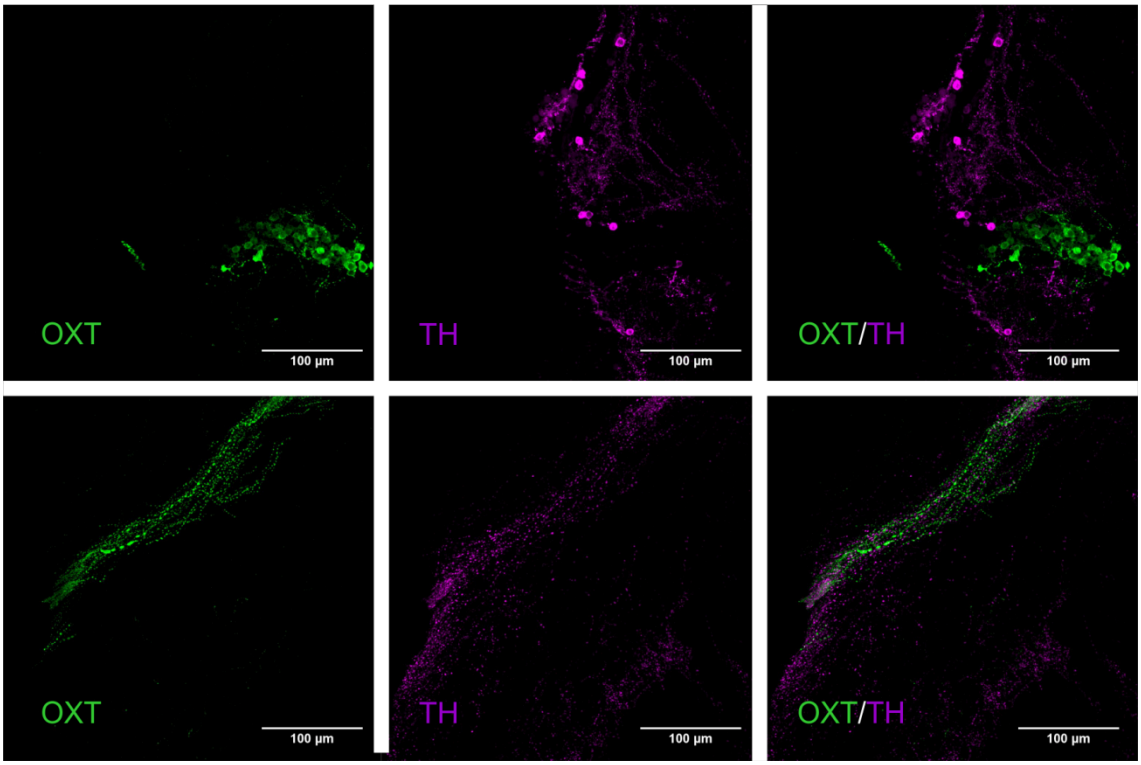

Figure 6. Oxytocin (OXT) and tyrosine hydroxylase (TH) were not colocalized in zebrafish brain. Sagittal cryosections of adult zebrafish brains were stained with mouse anti-TH antibody and rabbit anti-oxytocin antibodies and imaged with a confocal microscope to determine whether oxytocin colocalizes with TH. In the upper row are images from the cells in the preoptic area and in the lower row fibers in the hypothalamus. The left-most column represents the maximum projection images of oxytocin staining and the middle column the maximum projection images of TH staining. The right-most column represents the overlay images of the oxytocin and TH staining. There was no colocalization between oxytocin and TH staining either in the cells or the projections. In the figures anterior is up and ventral to the left.

Anti-oxytocin and anti-orexin-A-like immunoreactivities were colocalized in the zebrafish brain (Figure 7). Colocalization was evident in both the preoptic cells and the hypothalamic projections. The cells and projections recognized with oxytocin antibody were also recognized with the anti-orexin-A antibody. There were also distinct cells and projections that were recognized only with the anti-orexin-A antibody. These were in locations which are known to express orexin mRNA (Kaslin et al. 2004).

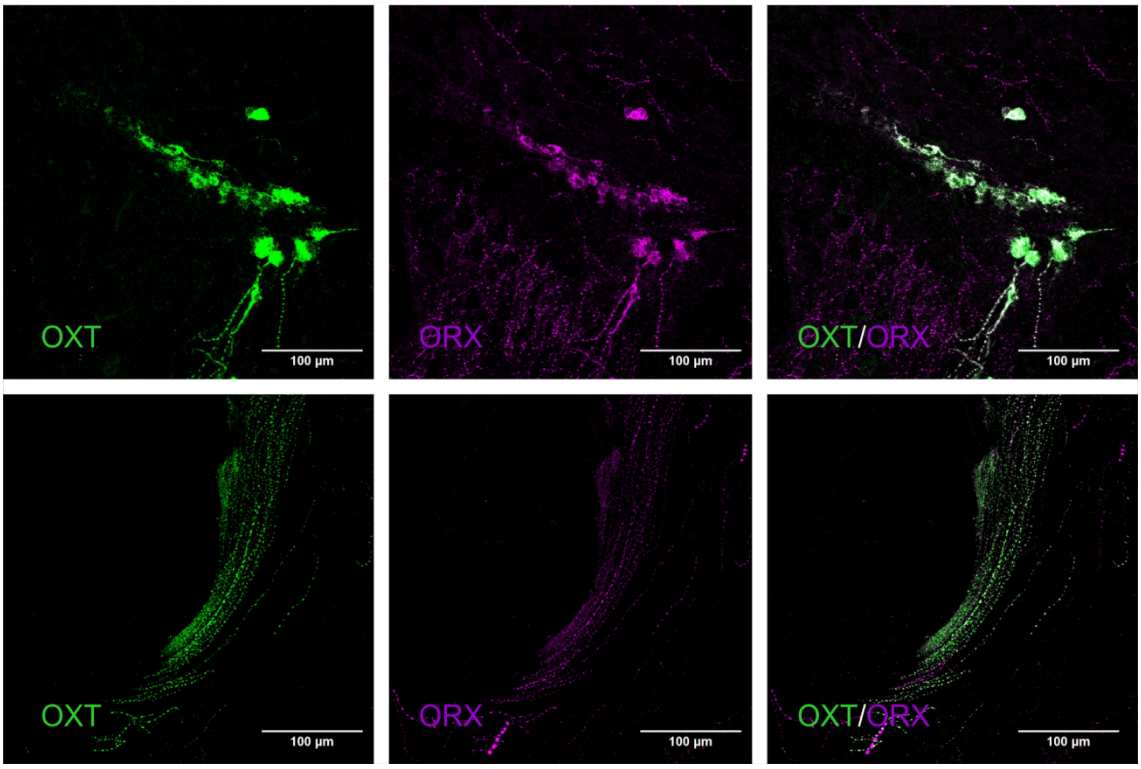

Figure 7. Oxytocin (OXT) colocalized with orexin-A (ORX) in zebrafish brain. Sagittal cryosections of adult zebrafish brains were stained with mouse anti-oxytocin antibody and rabbit anti-orexin-A antibody to determine whether oxytocin colocalizes with TH. In the upper row are confocal images from the cells in the preoptic area and in the lower row from fibers in the hypothalamus. The left-most column represents the maximum projection images of oxytocin staining and the middle column the maximum projection images of orexin-A staining. The right-most column represents the overlay images of both of the oxytocin and orexin-A staining. In the figures anterior is up and ventral to the left.

