THE ROLE OF DEVELOPMENTAL SOCIAL COMPLEXITY ON THE ADULT ZEBRAFISH BRAIN

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Tese submetida como requisito parcial para a obtenção do grau de:

MESTRE EM NEUROCIÊNCIAS COGNITIVAS E COMPORTAMENTAIS

2019

Dissertação de Mestrado realizada sob a orientação de Magda Teles, apresentada no ISPA – Instituto Universitário para obtenção de grau de Mestre na especialidade de Neurociências Cognitivas e Comportamentais

Agradecimentos

Este ano proporcionou-me uma experiência que me desafiou tanto a nível psicologico como fisico. Foram tantos altos e baixos, uma montanha russa que me atirou de frente para a realidade que é trabalhar em ciência. Em certas alturas o desespero tomou conta de mim mas, sem dúvida, que de modo a poder ultrapassar todos os obstáculos, existiram várias pessoas que me ajudaram diretamente e indiretamente, e que me deram sempre apoio:

Quero agradecer à Magda, que foi crucial na elaboração da minha tese, que me acompanhou em todos os passos e que me ajudou a navegar por todos os obstáculos que se impuseram à minha frente, estando sempre disponível para os meus problemas e dramas;

Ao Rui por me ter dado a oportunidade de trabalhar no seu grupo de investigação e por me ter ajudado a resolucionar problemas que foram surgindo na tese;

Ao pessoal do IBBG, em especial: à Manuela por ser a camarada que partilhou comigo o desafio interminável de contar células, à Cláudia por estar sempre preocupada como estavam a correr as coisas comigo, à Ibukun por me fazer rir nos melhores e piores momentos, ao Kyriakos por me ajudar na parte estatística e por me ter explicado o que é um café abatanado, à Vicky por me dar coragem durante todos os almoços, e à Rita que sempre fez questão de me fazer sentir integrado no grupo.

Ao pessoal da unidade de imagiologia do IGC, em particular: ao Nuno Pimpão e ao Gabriel Martins por arranjarem sempre tempo para resolver problemas que foram surgindo no microscópio e pela ajuda preciosa no desenvolvimento da macro.

Aos amigos que acompanharam o meu percurso académico, especialmente: à Leo, com que eu partilhei cada momento de felicidade e frustração, e que sem ela isto seria bem mais difícil de ultrapassar, à Patrícia e à Cláudia por todos os bons momentos produzidos pelos jogos de UNO intermináveis nos intervalos das aulas, e à Inês por me ajudar em todas as tecnicalidades desta tese e por compreender a minha crise existencial ao longo deste período.

À minha família: à minha irmã, que em tempos de maior aperto teve uma paciência de santa e me tentou facilitar ao máximo a minha vida, ao meu pai e à Teresa por todas as vezes que me deram coragem continuar a lutar pelos meus objectivos, e à Carla, por todas as vezes que me deixou trocar de turnos do trabalho para eu poder trabalhar na tese.

Acknowledgments

This year experience challenged me a lot in both a psychological and physical level. All those ups and downs felt a bit like a rollercoaster ride that threw me right to the reality of how hard it is to work in science. At certain points, some desperation took over me but, without a doubt, a lot of people were important to help me get through all those moments. So, I want to thank:

To Magda, who was crucial in my thesis elaboration, who guided me throughout all the steps of the process, and navigated me around all the obstacles that I had to face, being always available to all my problems and dramas;

To Rui, for giving me the opportunity of letting me work in his research lab, providing all the tools necessary, and for helping solve problems that I had to face throughout the thesis;

To the IBBG colleagues, especially: to Manuela, for being the comrade that shared with me daunting task of counting cells, to Cláudia for always being concerned about how my project was going, to Ibukun for making me laugh in good and bad moments, to Kyriakos for helping me in the statistical analysis and for explaining what is an abatanado coffee, to Vicky for always giving me courage during lunches, and to Rita for always making me feel integrated in the group.

To the guys of the IGC imaging unit, especially: to Nuno Pimpão and Gabriel Martins for always sparing time to help solve issues with the microscope and for their precious help in the macro development.

To my friends that walked this long academic journey by my side, especially: to Leo, whom I shared every bit of happiness and also moments of frustration, because without her this would have been a lot harder, to Patrícia and Cláudia for all the laughs prompted by lengthy games of UNO during the class breaks, to Inês for helping me with the thesis technicalities and for understanding my existential crisis throughout this period.

To my family, especially: to my sister for having the patience of a saint and for facilitating my life in the hardest times, to my father and Teresa for all the times that they gave me courage to try harder, and to Carla, for all the times that she let me change shifts at work in to let me work on this thesis.

Resumo

Crescer em diferentes ambientes sociais durante o desenvolvimento influencia as habilidades cognitivas posteriores de um indivíduo, podendo também alterar a sua estrutura cerebral. De modo a explorar esta relação, foram escolhidos os peixes-zebra como modelo social – conhecidos por serem animais altamente sociais que estabelecem relações sociais estruturadas com os seus conspecíficos. Diferentes complexidades sociais – tamanho do grupo e estabilidade – que um indivíduo experiencia durante o desenvolvimento, foram estudadas. A estrutura cerebral de peixes adultos, particularmente a quantificação do número de neurónios e não-neurónios, foram analisados de modo a perceber se existe variações entre os diferentes grupos sociais, e de que forma é que estes números se distribuem nas diferentes regiões cerebrais. Observou-se que indivíduos que cresceram num grupo pequeno (com 6 elementos) estável (a viver sempre com os mesmos elementos) tiveram uma densidade neuronal global superior relativamente ao grupo grande (com 12 elementos) instável (a viver num grupo cujo os conspecíficos são substiuídos todas as semanas), ao grupo pequeno instável e ao grupo grande estável. A densidade neuronal na região do diencéfalo foi significativamente superior nos grupos estáveis, em comparação com os instáveis. Estes resultados sugerem que viver em grupos pequenos e estáveis pode ser cognitivamente mais desafiante e que o diencéfalo pode ter um papel crucial na regulação do comportamento social. Estes resultados constituem uma evidência de que o ambiente social que um indivíduo experiencia durante o desenvolvimento provoca diferenças na sua estrutura cerebral e indica que diferentes programas de desenvolvimento são adoptados em diferentes situações ambientais.

Palavras-chave: Desenvolvimento, Complexidade Social, Neurónios, Diencéfalo, Fraccionador Isotrópico

Abstract

Living in different social environments throughout development lead to vastly distinct posterior cognitive abilities, and it may also change brain structure. To explore this relationship, zebrafishes, which are highly social animals that live in structured social relationships, were used as the social model in this thesis. Here, different social complexities – group size and stability – that individuals experience in early age were tested. Brain structures in adulthood, particularly the quantification of their neuronal and non-neuronal numbers, were checked to comprehend if there is a variation between treatments, and to understand how these numbers are spread throughout the several regions of the brain. Individuals who grew in a small (with 6 individuals) stable (living always with same conspecifics) group had a higher global density of neurons in comparison to the large (with 12 individuals) unstable (living in a group in which conspecifics change on a weekly basis) group, the small unstable and large stable group. The diencephalic neuronal density was also shown to be significantly higher in stable groups, in comparison with those individuals that grew in unstable groups. These main results suggest that living in small and stable groups could turn out to be cognitively challenging and the diencephalon might have a crucial importance in social behaviour regulation. Ultimately, this gives evidence to the fact that different social developmental environments play a relevant role in establishing differences in brain structure and indicate that different developmental programs are employed in different environment situations.

Key-words: Development, Social Complexity, Neurons, Diencephalon, Isotropic Fractionator

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Introduction

Brain Evolution

Described as one of the most energetically expensive organs in the body, the brain is still seen as an evolutive puzzle. The sole fact that it consumes about 20% of the entire energy produced by the organism, while only accounting for 2% of the total body mass, shows its relevance at the organismal level. Therefore, the quest for understanding its evolution in terms of size and complexity across species has been explored with different approaches (Dunbar, 1998).

From an epiphenomological and developmental perspective, the evolution of the brain is closely related to the way our biological developmental processes are organized, excluding external pressures. The epiphenomological view claims that brain evolution is the simple consequence of body size, while the developmental approach suggests that maternal metabolic inputs are the major factor for brain development. However, both hypotheses fall under the same mistake of ignoring the argument that evolution is the result of the balance between costs and benefits, and the cost of maintaining the brain is too expensive for the brain to grow just because the body also increases. Therefore, larger brains only evolve if the selection factor in its favor overshadows the cost gradient (Dunbar, 1998).

The ecological and the social brain hypothesis that appeared later, associate environmental pressures, on the development of larger brains, with abiotic factors. The ecological hypothesis postulates that selective pressures that our antecessors had to face, considering the available resources in the savanna and the lack of "weapons" (such as claws and canine teeth), favor the development of larger brains (Acedo-Carmona & Gomila, 2016). However, there is not any correlation between the neocortex volume and a set of ecological indicators, such as the style of foraging adopted, or the duration of daily displacements (Dunbar, 1998). The social brain hypothesis, on the other hand, views the social environment as a major selective force for brain and cognitive evolution in a group living context. (Acedo-Carmona & Gomila, 2016).

Social Brain Hypothesis

The Social Brain Hypothesis (SBH) argues brain size and its enhanced cognitive abilities arise from the complexity of group living, especially in new environments (Dunbar, 1998).

The social environment is less predictable than the physical environment, and it is under constant change, therefore, a continuous evaluation is needed for the individuals to adapt to these dynamic changes. In other words, the more complex a social environment is, the harder it is to follow its changes. As a consequence, the evolution of the brain reflects the complexity of the social environment (Chojnacka, Isler, Barski & Bshary, 2015). Moreover, this theory proposes that ecological problems, such as food foraging, are solved socially, and that mechanisms that improve social cohesion, ultimately lead to brain evolution (Dunbar & Shultz, 2007).

This hypothesis was first tested and confirmed in primates, where the size of the group, was positively correlated with brain size. It is presumed that social group size is limited by the primates' information processing abilities, and it seems that the neocortex ratio develops a crucial role in this information processing (Dunbar, 1998). The neocortex is a part of the cerebral cortex of the mammalian brain with an elaborated complexity. It is involved in several cognitive functions, such as the sensorial perception, spatial awareness, conscious though, and language (Dunbar, 1998). However, this relationship between group size and neocortex ratio does not seem to hold for other taxonomic groups, suggesting that this relationship might not be as straightforward as one might anticipate.

