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Neural mechanisms involved in visual learning in the cichlid fish *Pseudotropheus zebra*

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To my family,
*especially to my **wonderful** mom.*

Abstract

Over the last few decades, it has been shown that fish possess many cognitive abilities comparable to those of other vertebrates. Although several studies have investigated the cognitive abilities in fish, not so much is known about where the cognitive information is processed in the brain.

There are some information about areas involved in spatial and emotional memory, but the location of basic objects recognition and object memory areas need further investigation. For this PhD thesis I assessed visual learning and its neural substrates in the cichlid fish *Pseudotropheus zebra*. The aim of the project was to investigate brain areas involved in general visual learning tasks like object recognition and object memory.

The thesis contains three result chapters. The first chapter ("Brain areas activated during visual learning in the cichlid fish *Pseudotropheus zebra"*) has already been published in Brain Structure and Function. Immunohistochemical analysis of the pS6 expression in the fish brain was used to investigate the activation of areas associated with different sensory, motor and cognitive functions. In particular, the expression was measured in 19 brain areas of 40 individuals, and compared among 4 treatment groups, i.e. control, avoidance, trained and novelty groups, subjected to different behavioral situations. Although the behavioral tasks differed among the groups, common to all experimental groups, except the control, was a visual learning component. Results showed that different brain areas were active in response to the different treatments, but the nucleus diffusus of the inferior lobes was the area where a consistent activation of pS6 was observed in all three experimental groups compared to the control. It is known that, in fish, the optic tectum sends visual input via the nucleus glomerulosus to the inferior lobes, but nothing was known about the functional details of this pathway. My results indicate that the inferior lobes play a role in visual learning, particularly object recognition and memory.

The second chapter ("Activation Patterns of Dopaminergic Cell Groups Reflect Different Learning Scenarios in a Cichlid Fish, *Pseudotropheus zebra"*) has already been published by Journal of Chemical Neuroanatomy. For this study, I investigated the activation of dopaminergic cell groups in fish subjected to three behavioral contexts (control – avoidance – trained) by co-labeling tyrosine hydroxylase TH and pS6. The activation of the different dopaminergic cell groups was correlated with the different behavioral situations in the experimental groups. The preoptic-hypothalamic cell population responded to the stress component in the avoidance group. The raphe dopaminergic cell group was activated in both trained and avoidance groups, probably correlated with attention or arousal component present in both groups. A weak activation of the

periventricular pretectal nucleus was also present in both groups. This cell population projects to the optic tectum and may modulate tectal circuitry. The dopaminergic cells of the nucleus of the posterior tubercle, which projects to the inferior lobes, showed activation in both avoidance and trained groups and may be related to the strong activation of the inferior lobes in both groups. Finally, the co-activation patterns across all dopaminergic cell groups revealed robust differences across experimental groups, largely driven by cells in hypothalamic and midbrain regions, possibly encoding the valence and salience associated with the different stimuli in the three groups.

For the last chapter ("New neurons and reorganization of existing connections. Understanding the distribution of egr-1 and pS6 in the brain", submitted to Brain Research and currently under review), I compared the expression of the two most commonly used brain activity markers, i.e. the immediate early gene (IEG) egr-1, and pS6, in stimulated (avoidance group) and control animals. IEGs are cellular genes that are responsive to extracellular stimuli. They are first response genes, whose expressions are initiated immediately after stimulation in the absence of new protein synthesis. In contrast, pS6 is acting on the translation level. Both, the IEGs and ps6 are used as general activity markers, irrespective of their different intracellular functions. My comparison of egr-1 and pS6 stained areas in the brain revealed an important difference in their staining patterns. Egr-1 was exclusively associated with proliferation zones where new cells are generated and mature into functional neurons. PS6 was present also in many other areas far away from proliferation zones and may indicate increased synaptic plasticity in existing neurons. That would mean that egr-1 and pS6 can be used not only to study general activation of brain areas, but also to discriminate between two different learning mechanisms, memory formation due to addition of new neurons, and learning due to the modification of synaptic reorganization in existing neurons.

In summary, the obtained results provide important advances in understanding of the underlying neural substrates of fish cognitive behavior. The inferior lobes appear to be an important substrate for visual learning. Specific dopaminergic cell groups may control visual learning and memory in different areas of the brain and a comparison of the distribution of egr-1 and pS6 strongly suggests the existence of two different and separate mechanisms that contribute to visual learning and memory.

The results of my thesis open the way for further investigation. In particular, it is still not clear how the visual information from the optic tectum is processed on its way to the inferior lobes to allow object recognition. Furthermore, how the dopaminergic system is modulating object recognition and memory formation in the inferior lobes needs to be investigated more thoroughly. Finally, the

correlation between egr-1 and newborn immature neurons, and between pS6 and neuronal plasticity has to be substantiated to allow a better understanding of the involvement of the two neural markers in memory formation and synaptic plasticity.

Although many points still remain to be investigated, my results provide new information about the neural substrates involved in fish cognition. Studying fish cognition is important not only to understand its mechanisms in a major vertebrate group, but also to gain more insights into the evolution of cognition in general.

I. Index

II. Assistance received and resources used

II.I Behavioral experiments

I personally conducted part of the behavioral experiments on the animals. I also instructed the students Andy Lam, Elaine Osterkamp, Kevin Hsiung, and Ruben Eich who conducted behavioral experiments with the fish, under my supervision, during their internships.

II.II Processing of the animals and laboratory work

I personally processed each animal and conducted experiments in the laboratory. I killed the animals, I cut their brains with the cryostat and I performed immunohistochemistry. From February 2020 until April 2020 I worked at Caesar institute (The Max Planck Institute for Behavioral Neurobiology – Bonn, Germany) under the supervision of Dr. Pascal Malkemper, who helped me modify the protocol for the immunohistochemistry of IEGs.
From March 2022 until May 2022 I worked at the University of Texas at Austin (Austin, Texas, United States) under the supervision of Dr Hans Hofmann. At the University of Texas, I worked on part of my project (chapter 4), I carried out lab work for double labeling immunofluorescence and partial data analysis.

II.III Data analysis and manuscript writing

For the data analysis, I received assistance from Dr. Michael Hofmann who wrote the software programs (“Animal Explorer” and “Image Explorer”) for the analysis of the data of chapter 3 and chapter 4. Dr Hofmann also helped me with the interpretation of the data. For the writing of the manuscripts, I received assistance from Dr Schlüssel (chapter 3, chapter 4 and chapter 5), Dr Michael Hofmann (chapter 3, chapter 4 and chapter 5) and Dr Hans Hofmann (chapter 4).

III. List of abbreviations

A anterior thalamic nucleus

ATN anterior tuberal nucleus

CC crista cerebellaris

Cer cerebellum

CM corpus mamillare

Cor corpus cerebelli

CP central posterior thalamic nucleus

Dc central division of the dorsal telencephalon

Dcd dorsal subdivision of the central division of the dorsal telencephalon

Dcv ventral subdivision of the central division of the dorsal telencephalon

Dd dorsal division of the dorsal telencephalon

Dl lateral division of the dorsal telencephalon

Dld dorsal subdivision of the lateral division of the dorsal telencephalon

Dlv ventral subdivision of the lateral division of the dorsal telencephalon

Dm medial division of the dorsal telencephalon

Dmd dorsal subdivision of the medial division of the dorsal telencephalon

Dmv ventral subdivision of the medial division of the dorsal telencephalon

DON descending octaval nucleus

Dp posterior division of the dorsal telencephalon

DP dorsal posterior thalamic nucleus

E entopeduncular nucleus

EG eminentia granularis

fr fasciculus retroflexus

G granular layer of the cerebellum

Gl granular layer of the olfactory bulb

Hab habenula

IL inferior lobe

ILc inferior lobe, central nucleus

ILrec inferior lobe, nucleus of the lateral recess

IP nucleus interpeduncularis

ll lateral lemniscus

M molecular layer of the cerebellum

Ma Mauthner cell

Mg magnocellular octaval nucleus

ME median eminence of the hypothalamus

mlf medial longitudinal fascicle

mIII oculomotor nucleus

MON medial octavolateral nucleus

mV trigeminal motor nucleus

mX vagal motor nucleus

ND nucleus diffusus

NDl nucleus diffusus pars lateralis

NDm nucleus diffusus pars medialis

NG nucleus glomerulosus

NGT tertiary gustatory nucleus

NI nucleus isthmi

NLT nucleus lateralis tuberis

nlVal nucleus lateralis valvulae

OB olfactory bulb/granular layer

OT optic tectum

PGc commissural preglomerular nucleus

PGm medial preglomerular nucleus

POA preoptic area

PVO paraventricular organ

PTc pretectal area, centralis

PTco pretectal area, corticalis

PTpo pretectal area, nucleus of the posterior commissure

PTsm pretectal area, superficialis magnocellularis

PTsp pretectal area, superficialis parvocellularis

pV principle trigeminal nucleus

Ras raphe superior

RFi inferior reticular formation

RFm medial reticular formation

RFs superior reticular formation

SCN suprachiasmatic nucleus

SO stratum opticum of the optic tectum

SPV stratum periventriculare of the optic tectum

sVII sensory root of the facial nerve

Tel telencephalon

TL torus longitudinalis

TLat torus lateralis

TO optic tectum

TS torus semicircularis

Val valvula cerebelli

Vd dorsal nucleus of the ventral division of the telencephalon

VIII octaval nerve

Vl lateral nucleus of the ventral division of the telencephalon

VL vagal lobe

VM ventromedial thalamic nucleus

Vs supracommissural part of the ventral telencephalon

Vv ventral nucleus of the ventral division of the telencephalon

X vagal nerve

IV. List of figures

1. General introduction

Part of the introduction has been taken from the paper “Neural substrates involved in the cognitive information processing in teleost fish”, which has already been published by Animal Cognition (Calvo R, Schlüssel V. Neural substrates involved in the cognitive information processing in teleost fish. Anim Cogn 24, 923–946 (2021). https://doi.org/10.1007/s10071-021-01514-3).

Visual information processing involves features extraction, motion detection and image analysis, but higher visual information processing are associated with memory formation, learning and attention (Brown et al. 2011, Shettleworth 2010, Schlüssel 2015). Higher visual information processing are referred to as mental functions of higher order that make an animal capable of making decisions, and also play a role in mate-choice, foraging, orientation and navigation (Shettleworth 2001, Brown et al. 2011, Shettleworth 2010, Ebbesson and Braithwaite 2012, Schlüssel 2015, Marchetti 2018). It used to be a common idea that fish had cognitive abilities restricted to fixed action patterns, as suggested by Tinbergen (Tinbergen 1951). Over the last few decades though, many studies showed that fish present cognitive abilities and visual abilities challenging those of birds and mammals (Schlüssel 2015, Messina et al. 2020. For reviews see Brown et al. 2011, Calvo and Schlüssel 2021). There are many behavioral cognition studies available on fish (see for example Kotrschal et al. 1998, Bshary et al. 2002, Broglio et al. 2003, 2011, Brown et al. 2011, Maruska and Fernald 2018), but most of them are conducted on a few model species and, overall, they are still less numerous compared to the ones in birds and mammals. Nevertheless, due to their phylogenetic position and their unrivalled diversity, fish represent an excellent group to study cognitive visual behavior in.

Fish brains have been the subject of many different studies over the last century (e.g. Rupp et al. 1996, Hofmann 2001, Rodríguez et al. 2005, Salas et al. 2006, Ito et al. 2007, Ito and Yamamoto 2009, Hurtado-Parrado 2010, Vernier 2017, Yamamoto et al. 2017), suggesting that fish possess neural substrates involved in the processing of cognitive information that are analogous and homologous to those in mammals (Broglio et al. 2003, 2011). Considerably few studies have combined behavior and neuroanatomy, identifying the neural substrates involved in cognitive information processing in fish (e.g. Rodríguez et al. 2006, Kotrschal et al. 2013a, b, for reviews see Wullimann and Mueller 2004, Broglio et al. 2011, Ebbesson and Braithwaite 2012, Demski 2013, Maruska and Fernald 2018. For a review see Calvo and Schlüssel 2021). These suggest that fish

possess higher cognitive capabilities equivalent to those of mammals, including those of non-human primates (Brown et al. 2011).

In mammals and birds, a positive correlation between brain size and cognitive abilities has been suggested (e.g. Reader and Laland 2002, Sol et al. 2005, Deaner et al. 2007). Furthermore, the size of major brain areas may be associated with specializations in cognitive abilities (Tebbich and Bshary 2004). In cichlids, brain complexity, size and volume also seem to correlate with ecology, lifestyle and/or behavior (e.g. Anken and Bourrat 1998, Pollen et al. 2007, Pollen and Hofmann 2008, Burmeister et al. 2009, Shumway 2010, Gutiérrez-Ibáñez et al. 2011). A positive correlation between cognitive abilities and overall brain size was also found in guppies (*Poecilia reticulata* (Kotrschal et al. 2013 a, b, 2014)).

Different methods can be used to assess neural substrates involved in visual information processing. Lesion studies help determine the relationship between a specific brain structure and its function by testing what animals can do prior to and as a consequence of the lesion. Previous lesion studies have focused mainly on the involvement of the telencephalon in higher cognitive processing, such as avoidance learning and spatial memory (see also Overmier and Curnow 1969, Overmier and Savage 1974, Savage 1980, Laming and McKinney 1990, Rodríguez et al. 2006. Reviewed in Hofmann 2001 and Calvo and Schlüssel 2021). These studies showed that the removal of the telencephalon in teleosts is not as destructive as in mammals (e.g. Kaas 1987, Hofmann 2001), suggesting that most of the areas involved in cognitive information processing, in particular visual information processing and object recognition, are situated outside of the telencephalon. In goldfish (*Carassius auratus*) it has been demonstrated that the lateral zone of the dorsal telencephalon, which by some is considered to be homologous to the hippocampus of mammals, plays a key role in spatial memory and place learning (Broglio et al. 2011). Similar results were observed in sharks whose dorsomedial pallium (equivalent to the lateral pallium of teleosts) seems to play a role in processing complex place learning information (Fuss et al. 2014). Likewise, the medial zone of the dorsal telencephalon in goldfish and the lateral pallium in sharks, seem to be involved in avoidance learning tasks processed in the pallial amygdala of land vertebrates (reviewed in Broglio et al. 2011, Schwarze et al. 2013). In addition to the telencephalon, the cerebellum is the only brain structure that has been investigated in any detail in fish. Like the telencephalon, it is involved in spatial cognition (Durán et al. 2008; 2010, Rodríguez et al. 2005), emotional or avoidance learning (e.g. Kaplan and Aronson 1967, Álvarez et al. 2003, Rodríguez et

al. 2005) and in different conditioning tasks (Aronson and Herberman 1960, Karamian 1963, Álvarez et al. 2003).

Even though lesion studies have led to major discoveries, they have a range of shortcomings. First of all, it is difficult to target a specific area of interest, and to do so repetitively in different individuals. Furthermore, there is a high chance to damage non-target tissue in the process. In addition, the lesioning of any area could hyper-activate or hyper-inactivate other brain regions that may be connected to the target area (Fuster 1989, Damasceno 2010). As an alternative to lesion studies, in the last decades it has become popular to investigate the activation of immediate early gene (IEG) and/or phosphorylated ribosome marker (pS6). IEGs and pS6 are neural markers and have become a useful tool to study the activation of specific brain areas.

Cichlid fish are a great model to study visual learning in, since they are highly visual species (Fernald 1982, Carleton and Kocher 2001), and their visual abilities have already been well studied in several behavioral studies (e.g. Schlüssel et al. 2018, Fuss et al. 2018, Schlüssel et al. 2022).

1.1 Phylogeny and classification of Teleost fish

Chordates, which include all vertebrates, originated more than 500 million years ago. Vertebrates, as shown in Figure 1, are divided into two groups, the small group of jawless vertebrates called Cyclostomes (110 species) and the Gnathostomes. The Gnathostomes (jawed vertebrates) comprise about 54.650 species, split into Osteichthyes (bony fish) and Chondrichthyes (cartilaginous fish, which comprise over 1200 species) (Vernier 2017, Yamamoto et al. 2017, Ishikawa et al. 2022).

