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Ano 2017

Daniela Filipa Travanca dos Santos

The role of an oxytocin-like peptide in social reward in zebrafish

O papel de um péptido homólogo da oxitocina na recompensa social em peixe-zebra

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Rui Filipe Nunes Pais de Oliveira, Professor Catedrático com Agregação do Instituto Universitário de Ciências Psicológicas, Sociais e da Vida (ISPA), e da Doutora Maria de Lourdes Gomes Pereira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro

Apoio financeiro da FCT no âmbito do III Quadro Comunitário de Apoio.

"*Everybody is a genius. But if you judge a fish by its ability to climb a tree it will live its whole life believing that it is stupid.*"
Albert Einstein

Aos meus avós.

o júri

presidente

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professora auxiliar do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Comportamento social; peixe-zebra; isotocina; pS6; sistema de recompensa.

resumo

O peixe-zebra (*Danio rerio*) vive em cardumes e expressa uma preferência para se associar com conspecífios desde os primeiros estágios do seu desenvolvimento. Esta motivação social desempenha um papel importante para a sua sobrevivência, proporcionando protecção contra predadores e aperfeiçoando a eficiência na procura de alimento e no acasalamento. Assim, tem sido hipoteticado que os conspecíficos tenham adquirido uma propriedade de recompensa (também conhecida como recompensa social) em animais sociais, promovendo a vida em grupos. O objectivo deste projecto é instigar a ocorrência de recompensa social no peixe-zebra e caracterizar os seus mecanismos neuronais. Dado o papel da oxitocina na regulação do comportamento social nos vertebrados, pusemos a hipótese de que a isotocina (homólogo da oxitocina nos peixes) possa estar envolvida na recompensa social no peixe-zebra. Utilizámos, pela primeira vez, um paradigma social de preferência de lugar condicionada (CPP) nesta espécie, de forma a avaliar o valor de recompensa dos conspecíficos contra uma recompensa não social já estabelecida no peixe-zebra (por exemplo, comida). Subsequentemente, utilizámos uma linha ablada para o receptor da oxitocina para avaliar o impacto da oxitocina na recompensa social.

Fomos capazes de demonstrar que peixes-zebra machos adultos desempenham CPP para uma recompensa social de conspecíficos. Porém, esta experiência mostra que o estímulo social tem a mesma propriedade de recompensa do estímulo não social para peixes-zebra machos adultos, e que os péptidos homólogos da oxitocina parecem estar envolvidos no sitema de recompensa social. Por fim, utilizámos a expressão de um proteína de fosforilação (pS6) como um marcador de activação neuronal para mapear as regiões cerebrais involvidas na recompensa social no peixe-zebra, assim como a expressão da tirosina hidroxilase (TH) como um marcador do sistema dopaminérgico.

Ensaios imuno-histoquímicos adicionais são necessários para perceber o papel da oxitocina na regulação do Sistema de recompensa social. No entanto, os nossos resultados preliminares destacam a importância da estimulação social, no peixe-zebra, revelando diferenças relevantes na activação cerebral na área telencefálica ventral, mais especificamente no núcleo dorsal e no núcleo supracomissural.

keywords Social behaviour; zebrafish; isotocin; pS6; reward system.

abstract

Zebrafish (*Danio rerio*) live in shoals and express a preference to associate with conspecifics from very early on during their development. This social motivation plays an important role for their survival, providing protection against predators and improving foraging and mating efficiency. Thus, it has been hypothesized that conspecifics have acquired a reward property (as known as social reward) in social living animals, that promotes group living. The aim of this project is to investigate the occurrence of social reward in zebrafish and to characterize its neural mechanisms. Given the role of oxytocin in the regulation of social behaviour across vertebrates we hypothesized that isotocin (the fish homologue of oxytocin) can be involved in social reward in zebrafish. We have used, for the first time, a social conditioned place preference (CPP) paradigm in this species, in order to assess the rewarding value of conspecifics versus an established non-social reward in zebrafish (e.g. food). Subsequently, we used a knockout (KO) line for the oxytocin receptor to assess the impact of oxytocin on social reward.

We were able to demonstrate that adult male zebrafish perform CPP towards a social reward of conspecifics. Yet, this experiment showed that social stimulation has the same reward property as non-social stimulation for adult male zebrafish, and that oxytocin-like peptides seems to be involved in the social reward system.

Finally, we have used the expression of a phosphorylation protein (pS6) as a marker of neuronal activity to map the brain regions involved in social reward in zebrafish, as well as the expression of tyrosine hydroxylase (TH) as a marker of the dopaminergic system.

Further immunohistochemical assays need to be performed in order to uncover the role of oxytocin in the regulation of the social reward system. However, our preliminary results highlight the significance of social stimulation, in zebrafish, by revealing relevant differences in brain activation in the ventral telencephalic area, more specifically in the dorsal nucleus and in the supracommisural nucleus.

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List of abbreviations

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CNS	Central nervous system
CPP	Conditioned place preference
CS	Conditioned stimulus
DA	Dopamine
DAPI	4',6-diamidno-2-phenylindole
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
Dl	Lateral nucleus of dorsal telencephalic area
Dm	Medial nucleus of dorsal telencephalic area
DMSO	Dimethyl sulfoxide
HCl	Hydrochloric acid
HINGS	Heat-inactivated goat serum
IEGs	Immediate early genes
IHC	Immunohistochemistry
ISH	*In Situ* Hybridization
IT	Isotocin
KO	Knockout
L-DOPA	L-3,4-dihydroxyphenylalanine
NAcc	*Nucleus accumbens*
NaCl	Sodium chloride
NaOH	Sodium hydroxide
O.C.T	Optimum cutting temperature
OXT	Oxytocin

OXTR	Oxytocin receptor
PBS	Phosphate-buffered saline
PCIA	Phenol/chloroform/isoamyl alcohol
PCR	Polymerase chain reaction
PFA	Paraphormaldehyde
pS6	Phospho-S6 protein
SBN	Social behaviour network
SDM	Social decision making network
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SSC	Saline-sodium citrate
TBS	Tris-buffered saline
TBST	Tris-buffered saline tween
TH	Tyrosine hydroxylase
Tris-HCl	Tris hydrochloride
TU	*Tuebingen*
US	Unconditioned stimulus
Vc	Central nucleus of ventral telencephalic area
Vd	Dorsal nucleus of ventral telencephalic area
Vs	Supracommissural nucleus of ventral telencephalic area
Vv	Ventral nucleus of ventral telencephalic area
WT	Wild type

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1. Introduction

1.1. Social cognition and social learning

During their lives, animals are required to do rapid and adaptive decisions founded on the evaluation of the behaviour of their partners or opponents, as well as on memory of previous experiences. These decisions are very important for group-living animals, in order for them to survive and reproduce, hence having an effect on an individual's fitness. Therefore natural selection must have benefited individuals that have certain cognitive features to fit in a social environment in which they must make rapid decisions about when to engage in social interactions **(Platt *et al.,* 2016)**. This complex social environment has been hypothesized to be responsible for the evolution of larger brain structures due to the computational demands of living in large, complex societies (as known as social brain hypothesis) **(Dunbar & Shultz, 2007)**. Nevertheless, recent studies have contradicted this hypothesis, showing that small-brained animals, such as bees, ants or fish, may present complex social behaviour, emphasising that neural circuits underlying cognition and behaviour need to be understood, and not just the size of brain regions **(Chittka & Niven, 2009; Oliveira *et al.*, 2011; Robinson *et al.*, 2008)**. Many of the studies performed in these small-brained animals comprise social learning that, by definition, is "learning that is facilitated by observation, or interaction with, another individual or its products" **(Hoppitt & Laland, 2013)**.

Learning is an ubiquitous feature of all animals. This process can be achieved within only a fraction of the lifetime of the animal. Associative learning is a complex form of learning that has been widely studied. It requires the acquisition of temporal and/or causal relationships between at least two stimuli **(Gerlai, 2011)**. In the 1920 decade, Pavlov proposed a classical conditioning mechanism, which is composed by a neutral stimulus (Conditioned Stimulus - CS), which by itself will not produce a response, as well as a non-neutral stimulus (Unconditioned Stimulus - US). When these two stimuli are presented together, the animal eventually learns to associate them. After this association, the neutral stimulus (CS) by itself will produce the same response as the unconditional stimulus (US), called the conditioned response **(De Houwer *et al.*, 2001).** Associative learning has important human clinical relevance, as numerous central nervous system (CNS) disorders, such as neurodegenerative disorders including Alzheimer's disease, are associated with impaired associative learning **(Gerlai, 2011)**.

Classical conditioning can be converted into a social learning test when using a social US in reward-based conditioning paradigms, like the access to a group of individuals **(Al-imari & Gerlai, 2008).** Neuromodulators have been found to have a key role in the regulation of complex social behaviours **(Reddon *et al.,* 2012)**. The peptide oxytocin (OXT), which is conserved across vertebrate taxa **(Goodson & Kingsbury, 2011)**, has attracted significant scientific interest due to its important role in social cognition and behaviour **(Quintana *et al.*, 2016)**.

1.2. The role of oxytocin in social behaviour

The hypothalamus plays a crucial role in the body by synthesizing and secreting hormones that promote a link between the nervous and the endocrine systems via hypophysis (pituitary gland). All neurohypophysial hormones are nonapeptides with very similar amino acid sequence and structure. Depending on small amino acid residues variations, nonapeptides are organized into vasopressin and OXT families **(Gimpl & Fahrenholz, 2001)**.

OXT is a small peptide hormone, with nine amino acids, and is synthesized in the supraoptic and paraventricular nuclei of the hypothalamus in mammals. It is released in response to multiple physiological stimuli, by exocytosis, from the neurohypophysis and nerve terminals into the bloodstream, promoting, for example, the contraction of the uterus during labour and regulating milk let down **(Choe et al., 2015; Cochran *et al*. 2015)**. It has also been shown that OXT is also released into specific brain areas that control social behaviour, where it acts as a neuromodulator. Recent studies have demonstrated that the administration of OXT in humans promotes affective behaviours namely trust, empathy and bonding. However, the mechanisms through which OXT regulates social behaviour are not completely understood **(Insel & Young, 2001; Kosfeld *et al.*, 2005).**

Of all peptide hormones, this neurohypophysial hormone was the first to have its structure determined, to be chemically synthesized in an active form and the first one to be sequenced **(Gimpl & Fahrenholz, 2001)**.
This nonapeptide's actions are transduced by oxytocin receptors (OXTR). These receptors belong to the G-protein coupled receptor family (GPCR), and are distributed widely in the brain. In mammals there is only one isoform for the receptor, while in teleost fish there are two receptor isoforms (oxytocin receptor and oxytocin-like receptor), due to the occurrence of a duplication of the gene. Nevertheless, these two receptor isoforms are still not very well characterized **(Wircer *et al.*, 2015)**.

