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SOCIAL MODULATION OF NEURONAL COMPLEXITY IN ZEBRAFISH

VOLUME 1

Dissertation to obtain the Master degree in Biomedical Research, in the area of Neurobiology, under the supervision of Doctor Magda Cristina Cafum Teles Saturnino and co-supervision of Doctor Ana Paula Pereira da Silva Martins and presented to the Faculty of Medicine of the University of Coimbra.

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Social modulation of neuronal complexity in zebrafish

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Dissertation presented to the Faculty of Medicine of the University of Coimbra. The work was performed in the Rui Oliveira Lab of the Gulbenkian Science Institute, under the scientific supervision of Doctor Magda Cristina Cafum Teles Saturnino and co-supervision of Doctor Ana Paula Pereira da Silva Martins.

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ABREVIATION LIST

A

AVP Vasopressin

B

Bs Brainstem

C

Ce Cerebellum
CNS Central Nervous System

D

DAPI 4',6-diamidino-2-phenylindole
Di Diencephalon
DNA Deoxyribonucleic acid
dpf days post-fecundation

L

LS Large and Stable experimental group
LU Large and Unstable experimental Group

M

MAP-2 Microtubule Associated Protein 2

O

OB Olfactory Bulb
OXT Oxytocin
OXTR Oxytocin Receptors

P

PBS Phosphate Buffered Saline

R

RT Room Temperature

S

SDM Social Decision-Making
SEM Standard Error of the Mean
SS Small and Stable experimental group
SU Small and Unstable experimental group

T

Tel Telencephalon
TeO Optic Tectum

ABSTRACT

The social environment is the most complex external pressure that animals face since it includes interactions with other individuals, which have unpredictable outcomes. In response to variation in the social environment, individuals can alter their behavioural phenotypes, a phenomenon known as phenotypic plasticity, which is normally based on neural plasticity. Neural plasticity is the ability of the brain to reorganize its neural connectivity in response to environmental changes and might include changes in morphology, neurophysiological functions, as well as modifications in the neural networks. The main goal of this work was to assess the impact of environmental complexity in the dendritic arborization and the size of different brain regions in zebrafish reared in distinct social environments. To accomplish this objective the complexity of the social environment was induced through variation in group size (small vs. large) and group stability (stable vs. unstable) leading to four experimental treatments (SS, SU, LS, LU). The quantification of dendrites was performed in five different brain areas - Telencephalon, Diencephalon, Optic tectum, Cerebellum, and Brainstem -, using the microtubule-associated protein 2 has a dendritic marker. We found differences in brain size and dendritic density related to the complexity of the social environment, such that animals raised in less complex social environments (small and stable shoals) present a decrease in their brain area and dendritic density, which was paralleled by changes in their social behaviour. Our results indicate the relevance of the social environment in the modulation of neuronal complexity during development, which is paralleled by changes in behavioural performance. This modulation was also area-specific pointing to regional differentiation.

Keywords: Social environment; Adaptive neuroplasticity; Dendritic arborization; Behavior biology; Zebrafish.

RESUMO

O ambiente social é a pressão externa mais complexa que os animais enfrentam, uma vez que inclui interações com outros indivíduos, que produzem respostas comportamentais imprevisíveis. Em resposta à variação do ambiente social, os indivíduos podem alterar os seus fenótipos comportamentais, um fenómeno conhecido como plasticidade fenotípica, que se baseia normalmente na plasticidade neural. A plasticidade neural é a capacidade que o cérebro tem de reorganizar a sua conectividade neural em resposta às mudanças ambientais e isto pode incluir mudanças na morfologia, nas funções neurofisiológicas, assim como modificações nas redes neuronais. O principal objetivo deste trabalho é avaliar o impacto da complexidade ambiental na arborização dendrítica e no tamanho das diferentes regiões do cérebro em peixe-zebra que cresceram em ambientes sociais distintos. Para alcançar este objetivo, a complexidade do ambiente social foi induzida através da variação do tamanho do grupo (pequeno vs. grande) e da estabilidade do grupo (estável vs. instável) levando a quatro tratamentos experimentais (pequeno estável, pequeno instável, grande estável, grande instável). A quantificação das dendrites foi realizada em cinco áreas cerebrais diferentes - Telencéfalo, Diencéfalo, Tecto ótico, Cerebelo e Tronco cerebral - utilizando a proteína 2 associada a microtúbulos, que se define como um marcador dendrítico. Encontrámos diferenças no tamanho e densidade dendrítica do cérebro relacionadas com a complexidade do ambiente social, de tal forma que os animais que cresceram em ambientes sociais menos complexos (cardumes pequenos e estáveis) apresentam uma diminuição na sua área cerebral e densidade dendrítica, que é acompanhada por alterações no comportamento social. Os nossos resultados indicam a relevância do ambiente social na modulação da complexidade neuronal durante o desenvolvimento, que é coincidente com as alterações a nível do comportamento. Esta modulação é induzida de forma diferente dependendo das área do cérebro, indicando uma diferenciação regional.

Palavras-chave: Ambiente social; Plasticidade neuronal adaptativa; Arborização dendrítica; Biologia comportamental; Peixe-zebra.

CHAPTER 1:
GENERAL INTRODUCTION

1.1. The Evolution of Social Behaviour

The social environment is one of the most complex external pressures that animals face since it includes interactions with other individuals, and unpredictable outcomes. Without the ability to recall previous experiences and adapt the behavioural responses to the various social contexts, organisms will be unable to respond appropriately, and this would have consequences for their survival, making social behaviour a component of high adaptability [1;2;3].

In order to adjust their behavioural responses, animals must develop cognitive mechanisms to assess the internal state of others and adequately respond to the social complexity[1]. Social competency is defined as the capacity to optimise social connections while considering all social information in the environment, enabling animals to avoid or reduce the costs of risky social interaction. Therefore, it is expected that animals have the capacity to control the expression of social behaviour so that they can adapt their behavioural output to a particular situations in a complex and changing social world[1;2;3;4].

Because social competence allows animals to traverse the complexities of their social environment efficiently in order to survive, breed, and raise their young, it should be considered a significant predictor of their individual Darwinian fitness[1]. An individual's capacity to identify conspecific or other suitable mating and shoaling partners, as well as antipredator behaviour, migration, foraging, and mate choice, are influenced by social experiences[5;6].

At the evolutionary scale, the scientific community has long linked the evolution of the brain to changes in their ecology, which has been refuted, since the maintenance of a large brain is incredibly high cost, and primates and humans have much larger brains when compared to other species. In the late 1980s, a different hypothesis, the Social Brain Hypothesis, was proposed, defining brain and cognitive evolution as a result of the social characteristics and complexity of different environments, making social plasticity an adaptive trait that can be under positive selection when changes in the environment outpace the rate of genetic evolutionary change[7].

To be able to modify their social behaviour and interactions, animals must identify and respond to reliable social information, to extract key characteristics of the social environment, and integrate multiple sensory inputs[2;6]. Moreover, social knowledge can be obtained by interacting with others or by witnessing other behavioural agents (i.e., social learning)[2;3;8]. According to cognitive appraisal theory, the response to a stimulus depends on the meaning of the perceptual information to the organism at that specific time rather than just a result of the direct effects of the perceptual information alone[2;9]. Social environments can provide the opportunity to use knowledge generated by others to spot opportunities and challenges that

will trigger social plasticity, define as the capacity of animals to modify their social behaviour[2]. The cognitive system affects the behavioural performance of the animals by filtering the information from the environment, limiting what an animal can do, and controlling what it actually decides to do—the decision-making process—in face of its current motivation and environmental conditions. As a result, social learning abilities should have evolved in group living animals [6].

Social plasticity relies on different mechanisms and premises: (1) animals must continually be aware of their surroundings and gather pertinent social cues from encounters with other animals or from accessible sources of information to react correctly to social contextual changes, (2) Depending on the duration of exposure to relevant social signals, social stimuli cause neural activity-dependent mechanisms at the molecular and cellular levels, that leads to alter neurogenomic states, and (3) Different types of neuronal plasticity can be induced by hormones (such as sex steroids and glucocorticoids) and neuromodulators (such as neuropeptides and amines), enabling brain-body reactions to change in the social environment and life stage transitions[2].

Social plasticity results in behavioural flexibility, defined as variable, reversible, non-cyclical alterations in the individual's behaviour, giving room for potential modifications in the organism's physiology and morphology[2;3;9]. Because behavioural traits are more plastic than morphological and physiological ones, they can vary throughout life, and they can also trigger changes in other traits, making them a potent method for adaptive environmental changes. Instead of genetically fixed rules governing fixed responses, these enable the same genotype to produce distinct behavioural phenotypes[1;8]. Typically, an animal behaviour in reaction to social releasers varies depending on its social status, whether other conspecifics are present or not, and the environment in which the behaviour is being displayed[2;3;10]. In summary, behavioural plasticity, which underlies social competence, enables animals to adapt their behavioural responses to the perceived social environment, which can lead to short-term (i.e., behavioural flexibility) or long-term (i.e., behavioural consistency) changes in social behaviour, through different mechanisms of social plasticity[2].

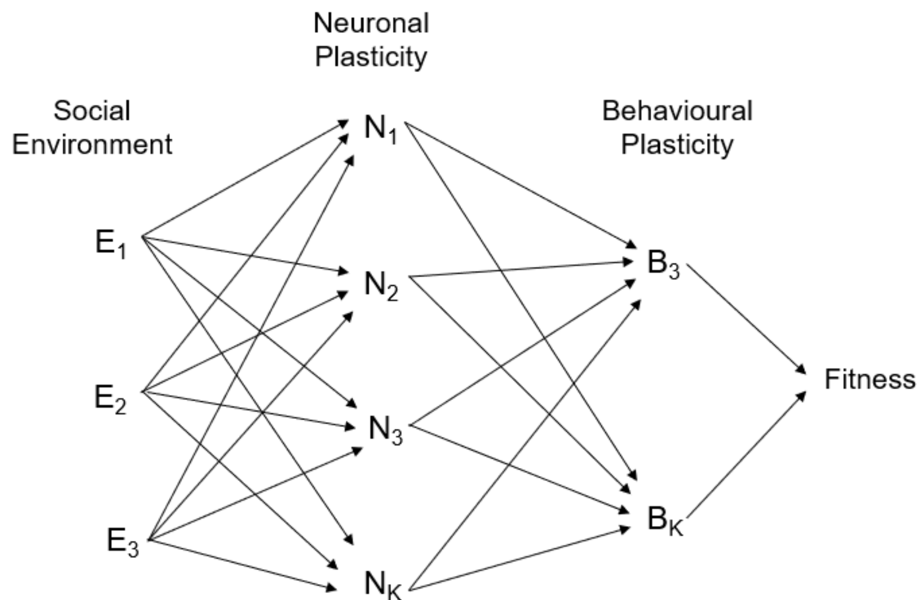


Figure 1| Impact of the social environment on individual's fitness. Representative diagram of the interaction between social environment, neuronal network, and behaviour. Changes in the social environment can induce changes in the neuronal structures like dendrites, that results in changes in the behaviour, which has an impact in the individual fitness. This way individuals with the same genotype can produce different behavioural phenotypes. Adaptation of the figure present in the article[3].