Primate relationships seem to differ from those of other taxa. In other vertebrate species, it has been observed that individuals who are in stable relationships (pair bonded relationships – bonds that extend further than the immediate mating season) exhibit a larger brain mass in contrast to other species of vertebrates that have different mating systems, i.e. a harem system which is composed by several adult females and one adult male during mating season; a multimale system where adult males are accompanied by adult females in a polygamous type of mating; or solitary species, who spend the majority of their year alone. The pair bonded relationship could be cognitively more demanding, not only because it requires the ability of conflict solving, but also because it compels the individual to optimize to the maximum the choice of their partner, so that the risk of a future betrayal is reduced. Furthermore, the maintenance of a bond requires the management of conflict that results from disputes of

resources, decisions about parental investment and time budgets (Shultz & Dunbar, 2007), making this type of interactions more demanding.

Several studies in primate species already indicate the relation between brain size and some dimensions of social complexity, such as: the size of social networks, group stability, strength of social bonds, social learning, and the complexity of the habitat (Dunbar & Shultz, 2007). On the other hand, in the Australian magpies, a correlation between group size and cognitive performance at an early age was found, suggesting that living in large groups favors enhanced cognition which was also linked to an increase in the reproductive success (Ashton, Ridley, Edwards & Thorton, 2018). Also in fish (in several ectodine species), the telencephalon and hypothalamus size seemed to be associated with the type of relationship. In monogamic species, telencephalon size is larger while the hypothalamus is bigger in polygamous relationships (Pollen et al., 2007). In a cooperative breeding cichlid, *Neolamprologus pulcher*, it was also showed that the relative brain size of specific macroareas was influenced by group-size rearing, for instance, hypothalamus and cerebellum increased in fish reared in large groups, whereas the optic tectum was bigger in fish reared in small groups, which may indicate some degree of specialization for each brain area (Fischer et al. 2015).

Apart from group size, environmental stability or familiarity was also investigated in several vertebrates indicating that stability also leads to enhanced cognitive abilities and perhaps brain size enlargement. For example, recovery after an episode involving fear in zebrafish is helped by the mere presence of conspecifics, and this effect is more significant if they are familiar. Besides the behavioural recovery, a faster suppression of the HPI axis, and an increase of central isotocin levels, also occurs in these individuals (Mathuru et al., 2017). Being in kin groups also provides an advantage in the growth rate to larvae and juvenile fish. This in turn, correlates with an increased fitness, especially for females, because if they grow faster, they can reach sexual maturity sooner leading to higher levels of reproductive output (Gerlach, Hodgins-Davis, MacDonald, Hannah, 2007).

However, there is also evidence that cast doubt into the role of stability on brain structure. Fedorova, Evans and Byrne (2017) analyzed the dynamic of group stability in a family of woodpeckers who were taxonomically and ecologically homogenous, but at the same time exhibit a wide spectrum of social relationships that vary from solitary species, that only get together during the mating season, to those who live in in pairs or group for long period of time. The authors observed that individuals who lived in large groups for a long time had

smaller brains, suggesting that it is natural that group living allows for disinvestment in cerebral tissue.

Neuronal numbers and not brain size

Since the SBH was proposed, the main focus on the field has been the study of brain size in different relevant regions. Although this seems to be an important measure in the context of the theory, it proves to be insufficient to fully explain the impact that social complexity has on brain structure. The cleaner wrasse (*Labroides dimidiatus*) is a cooperative species that displays cleaning mutualism behaviour, in which individuals remove ectoparasites from client reef fishes. Nevertheless, conflicts arise when the wrasse prefers their clients' mucus instead of their ectoparasites, and this strategic behaviour of mucus feeding, constitutes as cheating in the cooperative context. In a study with this species, authors demonstrated that there were no significant differences in the size of different areas of the brain, when comparing with other related species (of the Perciformes order) that did not use strategic behaviour. This could be a sign that cleaners reacts to selective pressures for an increase of cognitive abilities with a restructuration at a neuronal level, instead of an abrupt increase of brain mass (Chojnacka, Isler, Barski & Bshary, 2015). Therefore, a method that gives more relevance to structural brain organization is needed (Oliveira, 2013).

The development of the isotropic fractionator (Herculano-Houzel & Lent, 2005) was a step forward to measure with precision the total numbers of neuronal and non-neuronal cells in different regions of the brain, leading to a variety of studies that showcased the relevance of this method in a variety of species so far, such as chimpanzees (Miller, Balaram, Young & Kaas, 2014), rats (Bandeira, Lent & Herculano-Houzel, 2009), mole rats (Kverková et al., 2018) birds (Olkowicz et al., 2016), guppies (Marhounová, Kotrschal, Kverková, Kolm & Němec, 2019), crocodiles (Ngwenya, Patzke, Manger & Herculano-Houzel, 2016) and humans (Azevedo et al. 2009).

Herculano-Houzel, Messeder, Fonseca-Azevedo and Pantoja (2015), in a sample of mice, showed that individuals with larger brains do not have more neurons, in comparison with individuals with smaller brains. Interestingly, a correlation was observed in all brain structures, between the number of neurons and the neuronal densities, i.e. when an individual

has an increased number of neurons, it was also observed an increase in the neuronal density (and as a consequence, a smaller neuronal cell size). The same thing happened to non-neuronal cells.

More recently, Marhounová et al. (2019), used this same technique in female guppies (*Poecilia reticulata*) and they demonstrated that large-brained individuals have a higher overall number of neurons compared with small-brained individuals, but neuronal densities are very similar, indicating that selection for brain mass leads to matching changes in number of neurons and ultimately, brain size evolution is linked to the evolution of neuron numbers and cognition. Moreover, the authors found that different regions of the brain had widely different glial/neurons ratio. This relation was higher in the diencephalon/brainstem (0.83), followed by the telencephalon (0.65), tectum (0.55) and cerebellum (0.27). These numbers not only give an idea of what kind of numbers are to be expected in a fish species, but the high ratio of neurons in the cerebellum also emphasizes the importance of this structure in teleost fishes in functions like motor coordination and cognitive processes.

Developmental effects on the Central Nervous System

The complexity of the social environment in brain evolution has been widely tested in adult individuals with the main focus on evolution. However, ontogeny can also influence the way environmental complexity affects an individual during the development, leading to different developmental programs in order to let individuals adjust to the available environmental cues.

Rich environments experienced in early life stages could have a role in neurogenesis, leading to an enhancement in cognitive ability (Garthe, Roeder & Kempermann, 2015) and brain structure (Zarif, Nicolas, Petit-Paitel, Chabry & Guyon, 2018).

Living in different environments throughout development could lead to vastly different results in subsequent cognitive ability. Variation in rearing environment for instance (i.e. adding stones to a standard rearing tank) has an impact in brain differences that are normally attributed to the result of selection (Kihslinger & Nevitt, 2006). DePasquale, Neuberger, Hirrlinger and Braithwaite (2016) observed that zebrafish (*Danio rerio*) that grew in a richer environment (one that constantly had new structures and objects added to their tank) had a larger overall brain, compared to those who grew in simpler environments. Also, zebrafish

that lived in a rich/mildly anxious environment as juveniles were faster than others at habituating to a novel environment in a behavioural learning test, suggesting that enrichment is also important for learning abilities. In a study with Atlantic salmons (*Salmo salar*), similar results were observed - enrichment effects were correlated with overall brain sizes. However, the effects gradually disappeared after the treatment, which could be a sign that effects of environmental complexity on the neural system may not be maintained over time. It is known that fish body and brain continually grow throughout adulthood, which means that, even though environmental deprivation during juvenile life could lead to a smaller brain, placing them in a more challenging environment could compensate the initial flaw in their lives (Näslund, Aarestrup, Thomassen & Johnsson, 2012).

A study of ontogeny in cooperative breeding cichlids also showed the existence of plasticity in their developmental strategies. This highly social cichlid lives in groups that normally consist of a breeding pair, its offspring, and immature and mature brood care helpers. These helpers help guard the brood against potential predators and they do not engage in a direct relationship with the offspring. When comparing between juvenile fish that grew with breeders and helpers, with only breeders, without breeders or without breeder and helpers, it was observed that young individuals that were raised with their family members (+F, both breeders and helpers) displayed more appropriate behaviours in response to their assigned social roles, than those raised without family members (-F). Those individuals that were raised around family members were also capable of adjusting their behaviour to a social context. For example, when in a role of a shelter owner, these fish used a more restrained and form of aggression by threat displays, which is the adequate response of a dominant individual to defend his resource in case the social challenge is moderate. Interestingly, in the role of an intruder, an +F fish acted more often in submission when attacked than –F intruders. These differences indicate that a +F individual behave in order to reduce the potential costs of contests (Arnold & Taborsky, 2010). These differences were also found in one other study from the same authors (Taborsky, Arnold, Junker & Tschopp, 2012), with an additional evidence that early environment was also influential in a situation that the fish has never faced before in their life – integration into a social group as a subordinate group member – which in turn, suggests that fish who grew around adult members are better prepared to generalize across social situations and select the more appropriate behavioural response. Whole brain expression was performed for stress and sex hormones. Different rearing environments (social group or pair) cause significant changes in the expression of

neuroendocrine genes, including ARα, ERα and GR1, and it was suggested that brain regions that express these genes, along with regions of the social decision-making network, are probably sensitive to early-life effects and could cause permanent changes in behaviour (Solomon-Lane & Hofmann, 2019).

In zebrafish, few studies actually approached the impact of ontogeny in adulthood. By mixing different strains (TM1 and Nadia lines) into one and having pure lines, on the other hand, a study found that exposure to a mixed-strained environment at an early age impacted biting and shoaling behaviour (Moretz, Martins & Robison, 2007).

All these studies provide evidence that development has a crucial role in individuals' adult behaviour. Also, an impact in brain structure seems to occur, although this relation has been scarcely analyzed until now, and confounding results pose the question of whether brain size is a reliable measurement of cognition.

Zebrafish as a model organism for cognition

Zebrafish (*Danio rerio)* can provide an excellent social model species to analyze social behaviour. They belong to the teleosts class, and the order of the cypriniformes. They are known for being highly social animals that live in groups with structured social relationships that include shoal living, dominance hierarchies, and territoriality (Oliveira, 2013). According to Dreosti, Lopes, Kampff and Wilson (2015), zebrafish larvae are already born with a tendency for being social animals, and after the third week of life they begin to strongly prefer to stay close to their conspecifics.