OXT is present in all vertebrates, although it presents structural differences in one or two amino acids and, consequently, different designations (Figure 1) **(Donaldson & Young, 2008; Gutnick *et al.*, 2011)**. Fish are the largest vertebrate class, display an extensive and diverse array of social behaviours and express the nonapeptides vasotocin and isotocin (IT), homologues to vasopressin and oxytocin in mammals, respectively **(Lindeyer *et al.*, 2015; Reddon *et al.*, 2015).**

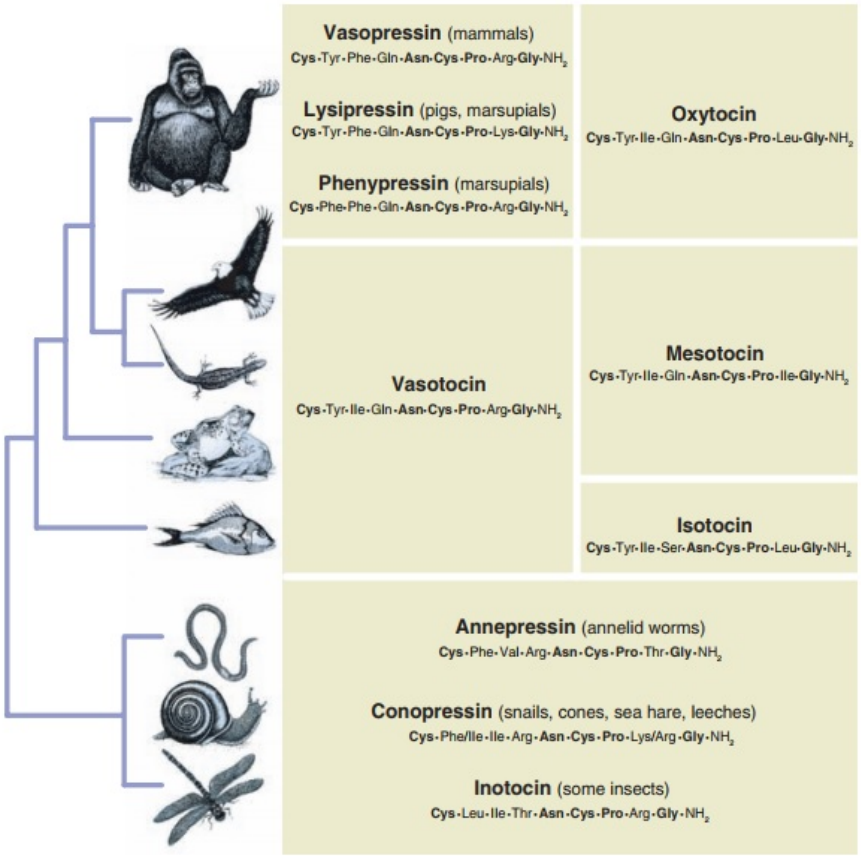

Figure 1. Scheme of oxytocin and vasopressin homologues through taxa. From Donaldson *et al.*, 2008.

Similarly to humans, in fish IT regulates contraction of smooth muscle in both the ovary and oviduct during oviposition, and promotes affiliative behaviours such as preference for conspecifics **(Braida *et al.*, 2012; Gutnick *et al.*, 2011)**.

Besides being a highly social species, zebrafish is a model organism with highly developed genetic tools in which OXT functions can be studied in depth **(Anderson & Adolphs, 2014)**.

1.3. Zebrafish (*Danio rerio*) as a model organism

"Model organism" denotes to a species used in an attempt to simplify and comprehend particular biological facts, targeting a vast range of systems and processes occurring in living organisms **(Maximino *et al.*, 2015; Levin & Cerutti, 2009)**

In current days, with the ongoing revolution in the development of genetically-based tools for studying the activity, anatomy and function of neural circuits, a broad variety of model organisms have been extensively used to study social cognition and related diseases **(Anderson & Adolphs, 2014)**. Among these organisms, rodents are still the most widely used, although other simpler organisms can be very useful to uncover fundamental brain processes, such as *Caenorhabditis elegans* and *Drosophila melanogaster*. However, a large research gap between the invertebrate and vertebrate model systems has been reported **(Hsu *et al.*, 2007)**. This hiatus can be filled by

other non-mammalian and more evolved organisms, namely zebrafish (*Danio rerio*) **(Ijaz & Hoffman, 2016)**.

In the past three decades, zebrafish has become a paramount system in life sciences and its ramifications into different biological fields, like behavioural neurosciences. As a vertebrate, this teleost fish shares a large portion of its genome with humans (70% genetic homology), and its diversity of social systems allows phylogenetic comparisons. Additionally, zebrafish has several advantages comparing to other vertebrate models, namely its small size, its low housing cost, its easy manipulation and the large amount of offspring generated from each cross. Furthermore, zebrafish presents rapid, external development of transparent embryos, allowing for early genetic manipulation **(Ijaz & Hoffman, 2016; Oliveira, 2013).**

As highly social animals, zebrafish lives in shoals and express a preference to associate with conspecifics from very early on during their development. This social motivation plays an important role for their survival, since living in a shoal provides protection against predators and improves foraging and mating efficiency. Besides these pro-social behaviours, zebrafish also exhibit conflict behaviours, such as aggression and hierarchy formation. Furthermore, other basic socio-cognitive processes have been described in zebrafish, such as the case of social recognition and social learning **(Oliveira, 2013; Oliveira *et al.*, 2011)**.

It has been hypothesized that, in group-living animals, conspecifics may have acquired a reward value (as known as social reward) in order to reinforce the expression of pro-social behaviours **(Oliveira, 2013)**. The reward value of specific stimuli can be assessed through behavioural assays, such as the conditioned place preference (CPP) paradigm **(Prus *et al.*, 2017)**.

1.4. Conditioned place preference as a tool to measure social reward

Both natural rewards and substances of addiction have the capacity to reinforce behaviours. However, it has been unclear whether identical neural pathways mediate the actions of both, and very little is known about these mechanisms **(Lau *et al.*, 2006)**.

Conditioned place preference paradigm, also known as CPP paradigm, is a standard behavioural assay for measuring the motivational effects (rewarding effects) of stimuli, and is usually used for modelling the rewarding and aversive effects of addictive drugs and alcohol. Despite different designs and setups, this paradigm is based on the association of a location (place) with a substance of addiction. A common version of this test consists of a three-chambered tank, with the chambers separated by removable gates to allow the focal fish to pass freely between them, and where the middle compartment is neutral and used as a start box **(Prus *et al.*, 2017)**. In this paradigm, the focal fish is exposed to two distinct environments, where only one of which is repeatedly paired with the administration of a substance of addiction (reward). If the subject develops a preference for the substance-associated environment by spending more time in that zone, it can be assumed that the substance has positive-reinforcing properties and that we are in the presence of a conditioned place preference (CPP), validating Pavlovian theory of classical

conditioning **(Millot *et al.*, 2014; Perathoner *et al.*, 2016; Prus *et al.*, 2017)**. On the other hand, if the subject spends significantly more time in the opposite environment, this is considered condition place aversion (CPA) **(Prus et al., 2017)**.

Hence, CPP paradigms can be used to assess both the reward and aversion value attributed by animals to several stimuli. In fish, this paradigm has been mostly used to determine the reinforcing effects of addictive drugs and alcohol, and it has been shown to express a preference for several stimulating substances, including nicotine, cocaine and D-amphetamine, among others **(Braida *et al.*, 2007; Kedikian*et al.*, 2013; Millot *et al.*, 2014; Trotha *et al.*, 2014)**. However, the value of social reward in these animals has been barely explored.

As mentioned before, conspecifics have acquired a reward effect (social reward) in social living animals, that promotes group living, and this reward value can be used as a positive-reinforcing property **(Oliveira, 2013).** The first goal of this project is to investigate the reward value of conspecifics and compare it with a non-social natural reward in zebrafish, such as food.

1.5. Reward system

During their lives, animals are frequently faced with circumstances that require decision making **(Conradt & Roper, 2003; Maruska *et al.*, 2013)**. In all these circumstances, environmental cues are managed by biological sensory systems into a signal while internal physiological cues and previous experience are integrated, which will result in adaptive behavioural responses **(Fernald & Maruska, 2012; O'Connell & Hofmann, 2011)**. These responses are accomplished through an evaluation of the salience of a stimulus by the animal's nervous system. In mammals, the nervous circuits responsible for this evaluation during social interaction are the mesolimbic reward system and the social behaviour network (SBN), that integrate an evolutionary ancient social-decision making (SDM) network (Figure 2) **(Maruska *et al.*, 2013; O'Connell & Hofmann, 2011)**. Reward system circuit consists of telencephalic brain regions and dopaminergic projections from the midbrain ventral tegmental area to *nucleus accumbens* (NAcc), through the median forebrain bundle. The mesolimbic dopaminergic system has been described in mammals and associated with addiction, depression and schizophrenia **(Goodson & Kingsbury, 2013; O'Connell & Hofmann, 2011)**.

The core nodes of SBN regulate several types of social behaviour (such as parental care), are mutually connected and contain receptors for sex steroid hormones, and there is evidence that some of these hypothalamic and amygdalar regions also regulates feeding behaviour **(O'Connell & Hofmann, 2011)**.

It has been shown that reward system and SBN are functionally connected, as both circuits play central roles in the regulation of behaviour. Moreover, these circuits are extensively interrelated in all vertebrate class, proposing that information can be easily transferred between these two networks. These networks share two nodes that play a role on social behaviour and reward processing: the lateral septum (LS) and bed nucleus of the stria terminalis (BNST). Both nodes are

thus well positioned to convey information about the significance of a social stimulus into an adaptive behavioural response. It is assumed that these mechanisms are not only integrated, but also highly conserved across vertebrates, and have played a fundamental role in vertebrate social evolution. However, these nodes are probably not the only regions involved in information trade between the two networks, since both the reward system and SBN are highly interrelated and may share related features of many social actions **(O'Connell & Hofmann, 2011)**.

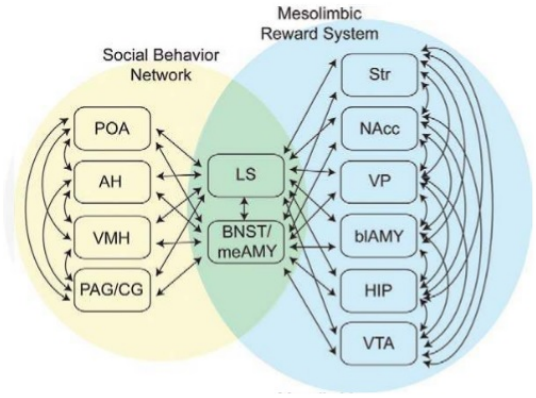

Figure 2. Schematic representation of the interactive nodes of the networks that regulate SDM. From O'Connell *et al.*, 2011.

Dopaminergic neurons have attracted significant interest based on their modulatory effect on many behavioural circuits and its association in neurodegenerative diseases **(PasterKamp, R, Smidt, & Burbach, 2009)**. Dopamine (DA) is one of the major catecholamine in the CNS **(Kobayashi, 2001)**. DA systems contribute to the control of motor activity, behaviour and perception and, at least in mammals, modulates reward, motivation and learning, among others **(Wise, 2004)**. Tyrosine hydroxylase (TH) catalyses the rate-limiting step in the synthesis of catecholamines, since it converts the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and is often used as a marker for catecholaminergic neurons **(PasterKamp, R *et al.*, 2009)**. Zebrafish has two paralogous of TH-encoding genes (*th*1 and *th*2), due to a genome duplication during teleost evolution. Besides its similar sequence, they are differentially regulated at the transcriptional level, and *th*2 is significantly more divergent from the human TH **(Filippi *et al.*, 2010; Meng *et al.*, 2008; PasterKamp, R et al., 2009).**

Reward pathways can be assessed through molecular assays, such as immunohistochemistry, using TH as a marker of the dopaminergic neurons **(Gaspar *et al.*, 1989; Lavoie *et al.*, 1989)**.