7.5 Morpholino oligonucleotides

OxtMO1 was lethal at a dose of 8 ng and doses between 0,75–5 ng led to abnormal development. At a dose of 0,6 ng the larvae developed normally but there was no effect on the production of oxytocin: The number of oxytocin producing cells did not significantly differ between the oxtMO1-injected larvae and the control groups (one-way ANOVA $F_{(2,10)}$=1,255, P>0,05). There was also no difference in the intensity of the oxytocin band in the RT-PCR or in the intensity of the oxytocin staining between groups (Figure 8).

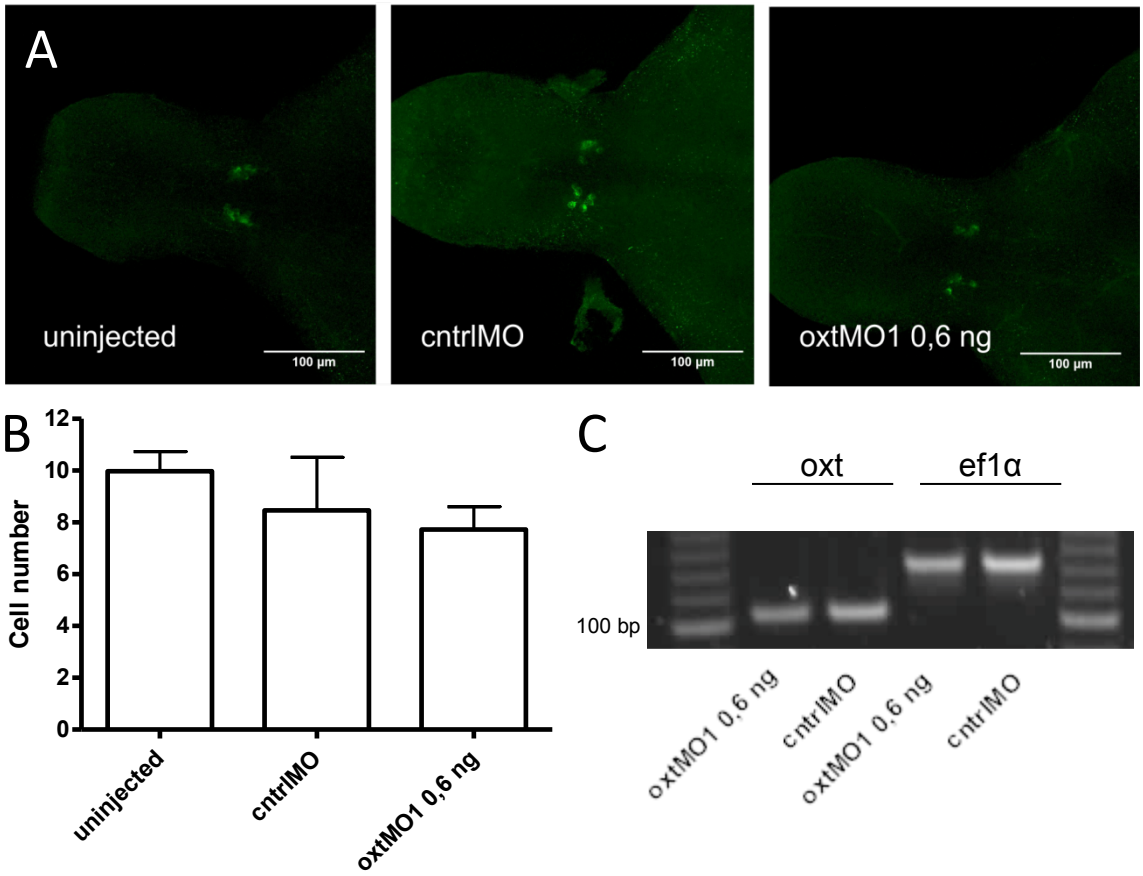

Figure 8. Oxytocin morpholino oligonucleotide 1 (oxtMO1) did not affect the production of oxytocin. To prevent the splicing of oxytocin precursor mRNA zebrafish embryos were microinjected with 0,6 ng of oxtMO1, which was the highest dose that did not cause developmental abnormalities. Uninjected and controlMO (cntrlMO) injected embryos were used as controls. Figure A: 7 dpf brain whole mounts were stained with mouse anti-oxytocin antibody and imaged with a confocal microscope. The figures represent the average staining pattern of all of the samples in one group. There was no difference in the intensity of the staining between groups. Figure B: The number of oxytocin immunoreactive cells was counted from the 7 dpf brain whole mounts. The number of samples per group was five in the uninjected group, three in the cntrlMO group and five in the oxtMO1 group. The cell numbers are presented as means ± SEM. The number of oxytocin immunoreactive neurons did not significantly differ between groups (one-way ANOVA $F_{(2,10)}$=1,255, P>0,05). Figure C: The amount of oxytocin mRNA was analyzed from 2 dpf larvae with RT-PCR. There was a minor difference in the oxytocin band intensity of the oxtMO1 group compared to cntrlMO group. However there was also a similar difference between ef1α bands which act as internal controls.

OxtMO2 reduced the production of oxytocin at doses of 2,5 ng and 4 ng. The number of oxytocin immunoreactive neurons was significantly lower in the oxtMO2-injected groups compared to the control groups (one-way ANOVA $F_{(3,47)}$=53,7, P<0.001). Also the intensity of the oxytocin band in RT-PCR and the oxytocin staining in the 7 dpf

brain whole mounts was reduced in the oxtMO2 injected groups (Figure 9). At a dose of 2,5 ng the morphology of the larvae was normal but increasing the dose to 4 ng led to developmental abnormalities such as pericardial edema and brain malformation (Figure 9). Control groups developed normally.