Social information is a key element for this species, as it gives them the chance of continually adjusting their behaviour to different situations in their social lives. This type of information can be of different kinds, such as; eavesdropping, where they eavesdrop other conspecifics in order to acquire social information about those individuals (e.g. to know who is the subordinate and the dominant as a result of fighting situation) (Abril-de-Abreu, Cruz & Oliveira, 2015); social buffering, the phenomenon in which individuals tend to have a better recovery from an aversive event when conspecifics are present. The sight of conspecific cues reduces their fear response to an alarm substance (Faustino, Tacão-Monteiro & Oliveira,

2017); and social memory, the ability to recognize fish who are familiar and unfamiliar to them, demonstrating the existence of long-term memory (Madeira & Oliveira, 2017).

Several brain areas seem to be related with the processing of social information in zebrafish. Stednitz et al. (2018) found that populations of forebrain neurons are necessary for social orientation. By performing a series of lesions at different dorsoventral locations of the telencephalon, it was found that the more lesions this area had, the more impaired was the social behaviour.

Also, Teles, Dahlbom, Winberg & Oliveira (2013), looked into how neuromodulators mediate changes in brain function underlying socially behavioural flexibility. Authors used a zebrafish aggressive behaviour paradigm and divided individuals into three types of interactions: (1) real opponent fight: the fish fought with a conspecific; (2) mirror fight: the fish fought with their own mirror image; (3) no fight: the fish had no agonistic interaction. From these three types of interactions, four experimental conditions were formed: winning the interaction, losing the interaction, fighting an unsolved interaction, or experience no interaction (control group). Serotonergic activity was observed to be significantly higher in the telencephalon of winners and optic tectum of losers. Dopaminergic activity was higher in the telencephalon of winners, leading to the possibility of the involvement of social reward. In the diencephalon, 5-HIAA (a metabolite of serotonin) was negatively correlated with aggressive behaviour, and dopamine positively correlated with submissive behaviour (in the real fight group). Moreover, the expression of aggressive behaviour was positively correlated with dopaminergic activity in the diencephalon in mirror fights. This suggests that there is an involvement of the diencephalic monoaminergic system in the regulation of aggressive and submissive behaviours in different social conditions. This hypothesis is further supported by the well-known role of different diencephalic nuclei in the regulation of species-specific behaviour across vertebrates. Both of these results point out the importance of forebrain areas, such as the telencephalon and diencephalon in the regulation of social behaviour.

Aims

The goal of this work is to study how social complexity influence brain development, by quantifying the number of neuron and non-neuronal cells, in five different brain regions from fish that grew in a gradient of environmental complexity.

Methods

Animals and brain collection

In order to create variation in the environmental complexity, group size (large and small) and group stability (stable and unstable) were manipulated in a factorial design producing four different treatments: small stable (SS), small unstable (SU), large stable (LS) and large unstable (LU). Large groups were composed by 12 individuals and small groups by 6 individuals. Group stability was manipulated on a weekly basis by swapping individuals between groups in order to disrupting social hierarchies and increasing the demand for individuals to adjust their behaviour to a changing social environment for the unstable treatments, whereas the stable treatments remain constant with the same group but were also manipulated weekly to control for handling stress. When animals reach adulthood (3-4 months), 52 Tuebingen zebrafish females (*danio rerio*) were collected from the different experimental tanks and used for neuronal quantification.

Animals were sacrificed, and their brains divided into six regions of interest (ROI), Olfactory bulbs (OB), Telencephalon (TL), Diencephalon (DE), Optic tectum (OT) Cerebellum (CB) and Brain stem (BS), placed in 4% PFA for 24 hours, and the regions weighted for future analysis. Due to the small size of zebrafish brain, for some ROI's the weight was not accurate, therefore, some values were replaced by the mean weight of the brain region in the corresponding experimental group (e.g. if the brainstem of an individual of SU group was negative, the number would be replaced by the mean weight of the brainstem of the SU group).

Isotropic Fractionator Method

In order to estimate the total number of neuronal and non-neuronal cells independently of tissue volume and anisotropy, a protocol developed by Herculano-Houzel & Lent (2005) was used. The Isotropic Fractionator Protocol is a fast, inexpensive, and reliable method that relies on the assumption that every cell in the brain contains one and only one nucleus. By transforming fixed and dissected brain structures into a homogeneous solution, in which

cytoarchitectural heterogeneities have been completely dissolved, one can count and identify cells morphologically and immunocytochemically as neuronal and non-neuronal. The total number of cells in the ROI is estimated by determining the density in small aliquots of suspension and then the proportion of neurons is calculated by doing an immunocytochemical detection of a nuclear antigen (NeuN), which is expressed in all nuclei of most neuronal cell types, but not in non-neuronal cells.

The protocol covers three major steps: tissue dissociation, total cell numbers counting and immunocytochemical identification of $NeuN^+$ cells.

1) Tissue collection and dissociation

Each sample was homogenized by mechanical friction, using a Wheaton 2ml Tenbroeck Tissue Grinder in a dissociation solution that dissolves the cell membranes while maintaining, the nuclear membranes intact, resulting in a suspension containing all cell nuclei of the structure in a graduated tube. In this process, the structure was removed from its original tube using a pair of stainless-steel forceps and placed on a paper to soak the excessive liquid surrounding the structure. Then, the structure was placed inside the homogenizer tube, with 500 µl of dissociation solution (1liter of 1% Triton XG100 in 40 mM sodium citrate). The piston was inserted in the homogenizer tube in a slow and controlled manner (to avoid the formation of foam) and homogenization of the tissue was done by making up-and-down and rotating movements with the piston until no tissue was visible. The resulting suspension was transferred to a new 1.5ml DNA lobind Eppendorf tube to avoid tissue deposits in the tube walls. The homogenization process is delicate, if it takes too long it can lead to cell destruction, and if it is short it can lead to the formation of several clumps of cells. The ideal amount of time to get free floating individual cells is situated between 15 to 20 minutes in the diencephalon, telencephalon and cerebellum. For the optic tectum and brainstem, an additional 5 minutes is required, due to a different texture type which seems to be harder to dissociate. (See Supplementary Figure 1 for work bench display with these materials).

2) DAPI counts

Samples were stained with 1ml/1mg diamino-phenyl-indol (DAPI), a fluorescence dye that emits a blue fluorescent stain when it binds to A-T regions of the DNA, therefore staining the nuclei of all cells. A Leica DMRA2 upright microscope was used to count the total cell numbers in each sample. To avoid the presence of cell clumps, nuclei suspension was made homogenous by inverting the graduated tube more than 20x times. After that, four aliquots of 10µl samples were collected with a pipette and placed into four different chambers of two hemocytometers, 0.0025mm^2 Neubauer Improved Brightline Chambers. After loading the chambers, two minutes were necessary to let nuclei sink in the chambers. Then, under a 40x 0.75NA objective, DAPI cells were counted. A total of 10 fields of the center grid were counted for each sample, and the four trials averaged to calculate the coefficient of variation (CV). If the CV was smaller than 0.15, the sample could proceed to the next step, if not, new aliquots had to be counted until that value was reached. Either four new trials were counted, or additional samples with the first ones were counted until the CV was lower than 0.15. The total number of nuclei for the ROI was then calculated by multiplying the number cells of nuclei per ml by the total suspension volume.

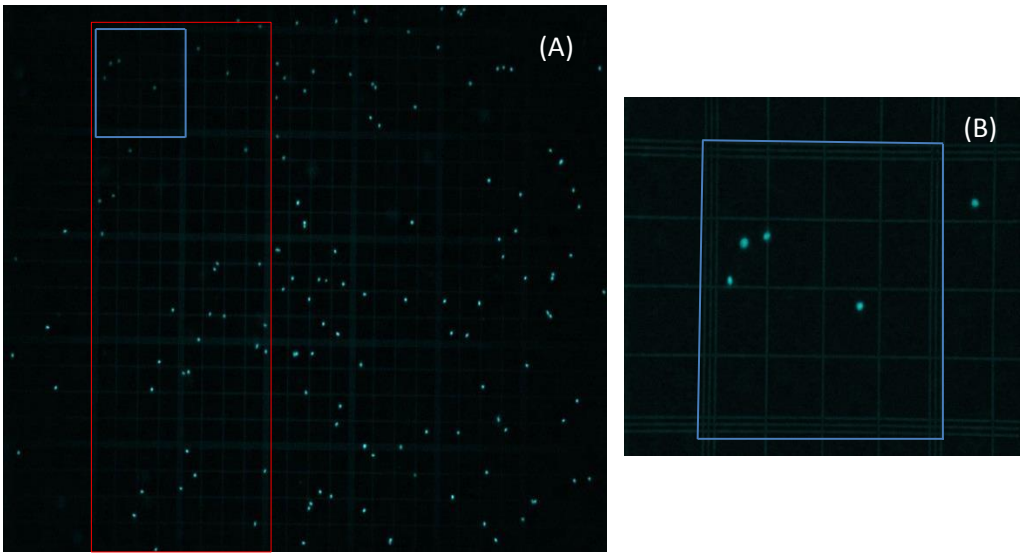

Figure 1. DAPI cells in the Neubauer central grid in a 10x and a 40x magnification. (A) The center grid is composed of 25 square fields, and the cells of the 10 first fields (red rectangle) are counted four times for each sample; (B) an individual field (blue square) taken from the center grid which contains 4 DAPI stained cells.

3) Immunocytochemistry for NeuN+

Neuronal nuclei were identified by the expression of the NeuN antigen. The neuronal nuclear protein (NeuN) is located in nuclei and perinuclear cytoplasm of most neurons of the central nervous system, and is commonly used as a biomarker for neurons. Therefore, an antibody for the NeuN protein was used to identify neuronal cells (however a small fraction of neuron cells, such as Purkinje cells do not express NeuN) (Gusel'nikova & Korzhevskiy, 2015). Immunocytochemistry is the technique used to detect the antigen of NeuN in the cells. Briefly, heat-induced epitope retrieval was necessary to break down the excess of aldehydes and the autofluorescence they cause. In this retrieval, the pellet was centrifuged for 10 minutes at 8000rpm, 4ºC, and 500µl of PBS was added to the pellet. Nuclei were centrifuged again, 5 minutes, 8000rpm at 4ºC, PBS was removed and 500µl of 0.2M boric acid, pH 9.0, was added to the pellet. After that, samples were incubated in a waterbath at 70ºC for 1 hour.