1.1.1. Phenotypic Plasticity

Since the environment in which the animal lives impose constraints, provides opportunities, and gives individual actions meanings, the environment plays a central role in the behaviour expressed by the animal. Thus, the nervous system, the body, and the environment, are all seen as a dynamic system always interacting with one another. Every living system's evolution and natural selection have traditionally relied on genetic variation to generate heritable phenotypic differences, however, when environmental changes occur too quickly for genetics to keep up, living systems must adapt fast[11]. This circumstance favours the evolution of phenotypic plasticity, where the same genotype can create different phenotypes based on the environmental cues perceived by the organism[12]. Hence, phenotypic plasticity can evolve within limits imposed by costs and constraints to plasticity. Therefore, social environmental changes may indirectly or passively result in physiological changes. Three of the mechanisms underlying phenotypic plasticity are developmental plasticity, alterations in the early life environment can have an impact on adults' behaviour, neuroplasticity, a small-scale variation in ecological complexity that may affect brain plasticity and cognitive performance, and behavioural plasticity, that is followed by neuronal changes in specific brain's areas [2].

1.1.2. Developmental Plasticity

An animal's early social environment may have a profound impact on its behaviour. Animals' ability to adapt their phenotype to the environment they were exposed to in early life is known as developmental plasticity. A wide variety of phenotypic features, including social behaviour, cognition, and life-history variables including dispersion choices, survival, and reproductive effort, can be influenced by the early social environment. During early development, animals are sensitive to environmental information, which may be used to reduce uncertainty about the current and expected future environmental conditions, and to fine-tune phenotypic development accordingly. For instance, animals raised without parental guidance may exhibit significant emotional dysregulation, poor social interaction skills, and a diminished capacity for social learning[13]. The early social environment can cause long-lasting modulations of brain gene expression, so in these early phases, significant behavioural variability is found [13;14;15]. Taborsky *et al.*[16] looked into how early social experiences may impact social skills. They investigated whether *Neolamprologus pulcher*, a cooperatively breeding cichlid, raised with older brood-caring conspecifics consistently outperformed individuals raised with older brood-caring conspecifics in a series of tasks including (1) simulating various social contexts, (2) assigning individuals various social roles, and exposing them to an unpredictable social situation. When aggressively competing with peers for a resource or when attempting to be accepted as a subordinate group member and potential brood care helper by an unfamiliar dominant pair, a situation they had never encountered before, fish that had been reared together with older conspecifics showed more appropriate behaviours both as winners (more aggressive displays) and as losers (more submissive displays). So, this study shows that the social environment experienced during the first 2 months of life had a long-term effect on the social behaviour of *N. pulcher* that had been raised either with or without older conspecifics. These findings imply that *N. pulcher* raised with older conspecifics were more socially competent than fishes raised without adults, which has also been confirmed by the outcome of social challenges for in a different context. These results were also validated for juvenile indicating the key role of the early social environment on social behaviour.

1.1.3. Neuronal Plasticity

Neuroplasticity is one of the mechanisms of phenotypic plasticity. It is defined as the brain's ability to reorganise neural connectivity in response to environmental change, while at the same time, deprivation or perturbation of some experiences can also impact the development of functions related to the nature of the deprivation. Although adult neurogenesis, the process

by which new neurons are produced, is frequently emphasised as one of the main mechanisms underlying structural reorganisation, it is important to recognise that neuroplasticity manifests itself in a variety of ways, including altered morphology (e.g., increased dendritic spines, modified dendritic branching), altered neurophysiological functions like long-term potentiation, and modified neural networks[11;17].

The brain networks that underlie social behaviour must have the capacity for neural plasticity such that, the same inputs to the network can result in a variety of different outputs depending on the animal's motivational state and previous experiences[1;3]. The structural remodelling of brain circuits, and the biochemical switching of neural networks are two of the primary neural mechanisms that have been postulated to mediate these changes in behaviour[18]. When motivational changes are gradual and long-lasting, structural rewiring of brain networks are expected, while in structural reorganisation, various structural modifications may occur. This can involve changing the connectivity between various network components (synaptic plasticity), adding new cells (neurogenesis), removing old cells (apoptosis), or altering the molecular components of the circuit to change how responsive the circuits are (e.g., differential expression of receptors)[2].

Adult neurogenesis has now been described in most vertebrate taxa, from fish to mammals, and it is thought that structural changes in behaviour can be achieved by either producing and incorporating new neurons into circuits or by producing new glial cells that regulate the neural environment in which the circuit operates[3].

An endless variety of neuronal states with corresponding behavioural states can be created by different combinations of activation and variation in the strength of connections of the nodes in the neural network behind social behaviour. Therefore, at the molecular level, neuronal plasticity depends on the social regulation of gene expression, so that different neurogenomic states correspond to different behavioural states and the switches between states are orchestrated by signalling pathways that interface the social environment and the genotype. Consequently, epigenetic markers have a critical role in maintaining behavioural states by preserving various neurogenomic states in the brain. These epigenetic changes are responsible for controlling functional and structural molecular states, thus enabling adaptive cellular expression patterns during development and differentiation, or plastic changes in adult organisms[8]. Thus, extremely different degrees of performance may result from the same neuronal parameters[3].

In summary, the brain networks that control social behaviours can be altered in order to adjusts behaviour output to the perceived social environment. Different types of neural plasticity can be engaged in the various nodes of the social behavioural network depending on the duration of exposure to relevant social signals, leading to temporary or permanent

changes in social behaviour that will ultimately improve the animal's fitness[2;13]. Social environment has already been reported as a major selective force that can induce structure modifications in the brain network, specifically in dendrite arborization and dendritic spines[19;20]. For example, Giuseppa *et al* [21] examined the dendritic arborization and spine density in a parietal cortex region of the brain that is primarily engaged in spatial learning in order to analyse the behavioural impacts of environmental complexity on several mechanisms of spatial function in rats. To achieve that, wistar rat pups were contained in either standard conditions, which correspond to two animals in a regular cage with no items, or enriched environments, which were generated by having 10 animals in a big cage with toys and a running wheel. After 3 months, morphological analyses on neurons of the parietal cortex were performed. As a result, the parietal cortex of the animals that were submitted to the enrichment environment presented a higher number of intersections, nodes, and spines of the apical and basal dendrites, furthermore, the basal dendrites exhibited a higher length, when compared to the parietal cortex of the animals that were under standard conditions. In parallel to these morphological changes, the rats from the most complex environment performed better in cognitive tasks. These results point for the importance of the environmental complexity in increasing dendritic arborisation as well as dendritic spine density in layer-III parietal pyramidal neurons.

1.1.3.1. Dendritic Arborization and Changes in the Dendritic Structure

Adjustments to existing circuits or the creation of new circuits are more likely to be reflected in plastic changes occurring in the neural circuits[22]. To investigated for this plastic changes in neural networks the connections between neurons, or synapses, was studied because neural networks are made up of individual neurons, an axon arborizations linked to another subset of other neurons to interconnect the networks[22;23]. The primary source where information enters neurons is through their dendrites, and various types of neurons have unique dendrite branching patterns[24]. Up to 95% of the receptor surface with which neurons interact is specifically made up of dendrites. In response to a variety of factors, including neuronal activity, the dendrites expand and contract, as a result, dendrites are one of the most sensitive markers of change in the central neuronal system[25].

Dendritic arbour form is one of the key parameters affecting how signals from individual synapses are integrated. A well-developed dendritic arbour is then established as a result of dynamic dendritic branching, neuronal activity, and synaptogenesis[24]. The following physiological conditions must be met by dendrites to guarantee optimal neuronal function; (1) the region that includes a neuron's sensory and/or synaptic inputs must first be covered by the neuron's dendrites, (2) The branching structure and dendritic density of the dendritic field must

also be appropriate for sampling and processing the signals that converge onto it, and (3) Dendrites must be adaptable to change as they develop and in response to experience, which means that the adult nervous system still retain cellular flexibility[24;25;26].

Reviewing, assumes that changes in the dendritic surface reflect changes in synaptic structure since there is an approximately linear relationship between the number of synapses and the area available for them (dendritic surface). Several researchers have demonstrated that placing animals in complex or simple environments results in significant changes in the number of synapses in particular brain areas[22].

By counting the number of connections per neuron in the brain of animals living in enriched environments, Turner and Greenough[27;28] explicitly tested this hypothesis that the number of synapses is closely associated with dendritic space. In the brains of enriched versus cage-reared rats, they discovered an increase in the number of synapses per neuron of roughly 20%. Therefore, even if the quantity of synapses in a segment of cortical tissue is almost the same, enriched cage-reared animals have higher dendritic space, which leads to more synapses per neuron. Kolb, Gibb, and Gorny[29] discovered that neurons in the motor and sensory cortical areas of adult and elderly animals kept in a complex environment had longer dendrites and a higher density of synapses relatively to a standard lab cage. They also reported that identical environmental changes had qualitatively different consequences on how neural circuitry were organised in juveniles and adults, since that under the same environmental conditions juveniles presented a higher dendritic length, but a lower spine density in comparison to adults.

1.2. Zebrafish

1.2.1. Historic Overview

The zebrafish was originally mentioned in a publication in the Web of Science in 1934[30], but it wasn't until the 1980s, when the developmental biologist George Streisinger chose it as a genetic model system, that the zebrafish was paid attention[31;32]. Streisinger was one of the key figures that bring about the contemporary era of molecular genetics[33;34]. His goal was to use molecular genetics to study vertebrate neural development at the level of individual cells and molecules[35]. Kimmel, also a pioneer in the use of zebrafish as a model organism, published detailed descriptions of cell differentiation and the nervous system organisation[36] .

In the following forty years, zebrafish gained preference among embryologists who appreciated its features, and became popular in the developmental biology field[31;32;34;36]. As a result, several genetic techniques tailored exclusively for zebrafish were developed, and among them the zebrafish genome project, based at the Sanger Institute in Cambridge, making the zebrafish genome available for the community[31;32]. Many milestones have been reached since the introduction of zebrafish into the laboratory settings that firmly establish him as a prominent genetic model organism for biology[34;35;36].

