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INSTITUTO UNIVERSITÁRIO

CIÊNCIAS PSICOLÓGICAS, SOCIAIS E DA VIDA

NEUROBEHAVIOURAL AND MOLECULAR MECHANISMS OF SOCIAL LEARNING IN ZEBRAFISH

Júlia Sabrina Ferreira Pinho

Dissertation presented to obtain the Ph.D. degree in

Behavioural Biology

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Júlia Sabrina Ferreira Pinho

Dissertation supervised by Rui Filipe Nunes Pais de Oliveira, Ph.D. and Professor at ISPA – Instituto Universitário, principal investigator at Instituto Gulbenkian de Ciência and associate researcher at Champalimaud Neuroscience Programme

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Palavras-chave

Aprendizagem social, circuitos neurais, Peixe-zebra

Keywords

Social learning, neural circuitry, zebrafish

PsycINFO Classification Categories and Codes

2400 Animal experimental & Comparative Psychology

- 2420 Learning & Motivation
- 2440 Social & Instinctive behaviour

2500 Psysiological Psychology & Neuroscience

- 2510 Genetics
- 2540 Physiological Processes

Dedicatória

Para os avôs Domingos e a avó Erminda

Resumo:

Os animais utilizam informação social e não social para tomarem decisões adaptativas que tem impacto no seu *fitness*. O uso de informação social traz vantagens como escapar a um predador, encontrar fontes de comida ou evitar lutas com indivíduos mais fortes, apenas por observação dos seus conspecíficos ou produtos relacionados com eles. A aprendizagem social ocorre quando os indivíduos observam o comportamento de outros ou as suas consequências para modificar o seu próprio comportamento. Esta estratégia comportamental é conservada entre espécies: os grilos, *Nemobius sylvestris,* adaptam o seu comportamento para evitar um predador depois de observar o comportamento de outros e mantem essas mudanças comportamentais, duradouramente, mesmo apos os demonstradores não estarem presentes; as abelhas operárias, *Apis Mellifera*, apresentam uma série de comportamentos motores estereotipados que informam outras operárias da localização precisa de uma fonte de comida.

Os mecanismos neuronais da aprendizagem social não estão claramente compreendidos, e são o centro de debate nesta área de investigação. Alguns autores hipotetizam que os mecanismos neurais da aprendizagem social são partilhados, e outros autores defendem que a aprendizagem social é um domínio geral presente até em espécies solitárias.

O principal objetivo deste trabalho é clarificar os mecanismos subjacentes a aprendizagem social e não social. Este trabalho subdivide-se em dois capítulos experimentais: o capítulo II, onde procuramos os circuitos neurais do condicionamento observado com um estímulo social ou não social; e capitulo III, no qual a eficácia de estímulos sociais químicos e visuais é testada num paradigma de condicionamento aversivo. Em ambos os capítulos, um gene de ativação imediata são usados como marcadores de atividade neuronal: no capítulo II utilizando a expressão de *c-fos*, por hibridação *in-situ*, para mapear as regiões do cérebro recrutadas em aprendizagem social e não social; e no capítulo III, a reação quantitativa em cadeia da polimerase foi utilizada numa abordagem com genes e regiões do cérebro candidatas para perceber o envolvimento do sistema olfativo em aprendizagem social olfativa.

No capítulo II, nós demonstramos que a aprendizagem social (SL) recruta diferentes regiões do cérebro quando comparada com a aprendizagem não social (AL): SL aumenta a expressão de *c-fos* nos bulbos olfativos, na zona ventral da área telencefálica ventral, na habénula ventral, no tálamo ventromedial e a AL diminui a expressão de *c-fos* na habénula dorsal e no núcleo tubercular anterior. Alem disso, conjuntos diferenciais de regiões cerebrais aparecem associados a aprendizagem social e não social depois de uma análise funcional da conectividade entre as regiões do cérebro.

No capítulo III, nós mostramos que pistas sociais visuais, como a observação de um conspecífico a exibir uma resposta de alarme, não é eficaz como um estimulo não condicionado (US), mas pistas sociais olfativas, como substância de alarme, foi altamente eficiente como US em aprendizagem aversiva. Além disso, identificamos os bulbos olfativos como uma área do cérebro essencial para condicionamento observado olfativo. Uma análise funcional da coesão e conectividade dos núcleos do cérebro envolvidos em processamento olfativo mostraram uma rede apurada para condicionamento observado olfativo.

Em resumo, a presente tese elucida o debate nesta área de investigação sobre os mecanismos da aprendizagem social. Este trabalho clarifica que ao nível comportamental a aprendizagem social requer um domínio geral e ao nível neuronal é necessária uma rede modular que permite a computação em simultâneo de várias informações com diferentes níveis de complexidade.

Abstract:

Animals use social and asocial information to take adaptive decisions that impact their fitness. The use of social information brings advantages as to escape a predator, to find a food source or to avoid fights with strongest individuals, only by the observation of conspecifics or their related products. Social learning occurs when individuals observe the behaviour of others, or its consequences, to modify their own behaviour. This behavioural strategy is highly conserved across taxa: the crickets, *Nemobius sylvestris,* adapt their predator-avoidance behaviour after having observed the behaviour of knowledgeable others, and they maintain these behavioural changes lastingly after demonstrators are gone; the foragers of honeybees, Apis mellifera, display a series of stereotypical motor behaviours which inform other foragers of the precise location of floral food.

The neuronal mechanisms of social learning are not clearly understood, and they are in centre of debate in the field. Some authors hypothesized that the neural mechanisms of social learning are shared and others that social learning is a general domain present even in solitary species.

The main goal of the present work is to clarify the mechanisms underlying social and asocial learning. This work subdivide in two experimental chapters: the chapter II, where we search for the neuronal circuits of reward observational conditioning with social or asocial stimuli; and the chapter III, in which the effectiveness of a chemical and a visual social stimulus are tested as unconditioned stimulus (US) in an aversive learning paradigm. In both chapters, an immediate early gene is used as a marker of neuronal activity: in chapter II using the expression of *c-fos*, by *in-situ* hybridization, to map the brain regions recruited in social and asocial learning; and in chapter III, the quantitative polymerase chain reaction (pPCR) was used in a candidate genes and brain regions approach.

In chapter II, we demonstrated that social learning (SL) recruit different brain regions than asocial learning (AL): SL increased the expression of *c-fos* in olfactory bulbs, in ventral zone of ventral telencephalic area, in ventral habenula, in ventromedial thalamus and AL decreased the expression of *c-fos* in dorsal habenula and in anterior tubercular nucleus. Moreover, differential sets of brain regions appear associated to social and asocial learning after a functional connectivity analysis.

In chapter III, we showed that the social visual cue, the sight of alarmed conspecifics, was not effective as an US; but social olfactory cue, the alarm substance, was highly efficient in aversive learning paradigm. Also, we identified the olfactory bulbs as an essential brain region to olfactory observational conditioning. A functional analysis of the cohesion and connectivity of the brain nuclei involved in olfactory processing were tuned to chemical observational conditioning.

In sum, the present thesis elucidated the debate in the field on the mechanisms of social learning. This work clarified that at the behavioural level social learning proved to be a general domain, and at the neuronal level a modular network is needed to allow the computation, at the same time, of high amount information with different levels of complexity.

Author Contributions

Julia Pinho (J.P.) and Rui Filipe Oliveira (R.F.O.) designed the experiments and established the behavioural protocols. J.P performed the behavioural experiments, processed all brains samples, quantified and analyzed the gene expression, and performed the functional connectivity analysis. The gene expression was measured by quantitative Polymerase Chain Reaction (qPCR) in collaboration to João Sollari (J. S.) or by *in-situ* hybridization in collaboration to Vincent Cunliffe (V.C.).

These studies presented in chapters II and III will be scientific papers in preparation to be submitted on international journal. J.P and R.F.O wrote the scientific papers with the collaboration from V.C.

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Chapter 1. General Introduction

1.1 Social information

We live in a world where individuals constantly interact with others, adjusting their behaviour to the surrounding environment, in order to reproduce and survive. They use social and asocial information to take adaptive decisions that impact their fitness. Social information, obtained either by observation or interaction with others, or by direct contact with their related products (e.g. a deformed object by other animal or an urine odor) (Heyes, 1994; Hoppitt and Laland, 2013), can be used later on to make important decisions. For instance, when a fish is wounded, it releases an alarm pheromone from its skin. Others will sense this substance signalling threat and will behave accordingly with a strong fear response characterized by erratic movements (a rapid zigzag movement to escape a predator) and freezing (a secondary strategy to hide themselves in the environment). In addition, there are also cues not related to individuals (so called asocial information) that influence the behaviour of animals, for instances, the migration intensity in several species is proved to be mainly predicted by weather changes (Doren and Horton, 2018).

Social and asocial cues are abundant in the environment, and individuals constantly select information and make decisions based on their previous experiences. Eavesdropping is a perfect example where animals shifts their behaviour towards others after observing them (Abril-de-Abreu et al., 2015). In addition, the increase in social information use is essential for the individual fitness. For instance, using social cues, animals can avoid predators and dangerous situations, reducing the escape costs and increasing reproduction success in safe environments (Seppanen et al., 2007).

This role of social information depends of the knowledge between individuals that share the same environment. Animals can be unreliable sources, commonly exhibiting false positive behaviours in safe environments (e.g. expressing fear behaviour without a presence of threat) and social groups are essential to identify who are trustable sources of relevant information. The origin of social information is also crucial since information out of time or space loses all the interest. For instance, an alarm call about a predator happens at a certain time, at a certain location, and is about specific things in the environment; it has little value for decision making a few years later, a kilometre away, or for an individual with a completely different set of predators (Seppanen et al., 2007). Also, each environment presents different sensory demands; the visual information is less value than auditory and chemical cues in low visibility environments (Seppanen et al., 2007). Hence, social information use is an adaptive trade-off to access accurate and reliable information with the less cost possible to better adapt to changing environments (Boyd and Richerson, 1985).

1.2 The role of social environment in brain and cognition

Social and asocial information is processed in the brain. The neuronal mechanism underlying each type of information is still not fully characterized. Pioneer work suggests that better cognitive performance is correlated with the complexity of the social environment (Buechel et al., 2018; Corral-López et al., 2017; Kotrschal et al., 2013, 2015, 2017a; Winer and Prater, 1966), one of the main driving forces for brain evolution (Social brain hypothesis SBH, (Dunbar, 1998), and this seems to be a conserved process across species (Costa et al., 2011; Garamszegi and Eens, 2004; Kotrschal et al., 2012, 2017b; Liedtke et al., 2011; Madden, 2001; Reader et al., 2011). Relative larger brain area sizes were observed in animals living in more complex environments (such as social environments) first in humans (Dunbar, 2009) and also in other taxa (Burish et al., 2004; Emery et al., 2007; Gonzalez-Voyer et al., 2009; Lihoreau et al., 2012; Pérez-Barbería et al., 2007; Pollen et al., 2007). For instance in the cichlid fish *Neolamprologus* pulcher, individuals reared in larger groups, exhibit larger hypothalamus, cerebellum and optic tectum, without changes in total brain size (Fischer et al., 2015).

Despite several works supporting the SBH, where brain area size could be a predictor for better performances in several cognitive tasks, this does not explain why some species present qualitative rather than quantitative differences (Dunbar and Shultz, 2007), neither how small-brained animals (such as bees) are able to perform cognitively demanding tasks in complex environments (Chittka, 2017; Chittka and Niven, 2009; Loukola et al., 2017; Oliveira, 2013).

Additional work suggests that basic cognitive abilities are formed by building blocks of cognitive processes conserved across taxa (Chittka and Skorupski, 2011; Oliveira, 2013; Theobald, 2014) and also that single neurons can be re-used as components of multiple circuits used in different processes (Niven and Chittka, 2010). The brain social decision-making (SDM) network is a clear example of functional modularity, where the expression of social behaviour, across vertebrates, can be better explain by a network than a single of its nodes (O'Connell and Hofmann, 2012). This network is composed by two interconnected neural circuits: the social behaviour network (Goodson, 2005; Newman, 1999) and the mesolimbic reward circuit (Adinoff, 2004), where the expression of genetic markers, hormone receptors, and neurochemical/ neurotransmitter systems establish homologies across taxa. The social behaviour network is composed by six nodes located in the forebrain and midbrain areas [i.e. bed nucleus of the stria terminalis / extended medial amygdala (BNST/meAMY), lateral septum (LS), preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus, and the periaqueductal gray (PAG)], that are reciprocally connected and that together regulate several dimensions of sociality (sexual behaviour, parental behaviour, and different forms of aggressive

behaviour) demonstrated in humans (Newman, 1999) and non-humans (Goodson, 2005). O'Connell and Hoffman (O'Connell and Hofmann, 2012) proposed the inclusion of the mesolimbic reward system, which is generally assumed to evaluate stimulus salience via dopaminergic signalling (Wickens et al., 2007) and shares overlapping nodes with the SBN (lateral septum and bed nucleus of the stria terminalis). According to the SDM network hypothesis, the same neural circuit may underlie the expression of different behaviours depending on social context (Teles et al., 2016), implying that each behavioural state is better represented by the overall profile of activation/ connectivity across the network rather than for the activity of a single node.

Living in complex environments force animals to adjust their behaviour, phenotypic plasticity, and this drives the evolution of brains capable to process cognitively demanding tasks that are conserved across taxa. Hence, the neural mechanisms underlie social behaviour can be dissected using less complex model organisms, such as zebrafish (Oliveira, 2009).

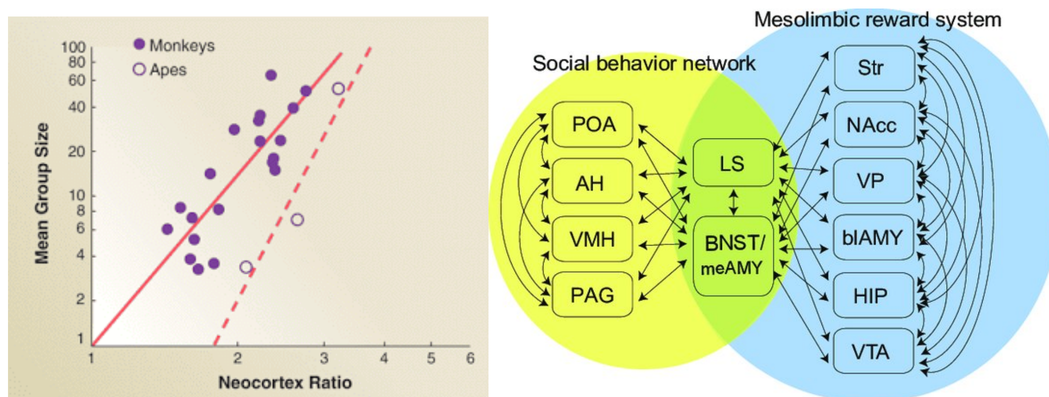

Figure 1.1 - Social information processing in the brain. A) Social Brain Hypothesis (SBH), where the increase of neocortex ratio were correlated with large groups of animals associated with a more complex environment (Dunbar, 1998). B) The vertebrate social decision making network where the social behaviour network and the mesolimbic reward system are enough to explain social behaviour (O'Connell and Hofmann, 2012).

1.3 Zebrafish as a model organism

Zebrafish is a species with a wide repertoire of social behaviour, including affiliative behaviour (as shoaling, mating and aggression) and capable of performing cognitive tasks (as attention and social learning) (Taborsky and Oliveira, 2012).

With the genome completely sequenced, we can nowadays target the function of specific genes due to the development of a wide genetic toolbox, which allows us to reveal the cellular and molecular

mechanisms underlying zebrafish behaviour (Koster and Sassen, 2015). Forward genetic screens have the potential to cover the entire genome and identify numerous mutations leading to the localization and identification of the genes and molecular pathways involved (Haffter et al., 1996). In addition, numerous genetic markers have been developed for the zebrafish (Patton and Zon, 2001) and along with other methods (Gerlai, 2010; Stewart et al., 2014), identification of the genes harbouring the mutations is within our reach.

Zebrafish development is relatively fast, reaching maturity at 3 month old (Spence et al., 2008). Due to low dimension of the zebrafish, we can grow large numbers of fish in a relatively small space when compared to rodents (Segner, 2009).

Several authors demonstrated zebrafish learning abilities: one-trial avoidance learning paradigm (Blank et al., 2009), olfactory conditioning (Braubach et al., 2009), place conditioning (Eddins et al., 2009), appetitive choice discrimination (Bilotta et al., 2005), active avoidance conditioning (Xu et al., 2007), visual discrimination learning in T-maze (Colwill et al., 2005), and an automated learning paradigm (Hicks et al., 2006). Hence, zebrafish has become a good model organism to study the neuronal mechanisms of social cognition, in particular learning processes.

Box 1 – Learning mechanisms definitions adapted by Heyes, C. (Heyes, 1994). Learning is a change in an animal caused by experience detected later in the animal´s behaviour (Rescorla, 1988). Different learning mechanisms are evolutionarily conserved and described in several species:

1) Habituation (H) and sensitization (S) is a non-associative learning where animals decreasing (H) or increasing (S) the response to a stimulus as result of repeated stimulation (Rose and Rankin, 2001; Thompson and Spencer, 1966). For instance, territorial individuals that show reduced aggression to familiar neighbours (Temeles, 1994).

2) Classical conditioning describes a form of associative learning in which animals exhibit a conditioned response (CR) to a conditioned stimulus (CS), after repetitive presentation to conditioned stimulus (CS, commonly a neutral stimulus) paired with and unconditioned stimulus (US, stimulus that induces unconditioned response). For example, Pavlov's dogs salivated (CR) in response to a sound (CS) that had been previously associated with food (US) (Pavlov, 1927)

3) Operant conditioning defines a form of associative learning in which behaviours are dependent on, or controlled by its rewards and consequences. The CS signals the conditioned behaviour, only after the animal operates, the individual receive the reward or the punishment (US). For example, conditioned rats press a lever to receive a food pellet, after hear a sound. If the animal press the lever, without this action is preceded by the sound, any reward or punishment is delivery (Skinner, 1937).

4) Latent learning is related to acquisition of latent information (information intentionally acquired and non-motivated by a reward or punishment) and latter this information would add advantageous in a particular situation (Jensen, 2006). For instance, animals familiarized with an environment escape better to a predator than non familiarized individuals (Metzgar, 1967).

5) Social learning is a form of learning that is influenced by observation of, or interaction with, another animal or their products (Heyes, 1994). This is a broad definition that includes several social learning mechanisms described below:

a. Stimulus enhancement refers to a type of single-stimulus learning where the actions of a demonstrator towards a stimulus causes the observer to increase the response towards that stimulus (Spence, 1937). For example, rats express preference for a specific type of food after interacting with other individuals that had just consumed that type of food and still have the smell on (Galef, 1988).

b. Local enhancement refers to a type of single-stimulus learning in which an observer visit or interact more with objects in a particular location, after a demonstrator appears, or interact with objects at this location (Thorpe, 1963). For instance, untrained guppies preferentially use the route of their demonstrators to find food, after share some days interacting with them (Laland and Williams, 1997).

c. Observational conditioning is a stimulus-stimulus learning in which the observation of the behaviour of others, or its products, is associated with a reward or a punishment in a Pavlovian conditioning or operant conditioning task (Heyes, 1994). For example, rhesus monkey (*Macaca mulatta*) presents a fear response towards snakes only after observing a fearful response of a demonstrator towards a snake (Hoppitt and Laland, 2013).

d. Social facilitation refers to the mere presence of a demonstrator be enough to affect the motivational level of the observer. For example, the presence of the female audience in ciclids, Oreochomis mossambicus, increased territorial defence in males (audience effect, 194).

e. Response facilitation occurs when the presence of a demonstrator performing an act increases the probability of an animal that sees it doing the same (Byrne, 1994). For example, groups of juvenile walleye pollock, *Theragra chalcogramma*, are more successful at exploiting spatially variable ephemeral food patches when compared with an individual alone (Ryer and Olla, 1992).

f. Contagion is an instinctive behaviour that triggered the performance of the same behaviour in another animal (Thorpe, 1963). For instance, yawns are triggered only by a video of another person yawning (Provine, 1986).

g. Imitation defines an observer that copies intentionally and accurately new actions by observation of a demonstrator (Byrne, 1992; Byrne and Tomasello, 1995). For example, a demonstrator grasping a fruit and then pull back to detach it from its stem facilitates an observer to copy the exact sequence of small actions (Hoppitt and Laland, 2013).

h. Emulation occurs when, after observing a demonstrator interacting with objects in its environment, an observer becomes more likely to perform any actions that have a similar effect on those objects (Whiten and Ham, 1992). For example, capuchin monkeys display only a way to open a plastic bottle after a demonstrator exhibit alternative actions to achieve this task (poking, pulling while twisting out a pair of smooth (Custance, D., Whiten, A., & Fredman, 1999).

i. Coaching represents the animals that encourage or discourage a response of an observer. For instances, dolphins copied a diver cleaning the windows of their aquarium (Moore, 1992).