Then, the samples were centrifuged, the supernatant was removed and 500µl of PBS was added to the pellet for a second wash. After PBS removal, 500µl blocking solution (50ml PBS 1x, 0,5g Albumin Bovine Fractionator, and 150ml Tween20) was used to prevent non-specific binding of antibodies to the cells. Samples were then incubated in an Eppendorf Thermomixer 5350 Mixer for 30 minutes, 350rpm at room temperature. Samples were washed again to remove the blocking reagent, and a dilution of 1:100 was used for the anti-NeuN (rabbit polyclonal) Cy3 Conjugate (ABN78C3). Samples were incubated overnight (between 15h to 18h) in the thermomixer at 4ºC.

In the day after, samples were centrifuged for 5 minutes, the supernatant removed and 500µl PBS was added. This step was repeated twice, albeit the final volume was selected taking in account the type of ROI (i.e. regions with less remaining cells should have smaller volumes, in order to obtain higher cell concentrations when quantifying neuronal and non-neuronal numbers). Final volume of optic tectum was eluted in 150µl, cerebellum and diencephalon in a volume of 100µl, and the telencephalon and brainstem in a final volume of 50µl.

Quantification of NeuN+

In order to determine the percentage of neuronal/non-neuronal cells, a 10µl sample (enough to fill the entirety of the chamber) was collected from each sample and placed in the hemocytometer. Images were acquired in the Leica DMRA2, which is equipped with a CoolSNAP HQ CCD camera. A 20x 0.70NA objective was used to collect images of DAPI staining and $NeuN^+$ TRITC fluorescence filtersets, controlled by the MetaMorph V7.5.1 software. For each sample, the chamber was analyzed, and images collected to both filters, first the DAPI filterset (which indicates the number of total cells) and then on the TRITC filterset (which indicate the cells that are $NeuN^+$, i.e. that are neurons). The composition of both images gives a clearer idea of which cells are $NeuN^+$ or $NeuN^-$. Around 500 DAPI marked cells were acquired. After that, a macro, built in ImageJ, and validated by us, automatically counted the cells in the two channels; DAPI (non-neuronal cells), and TRITC (neuronal cells).

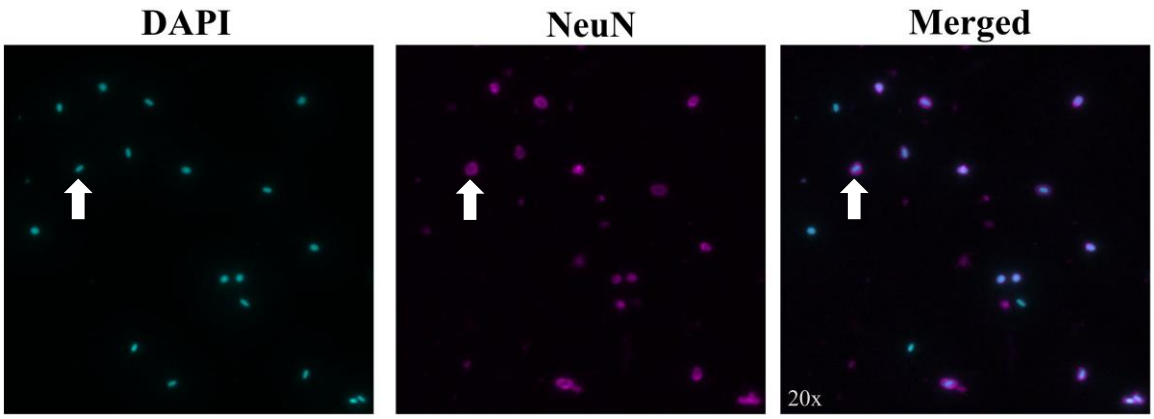

Figure 2. Images acquired, in a 20x 0.70NA objective, of an optic tectum sample. (A) DAPI stained cells in a DAPI channel, (C) $NeuN^+$ cells in a TRITC channel, and (C) Composition of both channels.

Statistical Analysis

Distribution of mass and cell numbers were calculated with Microsoft Excel 2010, by calculating the average, standard deviation, lowest and highest number of the samples of all groups.

Statistical analyses were conducted in the R software 3.5.2 (R Core Team 2015). Linear mixed models (LMMs) were used to test for differences between environmental treatments (small stable, small unstable, large stable and large unstable) and brain regions (Telencephalon, Diencephalon, Optic tectum, Cerebellum and Brainstem), with random effects for the subjects, nested with the treatment tank number. To assess the parametric assumptions of the mixed-effects models, plots of the residuals were used and the best model selected by stepwise backward elimination of nonsignificant effects, starting from the full model. Results are presented using the best-fitting model parameters and multiple comparison analysis were then used (Tukey post-hoc test) to evaluate specifically in which conditions were the differences observed. For all tests the significance level used was $p < 0.05$.

Results

41 zebrafish (9 individuals with missing values were excluded in this descriptive analyses) weighted between 0.34g and 0.67g (*M* = 0.51, *SD* = 0.059), their brain mass ranged between 1.9mg and 8.5mg (*M* = 4.89, *SD* = 1.20).

Total cell numbers ranged between 1.70 and 4.47 million cells (*M* = 3.06m, *SD* = 0.526m). Brains contained between 739 thousand and 2.84 million neurons (*M* = 2.11m, *SD* = 0.395m) and between 437 thousand and 2.21 million non-neuron cells (*M* = 0.955m, *SD* = 0.333m).

Neuronal density ranged between 1.58 and 6.76 million neurons per mg (*M* = 3.01m, *SD* = 1.37m) and non-neuronal density ranged between 0.46 and 5.31 million cells per mg (*M* = 1.30m, *SD* = 0.86m).

Neuronal cell numbers ranged between 1.64 and 2.84 million neurons per mg (*M* = 2.20m, *SD* = 2.89m) and non-neuronal cell numbers ranged between 0.43 and 2.21 million cells per mg (*M* = 0.93m, *SD* = 0.31m).

Glia/neurons ratio for the whole brain (excluding the olfactory bulb) was 0.424, the telencephalon had a 0.453 ratio, 0.566 for the diencephalon, 0.427 for the optic tectum, and 0.329 for the cerebellum. (detailed analysis in Table 1).

Table 1. Distribution of mass and cells in the zebrafish brain.

Structure	Mass (mg)	Nº Neurons	Neuronal Density (N/mg)	Nº Non-neurons	Non-neuronal density (N/mg)	Glia/neurons ratio
Whole Brain (without OB)	4.89 ± 1.20	2.20 x 106 ± 2.89 x 105	3.01 x 106 ± 1.37 x 106	9.33 x 105 ± 3.13 x 105	1.30 x 106 ± 8.62 x 105	0.424
Telencephalon	0.89 ± 0.48	1.88 x 105 ± 5.29 x 104	3.28 x 105 ± 3.30 x 105	8.52 x 104 ± 3.77 x 104	1.37 x 105 ± 1.16 x 105	0.453
Diencephalon	0.99 ± 0.62	4.01 x 105 ± 1.56 x 105	5.78 x 105 ± 4.49 x 105	2.27 x 105 ± 1.96 x 105	3.27 x 105 ± 3.68 x 105	0.566
Optic Tectum	1.13 ± 0.49	5.93 x 105 ± 1.56 x 105	7.13 x 105 ± 7.25 x 105	2.53 x 105 ± 1.32 x 105	3.34 x 105 ± 4.48 x 105	0.427
Cerebellum	0.89 ± 0.47	8.67 x 105 ± 1.87 x 105	1.17 x 106 ± 7.07 x 105	2.85 x 105 ± 1.10 x 105	3.74 x 105 ± 2.39 x 105	0.329
Brainstem	0.99 ± 0.47	1.51 x 105 ± 6.00 x 104	2.23 x 105 ± 2.24 x 105	8.27 x 104 ± 4.39 x 104	1.27 x 105 ± 1.42 x 105	0.548

Total number of neurons

A main effect of the total number of neurons was observed in the different ROI's (LMM, $F_{4,168} = 253.9$, $p < 0.001$). Every contrast between regions was significantly different (all $p < 0.001$) (Figure 3, (A)). Also, a main effect was detected for group size (LMM, $F_{1,46} = 10.4$, $p = 0.002$), as large groups had significantly more neurons than the small group (Figure 3, (B)).

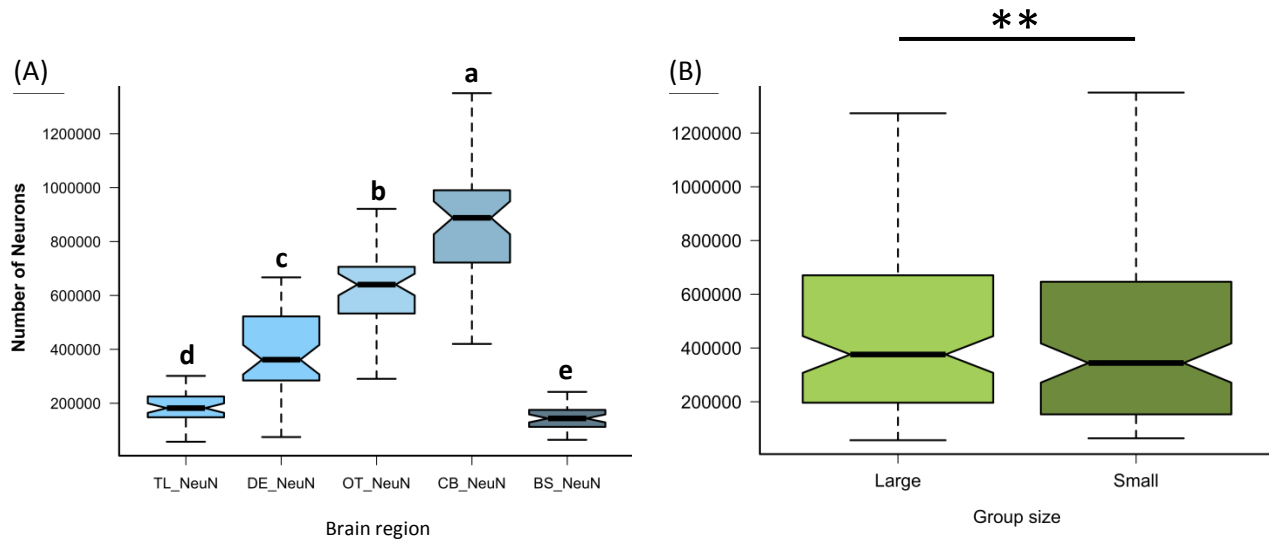

Figure 3. Graphs displaying the total number of neurons in the (A) brain regions and (B) between group sizes. The statistical significance level in all box plots is indicated as follows: *** $p < 0.001$, ** $p < 0.01$; * $p < 0.05$. For brain regions, letters were used to denote differences. If two regions are significantly different from each other, their letters are different.