1.6. Uncover neural mechanisms: *c-fos* and Phospho-S6 protein

To uncover the neural circuits underlying a certain stimulus, neuronal activity can be studied using different neuron activity markers, such as the transient expression of immediate-early genes

(IEGs). This type of markers is the most used in literature **(Bullitt, 1990; Fujita *et al.*, 2013; Minatohara *et al.*, 2016)**.
IEGs transcription is rapidly and transiently induced by many extracellular stimuli and is independent of previous protein synthesis. The expression of specific IEGs is induced by neural activity that produces stable changes in synaptic strength **(Spulber *et al.*, 2009)**.

IEGs are divided in two functional classes: one class encodes a diverse range of biological effector proteins which have more direct and defined effects on cellular function; the second class encodes regulatory transcription factors, whose products modulate downstream target genes (e.g. *c-fos*) **(Guzowski *et al.*, 2001).**

Since their first applications, *c-fos* became the most broadly used marker for identifying activated cells and CNS circuits **(Kovács, 1998, 2008).**

Generally, the kinetics of *c-fos* response to an acute stimulus is transient, with a peak of mRNA expression approximately at 30 minutes and c-Fos protein between 90-120 minutes **(Guzowski et *al.*, 2001)** after the exposure to the stimulus**.** As a marker for neural activation, *c-fos* has been extensively used to study the circuits and the brain regions activated during social interaction, resorting to techniques such as *in situ* hybridization (ISH) **(Gordon *et al.*, 2002; Martinez *et al.*, 2002**). ISH techniques allow the detection of specific nucleic acid sequences in morphologically preserved chromosomes, cells or tissue sections. The introduction of nonradioactive probes allowed easier and faster transcript visualization in whole-mounted tissues. Subsequent improvements permitted the detection of two or three different gene products using different colours within the same sample, through chromogenic stains or fluorescent dyes **(Machluf & Levkowitz, 2011)**.

On the other hand, phospho-S6 protein (pS6) is a component of the 40S ribosomal subunit. pS6 is of specific interest because it is phosphorylated by induction in response to stimuli and it is implicated in the regulation of translation initiation and protein synthesis **(Pirbhoy *et al.*, 2016)**.

pS6 is located near the mRNA/tRNA binding site junction between the 40S and 60S ribosomal subunits, making it the principle candidate to regulate the recruitment of mRNA into polysomes **(Pirbhoy *et al.*, 2016)**.
Phosphorylation of S6 ribosomal protein is associated with an increase in the translation of mRNA transcripts containing an oligopyrimidine tract in their 5' untranslated regions. These mRNA transcripts encode proteins involved in cell cycle regulation and translation essential elements like ribosomal proteins and elongation factors **(Biever *et al.,* 2015; Iwenofu *et al.*, 2008)**. Phosphorylation sites in pS6 include the Ser235, Ser236, Ser40 and Ser244 residues. Phosphorylation at Ser235/236 was detectable by 5 minutes and peaked at 30 minutes, being maintained for hours **(Biever *et al.*, 2015; Iwenofu *et al.*, 2008; Pirbhoy *et al.*, 2016)**.

As a marker for neural activation, pS6 has been used to study the circuits and the brain regions responding to several stimuli, resorting to techniques such as immunohistochemistry (IHC)

(Knight *et al.*, 2012). IHC allows the visualization of cellular components in tissue samples, such as proteins. This assay involves the detection of epitopes expressed by a single protein-target in a tissue sample using antibodies capable of binding those epitopes with high specificity. These antibodies are connected to a reporter molecule that produces a coloured precipitate at the site of the epitope-antibody complex **(O'Hurley *et al.*, 2014)**.

Several stimuli, like exploration of a novel environment, led to an increase in the phosphorylation of pS6 positive neurons that are activated, throughout the forebrain in a pattern reminiscent of IEGs induction **(Pirbhoy et al., 2016)**.
Knight *et al.* confirmed that S6 protein was phosphorylated in cells expressing c-Fos and showed that there is a wide variety of stimuli that results in extensive co-localization of pS6 and c-Fos.

1.7. Objectives

The aim of this project is to investigate the occurrence of social reward in zebrafish, to study the role of oxytocin in this process and to characterize its neural mechanisms in zebrafish. For this purpose, we have used a CPP paradigm to assess the rewarding value of conspecifics versus an established non-social reward in zebrafish (food). Subsequently, we used a knockout (KO) line for the OXTR to assess the impact of OXT signalling on social reward. Finally, we have used the expression of an immediate early gene (*c-fos*), as well as a phosphorylation protein (pS6) as markers of neural activity to map the brain regions involved in social reward in zebrafish, as well as the expression of tyrosine hydroxylase (TH) as a marker of the dopaminergic neurons.

2. Materials and Methods

All animal procedures were conducted in accordance with the Federation for Laboratory Animal Science Associations (FELASA) and approved by the Diração-Geral de Alimentação e Veterinária (DGAV).

2.1. Animal housing

All individuals tested in the CPP pilot experiment (n=18) were four to five months old wild type (WT) male zebrafish, generated from an outcross of *Tuebingen* strain. The subjects used in the conditioned place preference experiment (n=60 – 48 focal fish plus 12 reward stimulus fish) were four to five months old WT and KO male zebrafish, generated from an incross hetero OXTR_KO strain. All the animals were bred and held in Instituto Gulbenkian de Ciência, and were maintained in an enriched environment, with a water recirculation system, under controlled parameters, monitored every day, such as temperature established at 28ºC, 14h light-10h dark cycle, water concentration of nitrites <0.2ppm, nitrates <50ppm, ammonia 0.01-0.1ppm, pH=7.0 and conductivity at 700 µSm.

2.2. Genotyping

In order to group the individuals according to their OXTR genotype, male fish (n=150) and female fish (n=20) were genotyped at three months, before the behavioural assays. This line was generated using a TALEN-Based Genome Editing system (Weizmann Institute), and it is characterized by a single nucleotide deletion leading to a truncated IT receptor. In order to extract genomic DNA, fish were anesthetized with Tricaine (MS-222, 1X) to allow the extraction of a small portion of the caudal fin. Fin clips were collected into a tube containing 50 µL of NaOH 50 mM **(Meeker et al. 2007)**. The samples were incubated at 95ºC for 20-30 minutes, placed on ice and neutralized with 1/10 volume of Tris-HCl 1mM, pH=8.0. The genomic DNA was submitted to a PCR reaction, using specific primers (forward 5'-TGCGCGAGGAAAACTAGTT-3' and reverse 5'-TGACCATTCTGAGTGTCTGCT-3'). The PCR product was loaded in a 1% agarose gel, in order to cut the corresponding band (700bp) from the gel and purify the DNA using a commercial kit (NucleoSpinGel® and PCR Clean-Up (Macherey-Nagel)). The final product was then sent for sequencing. After DNA purification (see Annexes, **Figure S 1**), the product was sent for sequencing and the chromatograms were analysed **(Figure 3)**. Males were used both as focal fish and social reward stimulus, and females were used just as social reward stimulus. Only WT and KO individuals were used. Further genotyping was performed (n=40) to maintain a stock of breeders.

Figure 3. Sequencing results – chromatograms for the three possible genotypes for the OXTR_KO line. Each peak represents a nucleotide. (A) WT (+/+). (B) Heterozygote (+/-). (C) KO (-/-).

2.3. Tagging

In order for focal fish to be distinguished from the others that composed the social reward stimulus, the latter (n=12) were tagged according to **Dahlbom *et al.*, & Winberg, 2011.** Initially, animals were anesthetized with Tricaine (MS-222, 1X). A 27G needle with a nylon monofilament was pulled through the dorsal musculature, leaving the filament and painting its ends with nail polish **(Figure 4)**. The marked fish were returned to their home tanks and were allowed to recover for one week before the behavioural experiment.

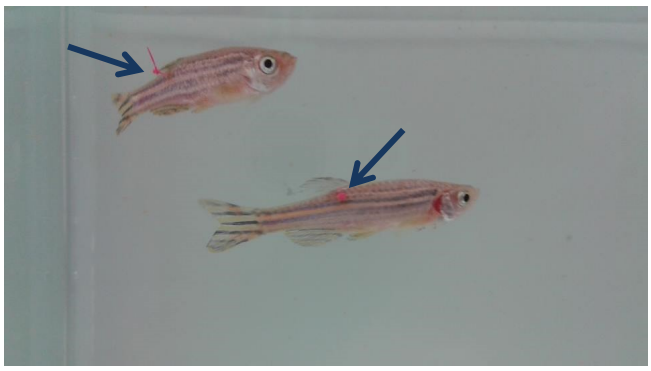

Figure 4. Fish tagged with nylon monofilament in the dorsal musculature painted with pink nail polish, indicated by the arrows.

2.4. CPP Paradigm

CPP paradigm was used to estimate the reward value given by fish to social and non-social stimuli, assessing if fish were able to associate one side of the tank (CS) with the reward stimulus (US). To evaluate the role of IT in this process, a KO line for its IT receptor was used. The social reward consisted of a mixed group of conspecifics (2 males and 2 females), tagged in the dorsal zone with nylon wire coloured with ink. The non-social reward was composed by three bloodworms, administered through a plastic tube with the aid of a plastic syringe. For the fish to associate the reward with one side of the tank, tanks were divided in two equal parts with different patterns, with a neutral zone in the middle divided with partitions.

A pilot test was performed using WT TU fish to evaluate the optimization of the setup and the protocol (see optimizations in Annexes, **Figure S 2**).

To test the OXTR_KO line, individuals were separated in six groups (n=8 in each group): three WT groups (control, social and non-social) and three KO groups (control, social and non-social). Individuals that showed an initial preference higher than 70% of time spent for either side were excluded from the study (according to **Mathur, Lau, & Guo, 2011**).

2.4.1. Tanks

Experimental glass tanks (30 x 15 x 18 cm) were divided in two equal parts and covered with self-adhesive paper of different patterns (half dotted and half white). The entire tanks were separated in three equally-sized compartments by two partitions, being the middle one a neutral zone, which was used as a start box **(Figure 5)**. During the pre-test and the test, transparent partitions were used, allowing the fish to see the entire experimental tank during the habituation period. On the contrary, during the conditioning sessions, opaque partitions lined with the same pattern of the tank compartment were used, conditioning the fish only to one pattern while they receive the reward, so that an association between the pattern and the reward could be accomplished.

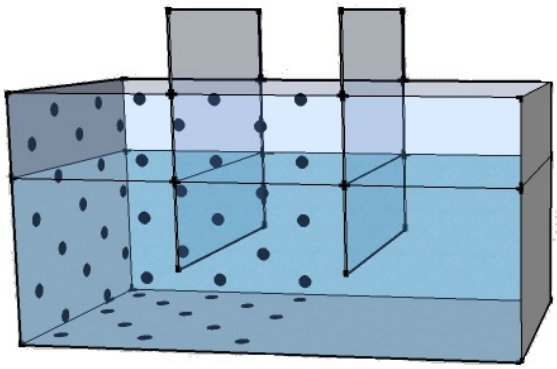

Figure 5. Schematic representation of CPP paradigm experimental tank.