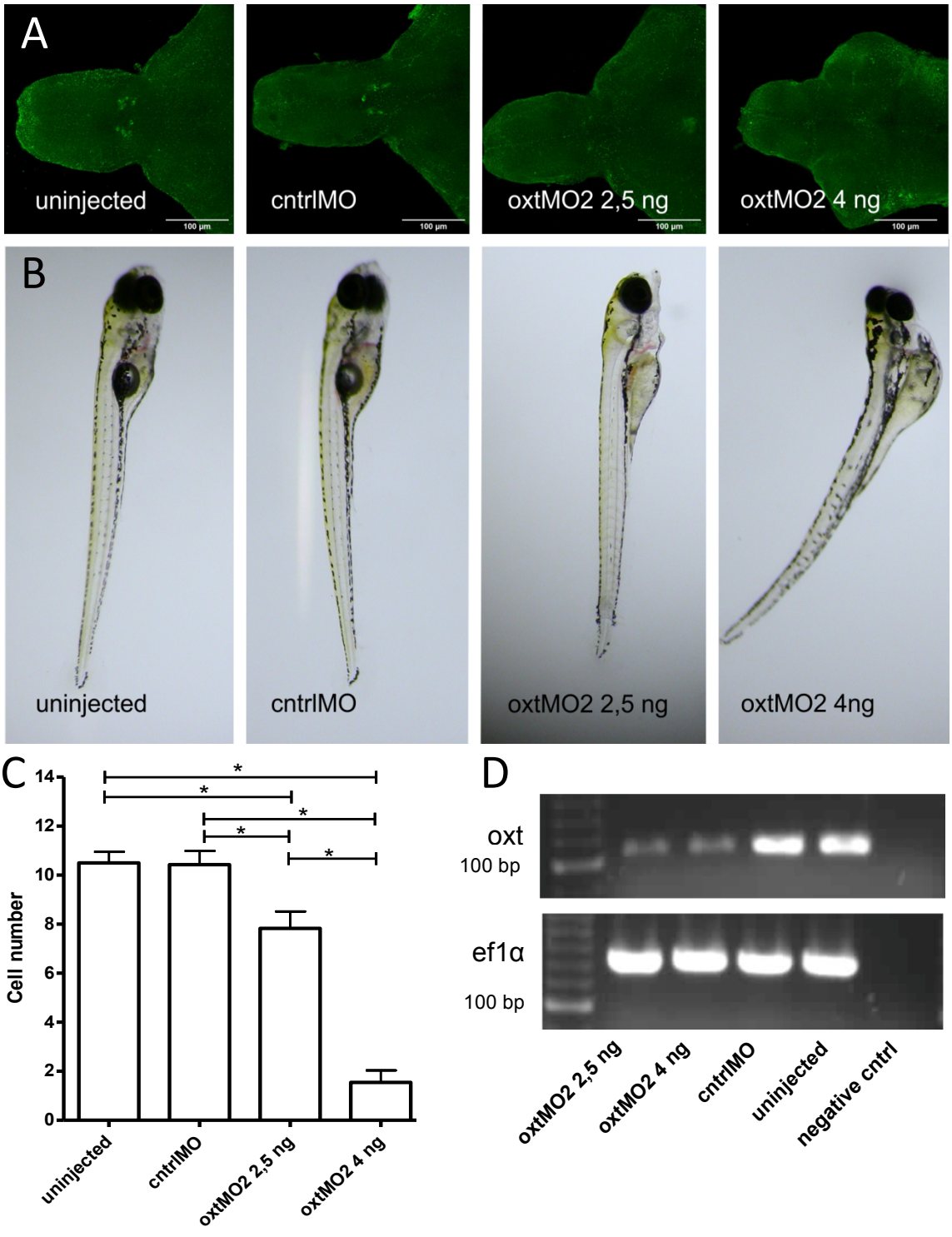

Figure 9. Oxytocin morpholino oligonucleotide 2 (oxtMO2) reduced the production of oxytocin. Zebrafish embryos were microinjected with 2,5 ng and 4 ng of oxtMO2 in order to prevent the splicing of oxytocin precursor mRNA. Uninjected and controlMO (cntrlMO) injected embryos were used as controls. Figure A: 7 dpf brain whole mounts were stained with mouse anti-oxytocin antibody and scanned with a confocal microscope. The figures represent the average staining patterns of all of the samples in one group. The intensity of the staining was lower in both oxtMO2-injected groups compared to controls. Figure B: The morphology of 5 dpf larvae was normal in all groups except the 4 ng oxtMO2 group. Figure C: The number of oxytocin immunoreactive cells was counted from 7 dpf brain whole mounts. The cell number is presented as means ± SEM. The number of samples per group was 14 in both the uninjected and cntrlMO group, 12 in the 2,5 ng of oxtMO2 group and 11 in the 4 ng of oxtMO2 group. The number of oxytocin producing cells differed significantly between groups (one-way ANOVA $F_{(3,47)}$=53,7, P<0.001). The cell number was significantly different between uninjected and 2,5 ng oxtMO2 (Tukey HSD P<0,05), between uninjected and 4 ng oxtMO2 (Tukey HSD P<0,001), between cntrlMO and 2,5 ng oxtMO2 (Tukey HSD P<0,05), between cntrlMO and 4 ng oxtMO2 (Tukey HSD P<0,001) and between 2,5 ng oxtMO2 and 4 ng oxtMO2 (Tukey HSD P<0,001) groups. Figure C: The amount of oxytocin RNA was analyzed from 2 dpf larvae with RT-PCR. The amount of oxytocin RNA was lower in the oxtMO2-injected groups compared to controls. *) P<0,05.

7.6 Locomotor activity

The locomotor activity of oxtMO2-injected groups was reduced (Figure 10). There was a significant difference in the total distance moved between groups (one-way ANOVA $F_{(3,84)}$=6,719, P<0,001). With a dose of 4,0 ng the total distance moved was significantly lower compared to the uninjected group (Tukey HSD p<0,01) and the cntrlMO group (Tukey HSD P<0,05). With a dose of 2,5 ng the total distance moved was significantly lower compared to the uninjected group (Tukey HSD P<0,01) but not compared to the cntrlMO group. There was no difference in the mean turn angle, mean meander or mean angular velocity among the groups.