Non-neuronal cells

A main effect was observed for brain regions (LMM, $F_{4,175} = 74.25$, $p < 0.001$), with the telencephalon total number of non-neuronal cells being significantly lower than the diencephalon, optic tectum and cerebellum ($p < 0.001$). Moreover, the diencephalon was significantly higher than the brainstem and lower than the cerebellum ($p < 0.001$). Finally, the brainstem was significantly lower than the cerebellum, diencephalon and optic tectum ($p < 0.001$) (Figure 4, (A)).

An interaction effect was detected between group size and group stability (LMM, $F_{1,44} = 4.08$, $p = 0.049$), however contrasts didn't show any significant differences (Figure 4, (B)).

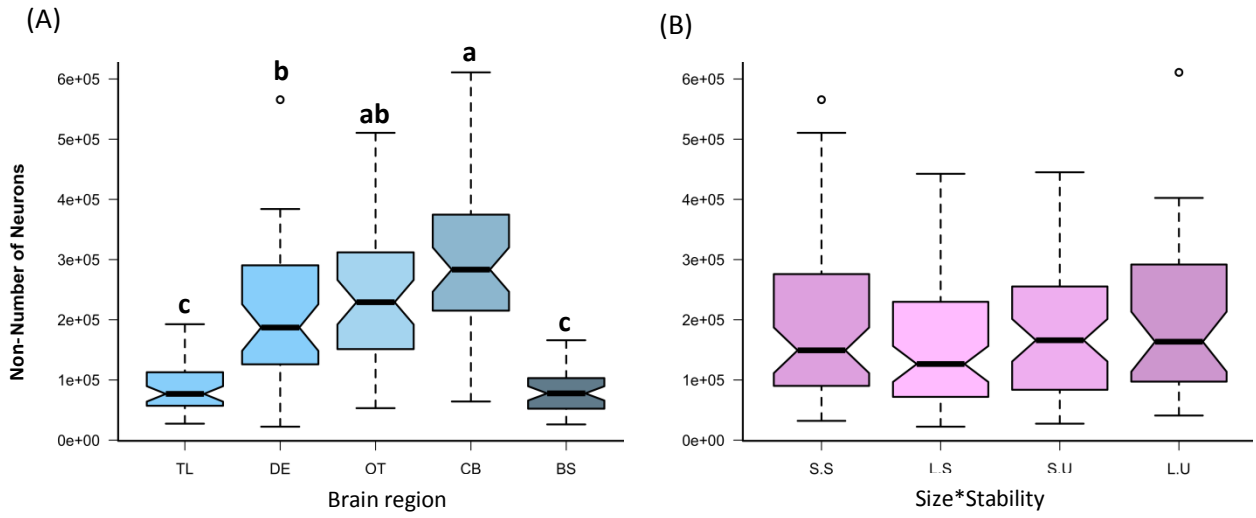

Figure 4. Graphs displaying the total number of non-neurons in (A) the several brain regions and (B) interaction between size and stability of groups.

Neuronal density

A main effect was observed for the brain region (LMM, $F_{4,159} = 84.69$, $p < 0.001$). Differences were found in the comparisons between all groups (always $p < 0.001$), except between the telencephalon and brainstem ($p = 0.086$), and the diencephalon and optic tectum ($p = 0.232$). Across all groups, the cerebellum was the region with a higher neuronal density, followed by the optic tectum, diencephalon, telencephalon and brainstem (Supplementary Figure 2).

A main effect was detected for group size (LMM, $F_{1,45} = 16.79$, $p < 0.001$), as the neuronal density in the larger group was significantly higher than the one presented in the smaller one ($p < 0.001$) (Supplementary Figure 3).

There was an interaction effect between size, stability and brain region (LMM, $F_{4,159} = 3.20$, $p = 0.015$). A Tukey post-hoc test showed that neuronal density had differences in the diencephalon between the SS and SU group ($p < 0.001$) (Figure 5). There was no interaction effect for group size and brain region (LMM, $F_{4,159} = 1.69$, $p = 0.154$), however interactions were observed between group stability and group size (LMM, $F_{1,45} = 6.61$, $p = 0.013$) and group stability and brain region (LMM, $F_{4,159} = 5.45$, $p < 0.001$). In the neuronal density interaction between group stability and group size, SS group was significantly higher than: LS ($p < 0.001$), SU ($p = 0.034$), and LU ($p = 0.012$) (Figure 6). For the interaction between group stability and brain region, there was an effect in the diencephalon between stable and unstable groups, with the stable treatment presenting higher neuronal density than the unstable one ($p < 0.001$) (Figure 7).

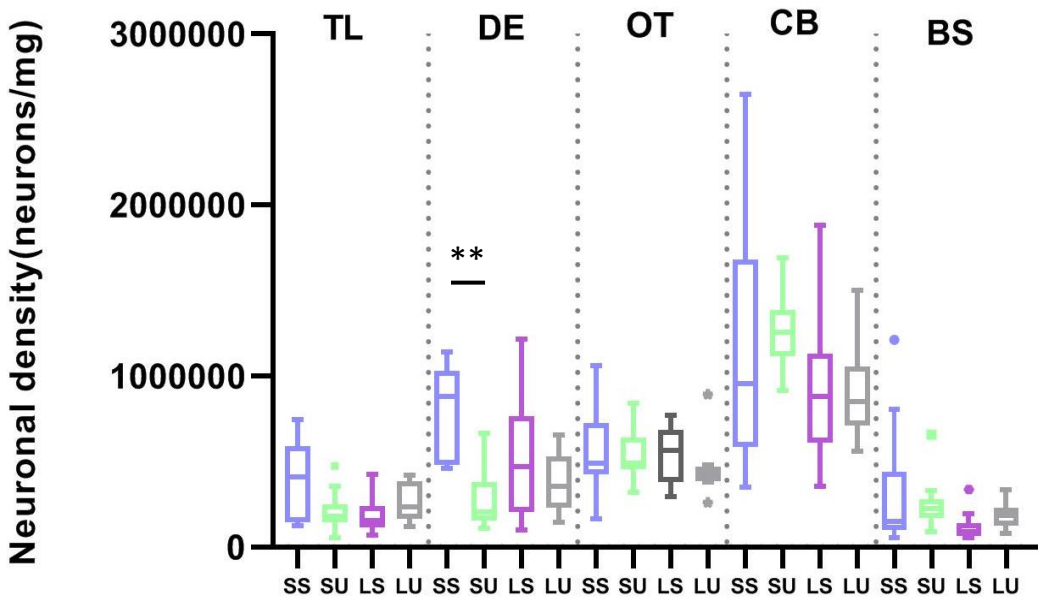

Figure 5. Graph displaying the neuronal density interaction between region of the brain, stability of group and size of group.

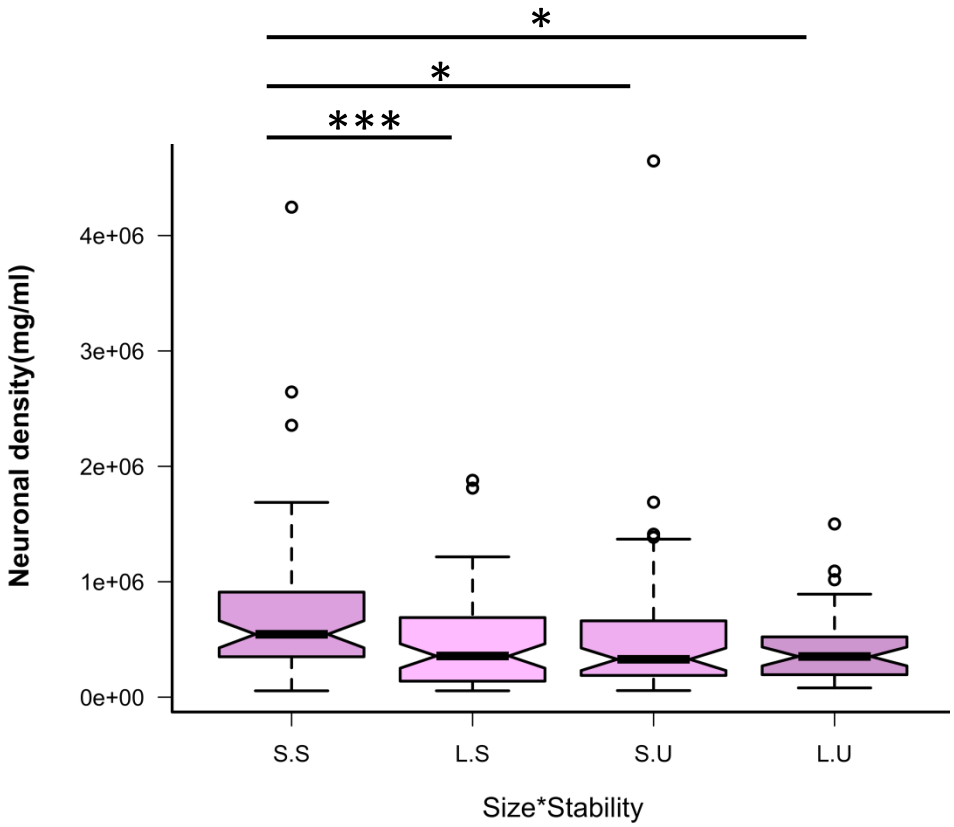

Figure 6. Graph displaying the neuronal density interaction of size of group with stability of group.