2.4.2. Housing and feeding

The focal fish were kept in isolation 24 hours before the experiment. During all the experimental period, the animals lived in individual tanks (15 cm x 15 cm). The control group and the group of fish tested with the social reward were fed in their home tanks, unlike the group of fish tested with the non-social reward. The group of fish used as social reward lived in group tanks (30 cm x 15 cm) and were fed every day. The tanks water was changed every day to maintain the water quality.

2.4.3. Protocol

The protocol used in this project was adapted from **Mathur et al., 2011**. This procedure has three stages: the pre-test (to assess the initial preference), the conditioning session (for training) and the final test (to evaluate if the individual reverted the initial preference) (**Figure 6**).

Day 1: Pre-test and 1st conditioning session

In the pre-test, the fish was placed in the neutral zone for 30 seconds, and then the partitions were opened. The fish was allowed to swim freely through the tank for 10 minutes, where the first 5 minutes were considered as a habituation period and only the last 5 minutes were analysed for the calculation of the CPP score. Immediately after the pre-test, the fish performed the first conditioning session, where first the fish was placed in the non-preferred zone for 30 minutes in the presence of a reward stimulus, and then he was moved to the preferred zone for another 30 minutes with no reward. Animals tested with social reward were introduced in the middle of the shoal and stayed with contact with it for the entire 30 minutes' session, while animals tested with non-social reward received 3 bloodworms, half of a bloodworm every 5 minutes, during the 30 minutes' session (**Table 1**).

Day 2: 2nd conditioning session

On the second day, the fish performed the second conditioning session, following the same protocol as the first one.

Day 3: Test

On the third day, the protocol was performed in the same way as the pre-test.
After the final test, fish was maintained in the experimental tank for 30 minutes, and then it was sacrificed and the brain was extracted for histological analysis.

Table 1. Representation of the experimental design. Each animal was exposed to the unconditioned stimulus (US) for 30 minutes.

	US SOCIAL	US NON-SOCIAL	CONTROL
WT	Shoal (2 males and 2 females)	Food (3 bloodworms/day: ½ bloodworm 5'/5')	No reward
KO	Shoal (2 males and 2 females)	Food (3 bloodworms/day: ½ bloodworm 5'/5')	No reward

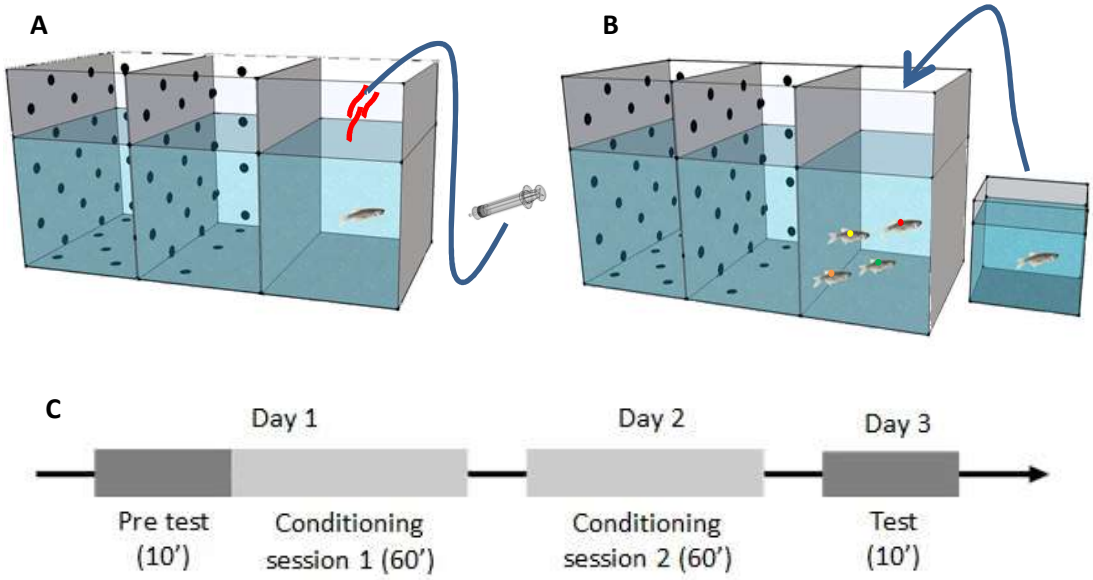

Figure 6. Schematic representation of the CPP paradigm: (A) non-social treatment conditioning phase; (B) social treatment conditioning phase; (C) protocol timeline.

CPP score was measured by the difference between the percentage of cumulative duration (% CD) spent by the fish in the non-preferred zone in the test and in the non-preferred zone in the pre-test, according to the following equation:

$$CPP\ Score\ (\%)\ = \%\ CD\ in\ the\ non\ preferred\ zone\ in\ test - \%\ CD\ in\ the\ non\ preferred\ zone\ in\ pre\ test$$

With this calculation it is possible to measure how much time (%) fish spent in one zone in the test in relation to the pre-test, concluding if the animal reverted his initial preference.

2.5. Sampling

For Immunohistochemistry, fish were sacrificed with an overdose of Tricaine (MS-222, 25X) 30 minutes after the final test, to reach the peak of *c-fos* expression, as well as serine phosphorylation of ribosomal pS6 **(Guzowski *et al.*, 2001; Pirbhoy *et al.*, 2016)**. During this period, the fish remained in the experimental tank and they were not exposed to the experimental stimulus.

After sacrificing the fish, the brain tissue was collected and fixated with 4% paraformaldehyde (PFA) at 4ºC overnight. On the following day, brains were cryoprotected using 34% sucrose solution and, finally, embedded in O.C.T. (Tissue-Tek®) and cryosected in 16 µm slices that were stored at -20ºC.

2.6. Video analysis

All behavioural tests were video-record using infra-red cameras with an acquisition rate of 30 fps connected to a laptop and using Pinnacle Studio 12 software (http://www.pinnaclesys.com). Ethovision XT11 from Noldus Inc. was used for automated videotracking of the behaviour of the individuals (http://www.noldus.com/animal-behavior-research/products/ethovision-xt).

2.7. Statistical analysis

2.7.1. CPP pilot test

The data regarding the CPP Score were analysed with a one-way ANOVA, while the data regarding the Time Spent were analysed with a two-way ANOVA. Post Hoc comparisons were performed using Fisher's Least Significance Different (LSD), due to the small sample size.

Descriptive statistics were plotted using the mean ± SEM. Significance levels used for inference tests were *p<0.05, **p<0.01, ***p<0.001, +p<0.1 (marginally significant). All the statistical analyses were performed with the STATISTICA (http://www.statsoft.com/Products/STATISTICA-Features) software package.

2.7.2. CPP test

All the data were analysed with a two-way ANOVA. Planned comparisons were performed: pre-test versus test in each treatment (control, food, shoal), and all treatments within the pre-test and the test.

Descriptive statistics were plotted using the mean ± SEM. Significance levels used for inference tests were *p<0.05, **p<0.01, ***p<0.001, +p<0.1 (marginally significant). All the statistical analyses were achieved with STATISTICA (http://www.statsoft.com/Products/STATISTICA-Features) software package.

2.8. Double technique: *In Situ* Hybridization – Immunohistochemistry

A double fluorescent *In Situ* Hybridization - Immunohistochemistry was performed to uncover the neural mechanisms modulated by oxytocin and how this neuropeptide regulates social behaviour (through *c-fos* mRNA activation), with dopaminergic neurons (through TH immunoreactive cells), revealing the reward system involvement.

2.8.1. Plasmidic DNA and probe synthesis

Bacteria (*Escherichia coli*) containing both the plasmid of interest and a resistance to the antibiotic kanamycin were grown in a LB medium containing kanamycin (direct inoculation). Plasmidic DNA was then extracted using a Plasmid Miniprep kit (Zyppy™).

After obtaining the plasmidic DNA, it was linearized through the incubation of the plasmid with a linearization mix (15 µl of plasmidic DNA, 10 µl of restriction enzyme buffer (10X), 3 µl of restriction enzyme (BAM HI), 0.5 µl of BSA (10mg/ml) and DEPC treated water up to 100 µl) overnight at 37ºC.

On the following day, the digestion of the plasmid was confirmed through a 1% agarose gel electrophoresis, and DNA was purified by extraction with PCIA. First, PCIA was added (200 µl of PCIA for 200 µl of DNA solution) and the mixture was centrifuged for 5 minutes, 1400 rpm at room temperature to separate the 2 phases. The aqueous upper phase was transferred to a fresh tube and 3M Sodium Acetate (pH 5.2) and 400 µl of absolute ethanol were added and the mixture was precipitated at -20ºC overnight. In the day after, the mixture was centrifuged for 30 minutes, 1400 rpm at 4ºC, and the supernatant was discarded. The mixture was washed with 1 ml of 70% ethanol and centrifuged for 5 minutes, 1400 rpm at room temperature. The ethanol was removed and the pellet was left to dry on the bench. Transcription reaction mix (2 µl of linear DNA, 2 µl of transcription buffer (10X), 2 µl of DIG labelling mix (10X), 2 µl RNA polymerase (T7 for the antisense probe), 1 µl of RNase inhibitor and DEPC treated water up to 20 µl) was prepared and incubated at 37ºC for 2h after a spin-down. After the incubation period, 1 µl of RNase-free DNase I was added and the mix was incubated for more 15 minutes. An aliquot was checked in a 1% agarose gel to assess if the DNA was efficiently removed. The sample was then precipitated by adding 180 µl of DEPC-treated water, 22 µl 3M Sodium Acetate (pH 5.2) and 500 µl of absolute ethanol, and stored at -20ºC overnight.

Next day, the pellet was precipitated in microfuge for 30 minutes, 1400 rpm at 4ºC and washed with 70% ethanol for 5 minutes. Finally, the pellet was resuspended in 30 µl DEPC-treated water and, if the probe could be seen in a 1% agarose gel, 30 µl of deionized formamide were added. The probe was stored at -20ºC until required for *in situ* hybridization.

2.8.2. *In situ* hybridization - Immunohistochemistry in slices

The slides were transferred from -20ºC to room temperature for 15 minutes and were post-fixated with 4% PFA for 10 minutes. Then, the slides were washed with PBS (3 x 5 minutes) and treated for 10 minutes with an acetylation mix (1ml of DEPC-treated water per slide with 11.2 µl triethanolamine and 2.5 µl acetic anhydride). Slides were washed again with PBS (3 x 5 minutes) and prehybridized horizontally with enough volume of prehybridization solution at 68ºC in a humidified chamber for approximately 5 hours. Next, 200 µl of probe were diluted in prehybridization solution and the slides were hybridized horizontally in a humidified box at 68ºC overnight. Slides were coverslipped to prevent probe's evaporation.