Apart from genetics, cell biology and anatomical research revealed the segmental organisation of the brain, the significance of cell lineage in the development of various neuronal subgroups, and that zebrafish and mammalian species share a syntenic connection[34;35]. The zebrafish emerged as one of the three main research species for geneticists by the beginning of the twenty-first century, alongside with the house mouse and the fruit fly. With the advent of contemporary zebrafish recombinant DNA techniques, other fields of biology, rather than embryology, have begun to pay attention to this small fish[33].

The zebrafish suddenly raised up to the top of the biomedical research, since the genome sequencing, and the interest in using this fish as a model organism for neuroscience has grown[33;34;37]. Nowadays, practically every subfield of biology has been represented in zebrafish research, including neurophysiology, medical research, ethology and behavioural neuroscience[31;32;33]. Being frequently used in over 400 labs worldwide for basic and applied research. There is also a growing interest in using zebrafish as a model organism to study the genetic basis of behaviour[36].

1.2.2. Zebrafish as a Model Organism in Neuroscience

In neuroscience research, zebrafish (*Danio rerio*) are quickly taking over as one of the most significant animals for examining the mechanisms underlying brain morphology and function[38]. Zebrafish is a model organism of choice for studying the development and function of neural circuitry and neuroanatomy because of the striking resemblance between the genetic blueprint for the nervous system of zebrafish and that of mammals[39]. Both larvae and adult zebrafish are widely used in central nervous system (CNS) research, even though they lose the transparency present in the first stages of development, and zebrafish is a newcomer in behavioural neuroscience [38;39].

Using this species as a model organism has many advantages, including high physiological and genetic homology to mammals, small and compact neuronal network, small adult brain size, external fertilisation, rapid development, transparency of the embryo and larvae, ease of genetic and other experimental manipulations, and cost- and space-effectiveness. Zebrafish also have methodological advantages for behavioural phenomics and brain imaging, in addition, to the detailed circuit analysis that is not limited to simple systems but can also be applied to higher-order brain areas. A range of experimental techniques can be used on the zebrafish brain to conduct rapid, low-cost, and high-resolution neuromorphology studies. Imaging this brain with a confocal microscope can generate high-resolution images, clearly revealing the anatomical relationships between zebrafish and other mammalian neuronal systems[38;39;40].

Zebrafish is a perfect organism for studies on neurophenotyping and brain imaging because they have "evolutionarily conserved" neuromodulator systems, brain architecture and neuropeptides with high similarity to other mammals. This model organism has great potential to be used in neurobiology, to understand the neural circuits underlying typical behaviours, in addition to the conservation of networks governing fundamental vertebrate physiological features[39;40].

Also, the advent of dependable video tracking tools greatly facilitates zebrafish neurobehavioral research. Since zebrafish prefer to swim in shoals and it is simple to measure how environmental factors affect this tendency to form groups, it is based on these species' extensive social behaviours that zebrafish are used to address social deficits. The neuroscience community has grown interested in using zebrafish to study increasingly sophisticated and complex behaviours like social interactions, learning, and memory in addition to visual and locomotor behaviours. There is no doubt that the zebrafish nervous system lacks some of the characteristics of a mammalian CNS and associated behavioural output, like the laminated cerebral cortex of mammals. Nevertheless, this model achieves the perfect balance between system complexity, practical simplicity and evolutionary conservation

that is necessary to establish the fundamental neuronal properties and core mechanisms that underlie behaviour in mammals, representing a good reductionist approach[38;39;41;42].

1.2.3. Zebrafish Social Development

Adult zebrafish are robustly social animals, whereas larvae are not, and the overt shoaling and schooling behaviours that are visible in adult zebrafish are not present in larval stages. While most 3-week-old zebrafish significantly prefer to stay in a compartment where they may observe conspecifics, 1-week-old zebrafish do not exhibit any apparent social preference[44].

Social preference is dependent on vision and requires the willingness to approach other conspecifics. Additionally, throughout 1-3 weeks, larval zebrafish develop an elementary social interaction known as a coordinated movement. Many other mammals and non-mammalian vertebrates share the propensity to observe and imitate conspecifics as one of their early social behaviours [44].

Zebrafish (*Danio rerio*) are highly sociable creatures and exhibit a wide range of non-reproductive social behaviours. Zebrafish embryos (between 0 and 5 days post-fecundation (dpf)) already have some basic sensory and locomotor functions. Larvae of 5 dpf forage for food and flee from predators. Then, between 10 and 16 dpf, zebrafish display progressively more sophisticated social behaviours, including location choice, orientation, and social cueing. It is suggested that attraction to conspecifics starts as early as 7 dpf, and relationships quickly become more complex. At 12 dpf, the first social behaviour is a preference for conspecifics in space, then, at 14 dpf, orientation towards others starts to appear, and it gets more precise with time, finally, by 16 dpf, they are responding faster to social cues. Altogether, these point out for the fast and successive acquisition of more complex social behaviours [14].

In the second and third weeks of life, brain systems begin to develop, and this is when social preference appears. The larvae already exhibit this visually mediated coupling of movement at one week of age, but it becomes significantly stronger during the subsequent weeks. For instance, it is known that some brain areas undergo extensive growth during this period, but social behaviour encompasses more than a preference to be near conspecifics. Individuals may coordinate their behaviour with other members of their social group, and this kind of coordination is clearly shown in schooling fish, where individuals coordinate their body orientation and time their movements, a trait that social mammals also exhibit. Social behaviours become strong by the 3 weeks of life when visual stimulation is adequate to promote social behaviour[44]. Additionally, the preference for shoaling is also influenced by early environmental factors, however, shoal-mates are not visually preferred until later, juvenile stages, and once this choice has been formed, it seems to be unchangeable [43].

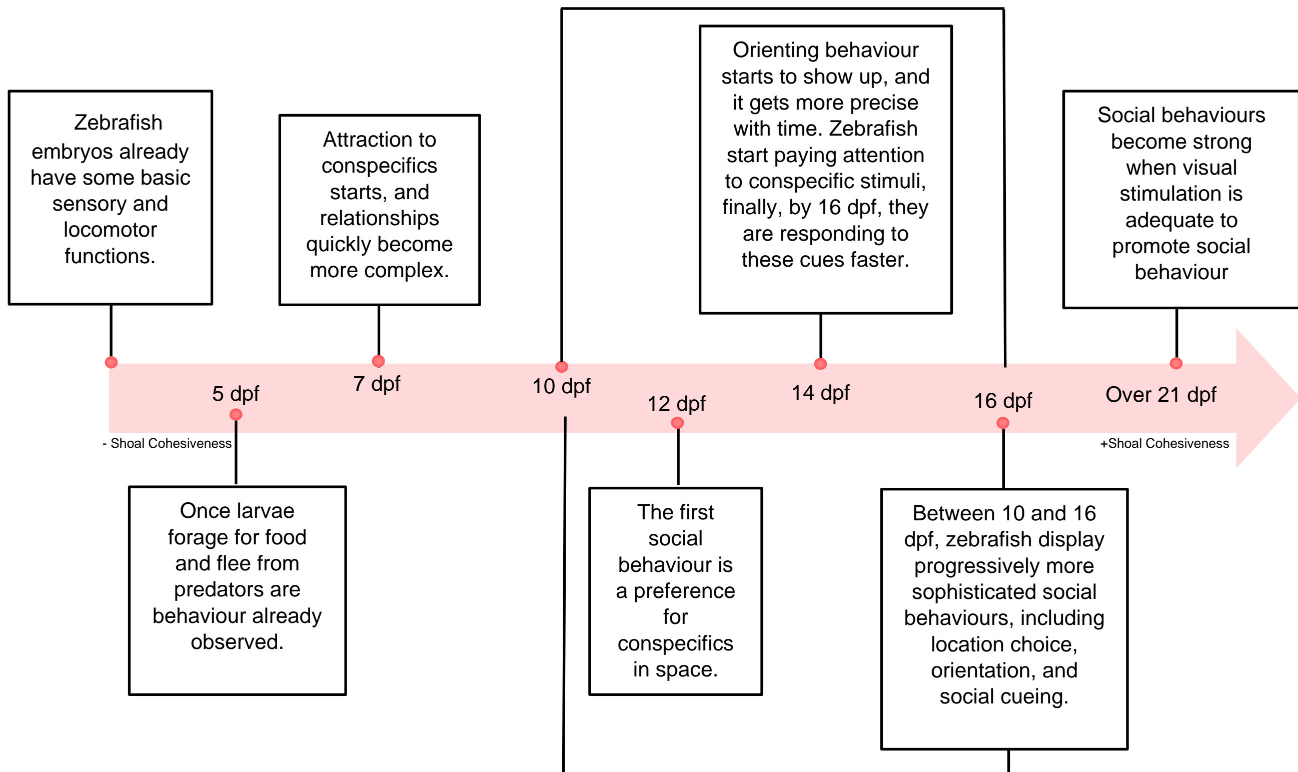


Figure 2| Social development of zebrafish. Zebrafish is a highly social animal.

1.2.4. Zebrafish's Brain

The mammalian brain develops via a process called evagination, and thus, for example, dorsal structures that are in the midline of the neural tube dive deeper inside as the mammalian brain develops, whereas the fish brain develops via eversion. Therefore, in the fish these structures remain on the surface and "slide down" as the brain grows, moving away from the dorsal part and ending up on the side or more ventrally while remaining on the surface[31;45]. Although the morphology of the brain is considerably different, major nuclei/brain regions (i.e., hippocampus, amygdala, locus coeruleus), neurotransmitters (glycine, gamma-aminobutyric acid, glutamate, monoamines: dopamine, acetylcholine, norepinephrin, epinephrin, serotonin, melatonin), neuropeptides (e.g., melanin concentrating hormone receptor 2), and the expression of immediate early genes (e.g., cFos, Jun, krox 24, brain-derived neurotrophic factor), are conserved. Brain neurochemistry is highly conserved across vertebrate species. As referred above, zebrafish have all major neuromediator systems, including transmitters, their receptors, transporters, and enzymes of synthesis and metabolism, similar to those observed in humans and rodents. The endocrine responses in zebrafish are generally homologous to those established in mammals and its neuroendocrine system is well-developed[39;45].

Despite the fact that the mammalian hippocampus is a complex structure, some neuroanatomists show that the fish brain has a homologous structure with the mammalian hippocampus, and that the cortex is present in the telencephalon and the diencephalon of zebrafish. The amygdala and dorsal hippocampus, which work together to produce a representation of context and track emotional valence, are crucial for the development of contextual memory in animals are also present in fish[46].