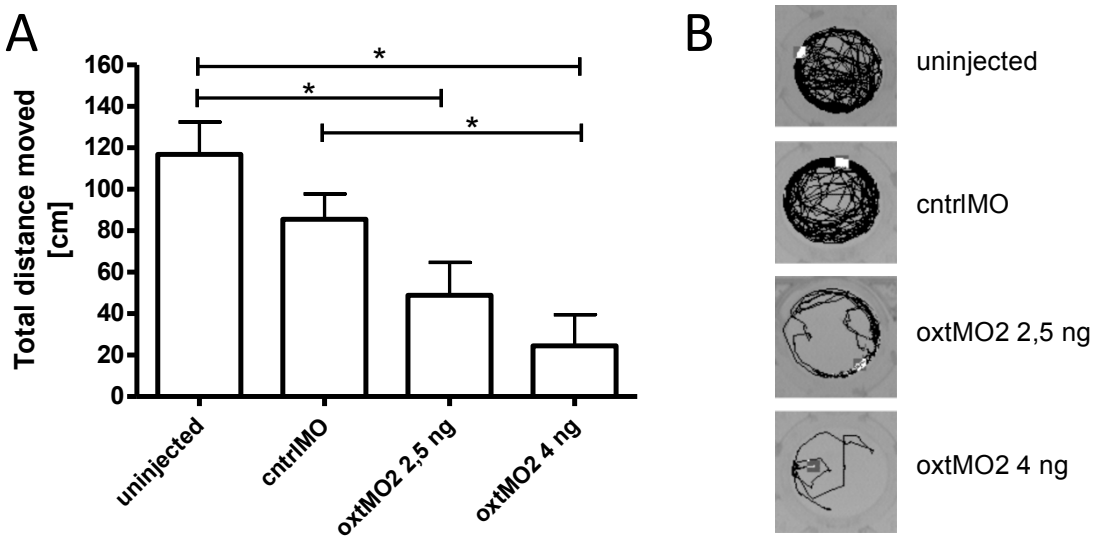

Figure 10. The locomotor activity of oxytocin morpholino oligonucleotide 2 (oxtMO2) injected larvae was reduced compared to controls. Zebrafish embryos were injected with 2,5 ng or 4 ng of oxtMO2 to prevent the splicing of oxytocin precursor mRNA. Uninjected and controlMO (cntrlMO) injected larvae were used as controls. The locomotor activity of 36 larvae per group was tracked for 15 minutes in a 48-well plate at 5 dpf. After excluding the data according to exclusion criteria set beforehand, the number of larvae per group was 20 in 2,5 ng of oxtMO2 group, 15 in 4 ng of oxtMO2 group, 29 in the cntrlMO group and 24 in the uninjected group. Figure A represents the total distance moved during the experiment. The values are presented as means ± SEM. The total distance moved differed significantly between groups (one-way ANOVA $F_{(3,84)}$=6,719, P<0,001). The total distance moved was significantly lower in the 4 ng of oxtMO2 group compared to both the uninjected group (Tukey HSD p<0,01) and the cntrlMO group (Tukey HSD P<0,05). The total distance moved was significantly lower in the 2,5 ng oxtMO2 compared to the uninjected group (Tukey HSD P<0,01) but not compared to the cntrlMO group (Tukey HSD P>0,05). Figure B represents the typical swimming pattern of each group. *) P<0,05

8 DISCUSSION

8.1 The structure of the zebrafish oxytocin system

The results of this study showed that in zebrafish oxytocin neurons are situated in two cell clusters on both sides of the diencephalic ventricle and they are found in both magnocellular and parvocellular preoptic nuclei in the preoptic area. This arrangement of oxytocin neurons is similar to the oxytocin neuron arrangement of other teleost

species (Holmqvist and Ekström 1995; Goodson et al. 2003; Saito et al. 2004). The detected neuroanatomy of oxytocin neurons is also in accordance with previous knowledge about the zebrafish oxytocin system: Unger and Glasgow (2003) showed with *in situ* hybridization that in zebrafish larvae aged 36–120 hpf oxytocin mRNA is expressed in bilateral cell clusters in the anterior hypothalamus, which gives rise to the adult preoptic nucleus.

The zebrafish brain had extensive oxytocin immunoreactive fiber projections. Besides the very prominent projections to the pituitary, extra-hypothalamic thinner oxytocin fibers were also detected. The structure of the zebrafish oxytocin fiber system resembles that of other teleost species. Based on previous studies, there are also differences in the oxytocin fiber structures between different teleosts. Projections to the pituitary, the thalamus and the pretectum seem to be a common feature for teleosts and they are reported, for example, for Atlantic salmon, plainfin midshipman and rainbow trout (Holmqvist and Ekström 1995; Goodson et al. 2003; Saito et al. 2004). These projections were also found in zebrafish.

In the forebrain of the rainbow trout and the plainfin midshipman, the oxytocin fibers run in both dorsal and ventral parts of the telencephalon, as was also the case in the zebrafish (Goodson et al. 2003; Saito et al. 2004). In the rainbow trout and the plainfin midshipman the oxytocin fibers terminate at the olfactory bulb but no oxytocin fibers were detected at the olfactory bulb in zebrafish. No oxytocin forebrain projections were reported for the Atlantic salmon (Holmqvist and Ekström 1995).

In the zebrafish, the plainfin midshipman and the rainbow trout oxytocin fibers are found in optic tectum and posterior tuberculum, but these projections were not reported for the Atlantic salmon (Holmqvist and Ekström 1995; Goodson et al. 2003; Saito et al. 2004). The rainbow trout have oxytocin fibers also in the raphe nuclei, the cerebellum and the spinal cord but these fiber projections were not detected in zebrafish nor have they been reported in the Atlantic salmon or in the plainfin midshipman.

The zebrafish oxytocin fiber structure is very similar to the fiber structure reported for the rainbow trout and plainfin midshipman (Goodson et al. 2003; Saito et al. 2004). There are, however, differences so it is possible that not all zebrafish oxytocin fibers were detected in this study. Even though a similar oxytocin projection pattern was found in all samples studied, the method might not have been sensitive enough for the detection of all oxytocin fibers: the signal of the thin extra-hypothalamic fibers was very weak so some fibers might have been undetected. This is further supported by the fact that fibers were detected going towards the areas innervated by oxytocin producing neurons in other teleosts although they were not detected directly in these areas in zebrafish.

Changes in the staining protocol, like longer incubation time with the primary and secondary antibodies, might have improved the sensitivity and revealed more fibers. The sensitivity might also be improved by using tyramide signal amplification (TSA), which has been used previously for signal amplification in zebrafish (Filippi et al. 2007). This study was conducted with sagittal and horizontal cryosections so studying coronal sections might have revealed more fibers.

Immunohistochemistry might not be the best method for studying the oxytocin system in teleosts. Duarte and others (2001) studied the oxytocin system of a teleost white seabream (*Diplodus sargus*) with a rabbit anti-teleost oxytocin antibody. No extra-hypothalamic projections were observed. Because extra-hypothalamic projections are reported for other teleost species, the authors suggest other methods to confirm the result. It would be interesting to know whether the result is correct because detecting the extra-hypothalamic oxytocin immunoreactive fibers proved to be demanding in this study.

Other methods than immunohistochemistry have been used previously to study the zebrafish oxytocin system. Coffey and others (2013) used a transgenic zebrafish line that expresses GFP from the oxytocin promoter. They recorded GFP expressing oxytocin cell bodies in the neuroendocrine preoptic area. An interesting result was that alcohol exposure induced oxytocin gene expression in a small group of hindbrain

neurons. The authors reported oxytocin fibers to the pituitary, the midbrain, the hindbrain and the spinal cord but they did not describe these projections in more detail. Contrary to the results of this study, Coffey an others (2013) were able to detect projections in the spinal cord with the transgenic zebrafish line. Therefore it is likely that zebrafish have oxytocin projections also in the spinal cord, as do other teleosts (Saito et al. 2004), although spinal projections were not detected in this study. There is no mention about oxytocin projections to the forebrain in the transgenic line (Coffey et al. 2013). Forebrain projections are, however, reported for other teleost species (Goodson et al. 2003; Saito et al. 2004) and they were also detected in this study. Different methods seem to give different results so combining different methods would be the best way to confirm the results. In addition to immunohistochemistry and transgenic approach one could also try for example anterograde tracing of the axons.

8.1.1 Antibodies made against mammalian oxytocin can be used to study zebrafish oxytocin system

Antibodies against the mammalian oxytocin were able to recognize zebrafish oxytocin but there were differences in the specificity of the antibodies. The mouse anti-oxytocin antibody was specific to oxytocin over vasopressin but the rabbit anti-oxytocin antibody recognised both peptides. Cryosections proved to be better than whole mounts for studying larval zebrafish with the mouse anti-oxytocin antibody. According to a previous study by Unger and Glasgow (2003) oxytocin mRNA is first detected at 36 hpf with *in situ* hybridization, so oxytocin might also be detected with immunohistochemistry around this age. In this study oxytocin immunoreactivity was not detected until 5–6 dpf using the whole mounts. With larval cryosections oxytocin was detected at 3 dpf. This was the only age of which cryosections were made so oxytocin could possibly be detected even earlier by staining cryosections.