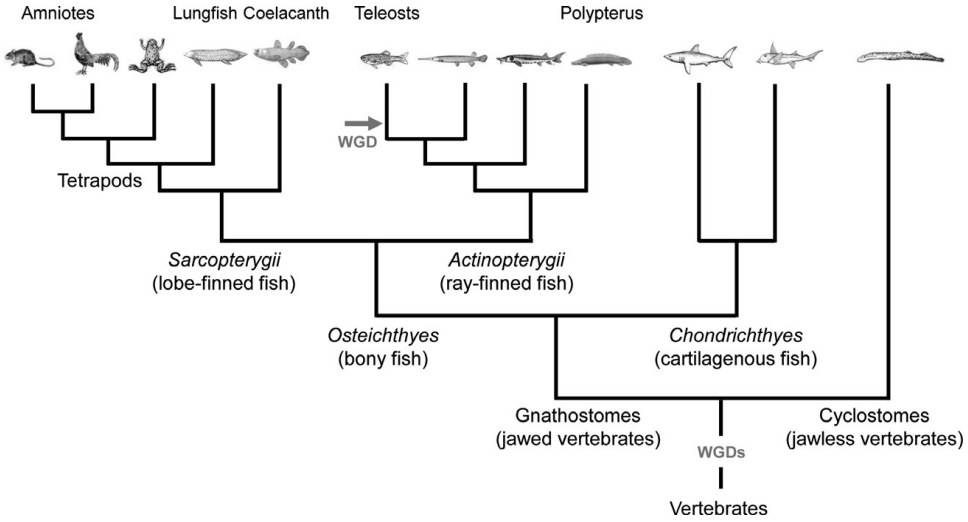

Figure 1: Simplified phylogeny tree of vertebrates (Yamamoto et al. 2017). WGD: whole genome duplication

The Osteichthyes (Figure 2) comprise almost 53.700 species and are divided into two superclasses, the sarcopterygians and the actinopterygians. The sarcopterygians (also called lobe-finned fishes), include all tetrapods, the largest group of animals with limbs. The actinopterygians (ray-finned fishes) mostly consist of the infraclass teleost (close to 99% of actinopterygians and 96% of all living fish species). There are only few non-teleost actinopterygians (12 species of polypteridæ (Cladistia), 28 of Chondrostei (sturgeons and related species), one Amidae species (bowfin), and 7 species of lepisosteidae (gars) (Eschmeyer et al. 2016)). Actinopterygians represent almost half of the extant vertebrate species (Vernier 2017, Yamamoto et al. 2017). Teleost, that probably arose in the Early or Middle Triassic (Nelson et al. 2016), underwent a specific whole-genome duplication and present a genome more complex of those of tetrapods (Ohno 1970).

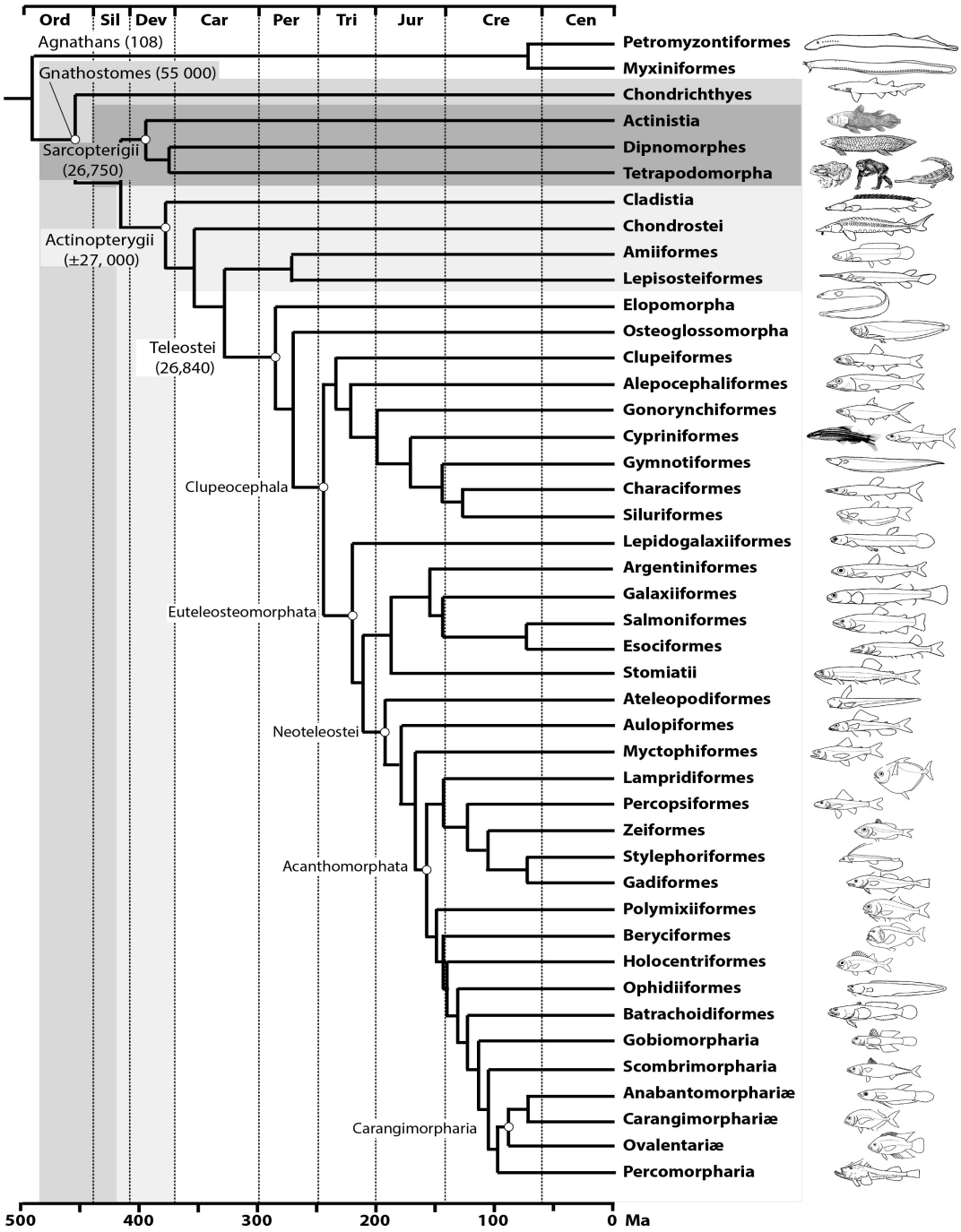

Figure 2: Phylogenetic relationships of the main groups of vertebrates, focusing on the diversity of teleosts. Abbreviations: Ord: Ordovician, Sil: Silurian, Dev: Devonian, Car: Carboniferous, Per: Permian, Tri: Triassic, Jur: Jurassic, Cre: Cretaceous, Cen: Cenozoic (Vernier 2017).

1.2 Cichlids

Animals used in this study belong to the species *Pseudotropheus zebra* (*Metriaclima zebra* or *Maylandia Zebra* (Boulenger, 1899)), also known as *Zebra Mbuna* (Konings & Stauffer 1997) (Figure 3). This species belongs to the family of Cichlidae (www.fishbase.org), which are part of the Ovalentaria within the derived Acanthomorpha. This species was assessed as "Least Concern" by the IUCN in 2018 (www.iucnredlist.org, 2018).

Figure 3: individuals of *Pseudotropheus Zebra* www.malawi-guru.de

In addition to more than 500 other cichlid species, estimated to have arisen from a common ancestor within the last million years, *Pseudotropheus zebra* is endemic to Lake Malawi in East Africa (Albertson et al. 1999), the southernmost of the three great lakes of East Africa (Gonfiantini et al. 1979). Figure 4 shows the distribution map of *Pseudotropheus zebra* (www.iucnredlist.org, modified).

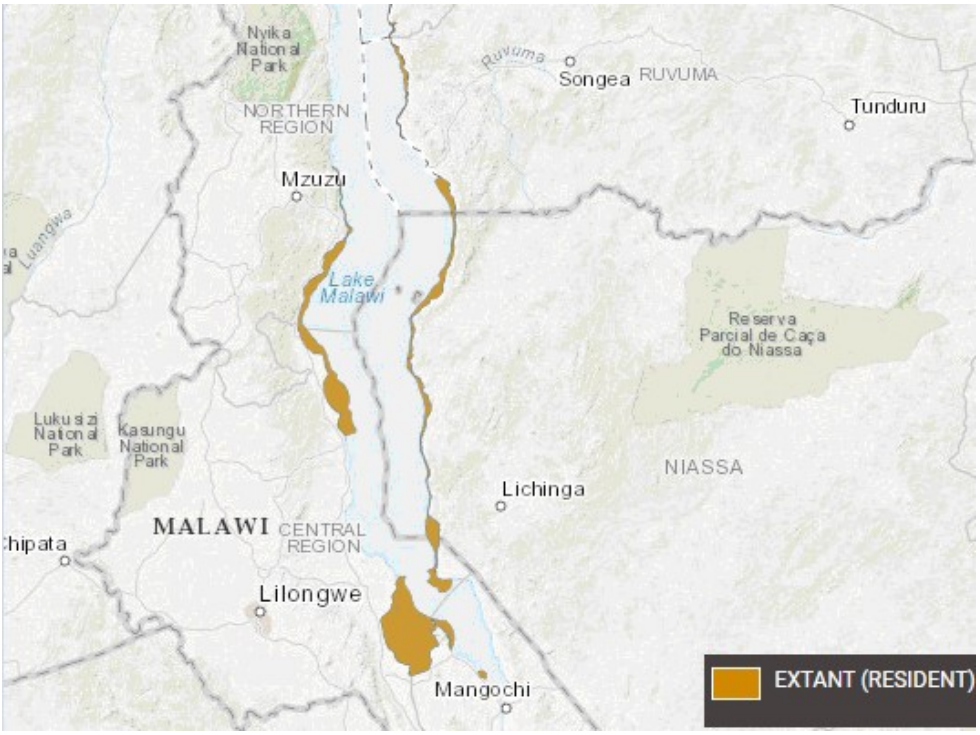

Figure 4: Distribution map of *P. zebra* (modified from www.iucnredlist.org)

The biodiversity of the family Cichlidae is a prime example of adaptive radiation, or explosive speciation, and is possible through sympatric speciation (Turner et al. 2001) as a result of natural (Dieckmann and Doebeli 1999) and sexual selection (Turner & Burrows 1995). Sympatric speciation is the emergence of new species without geographic isolation (Dieckmann and Doebeli 1999). For this reason, cichlids have attracted attention in evolutionary biology.

There is large variation in the body shape of cichlids with most species having a rather compressed body, while others possess elongated or disc-shaped bodies (Nelson et al. 2016). Characteristic of the genus *Pseudotropheus* is a steeply sloping face profile and a slightly subterminal mouth (Albertson et al. 1999).

Cichlids feature multiple reproductive and mating strategies. Some species are substrate brooders, monogamous individuals, and both sexes care for the eggs. Other species are mouthbrooders, polygamous individuals, and females carry the fertilized and newly hatched eggs in the mouth. Some species even combine both schemes, with individuals laying their eggs on the substrate and the young being carried in their parents' mouths (Nelson et al. 2016).

The *Pseudotropheus zebra* species complex is possibly the most species-rich of the Mbuna species complex (Allender et al. 2003). Within this complex, males have so-called mating patterns, which they can strengthen within minutes (Mcelroy et al. 1990).

1.3 Fish brain

The fish brain follows the general Bauplan of vertebrates (Vernier 2017). First, the neural tube generates three primary vesicles (three-vesicle stage): the forebrain (prosencephalon) - which in fish lacks the neocortex of mammals -, the midbrain (mesencephalon), and the hindbrain (rhombencephalon), continuous with the spinal cord. The three-vesicle stage develops into the five-vesicle stage (Yamamoto and Bloch 2017). Three different models have been proposed to describe the transition from the three- to five-vesicle stage (see Figure 5, modified from Yamamoto et al. 2017). In the "columnar model", the forebrain is subdivided into the diencephalon caudally and the telencephalon rostrally. The diencephalon is subdivided into the hypothalamus ventrally and the thalamus dorsally, while the telencephalon is divided into the pallium dorsally and the subpallium ventrally. The midbrain connects the forebrain to the hindbrain (rhombencephalon). The latter is divided into the metencephalon (containing the cerebellum) rostrally, and the myelencephalon (containing the medulla oblongata) caudally (Herrick 1910, Wullimann 1997, Simões et al. 2012, Yamamoto and Bloch 2017).

The "prosomeric model", proposed by Puelles and Rubenstein in the early 1990s, considers gene expression patterns to which it attributes morphological meanings (Puelles and Rubestain 2003). In this model, the forebrain is divided into the posterior diencephalon and the anterior secondary prosencephalon. The latter is located at the anterior end of the forebrain and contains the telencephalon dorsally and the hypothalamus ventrally, while the diencephalon is divided into the pretectum, thalamus, and prethalamus.

A new model was proposed by Affaticati et al. in 2015 and is based on the morphological analysis of the ventricular organization. This model is considered a modification of the "prosomeric model". Here, the secondary prosencephalon is divided into three parts: the telencephalon, hypothalamus, and optic recess region (Affaticati et al. 2015). Figure 5 shows a schematic summary of the three different models illustrating the different subdivisions of the forebrain.

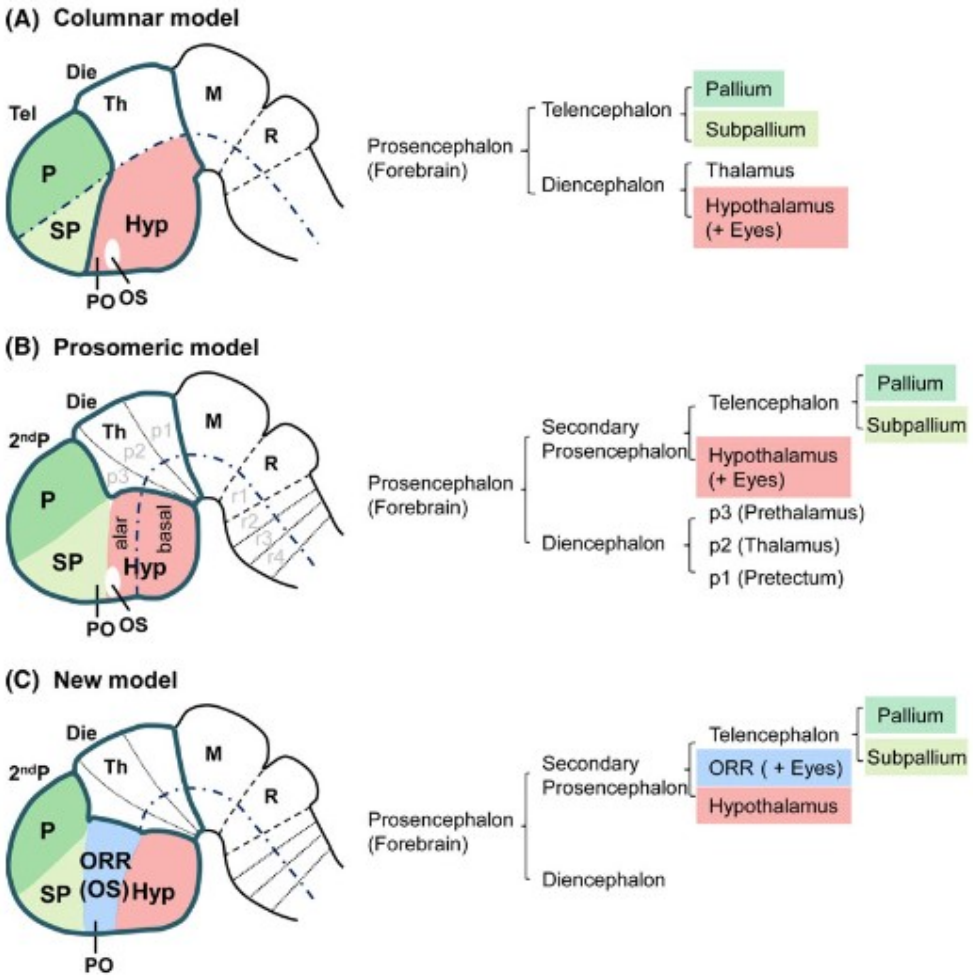

Figure 5: Schematic summary of the three different models illustrating the different subdivisions of the forebrain. a) The columnar model; here, the hypothalamus is considered to be the ventral half of the diencephalon, (Herrick 1910, Wullimann 1997, Simões et al. 2012, Yamamoto and Bloch 2017) b) The prosomeric model (Puelles and Rubenstein 2003); here, the hypothalamus is considered to be the ventral half of the most anterior part of the forebrain, and the telencephalon and hypothalamus consist of the secondary prosencephalon. c) the modification of the prosomeric model proposed by Affaticati et al. (2015); here, the secondary prosencephalon is divided into three parts, the telencephalon, the hypothalamus, and the optic recess region (ORR) (modified from Yamamoto et al. 2017).