On the following day, the slides were washed with a pre-warmed solution 1 (50ml 50% formamide, 25ml 5X SSC pH 4.5, 10ml 1% SDS and DEPC-treated water) up to 50ml, for 5 minutes at 68ºC. After this wash, another one was performed for 1 hour at 68ºC with the same solution. Next, the slides were washed with a pre-warmed solution 2 (50ml 25% formamide, 5ml 2X SSC pH 4.5, 0.5 ml 1% Tween20 and DEPC-treated water) up to 50ml, for 1 hour at 68ºC. Slides were washed twice with TBST for 5-10 minutes and transferred to a tray, where they were blocked with blocking buffer (10% HINGS in TBST) for 40 minutes at room temperature. Further, the slides were incubated in blocking buffer with anti-dig antibody (1:2000) for 80 minutes at room temperature and then washed with TBST for 5 minutes. After this, slides were post-fixated with 4% PFA for 10 minutes and then washed with TBST for 5 minutes. After, the tissue was blocked with TBS + 1% BSA for 40 minutes at room temperature and incubated in TBS + 1% BSA with primary antibody mouse anti-TH (1:400) overnight in a humidified chamber at 4ºC. On the next day, slides were

washed with TBST for 5 minutes and were incubated in TBS + 1% BSA with secondary antibody goat anti-mouse (1:1000) for 1 hour at room temperature, in a humidified chamber. After this period, slides were washed with TBST (2 x 5 minutes) followed by a wash with TBS (1 x 5 minutes). Then the slides were treated with DAPI (1:1000) in TBS, and washed with TBST (2 x 5 minutes). Finally, the slides were mounted with a fluorescence mounting medium from DAKO®.

2.9. Double Immunohistochemistry

A double fluorescent immunohistochemistry was performed to uncover the neural mechanisms modulated by OXT and how this neuropeptide regulates social behaviour (through phospho-S6 protein activation), as well as to assess the dopaminergic neurons, revealing the involvement of the reward system (through TH immunoreactive cells).

Initially, the slides were transferred from -20ºC to room temperature for 30 minutes and the tissue was post-fixed in 4% PFA for 10 minutes. Then, the slides were washed with TBS (3 x 5 minutes) and TBS 0.025% Triton X-100 (2 x 5 minutes). After, the tissue was blocked with TBS + 1% BSA for 1 hour at room temperature. Next, the slices were incubated in TBS + 1% BSA with primary antibody Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb and mouse anti-TH (1:400) overnight in a humidified chamber at 4ºC.

On the next day, slides were washed with TBS 0.025% Triton X-100 (2 x 10 minutes) and were incubated in TBS + 1% BSA with secondary antibody goat anti-rabbit and goat anti-mouse (1:1000) for 2 hours at room temperature, in a humidified chamber. After this period, slides were washed with TBS 0.025% Triton X-100 (2 x 5 minutes) followed by a wash with TBS (1 x 5 minutes). Then the slides were treated with DAPI (1:1000) in TBS, and washed with TBS 0.025% Triton X-100 (2 x 5 minutes). Finally, the slides were mounted with a fluorescence mounting medium from DAKO®.

2.10. Brain anatomy

Brain sections were studied using a Leica DMRA2 microscope at a magnification of 20 x and a digital camera (CoolSNAP HQ CCD). The manual counting of pS6 and TH immunoreactive cells on a computer screen was performed through the usage of the MetaMorph software. Counting was accomplished blind to experimental conditions. According to **Mayer *et al.*, 2017**, for counting, a 25.5 x 30.6 µm rectangle was positioned over spots of highest number of immunoreactive cells within brain areas of interest (namely: Vv, Vs, Vd, Vc, Dm and Dl), keeping the minimum distance from the border of a neighbouring subdivision and the edge of the brain section. Every activated cell within the sample areas was marked on the screen with an event marker tool of the referred software, which automatically calculated the total number of pS6 and TH immunoreactive cells. To measure cell density within a brain region two to ten sections of one hemisphere were selected by the shape and anatomical landmarks that correspond to section 50 to 114 of the zebrafish brain atlas **(Wullimann *et al.*, 1996)**. After concluding the counts, mean values from the different sections were calculated for each brain regions in each hemisphere, for all animals. Cell densities were standardized to 1 μm^2. Final results were considered indicators of the number of immunoreactive cells, and will be employed for further statistical analysis.

3. Results

3.1. Conditioned Place Preference assay – pilot experiment

To assess the reward value of a group of conspecifics, and the difference between this social reward with an established non-social reward (e.g. food), a CPP paradigm was performed using the *TU* line.

Fish presented higher CPP scores in both social ($F(1, 12) = 5.78$, $p<0.05$) and non-social ($F(1, 12) = 3.94$, $p<0.1$) treatments compared to the control group **(Figure 7 A)**.

Regarding the difference in time spent in the non-preferred zone between the pre-test and the test, a significant difference can be observed in the social treated group ($F(1, 18) = 7.91$, $p<0.05$), as well as a marginally significant difference in the non-social one ($F(1, 18) = 2.02$, $p=0.1$).

Within the test phase, a significant difference between the control group and the non-social treated group ($F(1, 18) = 5.17$, $p<0.05$) is also observed, as well as a marginally significant difference between the control group and the social one ($F(1, 18) = 3.78$, $p<0.1$). The control groups displayed no difference in the time spent in the non-preferred zone in the pre-test and the test, showing that there was no side bias in this paradigm **(Figure 7 B)**.

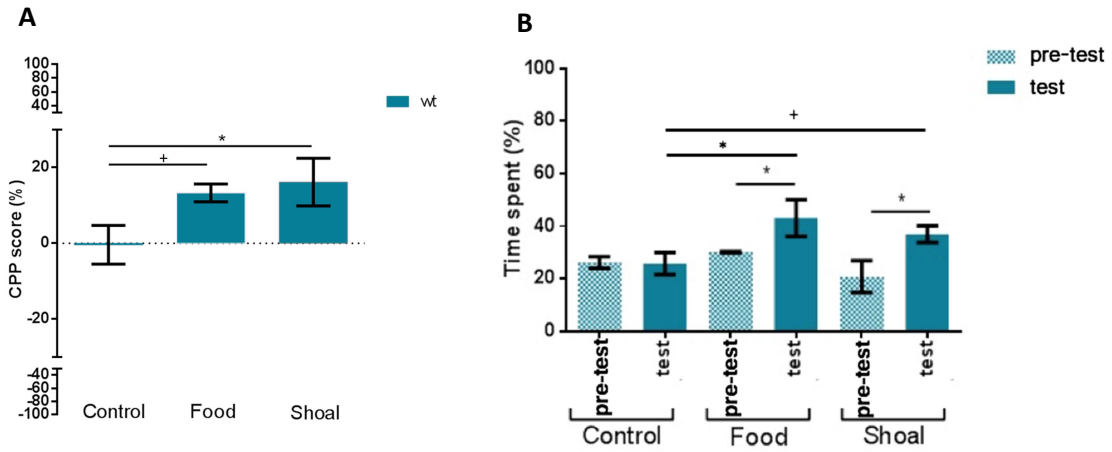

Figure 7. Zebrafish shows a significant response to social and asocial rewards: (A) CPP Scores for control, non-social and social rewards for TU line. The scores are given by the difference between the non-preferred zone in the pre-test and the non-preferred zone in the test; (B) Percentage of the time spent in the non-preferred zone in the pre-test and in the test. Statistical analysis using ANOVAs plotted with mean ± SEM, $*p<0.05$, $^{+}p<0.1$ (marginally significant).

Table 2. Main effect of experimental treatment (control, food and shoal) on the CPP Scores of pilot fish. One-way ANOVA.

Main effects CPP Score	F (DFn, DFd)	P value (p)
Treatment	F (2, 12) = 3.30	p<0.1

Table 3. Main effect of test phase (habituation and test) and treatment (control, food and shoal) on the time spent by the pilot fish. Two-way ANOVA.

Main effect Time Spent	F (DFn, DFd)	P value (p)
Test phase	F (1, 18) = 5.54	p<0.05
Treatment	F (2, 18) = 1.96	p>0.05
Interaction	F (2, 18) = 2.20	p>0.05

3.2. Characterization of the role of isotocin in social and non-social reward

To test if the ability to evaluate reward can be modulated by IT, the CPP paradigm was performed using the mutant homozygous genotypes (WT and KO) of a mutant line for the IT receptor.

As mentioned before (see Materials and Methods), individuals that showed an initial preference higher than 80% of time spent for either side were excluded from the study (according to **Lau *et. al* 2006**). This resulted in a final sample of 5 animals for the WT and 8 animals for the KO tratments.

WT subjects presented higher CPP scores in both social ($F(1, 32) = 14.15$, $p<0.001$) and non-social ($F(1, 32) = 4.62$, $p<0.05$) treatments in comparison to the controls.
On the contrary, KO subjects presented a higher CPP score in non-social treatment comparing to the control group ($F(1, 32) = 2.92$, $p<0.1$). Unlike the non-social treatment, the KO social treated group demonstrated no difference from the controls, showing a significant impairment compared to the WT social treated group ($F(1, 32) = 13.29$, $p<0.001$), **(Figure 8 A)**.

Regarding the difference in time spent in the non-preferred zone in the test, it can be seen that for the social treated group, WT fish present a significant difference compared to the controls ($F(1, 33) = 6.09$, $p<0.05$), as well as a marginally significant difference when comparing the non-social treated group with the controls ($F(1, 33) = 2.81$, $p>0.05$). For the KO animals a significant difference between the non-social treated group and the controls ($F(1, 33) = 5.89$, $p<0.05$) can be observed.
Within the social treated group, a significant difference between the WT and KO individuals ($F(1, 33) = 5.96$, $p<0.05$) can be observed. Both WT and KO control groups displayed no difference in the time spent in the non-preferred zone in both pre-test and test, showing that there was no side bias in this paradigm **(Figure 8 B)**.

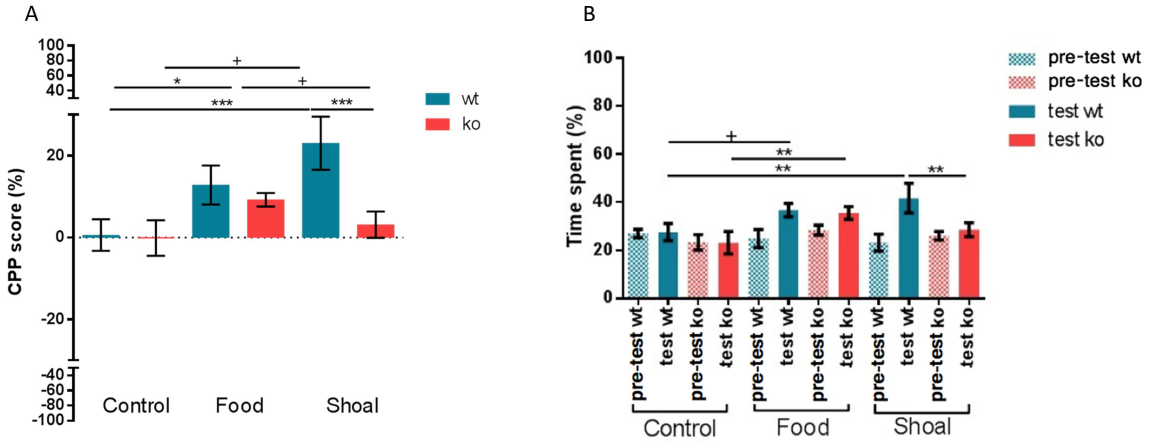

Figure 8. Wild type zebrafish shows a very significant response to social reward, unlike knockout genotype: (A) CPP Scores for control, non-social and social rewards for the oxytocin receptor mutant line. The scores are given by the difference between the non-preferred zone in the pre-test and the non-preferred zone in the test; (B) Percentage of the time spent in the non-preferred zone in the three groups. Statistical analysis using ANOVAs plotted with mean ± SEM, $*p<0.05$, $**p<0.01$, $***p<0.001$, $^{+}p<0.1$ (marginally significant).