8.1.2 Colocalization studies

TH is an enzyme that catalyses the rate-limiting step in the synthesis of catecholamines and it is often used as a marker of dopaminergic, noradrenergic and adrenergic cells

(Levitt et al. 1965; Molinoff and Axelrod 1971; Pickel et al. 1975). In humans almost 40% of the neurons in the paraventricular and supraoptic nucleus are immunoreactive for TH (Li et al. 1988). A subclass of these TH immunoreactive neurons express also oxytocin but most TH cells are distinct from the oxytocin or vasopressin neurosecretory cells. No colocalization between oxytocin and TH immunoreactivity was detected in zebrafish. In teleosts two genes coding for TH have been described: *th1 and th2* (Candy and Collet 2005). The anti-TH antibody used in this study detects only TH1 protein in zebrafish (Chen et al. 2009), so this study only shows that no colocalization exists between TH1 and oxytocin in zebrafish. The colocalization between TH2 and oxytocin should be studied to further evaluate the colocalization of TH and oxytocin in zebrafish.

Orexin-A is a neuropeptide, which is linked to feeding and sleep-wake states (Sakurai et al. 1998; Chemelli et al. 1999). In mammals it is produced in hypothalamic neurons. In a study by Kaslin and others (2004) orexin-A-immunoreactive neurons were detected in the preoptic area and in the anterior hypothalamus in zebrafish. However, with orexin-A *in situ* hybridization they were able to detect only the hypothalamic neurons. Thus, according to these results the neurons in the preoptic area were immunoreactive for orexin-A but not expressing orexin-A mRNA. The authors considered that the orexin-A-antibody might have recognized another epitope in the zebrafish orexin-like peptide or the antibody might have detected another structurally similar, unidentified peptide.

The mouse anti-oxytocin antibody was colocalized with the rabbit orexin-A antibody in most of the oxytocin immunoreactive neurons and fibers. However, it is not certain whether this detected colocalization is true colocalization or not. Kaslin and others (2004) used the same orexin-A antibody as was used in this study. The detected colocalization of oxytocin and orexin-A immunoreactivity suggests that the orexin-A antibody might recognize oxytocin or another peptide in these cells in addition to orexin-A. This would explain the colocalization found in this study and also why the orexin-A preoptic neurons identified in the study by Kaslin and others (2004) were only identified with immunohistochemistry. Furthermore, the orexin-A immunoreactive preoptic neurons correspond anatomically to the oxytocin immunoreactive neurons in the preoptic nucleus. Since oxytocin immunoreactive objects were also identified by

orexin-A antibody and there were more orexin-A immunoreactive objects than oxytocin immunoreactive objects, the results of this study indicate that the oxytocin antibody does not recognize orexin, but the orexin-A antibody recognises both orexin and oxytocin or another protein domain in the same cells. It is also possible that the orexin-A antibody recognises the neurophysin in the precursor protein of oxytocin instead of oxytocin itself. Absorption controls, in which the orexin-A antibody was incubated with oxytocin or its neurophysin, would be useful to study whether the orexin-A antibody binds to either of these peptides.

8.1.3 The structure of the zebrafish oxytocin system resembles that of mammals

In mammals oxytocin neurons are located in the supraoptic and paraventricular nuclei of the hypothalamus (Swaab et al. 1975; Buijs et al. 1978; Sawchenko and Swanson 1982). The zebrafish oxytocin neurons were found to be located in the preoptic nucleus. The preoptic nucleus of fish is considered to correspond to the supraoptic and paraventricular nuclei of mammals based on for example electrophysiological studies (Hayward 1974; Peter 1977). The homology of these nuclei is further supported by the fact that their development is regulated by the same regulatory genes, such as *Sim1 and Otp*, in both mammals and zebrafish (Eaton and Glasgow 2007; Eaton et al. 2008). Thus, oxytocin is produced in corresponding nuclei between mammals and zebrafish. Oxytocin is also produced in magnocellular and parvocellular neurons in both species.

Both mammals and zebrafish have oxytocin fibers projecting to the pituitary but they both also have extra-hypothalamic oxytocin fibers (Table 4). Some brain areas, such as the amygdala superior colliculus, thalamus, locus coeruleus and brainstem, are innervated in both zebrafish and mammals (Buijs 1978; Sofroniew 1980; Portavella and Vargas 2005; Griffin and Flanagan-Cato 2011; Knobloch et al. 2012). In mammals oxytocin fibers are found in the lateral septum, which is thought to correspond the ventral and lateral nuclei of the ventral telencephalic area (Vv/Vl) in zebrafish (Sofroniew 1980; Wullimann and Mueller 2004). In zebrafish oxytocin fibers were found in the Vv.

Oxytocin fibers have also been reported in the mammalian substantia nigra (Sofroniew 1980). The exact zebrafish homolog of the substantia nigra is currently unknown, but dopaminergic cells in the periventricular nucleus of posterior tuberculum (TPp) have been suggested to be homologous to the substantia nigra (Kaslin and Panula 2001). TPp was innervated by oxytocin in zebrafish so if this area proves to be susbtantia nigra homolog, it is innervated in both mammals and zebrafish.

There are also differences between the oxytocin fiber projections reported in mammals and those detected in zebrafish (Table 4). In mammals oxytocin fibers are found in the olfactory bulb, hippocampus, raphe nuclei, and spinal cord but these fibers were not detected in zebrafish. However, oxytocin fibers in the spinal cord have been reported previously in zebrafish and oxytocin fibers in the olfactory bulb, raphe nuclei and lateral zone of the dorsal telencephalic area (Dl, a hippocampus homolog) have been reported in the rainbow trout (Saito et al. 2004; Portavella and Vargas 2005; Coffey et al. 2013).

Oxytocin fibers in the bed nucleus of stria terminalis (BNST) have been reported in mammals (Knobloch et al. 2012). The ventral part of the postcommissural and supracommissural nuclei of the ventral telencephalic area (Vp and Vs) are thought to correspond to the BNST in zebrafish (Maximino et al. 2013). No oxytocin fibers were detected in this area in zebrafish but in other teleosts they have been described (Goodson et al. 2003; Saito et al. 2004). It is possible that not all zebrafish oxytocin fibers were detected in this study, which might explain some of the differences found between zebrafish and mammals and between zebrafish and other teleosts. In zebrafish oxytocin fibers were found in the pretectum and Vd, which is a partial striatum homolog, but these fibers have not been reported in mammals.