During embryogenesis, the telencephalon of Actinopterygians undergoes a process called 'eversion' (Gage 1893) which gives rise to a single ventricle separating two telencephalic hemispheres (Broglio et al. 2005), and a proliferative zone that lies at the dorsal part of the telencephalon (Mueller and Wullimann 2009). The 'eversion' process in this group is different from all other craniates (Figure 6), which undergo an 'evagination' process. The latter produces two telencephalic hemispheres, each one with its own ventricular cavity, and a proliferative zone that is oriented towards the ventricles (Muller and Wullimann 2009). Although the Actinopterygian telencephalon undergoes a different embryological development than that of other craniates, its overall organization is similar as a pallial and a sub-pallial zone can be differentiated (Northcutt & Braford 1980, Wulliman & Mueller 2004, Northcutt 2008, Braford 2009, Nieuwenhuys 2011), but potentially homologous areas are situated in different areas due to the two varying folding patterns (Yamamoto 2009).

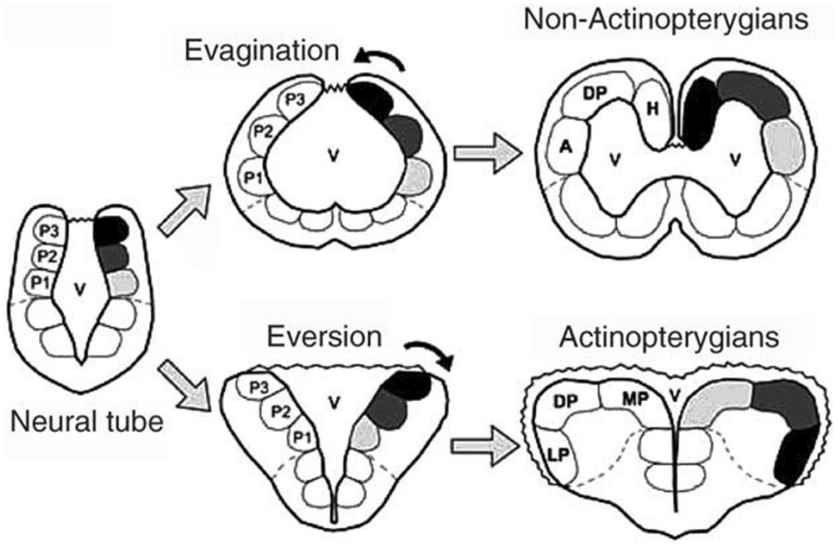

Figure 6: Schematic representation of the process of evagination in Non-Actinopterygians and eversion in Actinopterygians (modified from Broglio et al. 2005).

The nomenclature of the teleostean telencephalon was first introduced by Nieuwenhuys in 1963, and is based on topography. The telencephalon is divided into two areas: the dorsal and the ventral telencephalon, which correspond to the pallium and subpallium respectively. The dorsal telencephalon is then further subdivided into a medial, lateral, dorsal, posterior, and central area (Dm, Dl, Dd, Dp and Dc), which in turn present further subdivisions. The ventral telencephalon consists of a dorsal, ventral and lateral part (Vd, Vv and Vl) (Nieuwenhuys 1963, Yamamoto 2009).

The diencephalon features some areas or functional aspects of areas that are unique to teleosts. The dorsal thalamus does not seem to function as a sensory relay center as in tetrapods (Nieuwenhuys et al. 1998, Yamamoto and Ito 2005, Northcutt 2006). Instead, ventrolateral to the thalamus, there is the preglomerular complex, which functions as the sensory relay station to the telencephalon (Northcutt 2006, Yamamoto et al. 2007, Yamamoto and Ito 2005, 2008). Another unique characteristic of the teleost diencephalon is the ventrolateral presence of the inferior lobes, which are hypothesized to be involved in feeding and aggressive behavior (Demski & Knigge 1971). The inferior lobe is divided into three nuclei, the diffuse nucleus which is the most expanded part of the inferior lobe, the central nucleus, medial to the diffuse nucleus, and the periventricular nucleus (Ahrens and Wullimann 2002). There is a prominent visual pathway extending from the optic tectum over the nucleus corticalis and the nucleus glomerulosus to the inferior lobes of the hypothalamus (Wullimann and Meyer 1990, Butler et al. 1991, Ahrens and Wullimann 2002). The optic tectum, the mesencephalic roof, processes primary visual input receiving projections from contralateral retinal ganglion cells (Northcutt and Davis 1983). There are also some other highly derived areas, such as the corpus mamillare and the nucleus of the posterior tuberculum which, in turn, project to the medial part of the dorsal telencephalon (Murakami et al. 1983). In some teleost groups, other areas are also markedly elaborated, e.g. the lateral torus, which is related to gustatory functions (Ahrens and Wullimann 2002).
Figure 7 shows the cryostat sections of the *P. zebra* brain.

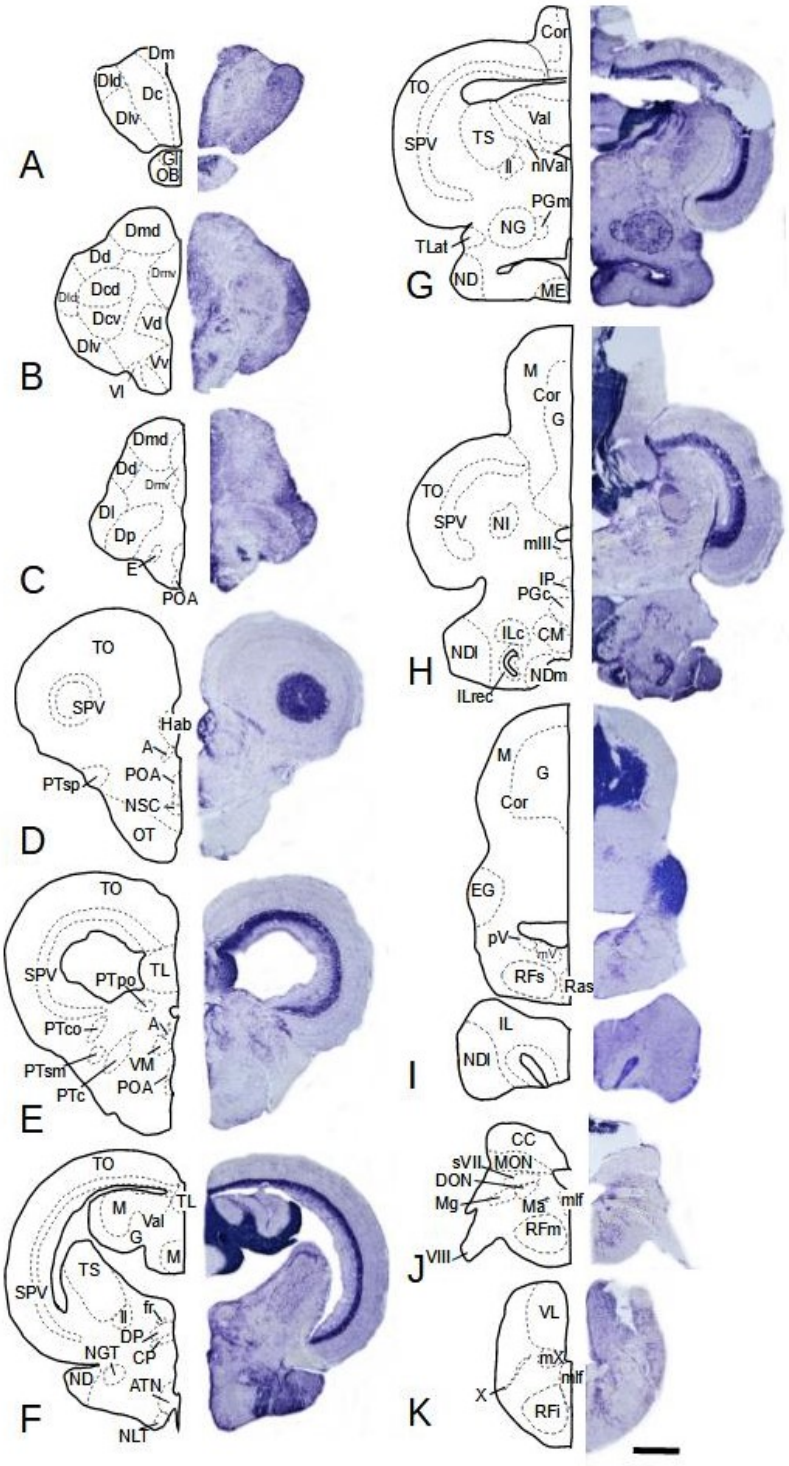

Figure 7: Cryostat sections of the *P. zebra* brain. The right side shows the microphotos of the original sections after Nissl staining, the left side shows a schematic drawing of identifiable areas and nuclei. See list of abbreviations. Scale bar: 500 µm for each section (Calvo and Schlüssel 2021)

1.4 Immediate early gene

The functions of the different brain areas are difficult to determine, especially 'higher' centers. Here, the recently discovered activity markers has been a valuable tool.

Immediate early genes (IEGs) are genes whose expression is induced without protein synthesis and are commonly used as markers of neuronal activity in the brain (Morgan and Curran 1991). Their activation can be visualized by various techniques, such as in situ hybridization or immunohistochemistry, and has been used to map activity patterns in response to sensory stimuli or behavioral tasks (Guzowski et al. 1999), and when investigating substrates underlying synaptic plasticity processes, such as long-term potentiation (LTP), long-term depression (LTD) and cognitive functions (Minatohara et al. 2016). The first evidence of the involvement of IEGs in neural functions came from studies conducted on PC 12 pheochromocytoma cells (Sheng and Greenberg 1990, Morgan and Curran 1991, Curran and Morgan 1995). IEGs own their name to the fact that the RNA transcriptional activation in the nucleus takes place very quickly, usually within five minutes of stimulation, and persists for 15 – 20 minutes. After that time, the transcripts are transferred to the cytoplasm (Greenberg and Ziff 1984, Greenberg et al. 1985, Guzowski et al. 1999). Their mRNA concentration levels reach their maximum around one hour after stimulation, and decline to baseline after two to five hours. The proteins reach their maximum concentrations 60–90 min after stimulation, and disappear within four hours after the treatment (Curran and Morgan 1995).

Various studies have demonstrated that very different types of stimuli (such as pharmacological agents, behavioral tests, seizures, etc.) can increase the expression of IEGs in the brain (Curran and Morgan 1995). The expression pattern indicates in which area of the brain the activation took place and what types of neurons were activated (Long and Salbaum 1998). Furthermore, IEGs expression codes for different classes of proteins playing several functions, such as cytoskeletal proteins, postsynaptic proteins, signaling molecules, metabolic enzymes, growth factors or transcription factors (Lanahan and Worley 1998). In neurons, about 30-40 different IEGs can be expressed, 10-15 of which function as transcription factors (Lanahan and Worley 1998). The latter regulate the expression of target genes and influence neuronal physiology (Curran and Morgan 1987, Curran and Franza 1988, Herdegen and Leah 1998, O'Donovan et al. 1999, Tischmeyer and Grimm 1999, Pinaud 2004, Pinaud et al. 2005, Gallo et al. 2018). C-fos and egr-1 are the most intensely studied and used IEGs that function as transcription factors for mapping neural activity. They play a crucial role in cognitive processes, including learning and memory, but are also

involved in proliferation and cell differentiation (Okuno 2011). In addition to their role in regulating cellular responses to extracellular stimuli, immediate early genes have also been implicated in various disease states. For example, alterations in their expression have been observed in numerous psychiatric and neurological disorders, including schizophrenia, bipolar disorder, and Alzheimer's disease (Lipska and Weinberger 2000).

1.4.1 C-FOS

C-fos is a proto-oncogene and belongs to the Fos family (Morgan and Curran 1989). Its induction, which was the first one to be shown as activity-dependent among the IEGs (Morgan and Curran 1988, Sagar et al. 1988, Gallo et al. 2018), rapidly occurs in response to different stimuli including cytokines, neurotransmitters, growth factors (Sheng and Greenberg 1990). C-fos encodes for the C-FOS nuclear protein, a 62-kDa transcription factor, which undergoes post-translational modifications that mainly consist of serine and threonine phosphorylation, and regulates gene expression by binding to DNA in the nucleus (Curran et al. 1984, Barber and Verma 1987, Morgan and Curran 1991). C-fos was the first ever IEG investigated in neurons (Schilling et al. 1991, Sheng et al. 1990, Okuno 2011) and there is little or no expression of the gene under baseline condition (Morgan and Curran 1989, Hoffman et al. 1993). Its activation brings to the expression of late-response genes involved in different processes (e.g. growth control, plastic changes) (Sukhatme et al. 1988, Williams et al. 2000, Bozon et al. 2003, Maddox et al. 2011, Gallo et al. 2018). C-fos can negatively regulate its own expression, characteristic required for a rapid decline in its expression (Morgan and Curran 1991).

1.4.2 EGR-1

Egr-1, also known as zif/268, krox-24, TIS8, NGFI-A or zenk, codes for the transcription factor EGR-1, a phosphorylated protein synthesized in the nucleus, where it then remains (Cao et al. 1990). The protein EGR-1 has the ability to auto regulate its own expression (Chen et al. 2010). In the brain, egr-1 is specifically expressed in neurons and synaptic activity regulates its activity (Worley et al. 1991). Unlike the other transcription factors (such as c-fos), whose expression declines after stimulation (Herdegen et al. 1995, Kaczmarek and Chaudhuri 1997), the expression of egr-1 is constantly induced by ongoing synaptic activity (Burmeister and Fernald 2005) due to the basal physiological synaptic activity (Worley et al. 1991). It seems that, under physiological stimulation

in vivo, synapsins (Thiel et al. 1994, Petersohn et al. 1995, Burmeister and Fernald 2005) and neurofilaments (Mello and Clayton 2004, Burmeister and Fernald 2005) are likely the targets that egr-1 regulates (Burmeister and Fernald 2005).

The egr-1 of the cichlid fish *Astatotilapia burtoni* was cloned by Burmeister and Fernald (2005) in order to investigate the evolutionary conservation of egr-1, whose protein sequence has been compared to other vertebrate groups. In this study, egr-1 was described for the first time in an organism other than a mammal or bird. *Astatotilapia burtoni* egr-1 shares 66% sequence similarity with mouse (*Mus musculus*) and 81% with zebrafish (*Danio rerio*) egr-1 (Burmeister and Fernald 2005). Furthermore, it was demonstrated that *A. burtoni* egr-1 reaches its highest expression levels about 30 minutes after stimulation, showing that expression kinetics are similar to those of mammals (Zangenehpour and Chaudhuri 2002) and birds (Mello and Clayton 1994) (Burmeister and Fernald 2005).

Cognitive processes are not the only factors that can induce IEGs' expression. Among them, pharmacological stimulation. For example, c-fos expression can be induced in the optic tectum and cerebellum of zebrafish larvae (*Danio rerio*) exposed to pentylenetetrazole (PTZ, a common convulsant agent) (Baraban et al. 2005). Similarly, egr-1 expression can be altered in the olfactory bulbs, ventral nucleus of the ventral telencephalon, central and lateral zone of the dorsal telencephalon, the optic tectum and in different regions of the diencephalon of *A. burtoni* by injecting kainic acid (a glutamate receptor agonist) (Burmeister et al. 2005). The expression of c-fos can be increased in the lateral and the medial zone of the adult zebrafish telencephalon by administrating d-amphetamine (von Trotha et al. 2014). Other studies combining IEG expression and pharmacological stimulation were conducted on rainbow trout (*Oncorhynchus mykiss*) (Matsuoka et al. 1998) and zebrafish (*Danio rerio*) (Ruhl et al. 2017).

IEGs analyses suffer from several potential shortcomings. First of all, the gene in question can vary considerably between species. Furthermore, it may be challenging to quantify learning effects and separate these from other contributing and potentially confounding factors, such as stress, not closely related to the treatment of interest.

For an overview of studies combining behavioral and IEGs studies in fish, please refer to Calvo and Schlüssel 2021.

1.5 Phosphorylated ribosome marker pS6

Like IEGs, the phosphorylated ribosome marker (pS6) acts as a marker for neural activation. S6 protein is a component of the 40S ribosomal subunit. Its inducible phosphorylation, which occurs in response to a large variety of stimuli, was the first post-translational modification described regarding ribosomal proteins (Gressner and Wool 1974, Meyuhas 2008) and has attracted attention since its discovery in 1974 (Gressner and Wool 1974).

The phosphorylation of the ribosomal protein S6 (rpS6) takes place in five evolutionary conserved serine residues in the carboxy-terminal sites in an ordered manner: first with Ser236 and followed sequentially by Ser235, Ser240, Ser244, and Ser247 (Martin-Pérez and Thomas 1983, Wettenhall et al. 1992, Meyuhas, 2008, 2015).

