The Role of the Preoptic Area in Social Interaction in Zebrafish

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Abstract

The proposed "Social Decision Making Network" (SDMN) is a network of conserved, interconnected brain areas believed to regulate social behaviour across vertebrate taxa. While the role of the SDMN in complex social behaviours is relatively well understood, we know comparatively little about the role of the SDMN in simple grouping and responses to social cues. While the existence of the SDMN in mammals is well supported, its role in social behaviour in other taxa is less well studied. By analysing neuronal activation in the preoptic area (POA), dorsal nucleus of the ventral telencephalic area (Vd), supracommissural nucleus of the ventral telencephalic area (Vs), and ventral nucleus of the ventral telencephalic area (Vv) in zebrafish (*Danio rerio*) I aimed to determine how these four nodes of the SDMN respond when shoaling and when exposed to different social cues. Combined visual and olfactory exposure to conspecifics significantly increased levels of the phosphorylated ribosome S6 protein (PS6) in the POA, however surprisingly there was no change in POA PS6 levels when fish were allowed to shoal, suggesting the POA may in fact be responding to social cues rather than shoaling itself. Other nodes of the SDMN showed no changes in PS6 levels. Based on these findings I investigated POA regulation of shoaling by analysing expression of isotocin (IT) neurons in the POA of two populations of zebrafish with different shoaling phenotypes. I found that more social fish had more magnocellular IT neurons than less social fish, but there were no differences in parvocellular or gigantocellular IT neuron numbers. My findings provide evidence that the POA is sensitive to social cues but is not necessarily activated by shoaling. Furthermore, my results demonstrate that adult plasticity in magnocellular IT neurons is associated with changes in shoaling tendencies in zebrafish.

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Contents:

Abbreviations:

AH	Anterior hypothalamus
AVP	Arginine Vasopressin
AVT	Arginine Vasotocin
blAMY	Basolateral amygdala
BNST/meAMY	Bed nucleus of the stria terminalis/medial amygdala
DAB	3,3′-Diaminobenzidine
GLMM	General linear mixed model
IEGs	Immediate early genes
IT	Isotocin
LS	Lateral septum
MS222	Tricaine methanesulfonate
NAcc	Nucleus accumbens
OCT	Optimal cutting temperature
OT	Oxytocin
PAG/CG	Periaqueductal grey/central grey
POA	Preoptic area
PS6	Phospho-S6 ribosomal protein
SDMN	Social Decision Making Network
TPp	Periventricular nucleus of the posterior tuberculum

Vd	Dorsal nucleus of the ventral telencephalic area
VMH	Ventromedial hypothalamus
VP	Ventral pallidum
Vs	Supracommissural nucleus of the ventral telencephalic area
VTA	Ventral tegmental area
Vv	Ventral nucleus of the ventral telencephalic area

Chapter One: Introduction

1.1 The Social Decision Making Network

The Social Decision Making Network (SDMN) is a proposed network of highly conserved and interconnected brains areas that is hypothesized to regulate social behaviour and reward in vertebrates (Crews, 2003; Goodson, 2005; O'Connell & Hofmann, 2011). The SDMN is comprised of two interconnected networks, the Social Behaviour Network (SBN) and the mesolimbic reward system (figure 1). The SBN regulates social behaviours including sexual behaviour and reproduction, aggression, and parental care (Newman, 1999). Social behaviour is regulated by neurons located in key regions of the SBN that secrete and contain receptors for nonapeptide and sex-steroid hormones (O'Connell & Hofmann, 2011). The mesolimbic reward system, including the dopaminergic system, is responsible for the evaluation of salience of an external stimulus (Deco & Rolls, 2005; Wickens et al., 2007). The mesolimbic reward system is interconnected with the SBN, regulating social behaviours through reinforcement of responses to external social stimuli in reproduction, dominance interactions, or caring for offspring (Numan, 2007; Paredes, 2009; Fuxjager et al., 2010). Through the integration of both the SBN and the mesolimbic reward system, the SDMN is understood to be an integrated and evolutionarily ancient circuit that regulates responses to both social and non-social salient stimuli (O'Connell & Hofmann, 2011).

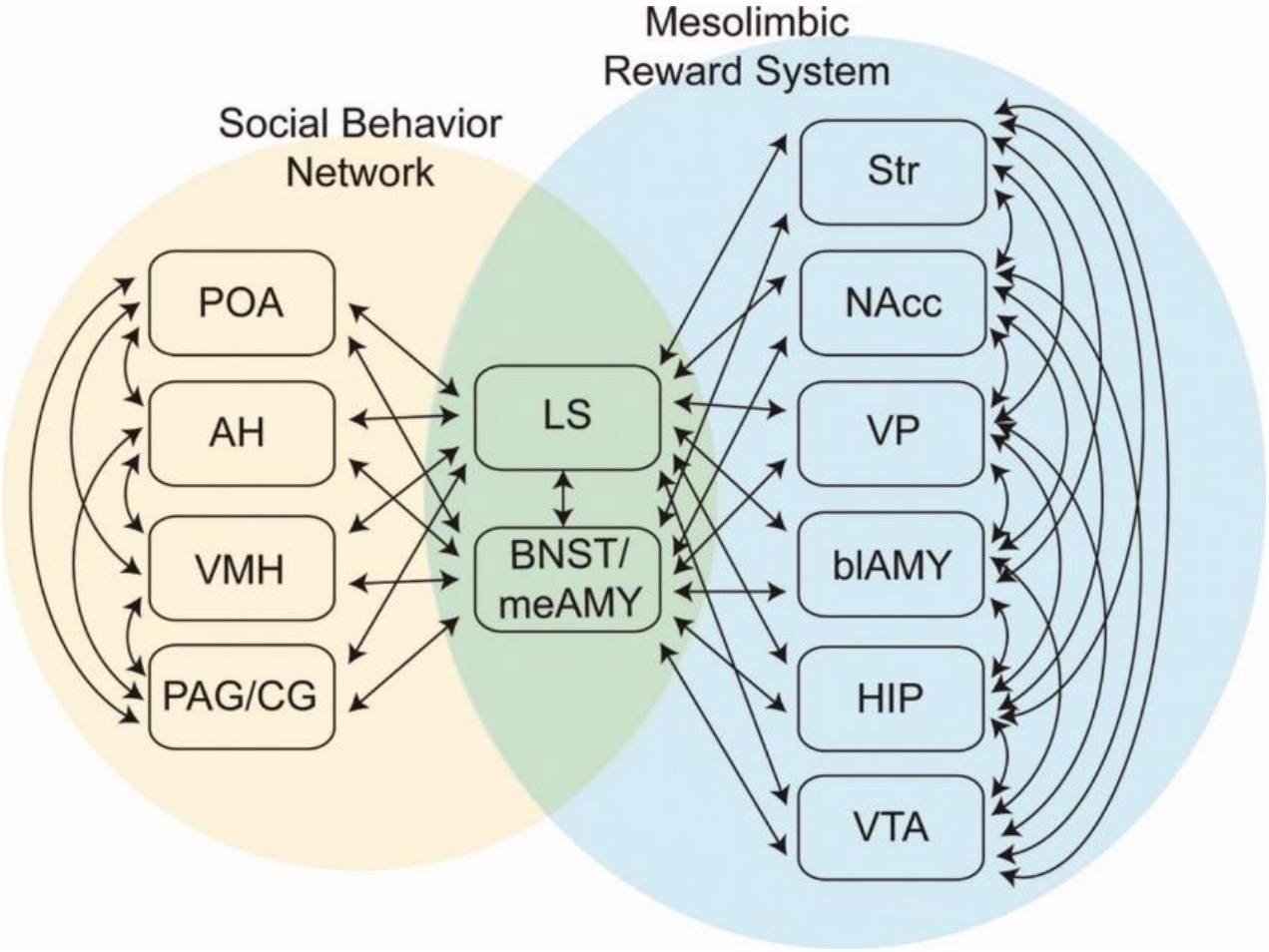

Figure 1. Representation of the SDMN and its constituent parts: the mammalian SBN (yellow), the mesolimbic reward system (blue), and brain regions that are implicated in both functions (green). Arrows represent connections between brain regions. Figure obtained from O'Connell & Hofmann, 2011).

1.1.1 SDMN in Mammals

Several key nodes of the SBN have been shown in mammals to mediate a wide range of social behaviours. Many of these nodes are either located within or are highly interconnected with the hypothalamus. A core region of the SBN is the preoptic area (POA), a brain region adjacent to the third ventricle in the hypothalamus and a key neuroendocrine region responsible for the regulation of a wide range of social behaviours including aggression, sexual behaviour, and parental care (Heimer & Larsson, 1967; Malsbury, 1971;

Hull & Dominguez, 2006; Lee & Brown, 2007). The POA is also involved in the processing of social olfactory cues, making it a key area for the processing of social information (Landgraf et al., 2003; Bielsky & Young, 2004). Other characterized regions in the SBN include the anterior hypothalamus (AH), ventromedial hypothalamus (VMH), and the periaqueductal grey/central grey (PAG/CG). The AH is believed to be a crucial node for the regulation of aggression as well as parental care (Ferris & Potegal, 1988; Bridges et al., 1999). The VMH is interconnected with both the amygdala and other regions of the hypothalamus and has been shown to be a regulator of aggression, feeding, and female receptivity in breeding (Panksepp et al., 1970; Saper et al., 1976; Malsbury et al., 1977). The PAG/CG is important for several social behaviours in both sexes including reproduction, aggression, and vocal communication (Floody & O'Donohue, 1980; Mos et al., 1982; Bandler & Carrive, 1988; Kollack-Walker & Newman, 1995; Lonstein & Stern, 1997; Jürgens, 2002; Schulz et al., 2005). Like many other regions in the SDMN, the PAG/CG also contains sex steroid receptors which are considered to be fundamental in the neural regulation of sexual behaviour and aggression (Rubinow et al., 1996; Murphy, et al., 1999).

The mesolimbic reward system is believed to mediate social behaviour and social decision-making through the regulation of reward (O'Connell & Hofmann, 2011). Reinforcing dopaminergic signalling induced by rewarding social cues is hypothesized to thus act as a mechanism driving social decision making. Key brain regions in the mammalian mesolimbic reward system include the striatum, nucleus accumbens (NAcc), ventral pallidum (VP), basolateral amygdala (blAMY), hippocampus, and the ventral tegmental area (VTA). The striatum is involved in reinforcement learning, thus mediating learning and goal-directed actions (Wickens et al., 2007). The NAcc integrates sensorimotor information in order to regulate evaluation and approach or avoidance of a stimulus (Ikemoto & Panksepp, 1999). It projects to the VP and receives projections from the blAMY and hippocampus, as well as

receiving a high level of dopaminergic projections from the VTA (Fallon & Moore, 1978; Beckstead et al., 1979; Heimer et al., 1997). These projections from the VTA are an important component of the mesolimbic reward system as dopamine release in the NAcc has been repeatedly implicated in the regulation of reward and reinforcement (Salamone et al., 2001; Salamone & Correa, 2002). The VP receives input from the striatum and NAcc and projects to several brain regions including the hypothalamus and VTA (Haber et al., 1985; Groenewegen et al., 1993; Ikemoto, 2007). The VP also regulates the behavioural output of goal-directed behaviours and reward processing (Mogenson et al., 1980; Smith et al., 2009). The blAMY projects to the hypothalamus through the stria terminalis, and to the striatum and NAcc (Russchen & Price, 1984; LeDoux et al., 1987; Turner & Herkenham, 1991; Petrovich et al., 1996; Wright et al., 1996; Risold et al., 1997). The blAMY is implicated in goal-directed behaviour by modulating the activation of dopaminergic neurons located in the VTA (Maeda & Mogenson, 1981). The hippocampus is responsible for memory, including the formation of memories of experiences and spatial representations of the environment (O'Keefe & Nadel, 1978; Andersen et al., 2007; Humphries & Prescott, 2010). In the context of social behaviour, the hippocampus is responsible for the encoding of social memory such as social recognition and facilitating social learning (Kogan et al., 2000). The VTA is the primary component of the dopaminergic system, releasing dopamine at the NAcc as a response to rewarding stimuli and facilitating reinforcement (Fallon & Moore, 1978; Phillipson, 1979; Domesick, 1988; Le Moal & Simon, 1991; Spanagel & Weiss, 1999). The VTA and NAcc have thus been implicated in the regulation of reproductive behaviour and parental care (Brackett & Edwards, 1984; Sirinathsinghji et al., 1986; Hansen et al., 1991; Hasegawa, 1991; Sotres-Bayón, 2001).

The mammalian SBN and the mesolimbic reward system overlap at two key nodes; the lateral septum (LS) and the bed nucleus of the stria terminalis/medial amygdala

(BNST/meAMY), regions that are hypothesized to regulate both social behaviour and reward. The mammalian LS receives projections from the hippocampus, AH, POA, and VMH, and projects to both the hypothalamus and midbrain (Meiback & Siegel, 1977; Swanson & Cowan, 1977; Swanson & Cowan, 1979; Staiger & Nürnberger, 1989; Risold & Swanson, 1997). The LS and VTA are reciprocally interconnected via the dopaminergic system, and LS stimulation of the VTA has been shown to produce excitatory effects on dopaminergic neurons located in the VTA (Maeda & Mogenson, 1981; Swanson, 1982). The meAMY projects to the hypothalamus through the stria terminalis, modulating reproductive, aggressive, and parental behaviours (Canteras et al., 1994; Risold et al., 1997; Swanson & Petrovich, 1998; Sheehan et al., 2001; Choi et al., 2005). Similarly, the BNST mediates aggression and reproductive behaviours (Valcourt & Sachs, 1979; Shaikh et al., 1986; Powers et al., 1987). However, the BNST is also largely involved in motivational behaviour through its excitatory effects on dopaminergic neurons located in the VTA (Delfs et al., 2000; Georges & Aston-Jones, 2001).

1.1.2 Teleost Homologues of the SDMN

O'Connell and Hofmann (2011) proposed the existence of a conserved vertebrate SDMN, involving homologues of mammalian SDMN brain regions based on structure, development, and morphology (*Figure 2*). Based on these suggested homologies I investigated neuronal activation in four key nodes of the proposed teleost SDMN. These four nodes included the teleost POA, the dorsal nucleus of the ventral telencephalic area (Vd), the supracommissural nucleus of the ventral telencephalic area (Vs), and the ventral nucleus of the ventral telencephalic area (Vv). The POA in teleosts is considered to be homologous to the POA in mammals and is similarly considered to be crucial for the regulation of a wide range of social behaviours including sexual behaviour and aggression (Demski & Knigge, 1971; Macey et al., 1974; Satou et al., 1984; Wong, 2000). The teleost POA is reciprocally

interconnected with the hypothalamus and telencephalon (Folgueira et al., 2004b), and also contains receptors for sex-steroid hormones (Forlano et al., 2005, 2010; Munchrath & Hofmann, 2010). The Vd is hypothesized to be a homologue of the mammalian NAcc and is associated with reward, as it contains dopamine receptors which are activated by dopaminergic projections from the periventricular nucleus of the posterior tuberculum (TPp), a putative teleost VTA homologue (Rink & Wullimann, 2001; O'Connell & Hofmann, 2011). In the context of social behaviour, the Vd is thought to be a reward centre that responds to social cues if they are considered rewarding (O'Connell & Hofmann, 2011). Evidence suggests that the Vs is a homologue of the mammalian meAMY and BNST due to shared gene markers and functional similarity in both sociality and reward (Demski & Knigge 1971; Satou et al., 1984; Alunni et al., 2004; O'Connell & Hofmann, 2011). The Vs also projects to several hypothalamic regions including the TPp (Folgueira et al., 2004a). Finally, the Vv is considered homologous to the mammalian LS and is associated with reproduction in both males and females (Kyle & Peter, 1982; Satou, et al., 1984). The Vv receives input from the TPp and is thus also thought to be involved in both sociality and reward (Rink & Wullimann, 2001).

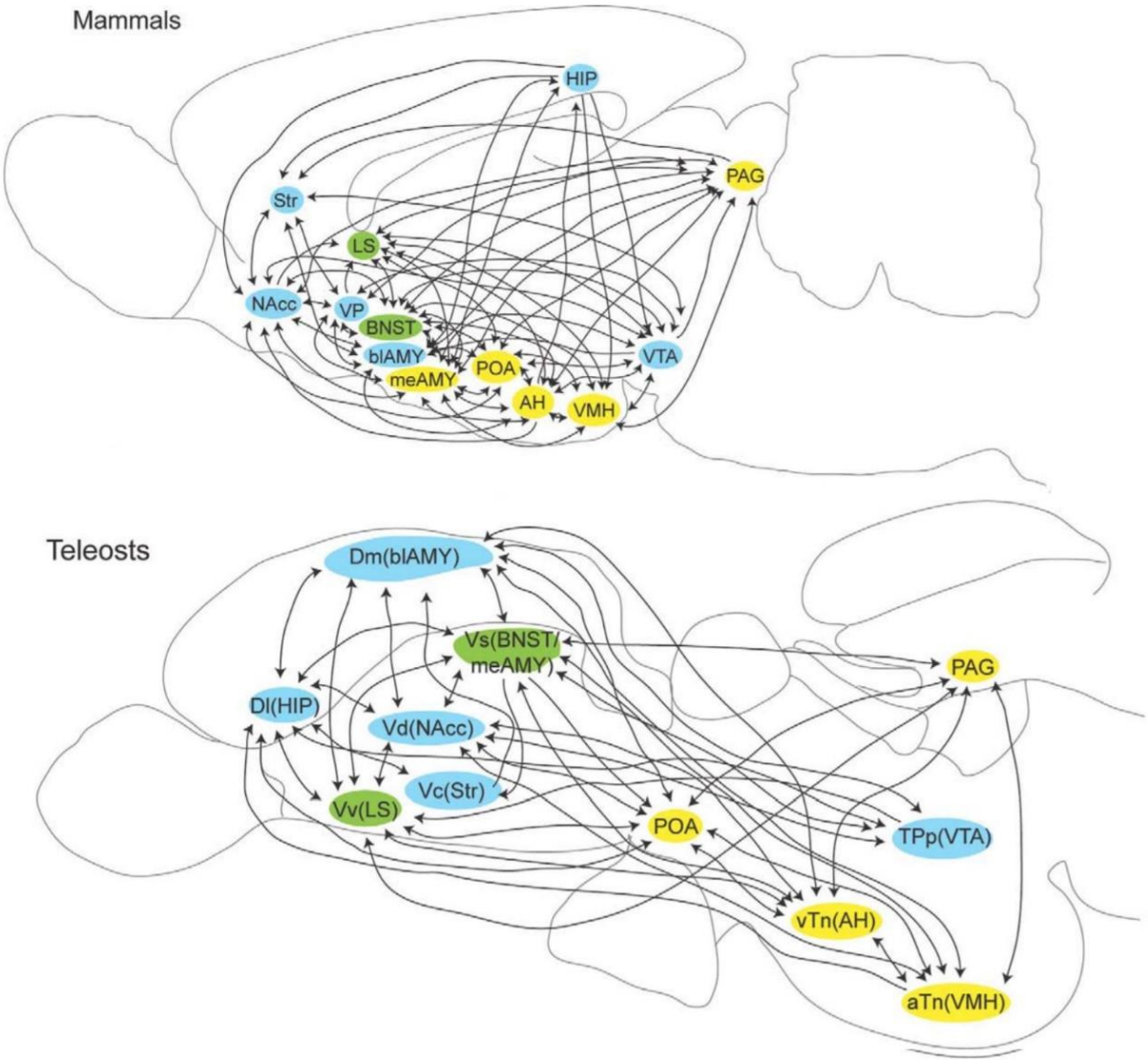

Figure 2. Proposed homologies of the SBN and mesolimbic reward system in mammals and teleosts represented in sagittal brain sections. Yellow regions represent regions associated with the SBN, blue with the mesolimbic reward system, and green regions associated with both networks. Figure obtained from O'Connell & Hofmann (2011).

1.2 Simple Social Behaviour

Grouping is a common behaviour exhibited in a wide range of species that benefits individual fitness, such as reducing predation risk, increasing foraging success, and increasing opportunities for mating (Krause & Ruxton, 2002). The role of the SDMN in the

context of complex social behaviours such as reproduction and aggression is relatively well-understood, however comparatively little work has been done on the role of the SDMN in simple social behaviour such as grouping. Investigation into simple social behaviour is important in order to establish whether the mechanisms that underpin this simple behaviour are shared with more complex social behaviours. Exposure to conspecifics elicited differences in immediate early gene (IEG) expression in the SDMN between gregarious and territorial birds (Goodson et al., 2005). IEG responses in the ventrolateral septum, AH, and lateral subdivision of the VMH were significantly higher in gregarious species of birds, while IEG expression in the extended AH was negatively correlated with sociality. Nonapeptide neurons located in the stria terminalis and LS have been hypothesized to promote flocking behaviour in estrildid finches (Goodson et al., 2009b). Similarly, pharmacological manipulation of nonapeptide receptors in the POA has indicated that V1a receptor antagonists decrease gregarious behaviour, suggesting that together these neurons and receptors promote preference for larger flock sizes (Kelly et al., 2011). These findings highlight the potential role of the SDMN in simple grouping behaviour and provide a novel opportunity to investigate how grouping and social cues affect neuronal activation in the SDMN.