Table 4. Main effect of experimental treatment (control, food and shoal) and genotype (WT and KO) on the CPP Scores of mutant line fish. Two-way ANOVA.

Main effect CPP Score	F (DFn, DFd)	P value (p)
Treatment	F (2, 32) = 5.80	p<0.01
Genotype	F (1, 32) = 6.56	P<0.05
Interaction	F (2, 32) = 3.59	p<0.05

Table 5. Main effect of experimental treatment (control, food and shoal) and genotype (WT and KO) on the time spent by the mutant line fish. Two-way ANOVA.

Main effect Time spent	F (DFn, DFd)	P value (p)
Treatment	F (2, 33) = 4.91	p<0.05
Genotype	F (1, 33) = 4.18	P<0.05
Interaction	F (2, 33) = 1.35	p>0.1

3.3. Characterization of the neural circuitry underlying social and non-social reward

In order to uncover which brain regions were activated in response to social and non-social rewards, and to relate this with the dopaminergic system, the expression of markers for neural activation and for dopaminergic neurons were used.

3.3.1. Results of *in situ* hybridization – immunohistochemistry procedure

To assess the expression of neural activation through *c-fos* mRNA and dopaminergic neurons through TH protein, a double *in situ* hybridization – immunohistochemistry was performed.

Although the molecular tests had shown the integrity of the probe (See Annexes, **Figure S 3**), this technique did not reveal the *in situ* hybridization marking. As it can be observed in **Figure 9**, only the TH signal was presented. Therefore, another marker of neuronal activity (pS6) was tested.

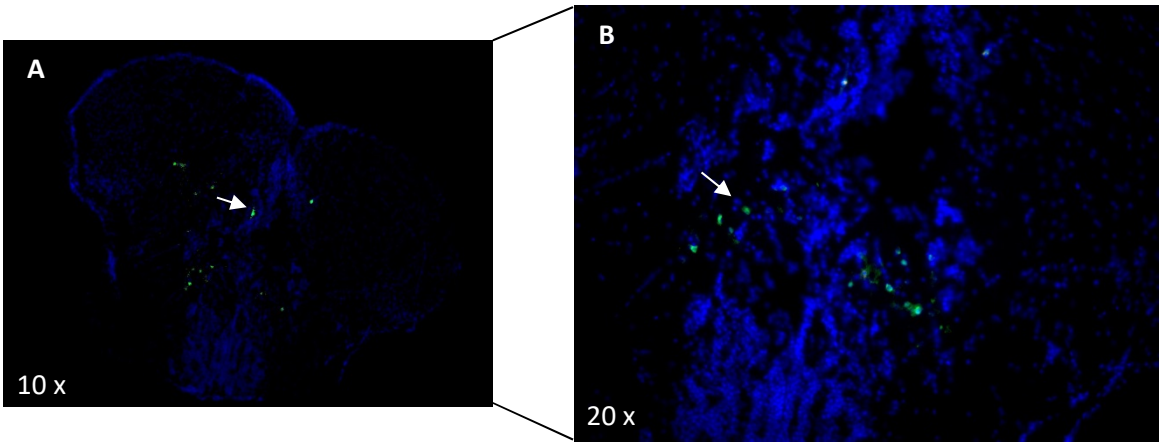

Figure 9. Double in situ hybridization-immunohistochemistry for c-fos and dopaminergic neurons in a WT fish treated with social stimulus: dopaminergic neurons expression (in green, indicated by the white arrow) in an adult male zebrafish brain exposed to a social reward stimulus 10 x magnification; colour blue indicates DAPI staining (A); Vd area highlighted in 20 x magnification (B).

3.3.2. Results of immunohistochemical procedure

We processed brains of all experimental groups. As we had to exclude animals from the behavioural assay due to a strong initial preference, this resulted in a final sample of 5 individuals for WT groups and 8 individuals for KO groups that were used for further analysis.

pS6 immunoreactive cells were stained red and dopaminergic cells were stained green. Cell nuclei were stained blue and thus different cells were easily distinguished. pS6 labelled cells appeared more or less homogeneously distributed over the extremities of the telencephalic brain areas. Dopaminergic neurons appeared either in clusters or isolated distributed over the interior zone of

telencephalic brain areas. Both pS6 immunoreactive cells and dopaminergic neurons varied considerably in location and density between individuals of different groups. However, no co-localization between these cells was observed.

Here is presented a representative example **(Figure 10)**, focusing on the section 71 from the zebrafish brain atlas **(Wullimann *et al.*, 1996)**, since this section includes most of the regions involved in the reward system and in the SDM network **(O'Connell & Hofmann, 2011).**

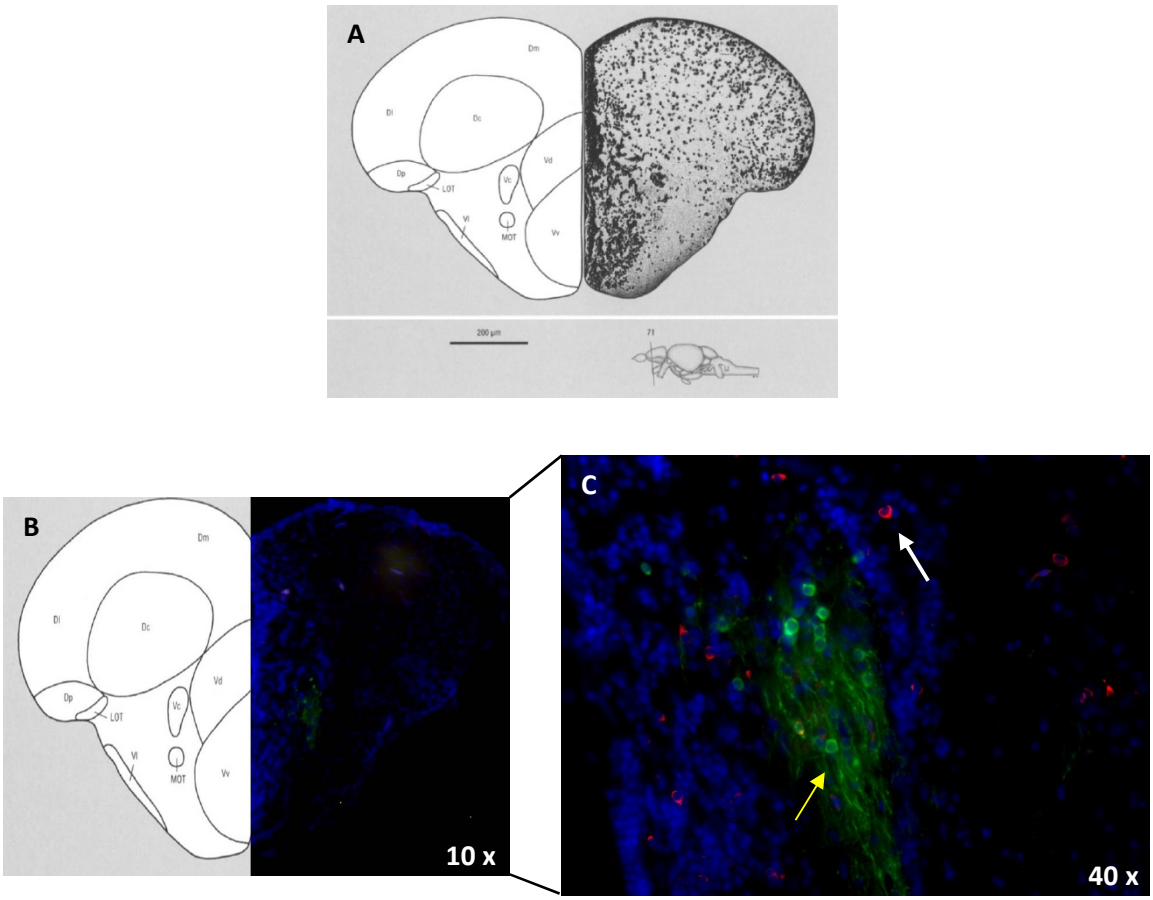

Figure 10. Double immunohistochemistry for pS6 and dopaminergic neurons in a WT fish treated with a social reward stimulus: (A) representation of coronal section 71 of the zebrafish brain atlas (Wulliman et al., 1996); (B) pS6 activated cells and dopaminergic neurons in the right brain hemisphere, and the corresponding schematic left brain hemisphere of section 71 from the zebrafish brain atlas, of an adult male WT zebrafish exposed to a social reward stimulus, 10 x magnification; (C) Vd area highlighted in 40 x magnification, showing expression of pS6 activated cells (in red, indicated by the white arrow) and dopaminergic neurons (in green, indicated by the yellow arrow).

3.3.3. Results of the quantitative analysis

Through a quantitative analysis, it can be ensured that there is a difference in the cell density within the brain regions between the different treatments of each genotype. This section presents merely representative examples of the three treatments (control, social and non-social) for the two tested genotypes (WT and KO) (n=1 for each group).

Regarding the KO individuals, the density of TH labelled cells in the dorsal nucleus of ventral telencephalic area (Vd) **(Figure 11 A)** was higher in the experimental groups compared to the control: 164% in the non-social group and 300% in the social group. A similar difference was presented in the supracommissural nucleus of ventral telencephalic area (Vs), with 100% higher TH labelled cell density in the non-social group and 250% in the social group, compared to the control. On the contrary, ventral nucleus of ventral telencephalic area (Vv) showed 50% less TH labelled cell density in the social group compared to the control group, and only 20% more density in the non-social group. Medial nucleus of dorsal telencephalic area (Dm) showed cell activation only in the non-social group, similarly to central nucleus of ventral telencephalic area (Vc) that only showed cell activation in the control group. On the opposite, lateral nucleus of dorsal telencephalic area (Dl) did not show cell activation in any group.

The density of pS6 activated cells also showed differences compared to the control groups in the KO individuals **(Figure 11 B)**, presenting less labelled cells in the majority of the cases. In the Vd the density was 33.3% less in the non-social group and 50.6% less in the social group compared to the control. Vv presented 42.8% less density in the non-social group and 10.7% less in the social group compared to the control. Dm showed 33.6% more density in the social, but 88.2% less density in the non-social group, equal to Dl, that showed 47.6% more density in the social group, but 13.5% less density in the non-social group, compared to the control. Vs showed cell activation only in the control and social groups, having 77.7% higher density in the control group. Vc only presented cell activation in the control group.