The zebrafish brain can be divided into six macroareas: Olfactory bulbs (OB) the telencephalon (Tel), diencephalon (Di), optic tectum (TeO), cerebellum (Ce) and the brainstem (Bs). It is known that these areas have a role in processing and perceiving olfactory and auditory stimuli. The Tel is reached by most sensory systems. The preotic area, also known as the telencephalo-diencephalic border zone, is frequently considered a component of the hypothalamus. The hypothalamus, posterior tuberculum, dorsal thalamus, ventral thalamus, and epithalamus are the five main divisions of the Di[45]. Since the amygdala, hippocampus, and cortex homologous structures are considered to be located there, both Tel and Di have been identified as being crucial areas when it comes to the processing of social information[46]. Different diencephalic nuclei have been shown to play a role in the regulation of species-specific behaviours in vertebrates, for instance, stimulation of the preoptic region in the bluegill fish (*Lepomis macrochirus*) inhibits aggressive behaviour and elicits courtship. It was also suggested that Tel and the ventral Di may also be involved in the social reward mechanism[18].

The TeO has the most intricately layered structure, being without a doubt the primary visual centre in the teleost brain, processing visual data related to motion, shape, and colour[43]. Previous research indicates that TeO may have a role in the control of visual and motor behaviour, multimodal sensory integration, and escape behaviours[18].

The vestibulolateralis lobe, corpus cerebelli, and valvula cerebelli are the three major regions of the Teleost Ce. The vestibulolateralis lobe and the corpus cerebelli have homologous in other vertebrates, but only ray-finned fishes have the valvula cerebelli[45]. The Ce was mentioned as a retinal input receptor, is involved in optokinetic oculomotor reflexes, and also in aggressiveness, since it was associated with strikes[18;45].

Hormones like oxytocin (OXT) have been implicated in the perception of social cues by increasing the salience and rewarding value of social stimuli[47]. This neuromodulator acts by binding to oxytocin receptors (OXTR) expressed in specific brain areas, including in Bs[48]. Additionally, the Bs is part of the histaminergic system in zebrafish, which has been involved in hormone regulation, feeding, drinking, sleep-wake cycle, consciousness, and memory[49].

Although the social perception is likely multimodal and additional sensory modalities, such as olfaction, auditory, and lateral line mechanoreception, the most important indicators for teleost fish to establish social behaviour are the visual cues[47;50]. The TeO, the thalamus, the

pretectum, the accessory optic system (found in the Ce), and the preoptic area are the five major CNS that receive primary retinal input in teleosts. These areas are critical in the caption of visual signals that will affect social behaviour[45].

CHAPTER 2:

OBJECTIVES

The aim of the present work is to study how the complexity of the social environment modulates the structural reorganization in the brain, by studying the dendritic density in the zebrafish.

Briefly, in unpublished data from the lab, it was verified that changing the complexity of the social environment had an impact on the zebrafish's brain. Changes in the number of neurons were found when the complexity of the social environment was changed. This previous research, conducted in Rui Oliveira's Lab by Doctor Magda Teles, revealed that in more complex social environments, there were lower neuronal numbers and smaller neuronal densities, leading to the hypothesis that lower neuronal densities could be related to an increase in dendritic complexity.

Therefore, the main goal of this project was to assess the impact of social environmental complexity on structural changes, specifically in the dendritic arborization of zebrafish brains reared in distinct social environments. To accomplish this, the dendrites were identified with the Microtubule Associated Protein 2 (MAP-2), one of the Microtubule Associated Protein, that is predominate in brain, mostly found in the dendrites. At various phases of neuronal development, this protein plays dynamic roles in the growth, differentiation, and neuronal plasticity, additionally, MAP-2 can also be found in non-mammalian vertebrates [53;54;55]. This allowed us to test our hypothesis, that in more complex social environments there is an investment in neuronal connections instead of neuronal numbers.

For that, we must consider some specific objectives:

- Determine if different environmental factors, like size and stability, have a significant impact on the neuronal complexity in zebrafish.

- Define how different social environments influence the size of the brain, and the density and ramification of dendrites in zebrafish.

- Describe the impact of the social environment on the different brain areas (Telencephalon, Diencephalon, Optic tectum, Cerebellum and Brainstem) of the zebrafish brain.

- Observe if increasing complexity of the social environment induces morphological changes in the dendrites in zebrafish.

CHAPTER 3:

EXPERIMENTAL WORK

3.1. Methods

3.1.1. Behavioural Experiment

In order to create differences in the social environmental complexity, fish were raised in different social conditions. Two different environmental factors were manipulated, group size and group stability. For the group size, we had large groups of 12 individuals, and small groups of 6 individuals, and for the stability they could be either stable or unstable. These two factors were then combined in a full factorial design given rise to four experimental treatments: small stable (SS), small unstable (SU), large stable (LS), large unstable (LU).

Group stability was manipulated on a weekly basis by swapping individuals between tanks (same treatment), disrupting social hierarchies, and increasing the demand for individuals to adjust their behaviour to a changing social environment. In the stable treatments, groups remained always the same, but individuals were also captured and reintroduced into the same group to control for handling stress.

After the fish reached adulthood, they were euthanized with a lethal dose of tricaine solution (MS222, Pharmaq; 500-1000 mg/L), their heads removed and fixed in 10% buffered formalin for 72h, and decalcified in EDTA (0.5M, pH=8.0) for 48h. After, the heads were paraffin included.

3.1.2. Immunocytochemistry

Seven brains per treatment were sliced at 6 µm. The deparaffination of the brains was performed in xylene for 30 minutes. Once deparaffinated, the slides were covered with sodium citrate buffer (0.001M) and placed in a preheated bath at 95ºC for 50 minutes. Then, the samples were washed on the shaker 3 times with distilled water for 5 minutes at room temperature (RT). The samples were then permeabilizated with PBST (PBS 0.1M and Tween 0.05%) for five minutes on the shaker, followed by incubation with the blocking solution (PBST, 1% bovine serum albumin and 2% goat serum) for 1 hour at the RT. The slides were incubated overnight, with the primary antibody, anti-MAP2 (Mouse Anti-MAP2 (2a+2b) (Sigma; Cat. No. 0000100853)), diluted at 1:100 in blocking solution inside a humid dark chamber at 4ºC.

On the second day, the slides were washed 3 times with PBST for 10 minutes under a gentle shake, followed by a 2-hour incubation in the humid chamber at RT, with the secondary antibody, Alexa 647 (Donkey Alexa Fluor 647 -conjugated anti-mouse IgG (H+L) (Invitrogen; Cat. No. A31571), diluted at 1:500 in blocking solution.

After, the slides were washed 3 times with PBS 0.1M for 10 minutes in the dark. A counterstaining with 4.6-diamidino-2-phenylindole dihydrochloride (DAPI: Sigma-Aldrich; 1

mg/ml), was also performed in a humid dark chamber for 20 minutes at RT to identify the brain nuclei. The slides were then washed 3 times in the dark with PBS 0.1M at RT.

The slides were mounted with EverBrite Hardset mounting medium (ref:23003), and after 2 hours at RT, were ready to be analysed at the fluorescent microscope.

This protocol was also made in a zebrafish brain sliced at 20 µm.

3.1.3. Identification of the Dendritic Density

Images were obtained on a Zeiss Imager Z2/ApoTome.2 system, equipped with a Hamamatsu Orca Flash 4.0 v2 CMOS camera [Axiocam 105 colour camera] at a magnification of 200x, with the acquisition software Zeiss's ZEN v3.1 (blue edition). All the slices corresponding to the entire brain were acquired.

For the dendritic quantification, every other slide was quantified to prevent quantification of the dendrites coming from the same cell. All the analysis was performed on Image J (1.53q). First, the images acquired on the same date were identified, the background of each was determined, and the mean of the background was calculated. For each image, the background corresponding to the date of the acquisition was subtracted from the image, a Gaussian blur filter applied (sigma=10) to retract the noise, and the outliers (pixels) of the image removed (block_radius_x=100000; block_radius_y=100000; standard_deviations=3).

To determine the fluorescence intensity and consequently, the dendritic density in the different brain areas (Tel, Di, TeO, Ce, and Bs), the areas were manually identified in the sections, and drawn using the zebrafish brain atlas[45]. The left and right hemispheres were quantified separately to test for lateralization effects. After, the measurement of the area and the Gray mean value of the drawn sections was obtained in order to quantify the intensity of fluorescence, which we used as a proxy of dendritic density. Lastly, the data was transferred to Excel to perform the calculation of the pondered mean of the Gray mean values of each slice, and to quantify the dendritic density of the different brain areas and the whole brain.

3.1.4. Statistical Analysis

Data analysis was performed in GraphPad Prism version 8. Firstly, the outliers were identified, and the Shapiro-Wilk normality test was performed to assess if the samples followed a normal distribution. When the samples presented a normal distribution, a two-way ANOVA was used to identify if there were significant differences between the fourth different treatments and to evaluate the influence of each main effect (i.e., size and stability) on the density of dendrites and the size area of each brain region. When the samples didn't present a normal distribution, the non-parametric Friedman test was used. To analyse the interaction between

the two treatments, a Tuckey post-hoc test was performed. To assess specific differences between the extremes of the treatments (SS vs LU), and the two brain hemispheres a student's t-test was used, two-tailed or one-tailed, respectively. The two-tailed test was performed to compare the extremes, when the interaction between the size and the stability, weren't significant, one-tailed test was done to evaluate the differences between the hemispheres. For non-parametric samples, Mann-Whitney test were performed. All graphic values are expressed as mean ± standard error of the mean (SEM). Differences were considered significant at $p<0.05$.

3.2. Results

3.2.1. Different Social Environments Influence Brain Size

3.2.1.1. Whole-brain Analysis

Considering the whole brain, only group size and the interaction between size and stability had a significant impact on the brain areas (mm^2) (Stability –F=0, p>0.999; **Size - F=5.399, p=0.0289**; **Interaction – F=6.392, p=0.0185**), with animals raised in larger groups showing larger brains compared to animals raised in smaller groups (Figure 3). When the different social groups (SS; SU; LS; LU) were compared between them with a post-hoc test, we observed a significant difference between the LS and the SS (p=0.0110), and no significant differences were found between the other treatments (SS:SU – p=0.3035; SS:LU – p=0.3747; SU:LS – p=0.3747; SU:LU – p=0.9989; LS:LU – p=0.3035) (Figure 3 c).