The first kinase that was identified, which is able to catalyze the phosphorylation of rpS6 in all the sites listed above, is p70/p85 S6 Kinase I (Krieg et al. 1988, Ferrari et al. 1991, Bandi et al. 1993, Meyuhas 2008, 2015).

The first evidence of the increase of rpS6 phosphorylation during synaptic plasticity was reported in 1991 by Klann and colleagues. Since then, a large number of electrical, chemical, pathological or pharmacological stimuli have been described to promote rpS6 phosphorylation in neurons, as well as the administration of antipsychotics and various other drugs (such as clozapine, morphine, methamphetamine) (Biever et al. 2015). It has also been demonstrated that most of the mouse models for neurological and neurodevelopmental disorders, including Rett syndrome, Down syndrome, and Fragile X display altered rpS6 phosphorylation, as well as in psychiatric disorders, such as schizophrenia (Biever et al. 2015). RpS6 phosphorylation has been shown to be directly involved in the control of cell size. Different cell types (pancreatic b-cells, interleukin 7-dependent cells derived from fetal livers and MEFs) derived from knockin mouse (rpS6P$^{-/-}$) mice, in which all serines residues were substituted by alanines, are significantly smaller than the same cells in wild-type animals (Meyuhas 2008). In particular, the protein kinase mammalian target of rapamycin (mTOR), is the central regulator of processes of cell growth and cell division. mTOR activates S6K (1 and 2), involved, among other things, in regulating protein synthesis, cell size and cell-cycle progression (Ruvinsky and Meyuhas 2006).

In the last few decades, pS6 has also become a popular marker to visualize neural activation in fish and it can be easily detected in both the cytosol and the nucleus (Pende et al. 2004).

In 2018, Fischer and colleagues analyzed the neural mechanisms involved in different social contexts in guppies (*Poecilia reticulata*) focusing on brain regions of social decision-making

networks. In particular, the immunohistochemical expression of pS6 in brains of fish assigned to three experimental groups (aggression, mating or isolation) was analyzed. Fish in the mating experimental group had higher pS6 activation in the preoptic area (POA), known to be involved in reproductive and sexual behavior, compared to fish in the aggression or isolation groups. Furthermore, the levels of pS6 immunoreactivity in the posterior tuberculum and in the lateral part of the ventral telencephalon were positively associated with the number of behaviors performed in both aggressive and mating contexts (Fischer et al. 2018).

PS6 has also been used as a neural marker to analyze mate-choice behavior in a cichlid fish (*Astatotilapia burtoni*). Butler and colleague (2019) tested whether ovulation resulted in increased neural activation in the retina when females were exposed to visual courtship signals from the male. Immunohistochemistry for pS6 was performed and it showed that ovulating females had greater activation in the inner nuclear layer and the ganglion cell layer of the retina than non-ovulating females. Furthermore, within the ovulating females, the ones injected with PGF2α (prostaglandinF2α), known to be produced in the ovary in response to ovulation in fish (Sorensen et al. 2018), had greater activation than naturally ovulating females, in both the nuclear layer and the ganglion cell layer. The pS6 activation in ovulating females seemed to be the consequence of the exposure to the visual courting behaviors from the dominant males (Butler et al. 2020).

The same animal model was used to test if galanin neurons of the anterior preoptic area and ventral part of hypothalamus are differentially activated during distinct parental care and feeding states in a mouthbrooding fish (Butler et al. 2020). In particular, females displaying post-release maternal care had the greatest percentage of activated galanin cells in the POA. In the ventral subdivision of the lateral tuberal nucleus of the hypothalamus, animals displaying brooding and post-release maternal care showed fewer activated galanin neurons than those in the fed, starved, and infanticide groups. Finally, in the intermediate subdivision of the lateral tuberal nucleus of the hypothalamus, maternal care and brooding females showed the lowest percentage of activated galanin neurons, while fed females had the highest percentage of activated galanin neurons and starved and infanticide females were an intermediate between brooding and fed groups (Butler et al. 2020). However, most of the studies using pS6 in fish focused on very specific behaviors (e.g. social and reproductive interactions, feeding behavior) (Travanca dos Santos 2017, Montesano et al. 2019, Tripp et al. 2019, 2020, Baran and Streelman 2020, Maruska et al. 2020, Chen et al. 2021, Dunlap et al. 2021, Scaia et al. 2022), with a very few studies focusing of sensory mechanisms (e.g. York et al. 2019, Schuppe et al. 2021).

1.6 Dopamine

Due to its involvement in different physiological functions (e.g. reward, motivation, motor control, stress-related response) and several human pathologies and psychiatric disorders (e.g. Parkinson's disease, schizophrenia, ADHD), the dopaminergic system has been thoroughly investigated, especially in mammals (Callier et al. 2003, O'Connell et al. 2011). The first evidence for the presence of dopamine-producing neurons came from the work of Annica Dahlström and Kjell Fuxe in the early 1960s, who also introduced the conventional numbering of the catecholaminergic cell populations from A1 to A12 in the rat (Dahlström and Fuxe 1964). Later on, additional groups were added (A13-A17) (Lindvall and Björklund 1984, Hökfelt 1984). The numbering of the DA cell populations is still used, as dopaminergic cells are not located in a specific nucleus and their locations and distributions vary between vertebrates' groups and species (Yamamoto and Vernier 2011). In general, DA cells are divided into retinal (A17), olfactory bulb (A16), diencephalic (A11–A15) and diencephalo-midbrain (A8–A10) groups (Smeets and González 2000).

It has been demonstrated that dopamine is not only present in invertebrates and vertebrates, but also in plants (Wintle and Van Tol 2001, Callier et al. 2003, Kulma and Szopa 2007). In the central nervous system (CNS) of vertebrates, dopamine plays several roles, acting as a modulatory neurotransmitter. It is involved in motor control (Harrington et al. 1996, Paus 2001), lactation (Demarest et al. 1984), motivation (Salamone and Correa 2012) reward/reinforcement (Schultz 1998, Berridge and Kringelbach 2008), and in cognitive functions (Paus 2001), to just name a few. In particular, in the CNS of mammals, dopamine is produced in the hypothalamus and midbrain, and it is distributed along four major pathways (mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular). The mesocortical and mesolimbic pathways originate from the A10 neurons of the ventral tegmental area (VTA) and project to the cortex and the nucleus accumbens, respectively (Horvitz 2000, Wise 2009). Together, they form the mesocorticolimbic system, which plays a role in reward and motivation (Kelley and Berridge 2002). Nevertheless, various studies have shown that stressful and aversive stimuli stimulate dopamine release from the mesolimbic system (Ikemoto and Panksepp 1999), suggesting the involvement of the dopaminergic system in response to stress (Salamone et al. 1997, Berridge and Robinson 1998). The nigrostriatal pathway is involved both in learning and motor functions and it's formed by the A9 dopaminergic neurons in the substantia nigra projecting to the striatum (Hikosaka et al. 2002). Finally, dopaminergic neurons from the arcuate and periventricular nuclei of the hypothalamus project to the pituitary

gland, resulting in the tuberoinfundibular pathway, which is involved in the regulation of the secretion of prolactin from the pituitary (Demarest et al. 1984).

Tyrosine-hydroxylase (TH) is the first and limiting enzyme involved in the synthesis of dopamine (Fernstrom and Fernstrom 2007). An important aspect of TH is its evolution. Candy and Collet in 2005 demonstrated that, during vertebrate evolution, the TH gene has been duplicated originating two paralogous genes (TH1 - orthologous of TH of placental mammals - and TH2) present in most vertebrates except for placental mammals (Candy and Collet 2005, Yamamoto et al. 2010). TH has been used as a marker in several species, including teleost fish (e.g. goldfish (Hornby et al. 1987), freshwater electric fish (*Apteronotus leptorhynchus*; Sas et al. 1990), zebrafish (*Danio rerio*; Kaslin and Panula 2001, Rink and Wullimann 2001), cichlid (*Astatotilapia burtoni;* O'Connell et al. 2013a)). However, it is possible that these studies did not distinguish between the localization of the two different paralogous. In particular, TH2 expressing cells have been described in the hypothalamic region of teleost fish (Chen et al. 2009) and new antibodies capable of detecting only TH2 were produced by Semenova and colleagues in 2014 (Semenova et al. 2014). Furthermore, dopamine can also be converted into norepinephrine (NE) by DA-β-hydroxylase (DBH) in neurons utilizing norepinephrine as transmitter (Fernstrom and Fernstrom 2007). Despite this, in the early 1990s it has been demonstrated by Ma that TH-immunoreactive cells in the forebrain (diencephalon and telencephalon) of teleost are dopaminergic. Using immunohistochemistry for DBH and TH, Ma (1994) showed that noradrenergic neurons rostral to the midbrain-hindbrain boundary are absent in zebrafish (Ma 1994, O'Connell et al. 2013a). This was first hypothesized in the late 1980s-early 1990 using a dopaminergic-specific antiserum in the European eel (*Anguilla anguilla;* Roberts et al. 1989) and the stickleback (Ekström et al. 1992).

In the last decades, teleost fish have become a popular model system in neuroscience. In particular, several studies have assessed the activation of different brain areas during different behavioral contexts using neural marker (i.e. immediate early genes – IEGs – for a review see Calvo and Schlüssel 2021). In order to get a more detailed description of the functions of dopaminergic system in teleosts, the aforementioned neural markers have been used to selectively mark the activation of dopaminergic cell populations. In 2013, O'Connell and colleagues investigated whether social stimuli would induce c-fos expression in dopaminergic populations in the brain of the cichlid fish *Astatotilapia burtoni*. They observed an increase of the induction of c-fos in the central region of the ventral telencephalon of both intruder challenge and reproductive opportunity contexts, compared to the control group (O'Connell et al. 2013b). The same animal

model was used by Weitekamp and Hofmann to assess whether cooperation would increase the activity of dopaminergic cell populations. Results of their work showed an increase activity of dopaminergic neurons in the preoptic area (Weitekamp and Hofmann 2017).

In larvae zebrafish, handling stress, chemical stressor and pH change were reported to induce c-fos expression in the dopaminergic cell populations of the posterior tuberculum and hypothalamus (Semenova et al. 2014).

1.7 Aim of the project

In the last century fish have been the subject of a wide range of cognition experiments which have well established that fish possess cognitive abilities challenging those of birds and mammals (for reviews see Brown et al. 2011, Schlüssel 2015). However, the neural correlates for most cognitive functions in fish are still unknown (for reviews see Rodríguez et al. 2006, Broglio et al. 2011, Ebbesson and Braithwaite 2012, Kotrschal et al. 2014, Calvo and Schlüssel 2021), with the exception of the telencephalon (in particular the medial and lateral division of its dorsal part). The telencephalon has for many years been considered the primary center of cognitive information processing (reviewed in Hofmann 2001) but lesion studies have shown that the lesion/ablation of this part in teleosts brain has little effect on various behaviors (for a review see Calvo and Schlüssel 2021), and basic behaviors (e.g. swimming, feeding) are not affected at all (Steiner 1888, Bethe 1899, Rizzolo 1929). However, few studies investigating the role of the telencephalon in learning demonstrated impairments in avoidance behavior (e.g. Flood et al. 1976, Davis and Kassel 1983, Overmier and Hollis 1983) but no involvement in object recognition tasks (Froloff 1925, 1928, Bull 1928, Nolte 1932). Moreover, the functions of other areas within the telencephalon are still unknown, as well as the diencephalon or brain regions outside of the forebrain. In particular, there is a prominent visual pathway extending from the optic tectum over the nucleus corticalis and the nucleus glomerulosus to the inferior lobes (Wullimann and Meyer 1990, Butler et al. 1991, Ahrens and Wullimann 2002), whose specific role in the visual pathway still has not been investigated so far. In fact, most of the studies have been conducted on spatial orientation or social cognition (for a review see Calvo and Schlüssel 2021).

The aim of my project was to investigate the brain areas involved in visual learning mechanisms, like object recognition and object memory, in the Malawi cichlid *Pseudotropheus zebra*.
In particular, my thesis consists of three result chapters, each focusing on a specific aspect involved in visual learning mechanisms:

- Localize the brain areas involved in visual learning, object recognition and object memory,
- Investigate the role of the dopaminergic system in visual learning,
- Analyze the mechanisms of learning and memory

The present thesis is a partially cumulative thesis. Its content consists mostly of a number of peer-reviewed publications to which I was the primary contributor.

2. Preliminary experiments

During my first year as a Ph.D. student, I had the opportunity to conduct several pilot experiments with the goal of learning and acquiring different techniques that could be effective for my research project. This period was a period of discovery and experimentation and proved crucial in building the solid foundation on which I would later develop my study. One of the most important aspects of research is choosing a methodology or technique that can guarantee reliable and effective results. Having the opportunity to conduct pilot experiments, I had the opportunity to test different methods and evaluate their performance. This allowed me to make a direct comparison of techniques while gaining a deep understanding of their applications and limitations. During this time, I was also able to analyze and compare the results obtained from each technique in order to evaluate its effectiveness in the context of my research questions. Each pilot experiment was a learning opportunity. Despite some difficulties encountered along the way, I learned to deal with them and learn from the results obtained. This allowed me to move forward in my research project with greater confidence and awareness of the choices I made. By conducting several pilot experiments, I was able to compare the different options available, learning about their characteristics and adapting them to my specific needs. This selection process allowed me to outline a robust research strategy that I subsequently followed throughout my Ph.D.

2.1 Testing different markers

From February 2020 to April 2020 I joined Dr Pascal Malkemper's laboratory (Center of Advanced European Studies and Research, CAESAR, Bonn Germany). During this time, I tested if polyclonal antibodies for c-fos and egr-1 available in the lab from previous studies would also work on the brain of *P. zebra*. Unfortunately, not enough antibody for egr-1 and c-fos was available to finish the project as production by the company Santa Cruz Biotechnology had been terminated in 2016. I switched then to a different IEG antibody (c-Jun, Santa Cruz Biotechnology, Inc.), but the antibody didn't stain anything specific in the fish brain. Similarly, another egr-1 antibody (Proteintech, 22008-1-AP) did not yield any results either. To find a new marker that would work on my animal model, I contacted Dr Hans Hofmann from the University of Texas (Austin, Texas, United States). Dr Hofmann suggested to switch to the phosphorylated ribosome marker (pS6) antibody, as they had already done in his laboratory in Austin. Indeed, in the last few decades pS6 has become a popular alternative to IEGs to visualize neural activation fish. Furthermore, as pS6 staining is cytoplasmic, it is more easily analyzed than c-fos and egr-1, which stain the nucleus of the cell. Figure 8 shows an example of c-fos (A1-A2-A3), egr-1 (B1-B2-B3), c-jun (C1-C2-C3) and pS6 (D1-D2-D3) staining in the brain of *P. zebra*. The first column (A1-D1) shows the staining of two areas in the telencephalon: Dmd and Dmv. The second column (A2-D2) shows the staining of the inferior lobes: ILrec, NDI, NDm. The third columns (A3-D3) shows the staining of the optic tectum, in particular the stratum periventriculare: SPV. As can be seen, c-fos and egr-1, mark cells in a very similar way (Figure 8, A1-B1-C1, A2-B2-C2). Scale bar: 50 µm applies for all. For abbreviations, see list of abbreviations.

The only major difference is that egr-1 presents a more pronounced background staining than c-fos, especially at the level of the optic tectum (Figure 8, B1-B2-B3). C-jun (Figure 8, C1-C2-C3) does not show any stained cells, only a light background staining can be observed. Unlike the three IEGs mentioned above, pS6 (D1-D2-D3) shows a more distinct staining pattern. In particular, the signal to noise ratio is very high, enabling the precise distinction of the marked cells.