Zebrafish (*Danio rerio*) make an ideal model organism for investigating the role of the SDMN in simple social behaviour as they are a highly social species that groups into shoals and find the presence of conspecifics socially reinforcing (Miklósi & Andrew, 2006; Saverino & Gerlai, 2008). It has also been suggested that a number of biological and behavioural properties are conserved between zebrafish and mammals, allowing for cross-species comparison of neural regulation of simple social behaviour (Norton & Bally-Cuif, 2010).

1.3 The Teleost Preoptic Area

Data compiled from multiple comparative studies provide evidence that there are specific types of conserved neurons located within the teleost POA that are homologous to neuron types identified in other species (Fox et al., 1997; Thompson & Walton, 2004; Aubin-Horth, 2007; Backström & Winberg, 2009; Walton et al., 2010; O'Connell & Hofmann, 2011; Oldfield & Hofmann, 2011). These neuron types include parvocellular, magnocellular, and gigantocellular nonapeptide neurons (Braford & Northcutt, 1983) that have been identified based on morphology, soma size, and localization (Godwin & Thompson, 2012).

These different types of neurons are hypothesized to regulate different types of social behaviours. Parvocellular neurons are expressed along the rostral and ventral regions of the POA with populations of small neurons located lateral to the third ventricle. These parvocellular neurons are typically thought to be involved in the regulation of stress responses, social approach, and subordinance (Godwin & Thompson, 2012). Teleost parvocellular neurons are considered to be homologous to the parvocellular neurons found in the supraoptic nucleus identified in tetrapod taxa and the paraventricular nucleus of the mammalian POA (Van den Dungen, 1982; Moore & Lowry, 1998).

Magnocellular and gigantocellular neurons expressed in the caudal region of the teleost POA are considered homologous to the magnocellular and gigantocellular neurons of the mammalian supraoptic nucleus and the paraventricular nucleus in tetrapods based on their colocalization with neurons that produce corticotropic releasing hormone and expression of the Nurr1 orphan receptor (Olivereau et al., 1988; Kapsimali et al., 2001). Magnocellular neurons are mid-sized neurons located adjacent to the third ventricle, while gigantocellular neurons are larger neurons located in the caudal and dorsal region of the POA (Godwin & Thompson, 2012). Both magnocellular and gigantocellular neurons have been implicated in

reproductive and aggressive behaviours (Godwin & Thompson, 2012). However, little is known about the role of these cell types in the regulation of simple grouping behaviour.

1.4 Study 1: Activation of the SDMN

The first experiment aimed to determine how different types of social exposure activate different nodes of the SDMN using the phosphorylated S6 ribosomal protein (PS6) as a proxy for detecting neuronal activity using immunohistochemical methods. Experimental fish were exposed to a stimulus shoal for 30 minutes with differing levels of social contact. Subjects were either only given Visual exposure to a shoal, were given Visual + Olfactory exposure, were placed in a Shoaling condition where subjects could physically interact with the stimulus shoal, or were placed in a no-shoal Control condition. By investigating multiple nodes within the SDMN I aimed to understand how different components of the SDMN process and integrate information and social cues, as well as determine whether the SDMN responds as a unitary structure, or if different nodes respond differently to different types of related social cues.

I predicted that as the level of social exposure increased, and subjects were provided access to increasingly rich social information, that PS6 levels would increase in the POA. This is due to evidence that expression of the IEG *Fos* increases in the POA in response to affiliative social stimuli but does not when exposed to aversive or aggressive social stimuli (Goodson & Wang, 2006; Goodson et al., 2009a; Bolborea et al., 2010; Ho et al., 2010). Additionally, exposure to female conspecifics, but not males, has been shown to elicit an increase in level of PS6 in the POA of male guppies (*Poecilia reticulata*) (Fischer et al., 2018). I further hypothesized to find increases in PS6 levels in the Vd, Vs, and Vv as the level of social exposure became increasingly rich. This is due to the involvement of these nodes in the mesolimbic reward pathway and the possibility that although the Visual and

Visual + Olfactory conditions may be socially stimulating, they may not necessarily be rewarding, and thus may not elicit strong responses in these brain areas, whereas I expected stronger responses in the Shoaling condition as evidence indicates that zebrafish find social stimuli rewarding and this condition provided the greatest social stimulus (Miklósi & Andrew, 2006; Saverino & Gerlai, 2008).

1.4.1 Phospho-S6 Ribosomal Protein

Ribosomal protein S6 is a component of the protein translation machinery which undergoes cellular activity-dependent phosphorylation. It has emerged as a method for assaying neuronal activity due to its rapid and transient phosphorylation in response to a stimulus, resulting in detectable levels of the phosphorylated S6 ribosomal protein (PS6). Neuronal activation elicits changes in gene expression, causing post-translational modification of specific amino acid residues on the S6 protein (Biever et al., 2015). S6 is sequentially phosphorylated across five serine residues: the Ser235, 236, 240, 244, and 247 residues (Krieg et al., 1988). Protein phosphorylation at Ser235 and Ser236 is detectable five minutes after exposure to a stimulus, and peaks at thirty minutes, after which expression is maintained for multiple hours (Iwenofu et al., 2008; Biever et al., 2015; Pirbhoy et al., 2016). Immunohistochemistry using antibodies for PS6 permits accurate detection, quantification, and localization of neuronal activation (Knight et al., 2012). While quantifying the expression of IEGs such as EGR-1 or *Fos* is a common proxy for detecting neuronal activation, interspecies variation in IEG gene sequences causes antibodies raised in mammals to not always work properly for immunohistochemistry in other taxa, such as fish. Conversely, ribosomal proteins are highly conserved between species and multiple mammal-origin antibodies have been validated for use in fish, and thus can be used with confidence in zebrafish (Shin et al., 2012; Schultz et al., 2017; Scheldeman et al., 2017).

1.5 Nonapeptide Regulation of Social Behaviour

My results from Study 1, as well as broader research into the neural basis of fish sociality highlights the importance of the POA as a mediator of social behaviour in teleosts (Cabrera-Álvarez et al., 2017). To further investigate the role that the POA plays in zebrafish grouping behaviour, I investigated variation in POA function in zebrafish populations with different social phenotypes. In order to do so I focused on nonapeptides, peptide hormones expressed in the teleost POA that are fundamental in the regulation of a wide range of social behaviours across vertebrate taxa (Urano & Ando, 2011).

1.5.1 Nonapeptides in Mammals

Evidence shows that the mammalian nonapeptides arginine vasopressin (AVP) and oxytocin (OT) are key regulators of behaviours including affiliation, parental behaviour, bonding, social recognition, and aggression in mammalian species (Melin & Kihlstroem, 1963; Williams et al., 1992b; Winslow et al., 1993b; Williams et al., 1994; Benelli et al., 1995; Gubernick et al., 1995; Engelmann et al., 1998; Kow & Pfaff, 1998; Wang et al., 2000; Meddle et al., 2007; Caldwell et al., 2008). Research investigating the role of OT in social behaviour suggests that OT may be functioning as a promoter of affiliative behaviour in mammals. In rodents, ventricular administration of OT promotes social affiliation and has been shown to reduce anxiety and aggressive behaviour (Witt et al., 1992; Dharmadhikari et al., 1997; Cho et al., 1999; Harmon et al., 2002; Lubin et al., 2003). OT has been found to facilitate affiliation and inhibit stress responses by instigating changes in the neural processing of external social stimuli in the amygdala (Kirsch et al., 2005; Petrovic et al., 2008).

Both AVP and OT are considered to be important in the neural processing of olfactory social information and for social discrimination (Bielsky & Young, 2004). Research shows

that OT specifically is crucial in social olfaction and social recognition through facilitating changes in neuronal activity and synaptic efficacy in the olfactory bulbs (Keverne, 1999; Brennan, 2001). Administration of OT to the amygdala in OT-knockout mice has been shown to restore social recognition (Ferguson et al., 2001). Furthermore, peripheral administration of low doses of OT has also been shown to facilitate social interaction in male rats (Popik et al., 1992). Together these findings suggest that OT may promote affiliative behaviour in mammalian species.

Despite the common perception of nonapeptides as ubiquitous regulators of social behaviours, a high level of disparity is found in nonapeptide function and expression between species and sex. Research investigating the role of AVP in affiliation and pair-bonding between two different species of voles has highlighted differences in AVP hormone and receptor function based on species-specific differences in social structure. Prairie voles (*Microtus ochrogaster*) are a highly social and sexually monogamous species that practices biparental care for offspring, while Montane voles (*Microtus montanus*) are a solitary and sexually promiscuous species (Young et al., 1999a). Centrally administered AVP was shown to increase affiliation in male Prairie voles but not in male Montane voles (Young et al., 1999a). In male Prairie voles affiliation and mating has also been shown to stimulate AVP release, facilitating pair-bonding, as well as promoting parental care and aggression towards unfamiliar males (Insel & Shapiro, 1992; Winslow et al., 1993a; Insel et al., 1994; Lim et al., 2004).

In general, expression of OT and OT receptors is higher in females than it is in males (Zingg & Laporte, 2003; Carter, 2007). Oestrus in female Prairie voles is activated by courtship behaviour and olfactory signalling from male voles, stimulating OT release (Williams et al., 1994; Cushing & Carter, 2000). Endogenous binding of OT released during mating to the NAcc facilitates the formation of pair-bonding in female Prairie voles,

nonapeptides thus performing seemingly complementary roles in mating behaviour between sexes (Williams et al., 1992a; Neumann, 2009). The administration of OT antagonists additionally blocks partner preference formation in female Prairie voles, suggesting that OT is essential for facilitating affiliation and selective pair-bonding in females (Cho et al., 1999).

Further research investigating species and sex differences in voles has suggested that differences in behaviour may be associated with nonapeptide receptor patterning. Ventral forebrain expression of the V1a receptor in males has been shown to be higher in monogamous California deer mice and marmoset monkeys, compared to related sexually promiscuous species, indicating that AVP mediation of social attachment in males of monogamous species is evolutionarily conserved (Bester-Meredith et al., 1999; Young, 1999; Young et al., 1999b). Male Prairie voles were found to have higher AVP receptor levels in the VP than male Montane voles (Young et al., 1999a), while female Prairie voles have also been found to have more OT receptors in the NAcc than female Montane voles (Young et al., 2001). Together, these findings in voles and other mammals illustrate sex differences in nonapeptide receptor expression are associated with specific social phenotypes across species.

Despite the abundance of research investigating the role of nonapeptides in complex social behaviours in mammals, there is comparatively little investigating their role in simple social behaviour such as grouping. This is perhaps a function of the difficulty of studying grouping in mammals compared to in smaller gregarious animals such as shoaling fish or flocking birds.

1.5.2 Nonapeptide Function in Teleosts

The function of nonapeptides in fish has received much less attention than in mammals despite the conservation of these neuropeptides across vertebrates, and the rich range of social behaviours seen in fish species. Grouping behaviour is also much easier to

study in small shoaling or schooling teleost species, such as zebrafish, providing opportunities to investigate the role of nonapeptides in simple social behaviour. By investigating POA nonapeptide signalling in the context of simple social behaviour in teleosts, we can further understand how variation in nonapeptide signalling might be involved in changes in shoaling tendencies.

The teleost homologues of AVP and OT are arginine vasotocin (AVT) and isotocin (IT) (Urano & Ando, 2011). In zebrafish, AVT and IT are primarily expressed in neurosecretory cells located along the third ventricle in the ventral POA, with additional expression in the caudal region of the hypothalamus (Godwin & Thompson, 2012). Nonapeptide neurons in teleosts that are located in the POA project to the posterior pituitary, which releases the nonapeptides into the periphery through the hypophysis, as well as to the median eminence of the anterior pituitary where they are implicated in adrenocorticotropin release. Further nonapeptide projections from the POA to the hindbrain additionally play a role in autonomic nervous system functionality (Goodson & Thompson, 2010). These projections to the periphery and hindbrain are thought to regulate physiological responses to external stressors, while central release of nonapeptides in the POA is hypothesized to mediate social behaviours (Goodson, 2008; Thompson & Walton, 2009).

Research investigating the role of nonapeptides in teleosts has primarily focused on behaviours such as aggression and dominance. Semsar and Godwin (2004) demonstrated that AVT is crucial for the formation of territorial status in both male and female bluehead wrasse (*Thalassoma bifasciatum*). Antagonism of the AVT V1 receptor blocked territorial dominance over vacated territories in both males and females. AVT administration has been shown to reduce aggression in dominant male zebrafish (Filby et al., 2010). Subordinate male *Neolamprologus pulcher* cichlids have been shown to express higher levels of AVT in the brain than dominant males. However, dominant males exhibit increased AVT expression in

the periphery, likely due to its role in the retention of urine, a social signal of submission (Reddon et al., 2015).

While AVT functions as a mediator of aggression in teleosts in multiple species, IT's role in aggression appears to vary by species. Exogenous IT did not impact aggression in damselfish (*Stegastes leucostictus*) (Santangelo & Bass, 2006), however, IT does seem to be implicated in the evaluation of conspecifics in the context of aggression. *Neolamprologus pulcher* cichlids demonstrated increased sensitivity to opponent size and exhibited more submissive displays following administration of IT (Reddon et al., 2012). O'Connell et al. (2012) also found that IT facilitated an increase in paternal care in *Amatitlania nigrofasciata* cichlids following mate removal while treatment with an IT antagonist blocked paternal care. Dominant false clownfish (*Amphirion ocellaris*) have been shown to have more parvocellular IT neurons than subordinates, indicating that neural IT varies based on social status (Iwata et al., 2010).

As in mammals, nonapeptides in teleosts are regulators of a wide range of social behaviours, though findings indicate that nonapeptide regulation of behaviour varies between species and context, indicating that the role of nonapeptides in teleost social behaviour is still not fully understood. Administration of an AVT/IT receptor antagonist has been shown to reduce affiliative behaviour and aggression in *Amatitlania nigrofasciata* cichlids during pair-bonding, but no effect was observed on fish with pre-established pair-bonds (Oldfield & Hofmann, 2011). Ventricular administration of AVT was shown to increase courtship behaviour in male white perch (*Morone americana*), but courtship vocalizations were inhibited in male plainfin midshipman fish (*Porichthys notatus*) following AVT administration (Goodson & Bass, 2000; Salek et al., 2002).

Little is currently known about the role of nonapeptides in simple shoaling behaviour in teleosts. Thompson & Walton (2004) showed that administration of IT facilitated social approach in goldfish (*Carassius auratus*). Conversely, AVT administration inhibited social approach while an AVT receptor antagonist was stimulatory on social approach. Furthermore, administration of both IT and AVT have been shown to increase shoaling preference in wildtype zebrafish (Braida et al., 2012). Nonapeptide signalling has also been shown to be plastic in response to social phenotypes. Territorial male cichlids have been shown to express higher total levels of AVT than non-territorial males (Greenwood et al., 2008). However, territorial males were found to have less expression of AVT in the parvocellular nucleus of the POA than non-territorial cichlids, indicating plasticity in nonapeptide expression based on social phenotypes. Further research in cichlids has shown that neural IT is higher in social *N. pulcher* than non-social *Telmatochromis temporalis* cichlids (O'Connor et al., 2016). These studies suggest that nonapeptide signalling in teleosts is plastic and is associated with specific social phenotypes. Such changes in nonapeptide signalling are not seen in mammals, where variation in social behaviour is more typically associated with changed in nonapeptide receptor abundance and patterning (Insel et al., 1994; Insel & Young, 2000; Donaldson & Young, 2008).

1.6 Study 2: Nonapeptide Expression in Different Populations

To investigate the role of IT in grouping behaviour in zebrafish, I characterized IT neurons from two populations of zebrafish with different social phenotypes using immunohistochemistry. These different populations were generated by exposing one population of zebrafish to an enriched physical environment at 5 months of age while the other population was kept in a standard control environment. After 4 weeks of enriched

housing populations reliably exhibited robust differences in shoaling behaviours when tested. Behavioural phenotyping indicated that zebrafish housed in an enriched environment expressed more dense shoaling behaviour with significantly more time spent within 2 body lengths of other fish, and shorter average path lengths to other shoal members. This is consistent with hypotheses that environmental enrichment has a generalized impact on neural function and activity in a broad range of taxa by promoting neural plasticity (van Praag et al., 2000; Salvanes et al., 2013).

I hypothesized that the more social population of zebrafish would express more IT neurons than the less social population of zebrafish. This was based on previous evidence that IT administration promotes affiliative behaviour in teleosts (Thompson & Walton, 2004; Braida et al., 2012). By quantifying expression of different types of IT neurons between populations of zebrafish I was able to determine whether IT's neuronal phenotype varies in response to environmental changes and whether this is associated with grouping behaviour phenotypes.

Chapter Two: Neuronal Activation in the Social Decision Making Network

2.1 Ethical Approval

All tests and procedures in the present studies were approved by the LJMU's Animal Welfare and Ethics Review Steering Group (Ethical Approval JK_WS2017-11) and all husbandry and euthanasia methods were in accordance with UK Home Office guidance.

2.2 Aim

My first experiment aimed to determine how different key nodes of the SDMN are activated in zebrafish in response to shoaling or social cues. By analysing levels of the PS6 protein using immunohistochemistry, I was able to quantify neuronal activation in the POA, Vd, Vs, and Vv under four contexts: a no-shoal Control, Visual exposure to a stimulus shoal, Visual + Olfactory exposure to a stimulus, or actual swimming with a shoal.

2.3 Subjects

Subjects were 48 adult (1-year old) male wild-type strain zebrafish (*Danio rerio)* taken from our breeding population. Zebrafish were originally obtained as a cohort of over 2000 larvae from multiple matings from the University of Manchester and reared at Liverpool John Moores University. Subjects in each housing tank were thus a mixture of fish from different matings. Subjects were housed in 4-litre clear polycarbonate tanks (dimensions 17cm width x 15cm height x 32cm length) with water temperature maintained at 27 ± 1 °C, and pH maintained at 7.4 ± 0.2. Water quality was tested once per week for pH, ammonia, nitrites, and nitrates. Tanks were placed in custom-built recirculating rack systems in which water constantly circulated through tanks into sumps on each rack. Sumps were fitted with biofilters, mechanical filters, UV lights to prevent pathogen growth, and two Eheim 150W water heaters per sump. Each rack system had a 30% water change once per week. Home tanks included one plastic plant and an image of gravel substrate placed beneath the

transparent tank bottoms. Fish were kept on a 14:10 hour light/dark cycle and fed a diet of flake food daily (Tetramin Tropical Flakes, Tetra, USA) as well as supplementary live artemia once weekly.

Testing took place in the surgical suite of the Life Sciences Building in 4-litre tanks similar to the subjects' home tanks (Figure 3). The testing tanks contained one plastic plant and an air bubbler, as well as a clear plastic partition between the left and right portions of the tank. The addition of the divider was necessary for separating experimental and stimulus shoal fish during the social exposure component of the experimental procedure. The partition was secured in place using aquarium-safe silicon sealant. I ensured that there were no leaks through the partition or sealant daily before testing in order to ensure that no water would cross the barrier, preventing the exchange of confounding olfactory information. A slotted partition was used for the Visual + Olfactory exposure condition in order to allow for the exchange of olfactory cues while still keeping experimental subjects separate from the stimulus shoal. This was accomplished through piercing small holes through a similar thin clear plastic partition. Tanks were tested before the experimental period to ensure that water would flow between sides of the tank, but fish could not fit through the holes.