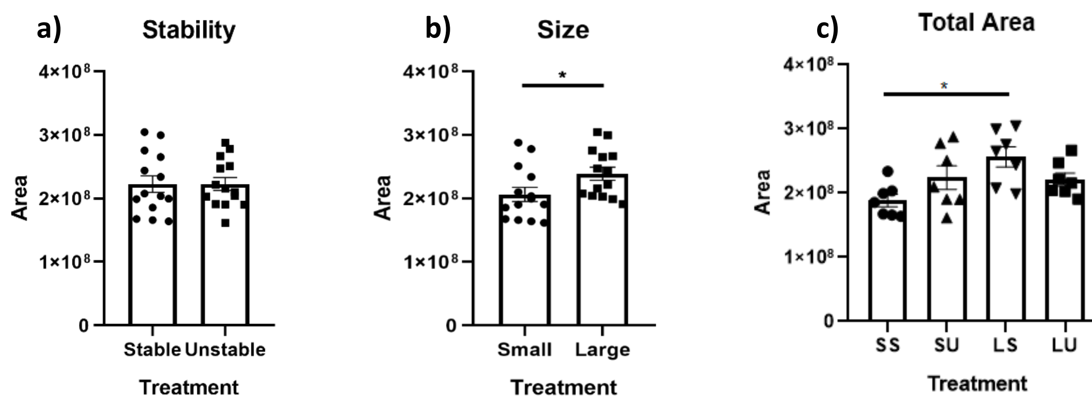


Figure 3| The impact of the social environmental complexity on the area of the whole-brain, in each behavioural groups (c), and the influence of the social environmental factors, group's size (a) and stability (b), on the whole-brain's area. The area of the brain is affected by group size, with animals raised in large groups displaying a larger brain region (a). The brain area is unaffected by the group's stability (b). The area of the brain is influenced by the environmental social complexity, making the LS group significantly different from the less complex environment, SS (c). Values are expressed as mean ± SEM. Asterisks indicate significant statistical differences, and differences were considered significant at p<0.05.

3.2.1.2. Macroareas Analysis

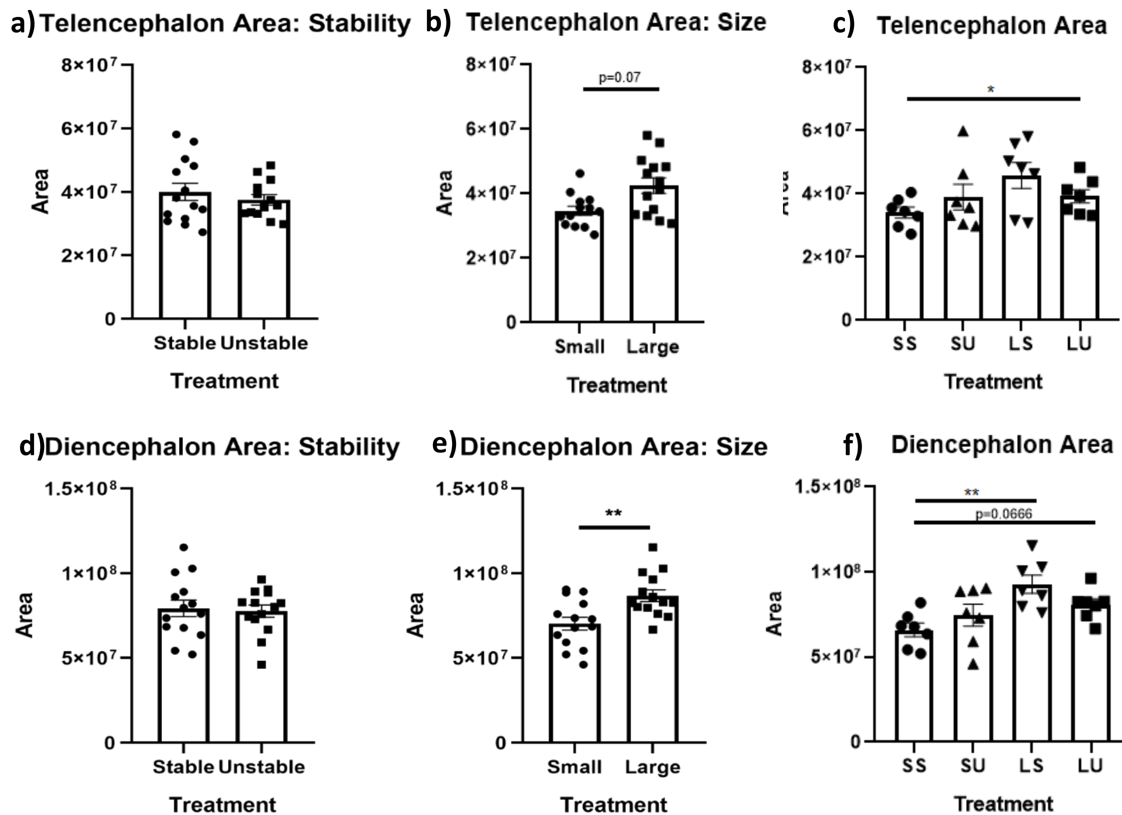
When we analyse each area individually, we found that Di and TeO were affected by group size and that none of the brain regions were affected by the stability of the group, in Tel, the results form the size and stability are close to significance (Tel: Stability – F=0.07244, p= 0.7901; Size - F=3.533**,** p=0.0723; Di: Stability – F= 0.1179, p=0.7343; **Size - F=11.32, p=0.026**; TeO: Stability – F=0.8283, p=0.3718**; Size - F=7.155, p=0.0132**; Ce: Stability – F=0.4690, p=0.500; Size0.3718 - F=0.2896, p=0.5984; Bs: Stability – F=0.9523, p=0.3398; Size - F=1.160, p=0.2932). In detail, it is visible that the large groups have a larger brain area

in all brain regions except in the Bs and Ce. The stability didn't have any effect on the brain size (Figure 4).

Regarding the interaction between the factors, some areas presented significant results, as the Di (F=4.478, p=0.0449) and the TeO (F=7.387, p=0.0120), in the Tel the results were close to significant (F=3.270; p=0.0831), and for the Ce and Bs no significant results were found (Ce: F=2.477, p=0.1286; Bs: F=1.879; p=0.1843).

In the significant interactions the post-hoc analysis identifies differences between SS and LS in the Di and TeO. For the Di it was also found differences between SS and LS, it as also notable that between the SU and LS the results are near significance (SS:SU – p=0.6002; **SS:LS - p=0.0038**; SS:LU – p=0.1704; SU:LS – p=0.0666; SU:LU – p=0.8136; LS:LU – p=0.3264) (Figure 4 f). For the TeO significant difference between SS and LS was found (SS:SU – p=0.0748; **SS:LS – p=0.0044**; SS:LU – p=0.0796; SU:LS – p=0.6035; SU:LU – p>0.9999; LS:LU – p=0.5850) (Figure 4 i).

Since the interaction between the factors was not significantly different in all brain region, and we wanted to test our hypothesis the two extremes of the treatment (lower complexity, SS and higher complexity, LU) differ we performed a one-tailed student's t test, in the Tel, Ce and Bs. These results indicate a significant difference between the SS and LU groups only in the Tel (**Tel: p=0.0435**; Ce: p=0.4444; Bs: p=0.4650).



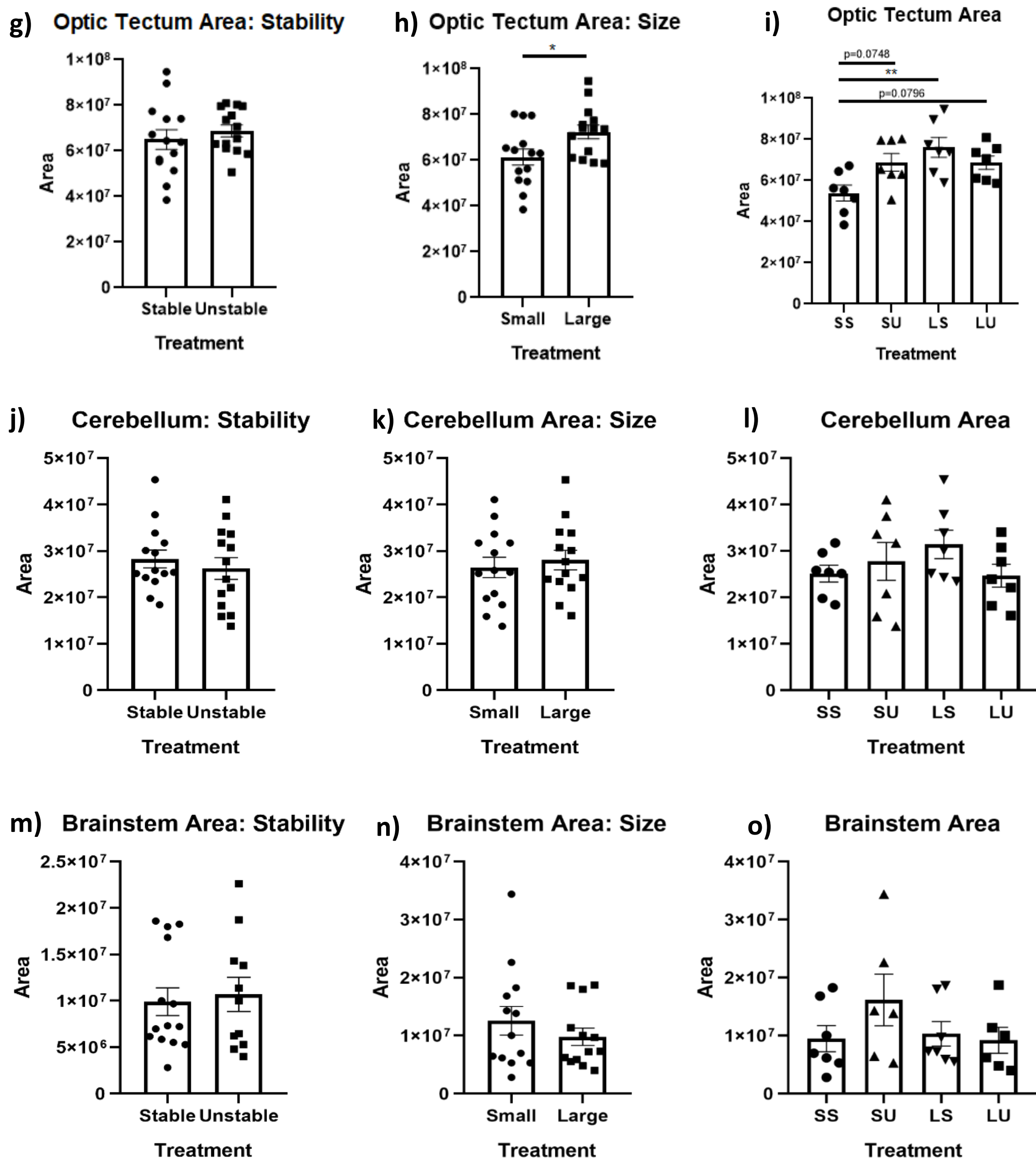


Figure 4| The impact of social environmental complexity on the area of different regions of the brain, in each behavior group (c; f; i; l; o) and the influence of each social environmental factor, the group's stability (a; d; g; j; m) and size (b; e; h; k; n), on the area of the different brain regions. All brain regions, except Tel, Bs and Ce, were affected by the size of the group, with the large groups presenting a higher measured area. The influence of group size in Tel's area is near significance. In contrast, none of the brain regions area seem to be significantly influenced by the stability of the experimental groups. Furthermore, comparing all the social groups between them, it is possible to observe that the area of the brain region is higher in the LS group, except in the Bs, where the higher area measured is present in the SU group (o). Furthermore, there are significant differences in the Tel when the SS and the LU are compared by a one-tailed student's t test and in the Di and TeO when the same behavioural groups are compared by a post-hoc test, (represented by c, f and i, respectively). Values are expressed as mean ± SEM. Asterisks indicate significant statistical differences, and differences were considered significant at $p<0.05$.