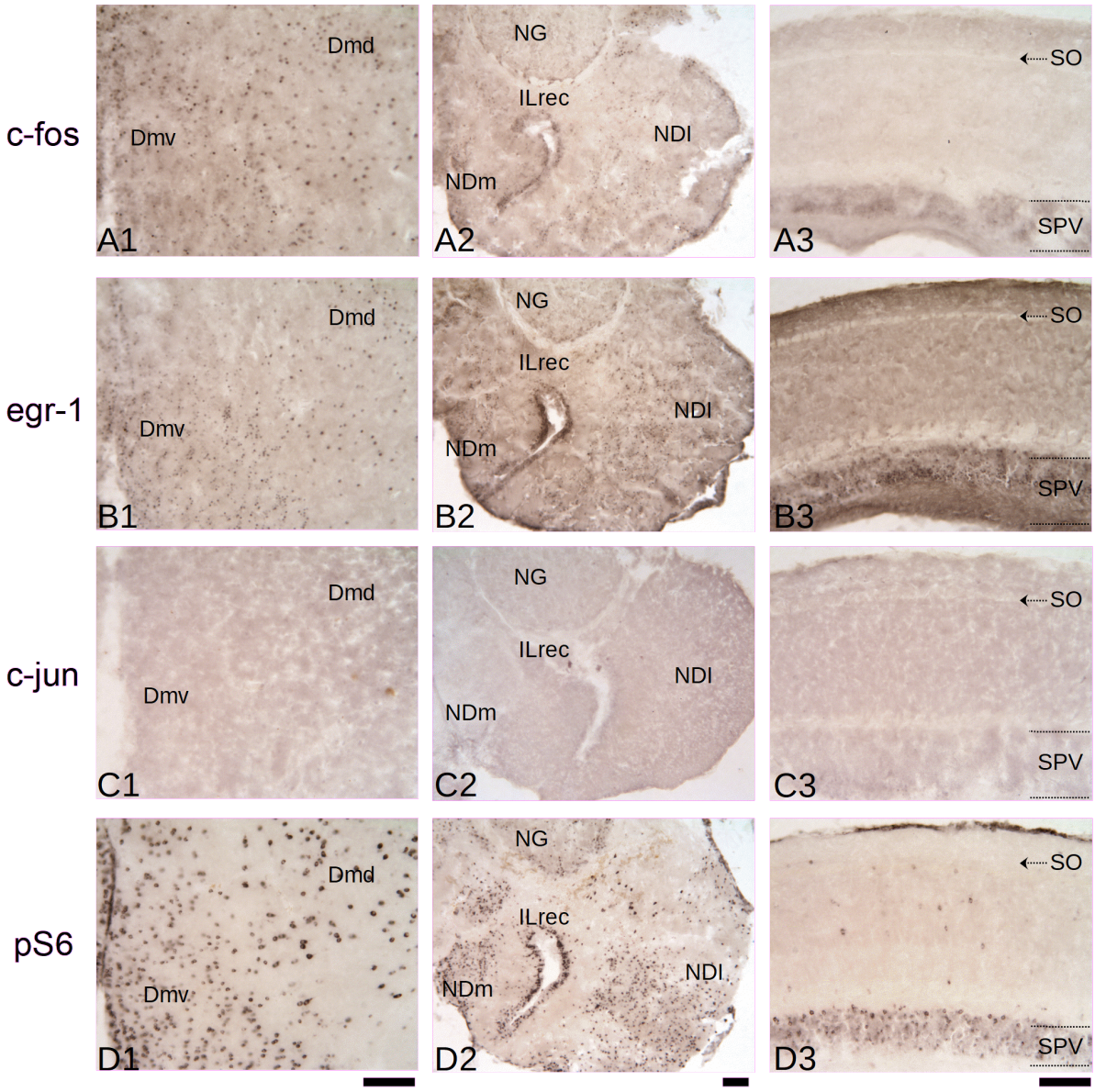

Figure 8: Example of c-fos (A1-A2-A3), egr-1 (B1-B2-B3), c-jun (C1-C2-C3) and pS6 (D1-D2-D3) staining in the brain of *P. zebra*. The first column (A1-D1) shows the staining of two areas in the telencephalon: Dmd and Dmv. The second column (A2-D2) shows the staining of the inferior lobes: ILrec, NDl, NDm. The third columns (A3-D3) shows the staining of the optic tectum, in particular the stratum periventriculare: SPV. Scale bar: 50 µm applies for all. For abbreviations, see list of abbreviations.

2.2 Staining procedures

During my time at CAESAR, I modified and adjusted the already available immunohistochemistry protocol for sharks to suit the cichlid *P. zebra*. Furthermore, I learned to perform the 'clearing technique' (Vigouroux et al. 2017) to investigate the expression of IEGs using light sheet microscopy. This technique allows for rapid volumetric imaging of specimens by illuminating a thin volume of the specimen by a sheet of laser light. Then, the fluorescence emitted is imaged onto a camera positioned at a right angle to the light sheet (for a review see Ueda et al. 2020). The final result is a three-dimensional view of the specimen.

In order to allow the antibodies to penetrate the specimen easily, a tissue permeabilization is performed. It consists of a series of different chemical solutions to gradually remove lipids and pigments and permeabilize the tissue for a better penetration of the antibodies. Aftere the antibody incubations, the tissue is cleared to enable a 'clean' view of the specimen under the microscope. Figure 9 shows an example of an egr-1 stained brain scanned with light sheet microscope. (A) a raw single section cut at a random angle (not a standard cross section) of the brain, (B) the rotated surface reconstruction of the brain, (C) a rotated cross section of the telencephalon (C), and (D) a magnification of the telencephalon (Dmd, Dmv, Dc). The dotted line in B shows the level and the angle of the cut for sections C and D. Scale bar in A and B: 1mm. Scale bar in C: 500 µm. Scale bar in D: 250 µm. For abbreviations, see list of abbreviations.

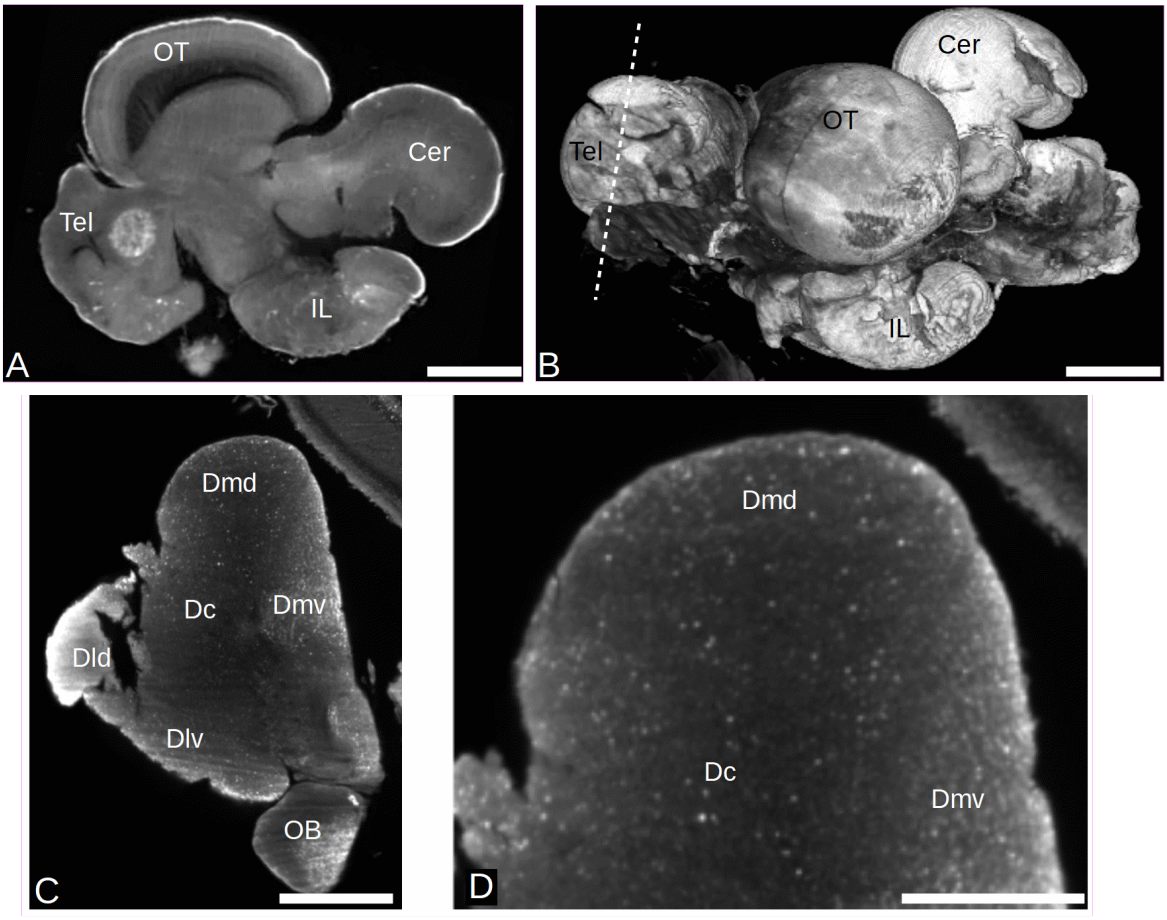

Figure 9: Egr-1 stained brain scanned with light sheet microscope (in collaboration with Pascal Malkemper, CAESAR institute, Bonn). A: raw single section cut of the brain. B: rotated surface reconstruction. C: cross section of the telencephalon. D: magnification of Dc, Dmd, Dmv of a. The dotted line in B shows the level and the angle of the cut for sections C and D. Scale bar in A and B: 1mm. Scale bar in C: 500 µm. Scale bar in D: 250 µm. For abbreviations, see list of abbreviations.

This technique is becoming popular for whole-brain profiling of stained cells but offers limited resolution while requiring a high volume of antibody for each brain (e.g. 30 uL of primary antibody for a single brain, compared to 6.5 uL of primary antibody for ten sliced brains during a normal immunohistochemistry on slides). As only little antibody was available, subsequent immunohistochemistry assessments were performed using bright field and immunofluorescence.

2.3 Cell counting methods

In order to count stained cells, I tested different programs, i.e. ImageJ, QuPath and Stereoinvestigator. ImageJ is the most popular freeware, Java-based image processing program available. It includes different tools for image processing, including histogram manipulations, background subtraction and standard image filters. There are two different ways to count cells with ImageJ, the manual and the automated ways. The first way enables cell counts by simply clicking on the cell on the image. Each click marks the cell with a colored square and adds the cell to a sheet. For this project, the manual way of counting was not really practical due to the high number of images to process and the high number of cells per image. However, the automated way is complicated, in particular in the case of images in bright field or multi-color (O'Brien et al. 2016). QuPath also is a freely available program and has been designed to analyze whole slide imaging data but could not be used for my project, as no counterstaining with hematoxylin was performed (Bankhead et al. 2017). 'Stereoinvestigator' is a commercially available software package, developed to help with stereological counting of cells in the tissue and requires a computerized microscope. The program is based on the optical fractionator workflow which estimates the total number of cells from the number of cells sampled with a Systematic Randomly Sampled set of unbiased virtual counting spaces covering the entire region of interest with uniform distance between unbiased virtual counting spaces in directions X, Y and Z. The Institute of Zoology neither owns the required microscope nor software, so Dr Hans Hofmann invited me to conduct the cell counting and required analysis at the University of Texas at Austin. Unfortunately, the time available was not enough to analyze my samples due to the high number of areas to be investigated (>3000). Back in Bonn, I used a program written by Prof Dr Michael specifically for this project. Details of the program can be found in Chapter 2 "Brain areas activated during visual learning in the cichlid fish *Pseudotropheus zebra*".

3. Chapter 3: Brain Areas Activated During Visual Learning in the Cichlid Fish *Pseudotropheus zebra*

Chapter 3 has already been published by Brain Structure and Function:
Calvo R, Hofmann MH & Schlüssel V. Brain areas activated during visual learning in the cichlid fish *Pseudotropheus zebra*. Brain Struct Funct 228, 859–873 (2023).
https://doi.org/10.1007/s00429-023-02627-w

3.1 Summary

In this paper, the activation patterns of 19 different brain areas were analyzed in cichlid fish subjected to three different behavioral tasks involving object recognition and memory formation. More specifically, fish (n=40) were assigned to four different groups. Fish in the control group were sacrificed with minimal interactions. Fish in the avoidance group were chased with a net for one hour, and then sacrificed. Fish in the trained group received daily training sessions to associate a visual object with food reward, and then they were sacrificed the day they reached learning criterion. Finally, fish in the novelty group were habituated to one set of visual stimuli, and then faced a change in stimulus type (novelty stimulus) before being sacrificed. Brains were then fixed in 4% PFA and thirty-five µm thick sections were cut at − 20 °C with a cryostat. Then, immunohistochemistry for the Ribosomal Protein S6 Phosphorylation (pS6) was performed. S6 protein is a component of the 40S ribosomal subunit and its inducible phosphorylation occurs in response to a different stimulus (Meyuhas 2008). Indeed, in the last decade pS6 has become a popular method to visualize neural activation in fish (e.g. Benitez-Santana et al. 2017, Butler et al. 2018, Fischer et al. 2018, York et al. 2018, 2019, Trip et al. 2019, 2020, Baran and Streelman 2020, Butler et al. 2020, Maruska et al. 2020, Schuppe et al. 2021, Suzuki et al. 2021, Scaia et al. 2022). For the current study, the specificity of pS6 antibody was checked by replacing either the primary or secondary antibodies with PBS, showing no reaction product. The same antibody from Cell Signaling has been used successfully in several studies on fish, including cichlids (Butler et al. 2018, 2019, 2020, Maruska et al. 2020). In cichlid, the antibody has been validated in *Astatotilapia burtoni* (Butler et al. 2020) by western blot.
The analysis of results consisted of counting the number of stained cells in the 19 brain areas in each fish. In order to be able to count cells in the most objective and blind way, an automated

costumed counting program was written by Dr Michael Hofmann. In the first step of the counting process (i.e. segmentation of the areas), the user selected a brain area for the segmentation and the program presented an image stack chosen randomly from the four groups without showing any labels, thus ensuring the blindness of the user. The segmentation was the only step with user intervention. Several areas were segmented from both sides of the brain or from different sections, resulting in more than 3000 individual areas defined for the 19 brain regions in the 40 animals. An automated image analysis workflow was used to measure the segmented areas. The ratio of the area stained by the pS6 antibody divided by the total area segmented for each brain part, averaged for each group was then calculated. A U-test was performed for each brain part to compare the three learning groups with the control group to check for significant differences. The activation in each brain area was calculated by dividing the pS6 stain level in the three learning groups by the staining level in the control group and log-transformed.

Unlike others, this study correlated the various components of the behavioral conditions with the activity in many different brain areas. The strong activation of the optic tectum and the inferior lobes was the only characteristic common to all experimental groups (avoidance, trained and novelty). The inferior lobes are present only in teleosts and are the target of a visual pathway originating in the optic tectum via the nucleus corticalis and the nucleus glomerulosus (Wullimann and Meyer 1990, Butler et al. 1991, Shimizu et al. 1999, Ahrens and Wullimann 2002, Yang et al. 2007). Furthermore, a recent study suggests that they are of mesencephalic origin (Bloch et al. 2019). My study showed for the first time that the inferior lobes are activated in all three visual learning situations. These results give the first evidence of a role of the inferior lobes, and the nucleus diffusus in particular, in visual discrimination, object recognition and memory formation, common components of the behavior experienced in all treatment groups except the control group.

3.2 Author's contributions

I am the primary author of the paper, Prof Dr Michael Hofmann and my supervisor Dr Vera Schlüssel are co-authors. Behavioral experiments were conducted under my supervision by students as part of their internship (see section 1. Assistance received and resources used). I took care of the killing of the fish, cutting their brains using the cryostat and performing immunohistochemistry. Prof Dr Hofmann wrote a costumed automated counting program and assisted me with the analysis of the data. Literature research and manuscript writing were taken care by myself. Dr Vera Schlüssel designed the behavioral experiments, provided all financial and logistical support and commented on the manuscript.

4. Chapter 4: Activation Patterns of Dopaminergic Cell Populations Reflect Different Learning Scenarios in a Cichlid Fish, *Pseudotropheus zebra*

Chapter 4 has already been published by Journal of Chemical Neuroanatomy:
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https://doi.org/10.1016/j.jchemneu.2023.102342.

4.1 Summary

In this paper, the distribution of the dopaminergic system was investigated in the cichlid *P zebra* and the activation patterns of 13 dopaminergic cell populations were analyzed in fish subjected to two different behavioral tasks involving object recognition and memory formation. More specifically, the same individuals were assessed in the previous study (chapter 3) concerning the activation of different brain areas by immunostaining with pS6. For this study, fish in the control, avoidance and trained groups were investigated (n=30). In order to investigate the distribution of the dopaminergic system in the brain of *P. zebra*, an immunohistochemistry for tyrosine hydroxylase (TH) was performed. Furthermore, a fluorescent double labeling rabbit anti-TH and mouse anti-pS6 antibodies was carried out at the University of Texas (Austin, Texas, United States) to analyze the specific activation of the dopaminergic cell populations. A rabbit anti-pS6 antibody has been used successfully in several studies on fish, including my previous publication (Calvo et al. 2023). In this study, anti-pS6 antibody made in mouse was used. Dr Ross DeAngelis, from Hans Hofmann's laboratory group in Austin, performed a double labeling rabbit anti-pS6 and mouse anti-pS6 showing no differences in the staining, that is rabbit anti-pS6 and mouse anti-pS6 stained the exact same cells.