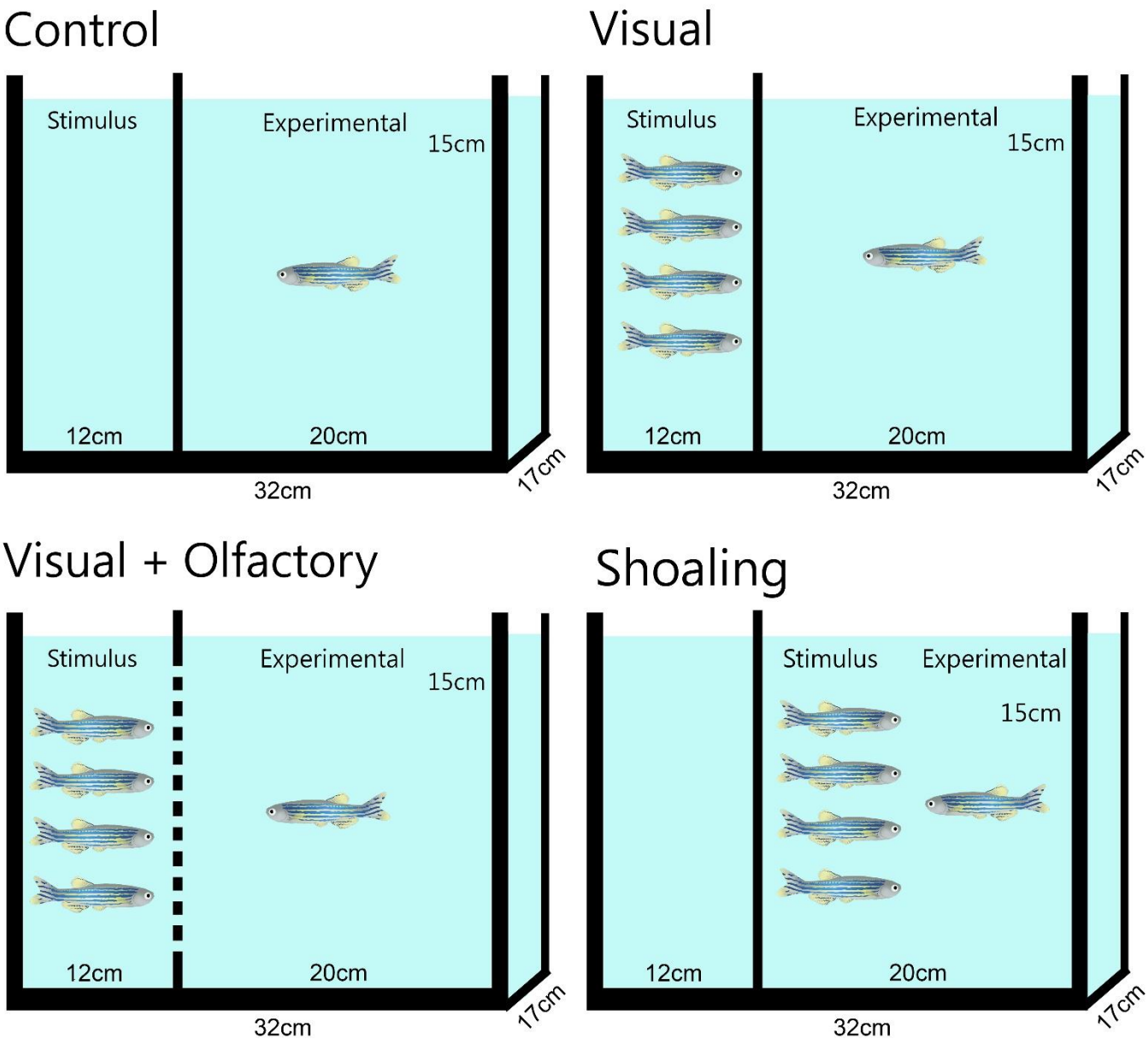

Figure 3. Visual representation of testing setup during each experimental condition. Exposure and stimulus portions of tanks were divided using a clear impermeable partition. This partition was slotted in the Visual + Olfactory condition and solid in all other conditions.

2.3.1 Experimental Groups

The experimental conditions consisted of four different contexts of social exposure with varying levels of social information: Visual exposure, Visual + Olfactory exposure, a Shoaling condition, and a no-shoal Control condition. In the Visual exposure condition subjects were exposed to a stimulus shoal with a clear impermeable plastic divider separating the fish so that they could see each other, but not could not physically interact. In the Visual + Olfactory condition subjects were separated from the stimulus shoal by a slotted clear plastic divider that allowed for water to flow between sides of the partition but were small enough to prevent subjects from crossing the barrier, allowing for the exchange of olfactory cues but preventing physical interaction. In the Shoaling condition, the stimulus shoal was placed in the subject side of the testing tank, meaning subjects were allowed to freely physically interact with the stimulus shoal. In the Control condition subjects were placed in an empty tank with no conspecifics. All conditions utilized tanks with partitions in order to control for free swimming space between groups. All subjects were housed in isolation the night before testing with obscuring dividers between tanks in order to control for confounding social exposure.

Subjects were divided into four groups of 12 randomly selected males and assigned to one of the four stimulus conditions. Sixteen female stimulus fish from our population were randomly selected for use in the stimulus shoals. This allowed for stimulus fish to be swapped between subjects, in order to minimize stimulus fish stress and potential changes in responding to the experimental subjects. The stimulus shoal size was determined based on early evidence indicating that shoals of as low of four zebrafish exhibit shoaling behaviour patterns that are indistinguishable from that of larger groups (Breder & Halpern, 1946). Female individuals were used as stimulus fish and males used as experimental fish in order to easily identify the male experimental subject at the end of the exposure period by visual

inspection, thus minimizing time between exposure and euthanasia, as well as avoiding stressful and potentially harmful invasive identification methods such as fin clipping or fluorescent tagging.

The exposure period began immediately after the experimental subjects were placed into the tank and lasted for 30 minutes. The length of this exposure period was chosen due to evidence indicating that while PS6 is detectable after 5 minutes, it peaks after 30 minutes and is then maintained for several hours (Biever et al., 2015). This exposure period was considered ideal for accurately measuring the level of PS6 in subjects' brain using immunohistochemistry.

2.4 Euthanasia & Tissue Processing

Following exposure, experimental subjects were removed from the testing tank and quickly euthanized using buffered tricaine methanesulfonate (MS222, 300mg/L, Sigma-Aldrich, Dorset, UK) dissolved in tank water, an approved Schedule One method of euthanasia. This euthanasia procedure was adopted from a procedure by Neiffer & Stamper (2009). MS222 was prepared in concentrated 50ml buffered frozen aliquots and adjusted to pH 7.4, identical to the pH of the home tanks. These aliquots were defrosted and combined with 200ml of tank water to dilute the MS222 to a working concentration of 300mg/L. Subjects were immersed in the MS222 for 5-10 minutes and observed until there had been an absence of opercular movement for 3 minutes, and no observable heartbeat. The subjects' brains were subsequently dissected using a standard protocol by Gupta & Mullins (2010).

2.4.1 Tissue Preservation & Sectioning

Following dissections, subjects' brains were submerged in Optimal Cutting Temperature (OCT) compound in moulds and then immediately snap-frozen in dry ice-chilled

hexane. Frozen tissue was stored at -20°C until sectioning. Sectioning was performed using a Leica CM3050 S Cryostat. Whole brains were sectioned coronally into slices 20μm thick and thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Loughborough, UK) in two parallel series. Slides were stored at -20°C until processed for immunohistochemistry.

Brain tissue for this project was not post-fixed or sucrose cryoprotected before freezing, but rather immediately snap-frozen and then post-fixed on the slides immediately before immunohistochemical staining. This was due to preliminary test stains with the PS6 antibody which indicated it was extremely sensitive to over-fixation. If whole brains were fixed or if the fixation period lasted any longer than 10 minutes, then no staining would be observed. Antigen retrieval techniques were also tested and proved unsuccessful in producing successful staining in over-fixed brain tissue. These fixation constraints additionally prevented any form of double-label staining with other primary antibodies that required overnight whole brain fixation.

2.5 Immunohistochemistry

Sections were thawed and air-dried before processing for staining. Slides were first immersed in 4% paraformaldehyde (4% paraformaldehyde powder + 1x PBS) for 10 minutes to post-fix brain tissue. Following immersion, sections were then washed in 0.1M Phosphate-buffered saline (1x PBS) three times for five minutes and outlined using a hydrophobic pen between the second and third washes in order to prevent liquid from running off the slides during incubations. Sections were then incubated in a blocking buffer comprised of 2% normal goat serum (Vector Laboratories, Peterborough, UK) diluted in PBST (1x PBS + 0.1% Triton X-100) for 60 minutes in a moist chamber in order to block nonspecific binding of the antibodies. The sections were then incubated in an anti-PS6 (Ser235/236) rabbit

polyclonal antibody (antibody #2211, Cell Signalling Technology, London, UK) diluted to 1:600 in blocking solution, and then stored at 4°C overnight in a moist chamber.

The following day the slides were washed three times in 1x PBS for 5 minutes, then incubated with a goat anti-rabbit IgG H&L AlexaFluor-594 fluorescent secondary antibody (ab150080, Abcam, Cambridge, UK) diluted to 1:500 in blocking solution for 1 hour at room temperature in a moist chamber in the dark. The slides were then washed three times in 1x PBS for 5 minutes in the dark and briefly dipped in de-ionized water to remove excess salt. Three to four drops of Fluoroshield + DAPI mounting medium (Abcam) were added before carefully mounting coverslips onto the slides using tweezers. Coverslips were then sealed on all sides using clear nail varnish in order to prevent mounting medium from drying out. Slides were stored in a sealed container at 4°C to prevent light exposure and accidental contact with the tissue throughout the duration of imaging.

2.5.1 Imaging

Imaging took place in the Imaging Suite of the Life Sciences Building using a fluorescent Leica LMD6 microscope at 20x magnification. Individual brain regions were imaged bilaterally using RHO (absorption peak = 541-551nm) and DAPI (absorption peak = 340-380nm) fluorescence filters. Brain regions were identified using an atlas by Wullimann et al. (1996).

In order to reliably and consistently quantify PS6-positive neurons in both hemispheres I analysed images using FIJI (ImageJ). I processed images using a custom macro. Images were loaded, contrast was enhanced, then the background removed. Images were then converted to black and white (8 bit) and threshold adjusted using auto threshold with a triangle method. Images were then de-speckled, and holes filled. Watershed was then run in order to subdivide overlapping neurons. Neurons were then counted using Analyse

Particles with size 240 pixels and circularity 0.10-1.00. Five representative samples of each brain region (examples Figure 4-7) were used per subject for the POA, Vd, and Vv, and two samples used for the Vs due to the small size of the brain region. Representative samples were chosen based on image clarity, minimal tissue damage, and lack of background autofluorescence that would lead to inaccurate neuron counts.

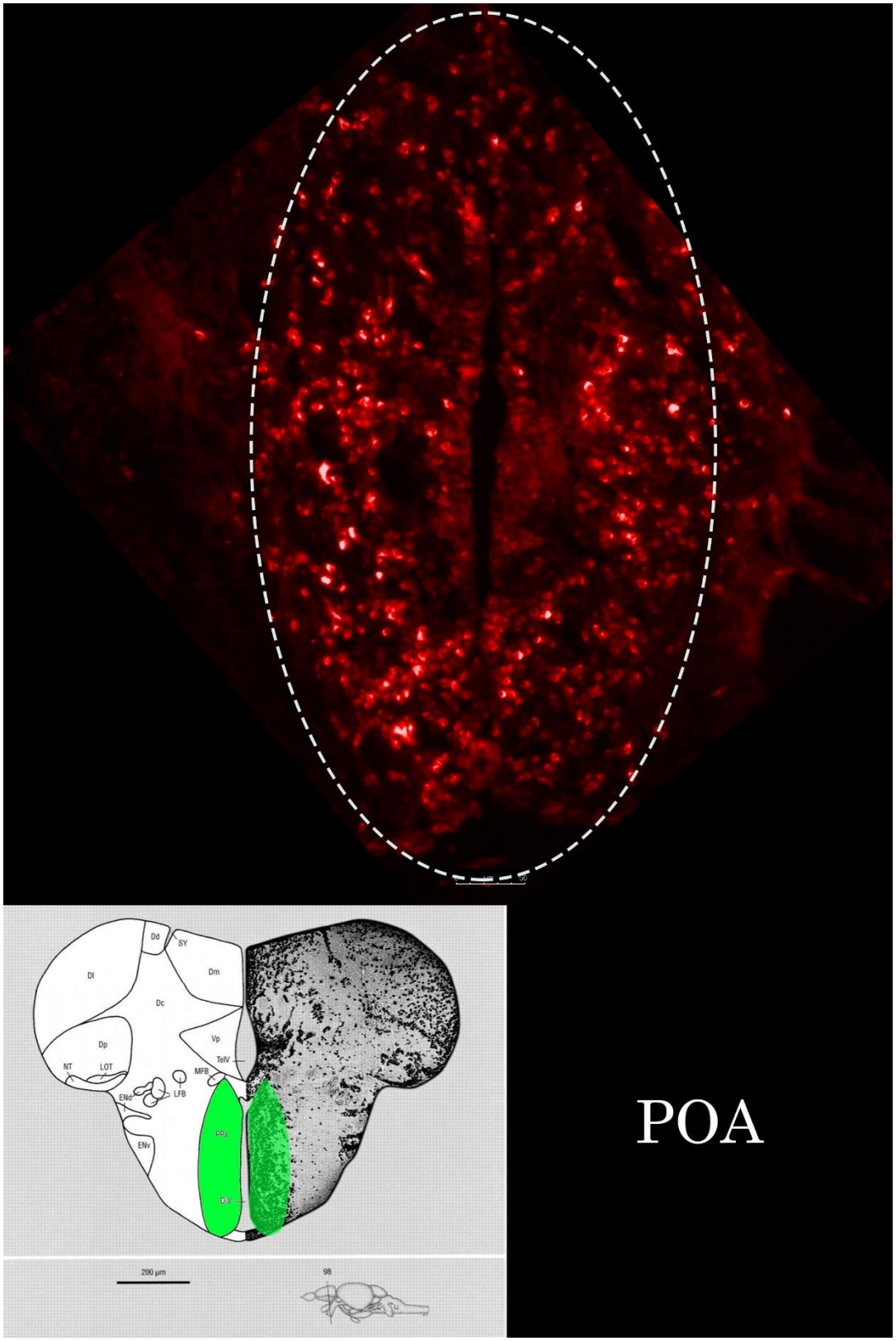

Figure 4. Sample image of the POA with anatomical reference (indicated in green). Brain atlas image taken from Wullimann et al. (1996).

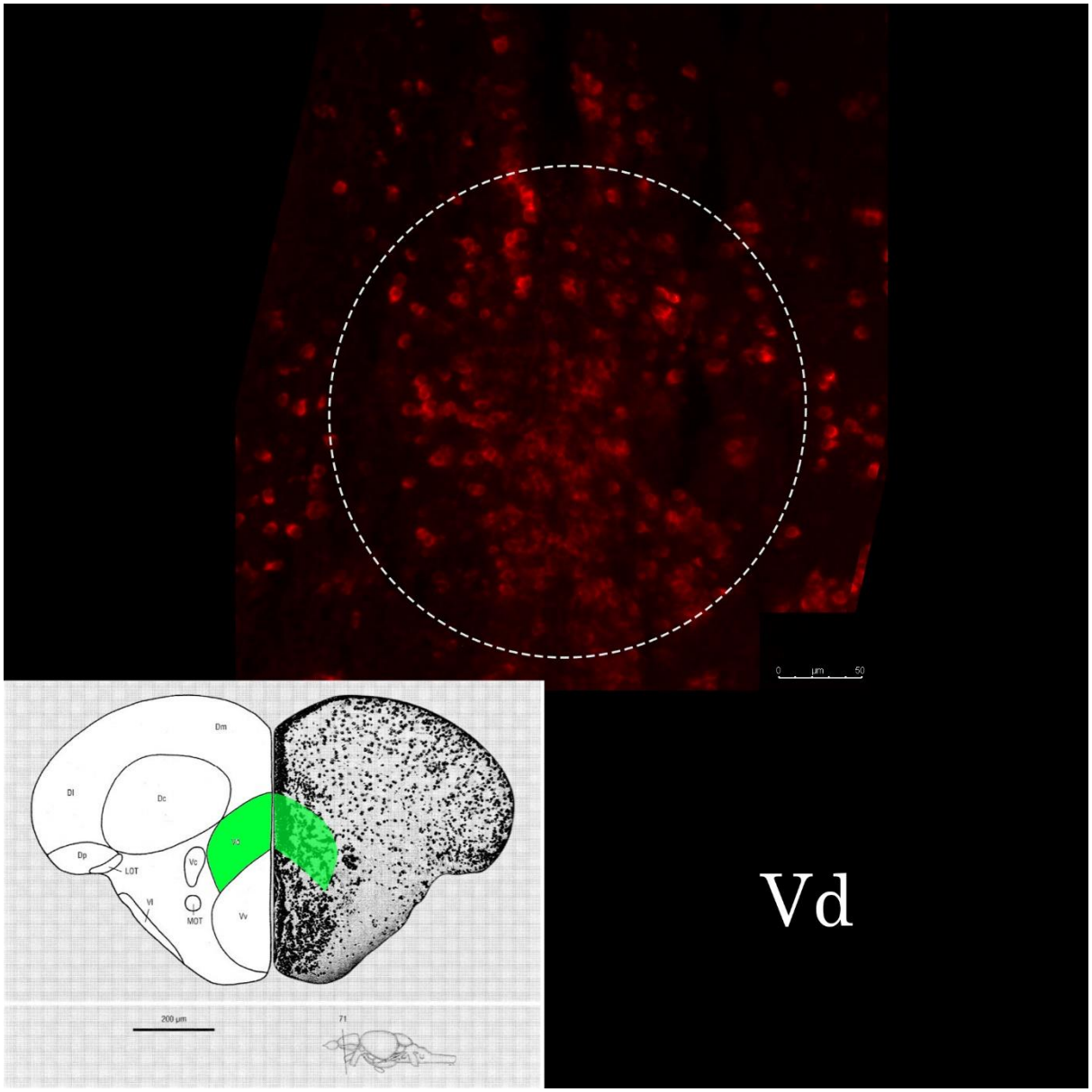

Figure 5. Sample image of the Vd with anatomical reference (indicated in green). Brain atlas image taken from Wullimann et al. (1996).

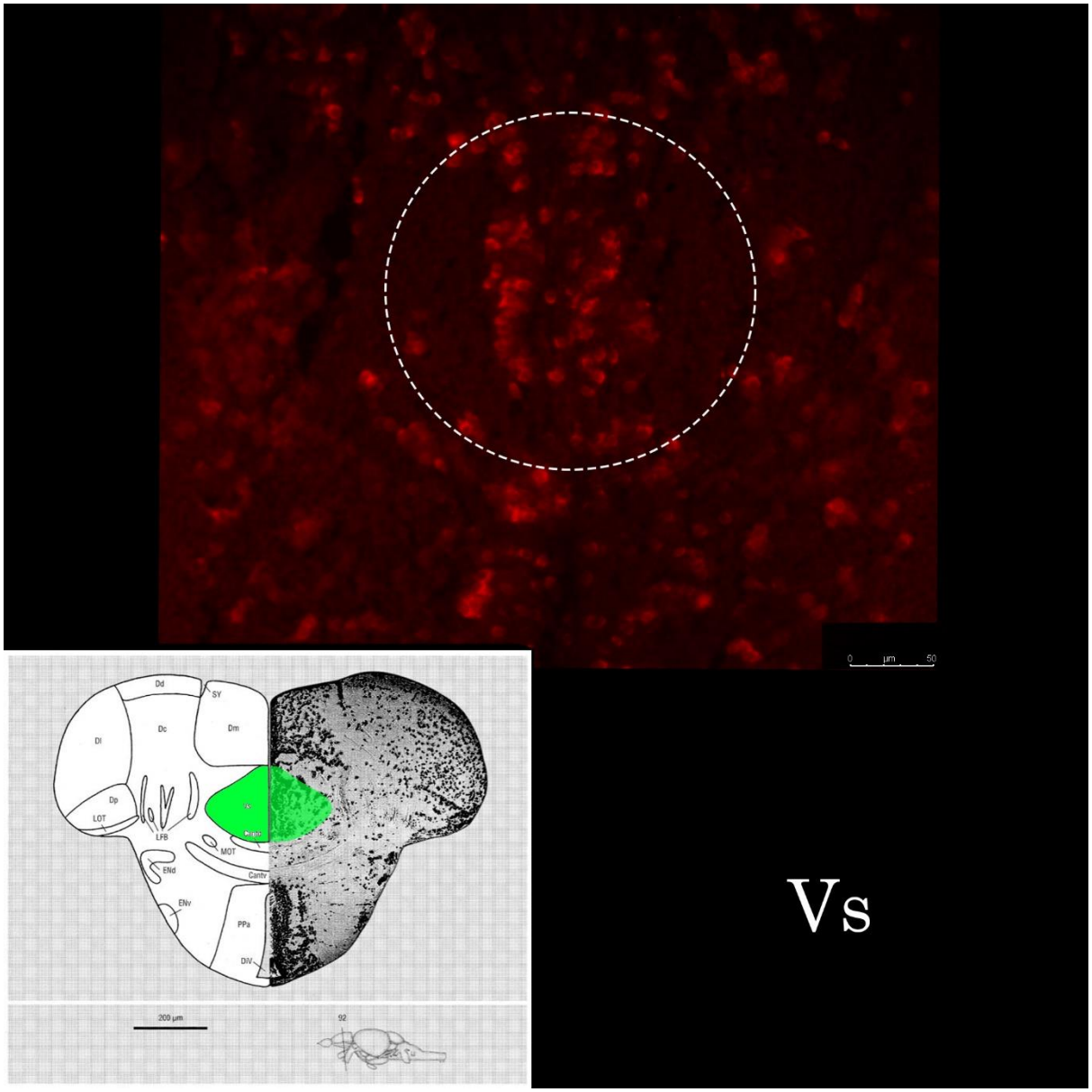

Figure 6. Sample image of the Vs with anatomical reference (indicated in green). Brain atlas image taken from Wullimann et al. (1996).