3.2.1.3. Brain's Area is Similar Between Hemispheres of Whole-brain Area

The data analysis did not show a significant difference in the size of the different hemispheres, this was verified in all experimental groups (SS – p=0.9524; SU – p=0.6757; LS – p=0.8462; LU – p=0.7604) (Figure 5).

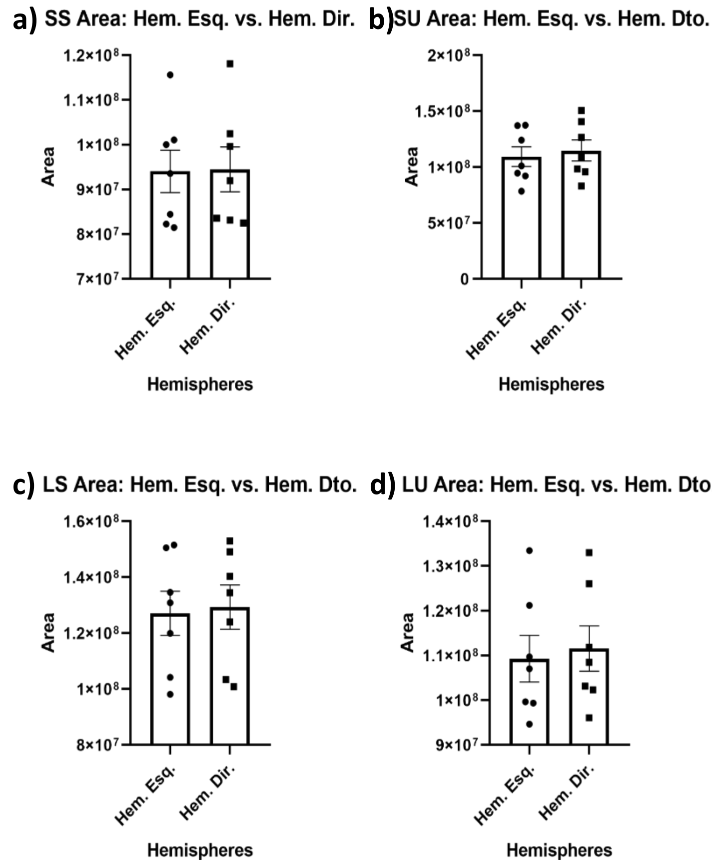


Figure 5| The impact of social environmental complexity on the area of both hemispheres considering the whole-brain area. There is no statistically significant variation in the area between the hemispheres. (a; b; c; d). Values are expressed as mean ± SEM. Asterisks indicate significant statistical differences, and differences were considered significant at $p<0.05$.

3.2.2. Stanning of Dendrites

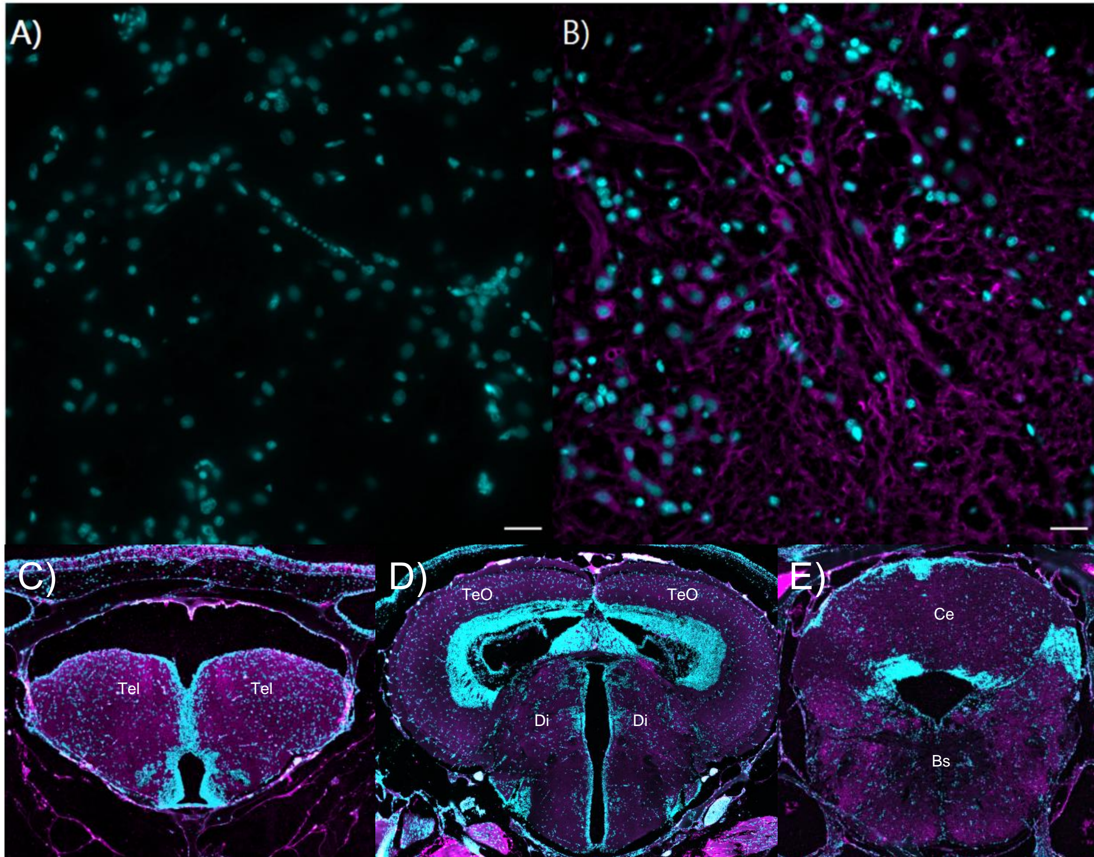


Figure 6| Representative image of immunocytochemistry of control (a) and dendrites (b), and from different regions of the brain (c;d;e). All Images were obtained on a Zeiss Imager Z2/ApoTome.2 system, equipped with a Hamamatsu Orca Flash 4.0 v2 CMOS camera [Axiocam 105 colour camera] at 200x drier magnification (c;d;e) and at 400x magnification in oil (a;b). The whole-brain was acquired with the acquisition software Zeiss's ZEN v3.1 (blue edition), and it was divided in different regions, Tel present in slices from the front part of the brain (c), Di and TeO, present in slices from the middle part of the brain (d) and Ce and Br, present in slices from the back part of the brain (e). The image a) and b) were acquired by Doctor Magda Teles.

3.2.3. Different Social Environments Modulate Dendritic Density

3.2.3.1. Whole-brain Analysis

Considering the whole brain, size had an impact on the dendritic density, and the stability of the group was close to the significant (Stability - F=3.823, p=0.0628; **Size - F=6.830, p=0.0155**; Interaction – F=2.189, p=0.1526), with the large and the unstable groups being the ones with higher dendritic density compared with the small and stable groups, respectively (Figure 7 a; b). In the interaction between the factors, the LU group shown to have a higher dendritic level. When the extremes were compared in a one-tailed student's t test, we observed a significant difference between LU and SS (p=0.0079) (Figure 7 c).

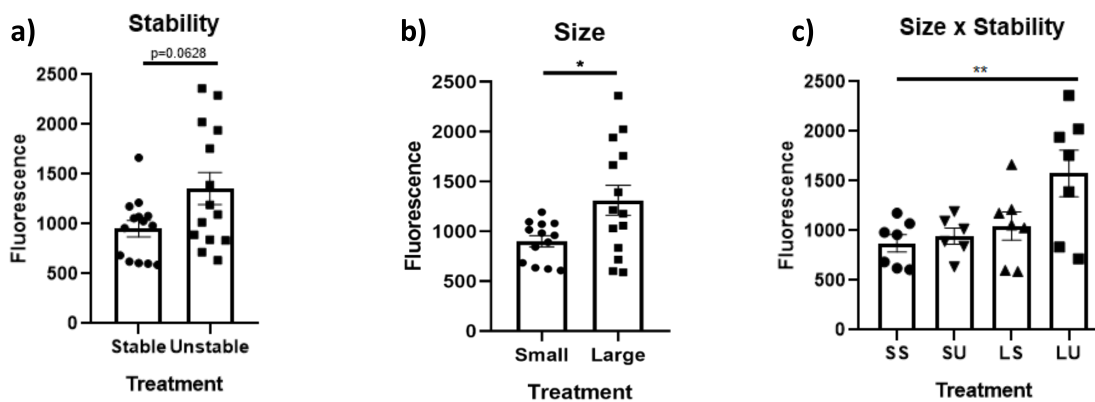
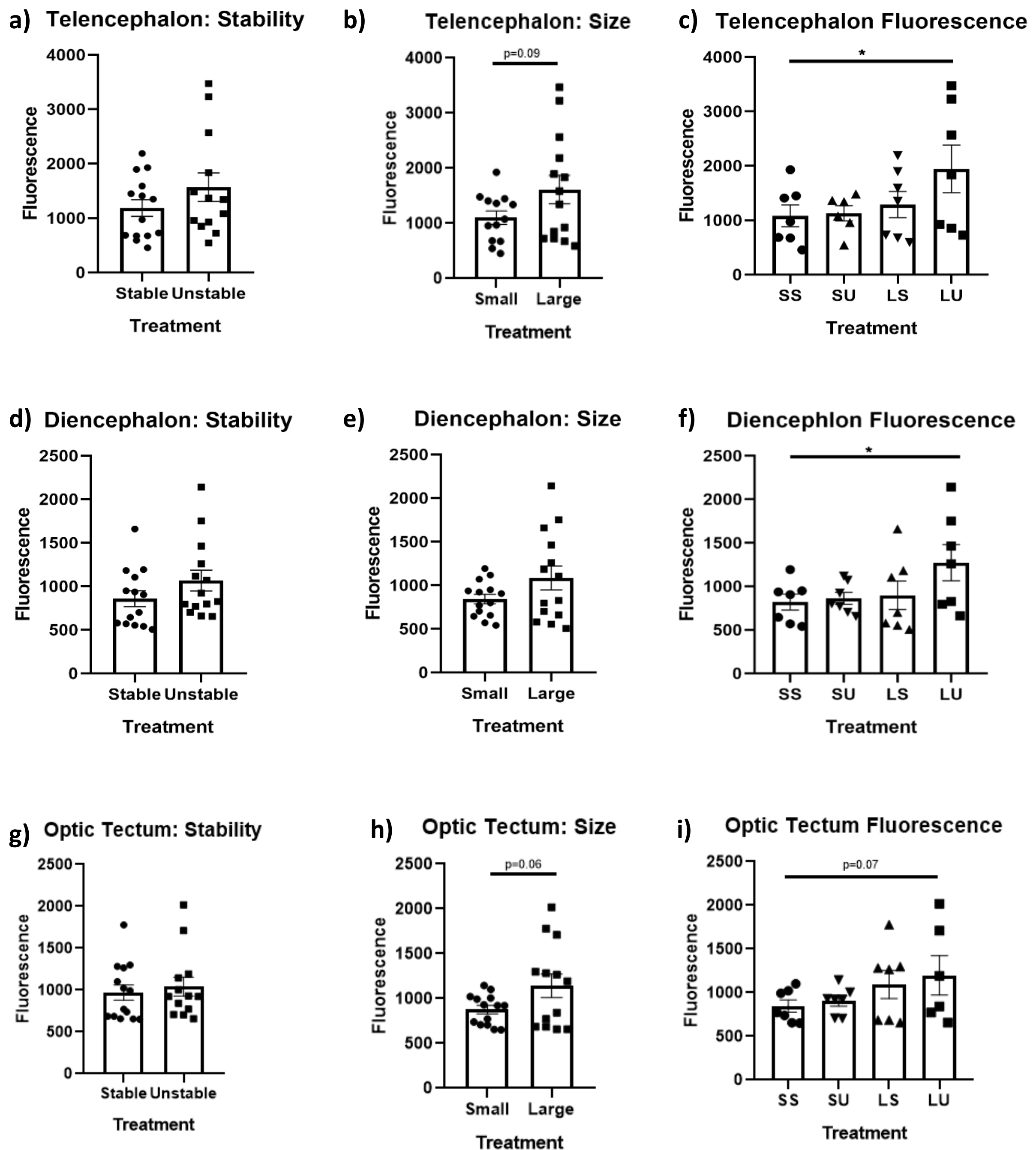