Dopamine is present in all vertebrates and is one of the most intensively investigated neurotransmitters due to its involvement in many physiological functions (e.g. motor control, reward, motivation) and various human diseases (e.g. Parkinson's, ADHD, schizophrenia) (Callier et al. 2003, O'Connell et al. 2011).

In the CNS of mammals, DA cell groups form five major pathways, mesocortical, mesolimbic, nigrostriatal, tuberoinfundibular, and spinal tract systems, which respectively are involved in reward and motivation (Kelley and Berridge 2002) and stress response (Salamone et al. 1997, Berridge and Robinson 1998, Ikemoto and Panksepp 1999), learning and motor function (Hikosaka et al. 2002), regulation of the secretion of prolactin from the pituitary (Demarest et al. 1984), and finally locomotion and modulation of the nociception in the spinal cord (Piña-Leyva et al. 2022).

The dopaminergic system has been also investigated in teleost fish (e.g. goldfish *Carassius auratus* (Hornby et al. 1987), the brown ghost knifefish (*Apteronotus leptorhynchus*; Sas et al. 1990), the zebrafish (*Danio rerio*; Kaslin and Panula 2001, Rink and Wullimann 2001), and Burton's mouthbrooder cichlid (*Astatotilapia burtoni*, O'Connell et al. 2011, O'Connell et al. 2013b)) and to get a more detailed understanding of the function of the dopaminergic system in teleost, the IEGs have been used to selectively mark the activation of dopaminergic cell populations (e.g. Kress and Wullimann 2012, O'Connell et al. 2013b, Semenova et al. 2014, Weitekamp and Hofmann 2017). Considering the diverse functions that dopamine plays as neurotransmitter and modulator, in the present study the activation of thirteen different dopaminergic cell populations was analyzed in fish subjected to two learning tasks compared to a control. In order to quantify the activation of the tyrosine hydroxylase positive (TH+) cells in the thirteen-cell group, the number of TH+ cells and the number of TH+ cells that were double-labeled with pS6 were counted in each area in each experimental group. Prof Dr Michael Hofmann helped me to design a specific program for the counting procedure. The proportion of double-labeled cell bodies in each area for the three groups was then calculated. A Fisher test based on the pooled cell counts for each group and brain part was made and the p-values were adjusted according to Bonferroni to show the significance that should be considered for a global null-hypothesis. In addition, the non-parametric Mann-Whitney-test was calculated to test for group differences. The results show a response of the preoptic (POA) -hypothalamic cell groups to the stress component in the avoidance group, together with a strong activation of the suprachiasmatic nucleus (SCN) and the nucleus of the posterior tubercle (nTP), due to the forced movement/locomotion. Interestingly, the hypothalamic nucleus of the posterior recess (nPR) dopaminergic cell population was the only area that presented less double-labeled cells in the avoidance group compared to the control. In the avoidance and trained groups, the activation was observed mainly in the periventricular pretectal nucleus (PP) and the nucleus of the posterior tubercle (nTP), suggesting a modulation of the optic tectum and the inferior lobes by the dopaminergic cell populations of PP and nTP, respectively.

Furthermore, activation of the dopaminergic populations of the raphe superior (Ras) was observed in both groups and may be correlated with the arousal during the training sessions.

Finally, a Principal Component Analysis (PCA) and a hierarchical clustering were conducted, showing robust differences across experimental groups, largely driven by hypothalamic and midbrain regions possibly encoding the valence and salience associated with stressful stimuli.

My results offer some insights into the different functions of the dopaminergic cell groups in the brain of a teleost specifically in correlation with different behavioral conditions, extending our knowledge for a more comprehensive view of the mechanisms of dopaminergic modulation in vertebrates.

4.2 Author's contributions

I personally proposed the idea of investigating the dopaminergic system due to its different physiological roles (e.g. involved in reward, motivation, learning and stress). I am the primary author of the paper, my supervisor Dr Vera Schlüssel, Dr Hans Hofmann and Prof Dr Michael Hofmann are co-authors. Behavioral experiments were conducted under my supervision by students as part of their internship (see section 1. Assistance received and resources used). I took care of the killing of the fish, cutting their brains using the cryostat and performing immunohistochemistry for tyrosine hydroxylase (TH). In order to investigate the activation of the DA cell groups, I also performed a fluorescent double labeling rabbit anti-TH and mouse anti-pS6 antibodies at the University of Texas (Austin, Texas, United States) were I worked on part of my project under the supervision of Dr Hans Hofmann. Literature research and manuscript writing were taken care by myself. Prof Dr Michael Hofmann helped with the designing of the counting program and with the writing of the manuscript. Dr Hans Hofmann provided me with antibodies and laboratory assistance for the double labeling and he helped me with part of the analysis of the data with R studio program. His PhD student, Jiawei Han, provided me with the R scripts for the analysis. Dr Vera Schlüssel provided financial and logistical support, and commented on the manuscript.

5. Chapter 5: New Neurons and Reorganization of Existing Connections. Understanding the Distribution of egr-1 and pS6 in the Brain.

Chapter 5 has been submitted to “Brain Research” and it is currently under review.

5.1 Summary

In this paper, the distributions of egr-1 and pS6 were investigated and compared in the brains of control and stimulated fish. Egr-1 and pS6 are neural markers commonly used to localize brain areas activated during different behavioral states but their expression is also often associated with morphological changes in the nervous system, e.g. proliferation of new neurons and synaptic reorganization. The latter are known to be the key steps in brain plasticity, essential process underlying learning and memory formation (Tischmeyer and Grimm 1999). It is already known that egr-1 presents a role in controlling proliferation in different cell types in response to mitogens (e.g. astrocytes, T-cells, glioma cells, glomerular mesangial cells, and keratinocytes (Perez-Castillo et al. 1993, Biesiada et al. 1996, Höfer et al. 1996, Kaufmann and Thiel 2001, 2002)), and its expression has also been connected with the development of cancer in humans (Eid et al. 1998), while RpS6 phosphorylation has been shown to be involved in the control of size of different cell types, among others (Meyuhas 2008). My results show that the expression of egr-1 is strongly correlated with proliferation zones, suggesting that egr-1 is involved in the maturation of new neurons. The distribution of pS6 is more widespread and may be associated with synaptic plasticity in existing neurons. In particular, almost the entire telencephalon shows proliferating cells and egr-1 positive (erg-1+) cells, as well as the POA which is associated with the diencephalic ventricle. Proliferation zones and egr-1+ cells are found in the dorsal diencephalon in the habenula, in the central posterior thalamic nucleus and dorsal posterior thalamic nucleus (CP and DP), as well as in the periventricular pretectal nucleus paracommissuralis, periventricular posterior tubercle and paraventricular organ (PVO). A larger system of proliferating cells is located along the ventricular system of the hypothalamic area and they extend into the posterior recess, and in the lateral recess that forms the inferior lobes which contain egr-1+ cells. In the mesencephalon, the most important proliferation zones are located in the optic tectum and torus longitudinalis. These also extend into the ventricular surface of the torus semicircularis and some proliferating material could be found also in vicinity of the nucleus lateralis valvulae. All those areas present erg1+ cells. Erg-1 is also present in the facial and vagal lobes, areas known to present proliferation zones.

Furthermore, Erg-1 is present in nuclei (e.g. torus lateralis and corpus mamillare) but it is not clear whether also receive newborn cells from proliferation zones.

PS6 is present in many more areas far away from proliferation zones. In some areas it is present even in control individuals. These cells (motor neurons, the Mauthner cell, locus coeruleus, and cerebellar Purkinje cells) are large in size and show ongoing synaptic plasticity and reorganization of their processes. For example, cells in the locus coeruleus are known to be able to dynamically change their synaptic terminals. Motor neurons are able to regrow their axons and make new synapses in case of injuries. Purkinje cells in the cerebellum, located in the caudal lobe and caudal corpus, are known to involved in vestibulo-ocular reflexes and stabilize the image on the retina during head movements. Since eye muscles have no muscle spindles, permanent feedback from the visual system is necessary to adjust the gain of the vesibular-oculomotor loop. This may be the reason why pS6 is present in the caudal lobe of the cerebellum even in controls.

In conclusion, results of our study first help to understand the significance of egr-1 and pS6 staining, but also give more information about the location of areas involved in learning and memory, due to the addition of new neurons, and about other areas that show predominantly plasticity, due to the reorganization of existing networks. In particular, on one hand there is a correlation between the distribution of egr-1 and the proliferation zones in the fish brain, suggesting a function of egr-1 in transforming immature neurons into functional neurons to get incorporated into existing networks. Therefore, egr-1 may be a marker of memory formation due to addition of new neurons in existing networks. On the other hand, pS6 is more widespread and may be the signal to change synaptic networks in existing, functional neurons. Thus, pS6 may be a marker for neurons that undergo synaptic plasticity.

5.2 Author's contributions

I am the primary author of the paper, my supervisor Dr Vera Schlüssel and Prof Dr Michael Hofmann are co-authors. Part of behavioral experiments was conducted by myself, part was conducted under my supervision by students as part of their internship (see section 1. Assistance received and resources used). I took care of the killing of the fish, cutting their brains using the cryostat and performing immunohistochemistry for both egr-1 and pS6. Prof Dr Hofmann assisted me with the analysis of the data, literature research and manuscript writing. Dr Vera Schlüssel provided financial and logistical support, and commented on the manuscript.

Copy of the manuscript submitted to "Brain Research"

New neurons and reorganization of existing connections. Understanding the distribution of egr-1 and pS6 in the brain.

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Abstract

Brain plasticity is the basis of learning and memory and is required for any structural changes within the central and peripheral nervous system. Morphological changes, like synaptic reorganization or structural change in dendrites or axonal branchings, are occurring constantly throughout development and beyond. In addition, new neurons are recruited and incorporated into existing networks. Many morphological changes are associated with the expression of key proteins that may act on the transcriptional level (e.g. c-fos and egr-1) or on the translational level (e.g. pS6). Such proteins can therefore be used as neuronal markers to identify brain areas involved in different kinds of behavioral tasks. However, it is not known what kind of changes in these 'activated' neurons occur. We studied the distribution of egr-1 and pS6 in the brains of controls and stimulated cichlids, Pseudotropheus zebra, and found that egr-1 is restricted to proliferation zones, while pS6 is found in many more areas also far away from proliferation zones. Furthermore, pS6 is permanently present in some cell populations like motor neurons, locus coeruleus, the reticular formation and cerebellar Purkinje cells. There is evidence that neurons in these areas are constantly readjusting their processes, suggesting that pS6 may be associated with synaptic plasticity in existing neurons. In contrast, egr-1 may play a role in the life cycle of neurons promoting their maturation and integration into existing networks. This offers the opportunity to use these markers not only to study neuronal activation, but also to localize where specific memory and learning events are taking place.

Keywords:

Cichlid, egr-1, pS6, proliferation, synaptic plasticity, learning

Footnote:

List of abbreviations

A anterior thalamic nucleus; *ATN* anterior tuberal nucleus; *CC* crista cerebellaris; *CM* corpus mamillare; *Cor* corpus cerebelli*; CP* central posterior thalamic nucleus; *Dc* central division of the dorsal telencephalon; *Dcd* dorsal subdivision of the central division of the dorsal telencephalon; *Dcv* ventral subdivision of the central division of the dorsal telencephalon; *Dd* dorsal division of the dorsal telencephalon; *Dl* lateral division of the dorsal telencephalon; *Dld* dorsal subdivision of the lateral division of the dorsal telencephalon; *Dlv* ventral subdivision of the lateral division of the dorsal telencephalon; *Dm* medial division of the dorsal telencephalon; *Dmd* dorsal subdivision of the medial division of the dorsal telencephalon; *Dmv* ventral subdivision of the medial division of the dorsal telencephalon; *DON* descending octaval nucleus; *Dp* posterior division of the dorsal telencephalon; *DP* dorsal posterior thalamic nucleus; *dV* descending trigeminal nucleus; *EG* eminentia granularis; *ETh* epithalamus; *fr* fasciculus retroflexus; *G* granular layer of the cerebellum; *Hab* habenula; *Hypo* hypothalamus; *Hc* caudal hypothalamus; *ll* lateral lemniscus; *I* intermediate thalamic nucleus; *IL* inferior lobes of the hypothalamus; *ILc* inferior lobes, central nucleus; *ILdl* inferior lobes, diffusus lateralis; *ILdm* inferior lobes, diffusus medialis; *ILrl* nucleus of the recessus lateralis; *IP* interpenduncular nucleus; *LC* locus coeruleus; *LVII* facial lobe; *LX* vagal lobe; *M* molecular layer of the cerebellum; *Ma* Mauthner cells; *mIII* oculomotor nucleus; *ME* medium eminence; *Mg* magnocellular octaval nucleus*; mIV* trochlear motor nucleus; *mIX* glossopharyngeal motor nucleus; *mlf* medial longitudinal fascicle; *MON* medial octavolateral nucleus; *mV* trigeminal motor nucleus; *mVI* abducens motor nucleus; *mVII* facial motor nucleus; *mX* vagal motor nucleus; *NC* nucleus corticalis; *ND* nucleus diffusus of the inferior lobes; *NG* nucleus glomerulosus; *NGT* tertiary gustatory nucleus; *NI* nucleus isthmi; *NLT* nucleus lateralis tuberis; *nPR* nucleus of the posterior recess; *NLV* nucleus lateralis valvulae*; nVII* sensory root of the facial nerve; *OB* olfactory bulb; *P* Purkinje layer of the cerebellum; *PAG* periaqueductal gray; *PGa* anterior preglomerular nucleus; *PGc* central preglomerular nucleus; *PGl* lateral preglomerular nucleus; *PGm* medial preglomerular nucleus*; POA* preoptic area; *POAa* preoptic area, anterior part; *POAp* preoptic area, posterior part; *PreT* pretectum; *PTc* pretectal area, centralis; *PTco* pretectal area, corticalis; *PTpa* pretectal area, nucleus paracommissuralis; *PTc* pretectal area, nucleus centralis; *PTpo* pretectal area, nucleus of the posterior commissure; *PTsm* pretectal area, superficialis magnocellularis; *PTsp* pretectal area, superficialis parvocellularis; *pV* principal trigeminal nucleus*; PVO* periventricular organ; *Ras* raphe superior; *RF* reticular formation; *RFi* inferior reticular formation; *RFm* medial reticular formation; *RFs* superior reticular formation; *RL* recessus lateralis; *SCN* suprachiasmatic nucleus; *SFGS* stratum fibrosum et griseum superficiale of the tectum opticum; *SGN* secondary gustatory nucleus; *SON* secondary octaval nucleus; *SPV* stratum periventriculare of the tectum opticum; *Teg* mesencephalic tegmentum; *TGN* tertiary gustatory nucleus; *TL* torus longitudinalis; *TLat* torus lateralis; *TPp* periventricular posterior tubercle; *TO* tectum opticum; *TPn* nucleus of the posterior tubercle; *TS* torus semicircularis; *Val* valvula cerebelli; *VAO* ventral accessory optic nucleus; *Vc* central nucleus of the ventral division of the telencephalon; *Vd* dorsal nucleus of the ventral division of the telencephalon; *VIII* octaval nerve; *Vl* lateral nucleus of the ventral division of the telencephalon; *VL* ventrolateral thalamic nucleus; *VM* ventromedial thalamic nucleus; *Vp* posterior nucleus of the ventral division of the telencephalon; *Vs* superior nucleus of the ventral division of the telencephalon; *VTN* ventral tuberal nucleus; *Vv* ventral nucleus of the ventral division of the telencephalon; *X* vagal nerve.