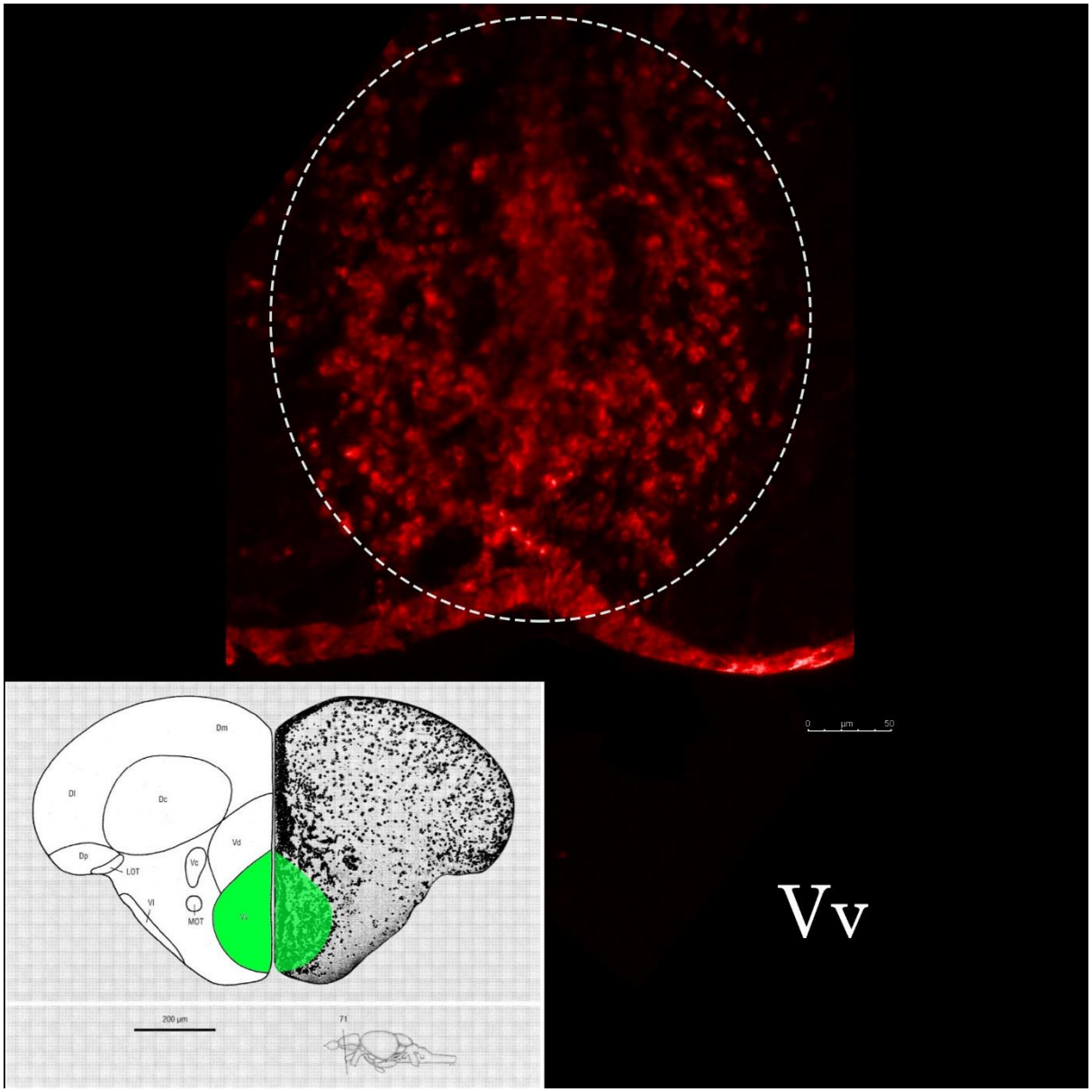

Figure 7. Sample image of the Vv with anatomical reference (indicated in green). Brain atlas image taken from Wullimann et al. (1996).

2.6 Analysis of Data

In order to analyse the level of PS6 in the sampled brain regions between conditions, I compared numbers of PS6-positive cells in each region individually. Counts were analysed using a Generalized Linear Mixed Model (GLMM) with a negative binomial error family for each brain area, using subject ID as a random factor in order to control for sampling of multiple sections per individual within each region. Models were fitted to the

negative binomial distribution as it is suitable for count data such as these. Overall effect of social exposure on PS6 counts was determined using type II Wald chi square tests. When there were significant overall effects of treatment, Tukey's post-hoc tests were utilized in order to determine which experimental conditions exhibited statistically significant differences. Statistical analyses and plots were performed using RStudio (RStudio Team, 2015) using the *lme4* (Bates et al., 2015), *car* (Fox & Weisberg, 2011), *multcomp* (Hothorn et al., 2008), and *ggplot2* (Wickham, 2016) packages.

2.7 Results

A significant effect of the social treatment condition on neuronal PS6 level was found in the POA (n=35, χ^2=9.925, p=0.019.) Post-hoc tests on cell counts in the POA showed that there were significantly more PS6-positive neurons in the Visual + Olfactory condition than the Control condition (p=0.031, means±SEM: Visual + Olfactory =159.980 ± 16.362, Control =114.867 ± 12.801), that there was a trend for less PS6 in the Shoaling condition than the Visual + Olfactory condition (p=0.073), but that there were no significant differences found between any of the other conditions (Shoal vs Control: p = 0.98, Visual vs Control: p = 0.24, Shoal vs Visual + Olfactory: p = 0.07, Visual vs Visual + Olfactory: p = 0.88, Visual vs Shoal: p = 0.42, Figure 8). No significant differences were found in any of the other sampled brain regions (Vd: n=35, χ^2=1.642, p=0.649; Vs: n=35, χ^2=4.481, p=0.214; Vv: n=35, χ^2=5.991, p=0.112).

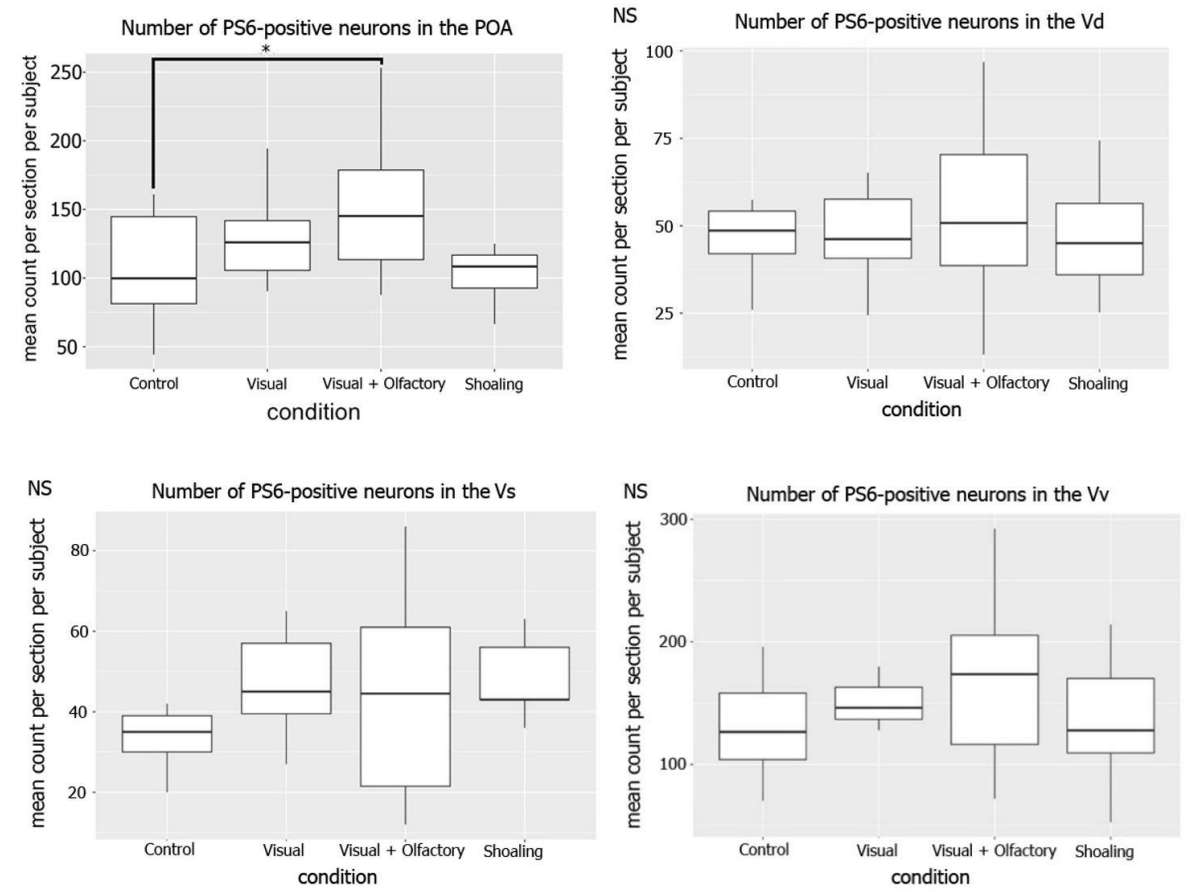

Figure 8. Boxplots of numbers of PS6-positive neurons per section in each sampled region of the SDMN between social exposure conditions. There was a significant effect of condition in the POA (p=0.019), where significantly more PS6 neurons (*p*=0.031) *observed between the Control and Visual + Olfactory conditions. No significant differences were found between other groups or in the other brain regions. Values are medians ± IQR.*

Chapter Three: Nonapeptide Levels in Different Populations

3.1 Aim

Findings from my first study highlighted the importance of the POA in the neural regulation of social behaviour. In order to determine whether changes in POA are associated with changes in behavioural phenotypes, I compared IT neurons in the POA between populations of zebrafish with different shoaling phenotypes, examining numbers of IT neurons of each different type found in the POA.

3.2 Population Behavioural Phenotypes

Previous research in our laboratory had shown that housing regime results in differences in shoaling behaviour after only 4 weeks (Michael Green, unpublished data). Briefly, 200 zebrafish from were given 15-minute shoaling tests in groups of 10, then split into two populations of 100 fish each, giving a sample size of n=10 tanks per treatment. They were then placed in control or environmentally enriched housing tanks and then their shoaling behaviour was tested again after 4 weeks of control/enriched housing. These tests were videoed, and movement and position information for each individual was then extracted using motion-tracking software. Data on time spent within 2 body lengths of other fish (a standard measure of shoaling tendency) and average path length to other fish were calculated, mean measures per group were calculated for each tank, and tank means were analysed using repeated measures ANOVA.

Analysis of these data showed that there were significant interactions between treatment and the week of testing in both time spent within 2 body lengths of another fish ($F_{(3,16)}$=5.834, p=0.021), and average path length ($F_{(3,16)}$=5.146, p=0.029) after 4 weeks of enriched housing (Figures 9 & 10). Based on the different behavioural phenotypes observed in these zebrafish populations as a result of environmental manipulation, and evidence from Study 1 that the POA is implicated in the regulation of social interaction in fish, I was interested in characterizing IT neurons in the two populations. My experimental

aim was to determine if there was an association between IT neuron number and increased shoaling behaviour in the enriched population of zebrafish, and which types of IT neurons might be involved in the changes in shoaling behaviour.

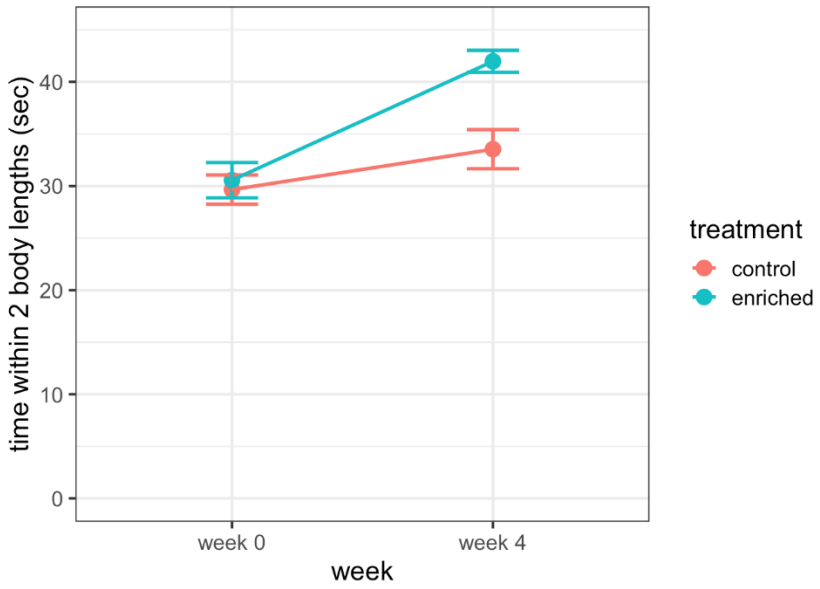

*Figure 9. Experimental subjects spent significantly more time within 2 body-lengths of conspecifics than control subjects after being housed in an enriched environment for 4 weeks (*p*=0.021). Values are means ± SEM.*

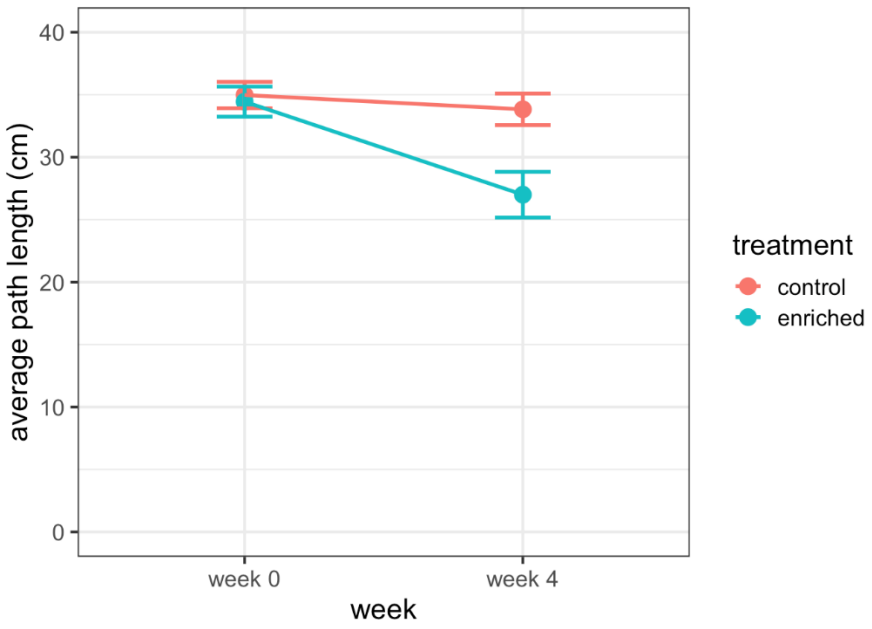

Figure 10. Experimental subjects demonstrated shorter average path lengths to the nearest conspecific in a shoal than control subjects after being housed in an enriched environment for 4 weeks (p=*0.029). Values are means ± SEM.*

3.3 Subjects

Subjects in this study were 22 adult (1.5-year old) female zebrafish *(Danio rerio)* (*n*=22), obtained from the same cohort of over 2000 larvae from the University of Manchester as the previous study and similarly reared at Liverpool John Moores University. Female subjects were used in this study due to evidence that levels of OT/IT are generally higher in females than males, allowing for more adequate analysis of IT expression (Zingg & Laporte, 2003; Carter, 2007). Subjects were housed in the same standard conditions as in the previous experiment: groups of 10, housed in 4-litre tanks with water temperature maintained at 27 ± 1 °C, and kept on a 14-hour light: 10-hour dark cycle. Fish were fed a daily diet of Tetramin flake food as well as supplementary live artemia once weekly. These fish were drawn from two different populations in our laboratory: one housed in standard tanks with a plastic plant and an image of gravel

substrate beneath the tank, and the other population housed in tanks with an enriched and physically dynamic environment. Twelve subjects were randomly selected from environmentally enriched tanks and twelve from standard tanks. However, usable immunohistochemical data was only collected from ten environmentally enriched fish due to poor-quality sections in two subjects, thus producing sample sizes of n=10 enriched and n=12 control fish.

The home tank arrangement for the control subject population followed the same standard tank setup as fish in the previous study. This included a single plastic plant and an image of gravel substrate placed beneath the transparent bottom of the tank. The enriched environment in the home tanks of the enriched subject population included 3 plastic plants, two different pieces of grey opaque plastic PVC piping (one straight tunnel and one 90° elbow, each with a diameter of approximately 2cm and approximately 7cm long), had physical gravel substrate at the bottom of each tank, and had an increased water flow rate from the rack system into each tank. Plastic plant types & colours and PVC pipe pieces were changed weekly in order to maintain a dynamic physical environment. Control environment tanks remained unchanged. Fish were housed in their respective tank environments 6 months before brain dissections.

3.4 Tissue Processing & Immunofluorescence

Subjects were removed from their home tanks individually and immediately euthanized using 300mg/L of buffered MS222 diluted in tank water. Fish were immersed in MS222 solution for 5-10 minutes until there was an absence of opercular movement for 3 minutes and no observable heartbeat. Brains were quickly dissected and fixed in 4% paraformaldehyde for 24 hours at 4°C. The following day the brains were sucrose cryoprotected (30% sucrose dissolved in 1x PBS) overnight in order to prevent cellular damage during snap-freezing (Rosene & Rhodes, 1990). Once the brains had sunk, they were

embedded in OCT moulds and snap-frozen in dry ice-chilled hexane as in Study 1. Whole brains were sectioned into slices 20μm thick and thaw-mounted onto Superfrost Plus slides. Slides were stored at -20°C until processed for immunohistochemistry.

3.4.1 Immunohistochemistry

Sections were thawed and air-dried before processing for staining. Sections were washed in 1x PBS three times for five minutes and outlined using a hydrophobic pen between the second and third washes. Sections were then incubated in a blocking solution containing 2% normal goat serum diluted in PBST for 60 minutes in a moist chamber in order to block nonspecific binding of the antibodies. The sections were then incubated in a mouse monoclonal anti-oxytocin primary antibody (Millipore MAB52096, Lot #2896413, Burlington MA, United States) diluted to 1:2000 in blocking buffer and stored at 4°C overnight in a moist chamber.

The next day the slides were washed three times in 1x PBS for 5 minutes. At this point sections were incubated with a Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) preabsorbed secondary antibody (ab150117, Abcam) diluted to 1:500 in blocking buffer for 1 hour at room temperature in a moist chamber in the dark. The slides were then washed three times in 1x PBS for 5 minutes in the dark. Subsequently the slides were incubated in a custom polyclonal rabbit anti-vasotocin primary antibody (a generous gift of Arja Sluiter, Netherlands Institute for Neuroscience) diluted at 1:2000 in blocking buffer overnight in the dark in a moist chamber.

On the third day the slides were washed three times in 1x PBS for 5 minutes in the dark and incubated in a Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594) preabsorbed secondary antibody (ab150080, Abcam) diluted to 1:1000 in blocking buffer for 1 hour at room temperature in a moist chamber in the dark. Following this incubation slides were

washed three times for 5 minutes in 1x PBS and briefly dipped in de-ionized water to remove excess salt. Three or four drops of Fluoroshield mounting medium were added before carefully mounting coverslips onto the slides. Slides were then sealed using clear nail varnish in order to prevent drying or moving of the sections under the microscope. Slides were stored in a sealed container at 4°C to prevent photobleaching prior to and during imaging.

While the sections were double-label stained for both AVT and IT, only IT-stained cells were counted and analysed as detailed antibody characterization carried out after staining indicated that while the anti-OT primary antibody reliably bound IT and not AVT, the anti-AVT primary antibody was not specific for AVT-expressing neurons and rather seemed to bind IT protein (see below for details).