Figure 7| The impact of social environmental complexity on dendritic density in the whole-brain, in each behavioural groups (c), and the influence of the social environmental factors, group's size (a) and stability (b), on the dendritic density. The dendritic density is significantly influenced by the size of the group, with the large groups exhibiting a higher dendritic level (b), the effect of the stability in the dendritic density is near the significance, with the unstable groups presenting a higher level of dendrites (a). Additionally, the environment's social complexity tends to increase dendritic density, with the most socially complex group (LU) differing significantly from the less complex group (SS). Values are expressed as mean ± SEM. Asterisks indicate significant statistical differences, and differences were considered significant at p<0.05.

3.2.3.2. Macroareas Analysis

Considering the brain macroareas individually, we can observe that the influence of the group size in the dendrites density in the Tel, TeO and Bs, are near the significance, in contrast, Di and Ce were not affected by group size, and only the Bs was affected by group stability (Tel: Stability – F=1.482, p=0.2359; Size - F=3.136, p=0.0898; Interaction – F=1.114; p=0.3021; Di: Stability – F=2.093, p=0.1609; Size - F=2.856, p=0.1040; Interaction – F=1.322, p=0.2615; TeO: Stability – F=0.3448, p=0.5628; Size - F=3.774, p=0.0644; Interaction – F=0.02898, p=0.8663; Ce: Stability – F=1.109, p=0.3032; Size - F=2.731, p=0.1120; Interaction – F=0.8368, p=0.3698; Bs: **Stability – F=14.73; p=0.001**; Size - F=3.065, p=0.0946; **Interaction – F=8.043; p=0.0099**). Furthermore, it is visible that the unstable and large groups have a higher density of dendrites in all brain regions (Figure 8).

Comparing all the social groups between them, it is possible to observe a general tendency for an increase in the dendritic density together with an increase in social complexity. Through an one-tailed student's t-test differences in the dendritic density between the extreme groups, SS and LU, were found only in the Tel and Di (Tel: p=0.0499; Di: p=0.0352; TeO: p=0.0702; Ce: p=0.0467) (Figure 8 c; f; i; l). In parallel, differences were found between the most social complex group (LU) and the other tree behavioural groups in Bs through a post-hoc analysis (SS:SU – p=0.8873; SS:LS - p=0.8617; **SS:LU – p=0.0032**; SU:LS – p=0.4844; **SU:LU – p=0.0214**; **LS:LU – p=0.0008**) (Figure 8 o).



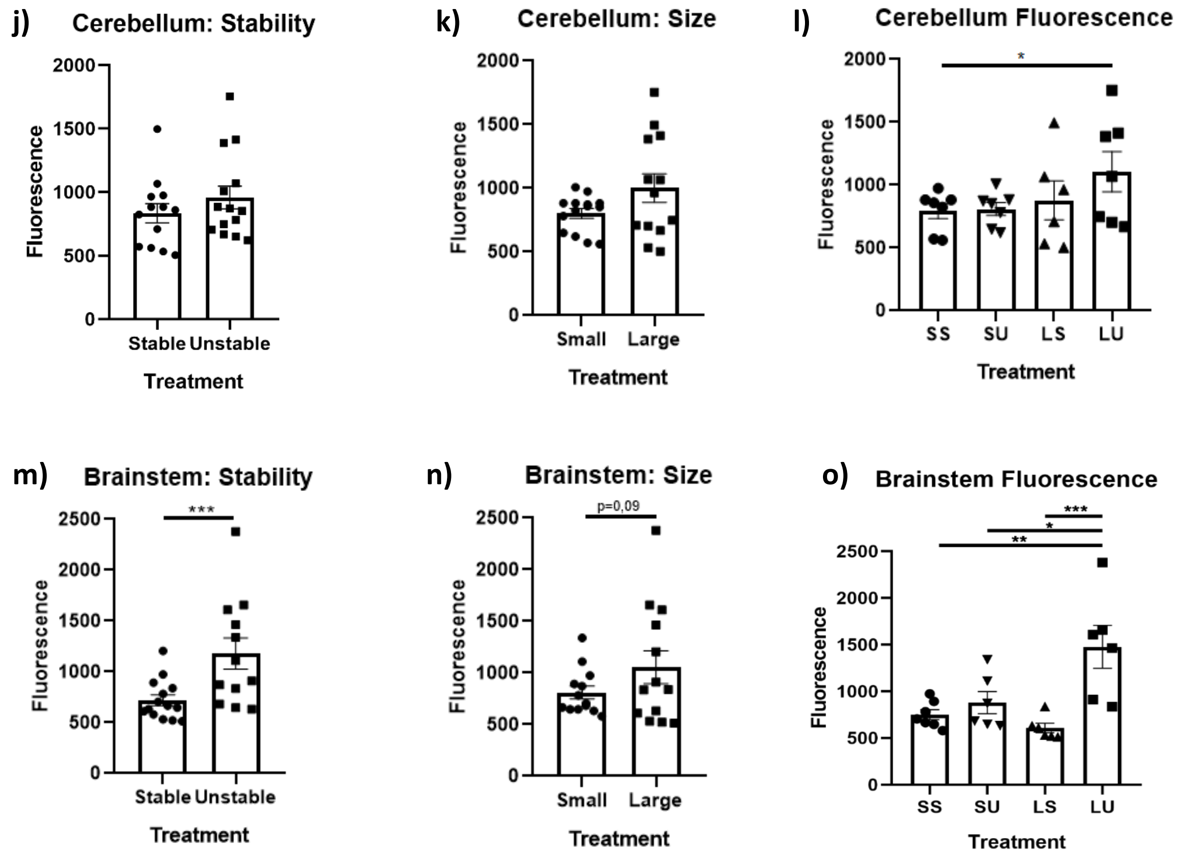


Figure 8| The impact of social environmental complexity on dendritic density in different regions of the brain, in each behavior group (c; f; i; l; o) and the influence of each social environmental factor, the group's stability (a; d; g; j; m) and size (b; e; h; k; n), on the area of the different brain regions. The influence of the group size in the dendrites' density in the Tel, TeO and Bs, are near the significance, in contrast, Di and Ce were not affected by group size (b; e; h; k; n). Only the dendritic density of the brainstem is impacted by the group's stability, with the unstable groups presenting a higher dendritic level in every region (m). Furthermore, dendritic density has a general tendency to rise with social complexity (c; f; i; l; o), in which Tel and Di presented significant differences between the extreme (SS and LU) through an one-tailed student t-test and Bs show a significant differences between LU and the other behavioural groups, (represented by c, f and o, respectively).Values are expressed as mean ± SEM. Asterisks indicate significant statistical differences, and differences were considered significant at $p<0.05$.

3.2.3.3. Dendritic density is similar between hemispheres in the whole-brain area

The data analysis did not show a significant difference in the dendritic density between hemispheres, this was verified in all experimental social groups (SS – p=0.9964; SU – p=0.6302; LS – p=0.5142; LU – p=0.413) (Figure 9).

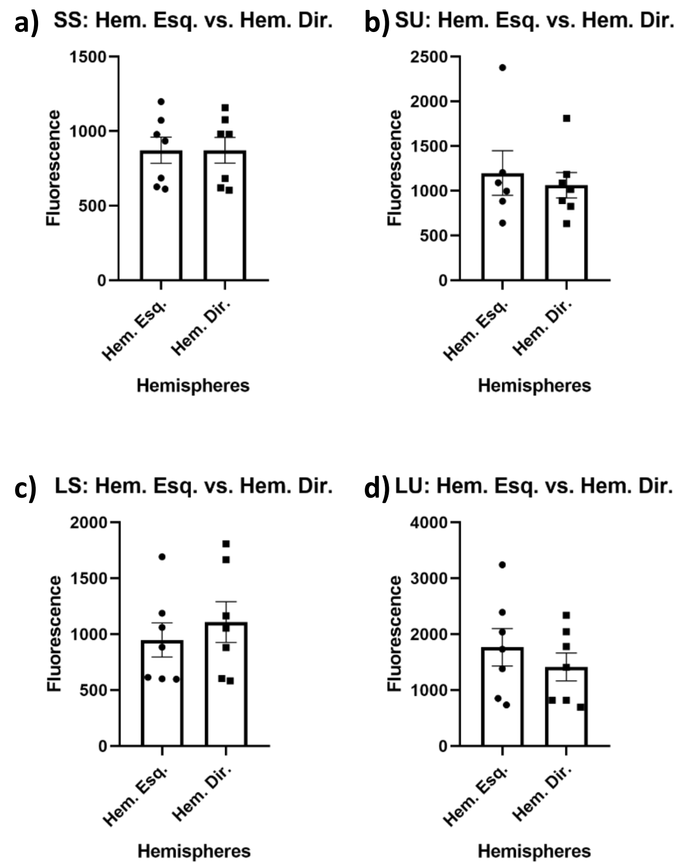


Figure 9| The impact of social environmental complexity on dendritic density in both hemispheres considering the whole-brain area. There is no statistically significant variation in dendritic density between the hemispheres (a; b; c; d). Values are expressed as mean ± SEM. Asterisks indicate significant statistical differences, and differences were considered significant at $p<0.05$.