1.4 Social cognition

Zebrafish is able to perform a set of cognitively demanding tasks in the social domain such as collecting information from others, and learning from or about others. This information is used to make decisions in challenging environments throughout their life. These choices drive the evolution of a competent brain able to learn new skills and also adjust their performance in accordance to the requirements of the environment (Georga and Koumoundouros, 2010).

1.4.1 Collecting information from others

Several works have demonstrated that zebrafish are attentive to their conspecifics, collecting information from them, an ability defined as social attention (Abril-De-Abreu et al., 2015; Braida et al., 2014; Echevarria et al., 2011). Different measures can be used to infer attention as the spatial distribution in the arena in relation to a stimulus fish, time spent near the stimulus and the orientation towards the stimulus fish (Abril-De-Abreu et al., 2015). Previous work also demonstrated that zebrafish display attention both to live conspecifics and to video playbacks of conspecific stimuli, without differences between both (Qin et al., 2014). For this reason, videos were an effective tool able to induce social attention and also they can be easily manipulated and standardized (Blake et al., 2003; Chouinard-Thuly et al., 2017; Fangmeier et al., 2018; Qin et al., 2014; Rosa-Salva et al., 2018; Saverino and Gerlai, 2008; Schultz et al., 2005; Vallortigara, 2018). Animals are attentive to others and collect information that can be used later on to make decisions when they need to perform more cognitively demanding tasks as learning from or about others.

1.4.2 Learning about others

Authors have also demonstrated that zebrafish learn about conspecifics and that this information is used when interacting with the observed animal in subsequent encounters (social eavesdropping) (Peake, 2005). The use of eavesdropped information in zebrafish depends on social status, with dominant bystander males paying more attention to losers than winners. It should be noted that, zebrafish are also able to recognize winners and losers, and they use this information to avoid fighting with the strongest individuals (Abril-de-Abreu et al., 2015).

Species recognition has also been shown in zebrafish, where individuals discriminate conspecifics from heterospecifics (Engeszer et al., 2007). For instance, animals placed in a tank with a

group of fishes tend to stay close to each other than to heterospecifics (Saverino and Gerlai, 2008). Also zebrafish perform individual-recognition within conspecifics, where they demonstrate a higher preference to novel rather than a familiar fish (Barba-Escobedo and Gould, 2012; Gerlach et al., 2008; Madeira and Oliveira, 2017; Saverino and Gerlai, 2008).

The use of previous information to infer unknown related relationships (social inference) is not yet demonstrated in zebrafish but clearly described in humans (Piaget, 1928) and in non-mammals (Grosenick et al., 2007). When animals learn about others (as in social eavesdropping), they can successfully make inferences on the hierarchy of a group by pairwise analysis of fights between rival and adjusting their own behaviour accordingly (Grosenick et al., 2007).

1.4.3 Learning from others

Individuals can learn from others the levels of threat in the environment or the sources of reward and ways to access to them (Nunes et al., 2017) through social learning.

One of the most used aversive cues in zebrafish is alarm cue or "Schreckstoff" (as proposed by Karl von Frisch who first described it in minnows; (Von Frisch, 1941)). This social cue is a chemical pheromone released from the skin of the fish when they are attacked by a predator. This substance can be obtained by extracts from the skin of a fish, inducing the same stereotyped response: a burst of rapid erratic swimming followed by freezing (Speedie and Gerlai, 2008).

The active compound that elicits that alarm response is still not clearly known. Hypoxanthine-3 N-oxide has been initially proposed as the active compound that elicits the alarm response (Parra et al., 2009) but it has not been the only one detected in the skin. More recently, a mixture that includes the glycosaminoglycan (GAG) chondroitin, has also been identified and has been proposed to be the major compound of the alarm substance in zebrafish skin (Mathuru et al., 2012).

Several work demonstrate that fish are able to use social cues to learn about danger, associating a neutral cue to fearful one (Suboski et al., 1990). For instance, individuals can learn from others the location of a predator (Lindeyer and Reader, 2010) and address the threat levels in the environment becoming more bold after interacting with domesticated zebrafish (Zala and Määttänen, 2013).

Other aversive stimuli can also be used to aversive learning, some of them non-related with social information (e.g. conspecifics or related products) such as alcohol (Morin et al., 2013), extreme water

parameter (Arthur and Levin, 2001), netting (Arthur and Levin, 2001), electric shocks (Kenney et al., 2017; Valente et al., 2012). Independently of the stimuli, aversive reinforcements are more effective than reward ones due to their potential negative impact on survival (Steel et al., 2016). Similarly, the magnitude of a stimulus plays a role in learning abilities because high and low intensity shock, for instances, have different cost (Rumbaugh et al., 2007).

In Zebrafish, the most used reward cue is food (asocial) and conspecifics (social). For instance, animals learn to prefer a red stimulus after a demonstrator associates food with this color (Zala and Määttänen, 2013); also animals approach more a red card red that it was paired with sighting conspecifics (Al-Imari and Gerlai, 2008). Several authors demonstrated that zebrafish perform different learning tasks but no ontogeny studies were made to assess the emergence of social learning across development (Bilotta et al., 2005; Blank et al., 2009; Braubach et al., 2009; Colwill et al., 2005; Eddins et al., 2009; Hicks et al., 2006; Xu et al., 2007). Despite some authors reveals that larvae turn toward each other with 7 days post fertilization (dpf) and decrease the distance between each other after 10 dpf pointing to a specific time window for the development of social skills (Buske and Gerlai, 2011; Hinz and de Polavieja, 2017). Asocial learning looks to appears around 3 weeks in classical and operant conditioning in aversive context (electric shocks) and 6 to 8 days in Pavlovian conditioning in reward context (visual access to conspecific) (Valente et al., 2012). These studies suggest the importance of social learning changes over time that is not well known yet.

Social learning is a specific type of learning, that can be reward or aversive, where a social cue is used (Heyes, 1994). It is a behaviour process than can be subdivided in several learning mechanisms (see box 1). Here, we will focus in observational conditioning in which a social cue such as an image of a conspecific or a eating-demonstrator is used in a Pavlovian conditioning paradigm. This behaviour is described for the first time in *Macaca mulatta*, young monkeys presents a fear response for snake only after observe a fearful response of a demonstrator to a snake (Hoppitt and Laland, 2013).

1.5 Neuronal activation mechanisms of learning

The neuronal mechanisms of learning have been the focus of intense research in the last decades. Reward and aversive learning have been shown to rely on different neuronal circuits (Arias-Carrián et al., 2010; Lattal and Abel, 2000; Nader et al., 2001). Here we will highlight the neural mechanism used to learn using social information. The techniques used to measure which brain region or types of neurons trigger are associated with learning will be described considering their evolution across the years.

1.5.1 How to measure neuronal activation?

The oldest neuronal activity marker is metabolic 2-deoxy-d-glucose (2-DG; (Sokoloff et al., 1977)) that is incorporated into tissues with energy consumption. This marker cannot undergo glycolysis and remains in the incorporated tissue. After this a less invasive method appears, blood oxygenation level dependent (BOLD), that enabled functional magnetic resonance imaging of live brain activity but with limited spatial resolution (Kawashima et al., 2014; Ogawa et al., 1990).

Immediate early genes (IEG) bring the cellular resolution and are the first genomic response upon cell depolarization. The expression of IEGs can be induced without requiring *de novo* protein synthesis or activation of any other responsive genes (Clayton, 2000). Pioneer work showed that the expression of an IEG (in this case *c-fos*) in cultured cells could be rapidly induced by the application of growth factors (Curran and Morgan, 1985; Muller et al., 1984; Ziff and Greenberg, 1984) simulating the activation of a neuron. The depolarization of neurons induce ions flowing into the cytoplasm [through N-metil-D-aspartate (NMDA) glutamate receptors (NMDARs) and voltage-gated calcium channels (VGCCs)] stimulates the activation of several calcium-dependent kinase cascades (Ca2+/calmodulin-dependent protein kinases (CaMKs; (Bito et al., 1996; Fujii et al., 2013)) and mitogen-activated protein kinases (MAPKs; (Dolmetsch et al., 2001; Zhai et al., 2013)) leading to the activation of these kinases cascades (as CREB (Bito et al., 1996), myocyte enhancer factor-2 (MEF2; (Mao et al., 1999)) and serum-responsive factor (SRF; (Norman et al., 1988)) thereby turning on rapid transcription of downstream IEGs.

Multiple publications have shown neuronal *c-Fos* activity in response to several stimuli, such as electrical seizure, tactile stimulation, and water starvation, either using immunostaining or *in situ* hybridization (Morgan et al., 1987; Sagar, S. M. Sharp, F.R. Curran, 1988). Other IEGs, such as *zif268/egr-1* (Saffen et al., 1988) and transcription factors as phosphorylated cAMP response element-binding protein (CREB) (Bito et al., 1996) have become accepted as proxies for neuronal activity and have been widely used to map activated circuits in response to specific behavioural tasks.

IEGs have been classified into rapid IEGs, where a stalling DNA polymerase II (Pol II) is in the vicinity of the promoter (expressed within a few minutes after stimulation), and delayed IEGs, in absence of Pol II (expressed later, ca.1-hour post-stimulation) (Saha et al., 2011). Depending on their function, IEG proteins can act themselves as transcription factors (e.g. *c-fos* and *egr-1*), or as effectors proteins (e.g. *arc* and *homer1a*) regulating synaptic function (Clayton, 2000).

The advent of genetic models allows the generation of fos-lacZ transgenic mice (and also cAMP response element (CRE)-lacZ (Impey et al., 1996), egr-1-lacZ (Tsai et al., 2000) and fos-tau-lacZ

(Wilson et al., 2002)) that enabled the activity mapping at a cellular scale based on β-gal staining (Smeyne et al., 1993). Also, the improvement of fluorescent proteins (as green fluorescent protein (GFP)) and bioluminescent protein (as firefly luciferase) allows the development of activity-dependent promoters in transgenic mice and virus vectors comparable with cfosIR and arcIR (Barth, 2004; Grinevich et al., 2009; Kawashima et al., 2008; Okuno et al., 2012; Wang et al., 2006)(Izumi et al., 2011; Wada et al., 2010).

Some proteins also started to be used as markers of neuronal activity (see in detail table 3): mitogen-activated protein kinase (MAPK/ERK), involved in the transduction of signals through a cascade of protein kinases in response to stimuli (Roberson et al., 1999; Wu et al., 2001); and later on phosphorylation of ribosomal protein S6 showed to be induced for diverse stimuli that were shown previously to induce *c-fos* expression (Cao et al., 2008; Valjent et al., 2011; Villanueva et al., 2009; Zeng et al., 2009).

An important study demonstrated that genes and proteins can have different dynamics measuring different scenarios of the same pathway. Adult fish reveals non-identical c-Fos protein and mRNA (von Trotha et al., 2014). Different neuronal markers present variation in cell-type and regional specificity of activity that can be explained by distinct transcriptional regulation and cellular calcium kinetics. For instance, in thalamic areas (Link et al., 1995; Lyford et al., 1995; Steiner and Gerfen, 1994) and cerebellum *c-fos* tend to be express rather than *arc* or *egr-1* (Guenthner et al., 2013). However, *c-fos* is expressed in both excitatory and inhibitory neurons (Staiger et al., 2002) and there are no IEGs that are expressed exclusively in a particular cell type. These studies allow to use IEG as marker of neural activity.

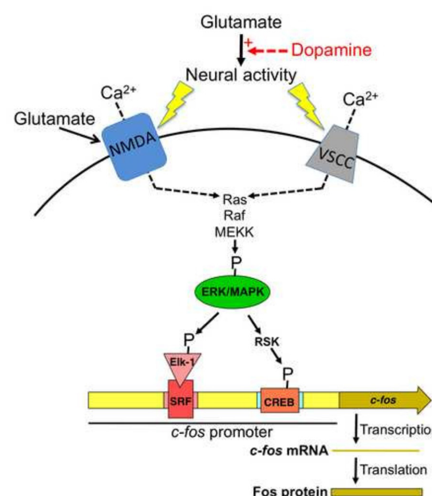

Figure 1.2- Illustration of the neurochemistry and molecular mechanisms after activation of *c-fos* and Fos expression. Glutamate is the main excitatory neurotransmitter that increases neural activity and dopamine can enhance (red arrow + sign) glutamate-mediated neural activation of a small proportion of neurons (Surmeier et al., 2007). Strong persistent neural activity induces calcium (Ca2+) influx

through NMDA-type glutamate receptors and voltage-sensitive calcium channels (VSCCs) to levels that are sufficient for phosphorylating and activating ERK/MAPK via the Ras-Raf-MEKK pathway. ERK/MAPK activation leads to phosphorylation of Elk-1 that is associated with serum response factor (SRF) as well as phosphorylation of CREB via ribosomal S6 kinase (RSK). Elk-1/SRF and CREB are transcription factors that, when phosphorylated, can induce transcription of the coding sequence for *c-fos*. Transcribed *c-fos* mRNA and the translated protein product Fos can be used as markers of strongly activated neurons (Cruz et al., 2013).

Table 1.1 - Examples of two different proteins with distinct mechanism of action accepted to be used as markers of neuronal activity.

Protein Neuronal Marker	Mechanism of action	Comparison with *c-fos* evidences
ERK	Activated via phosphorylation by its upstream kinase MEK (MAP kinase or ERK kinase) requiring a cascade that involves sequential activation of Ras, Raf, and MEK (Widmann et al., 1999). Upon activation, phosphorylated ERK (pERK) can be translocate into the nucleus to activate several transcriptional factors, such as cAMP-response element binding protein (CREB) that is required for the transcription of many neuronal genes (Gao and Ji, 2009). This transcription factor (Brindle et al., 1993) binds to the CRE site present in IEG promoter and acts as a key regulator of IEG expression activation.	Like *c-Fos*, the pERK expression is also very robust and requires high-threshold noxious stimuli. *c-Fos* is much more rapid and dynamic than pERK expression. Additionally, some authors demonstrated that *c-Fos* and pERK colocalized in neurons (Ha and Redmond, 2008) but others only found a partial co-localization (Gonzàlez et al., 2008; Gutierrez-Mecinas et al., 2011).

pS6	A structural component of the ribosome that is phosphorylated downstream of PI3-K/mTOR, MAPK, and PKA signaling (Meyuhas, 2008; Valjent et al., 2011). These same pathways regulate the transcription of activity-dependent genes such as *c-fos* (Flavell and Greenberg, 2008). Phosphorylation sites in pS6 include the Ser235, Ser236, Ser40 and Ser244 residues and different sites of phosphorylation can be regulated independently in various brain areas or different cell-types within the same brain region (Biever et al., 2015). Recent literature demonstrated that Ser235/236 was detectable by 5 minutes achieving the peaked of expression at 30 minutes, being maintained for hours similar to *c-fos* mRNA kinetics (Pirbhoy et al., 2016)	A wide range of stimuli induced overlapping neural expression of *c-Fos* and pS6 throughout the brain such as nutritional stimuli (including fasting, dehydration, salt challenge, and ghrelin treatment) demonstrated extensive co-localization of *c-Fos* and pS6 in hypothalamus; the control of body temperature showed colocalization of *c-fos* and ps6 in POA and cocaine (a stimulant), kainate (a convulsant), clozapine and olanzapine (antipsychotics) treatments induced co-localization of pS6 and *c-Fos* in a variety of brain regions (Knight et al., 2012) and also control of body temperature, pS6 immune activity co-localized with heat-induced *c-Fos* in the POA. However, this is a controversial issue in the field, some authors demonstrated *c-fos, npas, arc, egr1* and pS6 induced the similar patterns of neuronal activation but not exactly the same (Renier et al., 2016). Friedman also described that some stimuli like light induced strong pS6 but only scattered *c-Fos* within the suprachiasmatic nucleus (Knight et al., 2012). Martin G. Myers, Jr. et al described 54% of the pS6-IR neurons in fasted mice co-localized with c-Fos-IR in the medial basal arcuate nucleus (Villanueva et al., 2009).

1.5.2 Neuronal mechanisms of learning

The identification of the neuronal mechanisms of learning processes started to be dissected by the work develop in *Aplysia* at a cellular level in habituation, sensitization and classical conditioning (see table 2). These studies were of tremendous historical importance because they mapped, for the first time, behavioural learning phenomena onto a cellular process (Lattal and Abel, 2000).

In zebrafish, learning mechanisms of habituation were explored in the C-start reflex that is controlled by a bilateral pair of large command neurons, the Mauthner cells (Eaton et al., 2001); and the vestibulocular reflex (VOR), a reflexive eye movement in which vestibular signals are used to generate compensatory eye movements in the opposite direction from the head, in order to stabilize retinal images, that correlates with neurons in the inferior olive and cerebellum (Graf and Baker, 1983; Marsh and Baker, 1997; Pastor et al., 1994).

Others verified the occurrence of classical conditioning in juvenile and adult zebrafish using motor learning paradigms (Agetsuma et al., 2010; Aizenberg and Schuman, 2011; Aoki et al., 2013; Braubach et al., 2009; Karnik and Gerlai, 2012; Valente et al., 2012). These authors demonstrate that prior to training the CS and the US activated partially distinct populations of cerebellar neurons, and that as consequence of the learning process the number of CS-activated neurons in the cerebellum was increased (Aizenberg and Schuman, 2011).

Reward and aversive cues used to learn clearly recruit distinct neural mechanisms. In an aversive context, a circuit centred in the amygdala (a conglomerate of sub-nuclei) has been described to explain fear learning. Work in rodents described that sensory information arrives in the lateral nucleus of the thalamus and sensory cortices (Amaral, 1985; LeDoux et al., 1990) and the association between representations of the CS and US occurs in lateral nucleus of thalamus (Blair et al., 2001; Quirk et al., 1997; Romanski et al., 1993). This region project to the central nucleus and basal nucleus of the amygdala, which mediates the output to other regions that regulate expression of fear and anxiety (Nader et al., 2001). These regions of projection can modulate autonomic responses through hypothalamus (Price, 1981), expression of fear behaviour by ventral tegmental area and central gray (Simon et al., 1979) or avoidance behaviour via the basal ganglia from the basal nucleus (Hormigo et al., 2016). Thus, the amygdala, together with other functional regions within a circuitry, is important in fear conditioning. Other brain areas that have also been identified as relevant for learning processes include the hippocampus, a critical region for coding contextual information (e.g. timing of the events) (LaBar and Phelps, 2005; LaBar

et al., 1995), and the prefrontal cortex, which has a critical role in extinction observed in rats (Milad and Quirk, 2002) and humans (Phelps et al., 2004). Two studies have shown avoidance conditioning in larval to juvenile zebrafish mediated by habenula (Lee et al., 2010) the same region that is recruited in mammals (Shumake et al., 2010).

The positive valence of reward stimuli is dependent of the mesocorticolimbic system, central to process pleasurable information. This system is built by dopaminergic neurons located in substancia nigra (SNc), the ventral tegmental area (VTA) and the retrorubral field (RRF). The substancia nigra compacta extends its fibers into the striatum (caudate and putamen) playing an essential role in the control of voluntary movements known as a nigrostriatal system. The mesolimbic and mesocortical pathway arises from VTA, have been suggested to modulate emotion-related behavior; this area project to nucleus accumbens as well as olfactory tubercle innervating septum, amygdala, and hippocampus in mesolimbic pathway and the prefrontal cortex, cingulate and perirhinal cortex in the mesocortical pathway. These three pathways are mediated by the release of dopamine outside of the synaptic cleft. Dopamine is mediated by five distinct G protein-coupled receptor subtypes: two D1 like receptors couple to G proteins that activate adenylate cyclase (D1A and D1D) and three receptors subtypes belong to D2 like family and are Gi protein-coupled receptors that inhibit adenylate cyclase and activate K+ channels (D2, D3, and D4). These receptors have a similar pattern of distribution to that of dopaminergic fibers with D1 like receptors more expressed in prefrontal cortex and D2 like receptors elevated in caudate, putamen and nucleus accubens acting synergistically (Arias-Carrián et al., 2010). Several studies reveal a role of agonist of D2 receptor modulating working memory (faral1997), dopaminergic projections to striatum and frontal cortex mediating classical conditioning (Balleine et al., 2007), and also Parkinson individuals show impairments in associative learning at the level of frontal lobe lesioned patients (Grafman and Litvan, 1999).