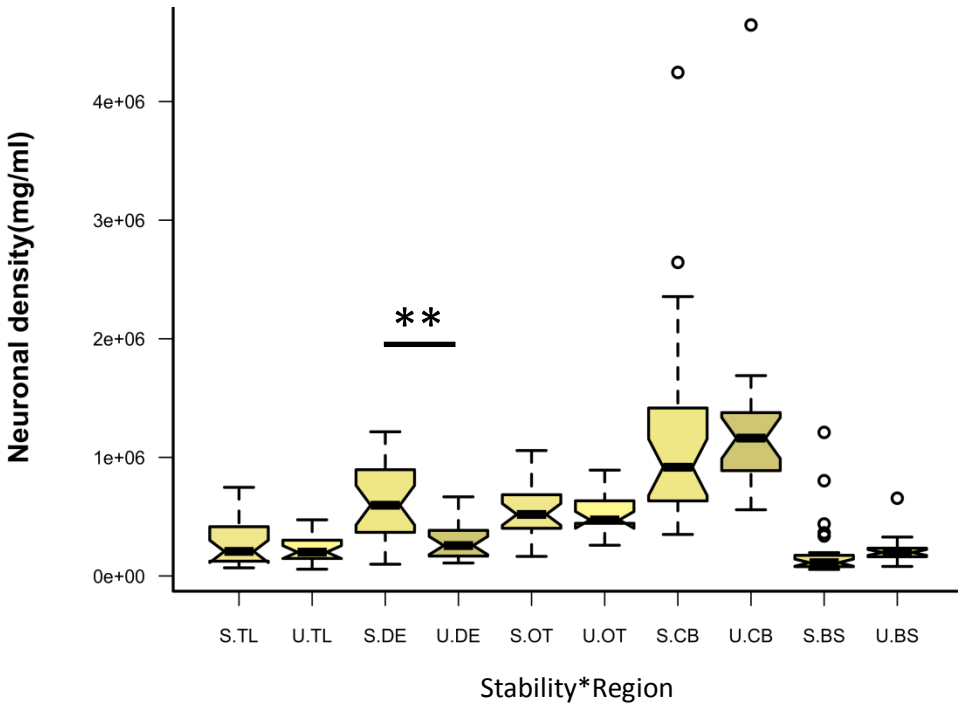

Figure 7. Graph displaying the neuronal density of the interaction of stability of group with region of the brain.

Non-neuronal density

A main effect was observed in the group size (LMM, $F_{1,45} = 5.02$, $p = 0.03$) (Supplementary Figure 4), and for brain region with differences observed between all groups, except for the diencephalon – optic tectum comparison ($p = 0.992$) and the telencephalon – brainstem ($p = 0.173$) (Supplementary Figure 5)

There was also an interaction effect for size, stability and brain region (LMM, $F_{4,152} = 3.09$, $p = 0.018$), albeit only one almost significant difference was observed in diencephalon between the SS and SU group ($p = 0.052$) and SS and LS groups (0.098) (Figure 8).

As it was observed for the neuronal density data, there was also no interaction between the group size and brain region (LMM, $F_{4,152} = 0.71$, $p = 0.587$), however interactions were observed for group stability with brain region and group stability with group size). An effect for group stability and brain region was found (LMM, $F_{4,152} = 4.49$, $p = 0.002$), the brainstem of the individuals of the unstable group was significantly higher than the ones in the stable groups ($p = 0.010$) (Figure 9). In the interaction between group stability and group size (LMM, $F_{1,45} = 13.63$, $p < 0.001$), SS and LU was significantly higher than the LS ($p = 0.002$, $p = 0.003$) respectively (Figure 10).

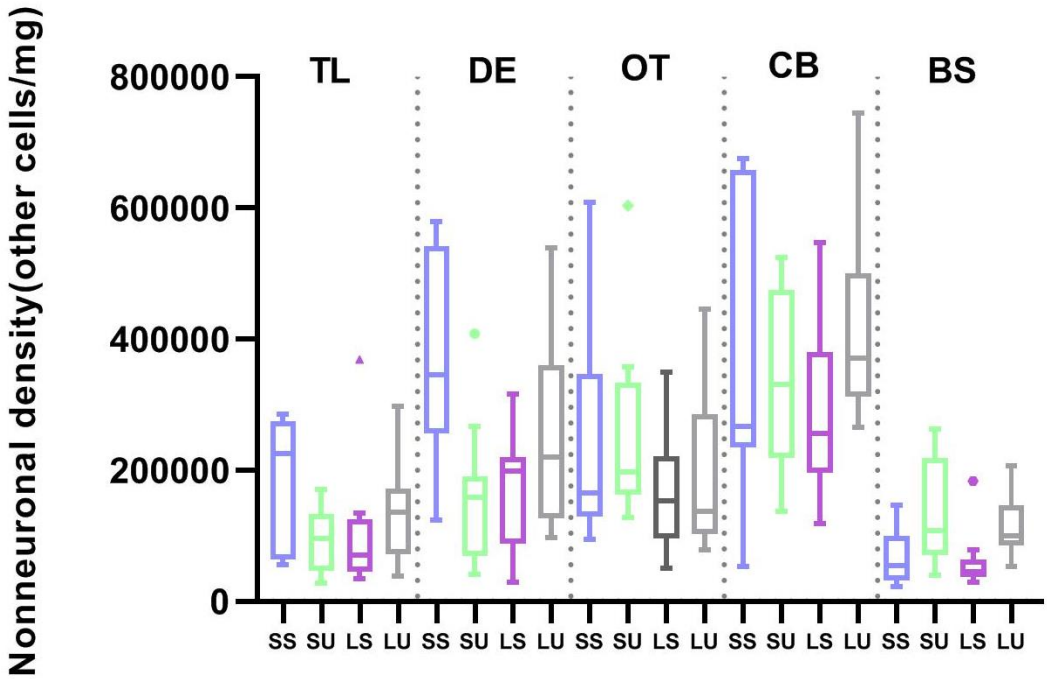

Figure 8. Graph displaying the non-neuronal density interaction between region of the brain, stability of group and size of group.

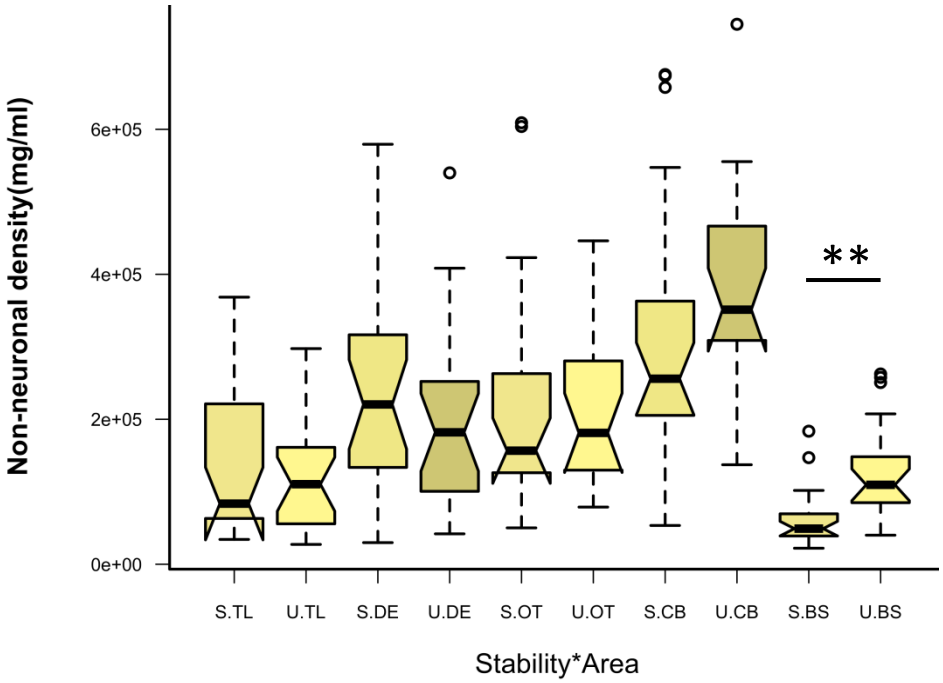

Figure 9. Graph displaying the non-neuronal density interaction of stability of group and brain region.

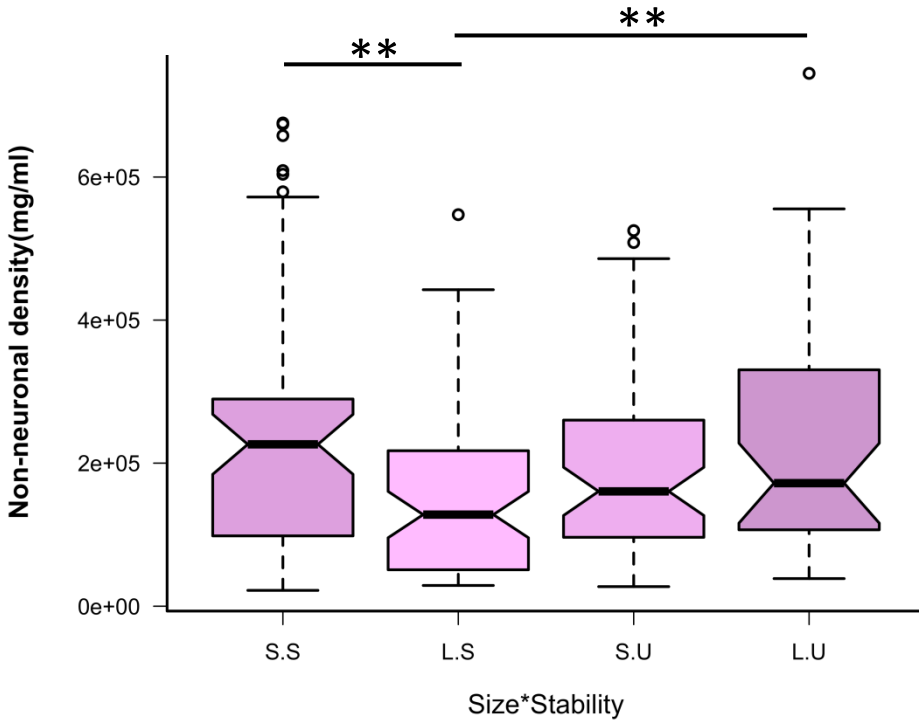

Figure 10. Graph displaying the non-neuronal density interaction between size of group and stability of group.

Neuronal Index

For the neuronal index (glia/neurons), only a main effect of the brain region was observed (LMM, $F_{4,157} = 14.082$, $p < 0.001$). The cerebellum index was significantly different from the telencephalon ($p = 0.0049$), diencephalon ($p < 0.001$) and brainstem ($p < 0.001$), and also significant differences were found between the optic tectum and diencephalon and brainstem (both $p < 0.001$) (Figure 13).