3.2.4. Morphological Analysis

Since the anti-body to marked MAP-2 didn't stain the whole brain slice, and it was impossible to discriminate the dendrites from the neurons and the dendrites from the microglia, there are no results to present regarding the morphological analysis.

3.3. Discussion

The social environment is the most complex external pressure that animals face since it includes interactions with other individuals, which have unpredictable outcomes. Social competence enables individuals to adapt their behavioural responses to changing social environment, which will have consequences for their survival [1;2;4]. In response to variation in the social environment individuals can alter their behavioural phenotypes, a phenomenon known as phenotypic plasticity, which allows individuals with the same genome to express different behavioural phenotypes. Phenotypic plasticity is normally based on neural plasticity, define as the ability of the brain to reorganize its neural connectivity in response to environmental changes and might include changes in cell morphology, in neurophysiological functions, as well as modification in the neural networks. In this present work, we focus on the structural changes in the brain network, specifically, in changes on dendritic arborizations[11;17]. Previous research, conducted in Rui Oliveira's Lab by Doctor Magda Teles, revealed that in more complex social environments, there were lower neuronal numbers and smaller neuronal densities, leading as to the hypothesis that lower neuronal densities could be related with an increase in dendritic complexity (unpublish data). Considering that, the main goal of this work was to assess the impact of social environmental complexity in the dendritic arborization of different regions of zebrafish brain reared in distinct social environments.

To accomplish this aim, the complexity of the social environment was induced through variation in group size (small versus large) and group stability (stable versus unstable) leading to four experimental treatments (SS, SU, LS, LU), in which the SS are considering the less complex group and the LU the most complex group. In the different groups we quantify the size of the brain area, and the dendritic density in the different macroareas of the brain (Tel, Di, TeO, Ce, Bs). We also analyse if there were any significant differences between the extreme environments (SS vs LU) and if there was any the laterization effects.

In this project, we found that group size has an impact on brain size, whole brain, and in the Tel, Di and in TeO, specifically. Additionally, the large stable (LS) group exhibit larger brain areas, precisely in the Tel, Di, TeO and Ce compared the small stable (SS) group. Di and TeO are the areas that presents most differences between the groups, with the SS differing from all the other groups in TeO and with the extremes, and the LS differing from SS, in the Di. As referring to the laterization effect we discovered that there is no significant difference between the right and left hemispheres. This data demonstrated that different social environments influence brain size in different brain regions, which reinforces results from previous studies. For instance, Kolb 1995 described that after 60 days of been kept in a

enriched habitat, changes in the total brain weight of young rats, on the range of 7%–10%, were observed[25]. Fischer et *al.*[54], analysed six brain structures to test if rearing-group size affected the size of these brain structures in Cichlid fish. The results of the study indicated that rearing-group size and time in the groups interactively affected brain architecture. Brain architecture was shaped by isolation day, and rearing-group size. Hypothalamus and cerebellum were larger in fish from small rearing groups isolated early, and in fish from large groups isolated late, whereas the opposite pattern applied to the optic tectum and as a tendency, also to the dorsal medulla. This suggests that there are indirect effects of group size on social behaviour.

Additionally, we found that different brain regions respond to different environmental factors, and group size affected all brain regions, except in the Ce and Bs, whereas group stability didn't influence any region indicating a regional differentiation. The differences found in the Tel and Di can be explained by the presence of specific nodes that are part of the social decision-making (SDM) network in this two macroareas. According to the SDM network hypothesis, it has been claimed that the expression of social behaviour in vertebrates is governed by an evolutionarily conserved SDM network that consists of two linked neural circuits: the social behaviour network and the mesolimbic reward circuit, which nodes are present in the forebrain and midbrain. Additionally, SDM involves the integration of multimodal sensory information about social status and social context with previous experience to produce an appropriate behavioural response that is adjusted to the perceived social environment[13;46;55]. A study from Rui Oliveira's Lab, Teles *et al.*[50], examined if the SDM network has any region-specific neuroplasticity processes in connection to the expression of social behaviour. The results showed that there was regional diversity, with specific neuroplasticity pathways being connected to social behaviour in particular parts of the SDM network. Different behavioural characteristics were associated with mechanisms such as neurogenesis, modifications in memory-related processes, cell proliferation, and synaptic plasticity in distinct SDM network regions. This suggests that there is not a single neuromolecular module underpinning behavioural flexibility and that social plasticity depends on a variety of neuroplasticity pathways present throughout the SDM network.

Other brain regions, like TeO, also variate with group size. Social organisms need to be very efficient at extracting clues about different conspecific traits from their surroundings and have sensory capacities suited to their social context. These sensory clues are therefore the basis of social behaviour and essential to survival[2]. Among the several sensory modalities, vision is crucial for social attachment in non-human primates, humans, and other animals like fish. In zebrafish, body shape and biological motion are the two primary visual cues that might indicate the existence of conspecifics. When they are exposed to conspecifics through visual

stimuli alone, either in real or in videos, zebrafish immediately approach the conspecifics to interact and form shoals[47;56]. These results confirm that the perception and process of visual cues are imperative for the individuals to respond adequately to the social environment.

Concerning the dendritic density, we found that the dendritic density increases with the complexity of the social environment, such that animals raised in less complex social environments (small and stable groups) present lower dendritic density, and there is a tendency for dendritic density to increase with the increase in social complexity. Furthermore, we also discovered that the level of dendrites in Tel, Di and TeO, are significantly affected by group size. As referring to the laterization effect, we noticed that there is no significant difference between the right and left hemispheres. In congruence with the results from the size of each brain region, changes in the complexity of the environment are followed by changes in the dendritic density in the Tel, Di and TeO, which can be linked to a reinforcement of different neuronal circuits, especially the ones from SDM network and the ones involved in processing visual cues[43;44;46;47].

Unexpectedly, only the in the Bs the dendritic density was affected by group stability. Here, the LU group differed from all the other experimental groups. These can be explained by the presence of receptors that activate OXT circuits and vasopressin (AVP) axonal projections in the brainstem[57]. OXT and AVP are neurohormones that have been linked to the perception of social cues and the control of a variety of intraspecific social behaviours, including aggression and social approach/withdrawal[3;47;57]. Additionally, the Bs is a component of the histaminergic system in zebrafish, which has been linked to awareness, memory, eating, drinking, hormone control, and the sleep-wake cycle[49]. Furthermore, a previous study found that the activation of hypothalamic neurons across cell classes promotes quick adaptive responses via peptide-independent glutamate release onto Bs neurons, which was observed in the presence of stimuli that threaten homeostasis[58;59]. Another interesting result that comes in congruence is that the stability of the group has a higher influence in Bs area than the size of the group.

Overall, our results indicate the relevance of the social environment in the modulation of neuronal complexity during development which is paralleled by changes in behavioural performance, so different social environments modulate dendritic density and brain size. However, as several of the statistics indicate a p-value around the significance, more samples from each group are required to draw more sustainable conclusions.

Relatively to the morphological analysis of this project, the experimental work failed in producing analyzable data. The brain slices were too thick to allow the antibody to stain throughout the tissue, and we were not able to acquire the z-stack images for further analysis. We start working on the optimization of the immunocytochemistry protocol. To improve the

permeabilization of the slice, the protocol was modified, we replaced Tween 20 by Triton X-100. We also changed the incubation periods by dividing it in two parts: the 2 first hours at RT and the rest of the period in the cold camera at 4ºC. We are currently working on a double immunocytochemistry using the HuC/HuD antobody [60], a neuron marker in zebrafish, and MAP-2 to identify the dendrites. Another possibility is to use beta tubulin 1, instead of MAP-2 which has been successfully used in rats[61]. However, previous studies report that beta tubulin 1 is only present in specific areas of adult zebrafish brain being important to previously select the brain regions that we want to analyze[62]. Due to time constrains, we couldn't complete the optimization protocol.

CHAPTER 4:
GENERAL CONCLUSIONS

4.1. General conclusions

The main goal of this study was to assess the impact of environmental complexity on structural changes, specifically in the dendritic arborization of zebrafish reared in distinct social environments.

Changes in the behaviour of zebrafish have been reported once complexity of the environment increases. In this study, we verify that this behaviour plasticity occurs in parallel with changes in brain size and in the dendritic arborization of the zebrafish brain, that differ depending on the macroareas where they are found.

Specifically, considering the analyse of the brain size, we verify that:

- Different social environments influence brain size in different brain regions.

- Animals raised in large and in stable groups had larger brains when the whole-brain area was analyzed.

- Different brain regions respond to different environmental factors, with a tendency for the group size to influence the size of all brain regions, except in the Ce and Bs. Group stability didn't affect any brain region indicating a regional differentiation.

Regarding the dendritic density, we found that:

- Different social environments modulate dendritic density.

- Large and unstable groups present higher dendritic densities when whole brain is analyzed.

- Different brain regions respond to different environmental factors, with a tendency for the influence of group size on dendritic density for all brain regions, except in the Di and Ce, and group stability only affects the Bs indicating again a regional differentiation.

The fundamental goal of this project was accomplished, but more data and a morphological analysis are needed to underpin the alterations that the social environment causes in the dendritic arborization. In conclusion, the current work offers insights into which brain areas might be altered by social contexts and how social environments regulate neural complexity.

4.2. Future perspectives

Regarding the results presented in this study, a follow up should be done were one:

- Define the changes in the morphology of the dendrites induced by social modulation, to confirm that the changes found in the dendritic density correlate with changes in the dendritic structure.

- Determinate which neuronal circuits are involved and reinforced when the social environment is manipulated. This way we can assess if the processes we suggest was being behind social modulation in zebrafish, specifically, the visual circuits, cognition circuits and circuits present in the SDM network, are reinforce following alterations on the social environment.

CHAPTER 5:

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