1. Introduction

Learning and memory formation are processes that depend on the capacity of the brain to remodel its functional and morphological states (Bruel-Jungerman et al., 2007). During these processes, cell proliferation and synaptic rearrangements are required in the central nervous system (Tischmeyer and Grimm, 1999). The activation of immediate early genes (IEGs) and the phosphorylation of the S6 ribosomal protein (pS6) are two processes known to be involved in the formation of new neurons and synaptic connections (for reviews see Gashler and Sukhatme, 1995; Meyuhas, 2008, 2015). IEGs are cellular genes responsive to extracellular stimuli. Their expression is immediately induced after stimulation following neural activity and does not require new protein synthesis (Morgan and Curran, 1991; Barry et al., 2016). One of the most commonly investigated IEG is the Early Growth Response Gene egr-1, also known as zif/268, krox-24, TIS8, NGFI-A or zenk. It codes for the transcription factor egr-1, a phosphorylated protein synthesized in the nucleus, where it remains (Cao et al., 1990). In the brain, the expression of egr-1 is specific to neurons and it is continually induced by ongoing synaptic activity as a consequence of the basal physiological synaptic activity (Worley et al., 1991; Burmeister and Fernald, 2005). This is a major difference between egr-1 and other inducible transcription factors (e.g. c-fos), whose expression declines after the initial stimulation (Herdegen et al., 1995; Kaczmarek and Chaudhuri, 1997). Furthermore, the egr-1 protein has the ability to autoregulate its own expression (Chen et al., 2010). It plays a crucial function in cognitive processes, particularly in learning and memory (Okuno, 2011). Egr-1 controls cell proliferation in response to mitogens, for example in astrocytes, T-cells, glioma cells, glomerular mesangial cells, and keratinocytes (Perez-Castillo et al., 1993; Biesiada et al., 1996; Hofer et al., 1996; Kaufmann and Thiel 2001, 2002). Furthermore, egr-1 has also been linked to the development of cancer in humans (Eid et al., 1998). Different genes have been identified as targets of egr-1, among them transforming growth factor-b1, insulin-like growth factor-II and platelet-derived growth factors A and B (Khachigian et al., 1995, 1996; Svaren et al., 2000), suggesting an involvement of egr-1 in the mitogenic signaling cascade. Studies suggest that expression of egr-1 can be mediated through the activation of MAP kinases, protein kinase A (PKA) and protein kinase C (PKC) (Mechta et al., 1989; Ginty et al., 1991; Vaccarino et al., 1992; Ferhat et al. 1993; Simpson and Morris, 1995).

Like IEGs, the phosphorylated ribosome marker pS6 is used to visualize neural activation. S6 protein is a component of the 40S ribosomal subunit. Its inducible phosphorylation, which occurs in response to a large variety of stimuli, was the first post-translational modification described

regarding ribosomal proteins (Gressner and Wool, 1974; Meyuhas, 2008) and has attracted attention since its discovery in 1974 in rat liver regeneration (Gressner and Wool, 1974). Today, pS6 phosphorylation is commonly used as a marker for neuronal activity. The first evidence for the increase of rpS6 phosphorylation during synaptic plasticity was reported in 1991 by Klann and colleagues. Since then, a large number of electrical, chemical, pathological or pharmacological stimuli have been described to promote rpS6 phosphorylation in neurons (Biever et al., 2015).

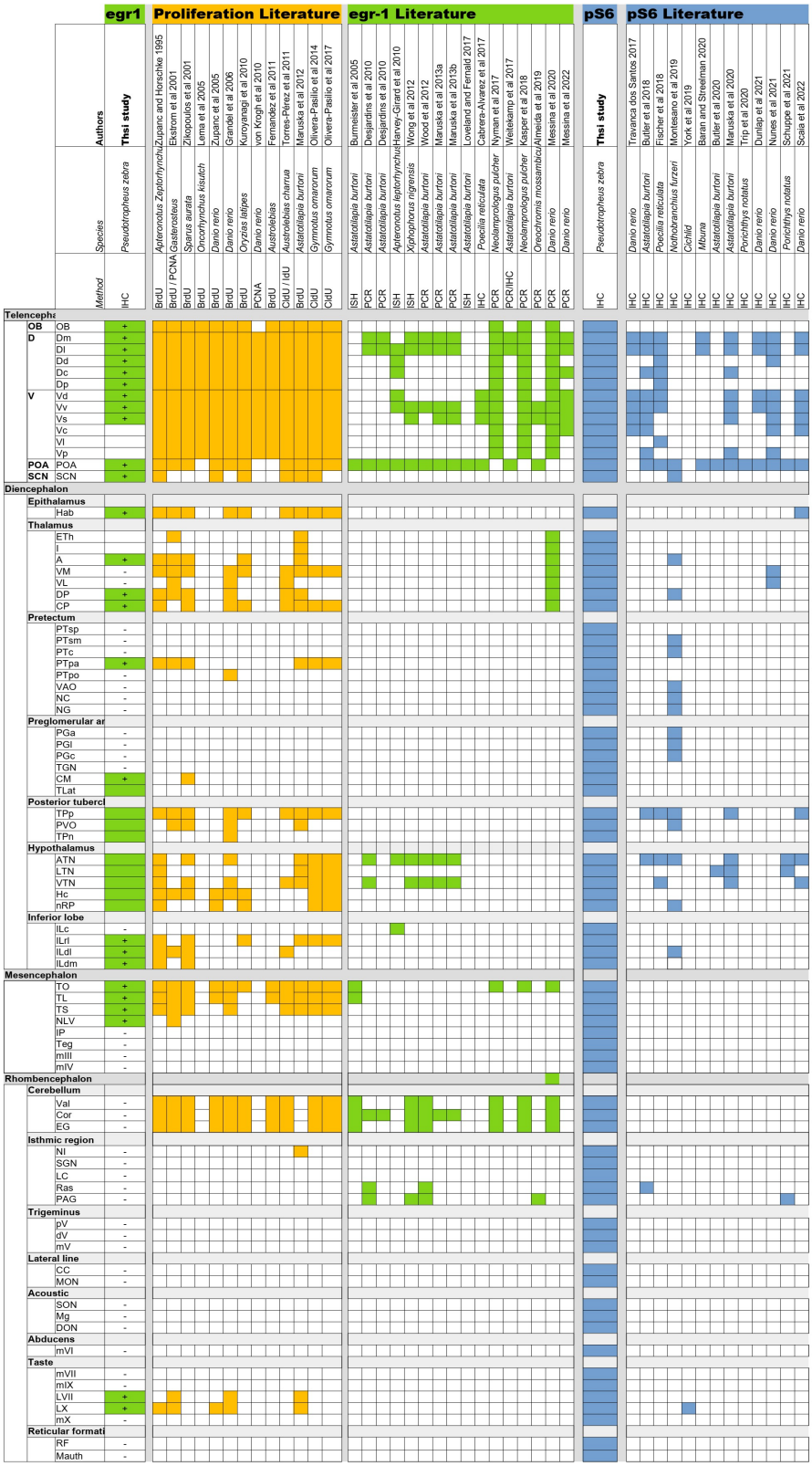
The factors that induce expression of egr-1 and pS6 and their effects on neuronal plasticity are similar (for reviews see Gashler and Sukhatme, 1995; Meyuhas, 2008, 2015), but functional differences are not clear. In most studies egr-1 or pS6 are used as tools, i.e. as functional markers to study the involvement of selected brain areas in specific processes. To our knowledge, there are no studies that describe their distribution throughout the entire brain of control and behaviorally stimulated animals. We do not know whether they are co-expressed in the same areas or neurons. We are also not aware whether all neurons or all areas can potentially be 'activated', or whether expression is limited to certain neuron types or areas.

Here, we investigated the distribution of egr-1 and pS6 throughout the entire brain of the cichlid *Pseudotropheus zebra* by comparing control animals (control group) with animals subjected to an avoidance learning task (avoidance group). Although there were areas that expressed both egr-1 and pS6, differences in their distribution gave insights into the unique role each of these markers play.

2. Results

The aim of this study was to describe the distribution of egr-1 positive (egr-1+) cells in comparison to pS6 positive (pS6+) cells. To see the maximum distribution, we will first describe the staining in the avoidance group because the animals received a stressful stimulation and showed activation, or increased levels of activation, of both egr-1 and pS6 in many areas. In contrast to previous studies, we did not count cells in selected areas, but describe the occurrence of labeled cells throughout the entire brain. Table 1 gives a list of all cell groups and the respective presence of egr-1 positive (egr-1+) and pS6 positive (pS6+) neurons found in the present study as well as data from previously published studies.

Table 1: List of all cell groups and the presence of erg-1 positive (egr-1+) and pS6 positive (pS6+) neurons found in this study and data from previously published studies for comparison. For abbreviations, see list of abbreviations.



2.1 Telencephalon

Egr-1 and pS6 stained cells were found in all areas of the telencephalon. In the olfactory bulb, they were mainly located in the internal cellular layer. All parts of the dorsal telencephalon showed both egr-1+ and pS6+ cells. However, the number of labeled cells was different. Dc showed many egr-1 stained nuclei whereas pS6+ cells were less frequent. The opposite was true for Dd (Fig 1B, C). This was similar in the ventral telencephalon where many egr-1+ nuclei were present in Vd, whereas less were found in Vv. PS6 stained cells had about equal numbers in Vd and Vv (Fig. 1 D, E). The only area that showed only a few stained cells was Vl and this applied to the egr-1 and pS6 staining.

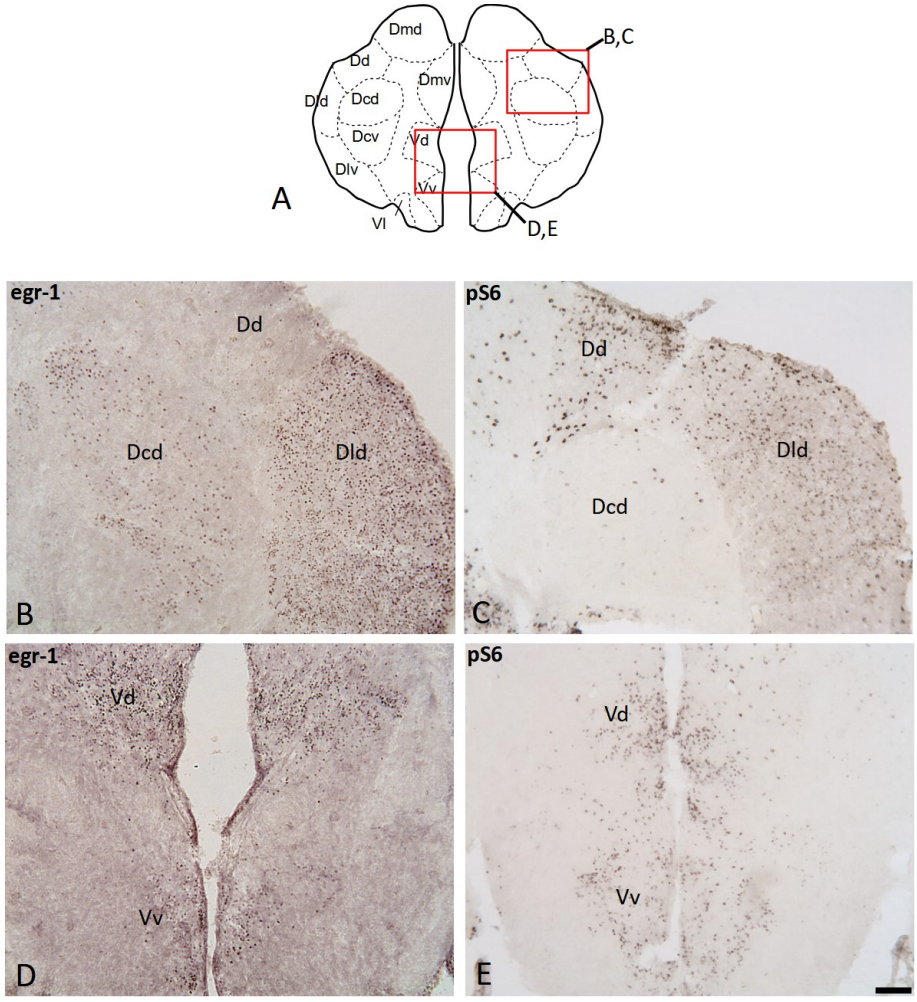


Figure 1: Sections through the telencephalon showing the distribution of egr-1 (B,D) and pS6 (C,E) stained cells. The drawing in A shows the location of the high magnification photographs (red boxes). B: egr-1+ cell nuclei in Dd, Dcd and Dld. C: corresponding section showing pS6+ cell bodies. D: egr-1+ cell nuclei in Vd and Vv. E: corresponding section showing pS6+ cell bodies. Scale bar in E: 50 µm applies for all. For abbreviations, see list of abbreviations.

2.2 Diencephalon

In the dorsal parts of the diencephalon, egr-1 staining was very restricted (Fig. 2). The habenula contained many egr-1 stained cells in the ventrolateral part. In the dorsal thalamus, nucleus A and DP and CP contained many egr-1+ nuclei, but the ventral thalamus (VM, VL) did not show staining. Most of the pretectal cell groups were devoid of any egr-1 staining except the nucleus paracommissuralis (PTpa). Some cells were labeled in the periventricular posterior tubercle (pTP). The migrated parts of the posterior tubercle consist of a number of important preglomerular nuclei, all of which were void of any egr-1 stains. This includes the tertiary gustatory nucleus (TGN). There were two other structure in that area, the torus lateralis (TLat) and the corpus mamillare (CM). Both showed many egr-1+ cells. Especially the CM showed a large number of egr-1+ cells in its granular layer.

The distribution of pS6 stained cells was much more widespread. They were present in all thalamic areas, including the ventral thalamus. The pretectum contained pS6 labeled cells not only in the periventricular nucleus paracommissuralis and nucleus of the posterior commissure (PTpo), but also in all of the migrated pretectal areas (PTsp, PTsm, NC, PTc, NG). The same was true for the region of the nucleus of the posterior tubercle (nTP). In addition to the egr-1 stained area, pS6 was present in all migrated preglomerular areas (anterior PGa, lateral PGl, medial PGm) and in the PGc, which is located directly dorsal to CM. Fig. 2 F and E show the striking difference between egr-1 and pS6 stains in these areas. Whereas PGc and NG were completely free of any egr-1+ cells, pS6 was present in many cells in these nuclei. The figures give the difference in staining pattern in CM, whereas egr-1 showed many stained cells in the granular part of the CM, pS6 stained only lateral parts of CM.

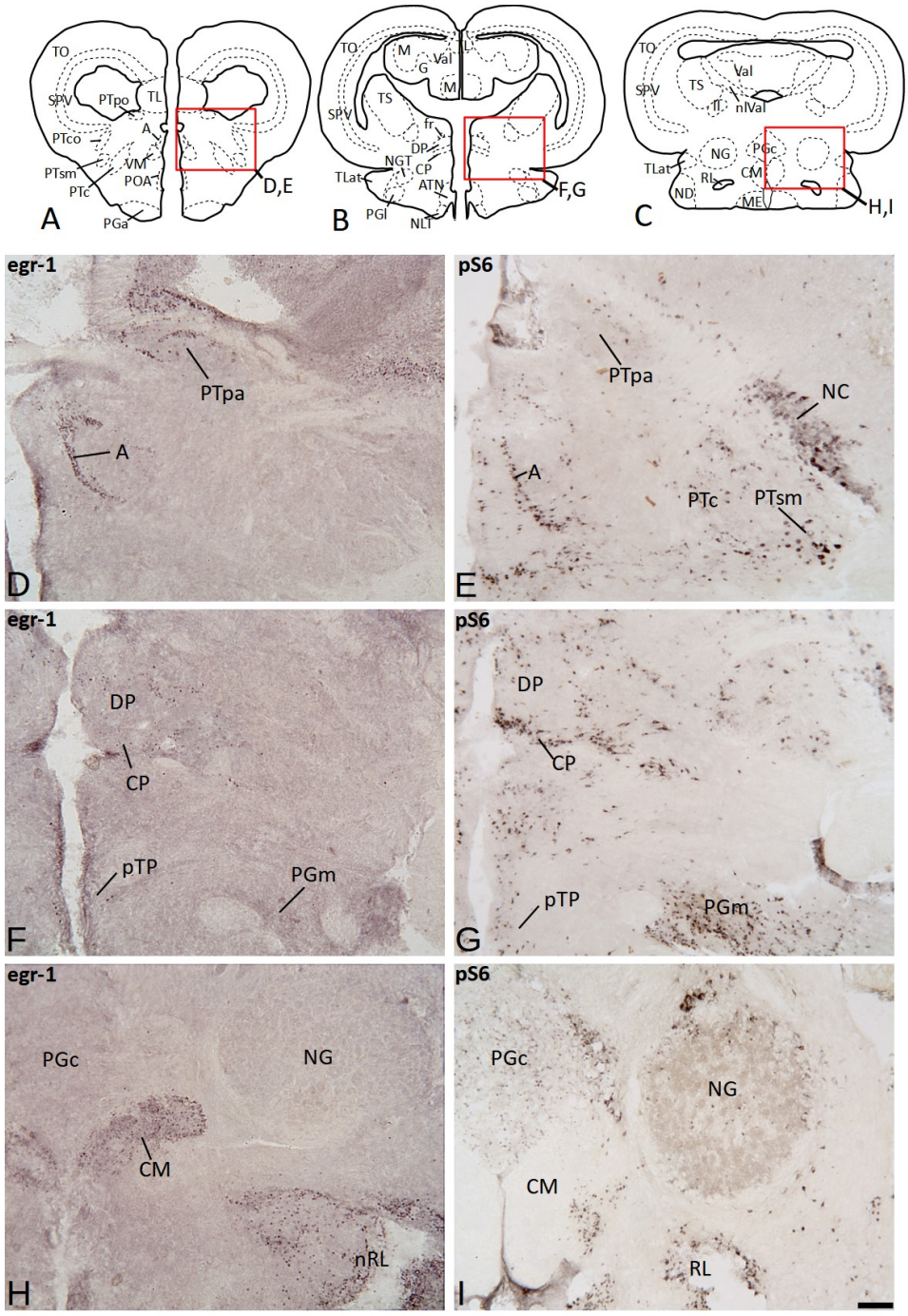


Figure 2: Sections through the diencephalon showing the distribution of egr-1 (D, F, H) and pS6 (E, G, I) stained cells. A, B, C: drawings showing the location of the high magnification photographs (red boxes). D: egr-1+ cell nuclei in nucleus A and PTpa. E: corresponding section showing pS6+ cell bodies also in NC, PTc, PTsm. F: egr-1+ cell nuclei in DP, CP, pTP and PGm. G: corresponding section showing pS6+ cell bodies. H: egr-1+ cell nuclei in PGc, NG, CM and nRL. I: corresponding section showing pS6+ cell bodies. Scale bar in I: 50 µm applies for all. For abbreviations, see list of abbreviations.