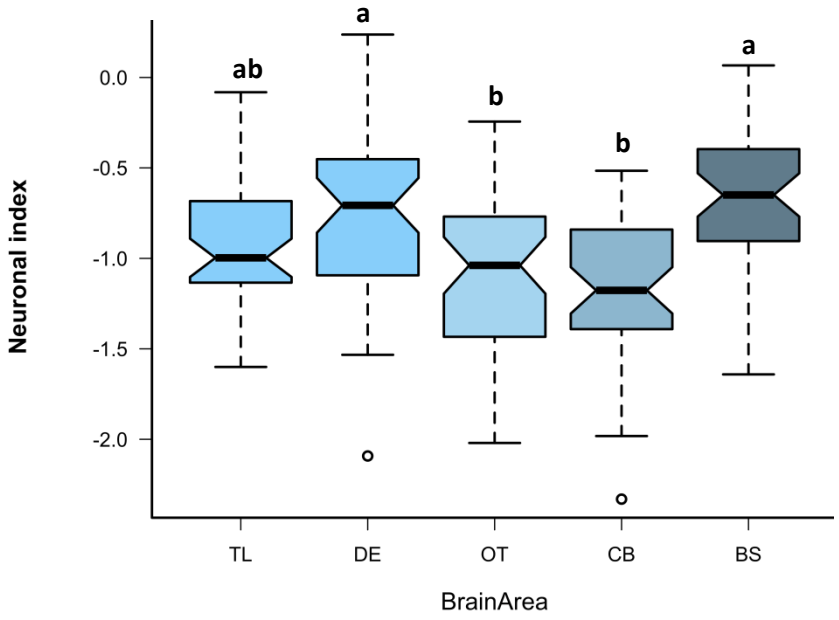

Figure 11. Neuronal index (Non-neuronal density/neuronal density) of the different regions of the brain.

Discussion

These results show that the environmental social complexity that an individual experience through his life has an influence on brain development. It is striking that by manipulating the social environment of individuals, their number of neurons change, which indicate that different developmental neurological programs are employed in different environment situations.

Higher density of neurons in the SS group

The SS group presented a significantly higher overall neuronal density, in comparison with the SU, LS, and LU group. Additionally, in the non-neuronal density, SS and LU groups also have greater densities than the LS group. In the SS group, individuals have to deal with the same group of individuals for the duration of three months, and maybe maintaining social cohesion and controlling aggressive behaviour towards conspecifics might be the main priority for the survival in the group. This, in turn, can affect cell numbers. It has been observed that the pairbonded type of relationship, which both consolidate the characteristics of being a small group (as it is composed of a dyad – two elements), and it is also stable (is long term relationship), could be more cognitively demanding because it requires that an individual should be able solve to conflicts (Shultz & Dunbar, 2007). Living in a stable group is also proven to be advantageous in a fear context as individuals recover faster of a fear induced episode if they surrounded by familiar conspecifics (Mathuru et al., 2017) and also being in kin groups helps juvenile fish grow rate (Gerlach et al., 2007).

Diencephalon neuronal density is higher in stable groups

The diencephalon neuronal density was higher in the stable groups in comparison to the unstable ones. Interestingly, when observing the density of non-neuronal cells of individuals, the SS group also had a tendency for a significant difference in the diencephalon with the SU group.

The diencephalon is a region where an interaction between the sensory, central, and endocrine systems occurs, and it seems to be a crucial element in fish social behaviour. This is one of the most elaborate structures in the vertebrate brain and, in zebrafish, is composed mainly by the prethalamus, thalamus, epithalamus (which contains the habenula), the pretectum, hypothalamus and the preoptic area (which is located in the intermediate region between the telencephalon and the diencephalon) (Puelles & Rubenstein, 2003). As it was mentioned previously, it has been demonstrated that the diencephalic monoaminergic system is involved with the regulation of aggressive and submissive behaviour in zebrafishes. 5-HIAA – metabolite of serotonin – is negatively related to submissive behaviour, and expression of aggressive behaviour is positively correlated with dopaminergic activity on individuals fighting a mirror (Teles et al., 2013). Also, when clients of cleaner fishes are exposed to either a cleaner or a conspecific, differences are noticed on the monoaminergic activity of some brain regions. 5-HIAA activity, for instance, is higher in the telencephalon and diencephalon when there is an exposition to a cleaner, instead of a conspecific. Additionally, a higher serotonergic activity in the diencephalon occurs in the duration of an interaction with a cleaner, similarly to what happens to a fish after the loss of a contest (Abreu, Messias, Thörnqvist, Winberg & Soares, 2018).

Looking at the individual structures that form the diencephalon, evidences demonstrates their importance in the regulation of social behaviour. The habenula – part of the epithalamus – is a region that is capable to control stressors, and whose disruption has been observed to contribute to anxiety disorders. When neural circuitry involving the dorsal habenula is disrupted, a deficit in active avoidance is caused in larval zebrafish. (Lee et al., 2010). This structure is divided by the lateral dorsal habenula (ldHb) and the medial dorsal habenula (mdHb). When inactivating both the ldHb (Agetsuma et al. 2010) and mdHb (Mathuru & Jesuthasan, 2013) separately, fish seem to always biased toward freezing rather than acting in a normal flight response to a conditioned fear stimulus. In the mdHb case, as more shocks are given to the fish, freezing decreases in control fish, but not in habenula lesioned fish.

Endothelins, a 21 aminoacid peptide neurotransmitters, act as both neurotransmitters and neuromodulators in CNS, more precisely, in the preoptic area of the anterior hypothalamus, which is part of the diencephalon. Gutiérrez et al. (2019) investigated the connection between endothelins and social behaviour in zebrafish. They discovered that zebrafish harboring a mutation in the endothelin receptor aa (ednraa) presents impairments in social behaviour. The loss of this receptor leads to higher levels of aggression and less social cohesion (higher

distance to nearest neighbors) when shoaling, suggesting that these individuals might display a dominant behaviour phenotype. This strong decrease in social interaction was also not dependent upon the number of individuals on the group, but the size of the test arena and the amount of time spent interacting, which indicates that mutants maximize their social spacing, and avoid other conspecifics. It was shown that adult mutants have a reduction in parvocellular arginine vasopressin (AVP) positive neurons in the preoptic area and an increase of the size of magnocellular AVP neurons. AVP concentration in the brain is known to be connected with sociality and social recognition, and its levels are usually lower in the brains of dominant fish in several species. Diencephalon differences between the stable and unstable groups that were found in our study might be related to the function in certain subregions of the structure, especially, the preoptic area and the habenula.

Contrary to what we hypothesized, no differences between groups were observed in the telencephalon, a structure known to be involved in the expression of social behaviour. For example, series of lesions at different dorsoventral locations of the telencephalon have been shown to impair social orienting behaviour (Stednitz et al., 2018).

Brainstem of unstable groups

Curiously, when looking at non-neuronal density, the brainstem of individuals in the unstable group was observed to contain a higher density than the individuals in the stable group. One recent interesting finding that links the role of glial cells in the brainstem with the switching of behavioural states in zebrafish might shed some light to these results. Mu et al. (2019) revealed that noradrenergic neurons encode swim failures and then radial astrocytes accumulate these failures signal and trigger passivity, because actions are futile and a behavioural switch is better strategy to, for example, conserve energy. This behavioural pattern has a familiar combination of features: trying to achieve a goal (fish swimming vigorously), failing several times despite trying harder (continuous ineffective swim attempts), giving temporarily (becoming passive), and then, later trying again (swim again). This futility-induced passivity belongs to a group of behaviours in which motor output is reduced, such as helplessness, passive coping, feigned death (which is an anti-predator behaviour), and break-point behaviour. A possible connection could be established with unstable groups in our study. Although this particular behaviour was not observed in

individuals of our unstable groups (nor in the stable), a similar cognitive process might also trigger a development of larger glial density. Glial cells seem to also have its own role in behaviour, not only neurons as it was once thought. However, the importance of glial cells (and the role of the brainstem) should be analyzed and explored more carefully in future studies.

Zebrafish brain in neuronal distribution and glial/neurons ratio

This study was also important to explore the distribution of neurons and non-neurons in the zebrafish brain. A similar pattern seems to unfold in both the number of neurons and non-neurons in the brain. The cerebellum is the structure that holds a higher number of neurons in the brain, followed by the optic tectum, diencephalon, telencephalon and brainstem. Considering previous studies, this was expected. While Marhounová et al. (2019) demonstrated, in female guppies, that the glial/neurons ratio was higher in the diencephalon/brainstem (0.83), followed by the telencephalon (0.65), tectum (0.55) and cerebellum (0.27), in our study, the ratio was also higher in the diencephalon (0.57), and brainstem (0.55), followed by the telencephalon (0.45), optic tectum (0.43), and the cerebellum also had clearly the lowest ratio (0.33). Even though the numbers between both studies have some variability among structures, a similar pattern is visible for small fish species. As an interesting comparison with humans, an adult male brain contains on average 86.1 billion of neurons and 84.6 billion of non-neurons, giving a glial/neurons ratio of 0.98, which seems distant from the number in zebrafish (0.51).

Conclusion and future direction

According to our results, different types of social complexity during development do indeed affect brain structure. Social brain areas, especially the diencephalon, are mainly affected by the variability of different group structures. It seems that group stability and group size change the way neuronal and non-neuronal cells numbers are organized throughout the brain.
In the forebrain, typically, more focus has been given to the telencephalon and its substructures, and the diencephalon is often seen as a secondary structure involved in social

behaviour. Our study indicates that, at least for the influence of the environmental complexity, this structure is very important and a higher priority should be given in future studies.

These results indicate that developmental plasticity is at play here. That is, different phenotypes result from the regulation of abiotic factors. For example, insect pigmentation has a role in thermal regulation and on visual communication with partners and, in particular, butterfly eyespots have a role dedicated to predation avoidance. This phenotype affects an individual performance in relation to its environment and also is affected by the environment conditions that individuals experience throughout development. In the case of the variation of butterfly eyespots, the abiotic factors are not from a social origin (as it happens in our study), but from an ecological origin because it is affected by factors such as temperature. However, in both cases, the same genotype can lead to distinct phenotypes (in our case neuronal restructuration) that are better adapted to the environmental conditions. (Beldade & Peralta, 2017).