Regarding the WT animals, the density of TH labelled cells in the Vd **(Figure 11 C)** was 90% higher in the non-social group and 25.7% higher in the social group, compared to the control. Vv showed 24% more density in the non-social group, but 30% less density in the social group, compared to the control. Vs didn't show activation in the control groups, contrary to what happened in the treated groups, being 300% higher in the non-social one. Vc presented cell activation only in the social group, and Dm in the control group. Dl did not present cell activation in any group.

The density of pS6 activated cells in the WT animals also showed major differences compared to the control group **(Figure 11 D).** Vd presented 78.1% more density in the social group, but 15.1% less density in the non-social group compared to the control, similarly to Vv, that showed 103.7% more density in the social group, but 6% less density in the non-social group. These differences also occurred in the Dm that showed 57.4% more density in the social group but 46.2% less density in the non-social group, as well as in the Dl that showed 46.6% more density in the social group but 25.1% in the non-social group, compared to the control. Vs only presented cell activation in the treated groups, having 120% higher density in the social group. Vc only presented cell activation in social and control groups, having 300% more density in the social one.

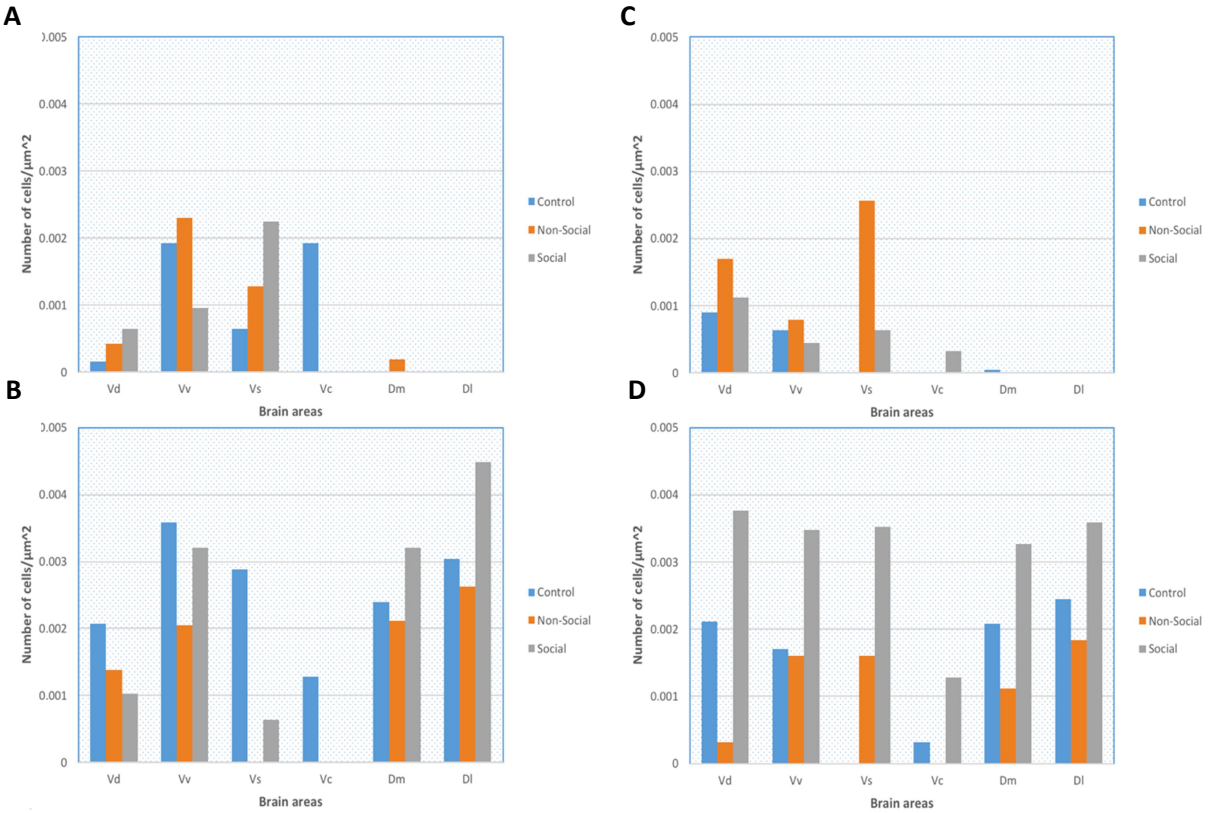

Figure 11. Measured pS6 activated cells and dopaminergic neurons in six different brain areas (Vs, Vv, Vs, Vc, Dm an Dl) in the six experimental groups (control, social and non-social groups for the two genotypes – WT an KO) (n=1 in each group): (A) dopaminergic neurons density in KO individuals; (B) pS6 activated cell density in KO individuals; (C) dopaminergic neurons density in WT individuals; (D) pS6 activated cell density in WT individuals

4. Discussion

In the present project we performed, for the first time, a social CPP paradigm, with a group of conspecifics as reward, in adult male zebrafish. Our results demonstrate that zebrafish revert their behaviour with this approach. Additionally, we showed that IT modulates the response to social reward.

Given the role of OXT in the regulation of social behaviour across vertebrates **(Donaldson & Young, 2008; Insel & Young, 2001)** we hypothesized that IT (the fish homologue of OXT) can be involved in social reward in zebrafish. To test this hypothesis, we have used a CPP paradigm to assess the rewarding value of conspecifics versus an established non-social reward in zebrafish (e.g. food). To assess the impact of IT in the social reward system, we used a KO line for the IT receptor.

CPP enables the study of rewarding effects of stimuli, through the assessment of the change in place preference of the individual, and is usually used to assess the effects of drugs and alcohol **(Prus et al., 2017)**. It has been shown that the visualization of a real shoal as a stimulus can be used as a reward in learning experiments **(Al-imari & Gerlai, 2008; Bee, 2009).** In this project, we used a real shoal as a stimulus not only with visual but also with physical contact with the focal fish.

Initial optimizations of the paradigm had to be performed in order to establish a protocol where a change in individual's place preference could be observed. Thus, we noticed that fish would learn better the place-reward association when visually restricted to one zone of the tank during the exposure to the reward. Also, fish responded better to the non-social stimulus (food) when it was administered every five minutes for the 30 minutes of conditioning phase, rather than when given at a single point in time.

We started by conducting a pilot test not only to evaluate the optimizations of the setup and the protocol, but also to confirm if zebrafish are more motivated to approach a social stimulus, rather than a non-social one. In this experiment, fish were isolated for 24 hours before the behavioural assay in order to increase the response towards the reward stimuli. The group of fish treated with a social stimulus was not able to be in contact (visual or physical) with other individuals, and the group of fish treated with a non-social stimulus was not fed during this period. As expected, it was observed that zebrafish reverted their first preference, spending more time in the zone of the tank where it previously received a social stimulus as a reward (group of conspecifics) as well as where it previously received a non-social stimulus as a reward (food), than where it did not receive any reward (control group). Although there were no significant differences between the treatments, indicating that these individuals attribute the same reward value to both stimuli.

These results show that the individuals can learn to associate the zone of the tank where they received the rewards, with the rewards themselves, either social or non-social. Moreover, they

indicate that individuals attribute a high reward value to the both presented rewards, and that a group of conspecifics can be used as a reward in this paradigm.
Given the behavioural results, we applied the same paradigm to the mutant line for the IT receptor to respond to our second question, if IT was involved in the social reward system. This peptide, which is conserved across vertebrate taxa **(Goodson & Kingsbury, 2011)**, has attracted significant scientific interest due to its important role in social cognition and behaviour across vertebrates **(Quintana et al., 2016).**

As the translation of IT is mediated for its receptor **(Wircer et al., 2015)**, KO individuals have the IT signalling pathway blocked. Thus, we are able to study the role of this peptide by comparing WT and KO individuals.

The protocol followed was the same as in the pilot test. This assay indicated that WT fish reverted their initial preference, spending more time in the zone of the tank where they previously received a social stimulus as a reward, as well as when they previously received a non-social stimulus as a reward. These results did not present significant differences between each other, indicating that WT individuals attribute the same reward value both to food and to conspecifics.

There were also significant differences when comparing the control group with the KO fish conditioned to the non-social stimulus, as these individuals reverted their initial preference, spending more time in the zone of the tank where they previously were exposed to the stimulus. However, the same did not happened when the KO fish were conditioned with the social stimulus, as they did not revert their initial preference and the CPP scores were similar to those of the control group. These results indicate that for the OXT_KO only the non-social stimulus have reward value, suggesting that OXT signalling is needed for social reward.

Although the results obtained in this experiment are in accordance to what was expected by the literature regarding OXT role in social behaviour, it is necessary to take into consideration that isotocin has cross-reactivity with vasotocin. Although with lower affinity, OXT can also bind to vasopressin receptors, and vice-versa, which makes it complicated to interpret data from social behaviour assays **(Engelmann *et al.*, 2000; Kelly & Goodson, 2014).**

On the other hand, this impairment of the KO individuals' social group may be explained by the interaction of the focal fish with the social stimulus (group of conspecifics) during the conditioning phase of the test. Several types of interactions between the individuals may be happening during this phase, as the case of aggression and stress behaviours, for example. Since these individuals have the OXT signalling pathway blocked, and since this hormone is so called the "love hormone", maybe the lack of it may be translated into these aggressive types of behaviours. So, it would be interesting to confirm this hypothesis with a supplementary analysis of the conditioning records. Advanced techniques of behavioural analysis like machine learning techniques applied to the detection of complex behavioural patterns in streams of behavioural data may be used to check if there is a pattern within the KO individuals' behaviours.

Understanding the role of IT in social interactions is very important for translational science. The significance of the basic social functions for individuals to survive and succeed is clarified in human disorders, such as autism, schizophrenia and social anxiety, in which OXT signalling is disrupted. This neuromodulator has been found to have a key role in the regulation of these complex social disorders **(Reddon *et al.*, 2012)**. Children with autism spectrum disorder, for example, show significantly lower plasma OXT compared with normally developing children **(Platt *et al.*, 2016)**.

The final aim of this project was to uncover the neural mechanisms underlying this social reward system by looking into the activated brain regions and the dopaminergic neurons, with the aim to identify the brain regions that encode reward and the role of dopamine in these regions.

As previously mentioned, the activation of brain regions can be assessed through *in situ* hybridization (ISH) or immunohistochemistry (IHC) techniques for neuronal activity markers, such as *c-fos* or pS6. The characterization of the dopaminergic system can be achieved through the marking of TH. However, we must have in mind that TH is a precursor of the catecholamine biosynthetic pathway and, thus, it is common to all catecholamines, not being specific to dopamine **(PasterKamp, R *et al.*, 2009)**.

As we had two molecular approaches available, we needed to accomplish a consensus time limit for the sampling of focal individuals' brains. Thus, we decide to sacrifice the animals at 30 minutes after the final test because it is the peak of expression of *c-fos* mRNA as well as of serine phosphorilation. In fact, this expression is maintained for hours in both *c-fos* and pS6 **(Guzowski *et al.*, 2001; Pirbhoy *et al.*, 2016)**. However, we preferred to sacrifice the animals at 30 minutes to be sure that the stimulus we would measure were due to the behavioural experiment. In this way we could use the samples for both molecular techniques.