Table 4. The brain areas innervated by oxytocin fibers in zebrafish according to the results of this study and in mammals (Buijs 1978; Sofroniew 1980; Wullimann and Mueller 2004; Portavella and Vargas 2005; Griffin and Flanagan-Cato 2011; Knobloch et al. 2012; Maximino et al. 2013). Abbreviations: Dm/Dl=medial and lateral zone of dorsal telencephalic area, VMH=ventromedial hypothalamus, TeO=optic tectum, Vv/Vl/Vd/Vc/Vs/Vp=ventral, lateral, dorsal, central, supracommissural and postcommissural nuclei of ventral telencephalic area, TPp=periventricular nucleus of posterior tuberculum, BNST=bed nucleus of stria terminalis. (*) reported in rainbow

trout (Saito et al. 2004) (**) reported in a previous study in zebrafish (Coffey et al. 2013)

Brain area (mammals/zebrafish homolog)	Zebrafish	Mammals
Olfactory bulb	– (*)	+
Amygdala/Dm	+	+
Hippocampus/DI	– (*)	+
Pituitary	+	+
Thalamus	+	+
Pretectum	+	–
Superior colliculus/TeO	+	+
Locus coeruleus	+	+
Raphe nuclei	– (*)	+
Septal formation/ Vv and Vl	+ (Vv)	+
Substantia nigra/TPp	+	+
Striatum/Vd and Vc	+ (Vd)	–
BNST/ventral Vs and Vp	– (*)	+
Brainstem	+	+
Spinal cord	– (**)	+

The oxytocin fiber system of zebrafish resembles the oxytocin fiber system reported for mammals. The results of this study combined to previous knowledge about the structure of the oxytocin system in different species indicate that the fiber projections of the oxytocin system are evolutionary conserved.

8.2 The role of oxytocin in zebrafish behaviour

Oxytocin affects behaviour in both mammals and teleosts. In mammals oxytocin for example reduces anxiety and stress and modulates social memory and pair bonding (Cho et al. 1999; Neumann et al. 2000; Ring et al. 2006; Savaskan et al. 2008). In teleosts oxytocin affects reproduction and social behaviour such as social approach response and reproduction-related vocalization (Goodson and Bass 2000; Thompson and Walton 2004). Oxytocin has been shown to modulate social and anxiety related behaviour also in zebrafish (Braida et al. 2012). In addition, oxytocin controls ion regulation by regulating ionocyte progenitor differentiation and proliferation in zebrafish and it is required for the formation of the neurovascular interface of the pituitary (Chou et al. 2011; Gutnick et al. 2011). However, the zebrafish oxytocin receptors have not been cloned and characterized.

In mammals the effect of oxytocin has been studied with oxytocin or oxytocin receptor knockout mice (Ferguson et al. 2000; Lee et al. 2008). Pharmacological studies with oxytocin and its agonists and antagonists have also been conducted (Ring et al. 2006; Savaskan et al. 2008). The pharmacological approach has also been used to study the role of oxytocin in zebrafish behaviour (Braida et al. 2012).

In this study a new approach was used and the effect of oxytocin on zebrafish behaviour was studied by transiently inhibiting the splicing of oxytocin with MOs. However, knocking down of oxytocin with MOs proved to be demanding. Of the two oxytocin MOs tested, only oxtMO2 was able to reduce the amount of oxytocin with a dose that did not cause developmental abnormalities. The oxtMO2 was able to significantly reduce the amount of oxytocin at doses of 2,5 ng and 4,0 ng. The reduction of oxytocin was greater in the higher dose group but it also led to severe morphological abnormalities. However, oxytocin knockout mice, which lack oxytocin entirely, develop normally (Nishimori et al. 1996). This indicates that at least in mammals the lack of oxytocin does not cause abnormal morphology. Therefore the morphological abnormalities observed were likely due to off-target effects of the MO. On the other hand it is possible that in zebrafish oxytocin is necessary for normal development for example through its effect on ion regulation.

8.2.1 Reduced locomotor activity with oxytocin morpholino oligonucleotide 2

With a dose of 4,0 ng the oxtMO2 significantly reduced the total distance moved compared to control groups. However the morphology of the 4,0 ng oxtMO2-injected fish was abnormal and this probably affected the swimming ability of the fish. Thus, the reduced swimming distance does not necessarily reflect reduction in the locomotor activity per se. In the 2,5 ng oxtMO2 group the total distance moved was reduced compared to the uninjected group but not compared to the cntrlMO group. The total distance moved did not significantly differ between the uninjected and cntrlMO groups but the distance moved was lower in the cntrlMO group. This leads to greater, and thus significant, difference between the oxtMO2 and uninjected group than the difference between the oxtMO2 and cntrlMO groups. The MO-injection might have caused some

small abnormality in the cntrlMO-larvae that slightly affected their swimming performance compared to the uninjected ones. Because the locomotor activity was also reduced in the cntrlMO group, the test should be repeated with a different batch of larvae to determine whether the total distance moved really is reduced in the 2,5 ng oxtMO2 group.

Although the morphology of the 2,5 ng oxtMO2 group seemed normal, it is possible that the detected reduction of locomotor activity was caused by some off-target effect rather than a specific reduction of oxytocin. Although the oxtMO2 successfully inhibited oxytocin formation, it may have additional unrelated effects. Thus, the specificity of the oxtMO2 should be studied. It could be studied by a rescue experiment in which oxytocin RNA that was modified to be resistant to the oxtMO2 would be injected simultaneously with the oxtMO2. If the effect of the oxtMO2 were specific, the phenotype would be rescued. This method has been used in other studies (Sundvik et al. 2011). Another method for assessing the specificity of MOs would be to study whether another MO directed against oxytocin caused a similar phenotype than oxtMO2. The specificity could also be studied by creating an oxytocin -/- CRISPR-Cas mutant. If the oxtMO2 proves to be specific in knocking down oxytocin, it would indicate that the reduction of oxytocin attenuates locomotor activity in zebrafish.

In mammals oxytocin acts as an anxiolytic: Oxytocin increases the number of punished crossings in the four-plate test and increases the time spent in the open quadrants in the elevated zero maze (Ring et al. 2006). In an open field test oxytocin decreases peripheral activity at low doses and reduces locomotion and rearing at high doses (Uvnäs-Moberg et al. 1994). The locomotor activity test used in this study corresponds to an open field test of mammals. If oxytocin had similar effect in zebrafish as it has in mammals, oxytocin should decrease the locomotor activity in an open field –setting. Thus, a decrease in oxytocin should increase the locomotor activity. However, reduction in the amount of oxytocin by oxtMO2, led to decrease in the total distance moved. So if this was due to specific blocking of oxytocin by oxtMO2, oxytocin would have an opposite effect on anxiety in zebrafish than it has on mammals.

In an open field –test a better indicator of an anxiolytic effect would be the increase in the time spent in the middle of the open field arena since the decrease in the distance moved might be caused by a sedative effect. Like mammals, also zebrafish prefer the edges of the arena to the centre (Colwill and Creton 2011). So if oxytocin would be anxiolytic, the oxtMO2 injected larvae should be spending more time in the edges and less time in the centre of the well. The time spent in different parts of the well should be studied to better evaluate the role of oxytocin in anxiety in zebrafish.

In oxytocin knockout mice total activity or frequency of rearing are not affected in an open field test (DeVries et al. 1997). This might imply that oxytocin acts as an anxiolytic but only after a certain stimulus, like a stressful event. This idea is further supported by the fact that stress increases the production of oxytocin and oxytocin reduces the release of stress hormones (Jezova et al. 1995; Stachowiak et al. 1995). Oxytocin also modulates the effect that restraint stress causes in spontaneous behaviour in an open field –test (Klenerova et al. 2009). Stress can affect fear response and anxiety also in zebrafish (Champagne et al. 2010) and thus it would be interesting to study how the oxtMO2-injected larvae react to a stressful stimulus in the locomotor activity test.