Box 2 – Cellular mechanisms of habituation, sensitization and classical conditioning described in Aplysia

Habituation is a presynaptic phenomenon and appears due to homosynaptic depression. This event results from the decrease of transmitter release that induced a reduction of the ca^{2+} availability needed to the neurotransmitters release due to repeated activation (Ocorr et al., 1986).

Sensitization induces the activation of a facilitator interneuron that acts on sensory neuron terminals to increase the level of intracellular cAMP, which activates cAMP-dependent protein kinase A (PKA) driving the phosphorylation of a set of targets, including a class of potassium channels in the sensory neuron (leading to the close of K+ channels and activation also PKC). The reduction of k+ efflux at the time of depolarization results in a longer period produced by each action potential (Ocorr et al., 1986). A longer-lasting form of sensitization occurs when stronger stimuli are used, or when weaker stimuli are applied repeatedly. The long-term facilitation differs in three main topics in comparison with short-term: requires protein synthesis in the presynaptic neuron, PKA is persistently active and translocate to the cell nucleus of the presynaptic neuron (not only transiently active), and cAMP response element binding protein 1 (CREB) is then activated in the cell nucleus resulting in the gene transcription.

Classical conditioning, the CS causes depolarization that enhances Ca^{2+} influx, which in turn enhances the synthesis of cAMP in response to a neuromodulator release by the US. Thus, increased cAMP levels by G-protein coupled receptors in the CS interneurons and ca^{2+} seem to provide biochemical mechanisms for encoding information about the temporal association of separate inputs to these cells. In short term, this results in an even greater enhancement of transmitter release from the CS interneuron and increase the PKA activity leading to an increase in gene transcription in the presynaptic neuron, with appears to be essential for long-term memory storage (Byrne, 1987). Murphy and Glanzman provide important evidence that postsynaptic neurons also contribute to Pavlovian conditioning showing that blockage of several receptors disrupting long-term acting (Murphy and Glanzman, 1997).

1.5.3 Neuronal mechanisms of learning: social learning as a particular case

The neuronal basis of social learning has been investigated in humans and some model organisms. Work done in humans suggests a central role for the amygdala in social and asocial transmission of pain (Olsson et al., 2007). Additional work, using the same paradigm demonstrated that the amygdala is involved only in associative learning, and social transmission of pain is mediated by the anterior cingulate cortex (Fan et al., 2016). Work in mice have demonstrated a specialized olfactory subsystem involved in the acquisition of socially transmitted food preference (STFPs) (Munger et al., 2010). In primates neurons in the lateral intraparietal area (LIP) respond specifically to the value of social information (Tremblay et al., 2017) and the anterior cingulate cortex is a key brain region underlying neural processing of social food foraging decision-making (Rushworth et al., 2011; Zhong et al., 2017).

As described this is a controversial issue, one of the main questions in the field is to understand if social learning is a general or a specific domain (Adolphs, 2009; Byrne and Bates, 2007; Chittka and Niven, 2009; Rosati, 2017; Shettleworth, 2010). These studies reveals some specialization of social learning that are congruent with the SDMN demonstrated to be enough to, at least in part, explain social behaviours. However, Heyes, C. postulates that the neuronal mechanisms of social and asocial learning were a general domain because the rules to learn with social and asocial cues are the same (Heyes, 2011; Heyes and Pearce, 2015); otherwise solitary species should not be able to use social information, and social learning would not be taxonomically general (Fiorito and Scotto, 1992).

The neuronal mechanisms underlying social learning are not fully understood, neither in terms of addressing the different types of social learning nor in terms of the sensory modalities of the stimuli involved.

1.6 Aim

The broader goal of the present work is to investigate the proximate mechanisms underlying social learning and to assess if they are shared with asocial associative learning or not. For this purpose, the behavioural and neural mechanisms involved in social learning will be investigated and contrasted with those of asocial learning.

The specific objectives are first to identify the role of social information as a CS in reward learning task and the neural mechanisms recruited (chapter II) and secondly to describe the neural mechanisms of social information as US in an aversive learning task (chapter III). These aims translate into the 2 experimental chapters of the thesis:

- Chapter II, we identify if the social learning circuitry is shared with the asocial one in a food reward learning paradigm. We start to develop a robust behavioural paradigm to compare social and asocial learning using Observational conditioning (OC). Then, we mapped the neuronal circuitry involved in social and asocial learning using the expression of IEG as a marker of neuronal activity, in order to address the neuronal mechanisms of social and asocial learning.
- Chapter III, we established an aversive associative learning paradigm where animals were trained to associate a light with the presence of an alarm pheromone and we searched for the circuitries to process chemical OC though the expression of IEG with a candidate gene and brain nuclei approach. In addition, we compared the learned valence of visual and chemical OC.

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Chapter 2 . Social and asocial learning recruit different neural circuits in zebrafish.

2.1 Abstract

In the environment, animals are exposed to social and asocial cues that can be used not only to trigger response but also to predict the presence of a reward through associative learning. The neuronal networks underlying innate behaviours have been well studied; however the neural circuits underlying social and asocial learning have not been well understood.

In this work, we use the expression of the immediate early gene *c-fos* to map the neural circuits recruited in social and asocial learning tasks.

We demonstrate that the ability to learn with social (fish image) and asocial (dot image) conditioned stimulus that have been paired with a reward (food) are similar. However, the neural mechanisms involved in both learning types, measured by the expression of the immediate early gene *c-fos* are distinct. Social learning is associated with an increase of *c-fos* expression in olfactory bulbs, ventral zone of ventral telencephalic area, ventral habenula and ventromedial thalamus. In contrast, asocial learning is associated with a decreased expression of *c-fos* in dorsal habenula and anterior tubercular nucleus. In addition, a functional network analysis allowed the identification of different sets of central brain nuclei associated with each treatment, with more anterior nuclei involved in social learning and more posterior in asocial learning.

Together these results suggest that social and asocial information is processed by different neuronal mechanisms in the zebrafish brain.

2.2 Introduction

The social environment constantly challenges individuals that need to adjust their behaviours accordingly, acting as a selective pressure for the development of cognitive abilities. As a result of new demands in the social environment, more complex cognitive abilities are needed for survival, driving the evolution of a social brain. For instance, the hypothalamus and cerebellum are enlarged in the African cichlid fish *Neolamprologus pulcher* reared in large groups and the optic tectum is bigger in fish reared in small groups, which may indicate some degree of specialization for each of these brain areas (Fischer et al., 2015).

In the environment, individuals can learn from and about others avoiding the costs of trial-and-error learning. By observing and interacting with others, animals retain information that they can use in subsequent encounters (social eavesdropping) and also to recall the route to find food or avoid a predator (social learning). In contrast, asocial learning refers to learning using asocial cues non-related with conspecific information (i.e. social: conspecifics or their vocalizations, footprints or pheromones; asocial: shocks, food, tones).

Where social learning is processed in the brain remains unclear and is in the centre of the debate on the modularity hypothesis, which postulates that social cognition is a domain-specific phenomenon (Adolphs, 2009; Byrne and Bates, 2007; Chittka and Niven, 2009; Rosati, 2017; Shettleworth, 2010). Some authors considered that social learning is modular because it is specifically based on social information (Kendal et al., 2018; Leadbeater and Dawson, 2017; Lefebvre and Giraldeau, 1996; Lotem and Halpern, 2012). Social behaviours can be described, at least in part, by changes in the activity of a brain social decision-making network and by its modulation by neuromodulators (O' Connell and Hofmann, 2012; Teles, 2015). For instance, research on mice demonstrated a specialized olfactory subsystem involved specifically in socially transmitted food preferences (STFPs) (Munger et al., 2010); another research on human subjects revealed that a node of this network is involved only in associative learning (amygdala) and the weaker connectivity strength from anterior insular cortex to superior frontal gyrus associated with social transmission of fear) (Fan et al., 2016). However, this is a controversial field of research with other authors on human subjects pointing to the amygdala as a central area to process social and asocial transmission of pain (Olsson et al., 2007). Some authors argue that social and asocial learning employ similar associative learning rules, being a general domain cognitive mechanism, and only input mechanisms are specialized, allowing social animals to be biased towards social information (Heyes, 2011, 2016). Otherwise, solitary species would not be able to learn using social information (Heyes, 2011; Webster and Laland, 2017), and social learning would not be conserved across species (Heyes, 2011; Reader and Laland, 2002).

Our goal in this work is to investigate the proximate mechanisms between social and asocial learning. We exposed fish to a social or asocial conditioned stimulus (2D photo of a zebrafish or a dot with the same visual target area and the same colour cue, respectively) using a plus-maze behavioural paradigm. All groups received food as a reward, the US can be paired with social or asocial CS in the same arm (Social Learning and (SL) and Asocial Learning (AL) treatments) or the food can be released in a pseudo-randomized arm unpaired with the social or asocial CS (Social Control (SC) and Asocial Control (AC) treatments). We then measured the expression of *c-fos* mRNA levels in several brain areas using *in situ* hybridization as a proxy for neural activity to assess the neural brain regions involved across treatments. Functional connectivity analyses were further performed to determined patterns of neuronal activation across treatments.

2.3 Methods

2.3.1 Animals

Zebrafish (*Danio rerio*) were 5 months old wild-type (Tuebingen strain) males, bred and held at Instituto Gulbenkian de Ciência Fish Facility (Oeiras, Portugal). Fish was kept in mixed-sex groups, at 28ºC, 750 μs, pH 7.0 pH in a 14L:10D photoperiod and fed twice a day (except on the day of the experiments) with freshly hatched *Artemia salina* and commercial food flakes. All experiments were performed in accordance with the relevant guidelines and regulations, reviewed by the Instituto Gulbenkian de Ciência Ethics committee, and approved by the competent Portuguese authority (Direcção Geral de Alimentação e Veterinária).

2.3.2 Behavioural paradigm

One day before the experiment, fish was moved to the home tanks (1.5L, 12.5 cm x 12.5 cm x 12.5 cm) where they only had visual and chemical access to a mix shoal of 4 animals (2 familiar males and 2 familiar females).

The experiment was subdivided into three phases: acclimatization, training and probe test. In the acclimatization phase, after one minute in the start box, animals were allowed to swim freely in the tank for 9 min, during which, they were attracted to all arms of the plus-maze with bloodworms, so that they became familiar with the whole maze. In the training phase, animals were trained in daily sessions of trials per session for 6 days. In the paired groups, animals had the CS and the US presented together in the same arm, and received a reward (bloodworm) when this arm was chosen (that changed on each trial in a pseudo-randomized way, within and between individuals); when another

arm was chosen, animals stayed one minute in the chosen arm, and then they were conducted to the right arm using the net where they receive the reward. In the unpaired groups, the animal spent 2 minutes in the chosen arm since the CS and US were never presented together. In both groups, when individuals reach the RoI (5 cm x 5 cm x 5 cm) of the chosen arm the start box was closed to avoid the animal change its decision. In the social treatments, the CS stimulus presented at the end of the arm was a static, 2D photography of a zebrafish. In the asocial treatments a digitally drawn circle with the same visual target area and the same mean colour of the zebrafish-stimulus was used. After each training session, individuals returned to their home tank.

In the probe test (24h after the last training trial), animals were only exposed to the CS for 2 min. The CS was then removed and the animal remained in the tank for 30 min to achieve the peak of expression for *c-fos* (Guzowski et al., 2001).

A preference test was performed to assess if individuals prefer social to asocial CS's. For this purpose, we used a rectangular tank (5L, 30cm x 15 cm x 15 cm) with the stimuli presented on each side (e.g. social stimulus in the left and asocial stimulus in the right side, in a randomized way between individuals). Individuals were placed in a start box for 2 min with transparent partition, and the time spent in both RoI's was compared.

To demonstrate that individuals can discriminate between the two CS used (i.e. social and asocial CSs), we performed a discrimination task. In this case, we trained fish (one-minute trial, 8 trials/day for 5 days) to associated one CS to a reward (food) and the other to a punishment (netting) (e.g. social stimulus in half of the animals was associated with food and in the other half it was associated with threat). In the probe test, only the CSs were presented, and we measured the duration spent by the focal fish in each arm; if individuals were able to discriminate between the two stimuli, they should prefer the arm associated with reward independently of their initial preference for the social stimulus.

In all experiments, the behaviour was recorded with a digital camera for subsequent analysis using a commercial video tracking software (EthoVisionXT 8.0, Noldus Inc. the Netherlands).

2.3.3 Brain collection

Animals were sacrificed with an overdose of Tricaine solution (MS222, Pharmaq; 500–1000 mg/L) and sectioning of the spinal cord. The brain was macrodissected under a stereoscope (Zeiss; Stemi 2000) and immediately collected to 4% PFA solution in 0.1M PB and kept overnight at 4º C. After cryopreservation (34% sucrose in 0.1M PB ON at 4ºC), the brains were embedded in mounting media (OCT, Tissue teck) and rapidly frozen on liquid nitrogen. The coronal sectioning was performed on a

cryostat (Leica, CM 3050S) at 16 um, sections were collected onto SuperFrost glass slides and stored at -20ºC.

2.3.4 *In situ* hybridization for the immediate early gene: *c-fos*

Chromogenic RNA in situ hybridization (CISH) was carried out according to a standard protocol available upon request from the lab of Professor Marysia Placzek, University of Sheffield. For the generation of c-fos probes, a pBK-CMV vector containing the c-fos cDNA (Genebank: CF943701) was cut with the restriction enzyme BamHI (antisense) and EcoRI (sense) to generate templates for in vitro transcription. Digoxigenin-labeled c-fos sense and antisense probes (11277073910, Merk (Roche), UK) were then synthesized through in vitro transcription of 1 mg template with T7 polymerases (M0251, New England Biolabs). The sections were fixated in 4% PFA, washed in PBS, rinsed in 0.25% acetic anhydride in 0.1 M tri-ethanolamine for 10 min and washed 3 times in phosphate buffer saline (PBS). Incubation in pre-hybridization buffer (hybridization solution without yeast RNA, minimum 3 hours) was done in order to prepare the tissue for receiving the probe diluted in hybridization solution (probe dilution: 1:40 ~4 ng/ul final concentration). The hybridization buffer contained 50% formamide, 5 x SSC (pH 7.0), 2% blocking powder, 0.1% triton X-100, 0.5% CHAPS, 1 mg/ml yeast RNA, 5mM EDTA and 50 ug/ml heparin. The hybridization incubation was performed at 68°C for 24 h. Following hybridization, the sections were treated with secondary antibody anti-dig-ap (1:1000, 11093274910, Merk (Roche), UK), after series of several washes decreasing concentrations of SSC, until 0.1× SSC. The tissue was then mounted onto GlicerolGel (GG1, Merk) coated slides and left to air dry.

2.3.5 Cell Counting

The slides were imaged using a tissue scanner (NanoZoomer Digital Pathology, Hamamatsu). A whole brain screening was performed to select the brain nuclei with higher *c-fos* activity to be counted. The areas were manually drawn and the signal automatically quantified using the Icy software (created by the Quantitative image analysis unit at Institut Pasteur). The sum of the cross sections was used as an individual measure to each area side.

2.3.6 Statistical Analysis

A N-1 chi-square test for proportions (Campbell, 2007) was used to compare the percentages of learners, non-learners or non-retention, relative to the total amount of individuals in each group. Non-parametric linear regressions were performed to compare the learning curves across the 4 experimental groups and a non-parametric test with the location on the tank (target, front, left or

right arms) as within factor and Social (social, asocial) and learning (learners, non-learners) as between factors, with planned comparisons followed by Benjamini and Hochberg's method for p-value adjustment to assess differences between the experimental treatments.

The effect of social learning on brain activity in the probe test was assessed by a non-parametric test with laterality (*c-fos* positive cells on left and right side of each brain nuclei) as repeated measure and social and learning as between factors area-by-area (OB, D, Vd, Vc, Vv, Vl, Dc, Dl, Dm, Dp, Vs, Dd, PPa, Vp, PM, PPp, Had, Hav, A, VM, VL, Hv, ATN, LH, Hd, CP, TPp, PGZ, Hc, DIL, CIL, DTN, NLV GC, CM). Planned comparisons followed by Benjamini and Hochberg's method for p-value adjustment were used to assess the brain areas associated with social learning (social learning vs social control) and asocial learning (asocial learning vs asocial control).

The functional connectivity was tested using Pearson correlations computed between the number of positive *c-fos* cells for each pair of brain nuclei, within each experimental treatment. These correlations were considered as indicative of co-activation between nuclei, such that positive correlations correspond to phasic activity and negative correlation to out-of-phase activity. The occurrence of different patterns of functional connectivity associated with different experimental groups was assessed by testing the association between any two matrices using quadratic assignment procedure (QAP) correlation test with 5000 permutations. The null hypothesis of the QAP test is that there is no association between matrices; a non-significant p-value indicates that the correlation matrices are different. Finally, we characterize the network structure in terms of centrality and cohesion. The eigenvector centrality, where relative scores were given to each node based on the assumption that connections to high-scoring nodes contribute more to the score of the node in question than equal connections to low-scoring nodes (a high eigenvector score means that a node is connected to many nodes who themselves have high scores). The proportion of all possible connections that was presented in the network was measured in density parameter. In order to compare the density of connections among experimental groups, we used a bootstrap t-test approach with 5000 sub-samples.

2.4 Results

2.4.1 Social and asocial classic conditioning in zebrafish

Pavlovian conditioning was assessed using a plus-maze paradigm divided into a training phase and a probe test. During the training phase, we paired a social and an asocial conditioned stimulus (CS) with an unconditioned stimulus (US; food = bloodworms) in a specific location (Fig. 1a). The

percentage of right choices per session (composed of 8 trials) was measured. In the probe test (24h after the last training session), individuals only had access to the CS, and the time spent in the region of interest (RoI) of the target arm was quantified to measure recall. Unpaired treatments were used as controls, where the CS (either social or asocial) was spatially unmatched with the US.

Animals learned both socially and asocially ($X^2_R(1)=28.44$, $p<0.0001$) as shown by the comparison the percentage of correct choices between paired CS-US (social learning (SL) and asocial learning (AL)) and unpaired CS-US (social control (SC) and asocial control (AC)) treatments for social and asocial CSs (SL vs SC:$X^2_R(1)=6.95$, $p=0.0089$; AL vs AC:: ($X^2_R(1)=28.44$, $p<0.0001$) (Fig. 1b). Animals in the social and asocial learning treatments (SL and AL) acquired information at the same rate, since no significant differences between social and asocial learning curves were found either in slope ($X^2_R(1)=1.53$, $p=0.22$) or elevation ($X^2_R(1)=0.001$, $p=0.97$) (Fig. 1b). It is worth mentioning that there were also no significant differences between the social and asocial control treatments (SC and AC) either in slope ($X^2_R(1)=2.0$, $p=0.16$) or elevation ($X^2_R(1)=0.14$, $p=0.70$) (Fig. 1b). Moreover, in control treatments, animals did not present any biased-behaviour towards one of the arms of the plus maze exhibiting a random proportion of right choices over the trials (25% in social and asocial treatments across the training sessions).

In the probe test, individuals from the learning treatments (SL and AL) spent more time in the target arm independently if they were trained using a social (SL: $X^2_F(1)=12.89$, $p=0.001$) or an asocial (AL: $X^2_F(1)=11.53$, $p=0.001$) CS, when compared to their control treatments (SC and AC, respectively). We did not observed any significant difference in the time spent in the other arms of the plus-maze indicating an absence of any spatial biases in the spatial use of the maze by the fish (opposite arm to the target arm: SL vs SC, $X^2_F(1)=1.22$, $p=0.276$, AL vs AC, $X^2_F(1)=0.32$, $p=0.577$; left of the target arm: SL vs SC, $X^2_F(1)=0.42$, $p=0.522$, AL vs AC, $X^2_F(1)=0.06$, $p=0.801$; right of the target arm: SL vs SC, $X^2_F(1)=0.25$, $p=0.617$, AL vs AC, $X^2_F(1)=0.21$, $p=0.649$) (Fig. 1c).