3.4.2 Imaging

Imaging took place in the Imaging Suite of the LSB using a Leica LMD6 fluorescent microscope using a 20x magnification. Images were taken of all sections in which IT-positive cells were stained using GFP (absorption peak = 460-500nm) and DAPI (absorption peak = 340-380nm) filters. Cell types were identified manually based on soma size and localization. Parvocellular cells were identified as densely-packed smaller neurons located in the rostral and ventral region of the POA, magnocellular neurons as mid-sized neurons located more caudal and dorsal throughout the POA, and gigantocellular neurons identified as large neurons in the caudal and dorsal region of the POA (Godwin & Thompson, 2012) (Figures 11-13). Parvocellular, magnocellular, and gigantocellular neurons in the POA were counted independently. Cell counts were quantified manually.

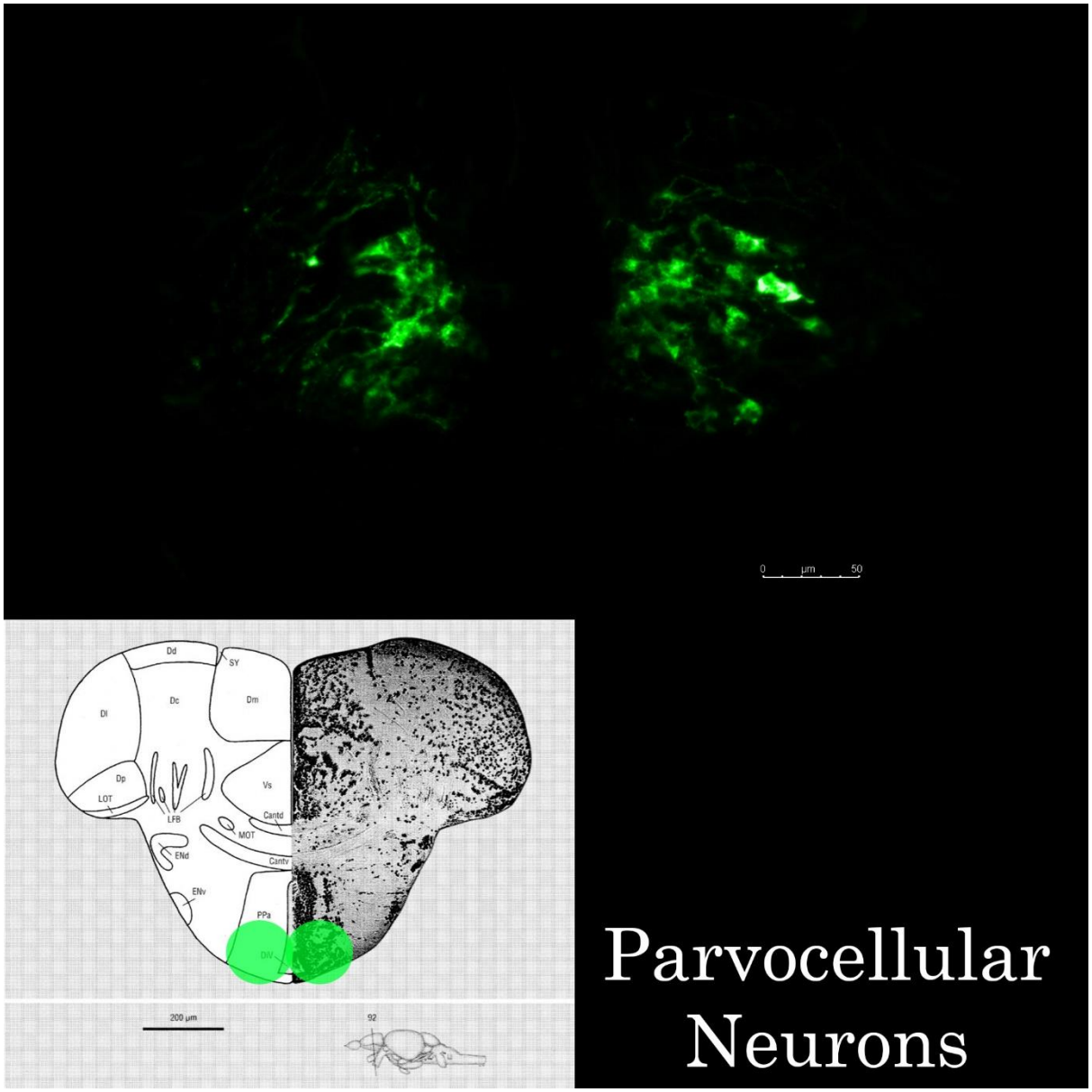

Figure 11. Sample image of parvocellular IT neurons in the POA with anatomical reference (indicated in green). Brain atlas image taken from Wullimann et al. (1996).

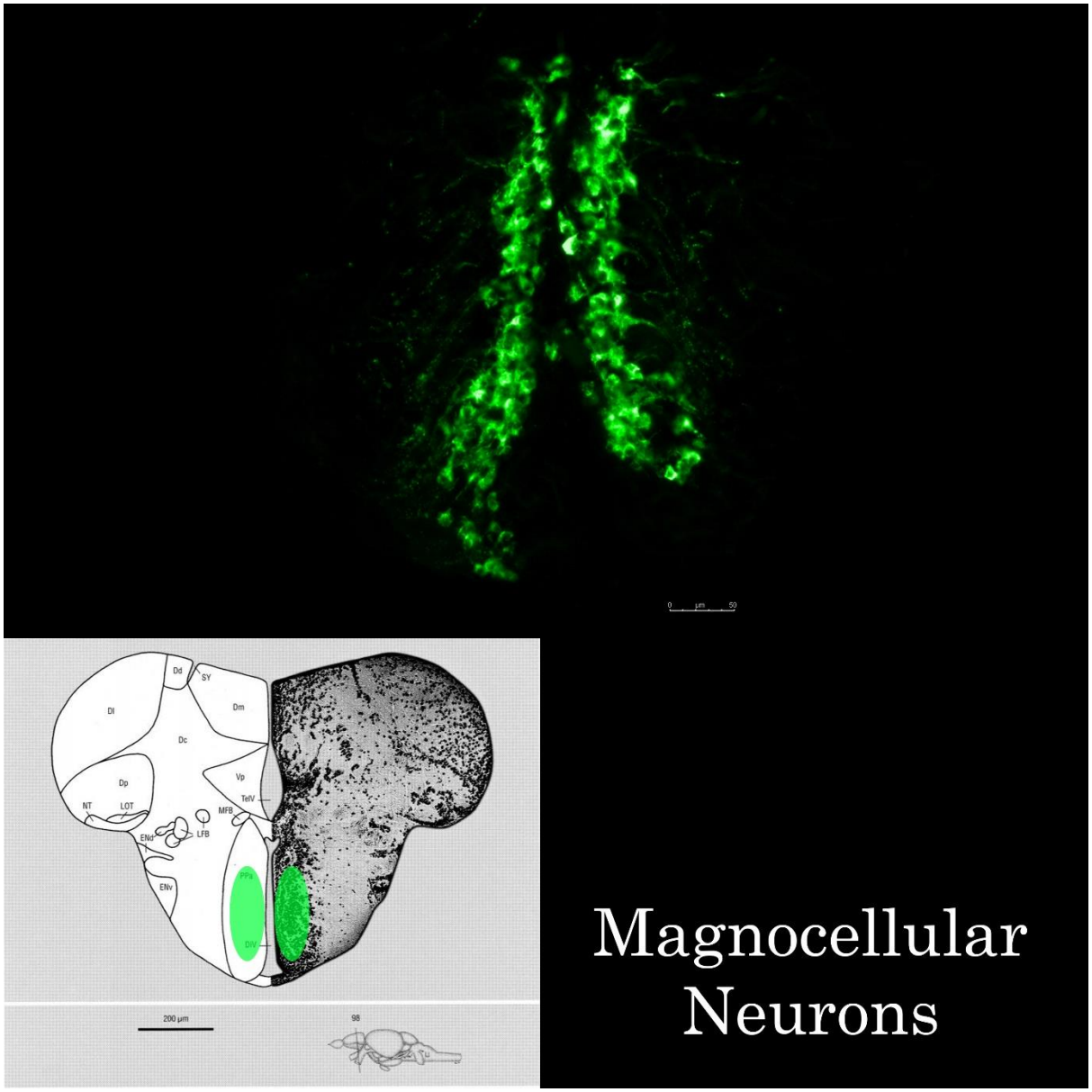

Figure 12. Sample image of magnocellular IT neurons in the POA with anatomical reference (indicated in green). Brain atlas image taken from Wullimann et al. (1996).

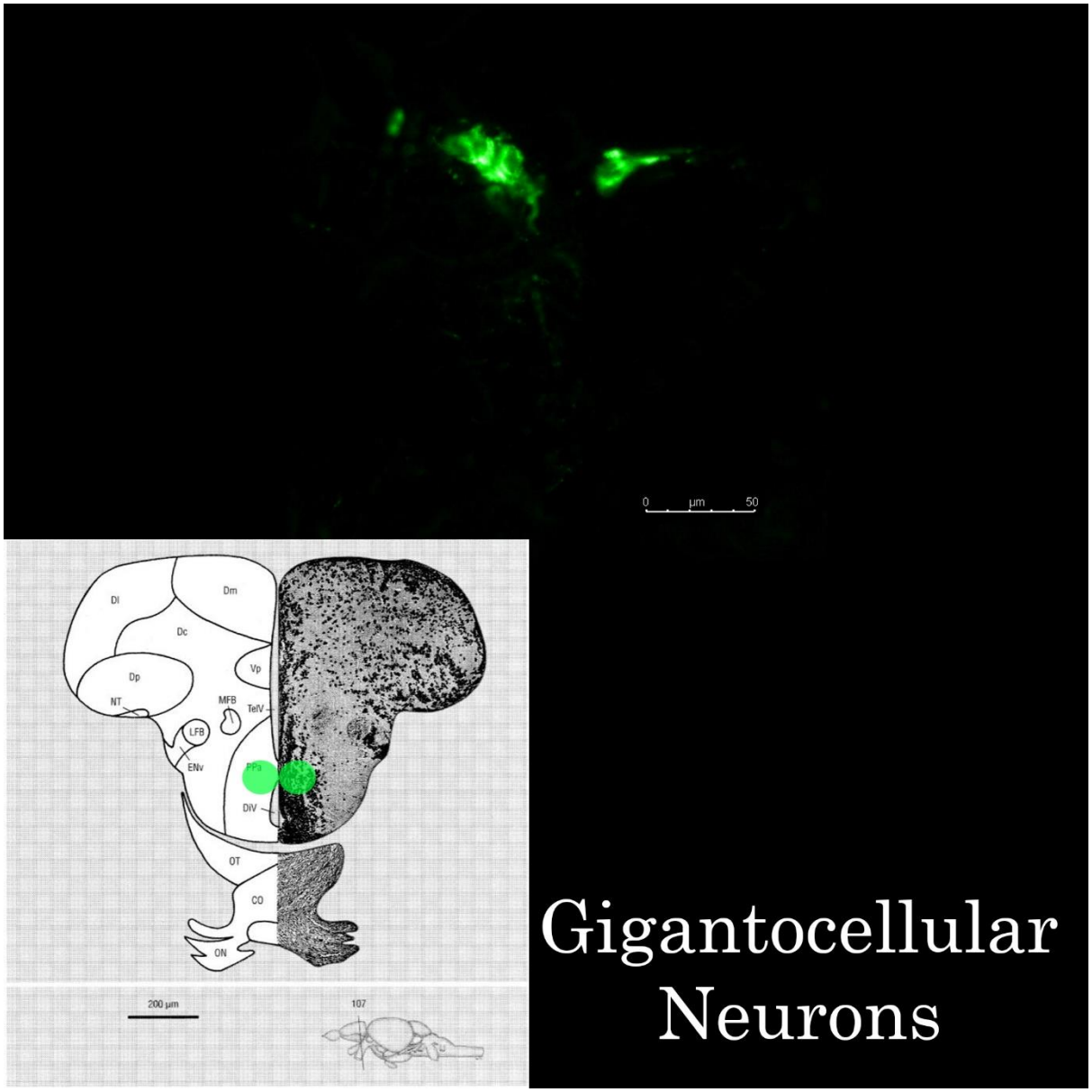

Figure 13. Sample image of gigantocellular IT neurons in the POA with anatomical reference (indicated in green). Brain atlas image taken from Wullimann et al. (1996).

3.4.3 Antibody Characterization: Pre-Incubation Staining

In order to validate antibody binding specificity, both pre-incubation staining and immunoblot dot blot procedures were carried out. For pre-incubation staining, the working dilution of both the anti-OT primary antibody and the anti-AVT primary antibody (1:2000, 1.67nMol) were pre-incubated with an excess of either 50µMol of pure AVT protein (Bachem, St Helens, UK), 47µMol of pure IT protein (Bachem), a combination of both

proteins, or a control stain with no pre-incubation. Immunohistochemistry was then carried out as described above but using the pre-incubated antibody solutions. If the primary antibodies reliably bound the target protein they were specific for, staining would be blocked by pre-incubation with the protein as the antibody would have already bound to the protein during pre-incubation. If staining was blocked by the alternate protein, this would indicate that the primary antibody had cross-reactivity for the other nonapeptide. If staining was not blocked during pre-incubation with either protein, then this would demonstrate that the primary antibody did not bind to either nonapeptide. Findings from pre-incubation staining indicated that the anti-OT primary antibody reliably bound IT and did not bind AVT. However, the anti-AVT primary antibody did not bind AVT, but rather bound IT. The anti-OT primary antibody was also previously validated in goldfish and confirmed to bind only to IT by Mangiamele et al. (2016), and shown to not bind to AVT by the manufacturer.

3.4.4 Antibody Characterization: Immunoblot

In order to further confirm antibody specificity, a dot immunoblot test was carried out. The dot blot procedure was performed using the same pure protein used for the pre-incubation staining following a protocol adapted from a standard Millipore immunoblot protocol.

PVDF membrane (Millipore) was cut into four 1cm wide strips and activated by immersion in 100% methanol for 15 seconds before being soaked in distilled water for 2 minutes until equilibrated. Ensuring that the membrane did not dry out, drops of four different concentrations of nonapeptide protein were blotted onto the membrane. For AVT I used 50µMol, 5µMol, .5µMol, and .05µMol concentrations. For IT, 47µMol, 4.7µMol, .47µMol, and .047µMol concentrations were used. The membranes were then allowed to dry

in order to properly absorb the protein for 1.5 hours at room temperature on a filter stack composed of a wet Whatman filter paper, a dry Whatman filter paper, and dry paper towels.

Following drying and absorption, membranes were re-activated by immersion in 100% methanol followed by distilled water as before. Membranes were blocked using 0.25% bovine serum albumin (BSA) in distilled water for 30 minutes at room temperature with agitation. The membranes were then incubated in primary antibodies for 1 hour at room temperature with agitation. Immunoblot strips of AVT and IT protein were incubated with either the anti-OT or anti-AVT primary antibody diluted to 1:2000. After primary incubation, membranes were washed 3 times for 5 minutes using 1x PBS.

Primary antibody binding was visualized using biotinylated secondary antibodies specific for the primary antibody used (biotinylated goat anti-rabbit IgG 1:200; biotinylated horse anti-mouse IgG 1:500, Vector Laboratories, Peterborough, England). Immunoblot strips were incubated with appropriate biotinylated secondaries for 1 hour with agitation, then washed 3 times for 5 minutes in 1x PBS. After washing, membranes were incubated in Vectastain ABC reagent (biotin-avidin-horseradish peroxidase complex, VECTASTAIN Elite ABC Universal PLUS Kit, Peroxidase Horse-anti-mouse/rabbit IgG, Vector Laboratories) for 30 minutes with agitation. After being washed 3 times for 5 minutes in 1x PBS, membranes were stained using SIGMA*FAST* 3,3′-Diaminobenzidine (DAB) solution (Sigma-Aldrich). The membranes were covered in the substrate for approximately 5 minutes to achieve optimal staining. At this point the reaction was stopped by washing with distilled water.

Immunoblot staining showed that the anti-OT primary antibody reliably bound to IT protein and not AVT, while the anti-AVT primary antibody did not bind AVT protein, but rather bound to IT protein. As both pre-incubation staining and immunoblot antibody validation procedures confirmed that the anti-OT primary antibody was specific for IT

protein and did not bind AVT, but that the anti-AVT primary antibody was not specific for AVT, only IT-positive neurons were counted and used for data analysis and the supposed AVT antibody staining was disregarded.

3.5 Analysis of Data

Normality of parvo-, magno-, and gigantocellular IT neuron counts was determined using Shapiro-Wilk tests. Normality tests indicated that both magnocellular and gigantocellular counts were normally distributed and these data were thus analysed using Welch Two Sample *t* tests. Parvocellular count data were not normally distributed and were thus analysed using a Wilcoxon Rank Sum test with continuity correction. All statistical tests and plots were carried out using RStudio (RStudio Team, 2015) and the *ggplot2* (Wickham, 2016) package.

3.6 Results

Analysis of cell counts between conditions showed that more social fish housed in an enriched environment had significantly more magnocellular IT neurons in the POA than control subjects ($t_{(11.837)}$=-2.444, means ±SEM: enriched=214.800±44.426, control=96.167±19.124, p=0.031) (Figure 14). There were no significant differences in cell count found between conditions in gigantocellular ($t_{(18.706)}$=-1.189, means ±SEM: enriched=6.8±1.474, control=4.5±1.217, p=0.249) (Figure 15) or parvocellular ($W(22)$=53, means ±SEM: enriched=30.6±11.458, control=21.833±5.491, p=0.667) neuron populations (Figure 16).

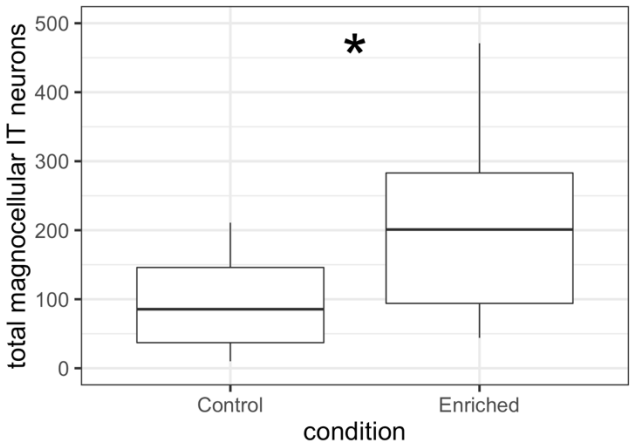

*Figure 14. More social zebrafish housed in an enriched environment had significantly more magnocellular IT neurons than control subjects (*p*=0.031). Values are medians ± IQR.*

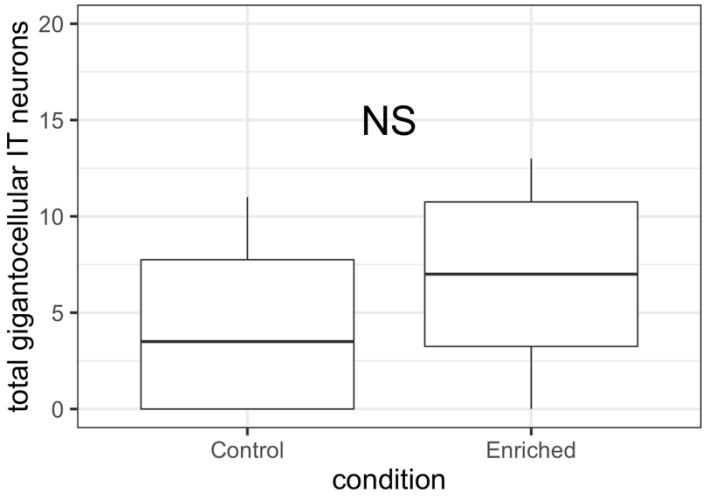

*Figure 15. There were no significant differences found in expression of gigantocellular IT neurons between populations of zebrafish (*p*=0.249). Values are medians ± IQR.*

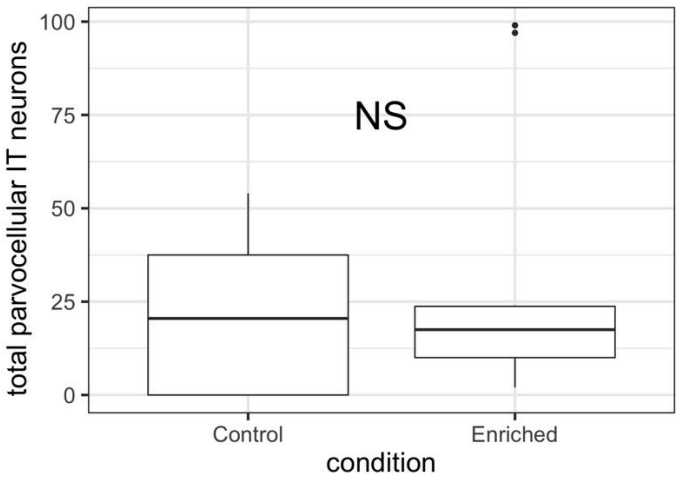

*Figure 16. There were no significant differences in expression of parvocellular IT neurons between populations (*p*=0.667). Values are medians ± IQR.*

Chapter Four: Discussion

4.1 SDMN Activation

In my first experiment I investigated the role of the SDMN in grouping behaviour in zebrafish, with consideration for how different levels of social cues may activate the SDMN in different ways. The results demonstrate that different contexts of social exposure to conspecifics leads to different levels of PS6 in the POA. Exposure to combined Visual + Olfactory social information significantly increased PS6 in the POA compared to Control conditions, but no significant differences were found between any other experimental conditions. No significant differences were found in other sampled nodes of the SDMN. My findings suggest that the POA is the most sensitive of the sampled nodes of the SDMN to social cues, and that it also appears to respond more strongly to social cues than to actual shoaling.