On the other hand, even if oxytocin requires stimuli to exert its anxiolytic effect, this does not explain why decrease in oxytocin reduced locomotor activity. It is possible, that even though the oxtMO2 blocked oxytocin synthesis, the seen effect may be due to some off-target effect. However, Gutnick and others (2011) used an oxytocin MO similar to oxtMO2 in their study. They showed that it was effective in reducing the amount of oxytocin and they did not report any off-target effects. There was, however, no mention about specificity controls for the MO. They also used a transgenic reporter line, oxtl:EGFP, whereas in this study the Turku strain was used. Although only a little is known about specific properties of different zebrafish strains, there are significant differences for example in gene expression and neurochemical levels between strains (Pan et al. 2012). It is therefore possible that differences between the strains could cause differences in the effect of the oxtMO2.

8.2.2 Oxytocin innervation and behaviour

The different aspects of behaviour that oxytocin modulates in mammals can be divided into three main groups: social behaviour, anxiety and memory. These are also the areas in which oxytocin might have therapeutic potential. Based on the similarities in the fiber projection pattern demonstrated between mammals and zebrafish in this study, it is likely that oxytocin modulates similar behaviour also in zebrafish. Only one article about the role of oxytocin in zebrafish behaviour has been published. Braida and others (2012) showed that in zebrafish oxytocin reduces fear to predator response and increases social preference. This indicates that oxytocin increases sociality and reduces anxiety also in zebrafish as it does in mammals.

Many brain areas are involved in the regulation of social behaviour in mammals. The core structure of the social behaviour network is thought to consist of seven limbic system areas: the medial amygdala, BNST, lateral septum, preoptic area, anterior hypothalamus, ventromedial hypothalamus and certain midbrain areas such as the periaqueductal gray (Newman 1999). In rodents oxytocin has been shown to modulate social behaviour, such as approach and avoidance behaviour and social recognition, especially through the medial amygdala (Ferguson et al. 2001; Choleris et al. 2007; Arakawa et al. 2010). Both oxytocin fibers and receptors are found in the medial amygdala of rats and oxytocin has an excitatory effect on these neurons (Tribollet et al. 1992; Terenzi and Ingram 2005; Knobloch et al. 2012). In humans oxytocin also affects social memory and social approach behaviour for example by increasing trust to other people (Kosfeld et al. 2005; Savaskan et al. 2008). However, although oxytocin fibers are detected in the human amygdala, oxytocin receptors are not (Fliers et al. 1986; Loup et al. 1989, 1991). The studies by Loup and others (1989, 1991) are the only studies that have studied the distribution of oxytocin receptors in the human brain. It is possible that high oxytocin concentration in the amygdala has led to downregulation of oxytocin receptors and therefore the oxytocin receptors were not detectable by autoradiography in the amygdala in these studies. However, many mismatches between the distribution of peptide fibers and their receptors have been reported (Herkenham 1987) and therefore the presence of oxytocin fibers in the amygdala does not necessitate the

presence of oxytocin receptors. Thus, it is possible that the social effect of oxytocin is mediated through a different brain area in humans than in rodents.

The social behaviour network in teleosts is very similar to mammals (Goodson 2005). In this study oxytocin fibers were detected in the zebrafish Dm, which is the area corresponding to the amygdala. The pro-social effect of oxytocin in zebrafish demonstrated by Braida and others (2012) could be mediated through these projections. Also fibers in the Vv, area corresponding to the lateral septum, could mediate this effect, as the lateral septum is known to affect social behaviour in mammals (Newman 1999). However, in rodents oxytocin does not affect social behaviour via the septum (Popik and van Ree 1991), so the probable target in zebrafish is also the Dm. If the effect was mediated through the Dm, it would require the existence of oxytocin receptors in this area. At the moment there is no knowledge about the distribution of oxytocin receptors in the zebrafish brain. Determining the location of oxytocin receptors would be crucial for determining the brain areas through which oxytocin exerts its effect in zebrafish.

The anxiolytic and fear reducing effect of oxytocin is mediated, at least partly, through the central amygdala in rodents (Bale et al. 2001; Viviani et al. 2011; Knobloch et al. 2012). The medial part of the central amygdala sends projections to the brainstem that evoke fear responses while activated (Hitchcock and Davis 1991; LeDoux 2000). Oxytocin has been shown to excite especially the neurons in the lateral part of the central amygdala (Huber et al. 2005). These neurons are γ-aminobutyric acidergic (GABAergic) and they have projections to the medial part of the central amygdala. Thus, the activation of the lateral neurons by oxytocin leads to inhibition of the neurons in the medial part and therefore inhibits the formation of the fear response.

In rodents oxytocin fibers are detected in the central amygdala (Knobloch et al. 2012). Similar to the medial amygdala, the oxytocin receptors in the central amygdala have been detected only in rodents, not in humans (Freund-Mercier et al. 1987; Loup et al. 1991). Yet oxytocin has been shown to reduce the activation of the amygdala in humans after looking at fearful faces (Kirsch et al. 2005). This was studied by functional

magnetic resonance imaging and it was not possible to distinguish the effects in different parts of the amygdala. However, it was demonstrated that oxytocin significantly reduced the connectivity between the amygdala and the brainstem, which indicates that the effect of oxytocin might be similar in humans and rodents.

Though there are many assays to test anxiety and fear in zebrafish, very little is known about the brain networks behind these traits. Many anxiolytic drugs, like diazepam and buspirone, have an anxiolytic effect also in zebrafish (Bencan et al. 2009; Egan et al. 2009). This indicates that regulation of anxiety could be similar between mammals and zebrafish, at least on a transmitter level. The anxiolytic effect of oxytocin in zebrafish could be mediated through amygdala also in zebrafish, as oxytocin fibers were detected in the Dm in this study. It is not known whether the Dm affects anxiety in zebrafish but it is likely since Dm affects many similar functions in teleosts that amygdala does in mammals (Portavella and Vargas 2005). Furthermore in another teleost species, Siamese fighting fish, lesion in the Dm leads to increase in the frequency of the startle response (Marino-Neto and Sabbatini 1983). This can be regarded as a sign of increased anxiety and therefore it suggests a link between Dm and anxiety in teleosts.

One brain area that has been proposed to contribute to fear and anxiety behaviour in zebrafish is habenula. The disruption of neural circuits involving the habenula inhibits avoidance learning: during conditioning the fish do not escape a shock but freeze (Lee et al. 2010). The fish also display a startle response more often than controls when subjected to shock. These results suggest that the habenula might be involved in modification of fear and anxiety in zebrafish. However, it is not likely that oxytocin affects anxiety through the habenula, because no oxytocin fibers were detected there. Future studies about the distribution of oxytocin receptors and especially about the involvement of different brain areas in anxiety in zebrafish are needed.