This paradigm allowed the classification of individuals in the learning treatments (SL and AL) into three different categories: non-learners, learners and learners that forget the learned information from the last learning session to the probe test (i.e. no-retention group: social non-retention (SNR) and asocial non-retention (ANR)). The learners were able to acquire the information and recall it (50% of individuals in both the social and asocial learning treatments); the “no-retention group” were animals that despite showing a learning curve during the training sessions did not recall the acquired information in the probe test (36.67% individuals in the social group (SNR) and 22.73% individuals in the asocial group (ANR)); and a small percentage of individuals that did not improve the performance over the training sessions (13.33% individuals in the social groups and 27.27% individuals in the asocial group) were classified as non-learners (Figs. 1d, e). The non-learners and no-retention animals were

identified using the interval of confidence in the training phase and duration in the RoI of the target arm during the probe test as criteria, respectively. The proportion of learners ($X^2(1)=0$, $p=1$), non-learners ($X^2(1)=1.56$, $p=0.21$) and non-retention ($X^2(1)=1.14$, $p=0.29$) individuals did not differed between social and asocial learning treatments (Figs. 1d, e).

Given the lack of difference in behavioural measures between social and asocial learning, it was important to make sure that the individuals discriminated the two CS stimuli used in this test. Thus, a visual discrimination task between the two stimuli (social and asocial CS) was used, where one stimulus was associated with a reward and the other with a punishment (e.g. social stimuli as a reward and asocial as a threat, and vice-versa). This test indicated that zebrafish distinguished between the social and asocial stimuli used in this study and that the learning curve for the acquisition of these discriminations was similar when either the social or the asocial CS was paired with the reward (slope $X^2_R(1)=1.74$, $p=0.22$; elevation ($X^2_R(1)=0.43$, $p=0.53$; Fig. 1f). Given that social animals usually have an innate preference for social cues, we tested the preference of zebrafish for the social stimulus used here to make sure that it had this social valence. Preference was assessed using a choice test, the time spent near the social vs. the asocial stimuli were used to assess preference. As expected, a preference for the social stimulus was observed (t $(15)=2.55$, $p=0.02$; Fig. 1g).

In summary, despite an innate preference for social cues, both social and asocial cues were equally efficient as CS in a classical conditioning paradigm.

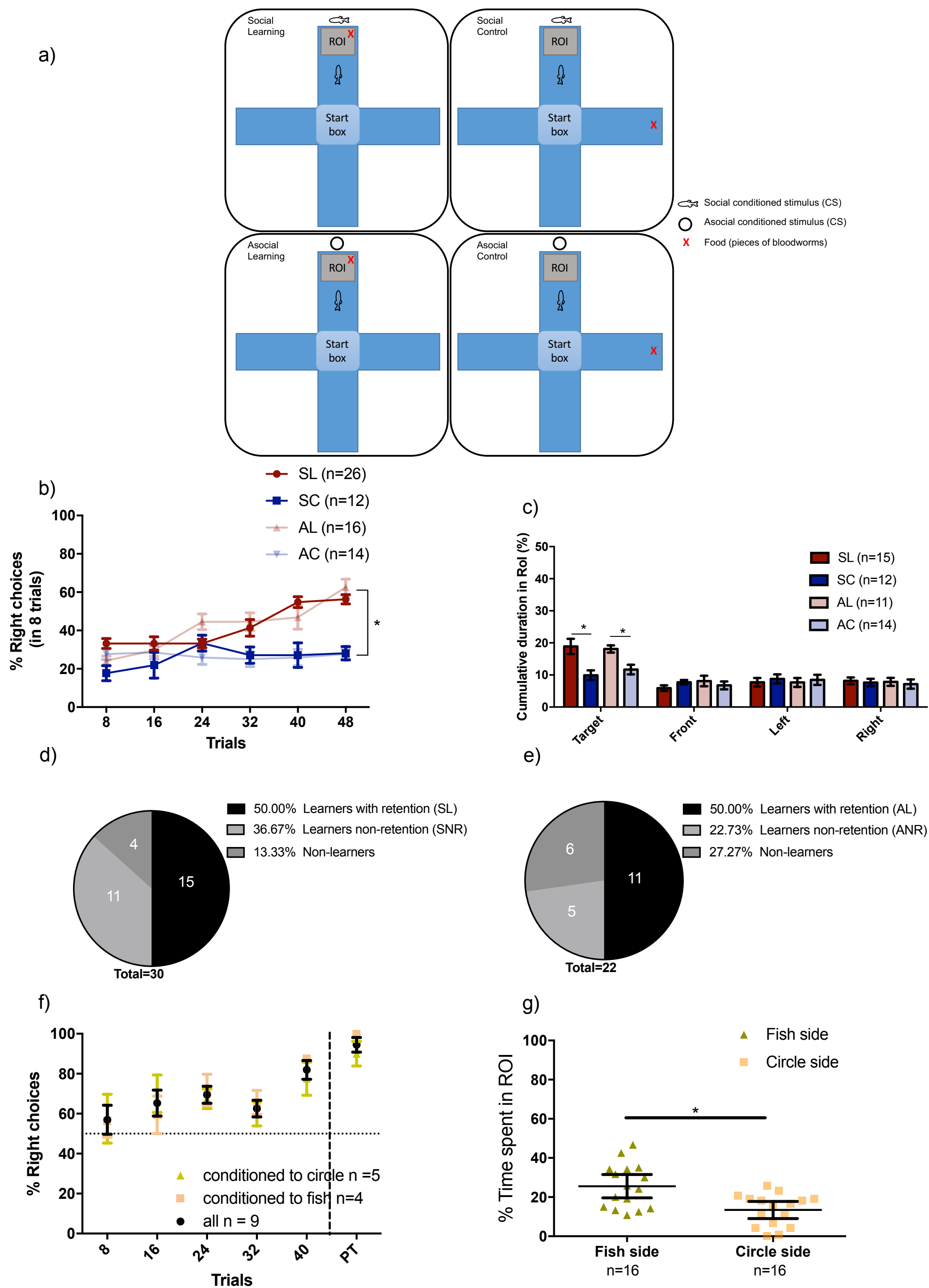


Figure 2.1 – Social and asocial classic conditioning in zebrafish. (a) Schematic representation of the plus-maze paradigm: 4 groups observed a CS (social or asocial cue) paired with a US (food: bloodworms) in the same arm (paired treatments: SL and AL) hence being able to establish the CS-US association; or in different arms (unpaired treatments: SC and AC) the controls of the experiment; (b) During the training phase animals increased significantly the percentage of right choices both in the social learning (SL, in red circles) and asocial learning (AL, in light red circles) treatments in comparison

with the respective unpaired treatments [in blue circles social unpaired control (SC) and in light blue circles the asocial unpaired control (AC)]. (c) In the probe test, the cumulative duration of time spent in the RoI indicates that learners (social and asocial) increased the time spent in the target arm. Pie graphs illustrating the proportion of learners, non-learners and non-retention animals in social (d) and asocial groups (e). The ability of the animals to distinguish between social and asocial stimuli were tested conditioning the animals to approach one stimuli and avoid other independent of their initial preference (in yellow triangles animals conditioned to approach asocial, in light pink squares individuals conditioned to approach social and in black circles the average of all individuals organized by reward) (f).The preference for the social and asocial stimulus (fish (yellow circle) or circle (grey square) static 2D picture, respectively) was assessed by a preference test (g). Asterisks indicate statistical significance at $p < 0.05$ using planned comparisons. In summary, despite the intrinsic positive valence of social stimuli, social and asocial learning is equally efficient in zebrafish.

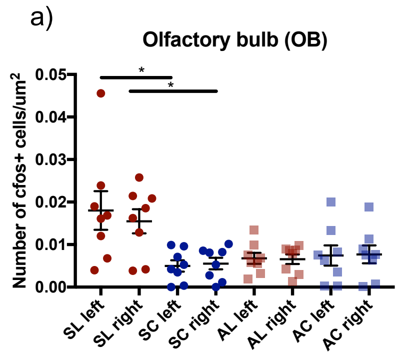
2.4.2 Brain regions involved in social and asocial classic conditioning in zebrafish

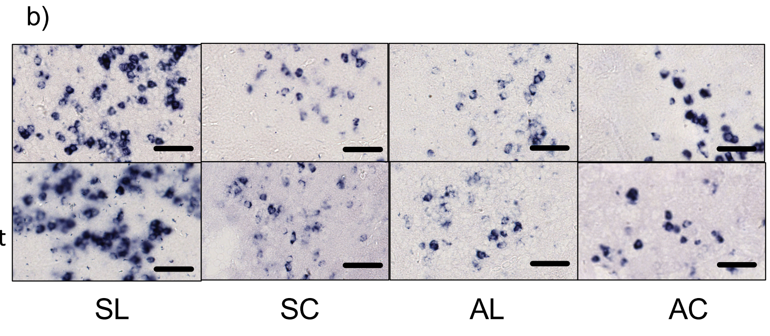
The brain regions associated with social and asocial learning were determined using the expression of the immediate early gene *c-fos* (by in situ hybridization) as a marker of neuronal activation. Because of possible laterality effects, the expression of *c-fos* was measured on both brain hemispheres (noted below as left or right for each brain region). We identified as brain nuclei involved in social (SL) or asocial (AL) learning those that presented significant differences in *c-fos* positive cells between animals of the paired treatments (SL or AL) that were able to acquire and recall the CS and the respective unpaired control treatments (SC or AC, respectively). The following areas showed increased expression of *c-fos* associated with social learning (SL): olfactory bulb (OB) (left: $X^2_F(1)=8.87$, $p=0.022$; right: $X^2_F(1)=7.35$, $p=0.022$), ventral nucleus of ventral telencephalic area (vV) (left: $X^2_F(1)=6.42$, $p=0.048$; right: $X^2_F(1)=5.72$, $p=0.048$), ventral habenular nucleus (Hav) (left: $X^2_F(1)=6.06$, $p=0.04$; right: $X^2_F(1)=10.28$, $p=0.012$) and ventral medial thalamic nucleus (VM) (left: $X^2_F(1)=6.20$, $p=0.038$; right: $X^2_F(1)=7.46$, $p=0.011$) (Table 1; Figs. 2a-g). In contrast, the following set of areas decreased expression of *c-fos* during asocial learning (AL): dorsal habenular nucleus (Had) (left: $X^2_F(1)=6.86$, $p=0.05$), anterior tubercular nucleus (ATN) (right: $X^2_F(1)=8.42$, $p=0.028$) (Table 1; Figs. 2i-l).

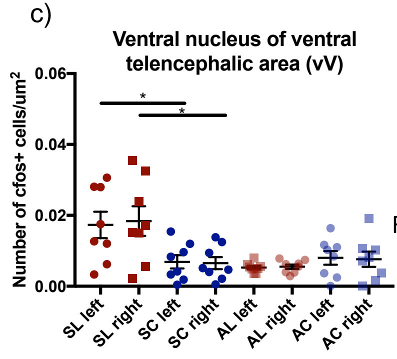
In summary, despite the behavioural similarities between social and asocial learning in zebrafish described in the previous section, learning from a social CS recruits different brain regions when compared to learning from an asocial CS.

Table 2.1 – Nomenclature of brain regions and their list of abbreviations used in this report.

Brain regions	Abbreviations
Olfactory bulbs (composed by external cellular layer, internal cellular layer, glomerular layer, primary olfactory fiber layer and medial olfactory tract)	OB
Dorsal telencephalic area	D
Dorsal nucleus of ventral telencephalic area	Vd
Central nucleus of ventral telencephalic area	Vc
Ventral nucleus of ventral telencephalic area	Vv
Lateral nucleus of ventral telencephalic area	Vl
Central zone of dorsal telencephalic area	Dc
Lateral zone of dorsal telencephalic area	Dl
Medial zone of dorsal telencephalic area	Dm
Posterior zone of dorsal telencephalic area	Dp
Supracommissural nucleus of ventral telencephalic area	Vs
Dorsal zone of dorsal telencephalic area	Dd
Anterior part of parvocellular preoptic nucleus	PPa
Postcommissural nucleus of ventral telencephalic area	Vp
Magnocellular preoptic nucleus	PM
Posterior part of parvocellular preoptic nucleus	PPp
Dorsal habenular nucleus	Had
Ventral habenular nucleus	Hav
Anterior thalamic nucleus	A
Ventromedial thalamic nucleus	VM
Ventrolateral thalamic nucleus	VL
Ventral zone of periventricular hypothalamus	Hv
Anterior tuberal nucleus	ATN
Lateral hypothalamic nucleus	LH
Dorsal zone of periventricular hypothalamus	Hd
Central posterior thalamic nucleus	CP
Periventricular nucleus of posterior tuberculum	TPp
Periventricular gray zone of optic tectum	PGZ
Caudal zone of periventricular hypothalamus	Hc
Diffuse nucleus of the inferior lobe	DIL
Dorsal tegmental nucleus	DTN
Central nucleus of the inferior lobe	CIL
Nucleus lateralis valvulae	NLV
Griseum central	GC
Corpus mamillare	CM


a)
Olfactory bulb (OB)
Number of cfos+ cells/um2
0.05
0.04
0.03
0.02
0.01
0.00
SL left
SL right
SC left
SC right
AL left
AL right
AC left
AC right


b)
Left
Right
SL
SC
AL
AC


c)
Ventral nucleus of ventral telencephalic area (vV)
Number of cfos+ cells/um2
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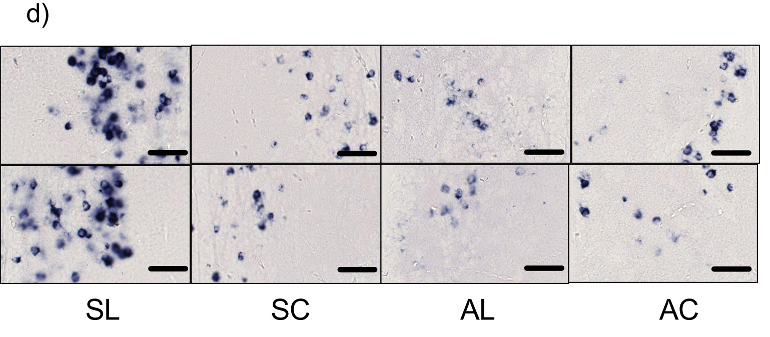
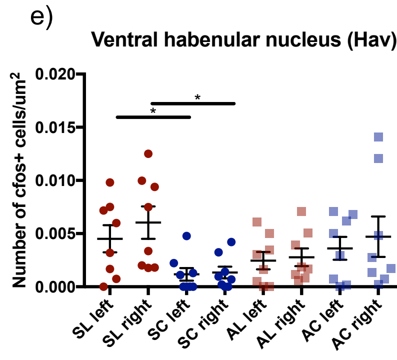
0.04
0.02
0.00
SL left
SL right
SC left
SC right
AL left
AL right
AC left
AC right


d)
Left
Right
SL
SC
AL
AC


e)
Ventral habenular nucleus (Hav)
Number of cfos+ cells/um2
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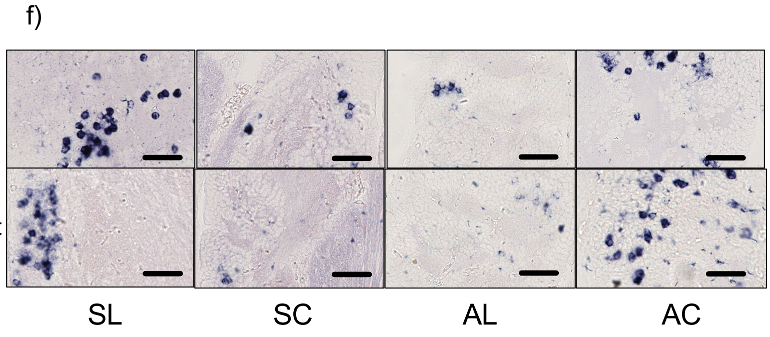
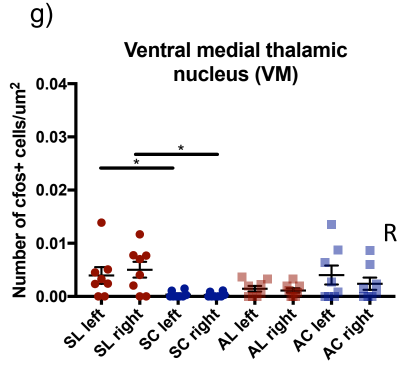
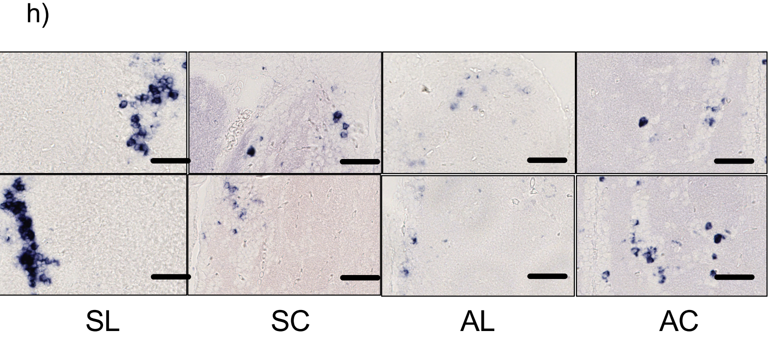
0.015
0.010
0.005
0.000
SL left
SL right
SC left
SC right
AL left
AL right
AC left
AC right


f)
Left
Right
SL
SC
AL
AC


g)
Ventral medial thalamic nucleus (VM)
Number of cfos+ cells/um2
0.04
0.03
0.02
0.01
0.00
SL left
SL right
SC left
SC right
AL left
AL right
AC left
AC right


h)
Left
Right
SL
SC
AL
AC

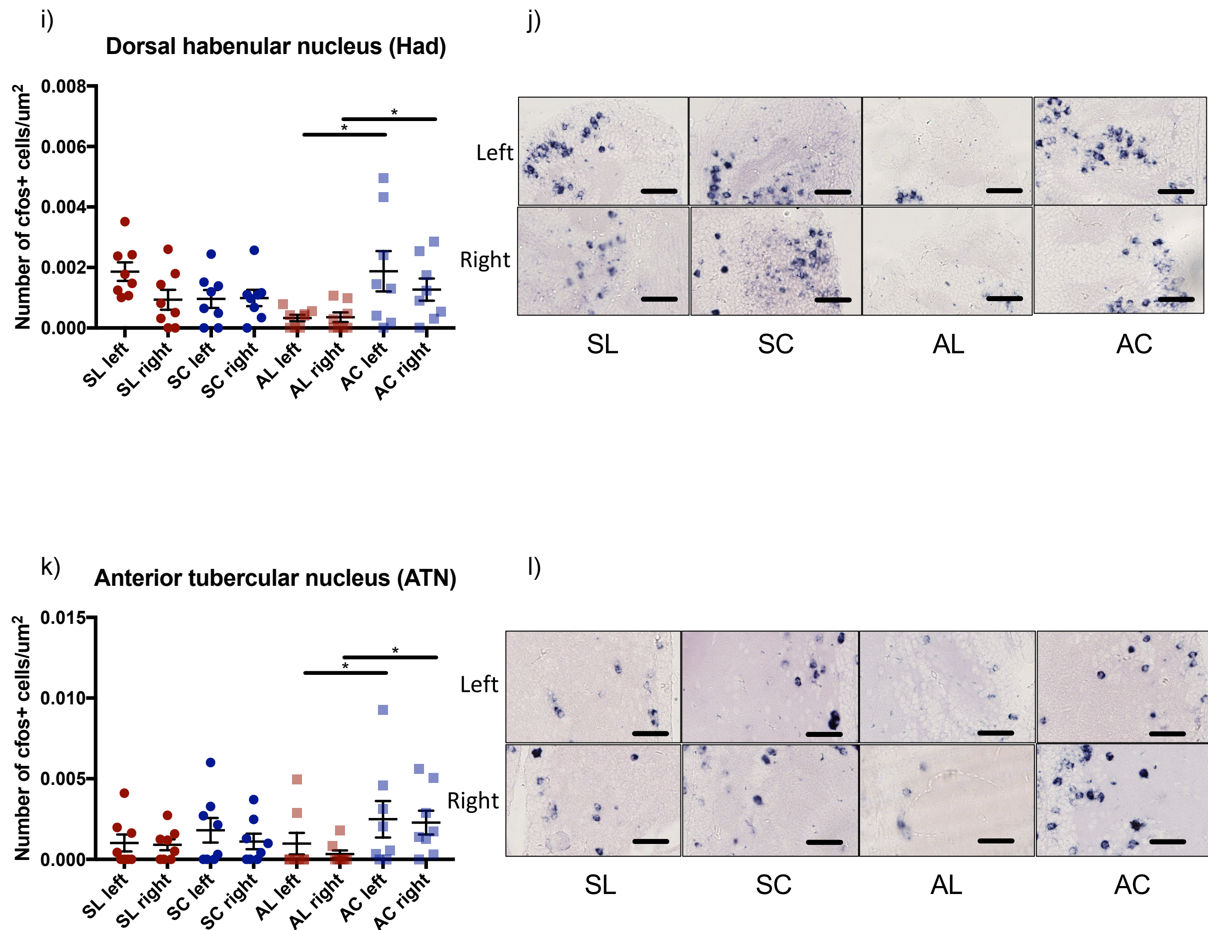


Figure 2.2 – Neuronal mechanisms of learning in zebrafish: the brain regions associated with social (a-d) and asocial (e-f) learning (SL and AL) were assessed by in situ hybridization to the immediate early gene *c-fos* comparing individuals from the learning treatments that learned (i.e that were successful in the probe test) with individuals of the relevant control treatments (SC and AC). Results revealed that different brain regions were associated with social (olfactory bulb, ventral nucleus of ventral telencephalic area, ventral habenular and ventral medial thalamic nucleus) and asocial learning (dorsal nucleus of ventral telencephalic area and anterior tubercular nucleus). Photomicrographs of representative *c-fos* in situ hybridization in OB (b), Vv (d), Hav (f), VM (h), Had (i) and ATN (k). Asterisks indicate statistical significance at $p < 0.05$ using planned comparisons followed by Benjamini and hochberg's method for multiple comparisons p-value adjustment. Scale bars represent 40 µm.