4.1.1 Context-Dependent Responses to Social Exposure

My findings that Visual + Olfactory exposure significantly increased PS6 level show that combined social information is more important for the neural processing of social information than only visual exposure to conspecifics. Cabrera-Álvarez et al. (2017) similarly found that expression of the IEG EGR-1 increased in the POA of guppies as a result of combined Visual + Olfactory exposure to a large shoal of conspecifics when compared to a small shoal or a no-shoal control, but no changes were seen in the Vd, Vs, or Vv. This context-dependent increase is possibly due to the role of the POA in the processing of social olfactory cues and social discrimination (Landgraf et al., 2003; Bielsky & Young, 2004). The POA, and OT specifically, have been shown to be critical for social olfaction and social recognition in other vertebrates, through the facilitation of changes in neuronal activity (Keverne, 1999; Brennan, 2001), thus suggesting that the more potent social cue of visual and olfactory information may be increasing neuronal activation in the POA in the Visual + Olfactory condition. It is possible that increased activation in the POA

in the Visual + Olfactory condition is a direct result of increased olfactory processing compared to other conditions and although no significant differences were seen, it is worth noting that PS6 levels in the Visual condition were intermediate between those in the Control and Visual + Olfactory conditions.

4.1.2 Social Exposure Did Not Affect the Vd, Vs, or Vv

My findings showed that while neuronal activation in the POA was context-dependent, I did not observe any significant differences in any other sampled nodes of the SDMN between conditions. This result is similar to findings by Cabrera-Álvarez et al. (2017) who found that there were no differences in the Vd, Vs, or Vv between combined Visual + Olfactory exposure to a shoal and Control conditions. It is well-documented that zebrafish find shoaling with conspecifics to be socially reinforcing (Saverino & Gerlai, 2008; Al-Imari & Gerlai, 2008). As the Vd, Vs, and Vv are thought to be parts of a teleost mesolimbic reward system and dopaminergic pathway (O'Connell & Hofmann, 2011), I predicted that PS6 level in these nodes would increase in the Shoaling condition. My results do not fit well with the SDMN hypothesis, suggesting that either reward in zebrafish is more complex than in mammals, or that the proposed teleost homologies in the SDMN are not completely accurate. It is possible that nodes of the teleost SDMN may be functionally different than mammalian nodes, or alternatively possible that some regions of the teleost SDMN have been misidentified and require further research in order to establish accurate homologues to mammalian reward pathways. Despite the Vd, Vs, and Vv having been proposed to play a role in social reinforcement, it is possible that social reward is not encoded by activation of these nodes, but instead involves activation of the SBN. Thus, these nodes of the SDMN may be less sensitive to variable social cues than the POA. More detailed neuroanatomical investigation of fish reward circuitry, including dopaminergic

circuits, is necessary to determine the role of the Vd, Vs, and Vv in reward and responses to social cues.

4.1.3 POA as a Regulator of Social Decision-Making

My findings are also contrary to my prediction that PS6 levels in the POA would increase as the level of social exposure grew increasingly rich. This was surprising as the Shoaling condition provided subjects with the highest amount of social exposure and greater potential for social interaction. There was a non-significant trend for increased activation of the POA in the Visual + Olfactory condition compared to the Shoaling condition, indicating that neurons in the POA may be being activated by more than just simple social cues from conspecifics. It is possible that the POA is involved in social decision-making but is not activated by shoaling itself. Thus, when the subject is unable to join the shoal but exposed to rich social cues the POA is activated, but when physically shoaling there is no decision to be made about whether to affiliate with the shoal.

Interestingly, Fischer et al. (2018) found that shoaling with female conspecifics significantly increased PS6 in the POA of male guppies compared to exposure to males or a no-shoal control, while my findings showed no difference between the Shoaling and Control conditions in zebrafish. It is possible that the POA responds to shoaling in a species-specific manner, illustrating the complexity and variance in teleost neural regulation of social behaviour. These differences in PS6 when exposed to female conspecifics between species could also be due to differences in social and breeding systems, where male guppies spend the majority of their time courting or attempting to mate with females, whereas zebrafish generally spawn at dawn (Breden et al., 1999; Breder et al., 1966; Nasiadka & Clark, 2012). These differences in social structure could lead to different types of social decision-making, or social information being processed differently between species, thus eliciting different responses in the POA. It is also possible that

guppies in the Fischer et al. study experienced an intruder effect that may have affected neuronal and behavioural responses as experimental fish were housed in testing tanks overnight before behavioural exposure, whereas in the present study both the experimental and stimulus fish were simultaneously introduced to an unfamiliar environment. These differences in findings suggest species-specific differences in neural regulation of social decision-making and highlight the importance of not assuming that the neurobiology of social behaviour is uniform across fish species.

It is also possible that sexual motivation may also be a factor in the present study. Subjects in the experiment were male fish exposed to female conspecifics. It is possible that the motivation to affiliate with the stimulus shoal is sexually-driven in addition to shoaling motivation. This is supported by the findings by Fischer et al. (2018) that shoaling with females, but not males increases PS6 expression in the POA of male guppies. This would further suggest that the POA is functioning as a regulator of social decision-making.

4.1.4 Social Stress Responses in the POA

While the difference between the Visual + Olfactory and Shoaling conditions was not significant, there was a non-significant trend for less PS6 in the Shoaling condition. This finding suggests that there may be more factors other than just simple social cues influencing the activation of the POA. It is possible that the increased neuronal responses to Visual + Olfactory exposure in the POA may be influenced by social stress. Social stress has previously been shown to lead to changes in the mammalian POA. Social isolation led to an increase in the number of post-synaptic dendritic spines in the medial POA of rats (Sánchez-Toscano et al., 1991). Defeat-induced social stress has also been shown to increase c-*fos* expression in several brain regions including the POA of hamsters (Kollack-Walker et al., 1997). The medial POA has also been shown to be crucial for the inhibitory role of circulatory testosterone on corticosteroid responses to stress in rats through the

inhibition of AVP (Viau & Meany, 1996). It is possible that the POA response in the Visual + Olfactory condition was a result of social stress when subjects were exposed to social cues but could not join the shoal. This effect would thus not be present in the Shoaling condition as swimming with the shoal would not be socially stressful, nor in the Control condition as while isolation is stressful to zebrafish, it is not necessarily directly socially stressful (Miklósi & Andrew, 2006; Saverino & Gerlai, 2008). This would fit with the idea that the POA is responding to social cues rather than to the act of grouping behaviour.

4.1.5 Implications for Current Research Methods

Shoaling is frequently used as a proxy for social behaviour in zebrafish through quantifying the preference of fish to swim near a stimulus shoal of conspecifics that has been separated by a barrier (Cabrera-Álvarez et al., 2017; Bass & Gerlai, 2008; Al-Imari & Gerlai, 2008). This experimental method is generally considered to have inherent drawbacks such, as restricting measurement of shoal dynamics and being an artificial construct (Miller & Gerlai, 2007). The barrier separating the stimulus and experimental fish may be in the form of a clear tank partition or by placing either the stimulus or the experimental fish inside of a cup placed in the tank. Whether or not this barrier is slotted to allow for olfactory information to be exchanged between experimental fish and the stimulus varies based on experimental methods. My findings indicate that neuronal responses are affected by the method of social exposure used, and thus it is possible that stimulus perception and behavioural responses of experimental subjects are likely to be affected.

My results indicate that when experimental subjects must be kept separate from a social stimulus, that researchers should use a combined Visual + Olfactory exposure as this type of exposure elicited the greatest response in the POA, whereas visual only exposure

was not as strong. Researchers should also consider whether stimuli used are purely social stimuli, or rather confound social stimuli and social stress, as my results suggest that at a neuronal level there appears to be a difference in activation depending on whether motivation to join a social group can be satisfied or not.

4.2 Isotocin and Shoaling Phenotypes

My second experiment sought to understand the role that the POA might be playing in social behaviour and specifically grouping behaviour by examining whether environmentally-driven differences in shoaling are associated with plasticity in POA nonapeptide neurons. By characterizing IT expression in two different populations of zebrafish with different levels of shoaling behaviour, as a result of housing in an enriched versus standard environment, I was able to determine the relationship between neuronal IT phenotypes and grouping behaviour.

Immunohistochemical analysis showed that zebrafish that were more social as a result of being housed in a tank with an enriched environment had significantly more magnocellular IT neurons in the POA than less social zebrafish that were housed in a standard control environment. These findings suggest that magnocellular IT neuron level is associated with shoaling tendencies in zebrafish and suggest that behavioural changes arising from environmental conditions may involve plasticity in nonapeptide circuitry. The positive correlation between affiliative behaviour and neural IT expression supports the idea that IT is a positive regulator of social behaviour, including grouping, in teleosts.

4.2.1 Isotocin in Grouping Behaviour

A general role of IT in teleost social behaviour is somewhat difficult to disentangle due to species and context-specific differences in function and expression. In general, OT is seen as a facilitator of affiliation in mammalian species. Chronic exposure to centrally-administered OT has been shown to significantly increase non-sexual affiliative behaviour

in male rats (Witt et al., 1992), while release of OT to the NAcc has been shown to be essential for pair-bond formation in female Prairie voles (Carter et al., 2008; Ross et al., 2009). In humans, OT promotes cooperative behaviour by decreasing activity in the cingulate cortex, amygdala, midbrain, and dorsal striatum (Baumgartner et al., 2008; Kosfeld et al., 2005). Further research has also shown that OT-induced inhibition of the amygdala promotes affiliative responses in humans by reducing social anxiety and fear (Petrovic et al., 2008; Kirsch et al., 2005). Mesotocin, the avian OT-homologue, has also been shown to increase preference for affiliation with larger groups and familiar same-sex stimuli in zebra finches (Goodson et al., 2009b).

In teleosts however we see a range of functional roles and patterns of expression of IT based on species. In similar fashion to effects seen in mammalian species, Thompson & Walton (2004) found that IT promotes social approach in male goldfish. Seasonal changes in free neural IT based on breeding cycle have been shown in gobies (*Neogobius melanostomus*) in which IT expression peaks during spawning but is lowest immediately after the spawning period ends (Sokołowska et al., 2015). Similarly, AVT- and IT-immunoreactive neuron number decreased after spawning in female medaka (*Oryzias latipes*), suggesting that social context has profound effects on neural IT expression (Ohya & Hayashi, 2006).

Research in cichlid fish has shown that neural IT expression was higher in the social *Neolamprologus pulcher* cichlid than in the non-social species *Telmatochromis temporalis*, and that IT level was correlated with social behaviour (O'Connor et al., 2016). However, research investigating the correlation between behavioural phenotypes and neural IT expression indicated that more social *N. pulcher* had lower levels of free neural IT than less social *N. pulcher* (Reddon et al., 2015). Furthermore, both high and low doses of exogenous IT reduced grouping behaviour in *N. pulcher*, while administration of an IT

receptor antagonist increased affiliation (Reddon et al., 2014). Interestingly, an intermediate dose of IT had no effect on affiliative behaviour, suggesting that the role of IT in teleost social behaviour is dose-dependent (Reddon et al., 2014). Further research investigating species-based differences in nonapeptide neurons between social systems in cichlids has shown that cooperatively-breeding species had fewer parvocellular IT neurons than independently-breeding cichlids (Reddon et al., 2017). There was no correlation between number of magno- or gigantocellular neurons and social system. This is interesting when considered in light of my findings that more social zebrafish have more magnocellular neurons, while no difference was seen in parvocellular neuron populations, further suggesting species-based variation in IT function. Combined, these findings highlight that the hypothesized role of the OT/IT family of neuropeptides in social behaviour is not as simple as sometimes portrayed, and that functional roles vary across a wide range of social and biological contexts.

4.2.2 Within-Species Variation in Nonapeptide Signalling

Within-species differences in nonapeptide neuron number is relatively common in fish. Large-bodied plainfin midshipman fish that express courting and territorial behaviours have been shown to have larger AVT-immunoreactive neurons than non-courting smaller conspecifics (Foran & Bass, 1998). While the number of these neurons did not vary between behavioural and physiological phenotypes, researchers concluded that smaller midshipman fish had more AVT-immunoreactive neurons when adjusted for size. Further research investigating the role of nonapeptide hormones as mediators of reproductive behaviour in midshipman fish showed that administration of AVT, but not IT, decreased courtship vocalizations in large courting males (Goodson & Bass, 2000). Interestingly, IT, but not AVT, decreased courtship vocalizations in females and smaller non-courting males (Goodson & Bass, 2000). This research demonstrates differences in nonapeptide and

nonapeptide neuronal regulation of behaviour within a species and suggests sexual and phenotypic dimorphism in neural mediators of reproductive behaviour.

In a study examining population differences in AVT regulation of behaviour in pupfish (*Cyprinodon nevadensis*) between populations with different aggressive phenotypes it was found that the more aggressive population of pupfish had more magnocellular AVT neurons and had larger, but fewer AVT parvocellular neurons than the less aggressive population (Lema, 2006). Research investigating the role of AVT and IT in aggression, courtship, and parenting behaviour using male stickleback fish (*Gasterosteus aculeatus*) shows that aggressive males who care for eggs and demonstrate competitive aggression in a group express increased levels of free AVT, while dominant males that exhibit territorial and courtship behaviour express higher levels of free IT (Kleszczyńska et al., 2012). These findings combined with my own indicate that nonapeptide neurons are often plastic in teleosts and that variation in nonapeptide levels are associated with different behavioural phenotypes within-species.

In addition to phenotypic associations, nonapeptide neurons have been shown to be plastic in response to the environment. Environmental factors such as salinity and temperature have been shown to affect the size and number of AVT neurons in pupfish. A higher salinity environment decreased the number of magnocellular AVT neurons, while the size of these neurons was affected by salinity in a temperature-dependent manner (Lema, 2006). Interestingly, the effects of salinity on parvocellular AVT neuron number differed between populations of pupfish. Rearing in a low-salinity environment decreased the number of parvocellular AVT neurons in aggressive pupfish, while this condition increased parvocellular AVT neuron number in less aggressive pupfish (Lema, 2006). These findings demonstrate plasticity in the POA and social behaviour in response to environmental manipulation.

4.2.3 Parvo-, Magno-, and Gigantocellular Neurons in Grouping Behaviour

My results demonstrate a positive correlation between grouping behaviour and the number of magnocellular IT neurons. In general, both magnocellular and gigantocellular AVT neurons are hypothesized to be responsible for complex behaviours such as the regulation of aggressive, reproductive, and dominant behaviours (Godwin & Thompson, 2012; Iwata et al., 2010). However, comparatively little is known about the role of IT magnocellular neurons in teleost social behaviour. My data combined with related evidence that IT promotes social approach in goldfish (Thompson & Walton, 2004) suggests that IT, whether exogenous or endogenous magnocellular IT, may be acting as a regulator of simple grouping behaviour in zebrafish, and that magnocellular neurons are sensitive and plastic to environmental and behavioural changes within a population.

While I found that more social zebrafish housed in an enriched environment had more magnocellular IT neurons than less social control subjects, there were no significant differences in parvocellular or gigantocellular IT neurons between conditions. While little is known about the function of gigantocellular IT neurons, parvocellular IT neurons have been found to regulate paternal care in *Amatitlania nigrofasciata* cichlids and are positively correlated with dominance rank in false clown anemonefish (O'Connell et al., 2012; Iwata et al., 2010). Research in cichlids indicates a negative correlation between both number of parvocellular IT neurons and free IT levels with affiliation (Reddon et al., 2015; 2017). These differences compared to my own findings suggest further complexities to the role of IT in regulating social behaviour in fish. Further characterization of different types of IT neurons is necessary in order to determine the precise role of magnocellular IT neurons in shoaling responses in zebrafish. This will allow us to determine whether magnocellular IT neurons have a causal role in shoaling tendencies, or if this neuronal change is due to a

related but different aspect of social behaviour that is also affected by environmental enrichment.

It is noted that counts of parvocellular neurons in the present study are abnormally low relative to magnocellular neuron counts, with zeroes for some counts in some individuals. It is possible that poor-quality or damaged sections compounded with inadequate microscopy may have obstructed data collection and skewed cell counts. Although unlikely, it is also possible that the antibody, while specific for IT, does not bind to parvocellular neurons in zebrafish as reliably as it binds to magnocellular and gigantocellular neurons.

4.3 Future Research Directions

The questions raised by these results mean that the next logical steps would be to determine whether the POA has a role in regulating shoaling behaviour, and if so, if IT signalling is important in this regulation. This could be accomplished through measuring IT release in response to social cues, or by conducting electrophysiology assays in the POA during social exposure. Other approaches could include lesioning the POA or using inhibitory DREADD (Designer Receptor Exclusively Activated by Designer Drugs) technology to selectively inhibit the activation of different populations of IT neurons during behavioural assays in order to see how shoaling behaviour is affected.

In order to properly characterize the role of nonapeptide neurons in the POA in social behaviour it is necessary to further investigate the response of nonapeptide neurons to shoaling cues and shoaling itself. This investigation would determine whether or not neurons activated by Visual + Olfactory exposure in the POA are nonapeptide neurons, and whether or not these nonapeptides are responsive to these simple social cues. If antibody incompatibility issues arising from differing fixation requirements could be resolved, co-expression of nonapeptide neurons and PS6 using double-label immunohistochemistry

would help determine how different types of nonapeptide neurons respond to social stimuli, elucidating the role of nonapeptide hormones in simple social behaviour, and allowing for further investigation into the role of nonapeptide neurons in shoaling phenotype variation.

4.4 Conclusion

The results of my studies show that the teleost POA is a crucial node of the SDMN in the regulation of social behaviour. However, the precise role of the POA in shoaling is not fully clear. My data shows that neurons in the POA responded to combined Visual + Olfactory information, but not to any other form of social exposure. This indicates that the POA is involved in social behaviour, but that it's not responding simply to the strength of the social stimulus, but the nature of the social cue. This suggests that the POA is responding to specific social cues rather than to social interaction directly, and that the POA may be involved in social decision-making. Furthermore, more social zebrafish were also found to have more magnocellular IT neurons in the POA than subjects with lower shoaling tendencies. These changes in behaviour were a result of environmental manipulation, suggesting that environmentally-driven changes in adult social phenotypes in zebrafish may involve changes in IT signalling in the POA. Magnocellular IT neurons may also act as plastic mediators of grouping behaviour in zebrafish.

When considering both studies together I am presented with an interesting conundrum. My first experiment shows that the POA is not activated by shoaling, but rather by shoaling-related cues, yet in the second experiment I show that fish with increased shoaling tendencies have more IT neurons in the POA. My combined evidence suggests that IT and magnocellular neurons are important for grouping behaviour, but that neurons in the POA respond in a social context-dependent manner. If the POA is responding to social cues, then it is possible that the increase in IT neurons increases the capacity for encoding and

responding to social information in the POA. My findings demonstrate the importance and plasticity of the teleost POA and IT in simple social behaviour and show how adult behavioural phenotypes are plastic in response to environmentally-driven changes.

References

Al-Imari, L., Gerlai, R. (2008). Sight of conspecifics as reward in associative learning in zebrafish (*Danio rerio*). *Behavioural Brain Research*, 189(1): 216-219.

Alunni, A., Blin, M., Deschet, K., Bourrat, F., Vernier, P., Rétaux, S. (2004). Cloning and developmental expression patterns of Dlx2, Lhx7 and Lhx9 in the medaka fish (*Oryzias latipes*). *Mechanisms of Development*, 121: 977–983.

Andersen, P., Morris, R., Amaral, D.G., Bliss, T., O'Keefe, J. (2007). *The hippocampus book*. New York: Oxford University Press.