We performed double ISH-IHC technique as a first approach because it is widely used and has obtained good results **(Escobar *et al.*, 2013; Servili *et al.*, 2011)**. Also, *c-fos* is the most commonly neuronal activity marker used in neuroscience **(Kovács, 1998, 2008)**.

As mentioned in the Results, although the integrity of the probe had been proved, the double ISH-IHC technique only revealed the IHC marking. These results may translate a weak signal generated from the stimuli, or even "weak" stimuli, in the sense that the stimuli used in this experiment are already known and experienced for the focal fish, and thus they might not generate high response in the brain. Other explanations for this protocol not to work might be related with the ISH protocol or even with the transition step of the two techniques.

These results probably indicate that the ISH-IHC double technique assay still needs further optimization, namely hybridization temperature and post-hybridization washes salt concentration. Among other possible problems, reagents contaminations with RNase enzyme or low concentration of the probe may also explain the obtained results.

Since this technique didn't show results, and due to the lack of time to perform optimizations, we followed a second approach that was implemented in the lab.

The obtained results demonstrated different activation of the telencephalic area not only between genotypes, but also between experimental groups. However, it is important to have in account that the presented results are merely representative examples since they correspond only to one animal per group.

We analysed six brain areas of interest related with the social decision making network (SDM) – Vv (the homologue for lateral septum in mammals) and Vs (the homologue for medial amygdala and BNST in mammals) – as well as to the mesolimbic reward system – Vd (the homologue for NAcc in mammals), Vc (the homologue for striatum in mammals), Dm (the homologue basolateral amygdala in mammals) and Dl (the homologue for hippocampus in mammals) **(O'Connell & Hofmann, 2011)**.

pS6 is induced by the biochemical activation of neurons, and therefore constitute a marker that may be most sensitive to certain stimuli that modulate neurons, such as neuropeptides. Besides many stimuli that activate neurons induce pS6, brain regions that have a high level of this ribosomal protein at baseline may be less amenable to variations in treated groups **(Knight et al., 2012)**. This can be observed in the density of pS6 activated cells in the KO animals that do not present significant differences between the control and treated groups. However, this variation can be observed in the WT animals. The impairment between the control and social groups reveals the great importance of social stimulation. These results support the literature, since it has been proved that the deletion of genes related with social disorders, which are related with lower levels of OXT in the system, increases the pS6 activation **(Lipton & Sahin, 2014)**.

The main difference observed refers to the social treatment between genotypes. Although Vc area presents activation in the WT social treated group contrary to what happens in the KO social treatment, the major difference can be detected in the Vd and Vs areas. These areas present a much higher cell density in the WT animals, being cell density 3.7 times higher in Vd and 5.5 times higher in Vs, comparing to the KO animals.

As mentioned before, Vd area is the homologue for NAcc in mammals, that is the central integrator of sensorimotor information that facilitates a favourable behavioural output of either approach or avoidance of a stimulus. In teleosts, this area is rich in GABA immunoreactivity and dopamine receptors. However, this area is also a striatal-like region of the mammalian basal ganglia, playing an important role in reinforcement learning, suggesting a homology relationship between the Vd area in zebrafish and striatum and NAcc in mammals. Regarding Vs, the homologue for medial amygdala and BNST in mammals, it plays a role in motivational aspects, can generate long excitatory effects on dopaminergic neurons and modulates bonding behaviours **(O'Connell & Hofmann, 2011)**.

Despite these results, no co-localization between pS6 and TH labelled cells was observed. Yet, since this quantitative analysis is still ongoing, and we only analysed one animal per group, it is not possible to conclude if there are, in fact, differences in the density of cells activated in the different brain regions between the two genotypes, as well as co-localization of the referred activated cells. However, looking into the major obtained differences, we can hypothesize that Vd and Vs areas are key points for the interaction regulation of the social reward system with OXT.

The performance of a treatment with dopamine receptor antagonists in the WT fish would be a hypothesis to test if these individuals' social reward would be abrogated.

Yet, since the zebrafish has two receptor isoforms for IT, and the mutant line used in this experiment was KO to only one of them, it would also be interesting to conduct a triple-immunohistochemistry assay for the IT receptor together with TH and pS6. This assay would assess if the KO individuals still had activation of the isotocinergic pathway, focusing in brain regions that have been shown to present receptors for IT in social fish **(Huffman et al., 2012)**.

5. Conclusion

The present project leads us to infer that a social stimulus has the same reward property as a non-social stimulus for TU male zebrafish.

We performed, for the first time, a social conditioned place preference with zebrafish species and we were able to demonstrate that adult TU zebrafish perform conditioned place preference towards a social reward of conspecifics.

Nevertheless, we were also able to determine that KO animals for the isotocin receptor show an impairment only in social tasks in the CPP paradigm, showing that oxytocin-like peptides may be involved in social reward.

Additionally, this study highlights the significance of social stimulation by revealing important differences in brain activation.

Nevertheless, further immunohistochemical assays need to be performed in order for us to understand the action of oxytocin in the regulation of the social reward system. However, the obtained results propose that the dorsal nucleus of ventral telencephalic area (the homologue for NAcc in mammals) and the supracommissural nucleus of ventral telencephalic area (the homologue medial amygdala and BNST in mammals) are key points for understanding the role of this peptide in the referred system.

Future perspectives include the consolidation of the behavioural data and the performance of a treatment of WT fish with dopamine receptor antagonists to verify if it will abolish social reward.

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7. Annexes

7.1 Genotyping

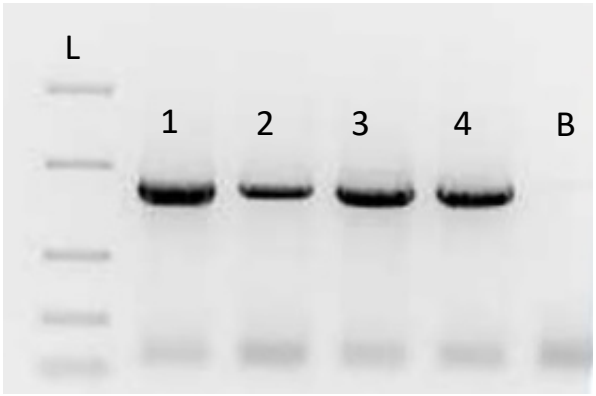

Figure S 1. PCR analise in a 1% agarose gel electrophoresis of genomic DNA for further genotyping (L) DNA ladder (Gene Ruler – Thermo Fisher); (1, 2, 3, 4) genomic samples; (B) blanck.

7.2 Optimization of setup procedures and protocols

Initial optimization of the setup procedures and protocols of the behavioural assays were performed (n=10), namely the position of the recording cameras, the illumination of the setup box, the administration protocol of the non-social reward (food) **(Figure S 2 A)** and the type of partitions used during the conditioning phase (opaque or transparent) **(Figure S 2 B)**. As it can be observed, fish had a tendency to learn better when they performed two conditioning sessions in different days and half bloodworm was administered every five minutes. Individuals also had a tendency to associate one tank environment with the reward when the partitions were opaque in this part of the test, as they were both physically and visually restricted to that environment.

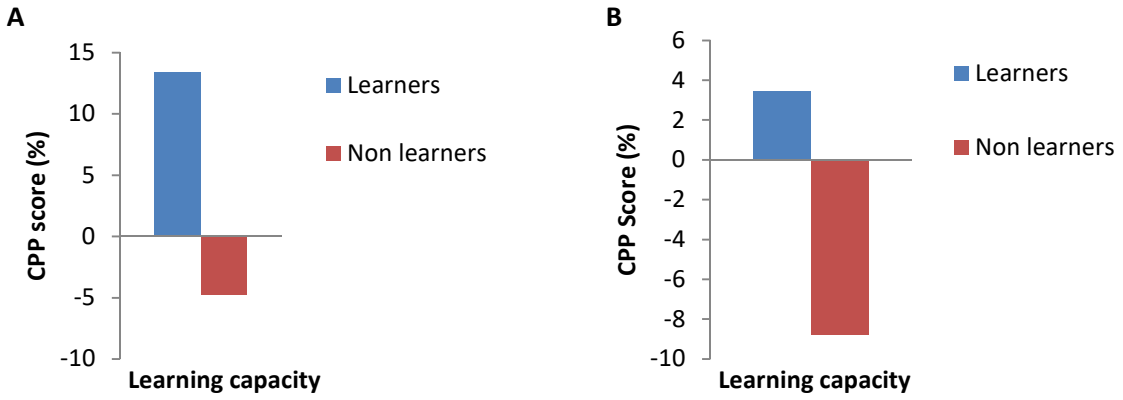

Figure S 2. Optimization of CPP protocol: (A) optimization of zebrafish's response to the non-social stimulus: administration od half bloodworm every 5 minutes for 30 minutes versus administration of one bloodworm every 10 minutes for 30 minutes; (B) optimization of the CPP setup during the conditioning phase: opaque versus transparent partitions.

7.3 Double *In Situ* Hybridization - Immunohistochemistry

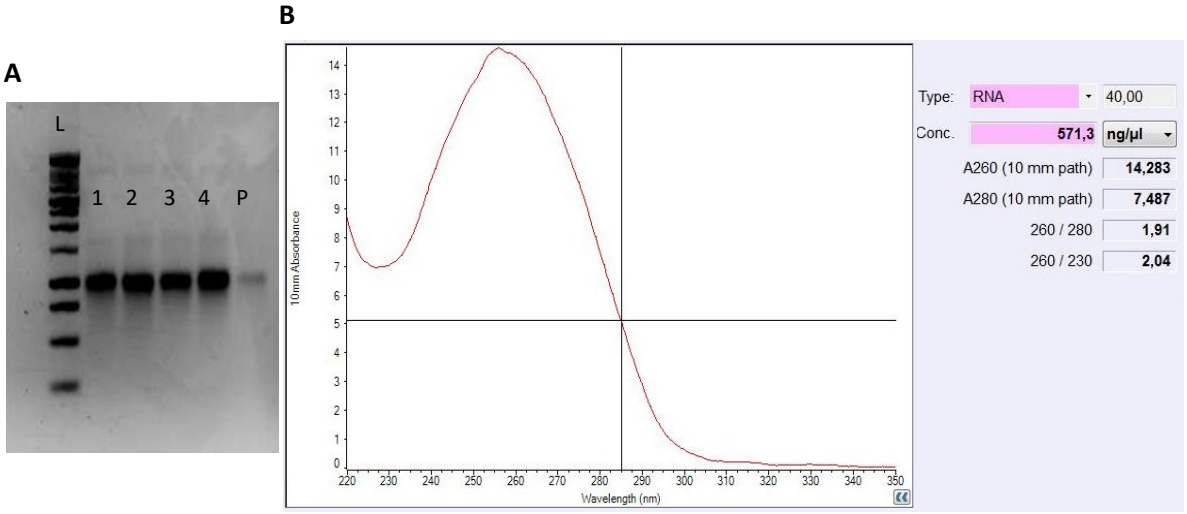

Figure S 3. Verification of probe's integrity: (A) 1% agarose gel electrophoresis of bacterial linear DNA (1,2,3,4) and final probe (P), (L) DNA ladder (Gene Ruler – Thermo Fisher); (B) nucleic acid quantification: spectrum of purified RNA without contamination.