Table 2.2 - Effect of social and asocial learning assessed by measure *c-fos* positive cells in different brain nuclei. Main effects, interactions and multiple comparisons were calculated using the non-parametric Friedman Test. SL, social learners (SL); SC, social control; AL, asocial learners; AC, asocial control.

Areas	Factor														Planned comparisons followed by Benjamini and Hochberg's							
	Within								Between													
	Laterality		Laterality x Social		Laterality x Learning		Laterality x SocialxLearning		Social		Learning		Social x Learning		L: SL-SC		R: SL-SC		L:AL-AC		R:AL-AC	
	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p
OB	0.000	1.000	0.005	0.944	0.020	0.889	0.125	0.726	1.116	0.300	4.283	0.048	4.555	0.042	8.870	0.022	7.350	0.022	0.008	1.000	0.000	1.000
D	0.000	1.000	0.966	0.334	0.075	0.786	1.255	0.272	2.972	0.096	0.064	0.801	1.531	0.226	2.060	0.652	0.270	0.718	0.850	0.718	0.133	0.718
Vd	0.000	1.000	3.022	0.093	0.247	0.623	0.062	0.806	1.717	0.201	0.101	0.753	3.785	0.062	2.860	0.269	2.170	0.269	1.270	0.269	1.310	0.269
Dm	0.000	1.000	0.804	0.378	0.651	0.426	0.008	0.929	3.006	0.094	0.823	0.372	2.166	0.152	3.290	0.302	2.180	0.302	0.080	0.779	0.240	0.779
Dl	0.000	1.000	0.353	0.557	1.630	0.213	1.414	0.244	1.171	0.201	0.138	0.713	1.874	0.182	2.650	0.460	0.630	0.499	0.457	0.499	0.470	0.499
Dc	0.000	1.000	0.763	0.390	0.221	0.642	0.072	0.790	0.004	0.953	0.206	0.653	3.298	0.080	1.920	0.354	2.880	0.354	0.918	0.381	0.790	0.381
Dp	0.000	1.000	0.070	0.793	0.040	0.844	0.215	0.646	0.979	0.331	2.444	0.129	0.207	0.653	2.010	0.396	1.740	0.396	0.378	0.544	0.789	0.509
Vc	0.000	1.000	0.065	0.801	1.367	0.252	0.041	0.840	1.173	0.288	0.491	0.489	4.510	0.043	5.110	0.128	2.070	0.323	0.460	0.503	1.370	0.336
Vv	0.000	1.000	0.111	0.741	0.198	0.660	0.607	0.443	2.605	0.118	1.689	0.204	5.030	0.033	6.420	0.048	5.720	0.048	0.680	0.554	0.240	0.628
Vl	0.000	1.000	0.001	0.970	0.006	0.940	0.423	0.521	0.248	0.623	0.709	0.407	1.596	0.217	1.810	0.760	0.530	0.850	0.220	0.850	0.010	0.936
Dd	0.000	1.000	0.002	0.965	0.604	0.444	0.675	0.418	0.033	0.857	1.500	0.231	0.054	0.818	2.040	0.648	0.210	0.648	0.400	0.648	0.410	0.648
Vs	0.000	1.000	0.082	0.776	1.429	0.242	1.209	0.281	2.147	0.154	0.314	0.580	3.761	0.063	2.550	0.162	2.640	0.162	0.047	0.830	2.540	0.162
Ppa	0.000	1.000	0.276	0.603	2.219	0.147	0.008	0.931	3.901	0.058	0.618	0.438	1.320	0.260	1.190	0.568	2.500	0.500	0.240	0.836	0.000	1.000
Vp	0.000	1.000	1.065	0.311	0.067	0.798	0.674	0.419	1.483	0.233	0.643	0.429	0.045	0.833	0.450	0.836	0.006	0.936	24.000	0.836	0.690	0.836
PM	0.000	1.000	0.162	0.690	0.131	0.720	0.364	0.551	2.642	0.115	0.352	0.558	0.892	0.353	0.310	0.779	0.012	0.915	1.110	0.779	0.740	0.779
PPp	0.000	1.000	0.006	0.941	0.000	1.000	0.006	0.941	9.664	0.004	0.613	0.440	5.197	0.030	4.230	0.098	4.340	0.098	0.990	0.327	1.050	0.327
Hav	0.000	1.000	0.122	0.729	0.667	0.421	0.035	0.853	0.069	0.794	3.065	0.091	7.292	0.012	6.060	0.040	10.280	0.012	0.610	0.586	0.170	0.681
VM	0.000	1.000	0.145	0.706	0.045	0.834	0.007	0.933	0.097	0.758	2.424	0.131	7.196	0.012	6.200	0.038	7.460	0.011	0.540	0.470	0.430	0.519
A	0.000	1.000	2.791	0.106	0.789	0.382	0.280	0.601	0.404	0.530	1.116	0.300	1.645	0.210	0.001	0.987	0.060	0.987	3.840	0.240	1.010	0.648
Had	0.000	1.000	0.878	0.357	2.005	0.168	2.419	0.131	2.662	0.114	2.204	0.149	6.512	0.016	3.390	0.100	0.190	0.666	6.860	0.050	5.140	0.060
Hv	0.000	1.000	1.312	0.262	0.390	0.537	0.011	0.918	1.586	0.218	0.206	0.653	6.240	0.019	2.260	0.144	1.770	0.194	3.830	0.120	4.580	0.120
ATN	0.000	1.000	0.015	0.905	0.132	0.719	1.465	0.236	0.005	0.943	4.921	0.035	2.187	0.150	0.540	0.620	0.020	0.882	2.680	0.226	8.420	0.028
VL	0.000	1.000	0.340	0.564	0.012	0.912	0.093	0.762	0.031	0.861	0.106	0.747	6.712	0.015	1.810	0.760	0.530	0.850	0.220	0.850	0.007	0.936
Tpp	0.000	1.000	0.190	0.666	0.639	0.431	1.189	0.285	5.289	0.029	1.857	0.184	1.857	0.180	0.140	0.712	0.130	0.712	3.250	0.164	3.600	0.164
PGZ	0.000	1.000	0.009	0.924	0.083	0.776	0.000	1.000	0.042	0.839	0.017	0.897	1.342	0.257	0.450	0.506	0.560	0.506	0.870	0.506	0.730	0.506
CP	0.000	1.000	0.406	0.529	1.210	0.281	2.266	0.143	2.164	0.152	0.004	0.951	3.175	0.086	0.260	0.611	3.240	0.332	1.230	0.368	1.820	0.368
Hd	0.000	1.000	0.161	0.692	1.183	0.286	0.118	0.734	1.733	0.194	0.143	0.709	0.987	0.329	0.540	0.620	0.010	0.937	0.540	0.620	1.200	0.620
LH	0.000	1.000	0.521	0.476	0.301	0.588	0.003	0.954	1.108	0.302	0.805	0.377	1.277	0.268	1.580	0.438	2.010	0.438	0.100	1.000	0.000	1.000
DTN	0.000	1.000	0.161	0.692	0.085	0.773	5.612	0.025	0.736	0.398	0.334	0.568	0.985	0.329	2.550	0.488	0.330	0.757	0.460	0.757	0.008	0.929
Hc	0.000	1.000	1.845	0.185	0.780	0.385	4.433	0.044	1.353	0.255	1.400	0.247	1.307	0.263	1.680	0.410	3.520	0.284	0.370	0.561	0.350	0.561
CM	0.000	1.000	3.345	0.078	2.208	0.148	0.640	0.430	1.215	0.280	0.137	0.714	0.007	0.932	0.006	0.940	0.520	0.940	0.006	0.940	0.100	0.940
Dil	0.000	1.000	0.381	0.542	3.657	0.066	0.460	0.503	3.912	0.058	0.167	0.686	4.171	0.051	0.230	0.636	2.990	0.190	3.560	0.190	1.900	0.240
Cil	0.000	1.000	2.451	0.129	0.002	0.967	0.458	0.504	2.212	0.148	3.075	0.090	0.126	0.725	2.580	0.400	1.110	0.400	0.470	0.497	1.110	0.400
GC	0.000	1.000	0.068	0.796	0.772	0.387	0.273	0.605	0.006	0.937	1.077	0.308	0.042	0.839	0.990	0.684	0.010	0.922	0.870	0.684	0.440	0.684
NLV	0.000	1.000	1.526	0.227	0.226	0.638	1.770	0.194	0.144	0.707	2.439	0.130	2.059	0.162	0.099	0.866	0.030	0.866	1.860	0.368	6.570	0.064

2.4.3 Neural networks and patterns of functional connectivity involved in social and asocial classic conditioning in zebrafish

The correlation matrix for *c-fos* positive cells across all measured brain regions was used to assess co-activation patterns between brain regions (Figure 3). Quadratic Assignment Procedure (QAP) correlations detected significant differences between learners and unpaired groups (SL vs SC ($r=0.125$, $p=0.001$), AL vs AC ($r=0.081$, $p=0.006$) and also between social and asocial learning (SL vs AL ($r=0.070$, $p=0.017$)), meaning that in general the networks are not significantly different. A structural characterization of the functional network of associations between brain regions revealed that different nodes were central in the brain functional connectivity networks for the different experimental treatments (Figure 3): D_{lr}, Vd_{lr}, Dc_{lr}, Dd_{l}, Vp_{l}, PPp_{l}, Vc_{r} and Vv; for social learners (SL); Dm_{l}, PPa_{lr}, Hc_{l}, CIL_{l}, Vc_{r}, Vs_{r} for social controls (SC); Vp_{lr}, PM_{l}, CIL_{l}, NLV_{l}, Dm_{r}, Dd_{r}, PPa_{r}, CP_{r} and Hd_{r} for asocial learners (AL); and finally, Vd_{lr}, Dm_{l} and Hd_{r} for asocial controls (AC) (Table 2). Regarding cohesion, the density of *c-fos* networks was significantly higher in social control than in the social learning treatment (SL vs SC ($t=2.85$, $p=0.002$)). Social learning presented a close-to-significant denser network when compared with asocial learning ($t=1.26$, $p=0.10$). The control groups did not show a statistically significant difference between them ($t=0.18$, $p=0.43$). Visual inspection of functional connectivity networks suggests recruitment of more rostral telencephalic nuclei in social learning (Fig. 3a), and of ventro-posterior nuclei in asocial learning (Fig. 3c).

a)

Social Learning (SL)

Dorsal

Posterior

Anterior

Ventral

b)

Social Control (SC)

Dorsal

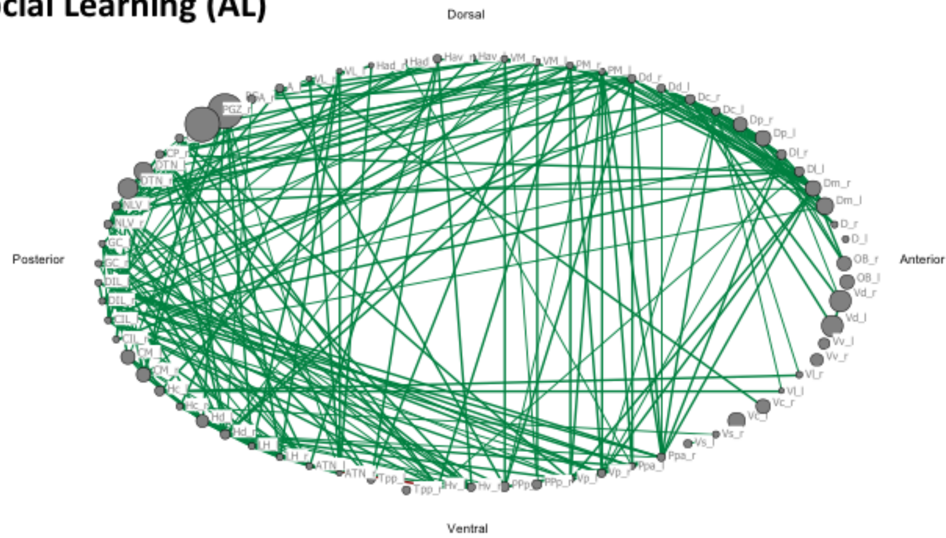
Posterior

Anterior

Ventral

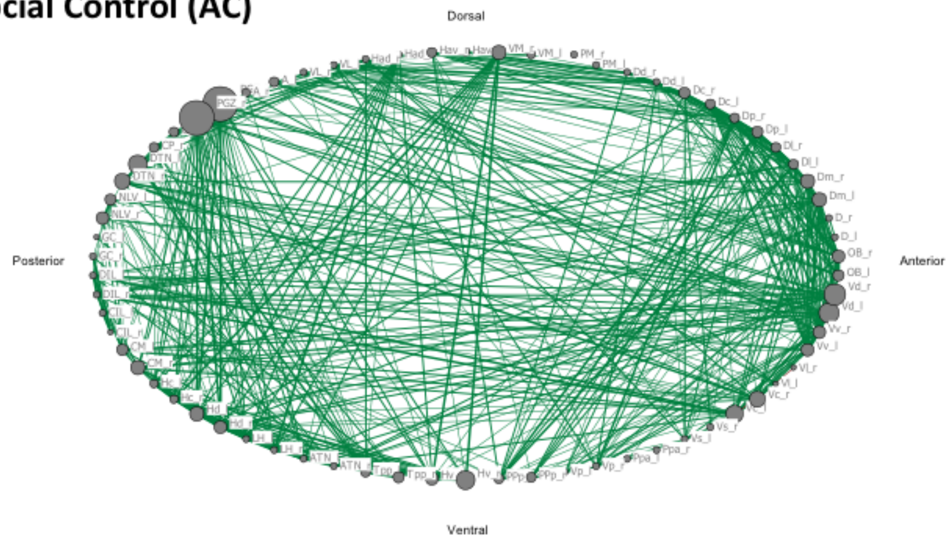
c)

Asocial Learning (AL)



d)

Asocial Control (AC)



e)

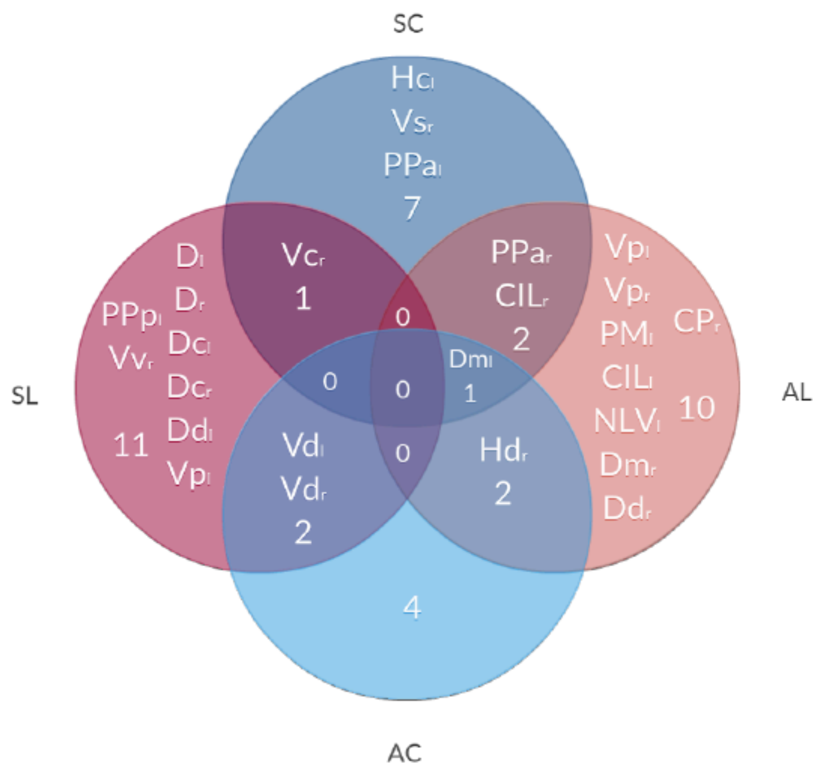


Figure 2.3 – Functional connectivity measured by Pearson correlations (r) of *c-fos* positive cells between pairs of brain nuclei for each experimental treatment: a) Social learning (SL); b) Social control (SC); (c) asocial learning (AL); d) asocial control (AC); colour scheme represents significant positive (green) and negative (red) r values ($p < 0.05$) and size of each node the average of *c-fos* positive cells: e) Venn diagram representing the central areas in the functional connectivity network of each experimental treatment.

Table 2.3 - Quantitative characterization of the functional connectivity networks for each experimental treatment, using *c-fos* positive cells as reporters of neuronal activity. Values correspond to centrality measures (eigenvalues) for each network node (legend in figure 3) and cohesion (density) across treatments (SL, AL, SC and AC).