Backström, T., Windberg, S. (2009). Arginine vasotocin influence on aggressive behavior and dominance in rainbow trout. *Physiology and Behavior*, 96(3): 470-475.

Bandler, R., Carrive, P. (1988). Integrated defense reaction elicited by excitatory amino acid microinjection in the midbrain periaqueductal grey region of the unrestrained cat. *Brain Research*, 439: 95–106.

Bass, S.L.S., Gerlai, R. (2008). Zebrafish (*Danio rerio*) responds differentially to stimulus fish: the effects of sympatric and allopatric predators and harmless fish. *Behavioural Brain Research*, 186(1): 107-117.

Bates, D., Maechler, M., Bolker, B., Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1): 1-48.

Baumgartner, T., Heinrichs, M., Vonlanthen, A., Fischbacher, U., Fehr, E. (2008). Oxytocin shapes the neural circuitry of trust and trust adaptations in humans. *Neuron*, 58(4): 639-650.

Beckstead, R.M., Domesick, V.B., Nauta, W.J. (1979). Efferent connections of the substantia nigra and ventral tegmental area in the rat. *Brain Research*, 175: 191–217.

Benelli, A., Bertolini, A., Poggioli, R., Menozzi, B., Basaglia, R., Arletti, R. (1995). Polymodal dose-response curve for oxytocin in the social recognition test. *Neuropeptides*, 28: 251-255.

Bester-Meredith, J., Young, L., Marler, C. (1999). Species differences in paternal behavior and aggression in *peromyscus* and their associations with vasopressin immunoreactivity and receptors. *Hormones and Behavior*, 36: 25-38.

Bielsky, I.F., Young, L.J. (2004). Oxytocin, vasopressin, and social recognition in mammals. *Peptides*, 25(9): 1565-1574.

Bolborea, M., Ansel, L., Weinert, D., Steinlechner, S., Pévet, P., Klosen, P. (2010). The bed nucleus of the stria terminalis in the Syrian hamster (*Mesocricetus auratus*): absence of vasopressin expression in standard and wild-derived hamsters and galanin regulation by seasonal changes in circulating sex steroids. *Neuroscience*, 165: 819-830.

Brackett, N.L., Edwards, D.A. (1984). Medial preoptic connections with the midbrain tegmentum are essential for male sexual behavior. *Physiology and Behavior*, 32: 79–84.

Braford, M.R. Jr, Northcutt, R.G. (1983). *Organization of the diencephalon and pretectum of ray-finned fishes*. In: David, R.G., Northcutt, R.G., editors. *Fish neurobiology*, vol 2. Ann Arbor: University of Michigan Press. p 117–163.

Braida, D., Donzelli, A., Martucci, R., Capurro, V., Busnelli, M., Chini, B., Sala, M. (2012). Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in zebrafish. *Psychopharmacology*, 220: 319-330.

Breden, F., Ptacek, M.B., Rashed, M., Taphorn, D., Figueiredo, C.A. (1999). Molecular phylogeny of the live-bearing fish genus *Poecilia* (Cyprinodontiformes: Poeciliidae). *Molecular Phylogenetics and Evolution*, 12(2): 95-104.

Breder, C.M., Halpern, F. (1946). Innate and acquired behavior affecting the aggregation of fishes. *Physiological Zoology*, 19(2): 154-190.

Breder, C.M., Rosen, D.E., American Museum of Natural History (1966). *Modes of reproduction in fishes*. Garden City, New York: Natural History Press.

Brennan, P. (2001). The vomeronasal system. *Cellular and Molecular Life Sciences*, 58: 546-555.

Bridges, R.S., Mann, P.E., Coppeta, J.S. (1999). Hypothalamic involvement in the regulation of maternal behaviour in the rat: inhibitory roles for the ventromedial hypothalamus and the dorsal/anterior hypothalamic areas. *Journal of Neuroendocrinology*, 11: 259–266.

Cabrera-Álvarez, M.J., Swaney, W.T., Reader, S.M. (2017). Forebrain activation during social exposure in wild-type guppies. *Physiology & Behavior*, 182: 107-113.

Caldwell, H.K., Lee, H.J., Macbeth, A.H., Young III, W.S. (2008). Vasopressin: behavioral roles of an "original" neuropeptide. *Progress in Neurobiology*, 84: 1-24.

Canteras, N.S., Simerly, R.B., Swanson, L.W. (1994). Organization of projections from the ventromedial nucleus of the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. *Journal of Comparative Neurology*, 348: 41–79.

Carter, C.S. (2007). Sex differences in oxytocin and vasopressin: implications for autism spectrum disorders? *Behavioral Brain Research*, 176: 170-186.

Carter, C.S., Grippo, A.J., Pournajafi-Nazarloo, H., Ruscio, M.G., Porges, S.W. (2008). Oxytocin, vasopressin and sociality. *Progress in Brain Research*, 170: 331-336.

Cho, M., DeVries, A., Williams, J., Carter, C. (1999). The effects of oxytocin and vasopressin on partner preferences in male and female prairie voles (*Microtus ochrogaster*). *Behavioral Neuroscience*, 113: 1071-1080.

Choi, G.B., Dong, H.W., Murphy, A.J., Valenzuela, D.M., Yancopoulos, G.D., Swanson, L.W., Anderson, D.J. (2005). Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. *Neuron,* 46: 647–660.

Crews, D. (2003). The development of phenotypic plasticity: where biology and psychology meet. *Developmental Psychobiology,* 43: 1–10.

Cushing, B., Carter, C. (2000). Peripheral pulses of oxytocin increase partner preference in female, but not male, prairie voles. *Hormones and Behavior,* 37: 49-56.

Deco, G., Rolls, E.T. (2005). Attention, short-term memory, and action selection: a unifying theory. *Progress in Neurobiology,* 76: 236–256.

Delfs, J.M., Zhu, Y., Druhan, J.P., Aston-Jones, G. (2000). Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. *Nature,* 403: 430–434.

Demski, L.S., Knigge, K.M. (1971). The telencephalon and hypothalamus of the bluegill (*Lepomis macrochirus*): evoked feeding, aggressive and reproductive behavior with representative frontal sections. *Journal of Comparative Neurology,* 143: 1–16.

Dharmadhikari, A., Lee, Y., Roberts, R., Carter, C. (1997). Exploratory behavior correlates with social organization and is responsive to peptide injections in prairie voles. *Annals of the New York Academy of Sciences*, 807: 610-612.

Domesick, V.B. (1988). Neuroanatomical organization of dopamine neurons in the ventral tegmental area. *Annals of the New York Academy of Sciences*, 537: 10–26.

Donaldson, Z.R., Young, L.J. (2008). Oxytocin, vasopressin, and the neurogenetics of sociality. *Science,* 332: 900-904.

Engelmann, M., Ebner, K., Wotjak, C.T., Landgraf, R. (1998). Endogenous oxytocin is involved in short-term olfactory memory in female rats. *Behavioral Brain Research*, 90: 89-94.

Fallon, J.H., Moore, R.Y. (1978). Catecholamine innervation of the basal forebrain. IV. Topography of the dopamine projection to the basal forebrain and neostriatum. *Journal of Comparative Neurology*, 180: 545–580.

Ferguson, J., Aldag, J., Insel, T., Young, L. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. *Journal of Neuroscience*, 21: 8278-8285.

Ferris, C.F., Potegal, M. (1988). Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. *Physiology and Behavior*, 44: 235–239.

Filby, A.L., Paull, G.C., Hickmore, T.F., Tyler, C.R. (2010). Unravelling the neurophysiological basis of aggression in a fish model. *BMC Genomics*, 11: 498.

Fischer, E.K., Westrick, S.E., Hartsough, L., Hoke, K.L. (2018). Differences in neural activity, but not behavior, across social contexts in guppies, *Poecilia reticulata*. *Behavioral Ecology and Sociobiology*, 72(8). doi:10.1007/s00265-018-2548-9

Floody, O.R., O'Donohue, T.L. (1980). Lesions of the mesencephalic central gray depress ultrasound production and lordosis by female hamsters. *Physiology and Behavior*, 24: 79–85.

Folgueira, M., Anadón, R., Yáñez, J. (2004a). An experimental study of the connections of the telencephalon in the rainbow trout (*Oncorhynchus mykiss*). I: Olfactory bulb and ventral area. *Journal of Comparative Neurology*, 480: 180–203.

Folgueira, M., Anadón, R., Yáñez, J. (2004b). Experimental study of the connections of the telencephalon in the rainbow trout (*Oncorhynchus mykiss*). II: Dorsal area and preoptic region. *Journal of Comparative Neurology*, 480: 204–233.

Foran, C.M., Bass, A.H. (1998). Preoptic AVT immunoreactive neurons of a teleost fish with alternative reproductive tactics. *General and Comparative Endocrinology*, 111: 271-282.

Forlano, P.M., Deitcher, D.L., Bass, A.H. (2005). Distribution of estrogen receptor alpha mRNA in the brain and inner ear of a vocal fish with comparisons to sites of aromatase expression. *Journal of Comparative Neurology*, 483: 91–113.

Forlano, P.M., Marchaterre, M., Deitcher, D.L., Bass, A.H. (2010). Distribution of androgen receptor mRNA expression in vocal, auditory, and neuroendocrine circuits in a teleost fish. *Journal of Comparative Neurology*, 518: 493–512.

Fox, H.E., White, S.A., Kao, M.H., Fernald, R.D. (1997). Stress and dominance in a social fish. *Journal of Neuroscience*, 17: 6463-6469.

Fox, J., Weisberg, S. (2011). *An {R} companion to applied regression, second edition*. Thousand Oaks CA: Sage.

Fuxjager, M.J., Forbes-Lorman, R.M., Coss, D.J., Auger, C.J., Auger, A.P., Marler, C.A. (2010). Winning territorial disputes selectively enhances androgen sensitivity in neural pathways related to motivation and social aggression. *Proceedings of the National Academy of Sciences of the United States of America*, 107: 12393–12398.

Georges, F., Aston-Jones, G. (2001). Potent regulation of midbrain dopamine neurons by the bed nucleus of the stria terminalis. *Journal of Neuroscience*, 21: RC160.

Godwin, J., Thompson, R. (2012). Nonapeptides and social behavior in fishes. *Hormones and Behavior*, 61(3): 230-238.

Goodson, J.L., Bass, A.H. (2000). Vasotocin innervation and modulation of vocalacoustic circuitry in the teleost *Porichthys notatus*. *Journal of Comparative Neurology*, 422: 363-379.

Goodson, J.L. (2005). The vertebrate social behavior network: evolutionary themes and variations. *Hormones and Behavior*, 48(1): 11-22.

Goodson, J.L., Evans, A.K., Lindberg, L., Allen, C.D. (2005). Neuro-evolutionary patterning of sociality. *Proceeds of the Royal Society B: Biological Sciences*, 272: 227-235.

Goodson, J.L., Wang, Y. (2006). Valence-sensitive neurons exhibit divergent functional profiles in gregarious and asocial species. *Proceedings of the National Academy of Sciences of the United States of America*, 103: 17013-17017.

Goodson, J.L. (2008). Nonapeptides and the evolutionary patterning of sociality. *Progress in Brain Research*, 170: 3-15.

Goodson, J.L., Rinaldi, J., Kelly, A.M. (2009a). Vasotocin neurons in the bed nucleus of the stria terminalis preferentially process social information and exhibit properties that dichotomize courting and non-courting phenotypes. *Hormones and Behavior*, 55: 197-202.

Goodson, J.L., Schrock, S.E., Klatt, J.D., Kabelik, D., Kingsbury, M.A. (2009b). Mesotocin and nonapeptide receptors promote estrildid flocking behavior. *Science*, 325(5942): 862-866.

Goodson, J.L., Thompson, R.R. (2010). Nonapeptide mechanisms of social cognition, behavior and species-specific social systems. *Current Opinion in Neurobiology*, 20(6): 784-794.

Greenwood, A.K., Wark, A.R., Fernald, R.D., Hofmann, H.A. (2008). Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behavior in an African cichlid fish. *Proceedings of the Royal Society B: Biological Sciences*, 275: 2393-2402.

Groenewegen, H.J., Berendse, H.W., Haber, S.N. (1993). Organization of the output of the ventral striatopallidal system in the rat: ventral pallidal efferents. *Neuroscience*, 57: 113–142.

Gubernick, D.J., Winslow, J.T., Jensen, P., Jeanotte, L., Bowen, J. (1995). Oxytocin changes in males over the reproductive cycle in the monogamous, biparental California mouse, *Peromyscus californicus*. *Hormones and Behavior*, 29: 59-73.

Gupta, T., Mullins, M.C. (2010). Dissection of organs from the adult zebrafish. *Journal of Visualized Experiments* (37), e1717, doi:10.3791/1717

Haber, S.N., Groenewegen, H.J., Grove, E.A., Nauta, W.J. (1985). Efferent connections of the ventral pallidum: evidence of a dual striato pallidofugal pathway. *Journal of Comparative Neurology*, 235: 322–335.

Hansen, S., Harthon, C., Wallin, E., Löfberg, L., Svensson, K. (1991). Mesotelencephalic dopamine system and reproductive behavior in the female rat: effects of ventral tegmental 6-hydroxydopamine lesions on maternal and sexual responsiveness. *Behavioral Neuroscience*, 105: 588–598.

Harmon, A., Huhman, K., Moore, T., Albers, H. (2002). Oxytocin inhibits aggression in female Syrian hamsters. *Journal of Neuroendocrinology*, 14: 963-969.

Hasegawa, T., Takeo, T., Akitsu, H., Hoshina, Y., Sakuma, Y. (1991). Interruption of the lordosis reflex of female rats by ventral midbrain stimulation. *Physiology and Behavior*, 50: 1033–1038.

Heimer, L., Larsson, K. (1967). Impairment of mating behavior m male rats following lesions in the preoptic-anterior hypothalamic continuum. *Brain Research*, 3: 248-263.

Heimer, L., Alheid, G.F., de Olmos, J.S., Groenewegen, H.J., Haber, S.N., Harlan, R.E., Zahm, D.S. (1997). The accumbens: beyond the core-shell dichotomy. *The Journal of Neuropsychiatry and Clinical Neurosciences*, 9: 354–381.

Ho, J.M., Murray, J.H., Demas, G.E., Goodson, J.L. (2010). Vasopressin cell groups exhibit strongly divergent responses to copulation and male-male interactions in mice. *Hormones and Behavior*, 58: 368-377.

Hothorn, T., Bretz, F., Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal*, 50(3): 346-363

Hull, E.M., Dominguez, J.M. (2006). Getting his act together: roles of glutamate, nitric oxide, and dopamine in the medial preoptic area. *Brain Research*, 1126: 66–75.

Humphries, M.D., Prescott, T.J. (2010). The ventral basal ganglia, a selection mechanism at the crossroads of space, strategy, and reward. *Progress in Neurobiology*, 90: 385–417.

Ikemoto, S., Panksepp, J. (1999). The role of nucleus accumbens dopamine in motivated behavior: a unifying interpretation with special reference to reward-seeking. *Brain Research Reviews*, 31: 6–41.

Ikemoto, S. (2007). Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Research Reviews*, 56: 27–78.

Insel, T., Shapiro, L. (1992). Oxytocin receptor distribution reflects social organization in monogamous and polygamous voles. *Proceedings of the National Academy of Sciences of the United States of America*, 89: 5981-5985.

Insel, T., Wang, Z., Ferris, C. (1994). Patterns of brain vasopressin receptor distribution associated with social organisation in microtine rodents. *Journal of Science*, 14: 5381-5392.

Insel, T.R., Young, L.J. (2000). Nonapeptides and the evolution of social behavior. *Current Opinion in Neurobiology*, 10: 784-789.

Iwata, E., Nagai, Y., Sasaki, H. (2010). Social rank modulates brain arginine vasotocin immunoreactivity in false clown anemonefish (*Amphiprion ocellaris*). *Fish Physiology and Biochemistry*, 36: 337-345.

Jürgens, U. (2002). A study of the central control of vocalization using the squirrel monkey. *Medical Engineering and Physics*, 24: 473–477.

Kapsimali, M., Bourrat, F., Verneier, P. (2001). Distribution of the orphan nuclear receptor Nurr1 in medaka (*Oryzias latipes*): cues to the definition of homologous cell groups in the vertebrate brain. *Journal of Comparative Neurology*, 431: 276-292.

Kelly, A.M., Kingsbury, M.A., Hoffbuhr, K., Schrock, S.E., Waxman, B., Kabelik, D., Thompson, R.R., Goodson, J.L. (2011). Vasotocin neurons and septal V1a-like receptors potently modulate songbird flocking and responses to novelty. *Hormones and Behavior*, 60: 12-21.

Keverne, E. (1999). The vomeronasal organ. *Science*, 286: 716-720.

Kirsch, P., Esslinger, C., Chen, Q., Mier, D., Lis, S., Siddhanti, S., Gruppe, H., Mattay, V.S., Gallhofer, B., Meyer-Lindenberg, A. (2005). Oxytocin modulates neural circuitry for social cognition and fear in humans. *Journal of Neuroscience*, 25: 11489-11493.

Kleszczyńska, A., Sokołowska, E., Kulczykowska, E. (2012). Variation in brain arginine vasotocin (AVT) and isotocin (IT) levels with reproductive stage and social status in males of three-spined stickleback (*Gasterosteus aculeatus*). *General and Comparative Endocrinology*, 175(2): 290-296.

Knight, Z.A., Tan, K., Birsoy, K., Schmidt, S., Garrison, J.L., Wysocki, R.W., Friedman, J.M. (2012). Molecular profiling of activated neurons by phosphorylated ribosome capture. *Cell*, 151(5): 1–22.

Kogan, J.H., Frankland, P.W., Silva, A.J. (2000). Long-term memory underlying hippocampus-dependent social recognition in mice. *Hippocampus*, 10(1): 47-56.

Kollack-Walker, S., Newman, S.W. (1995). Mating and agonistic behavior produce different patterns of Fos immunolabeling in the male Syrian hamster brain. *Neuroscience*, 66: 721–736.

Kollack-Walker, S., Watson, S.J., Akil, H. (1997). Social stress in hamsters: defeat activates specific neurocircuits within the brain. *Journal of Neuroscience*, 17(22): 8842-8855.

Kosfeld, M., Heinrichs, M., Zak, P.J., Fischbacher, U., Fehr, E. (2005). Oxytocin increases trust in humans. *Nature*, 435: 673-676.

Kow, L.M., Pfaff, D.W. (1998). Mapping of neural and signal transduction pathways for lordosis in the search for estrogen actions on the central nervous system. *Behavioral Brain Research*, 92: 169-180.

Krause, J., Ruxton. G.D., *Living in groups*. Oxford University Press, Oxford (2002), 10.1093/sysbio/sys022

Krieg, J., Hofsteenge, J., Thomas, G. (1988). Identification of the 40 S ribosomal protein S6 phosphorylation sites induced by cycloheximide. *The Journal of Biological Chemistry*, 263: 11473-11477.

Kyle, A.L., Peter, R.E. (1982). Effects of forebrain lesions on spawning behaviour in the male goldfish. *Physiology and Behavior*, 28: 1103–1109.

Landgraf, R., Frank, E., Aldag, J.M., Neumann, I.D., Sharer, C.A., Ren, X., Terwilliger, E.F., Niwa, M., Wigger, A., Young, L.J. (2003). Viral vector-mediated gene transfer of the vole V1a vasopressin receptor in the rat septum: improved social discrimination and active social behaviour. *European Journal of Neuroscience*, 18: 403-411.

LeDoux, J.E., Ruggiero, D.A., Forest, R., Stornetta, R., Reis, D.J. (1987). Topographic organization of convergent projections to the thalamus from the inferior colliculus and spinal cord in the rat. *Journal of Comparative Neurology*, 264: 123–146.

Lee, A.W., Brown, R.E. (2007). Comparison of medial preoptic, amygdala, and nucleus accumbens lesions on parental behavior in California mice (*Peromyscus californicus*). *Physiology and Behavior*, 92: 617–628.

Lema, S.C. (2006). Population divergence in plasticity of the AVT system and its association with aggressive behaviors in a Death Valley pupfish. *Hormones and Behavior*, 50(2): 183-193.

Le Moal, M., Simon, H. (1991). Mesocorticolimbic dopaminergic network: functional and regulatory roles. *Physiological Reviews*, 71: 155–234.

Lim, M., Murphy, A., Young, L. (2004). Ventral striatopallidal oxytocin and vasopressin V1a receptors in the monogamous prairie vole (*Microtus ochrogaster*). *Journal of Comparative Neurology*, 468: 555-570.

Lonstein, J.S., Stern, J.M. (1997). Role of the midbrain periaqueductal gray in maternal nurturance and aggression: c-fos and electrolytic lesion studies in lactating rats. *Journal of Neuroscience*, 17: 3364–3378.