In mammals oxytocin attenuates memory and learning: oxytocin for example shortens step-down latency (Kovács et al. 1978). A probable target of these effects is the hippocampus, which is known to have an important role in memory formation (Zola-Morgan et al. 1986). This is supported by the fact that the hippocampus is innervated by

oxytocin fibers, oxytocin receptors are detected in the hippocampus and oxytocin can excite hippocampal neurons (Mühlethaler and Dreifuss 1983; Insel et al. 1991; Knobloch et al. 2012). Oxytocin has also been shown to enhance long-term synaptic potentiation in the hippocampus, which is an essential mechanism in memory formation and learning (Bliss and Collingridge 1993; Tomizawa et al. 2003). Behavioural studies also indicate that oxytocin affects memory through the hippocampus: oxytocin attenuates passive avoidance behaviour when administered into the hippocampal dentate gyrus (Kovács et al. 1979). Interestingly, oxytocin also attenuates passive avoidance when injected into raphe nucleus. Serotonin affects the electrical activity of hippocampal cells (Colino and Halliwell 1987), so oxytocin might modulate memory also through affecting the serotonin release from the raphe nucleus. This is possible because oxytocin receptors are detected in the raphe nucleus (Yoshimura et al. 1996). Other brain areas may also contribute to the memory attenuating effect of oxytocin. For example, oxytocin inhibits spatial learning in the cholinergic nucleus basalis of Meynert where also oxytocin receptors are expressed (Loup et al. 1991; Wu and Yu 2004).

In teleosts the Dl is considered to be a hippocampus homolog. Lesions in this area attenuate spatial learning and conditioned avoidance response (Portavella et al. 2002), which suggests that the Dl has a role in memory processes. It is not known whether oxytocin affects memory and learning in zebrafish or in other teleosts. In zebrafish oxytocin fibers were not detected in the Dl or in the raphe nucleus. If future studies show that oxytocin affects memory also in zebrafish, the results of this study suggest that the effect is not mediated through hippocampus, at least not directly.

8.3 The applicability of zebrafish for oxytocin research

Based on the similarities between mammalian and zebrafish oxytocin systems zebrafish seems to be a suitable model organism for oxytocin research. The structure of the zebrafish oxytocin system resembles that of mammals in terms of oxytocin cell location and fiber projections. Oxytocin has also been shown to affect similar functions in zebrafish as it does in mammals, most importantly to reduce anxiety and increase sociality (Braida et al. 2012). Furthermore, it is possible that some of the behavioural

effects might be mediated through similar structures in both species, although this requires further studies. Especially the oxytocin receptor distribution should be studied.

The highly social nature of zebrafish and the availability of many behavioural tests also support the use of zebrafish in the oxytocin research. Zebrafish is a social, shoaling fish and is therefore well suited for studies concerning the effect of oxytocin on social behaviour. Many different things, like shoal joining tendency, dominant-subordinate relationship and social interactions can be analysed to evaluate social behaviour (Delaney et al. 2002; Saverino and Gerlai 2008; Pavlidis et al. 2011). Besides the tests that measure social behaviour, tests to assess non-social behaviour like anxiety and learning are also available for zebrafish (Bilotta et al. 2005; Bencan et al. 2009). However, oxytocin has also been shown to affect higher functions, like trust, in humans and these traits are difficult, if not impossible, to model in zebrafish.

Another advantage of using zebrafish is the possibility to easily modify the oxytocin system. The transient inhibition of oxytocin production with MOs proved to be difficult in this study and other methods will probably be more effective. There are many, both peptide and non-peptide, oxytocin agonists and antagonists available (Manning et al. 2012). Only a little is known about the zebrafish oxytocin receptor system but unlike mammals zebrafish seems to have two genes coding for oxytocin receptor, i*tnpr-like 1* and *itnpr-like 2* (Chou et al. 2011). A highly selective oxytocin antagonist desGly-NH2-d(CH2)5-[D-Tyr2,Thr4]OVT is the only oxytocin antagonist that has been studied in zebrafish but it was effective and selective in zebrafish (Braida et al. 2012). An advantage of using a pharmacological approach is its feasibility for HTS because many of the non-peptide agents could be dissolved to the swimming water. Another approach would be to create a mutant fish line lacking oxytocin for example using the CRISPR-Cas method.

The pharmacological agents and the mutant fish could be used to study the role of oxytocin on further aspects of zebrafish behaviour. Then the achieved phenotypes could be compared with rodent and human disease phenotypes to find out whether or not there is a resemblance. They could also be used in the discovery of new drugs for oxytocin

related disorders by evaluating the effect of compounds on the achieved disease phenotypes.

Behavioural tests should be suitable for HTS to really take advantage of using zebrafish instead of rodent models in the oxytocin research. Sociality, memory and anxiety are probably the most studied effects of oxytocin in relation to its therapeutic potential, so it is useful that there are high-throughput methods for zebrafish to measure learning, anxiety and social behaviour (Blaser and Gerlai 2006; Gerlai et al. 2009; Pather and Gerlai 2009). However, the tests need to be developed further and validated for them to be really useful in the actual screening processes.

Most of the zebrafish HTS assays rely on the use of embryos or larvae instead of adult zebrafish. The results of this study showed that in zebrafish oxytocin is expressed already at early stages of development. Also oxytocin receptors are expressed early in the development (Chou et al. 2011). However, the behavioural studies concerning oxytocin have been conducted only on adult zebrafish so it is not certain whether the behavioural effects of oxytocin would be seen already at larval stages. It is also possible that not all the effects can be studied with larvae because all the behavioural characteristics are not necessarily present at that age. For example shoaling behaviour is age-dependent and young larvae only form loose aggregates (Buske and Gerlai 2011). The larvae may thus not be as good as adults when studying the effect of oxytocin on sociality through shoaling behaviour. Therefore, high or medium throughput assays using adult zebrafish might be best suited for oxytocin research.

The use of zebrafish in the oxytocin research offers many advantages compared to other model organisms but it also has its drawbacks. More information about the structure and function of the zebrafish oxytocin system are needed to further assess its similarity to mammals. Additional high-throughput methods to analyse different aspects of zebrafish behaviour are needed to fully exploit the potential of this unique model organism in the oxytocin research.

9 CONCLUSIONS

The zebrafish oxytocin system resembles the mammalian oxytocin system both structurally and functionally: zebrafish have both hypophyseal and extra-hypothalamic oxytocin projections like mammals and oxytocin affects similar functions in zebrafish and mammals. The specificity of antibodies made against mammalian oxytocin to recognize zebrafish oxytocin differs, but the mouse anti-oxytocin antibody described in this study is specific for zebrafish oxytocin. A transient knockdown of oxytocin using MO successfully prevented oxytocin synthesis and reduced locomotor activity, but the specificity of the MO has to be studied further. The structure of the zebrafish oxytocin receptor system and the effect of oxytocin on different aspects of zebrafish behaviour should be studied in order to further assess the applicability of zebrafish for oxytocin research.

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APPENDIX 1

Solution recipes

Phosphate-buffered saline (PBS)

10xPBS:
80 mg NaCl (anhydrous)
2 g KCl (anhydrous)
14 g Na_2HPO_4 x 2 H_2O
2 g KH_2PO_4 (anhydrous)

Add mQ-H_2O to a final volume of 1000 ml (the pH will be 6,8; pH adjustment is not needed). Dissolve 1:10 to make 1xPBS. The pH for 1xPBS will be 7,4.

0,1 M phosphate buffer (PB)

Solution 1:
Na_2HPO_4 x 2 H_2O 35,6 g/2 l
Solution 2:
NaH_2PO_4 x H_2O 6,9 g/500 ml

The pH of the solution 1 is adjusted to 7,4 with solution 2.