Brain nuclei		c-fos positive cells							
		SL (left)	SL (right)	SC (left)	SC (right)	AL (left)	AL (right)	AC (left)	AC (right)
Eigenvalues	OB	0.014	0.150	0.032	0.020	0.000	0.044	0.117	0.166
	D	0.215	0.207	0.193	0.088	-0.001	0.028	0.012	0.025
	Vd	0.270	0.243	0.094	0.153	0.013	0.029	0.229	0.226
	Dm	0.000	0.160	0.204	0.125	0.076	0.232	0.223	0.184
	Dl	0.092	0.114	0.139	0.176	0.162	0.197	0.178	0.137
	Dc	0.259	0.257	0.126	0.177	0.101	0.083	0.177	0.069
	Dp	0.131	0.066	0.176	0.181	0.032	0.082	0.142	0.145
	Vc	0.181	0.277	0.158	0.218	0.000	0.001	0.163	0.185
	Vv	0.183	0.214	0.152	0.061	0.000	0.000	0.198	0.179
	Vl	0.166	0.000	0.000	0.000	0.011	0.011	0.016	-0.008
	Dd	0.237	0.094	0.128	0.110	0.103	0.215	0.101	0.067
	Vs	0.041	0.105	0.010	0.268	0.000	0.001	0.070	0.015
	Ppa	0.087	0.150	0.224	0.231	0.199	0.256	0.001	0.000
	Vp	0.222	0.067	0.010	0.009	0.220	0.219	0.072	0.101
	PM	0.001	0.004	0.006	0.061	0.321	0.193	0.003	0.000
	PPp	0.261	0.136	0.005	0.025	0.152	0.017	0.103	0.070
	Hav	0.042	0.003	-0.010	-0.001	-0.002	0.107	0.027	0.065
	VM	0.086	0.000	0.022	-0.001	0.078	0.097	0.007	0.098
	A	0.030	0.004	0.000	0.163	0.049	0.072	0.066	0.018
	Had	0.087	0.099	0.003	-0.001	0.032	0.010	0.110	0.077
	Hv	0.000	0.000	0.113	0.076	0.161	0.145	0.127	0.098
	ATN	0.005	0.000	0.036	0.149	0.153	0.015	0.133	0.107
	VL	0.012	0.034	0.000	0.004	0.009	0.010	0.105	0.044
	Tpp	0.105	0.082	0.041	0.042	0.071	-0.001	0.064	0.013
	PGZ	0.000	0.000	0.100	0.106	0.057	0.038	0.191	0.196
	CP	0.051	0.000	0.048	0.027	0.137	0.239	0.077	0.092
	Hd	0.000	0.000	0.171	0.137	0.069	0.230	0.190	0.204
	LH	0.000	0.000	0.171	0.180	0.031	0.035	0.113	0.113
	DTN	0.000	0.000	0.064	0.185	0.029	0.056	0.072	0.137
	Hc	0.004	0.044	0.200	0.171	0.007	0.008	0.136	0.114
	CM	0.000	0.000	0.044	0.041	0.049	0.086	0.159	0.146
	DIL	0.007	0.164	0.026	0.086	0.104	0.140	0.128	0.157
	CIL	0.091	0.058	0.251	0.025	0.234	0.024	0.017	0.018
	GC	0.000	0.000	0.000	0.057	0.098	0.049	0.001	0.027
	NLV	0.000	0.000	0.045	0.012	0.219	0.113	0.093	0.067
Density		0.094		0.154		0.071		0.158	

2.5 Discussion

In this study, animals learned using both social and asocial cues, and there were no significant differences in learning, either during training or during recall in the probe test, between the social and asocial learning treatments. However, individuals were able to discriminate between the social and asocial cues and, as previously described, they preferred the social cue (Dreosti et al., 2015; Saverino and Gerlai, 2008). Thus, together these results allow us to interpret the observed differences in the patterns of brain activation between social and asocial learning as indicating different neural circuits involved in each of these two types of learning rather than differences in the valence of the stimuli. We found that social learning recruits the activity of olfactory bulbs (OB), ventral zone of ventral telencephalic area (Vv), ventral habenular nuclei (Hav) and ventromedial thalamic nuclei (VM), whereas asocial learning is associated with a decrease in activity in the dorsal habenular nuclei (Had) and anterior tubercular nucleus (ATN).

Interestingly, all the brain regions identified as essential in social learning have been previously implicated in learning tasks. The OB has been described as an important brain region for social learning. The cryptic cells (a subtype of cells in olfactory bulbs) are recruited in kin recognition (Biechl et al., 2016), an increased of GABA and glutamate in mitral cells is observed after training in social transmission of food preference (Brennan et al., 1995; Burne et al., 2010; Kendrick et al., 1992; Nicol et al., 2014). These evidences are in agreement with our results where social learning, but not asocial learning, recruited OB. However, these results are controversial, because learning has been viewed as being processed by central brain regions (e.g. amygdala) rather than by more peripheral sensory brain areas (Haber and Knutson, 2010; Olsson et al., 2007; Twining et al., 2017).

The olfactory bulbs, in zebrafish, project to ventral zone of ventral telencephalic area (Vv) (homologous of the lateral septum in mammals) by primary and secondary olfactory projections (Kermen et al., 2013). In the present work, we demonstrated that Vv was a key brain region in social learning. This involvement of Vv can be through this direct projection that it receives from OB (Kermen et al., 2013), or by the efferent of the ventral telencephalon (Rink and Wullimann, 2004). In rodents, the lateral septum has been demonstrated to be involved in learning processes: the neurons of dorsal Lateral septum have been specifically associated with auditory fear learning (Calandreau et al., 2007, 2010), neurons of medial and lateral septum have been associated with contextual learning (Calandreau et al., 2007; Sparks and LeDoux, 1995), medial and lateral septum lesions impair working memory (M'Harzi and Jarrard, 1992). Others studies revealed the role of Vv in the processing of social information, such as social orientation

(Stednitz et al., 2018), audience effects (Roleira et al., 2017) and social exploration (Menon et al., 2018). Together, this evidence is congruent with our findings, where Vv is crucial to learning related from social cues.

Our results also revealed that social learning increased the expression of *c-fos* in the ventral habenula (vHb), a brain region that has been shown to mediate learning and memory. For instance, the inhibition of lateral habenula (LHb) led to deficits in spatial memory (Thornton and Davies, 1991), object recognition (Lecourtier et al., 2004), spatial working memory (Mathis et al., 2017), aversive conditioning to cocaine (Gill et al., 2013), and complex conditioning task (Flagel et al., 2011). Social behaviours can also be modulated by LHB: social isolation decreases the expression of *c-fos* in LHb, social play reduces the expression of *c-fos* in lateral habenula (Kerkhof et al., 2013), and the activation of LHb or PFC neurons and PFC-LHb projections impairs social behaviours (Benekareddy et al., 2018; Kim and Lee, 2012; Proulx et al., 2014; Yizhar et al., 2011). Animals without a defined PFC, such as zebrafish, are able to perform tasks that are PFC-dependent revealing that the function of the habenula complex is highly conserved across vertebrates (Parker et al., 2012). Two other circuits have been described in LHb: the GPH-LHb projection, that plays a role in the representation of valence through VTA (Baker et al., 2016), and VTA neurons or VTA-Nac projection, whose activation increases social interactions, but not interactions with objects (Gunaydin et al., 2014)); and the VP-LHb projections, which are essential to control the motivational valence and salience of the stimuli (Stephenson-Jones et al., 2019), that projects to dorsal raphe, which in turn has been demonstrated to induce sociability when photo activated and social isolation when photo inhibited (Matthews et al., 2016). These circuits are essential for the evaluation of the stimuli allowing the animals to avoid or approach aversive or rewarding cues, respectively (Stephenson-Jones, 2019; Stephenson-Jones et al., 2016, 2019). We observed an increased of expression of *c-fos* in LHb in social learning, which is controversial when compared with the literature: activation of LHb has been associated with improvement of learning skills (similar with our findings) but deficits of social behaviour have been associated with the activation of LHb. However, all these brain regions act as parts of networks and probably this effect is mediated by one of these projections or by all of them acting on circuits.

The lateral habenula, is a brain region part of the thalamus complex which is composed by thalamus proper (the anterior thalamic nucleus (A), the dorsal posterior thalamic nucleus (DP), the central posterior thalamic nucleus (CP)); the habenula (ventral (Hav) and dorsal (Had) habenular nuclei), and the prethalamus structures (the intermediate (I), the ventromedial (VM), and the ventrolateral (VL) thalamic nuclei) (Mueller, 2012). In the present work, the expression of *c-fos* in the ventromedial nucleus of the

thalamus also increased in social learning. This brain region belongs to the cortico-basal ganglia-thalamic loop circuit; the basal ganglia receives neural information from cortex and transfer them back to frontal and motor cortex via VM (Kase et al., 2015). The Gpi neurons, a motor output of the basal ganglia, projected to VM through GABAergic neurons recruiting migro-thalamic synapses to control excitation and inhibition of VM (Kase et al., 2015). This circuitry is also connected with LHb allowing animals to adjust the salience and valence of the stimuli.

In summary, social learning looks to be dependent on two main circuits: the olfactory system (OB-Vv, OB-Dp-vHb, Vv-hypothalamus) and the basal ganglia circuit (Gph-vHB-VTA-Nac, VP-vHb-DR and Gpi-VM) that together integrate the importance of social information to animals in a learning task with social cues. In asocial learning the circuits recruited imply other brain regions: the dorsal habenular nuclei (dHb) and the anterior tubercular nucleus (ATN).

The dorsal habenular nuclei (homologous of medial habenula in mammals) receive inputs mainly from the limbic system, and sends outputs to the interpeduncular nucleus, which in turn regulates activity dopamine (DA) and serotonin (5HT) neurons (Hikosaka, 2010; Kobayashi et al., 2013; Viswanath et al., 2014). Evidence in both mice and zebrafish supports our results that suggested dHb to be related to asocial learning. Ablation of mHb induces deficits in long-term spatial memory (Kobayashi et al., 2013), complex learning paradigms (Kobayashi et al., 2013) and fear learning (Agetsuma et al., 2010; Mathuru and Jesuthasan, 2013). In contrast, our findings imply a decreased expression of *c-fos,* probably due to a disinhibitory mechanism. However, mHb is also has been associated to stress responses: restraint (Sugama et al., 2002), aggression (Cirulli et al., 1998) and cold or hypoxia (Ebner and Singewald, 2006).

We demonstrated that the anterior tubercular nucleus (ATN) (homologous of ventromedial hypothalamus (VMH) in mammals) was associated with asocial learning. Commonly, VMH is associated with aggression (Lin et al., 2011) (Hashikawa et al., 2017; Lee et al., 2014; Yang et al., 2017), sexual behaviour (Hashikawa et al., 2017), defensive behaviour (Dhillon et al., 2006; Kunwar et al., 2015), fear (Beck and Fibiger, 1995; Zagrodzka et al., 2000) and satiety (Gaur et al., 2014). This brain region has been also related to learning processes with strong *c-fos* expression after fear conditioning (Trogrlic et al., 2011) and recall of conditioned fear (Campeau et al., 1997). The role of VMH in learning processes can be explained by the afferents from the amygdala (BLA and MEA), a brain region clearly shown to be involved in learning processes (Trogrlic et al., 2011). The amygdala is connected to the basal ganglia circuit through the striatum (Cho et al., 2013). Our results indicated that social and asocial learning recruited this circuit through different brain regions (social learning through ventral habenula and asocial learning by ATN-

amygdala-striatum). In summary, asocial learning triggered two other projections (ATN-amygdala-Striatum, dHb-IPN) that seem to not be directly connected to the same circuitry.

The functional connectivity analysis, shows a tuning effect of the general correlation between activity in brain nuclei with the learning groups (social and asocial learning) reducing the number of connections in the network when compared with control groups (social and asocial unpaired treatments). This phenomenon has been described as *default mode network* (Raichle, 2015) where a task-induced activity decreases functional connectivity in comparison with a resting state (Md et al., 2003). Our results also indicate that the central areas that operate in each functional network differ clearly across treatments supporting the hypothesis that social and asocial learning recruit different neural mechanisms.

In the present work, we demonstrated that social and asocial learning recruit different brain regions and those different patterns of functional connectivity parallel the different treatments. Together, our results are the first experimental evidence that social learning is a modular and not a general domain.

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Chapter 3. Mechanisms of social fear learning in zebrafish

3.1 Abstract

Detecting and responding to the presence of danger in the environment is a key component of Darwinian fitness. Group living animals can use social information to detect threat in the environment. In particular social learning allows animals to learn about dangers in the environment without incurring in the costs of trial-and-error learning about dangerous stimuli. In observational fear conditioning, the pairing of a social cue of danger (unconditioned stimulus, US) with a previously neutral stimulus (conditioned stimulus, CS), results in a conditioned alarm (fear) response elicited by the CS alone. In zebrafish both chemical and visual social cues of the presence of a threat in the environment elicit an innate alarm response, which consists of erratic movement followed by freezing behaviour. Injured zebrafish release an alarm substance from their skin into the water that is detected through olfaction and elicits the alarm response. Similarly, the sight of conspecifics displaying the alarm response can also elicit the expression of this response in observers.

In this study, we investigated if these two social cues of danger can also be used by zebrafish as unconditioned stimulus (US) in social fear conditioning. We found that only the chemical cue (alarm substance) was effective as an US in the observational conditioning task.

We suggest that this differential efficacy of social cues in the conditioning process results from the fact that the alarm cue is a more reliable indicator of the presence of threat in the environment (because it requires a physical injury of a conspecific), than the sight of an alarmed conspecific. Therefore, although multiple social cues may elicit innate responses not all have been evolutionarily co-opted to act as US in social associative learning. Furthermore, the use of the expression of the immediate early genes *c-fos, egr-1, bdnf* and *npas4* as markers on neuronal activity revealed that chemical observational conditioning is paralleled by a differential activation of the olfactory bulbs and by a different pattern of functional connectivity across brain regions involved in olfactory processing.

3.2 Introduction

A key component of Darwinian fitness is the ability of animals to detect and respond to the presence of danger in the environment, namely predators. Given that typically threat cues used by animals to detect danger have some overlap with background ambient noise in the sensory modality used to monitor the environment (e.g., an individual may have to decide if a rustle in the grass indicates the presence of a predator or if it is just the wind), according to signal detection theory individuals need to set a signal detection threshold that they use to make a decision that they are in the presence of a threat and activate the appropriate behavioural response (Wiley, 2013, 2006). If individuals set a high threshold they will fail to detect a real threat frequently (miss), but they will activate few false alarms. On the other hand, if they set a low threshold they will miss fewer real threats, but at the cost of more frequent false alarms. Therefore, there is a trade-off between misses and false alarms and the setting of a threat detection threshold is critical for survival (Oliveira and Faustino, 2017). Group living animals can use social information to detect threat in the environment. In group living species individuals can use social information provided by others to detect threat cues, and it has been demonstrated that the above mentioned trade-off between misses and false alarms present in individual decision-makers, can be overcome in a group of decision-makers using a quorum decision rule (Max et al., 2013). Therefore, the use of social information in threat perception allows to overcome this basic trade-off in individual signal detection theory. Thus, the use of social information in threat perception is expected to be widespread in social living organisms.

There is ample evidence that animals indeed use social information to modulate their threat perception. The exposure to social cues signalling threat, such as the sight, sound, or smell of an alarmed conspecific usually trigger a fear response (e.g. (Inagaki et al., 2014; Kim et al., 2010; Pereira et al., 2012), a phenomenon known as social contagion of fear (Dezecache et al., 2015; Keum and Shin, 2016). Conversely, the presence of a non-alarmed familiar conspecific may signal safety and it has been shown to attenuate fear response, which has been termed social buffering of fear (e.g. (Edgar et al., 2015; Kiyokawa et al., 2014; Smith and Wang, 2014)). Moreover, these social cues of threat can be used as unconditioned stimulus (US) in a classic conditioning paradigm, such that when paired with a conditioned stimulus (CS) may reinforce the establishment of conditioned fear responses to this CS, a phenomenon referred to as social fear learning (aka vicarious fear learning, vicarious aversive conditioning, or observational fear learning (Debiec and Olsson, 2017; Olsson and Phelps, 2007)). One of the first documented cases of social fear learning has been described in rhesus monkeys (*Macaca mulatta*) in

which naïve individuals have no fear of snakes but acquire a fear response towards snakes after observing a demonstrator reacting fearfully to a snake (Cook and Mineka, 1988; Mineka et al., 1984). Although this phenomenon has been initially termed observational conditioning, it is not restricted to visual cues, and examples of social fear learning have been documented with odour cues, such as alarm pheromones in fish (Brown and Chivers, 2006), or acoustic cues, such as mobbing calls in birds (Curio, 1988), being used as US. The ubiquity of social fear learning across different taxa and using different sensory modalities reflects its adaptive importance since it allows individuals to learn about threat without using trial-and-error learning in an ecological domain where the cost of misses would be very high, most probably death (Hoppitt and Laland, 2013) .

Zebrafish uses both chemical and visual social threat cues to assess the presence of danger in the environment, and responds to these with an innate alarm response, which consists of erratic movement followed by freezing behaviour. Like in many other fish, injured zebrafish release an alarm substance from their skin into the water that is detected through olfaction and elicits the alarm response (Jesuthasan and Mathuru, 2008; Speedie and Gerlai, 2008). The alarm substance, originally termed Schreckstoff by Karl von Frisch, who first described it in minnows (*Phoxinus phoxinus*) (Von Frisch, 1941), is produced in specialized epidermal club cells, and is released upon skin injury (Smith, 1992). The molecular identity of the alarm substance has not been yet clearly established, but the available evidence suggests it is a mixture of compounds, and two putative active compounds have been suggested so far: hypoxanthine-3 N-oxide and the glycosaminoglycan chondroitin (Brown et al., 2000; Lebedeva et al., 1975; Mathuru et al., 2012; Parra et al., 2009; Pfeiffer et al., 1985). The sight of conspecifics displaying the alarm response can also elicit the expression of this response in observer zebrafish (Al-Imari and Gerlai, 2008). Although there have been previous publications reporting socially learned alarm response in zebrafish a closer examination of the original findings reveals some weaknesses, namely the use of very small sample sizes lacking statistical power (N=2 in (Suboski et al., 1990); N= 3 in (Hall and Suboski, 1995)), the use of group behavioural measures rather than individual behaviour, thus, ignoring individual variation in the response, and a higher alarm response in observers than demonstrator (Suboski et al., 1990; Hall and Suboski, 1995). Despite these weaknesses this seminal work, the occurrence of social transmission of fear in zebrafish has been propagated in secondary sources in the literature and given the attention that this field of research has been receiving recently it needs an urgent reassessment.

Here, we examine the efficacy of two social cues of danger (alarm substance and alarmed conspecifics) as unconditioned stimulus (US) in social fear learning in zebrafish, and in positive cases we

will also describe the neuronal mechanisms involved using the expression of the immediate early gene *c-fos* as a reporter of neuronal activity.

3.3 Material and methods

3.3.1 Animals and housing

Tubingen's adult wild-type Zebrafish (*Danio rerio*) (n=72) were bred and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). Fish was kept in a recirculation system (ZebraTec, 93 Tecniplast) at 28ºC with 14 light: 10 darkness photoperiod until four months of age. The water system was maintained at less than 0.2 ppm nitrites, 50 ppm nitrates and 0.01 ppm ammonia, while pH and conductivity were maintained at 7 and 700 µSm respectively. Fish was fed twice a day with commercial food flakes (Bionautic) and *Artemia salina*.

3.3.2 Experimental protocol

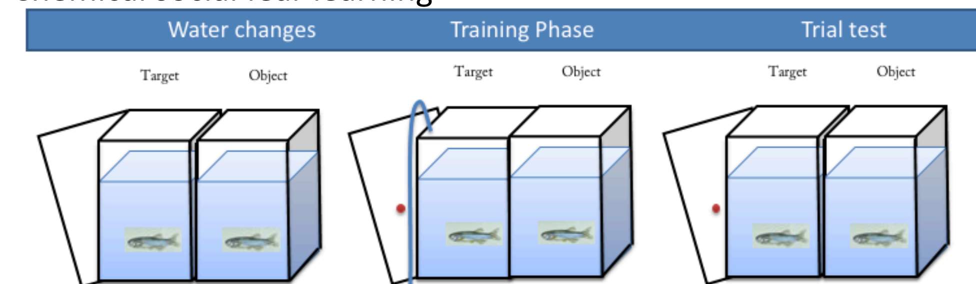
In the innate response to threat experiment, each animal was exposed to one of 4 treatments for 5 minutes: alarm substance (alarm), pre-trained conspecific (conspecific), distilled water (control) and light (L). The threat response is a stereotyped behaviour where adult zebrafish exhibit erratic movement and freezing. Erratic movement is characterized by multiple darts (fast acceleration bouts and stochastic changes in direction) and is normally the first response to danger (Kalueff et al., 2013). Freezing is a complete cessation of movement (except for gills and eyes) while the fish is at the bottom of the tank and showing abundant opercular movements (respiration/gill movements) (Kalueff et al., 2013).

A behavioural paradigm was designed to characterize chemical and visual social fear learning in zebrafish. In visual social fear learning, a demonstrator fish was trained to pair a light with alarm substance (CS + D) or distilled water (CS - D). This demonstrator training phase lasted for 3 days, with 3 trials/day. During this phase, the observer did not have contact with the demonstrator. The observer training phase followed, during which the demonstrator and observer were presented with the CS for 3 trials/day for 3 days, which elicited a conditioned alarm response in the demonstrator (CS + O and CS- O). On the seventh day (trial test), the response of the observer towards the CS was tested in the absence of the demonstrator. In chemical social fear learning, the observer fish was subjected to water changes for 3 days in order to standardize conditions between experiments. During the training phase, the observer

was trained (3 trials/day for 3 days), in a similar way to the training of demonstrators in the previous experiment, to pair the light (CS) with the alarm substance (CS +) or with distilled water (CS -). On the trial test (7th day), the response of the observer towards the CS was tested in the absence of the alarm substance administration.