Lubin, D., Elliott, J., Black, M., Johns, J. (2003). An oxytocin antagonist infused into the central nucleus of the amygdala increases maternal aggressive behavior. *Behavioral Neuroscience*, 117: 195-201.

Macey, M.J., Pickford, G.E., Peter, R.E. (1974). Forebrain localization of the spawning reflex response to exogenous neurohypophysial hormones in the killfish, *Fundulus heteroclitus*. *Journal of Experimental Zoology*, 190: 269–280.

Maeda, H., Mogenson, G.J. (1981). Electrophysiological responses of neurons of the ventral tegmental area to electrical stimulation of amygdala and lateral septum. *Neuroscience*, 6: 367–376.

Malsbury, C.W. (1971). Facilitation of male rat copulatory behavior by electrical stimulation of the medial preoptic area. *Physiology and Behavior*, 7: 797–805.

Malsbury, C.W., Kow, L.M., Pfaff, D.W. (1977). Effects of medial hypothalamic lesions on the lordosis response and other behaviors in female golden hamsters. *Physiology & Behavior*, 19(2): 223-237.

Mangiamele, L.A., Gomez, J.R., Curtis, N.J., Thompson, R.R. (2016). GPER/GPR30, a membrane estrogen receptor, is expressed in the brain and retina of a social fish (*Carassius auratus*) and colocalizes with isotocin. *Journal of Comparative Neurology*, 525: 252-270.

Meddle, S.L., Bishop, V.R., Gkoumassi, E., van Leeuwen, F.W., Douglas, A.J. (2007). Dynamic changes in oxytocin receptor expression and activation at parturition in the rat brain. *Endocrinology*, 148: 5095-5104.

Melin, P., Kihlstroem, J.E. (1963). Influence of oxytocin on sexual behavior in male rabbits. *Endocrinology*, 73: 433-435.

Miklósi, Á., Andrew, R.J. (2006). The zebrafish as a model for behavioral studies. *Zebrafish*, 3(2). doi: 10.1089/zeb.2006.3.227

Miller, N., Gerlai, R. (2007). Quantification of shoaling behaviour in zebrafish (*Danio rerio*). *Behavioural Brain Research*, 184(2): 157–166.

Mogenson, G.J., Jones, D.L., Yim, C.Y. (1980). From motivation to action: functional interface between the limbic system and the motor system. *Progress in Neurobiology*, 14: 69–97.

Moore, F., Wood, R., Boyd, S. (1992). Sex steroids and vasotocin interact in a female amphibian *Taricha granulosa* to elicit female-like egg-laying behavior or male-like courtship. *Hormones and Behavior*, 26: 156-166.

Moore, F.L., Lowry, C.A. (1998). Comparative neuroanatomy of vasotocin and vasopressin in amphibians and other vertebrates. *Comparative Biochemistry and Physiology C*, 119: 251–260.

Mos, J., Kruk, M.R., van der Poel, A.M., Meelis, W. (1982). Aggressive behavior induced by electrical stimulation in the midbrain central gray of male rats. *Aggressive Behavior*, 8: 261–284.

Munchrath, L.A., Hofmann, H.A. (2010). Distribution of sex steroid hormone receptors in the brain of an African cichlid fish, *Astatotilapia burtoni*. *Journal of Comparative Neurology*, 518: 3302–3326.

Murphy, A.Z., Shupnik, M.A., Hoffman, G.E. (1999). Androgen and estrogen (alpha) receptor distribution in the periaqueductal gray of the male rat. *Hormones and Behavior*, 36: 98–108.

Neumann, I.D. (2009). The advantage of social living: brain neuropeptides mediate the beneficial consequences of sex and motherhood. *Frontiers in Neuroendocrinology*, 30: 483-496.

Newman, S.W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. *Annals of the New York Academy of Sciences*, 877: 242–257.

Norton, W., Bally-Cuif, L. (2010). Adult zebrafish as a model organism for behavioural genetics. *BMC Neuroscience*, 11(90). doi: 10.1186/1471-2202-11-90

Numan, M. (2007). Motivational systems and the neural circuitry of maternal behavior in the rat. *Developmental Psychobiology,* 49: 12–21.

O'Connell, L.A., Hofmann, H.A. (2011). The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *The Journal of Comparative Neurology*, 519(18): 3599-3639.

O'Connell, L.A., Matthews, B.J., Hofmann, H.A. (2012). Isotocin regulates paternal care in a monogamous cichlid fish. *Hormones and Behavior*, 61(5): 725-733.

O'Connor, C.M., Marsh-Rollo, S.E., Aubin-Horth, N., Balshine, S. (2016). Species-specific patterns of nonapeptide brain gene expression relative to pair-bonding behavior in grouping and non-grouping cichlids. *Hormones and Behavior*, 80: 30-38.

Ohya, T., Hayashi, S. (2006). Vasotocin/isotocin-immunoreactive neurons in the medaka fish brain are sexually dimorphic and their numbers decrease after spawning in the female. *Zoological Science*, 23: 23-29.

O'Keefe, J., Nadel, L. (1978). *The hippocampus as a cognitive map*. New York: Oxford University Press.

Oldfield, R.G., Hofmann, H.A. (2011). Neuropeptide regulation of social behavior in a monogamous cichlid fish. *Physiology & Behavior*, 102(3-4): 296-303.

Olivereau, M., Moons, L., Olivereau, J., Vandesande, F. (1988). Coexistence of corticotropin-releasing factor-like immunoreactivity and vasotocin in perikarya of the preoptic nucleus in the eel. *General and Comparative Endocrinology*, 70: 41-48.

Panksepp, J., Gandelman, R., Trowill, J. (1970). Modulation of hypothalamic self-stimulation and escape behavior by chlordiazepoxide. *Physiology and Behavior*, 5: 965–969.

Paredes, R.G. (2009). Evaluating the neurobiology of sexual reward. *ILAR Journal* 50: 15–27.

Petrovic, P., Kalisch, R., Singer, T., Dolan, R.J. (2008). Oxytocin attenuates affective evaluations of conditioned faces and amygdala activity. *Journal of Neuroscience*, 28: 6607-6615.

Petrovich, G.D., Risold, P.Y., Swanson, L.W. (1996). Organization of projections from the basomedial nucleus of the amygdala: a PHAL study in the rat. *Journal of Comparative Neurology*, 374: 387–420.

Phillipson, O.T. (1979). Afferent projections to the ventral tegmental area of Tsai and interfascicular nucleus: a horseradish peroxidase study in the rat. *Journal of Comparative Neurology*, 187: 117–143.

Popik, P., Vetulani, J., van Ree, J.M. (1992). Low doses of oxytocin facilitate social recognition in rats. *Psychopharmacology*, 106(1): 71-74

Powers, J.B., Newman, S.W., Bergondy, M.L. (1987). MPOA and BNST lesions in male Syrian hamsters: differential effects on copulatory and chemoinvestigatory behaviors. *Behavioral Brain Research*, 23: 181–195.

RStudio Team (2015). *RStudio: Integrated Development for R*. RStudio, Inc., Boston, MA. http://www.rstudio.com/.

Reddon, A.R., O'Connor, C.M., Marsh-Rollo, S.E., Balshine, S. (2012). Effects of isotocin on social responses in a cooperatively breeding fish. *Animal Behaviour*, 84(4): 753-760.

Reddon, A.R., Voisin, M.R., O'Connor, C.M., Balshine, S. (2014). Isotocin and sociality in the cooperatively breeding cichlid fish, *Neolamprologus pulcher*. *Behaviour*, 151(10): 1389-1411.

Reddon, A.R., O'Connor, C.M., Marsh-Rollo, S.E., Balshine, S., Gozdowska, M., Kulczkowska, E. (2015). Brain nonapeptide levels are related to social status and affiliative behaviour in a cooperatively breeding cichlid fish. *Royal Society Open Science*, 2(2), 140072. doi: 10.1098/rsos.140072

Reddon, A.R., O'Connor, C.M., Nesjan, E., Cameron, J., Hellmann, J.K., Ligocki, I.Y., Marsh-Rollo, S.E., Hamilton, I.M., Wylie, D.R., Hurd, P.L., Balshine, S. (2017). Isotocin neuronal phenotypes differ among social systems in cichlid fishes. *Royal Society Open Science*, 4(5), 170350. doi:10.1098/rsos.170350

Rink, E., Wullimann, M.F. (2001). The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). *Brain Research*, 889: 316–330.

Risold, P.Y., Swanson, L.W. (1997). Connections of the rat lateral septal complex. *Brain Research Reviews*, 24: 115–195.

Risold, P.Y., Thompson, R.H., Swanson, L.W. (1997). The structural organization of connections between hypothalamus and cerebral cortex. *Brain Research Reviews*, 24: 197–254.

Rosene, D.L., Rhodes, K.J. (1990). Cryoprotection and freezing methods to control ice crystal artifact in frozen sections of fixed and unfixed brain tissue. *Methods in Neurosciences*, 3: 360-385.

Ross, H.E., Freeman, S.M., Spiegel, L.L., Ren, X., Terwilliger, E.F., Young, L.J. (2009). Variation in oxytocin receptor density in the nucleus accumbens has differential effects on affiliative behaviors in monogamous and polygamous voles. *Journal of Neuroscience*, 29(5): 1312-1318.

Rubinow, D.R., Schmidt, P.J. (1996). Androgens, brain, and behavior. *American Journal of Psychiatry*, 153(8): 974-984.

Russchen, F.T., Price, J.L. (1984). Amygdalostriatal projections in the rat. Topographical organization and fiber morphology shown using the lectin PHA-L as an anterograde tracer. *Neuroscience Letters*, 47: 15–22.

Salamone, J.D., Wisniecki, A., Carlson, B.B., Correa, M. (2001). Nucleus accumbens dopamine depletions make animals highly sensitive to high fixed ratio requirements but do not impair primary food reinforcement. *Neuroscience*, 105: 863-870.

Salamone, J.D., Correa, M. (2002). Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. *Behavioural Brain Research*, 137(1-2): 3–25.

Salek, S.J., Sullivan, C.V., Godwin, J. (2002). Arginine vasotocin effects on courtship behavior in male white perch (*Morone americana*). *Behavioral Brain Research*, 133: 177-183.

Salvanes, A.G.V., Moberg, O., Ebbesson, L.O.E., Nilsen, T.O., Jensen, K.H., Braithwaite, V.A. (2013). Environmental enrichment promotes neural plasticity and cognitive ability in fish. *Proceedings of the Royal Society B: Biological Sciences*, 280(1767), 20131331. doi:10.1098/rspb.2013.1331

Sánchez-Toscano, F., Sánchez, M.M., Garzón, J. (1991). Changes in the number of dendritic spines in the medial preoptic area during a premature long-term social isolation in rats. *Neuroscience Letters*, 122(1): 1-3.

Santangelo, N., Bass, A.H. (2006). New insights into neuropeptide modulation of aggression: field studies of arginine vasotocin in a territorial tropical damselfish. *Proceedings of the Royal Society of London B: Biological Sciences,* 273(1605): 3085-3092.

Saper, C.B., Swanson, L.W., Cowan, W.M. (1976). The efferent connections of the ventromedial nucleus of the hypothalamus of the rat. *Journal of Comparative Neurology,* 169: 409–442.

Saverino, C., Gerlai, R. (2008). The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. *Behavioural Brain Research,* 191: 77-87.

Satou, M., Oka, Y., Kusunoki, M., Matsushima, T., Kato, M., Fujita, I., Ueda, K. (1984). Telencephalic and preoptic areas integrate sexual behavior in hime salmon (landlocked red salmon, *Oncorhynchus nerka*): results of electrical brain stimulation experiments. *Physiology and Behavior*, 33: 441–447.

Scheldeman, C., Mills, J.D., Sierkierska, A., Serra, I., Copmans, D., Iyer, A.M., Whalley, B.J., Maes, J., Jansen, A.C., Lagae, L., Aronica, E., de Witte, P.A.M. (2017). mTOR-related neuropathology in mutant tsc2 zebrafish: Phenotypic, transcriptomic and pharmacological analysis. *Neurobiology of Disease*, 108: 225-237.

Schultz, L.E., Solin, S.L., Wierson, W.A., Lovan, J.M., Syrkin-Nikolau, J., Lincow, D.E., Severin, A.J., Sakaguchi, D.S., McGrail, M. (2017). Vascular Endothelial Growth Factor A and Leptin Expression Associated with Ectopic Proliferation and Retinal Dysplasia in Zebrafish Optic Pathway Tumors. *Zebrafish*, 14(4): 343-356.

Schulz, G.M., Varga, M., Jeffries, K., Ludlow, C.L., Braun, A.R. (2005). Functional neuroanatomy of human vocalization: an H215O PET study. *Cerebral Cortex*¸ 15: 1835–1847.

Semsar, K., Godwin, J. (2004). Multiple mechanisms of phenotype development in the bluehead wrasse. *Hormones and Behavior,* 45: 345-353.

Shaikh, M.B., Brutus, M., Siegel, H.E., Siegel, A. (1986). Regulation of feline aggression by the bed nucleus of stria terminalis. *Brain Research Bulletin*, 16: 179–182.

Sheehan, T., Paul, M., Amaral, E., Numan, M.J., Numan, M. (2001). Evidence that the medial amygdala projects to the anterior/ventromedial hypothalamic nuclei to inhibit maternal behavior in rats. *Neuroscience*, 106: 341–356.

Shin, J., Padmanabhan, A., de Groh, E.D., Lee, J.S., Haidar, S., Dahlberg, S., Guo, F., He, S., Wolman, M.A., Granato, M., Lawson, N.D., Wolfe, S.A., Kim, S.H., Solnica-Krezel, L., Kanki, J.P., Ligon, K.L., Epstein, J.A., Look, A.T. (2012). Zebrafish neurofibromatosis type 1 genes have redundant functions in tumorigenesis and embryonic development. *Disease Models & Mechanisms*, 5(6): 881-894.

Sirinathsinghji, D.J., Whittington, P.E., Audsley, A.R. (1986). Regulation of mating behaviour in the female rat by gonadotropin-releasing hormone in the ventral tegmental area: effects of selective destruction of the A10 dopamine neurones. *Brain Research*, 374: 167–173.

Smith, K.S., Tindell, A.J., Aldridge, J.W., Berridge, K.C. (2009). Ventral pallidum roles in reward and motivation. *Behavioral Brain Research,* 196: 155–167.

Sokołowska, E., Kleszczyńska, A., Nietrzeba, M., Kulczykowska, E. (2015). Annual changes in brain concentration of arginine vasotocin and isotocin correspond with phases of reproductive cycle in round goby, *Neogobius melanostomus*. *Chronobiology International*, 32(7): 917-924.

Sotres-Bayón, F., Torres-López, E., López-Avila, A., del Angel, R., Pellicer, F. (2001). Lesion and electrical stimulation of the ventral tegmental area modify persistent nociceptive behavior in the rat. *Brain Research*, 898: 342–349.

Spanagel, R., Weiss, F. (1999). The dopamine hypothesis of reward: past and current status. *Trends in Neuroscience*, 22: 521–527.

Staiger, J.F., Nürnberger, F. (1989). Pattern of afferents to the lateral septum in the guinea pig. *Cell Tissue Research*, 257: 471–490.

Swanson, L.W., Cowan, W.M. (1977). An autoradiographic study of the organization of the efferent connections of the hippocampal formation in the rat. *Journal of Comparative Neurology*, 172: 49–84.

Swanson, L.W., Cowan, W.M. (1979). The connections of the septal region in the rat. *Journal of Comparative Neurology*, 186: 621–655.

Swanson, L.W. (1982). The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Research Bulletin*, 9: 321–353.

Swanson, L.W., Petrovich, G.D. (1998). What is the amygdala? *Trends in Neuroscience*, 21: 323–331.

Thompson, R.R., Walton, J.C. (2004). Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). *Behavioral Neuroscience*, 118: 620-626.

Thompson, R.R., Walton, J.C. (2009). Vasotocin immunoreactivity in goldfish brains: characterizing primitive circuits associated with social regulation. *Brain, Behavior and Evolution*, 73: 153-164.

Turner, B.H., Herkenham, M. (1991). Thalamoamygdaloid projections in the rat: a test of the amygdala's role in sensory processing. *Journal of Comparative Neurology*, 313: 295–325.

Urano, A., Ando, H. (2011). Diversity of the hypothalamo-neurohypophysial system and its hormonal genes. *General and Comparative Endocrinology*, 170: 41-56.

Valcourt, R.J., Sachs, B.D. (1979). Penile reflexes and copulatory behavior in male rats following lesions in the bed nucleus of the stria terminalis. *Brain Research Bulletin*, 4: 131–133.

Van den Dungen, H.M., Buijs, R.M., Pool, C.W., Terlou, M. (1982). The distribution of vasotocin and isotocin in the brain of the rainbow trout. *Journal of Comparative Neurology*, 212: 146-157.

van Praag, H., Kempermann, G., Gage, F.H. (2000). Neural consequences of environmental enrichment. *Nature Reviews Neuroscience*, 1(3): 191–198.

Viau, V., Meaney, M.J. (1996). The inhibitory effect of testosterone on hypothalamic-pituitary-adrenal responses to stress is mediated by the medial preoptic area. *Journal of Neuroscience*, 16(5): 1866-1876.

Walton, J.C., Waxman, B., Hoffbuhr, K., Kennedy, M., Beth, E., Scangos, J., Thompson, R.R. (2010). Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic. *Neuropharmacology*, 58: 126-134.

Wang, Z., Liu, Y., Young, L.J., Insel, T.R. (2000). Hypothalamic vasopressin gene expression increases in both males and females postpartum in a biparental rodent. *Journal of Neuroendocrinology*, 12: 111-120.

Wickens, J.R., Budd, C.S., Hyland, B.I., Arbuthnott, G.W. (2007). Striatal contributions to reward and decision making: making sense of regional variations in a reiterated processing matrix. *Annals of the New York Academy of Sciences,* 1104: 192–212.

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer-Verlag, New York.

Williams, J.R., Carter, C.S., Insel, T. (1992a). Partner preference development in female prairie voles is facilitated by mating or the central infusion of oxytocin. *Annals of the New York Academy of Sciences*, 652(1): 487–489.

Williams, J.R., Catania, K.C., Carter, C.S. (1992b). Development of partner preferences in female prairie voles (*Microtus ochrogaster*): the role of social and sexual experience. *Hormones and Behavior,* 26: 339-349.

Williams, J.R., Insel, T.R., Harbaugh, C.R., Carter, C.S. (1994). Oxytocin administered centrally facilitates formation of a partner preference in female prairie voles (*Microtus ochrogaster*). *Journal of Neuroendocrinology,* 6: 247-250.

Winslow, J., Hastings, N., Carter, C., Harbaugh, C., Insel, T. (1993a). A role for central vasopressin in pair bonding in monogamous prairie voles. *Nature*, 365: 545-548.

Winslow, J.T., Shapiro, L., Carter, C.S., Insel, T.R. (1993b). Oxytocin and complex social behavior: species comparisons. *Psychopharmacology Bulletin*, 29: 409-414.

Wright, C.I., Beijer, A.V., Groenewegen, H.J. (1996). Basal amygdaloid complex afferents to the rat nucleus accumbens are compartmentally organized. *Journal of Neuroscience*, 16: 1877–1893.

Witt, D., Winslow, J., Insel, T. (1992). Enhanced social interactions in rats following chronic, centrally infused oxytocin. *Pharmacology Biochemistry and Behavior*, 43: 855-861.

Wong, C.J. (2000). Electrical stimulation of the preoptic area in Eigenmannia: evoked interruptions in the electric organ discharge. *Journal of Comparative Physiology A,* 186: 81–93.

Wullimann, M.F., Rupp, B., Reichert, H. (1996). *Neuroanatomy of the zebrafish brain: A topological atlas*, Basel: Birkhäuser Verlag.

Young, L. (1999). Oxytocin and vasopressin receptors and species-typical social behaviors. *Hormones and Behavior*, 36: 212-221.

Young, L.J., Nilsen, R., Waymire, K.G., MacGregor, G.R., Insel, T.R. (1999a). Increased affiliative response to vasopressin in mice expressing the V1a receptor from a monogamous vole. *Nature*, 400: 766-768.

Young, L., Toloczko, D., Insel, T. (1999b). Localization of vasopressin (V1a) receptor binding and mRNA in the rhesus monkey brain. *Journal of Neuroendocrinology*, 11: 291-297.

Young, L.J., Lim, M.M., Gingrich, B., Insel, T.R. (2001). Cellular mechanisms of social attachment. *Hormones and Behaviour*, 40(2): 133-138.

Zingg, H.H., Laporte, S.A. (2003). The oxytocin receptor. *Trends in Endocrinology and Metabolism*, 14: 222-227.