The nuances of social behaviour and a wide range of social complexities can be tested using this method, and future approaches should proceed to explore how different forms of social behaviours like different shoaling structures affect brain structure. However, it's important to keep in mind that, in order to understand the full picture of a certain phenomenon, one should also look to chemical signals that underlie certain behaviours (e.g. neurotransmitters), as those might explain how structures of brain change as a response to the social world.

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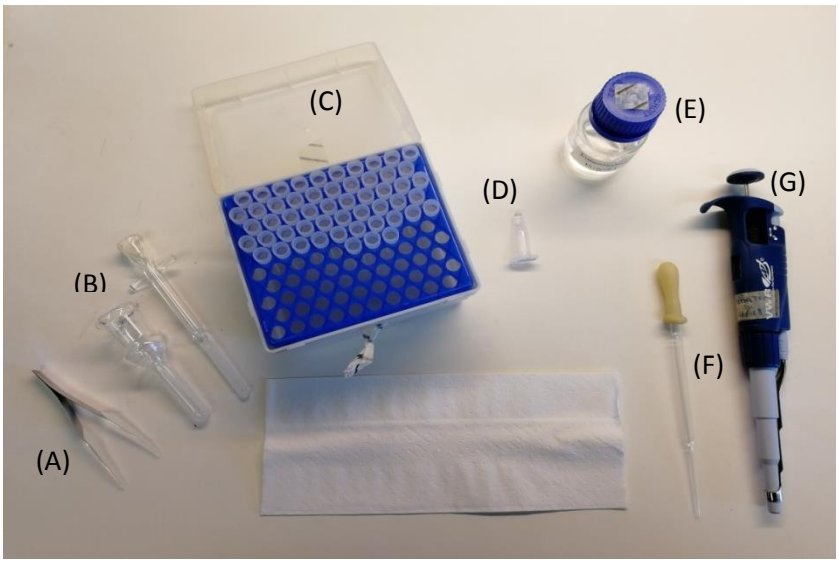
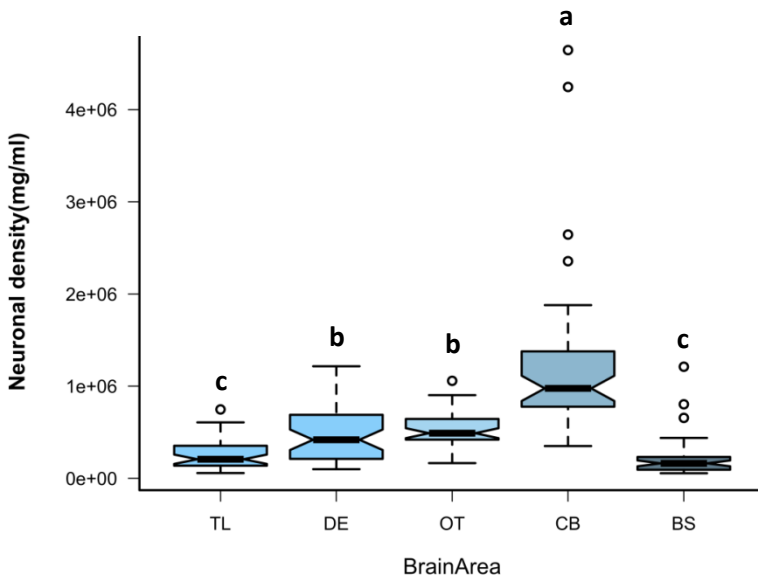
Attachments

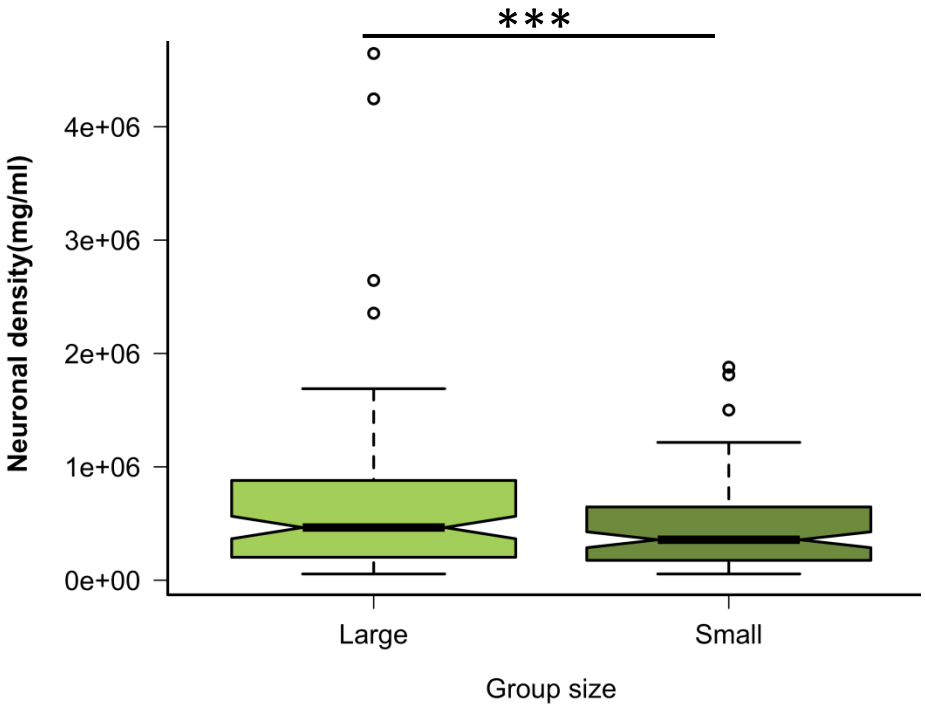
Supplementary figures



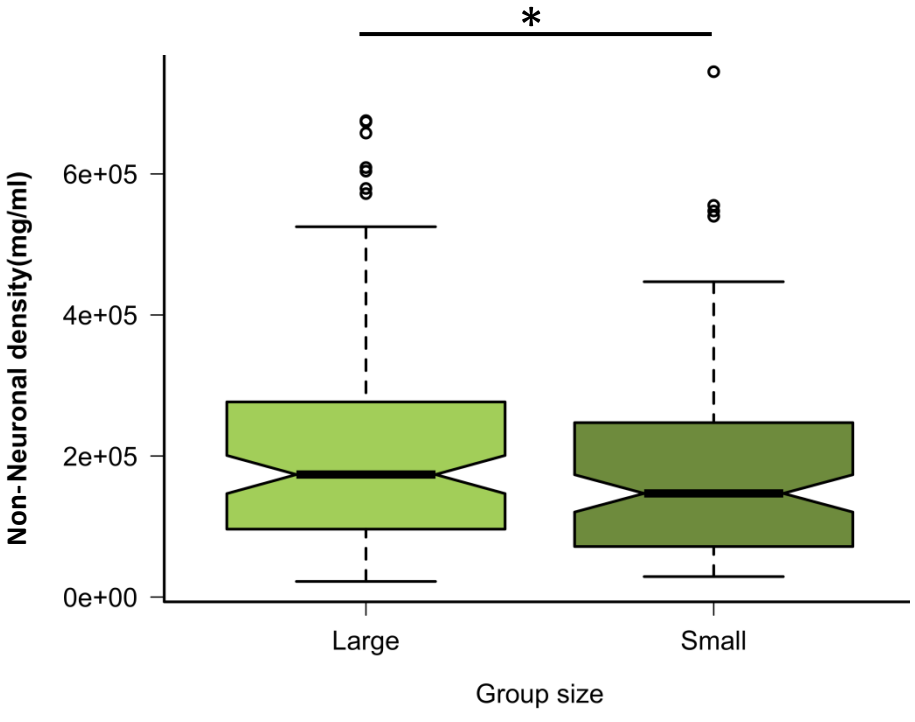
Supplementary Figure 1. Display of materials used in the tissue dissociation: (A) FST Forceps, (B) 2ml Wheaton Tenbroeck Grinder, (C) Tips, (D) DNA lobind 1.5ml Eppendorf tube, (E) Dissociation Solution, (F) VWR 150mm glass Pasteur pipette, (G) VWR Signature Ergonomic Single Channel Pipette 100µl - 1000µl.



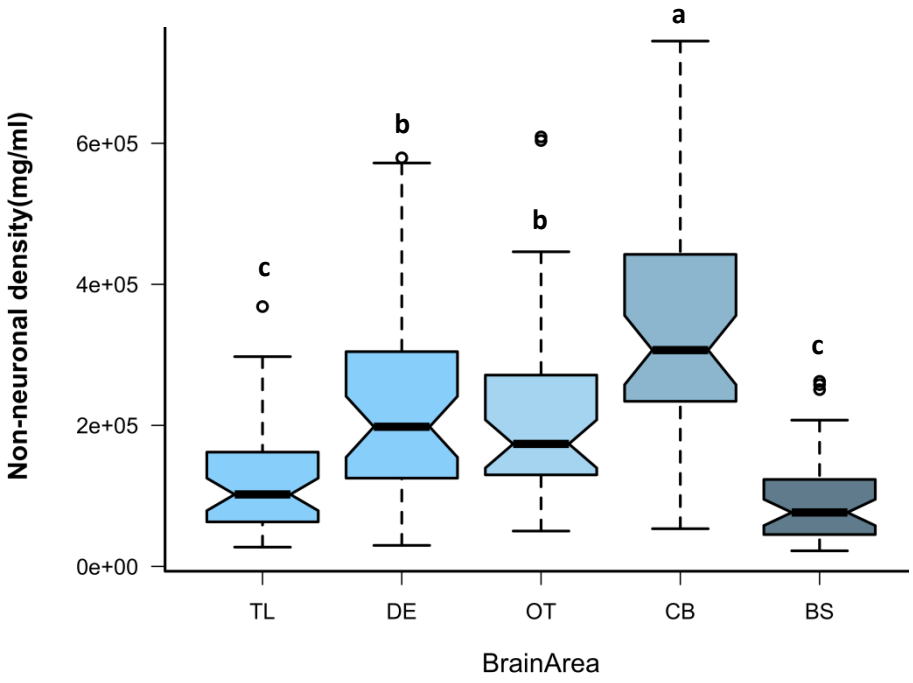
Supplementary Figure 2. Graph displaying the neuronal density in the several brain regions.



Supplementary Figure 3. Graph displaying the neuronal density by group size type.



Supplementary Figure 4. Graph displaying the non-neuronal density of the group size types.



Supplementary Figure 5. Graph displaying non-neuronal density between different brain regions.