All fishes were isolated in individual tanks one day before the experiments. Alarm substance and control (US) were introduced into the tanks using a plastic tube (0.8 mm internal diameter, Kartell, UK) connected to a 5 ml syringe (Terumo, Japan). The alarm substance was prepared from skin extracts of zebrafish following a modified protocol described by Speedie and Gerlai (2008). The alarm substance donors were commercial wild-type zebrafish (half were males and half females) captured and quickly sacrificed by decapitation using surgical scissors. Light (CS) was presented at the side of the tank in the middle of the water column. The experiments were videotaped in side view. The behaviours were recorded using a multi-event recorder (Observer XT 9, Noldus Technology) and an automatic tracking system (Ethovision XT 12, Noldus Technology).

Chemical social fear learning



Visual social fear learning

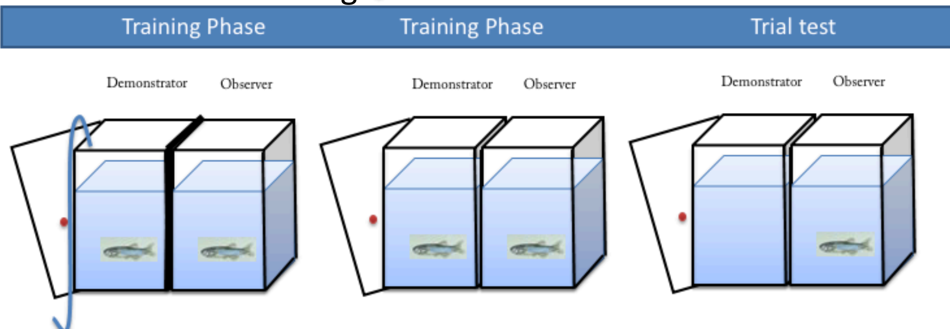


Figure 3.1 – Experimental design of visual and chemical social fear learning. In chemical social fear learning, after 3 days of water changes the training phase consisted of pairing a light (CS) with alarm substance (CS+) or control (CS-). In the trial test, the CS alone was presented to evaluate if the fish have

learned the association. The fish in the contiguous tank was used to standardize conditions between the visual and the chemical social fear learning experiments. In visual social fear learning, the demonstrator was trained in the same way as the observer in the chemical social fear learning protocol. During this period, an opaque partition was used to avoid visual access of the observer to the demonstrator. In the observer training phase, the light elicited an alarm response in the demonstrator, hence from the observers perspective the light was paired with the sight of an alarmed conspecific (CS+). In the trial test, demonstrators were not present and the observer was exposed by themselves to the light (CS).

3.3.3 Microdissection of the zebrafish brain

After the behavioral experiment, the animals were quickly sacrificed with an excess of anesthesia (MS-222, Pharmaq; 300-400ppm) followed by decapitation. The head was incorporated in Optical Cutting Temperature (OCT) (Tissue-Tek, Sakura, Netherlands) and frozen at -80ºC. Coronal head slices (150µm) were cut in a cryostat and stored at -20ºC on microscope slides (Thermo Scientific, USA). Regions of interest were micropunched from the brain slices using a modified 27G needle (the bevel and the external diameter were removed) following a zebrafish brain atlas (Wullimann, M.F., Rupp, B., Reichert, 1996) to localize their anatomical position. The following brain regions were micropunched: Olfactory bulb (OB), medial zone of Dorsal Telencephalic area (Dm: homolog of cortical amygdala in mammals) (Friedrich et al., 2010), posterior zone of Dorsal Telencephalic area (Dp: homolog of olfactory cortex) (Friedrich et al., 2010), ventral nucleus of Ventral Telencephalic area (Vv: homolog of septal formation in mammals) (Friedrich et al., 2010)and Habenula (Ha: homolog of mammalian lateral Habenula) (Amo et al., 2010). The samples were stored in Eppendorfs with 50 µl of Quiazol (Quiagen, USA) at -80ºC.

3.3.4 Quantitative RNA expression of immediate early genes

The following genes were used as markers of neuronal activity (*c-fos* and *egr-1*) or of different types of neuronal plasticity: brain-derived neurotrophic factor (bdnf), involved in changes in synaptic plasticity by increasing synaptic strength in response to excitatory transmission (Leal et al., 2013) and neuronal PAS domain protein 4a (npas4), involved in homeostatic plasticity, by enhancing inhibitory synapses in response to excitatory transmission (Lin et al., 2008).

Total RNA from each brain microarea was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, USA) and then stored at -80ºC. The integrity of the RNA extracted was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, UK). First-strand cDNA was prepared using iScript cDNA synthesis kit

(Biorad, USA) based on RNase H+ and oligo(dT) and random hexamer primers and stored at -20ºC. Quantitative PCR (qPCR) was performed in the ABI7900HT (Applied Biosystems, Life Technologies, USA) using 384 well-plates where cDNA, primers (Table 1) and SYBR green PCR master mix (Applied Biosystems, Life Technologies, USA) were added. qPCR data were collected using Sequence Detection Systems (SDS 2.4) (Applied Biosystems, Life Technologies, USA) with 1 cycle 95°C for 5 minutes; 40 cycles 95°C for 30 seconds, annealing temperature of the primers (Table 1) for 30 sec and 72°C for 30 sec). The threshold was defined by gene and a table of Ct values for each of the 384 reactions exported for data analysis. The Ct analysis were performed using 2 ^ (Ct housekeeping - Ct target gene) assuming that the efficiency of the genes was 100%. To validate this assumption, we measured the efficiency of each gene and we have only used primers with efficiencies above 90%.

Table 3.1 – Sequence, annealing temperature and efficiency of the primers used to measure immediate early gene expression using qPCR.

Primer	Sequence	Annealing Temperature (°C)	Efficiency (%)
elf1a_F	5'CAAGGAAGTCAGCGCATACA3'	59	96.2
elf1a_R	5'TCTTCCATCCCTTGAACCAG3'		
c-fos_F	5'CCGATACACTGCAAGCTGAA3'	59	99
c-fos_R	5'CGGCGAGGATGAACTCTAAC3'		
egr1_F	5'GTGAGCCAACCCCATCTAT3'	60	99.5
egr1_R	5'CCAGGCTGATCTCACTTTGC3'		
bdnf_F	5'GCTGCCGAGGAATAGACAAG3'	61	99.2
bdnf_R	5'CTGCCCCTCTTAATGGTCAA3'		
npas4_F	5'GACACGGGTTGAGAATGGTT3'	59	99.1
npas4_R	5'GCACCAAGCACCCTGTAAAT3'		

3.3.5 Statistical analysis

The behavioural effects of innate responses to alarm cues were tested using a non-parametric ANOVA (Kruskal-Wallis) followed by post-hoc tests where equal variances were not assumed (Tamhane T2 post-hoc). The occurrence of chemical social fear learning was tested using non-parametric t-tests

(Mann-Whitney) for each measure (i.e. erratic movement and freezing). The occurrence of visual social fear learning was tested using a non-parametric ANOVA (Kruskal-Wallis) followed by post-hoc comparisons where equal variances were not assumed (Tamhane T2 post-hoc) for each measure. The effects of social fear learning (trained animals with alarm cue or distilled water) and brain region (OB, Dm, Dp, Vv, Ha) in the expression of immediate early genes (*c-fos, egr-1, bdnf, npas4*) were tested using between-subject linear mixed models (LMMs) with the subject as a random effect. Parametric assumptions were checked using Shapiro–Wilk and Jarque-Bera adjusted multiplier tests (to test for normality), Bartlett, Levene and Fligner–Killeen tests (to test for homoscedasticity), and plots of the residuals, fitted values and estimated random effects in the LMM. Gene expression data were log-transformed before the analyses to fit parametric assumptions. Planned comparisons among social fear learning treatments within each brain nucleus and for each IEG were computed to test for differential activation of each brain region in response to social fear learning.

Functional connectivity among the sampled brain regions was tested with Pearson correlation matrices computed between the IEG expression for each pair of brain regions in social fear learning treatment. Two regions correlated positively indicate co-activation in response to that treatment, whereas two regions correlated negatively indicate reciprocal inhibition in response to the treatment. Quadratic assignment procedure (QAP) correlation tests with 5000 permutations were used to test for differences between the correlation matrices (that portrait co-activation/co-inhibition among brain regions) for each treatment (Borgatti et al., 2013) . In QAP tests a significant p-value is indicative of association between the matrices; that is, different patterns of functional connectivity captured by the matrices correspond to non-significant QAP test p-values.

The structure of the neural network composed of the sampled brain regions in this study was characterized using measures of centrality and cohesion. Centrality of each node in the network was measured using eigenvector centrality, which integrates every link a node receives with the relevance of each node of the network. Cohesion of the whole network was measured by density, which is the average of connections quantified for each network. Density was assessed using a bootstrap t-test approach with 5000 sub-samples.

Statistical analyses were performed on SPSS (version 22) and R (version 3: www.R-project.org) using the following packages: car (Levene test), cluster (PAM), fBasics (Jarque – Bera test), Hmisc (correlations), lattice (heatmaps), multcomp (planned comparisons) and nlme (LMMs). The network analysis parameters were estimated using UCINET v. 6. Network representations were produced using Python.

3.4 Results

3.4.1 Innate response to threat cues

Both the alarm substance and the sight of alarmed conspecifics induced a peak in swimming speed followed by a decrease, which correspond to the erratic movement and freezing phases of the zebrafish alarm response. A shorter distance from the bottom is also observed during the alarm response, and despite some inter-individual variation the alarm response to both alarm cues is quite robust (fig 2a and 2b). Importantly, neither distilled water (used here as a CS-) nor light (used as a CS+) triggered by themselves any such behavioural responses. A single exposure to the alarm substance elicited an alarm response composed of 20 % time in erratic movement and 80 % in freezing (triangles, fig 2c). The sight of alarmed conspecific induced an alarm response with a bimodal distribution in erratic movement (i.e. with low and high responders) and the freezing was almost absent (there were no significant differences in the time in freezing between treatments) (fig 2c). Control and light never elicited erratic movements or freezing (fig 2c). For detailed statistical information check table 2.

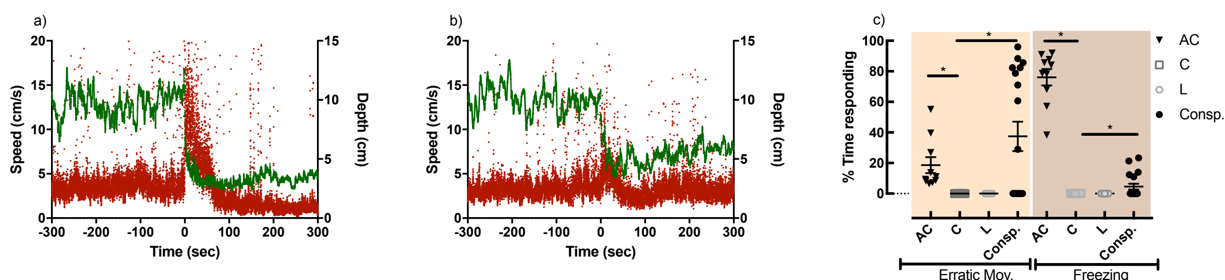


Figure 3.2 – Innate response to visual (sight of alarmed conspecifics (Consp.)) and chemical (alarm substance (AC)) social cues of threat, control (distilled water (C)) and light (L). Speed (red) and depth (green) are plotted along time in response to alarm substance (a) and sight of alarmed conspecific (b); t=0 is when the social cue is delivered. The percentage of time in erratic movement and freezing was measured in all experimental treatments (c). * represents p-value < 0.05.

3.4.2 Visual and Chemical social fear learning

In visual social fear learning, 50% of the animals respond to the alarmed demonstrator on the first trial (US in this experiment) (in black fig 3a). Observer did not seem to have learned by observation that light (CS) predicts the alarmed conspecific (US), since there was no reduction in latency to respond to the CS along the training trials (full black circle, fig 3c). Moreover, in the probe test, observers did not exhibited a conditioned alarm response to the CS alone (erratic movement: KW=382, p<0.0001, freezing: KW=5.693, p=0.002) (full black circle, fig 3d). Demonstrators and observers trained with distilled water (CS - D or CS - O) did not exhibit alarm responses (table 2; empty black triangle and circle respectively, fig 3d). Demonstrators showed the conditioned alarm response in response to the light (table 2; in full black triangle, fig 3d).

In chemical social fear learning, 100% of the animals exhibited the alarm response from the first trial onwards (in grey, fig 3a), and during training, they started to express the alarm response before the US is present from the third trial onwards (in full grey squares, fig 3b). Animals exposed to the CS- (control group) did not express the alarm response all over the training phase (in empty grey squares, fig 3b). In the probe test, animals expressed the alarm response towards the CS+ alone, but not towards the CS- (erratic movement: MW=11, p=0.0001, freezing: MW=11, p<0.0001).

These results show that although zebrafish has an innate response both to chemical and to visual social cues of danger, it only learns from chemical but not from visual cues. Since only the chemical social cue acted as a US only the brains of animals from this experiment were collected to characterize the putative neural circuits underlying social fear learning.

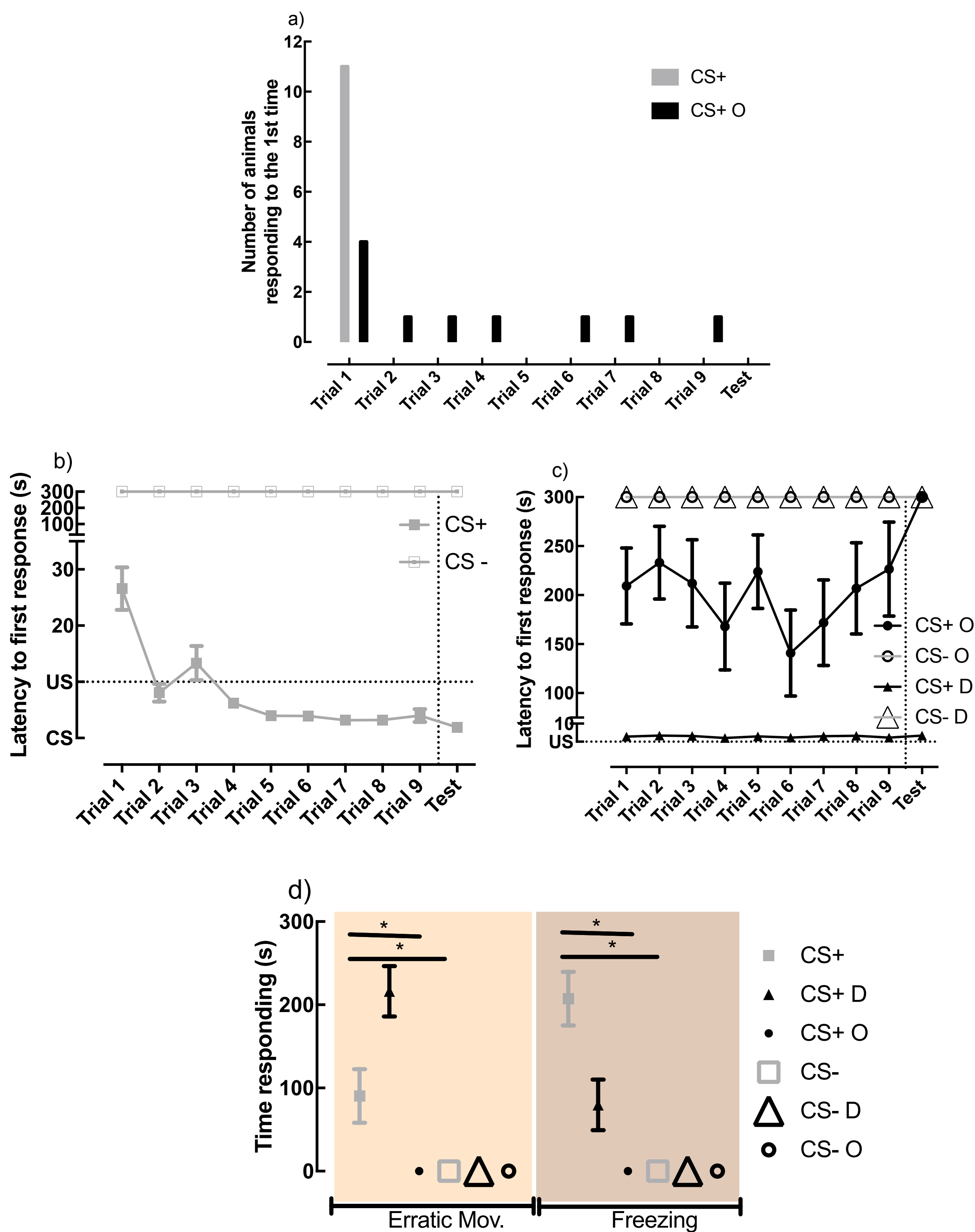


Figure 3.3 – Chemical and visual social fear learning in Zebrafish. a) Number of animals responding for the first time to CS + (in the grey bars) of social fear learning and CS + O (in black bars) of social fear learning.

b) Latency to the first response to CS + (in grey filled square) and CS - (in grey open square) of social fear learning. c) Latency to the first response to CS + O (in black filled circle) an CS -O (in black open circle) of the social fear learning (demonstrators' values are shown in black filled triangle (CS + D) and black empty triangle (CS - D)). d) Time responding in seconds during trial test with erratic movement (left) and freezing (right) by the same experimental groups described in the previous graph. Values are means ± se. * represents p value < 0.05.

3.4.3 Neural correlates of social fear learning

Transcriptional expression of immediate early genes (*c-fos, egr-1, bdnf* and *npas4*) were measured in brain regions of interest (OB, Dm, Dp, Vv and Ha) as markers of neuronal activation. *Egr-1* and *npas4* RNA expression levels (normalized to the housekeeping gene *elf1a*) in olfactory bulbs in response to the CS+ in the probe test were significantly reduced when compared to their response to the CS- (egr1 (z=2.48, p=0.013) and npas4 (z=2.19, p=0.028). No other significant differences in RNA expression of IEGs were found in the sampled brain regions (table 2).

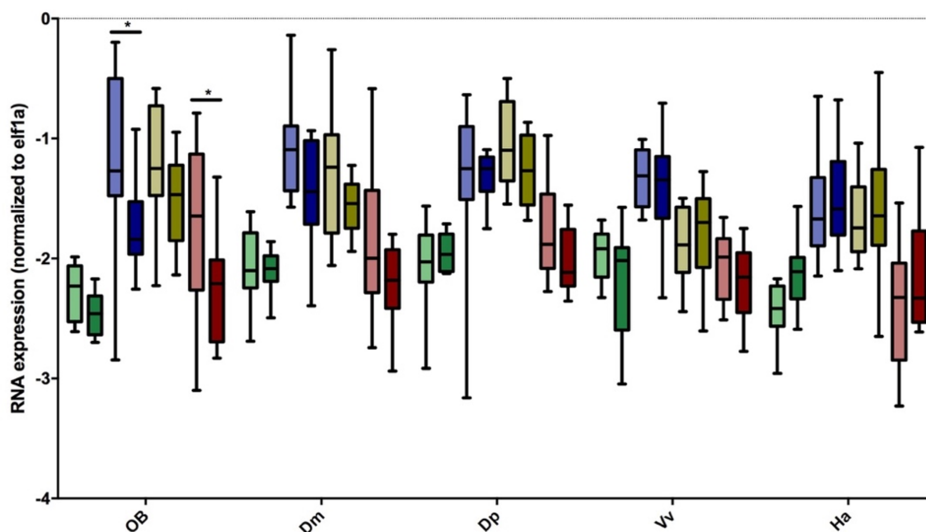


Figure 3.4 – Transcriptional pattern of RNA expression of the *c-fos* (green), *egr-1* (blue), *bdnf* (yellow) and *npas4* (red) were measured in the probe test in response to CS + (in dark colours) and CS - (in light colours) across the brain regions of interest (OB, Dm, Dp, Vv and Ha). Values are median ± interquartiles ranges. * represents a significant difference between the indicated groups.

3.4.4 Changes in functional connectivity of brain regions of interest in response to social fear learning

In chemical social fear conditioning, the co-activation matrices for CS + treatment were significantly different from CS- (control) for *c-fos* (r= 0.51 p= 0.186), *egr-1* (r = 0.32 p= 0.337), *bdnf* (r= 0.67 p= 0.173) and *npas4* (r= 0.23 p= 0.385). The structure of the gene expression networks was characterized through density and cohesion. The density of gene expression induced by the CS + was significantly lower than that induced by CS- (control) for *bdnf* (t= 1.77 p=0.041) and *npas4* (t= 1.62 p=0.037). The centrality of the different brain regions was also different between CS + alarm and CS- (control) treatments for the different IEG's.

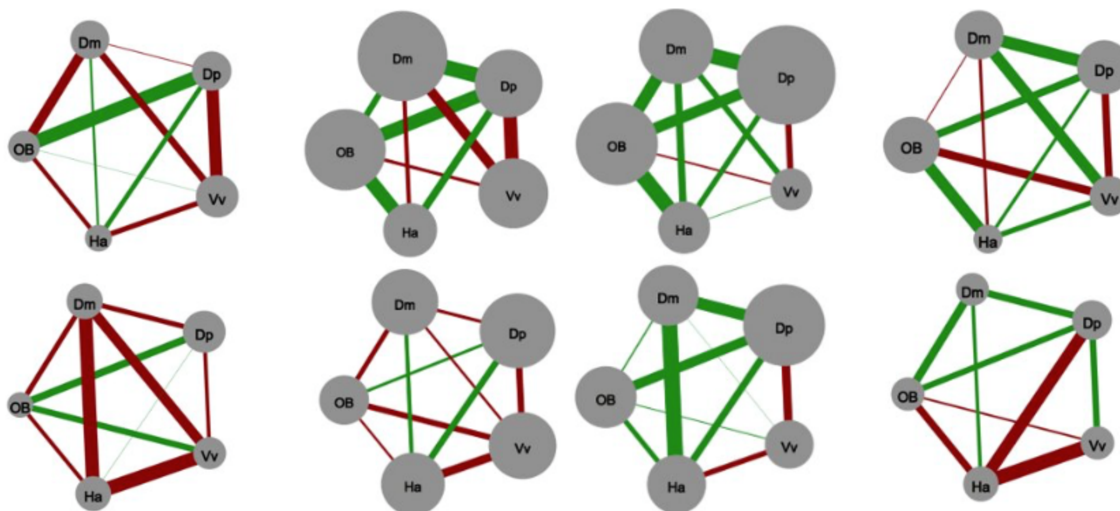


Figure 3.5 – Functional connectivity of gene expression networks across brain regions of interest induced by social fear learning (CS - (first row) and CS + (second row) and *c-fos* (first column), *egr-1* (second column), *bdnf* (third column) and *npas4* (fourth column). The size of the circles indicates of the level of RNA expression of each node. Arrows thickness illustrate the magnitude of correlation coefficient (r) between pairs of brain nuclei and arrow colour represents the sign: positive (green) and negative (red).

Table 3.2 – Descriptive statistics of the innate, chemical and visual social fear learning and transcriptional expression of IEG in chemical social fear learning.

Experiment	Behavior	Main effect	Multiple comparisons Tamhane T2 Post-hoc					
			C-AC	L-AC	AC-Consp.	C-L	C-Consp.	L-Consp.
Innate Response	Erratic movenment	KW=15.41, p<0.0001	p<0.0001	p<0.0001	p=0.544	p=1	p=0.005	p=0.005
	Freezing	KW=55.17,p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=1	p=0.118	p=0.118

Experiment	Behavior	Main effect
Chemical social fear learning		
	Erratic movenment	MW=11,p<0.0001
	Freezing	MW=11,p<0.0001

Experiment	Behavior	Main effect	Multiple comparisons Tamhane T2 Post-hoc			
			CS+alarm O - CS+ct O	CS+ D - CS- D	CS+ O - CS+ D	CS- O - CS- D
Visual social fear learning	Erratic movenment	KW=382, p<0.0001	p=1	p<0.0001	p<0.0001	p=1
	Freezing	KW=5.693,p=0.002	p=1	p=0.110	p=0.110	p=1

	LMM	c-fos CS+Ct - CS+alarm	egr1 CS+Ct - CS+alarm	bdnf CS+Ct - CS+alarm	npas4 CS+Ct - CS+alarm
Transcriptomic patters of IEG in social fear learning	OB	z=0.668, p=0.504	z=2.478, p=0.013	z=1.529, p=0.126	z=2.195, p=0.028
	Dm	z=0.215, p=0.830	z=1.478, p=0.139	z=1.178, p=0.239	z=1.616, p=0.106
	Dp	z=-0.455, p=0.649	z=0.552, p=0.581	z=1.030, p=0.303	z=1,197, p=0.231
	Vv	z=1.055, p=0.291	z=0.454, p=0.650	z=-0.396, p=0.692	z=-0.646, p=0.518
	Ha	z=-1.179, p=0.238	z=-0.494, p=0.621	z=-0.355, p=0.722	z=-1.120, p=0.263

3.5 Discussion:

Our results demonstrate for the first time, that zebrafish learn a conditioned fear response using alarm substance but not the sight of an alarmed conspecific as a US. Thus, although zebrafish innately respond both to chemical and visual alarm cues, only chemical cues are efficient as an US in fear conditioning. These results suggest that chemical alarm cues have a higher threat value than the visual ones. Indeed, since the content of club cells (that produce the alarm substance) cannot be released voluntarily, and these cells are among the first to be damaged upon a predator attack (due to their superficial location in the epidermis), the release of the alarm substance is a reliable social cue for the presence of an active predator in the environment (Chivers et al., 2007; Smith, 1992). In contrast, alarm responses in conspecifics do not request a predator attack to have happened and must be more variable across individuals and hence less reliable. However, this result is somewhat surprising since social fear learning is highly adaptive, given the cost to learn by trial-and-error the consequences of interacting with threat sources (e.g. predators). For instance, in the damselfish *Acanthochromis polyacanthus*, individuals that socially learned the odour of the predator increased their probability of surviving 5-fold (Manassa and McCormick, 2012).

Different sensory modalities can be used to detect alarm cues in the environment. Both chemical and visual alarm cues are well documented in fish (Brown and Laland, 2003; Elvidge and Brown, 2012). In 2001, a work reveals that chemical cues seem to have a role in to warn the presence of a predator and visual ones to assess the risk in sculpin (Chivers et al., 2001). While studies have shown that visual information is enough to elicit learning(Ferrari et al., 2007) others highlighted that in the absence of light, social recognition can still occur in damselfish (Manassa et al., 2013) indicating the importance of chemical cues to learning processes. The salience and the valence of the stimuli is an important factor in learning success. Individuals learn faster with aversive cues since the cost of receiving a punishment is higher than losing a reward (Steel et al., 2016). Also, the salience of a stimulus plays a role in learning abilities; high and low intensity shock, for instances, have different costs (Rumbaugh et al., 2007), and in zebrafish the intensity of the alarm reaction varies directly with the concentration of the alarm substance that the fish is exposed to (Speedie and Gerlai, 2008). Moreover, it has been established that animals are evolutionary predisposed to learn some associations better than others, a phenomenon named prepared learning (Dunlap and Stephens, 2014). Together these facts suggest that chemical alarm cues are more reliable than visual ones, and hence became more salient and as a result zebrafish became predisposed to learn better a fear conditioned responses triggered by a chemical US than by a visual US.

The neural mechanism associated with chemical social fear learning were also assessed though IEG expression. Our results point to the involvement of the olfactory bulbs in chemical social fear learning, a brain region that has also been described to process the innate response to alarm substance (Enjin and Suh, 2013). The involvement of the OB in odour fear conditioning has also been reported in rodents (Jones et al., 2007) as well as appetitive odour learning (Sullivan and Leon, 1987).

The olfactory bulbs are a brain sensory area that is involved in the detection of olfactory cues in the environment, and that projects (through OB mitral cells, which are second-order sensory neurons of the OB) to the olfactory higher processing areas (e.g. piriform cortex in mammals). Antagonists of norepinephrine receptor in OB impaired conditioned odour preference learning, memory recognition and odour identification (Escanilla et al., 2012; Guerin et al., 2008; Linster et al., 2011; Manella et al., 2013). Also, some authors suggest that OB circuits display functional plasticity, as other brain regions, including long-term synaptic potentiation (Gao and Strowbridge, 2009), adult neurogenesis (Lledo et al., 2006) and reconfiguration by neuromodulators (Devore et al., 2012). Together, these data support the role of the OB as a plastic brain nucleus involved in learning besides its role as a sensory region (Tong et al., 2014).

Different patterns of functional connectivity among brain regions involved in olfactory processing were also observed between chemically conditioned animals and their controls for all IEGs tested (*c-fos, egr-1, bdnf*, and *npas4*). The central areas in each network were also different between chemical social fear learning and its controls for all genes. *Bdnf* and *npas4* significantly decreased the average number of connections in chemical conditioned individuals in relation to their controls. Similarly, authors described the role of *bdnf* as neuromodulator in mammalian hippocampus and its implications for learning and memory (acting on long-term potentiation) (Kovalchuk et al., 2002; Park and Poo, 2013). Also, *npas4* has been implied in the formation of contextual memories in the hippocampus in rodents (Ramamoorthi et al., 2011). These genes were used as markers of neuroplasticity (*bdnf* - synaptic plasticity (Leal et al., 2013) *npas4* - homeostatic plasticity (Lin et al., 2008)).

In summary we have confirmed the occurrence of social fear learning in zebrafish only for chemical cues, and we have characterized its underling neural circuits.

3.6 Acknowledgments

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Chapter 4 . General discussion

4.1 Overview of results

In the present thesis were identified the behavioural and neuronal mechanisms underlying social learning in zebrafish. Social learning was behaviourally tested through aversive and reward observational conditioning. In aversive observational conditioning, we used a red light paired with alarm cue or sighted alarm conspecific. In reward observational conditioning, the food was paired with social and asocial cues (image of a conspecific or a dot). At the neuronal level, *c-fos* (an immediate early gene, marker of neuronal activity) was used to characterize the brain nuclei involved in processing social and asocial learning in zebrafish. In addition, we characterized changes in olfactory system in response to olfactory social learning.

In chapter II, reward Observational conditioning was evaluated. Behaviourally, no differences were observed between social and asocial learners. *C-fos* expression was used to map the brain nuclei recruited to process social and asocial observational conditioning in zebrafish. Social learners found the location of the food using a social cue (picture of a conspecific) and showed differential activation of the Olfactory Bulbs (OB), ventral zone of ventral telencephalic area (Vv), ventral habenula (Hav) and ventromedial thalamus (VM). Asocial learners used dorsal habenula (Had) and anterior tubercular nucleus (ATN) to find the food source through asocial cues (picture of a dot matched in size and colour to the fish image used for social learning). Also, social and asocial learning reveals distinct networks of correlation of the brain nuclei in functional analysis. Thus, specific neuronal circuits were associated with social and asocial learning.

In the chapter III, the effectiveness of a social cue processed through two distinct sensory modalities was assessed using an aversive learning paradigm. The social visual cue, the sight of an alarm conspecific, was not effective as an US. In contrast, the social olfactory cue, the alarm substance, was highly efficient as an US. Using a candidate brain nuclei approach and the expression of immediate early genes as markers of neuronal activity, the olfactory bulbs were identified as an essential brain region for olfactory observational conditioning. Moreover, the connectivity and cohesion of the brain nuclei involved in olfactory processing were tuned in response to chemical observational conditioning.

4.2 Observational conditioning as a behavioural mechanism

Observational conditioning is defined as a learning mechanism that is influenced by

observation of, or interaction with, another animal (typically a conspecific) or its products (Galef, 1988), and has been demonstrated in several species (humans (Burke et al., 2010; Haaker et al., 2017), rhesus monkeys (*Macaca mulata*) (Mineka et al., 1984), rats (Galef and Whiskin, 2003), birds (European blackbirds (*Turdus merula)* and Australian zebra finches *(Tueniopygia guttata))* (Curio, 1988), octopuses *(Octopus vulgaris)* (Fiorito and Scotto, 1992), fish (*Paralichthys olivaceus*) (Arai et al., 2007) and bumblebees (*Bombus terrestris*) (Chittka and Avargue, 2014). Controversially, some authors consider that social learning only happens when animals acquire a new behaviour using social information (Brown and Laland, 2003), and others defend a restrictive definition where observational conditioning occur only with the association of a demonstrator in aversive contexts, despite several evidences of observational conditioning in appetitive contexts (Heyes, 1994; Slater et al., 1993). Also, second order conditioning could be considered an extension of classical conditioning but is also classified as a different mechanism (Dawson et al., 2013).

Observational conditioning is commonly described when, at least, one of the learning cues (CS or US) is a demonstrator (Arai et al., 2007; Chittka and Avargue, 2014; Fiorito and Scotto, 1992; Koksal and Domjan, 1998; Mineka et al., 1984). However, products of animals or their outcomes (such alarm substance or a deformed object by action of a demonstrator), when used as stimuli to learn, have been labelled differently, such as associative learning, social learning, experienced learning or classical conditioning, and regarded as a different learning mechanism (Arai et al., 2007; Chivers and Smith, 1994; Karnik and Gerlai, 2012; Ruhl et al., 2017). This problem was highlighted when social food preference was described as a special case of observational conditioning (Zentall, 2011). Rats change an initial preference for a food type after interacting with a demonstrator that had eat a different food. This behavioural shift is mediated by carbon disulphide released from the mouth of the demonstrator (so it is not the demonstrator itself but a chemical cue) (Munger et al., 2010). This ambiguity of the observational conditioning definition is due to the multidisciplinary of the field, which conduct to loss of information being the same learning mechanism is considered a different one (Heyes and Street, 1994).

Social learning can be explained as an exaptation, where an existing trait gains a new use when the right circumstances arise (Leadbeater, 2014). For example, feathers appear in the fossil record on the flightless ancestors of birds, but later became instrumental to bird's fight (Gould, 1991). The same can be applied to social learning, where learning abilities were present but only were used in social contexts when the environment requires. This phenomenon differs from

adaptation, where the environment pressures the development of a new characteristic. Social learning mechanisms can be described as Pavlovian processes (Heyes and Pearce, 2015; Leadbeater, 2014; Zentall, 2011). Social stimuli become conditioned to an asocial one independently if the social information is presented in the US or in the CS. For instance, bumblebees learn a negative association between conspecific footprints (CS) and nectar reward (US) with social information in the CS (Leadbeater and Chittka, 2011) and tadpoles show activity reduction to conspecific stress cues (US) when paired with the predator cue (CS), with the social information in the US (Gonzalo et al., 2006). These associative learning processes can be the result of selective history, such as the case of pups that are deprived of maternal licking in early life that fail to learn food preferences from conspecifics (Lindeyer et al., 2012), or biologically relevant associations seem to be more easily learnt than others (Dunlap and Stephens, 2000; Ohman et al., 2001).

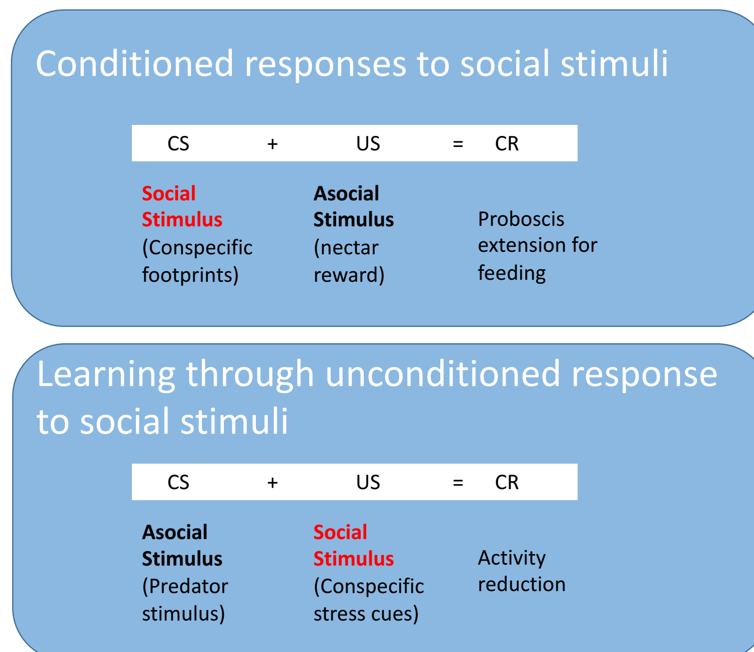

Figure 4.1 - Social information use in learning processes. (a) Conditioned responses in which animals learn to associate a social stimulus with a reward. (b) Animals might learn about features of the environment through unconditioned social stimuli. Adapted from Leadbeater E. (Leadbeater, 2015).

In both chapters, we used social cues as stimuli in learning processes: in chapter II social information was presented as the CS and in the chapter III as the US. Our results indicate that social and asocial learning (observational conditioning and classical conditioning, chapter II) uses similar

behavioural mechanism. These results demonstrate that animals learn at the same rate independently of the social nature.

A recent review hypothesized that cognitive processes may present different architectures: (1) animals can develop a general intelligence if the mechanisms of information processing are shared; (2) modular intelligence if different types of environmental information recruit specific mechanisms; or (3) mixed intelligence if only some mechanisms of information processing are shared (Varela et al., in press). This perspective corroborates our results that point to shared behavioural mechanisms between social and asocial learning. We propose a higher level of complexity adding that behavioural and neuronal mechanisms can present distinct architectures for the same cognitive process (cognitive and functional architectures).

4.3 Neuronal Mechanisms of Social learning

The cognitive process can be mapped onto the brain but the functional architecture of neuronal networks is still poorly understood. “Small world architecture”, where neurons promote local connections in detriment of long distance connections results in the formation of modules (Rubinov and Sporns, 2010). Long-distance connections between cells in different modules are needed to integrate the information across modules. This modularity allows to process complex cognitive functions that are associated with distributed brain networks rather than with single brain regions. These networks are also dynamic so that each node (i.e. brain region) may participate in multiple cognitive functions by rapid functional connectivity reconfigurations (Rubinov and Sporns, 2010). The combination of functional specialization in domain-specific modules with the integration at the neural network level allows the expression of complex and flexible behaviour.

The existence of social domain-specific modules within these networks has been demonstrated when social odour is processed by the mammalian vomeronasal system relative to asocial odours processed by the main olfactory system (Doving and Trotier, 1998), and by the specialized face recognition areas in the brains of humans, macaques (*Macaca mulatta*) and sheep (Doris and Livingstone, 2008; Kanwisher et al., 1997; Kendrick and Baldwin, 1987). In addition, an evolutionary conserved social decision-making network in vertebrates has been proposed, based on conserved patterns of expression of developmental genes and neurochemical systems in the forebrain responsible to multiple forms of social behaviour (O’ Connell and Hofmann, 2011a, 2011b; O’Connell and Hofmann, 2012). Several studies provided supporting evidence in favour of the social

brain network hypothesis. In the African cichlid *Astatotilapia burtoni* subordinate males that raise in social rank show higher expression of immediate early genes in all nodes of the social behaviour network when compared either to stable subordinate or dominant males (Maruska et al., 2013); in the green anole lizard (*Anolis carolinensis*) repeated exposure to video-playbacks of aggressive displays by conspecific males induce changes in functional connectivity within the network (Yang and Wilczynski, 2006); in 4 species of birds, different nodes of the network are differentially activated in response to the presence of a conspecific, in a way that is related to inter-specific differences in sociality (Goodson et al., 2005); and in zebrafish (*Danio rerio*) the outcome of aggressive interactions elicits rapid shifts in functional connectivity in the SDM network (Teles, 2015).

In both chapters (II and III), we demonstrate changes in immediate early genes expression networks after social learning. In chapter III, we observed a shift in the olfactory neuronal network of social learners after associated a red light with an alarm pheromone. In chapter II, we clearly showed distinct neural states to social and asocial learning through *c-fos* expression network. The neuronal mechanisms of social learning are based in a modular intelligence where different nodes are recruited to express social and asocial learning supported by distinct patterns of functional connectivity.

4.4 Concluding remarks

In this work, we proposed that behavioural and neural mechanisms of observational conditioning can present distinct architectures. At the behavioural level, the mechanisms of observational conditioning seem to be shared (general intelligence), in contrast the patterns of brain activation reveal modular mechanisms. At the neural level, several processes can be executed at the same time and complexity and amount of information recruits functional networks. This new perspective helps to elucidate the debate in the field on the mechanisms of social learning being domain specific or general-domain.

In addition, we clearly demonstrated an individual variability to learn in a reward context (chapter II). Several other works have shown this intraspecific variation in learning tasks, but it is not clear if this intraspecific variation is due to prior experience, personality, immune, metabolic, microbiome state or even a complex view where several factors act in the phenotype. The implications of this intraspecific variation at the neural level is still not demonstrated. This is a new research perspective in the field that open new avenues for future research.

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