2.3 Mesencephalon

In the mesencephalon, egr-1 stained cells were restricted to TO, TL, TS, and NLV. In the TO, they were found only in the periventricular cell layer. In the TS, most cells were located in the periventricular layer, but some were found also in deeper parts. Egr-1+ nuclei were very weakly stained in NLV and TL. The tegmentum (Teg) did not show any egr-1+ cells.

PS6 labeled cells were present in all areas with egr-1+ cells, but also in many other mesencephalic areas. In the TO, pS6+ cells were found also in more superficial layers and were more abundant in the deeper layers of the TS. In addition, all of the oculomotor and trochlear motor neurons (mIII and mIV) were stained, as well as many cell groups in the tegmentum, including the interpeduncular nucleus. These tegmental areas did not show any egr-1 staining.

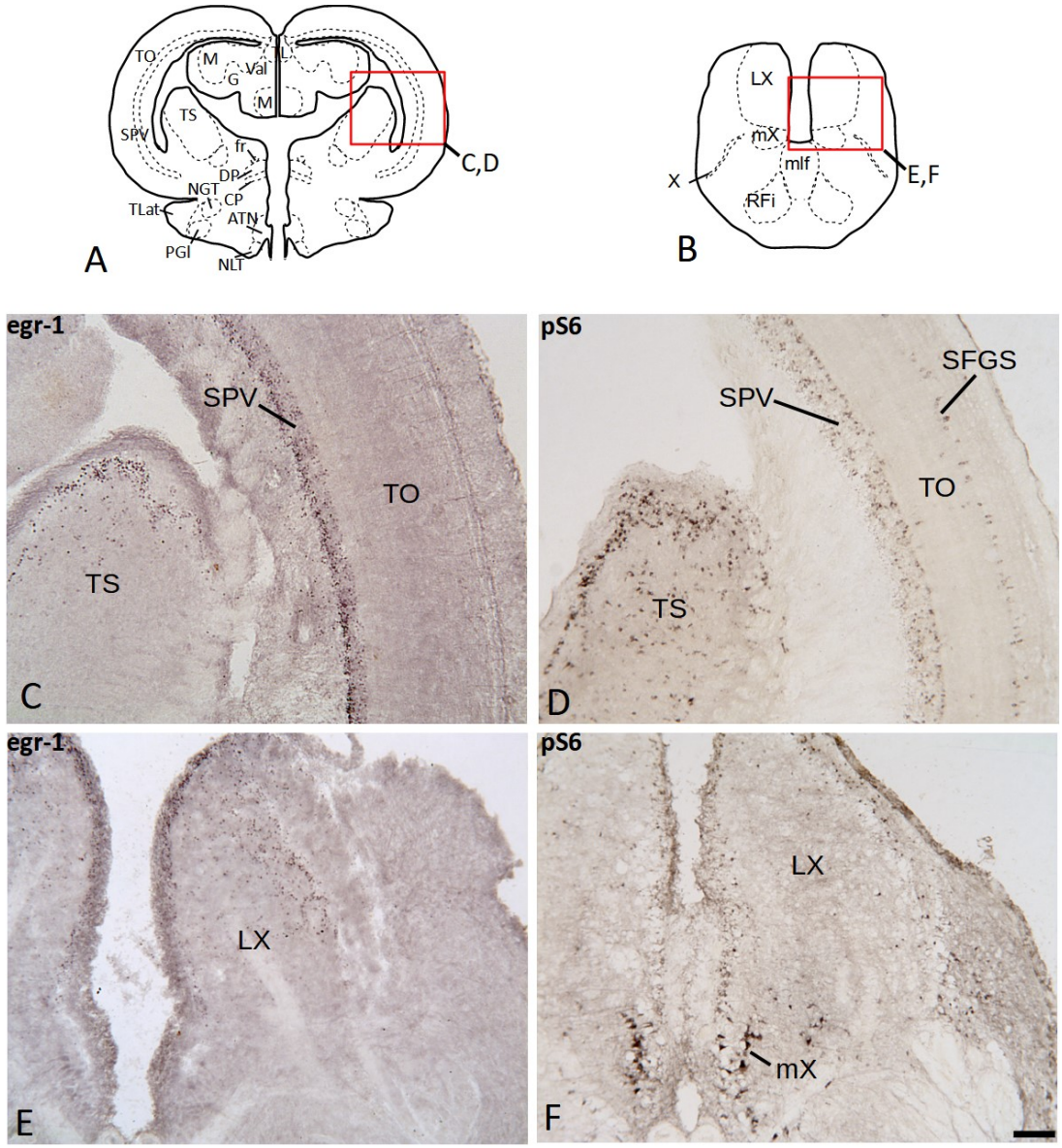


Figure 3: Sections through the midbrain and rhombencephalon showing the distribution of egr-1 (C, E) and pS6 (D, F) stained cells. A, B: drawings showing the location of the high magnification photographs (red boxes). C: egr-1+ cell nuclei in the TL and SPV of the TO. D: corresponding section showing pS6+ cell bodies. E: egr-1+ cell nuclei in the sensory layers of the LX. F: corresponding section showing pS6+ cell bodies. Scale bar in F: 50 µm applies for all. For abbreviations, see list of abbreviations.

2.4 Rhombencephalon

The rostral rhombencephalon, including the cerebellum, contained no cells labeled with egr-1 antibodies. More caudally, some stained cells were present in the facial lobes and they continued into the vagal lobes, which showed many egr-1+ nuclei in the sensory layers. No labeled cells were found in any of the motor areas or the reticular formation. There were also no labeled cells in any of the other sensory areas in the rhombencephalon.

PS6+ cells were found in the facial and vagal lobes, as well as in many additional rhombencephalic areas. In the cerebellum, some Purkinje cells were deeply stained, whereas the granular cells were only weakly stained. This may be due to the fact that granular cells present extremely small cell bodies. Not all areas of the cerebellum showed pS6 stain. They were usually found in the caudal part of the corpus, including the eminentia granularis, but some were also found in the anterior corpus and in the valvula.

Besides the gustatory areas, pS6 was found also in the sensory lateral line and octaval areas. In the MON, smaller interneurons were stained. Many other cell types were stained in the octaval areas.

Motor neurons were also well stained with the pS6 antibody. The oculomotor and trochlear motor neurons in the mesencephalon were already mentioned above. In the rhombencephalon, pS6 was present also in the trigeminal, abducens, facial, and vagal motor neurons (mV, mVI, mVII, mX). In addition, many cells in the reticular formation were also stained, both small and large. The Mauthner cells also showed Ps6 staining, together with the locus coeruleus.

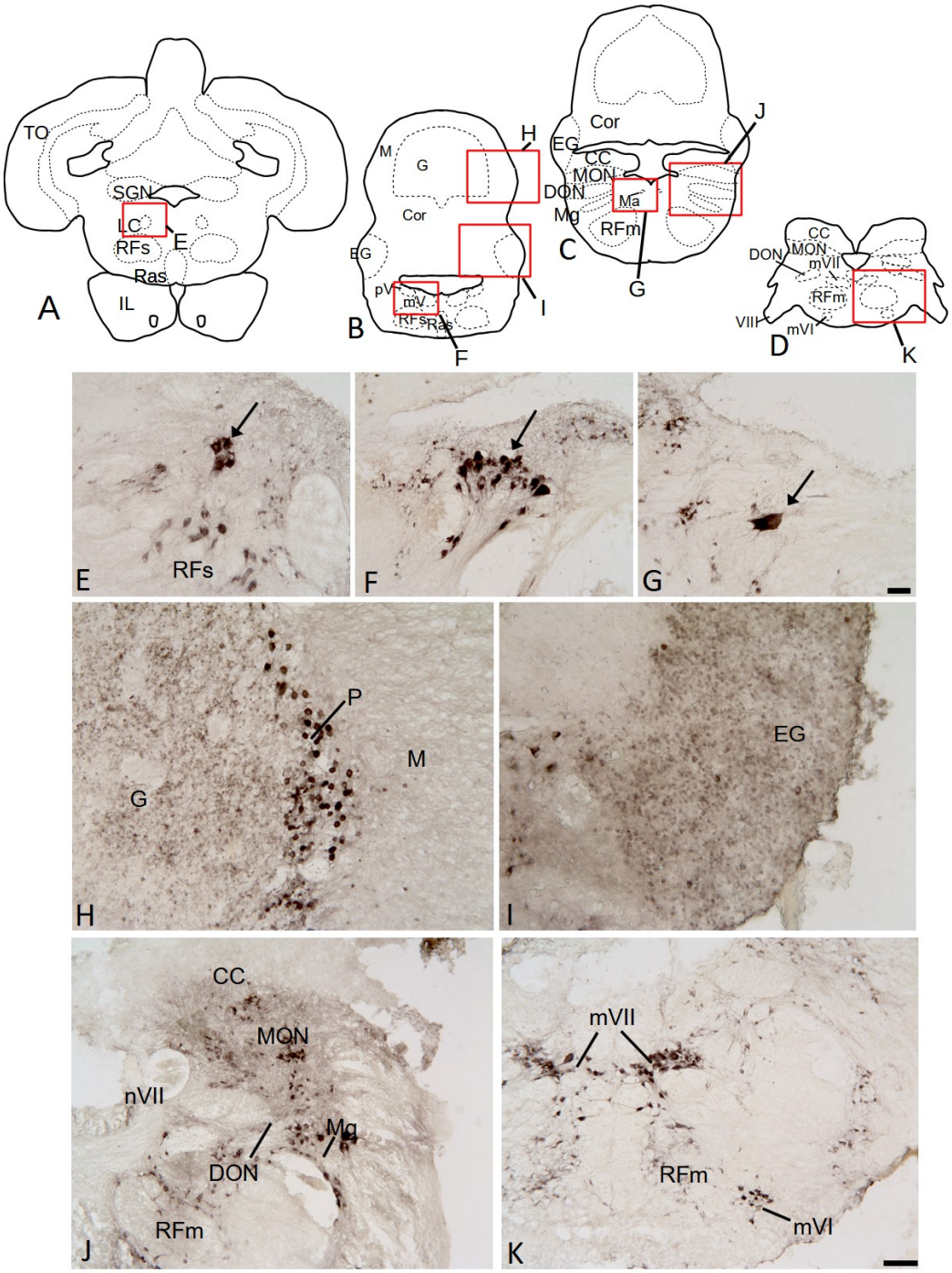


Figure 4: Section through the rhombencephalon showing the distribution of pS6 stained cells. A, B, C, D: drawings showing the location of the high magnification photographs (red boxes). E: pS6+ cell bodies in RFs. F: pS6+ cell bodies in mV. G: pS6+ cell bodies in Ma. H: pS6+ cell bodies in P, M, G of the cerebellum. I: pS6+ cell bodies in EG. J: pS6+ cell bodies in CC, MON, nVII, DON, Mg and RFm. K: pS6+ cell bodies in mVII, RFm, mVI. Scale bar in G: 25µm applies for E, F, G. Scale bar in K: 50µm applies for H, I, J, K. For abbreviations, see list of abbreviations.

2.5 Baseline level of egr-1+ and pS6+ cells

The previous description referred to the avoidance group that received strong stimulation. In the control group, the number of stained cells was much less, but some egr-1+ cells were still found in all of the same areas that showed egr-1+ staining in the avoidance group. Also, the number of pS6 positive cells was much lower in the control, but in some cell groups, pS6 was present in all cells even in the controls. All of the motor neurons in the mes- and rhombencephalon were always stained, as well as the locus coeruleus and the Mauthner cell. In the cerebellum, the caudal part of the corpus showed pS6+ Purkinje cells in all control animals. The more rostral parts of the corpus and the valvula showed a few Purkinje cells stained. Granular cells were well stained in the avoidance group, but little staining was present in the controls group.

3. Discussion

Our results show that the distribution of egr-1+ and pS6+ cells was very different. Whereas pS6+ cells were found in almost all areas throughout the brain, the distribution of egr-1 was restricted to the forebrain, dorsal mesencephalon, and the rhombencephalic taste systems. A closer examination of these areas suggests that these are associated with cell proliferation zones in the fish brain.

3.1 Egr-1 and proliferation zones

Proliferation zones in the fish brain have been described in a number of species (*Apteronotus Zeptorhynchus*, Zupanc and Horschke, 1995; *Gasterosteus*, Ekstrom et al., 2001; *Sparus aurata*, Zikopoulos et al., 2000; *Salmo trutta fario*, Candal et al., 2005; *Oncorhynchus kisutch*, Lema et al., 2005; *Danio rerio*, Zupanc et al., 2005; Grandel et al., 2006; von Krogh et al., 2010; *Oryzias latipes*, Kuroyanagi et al., 2010; *Austrolebias*, Fernandez et al., 2011; Torres-Pérez et al., 2011; *Gymnotus omarorum*, Olivera-Pasilio et al., 2014, 2017), including cichlids (Maruska et al., 2012). A comparison of the distribution of egr-1 and the location of proliferation zones shows great overlap as seen in Table 1.

Proliferation zones are usually located along ventricles. Cells that no longer divide, move to their target sites and differentiate either into glia cells or neurons. Most studies on proliferation zones used BrdU to tag cells that are undergoing mitosis. Depending on the time after BrDU injection, cells could be seen also at some distance to the proliferation zones on their way to their targets. In Table 1, we listed all areas mentioned in the proliferation studies regardless of survival time after BrDU injections. In most studies, isolated scattered BrDU positive cells were seen in areas not marked as positive in our table. Unless otherwise stated, the following section is based on references in table 1.

Almost the entire telencephalon is surrounded by ventricular zones that show proliferating cells and egr-1+ cells could be found in all areas of the telencephalon. A proliferation zone in the ventral part of the telencephalon is continuing into the diencephalic ventricle along the POA where egr-1+ cells were regularly found. There are also some small proliferation zones in the dorsal diencephalon, that correspond to the egr-1+ cells in nucleus A, Hab, and DC/DP. Another zone in this area may give rise to the paracommissuralis nucleus, which is part of the periventricular pretectum. There are two different periventricular pretectal nuclei in this area, the

paracommissuralis and the nucleus of the posterior commissure. We found egr-1 cells only in the paracommissuralis. None of the other migrated pretectal areas contained any egr-1+ cells.

Ventral to these areas, a proliferation zone is located in the periventricular posterior tubercle (pTP) and periventricular organ (PVO) that were associated with egr-1+ nuclei. A larger system of proliferating cells is located along the hypothalamic ventricular system. These extend into the posterior recess, but also into the lateral recess that forms the inferior lobes. Egr-1+ cells were found in all areas that are located along those ventricular proliferation zones. The lateral recess extends into the inferior lobes and all areas of inferior lobes contained egr-1+ nuclei. Egr-1 was also present in the TLat and CM, but it is unclear at the moment whether those nuclei also receive newborn cells from hypothalamic proliferation zones.

In the mesencephalon, the most important proliferation zones are located in the tectum opticum and torus longitudinalis. These extend into the ventricular surface of the torus semicircularis and some proliferating material was found close to the nucleus lateralis valvulae. All those areas also featured egr-1+ cells.

In the rhombencephalon, egr-1 was present in the facial and vagal lobes and those areas are also known to have proliferation zones. Most other areas of the rhombencephalon are free of proliferation zones and were void of egr-1+ cells.

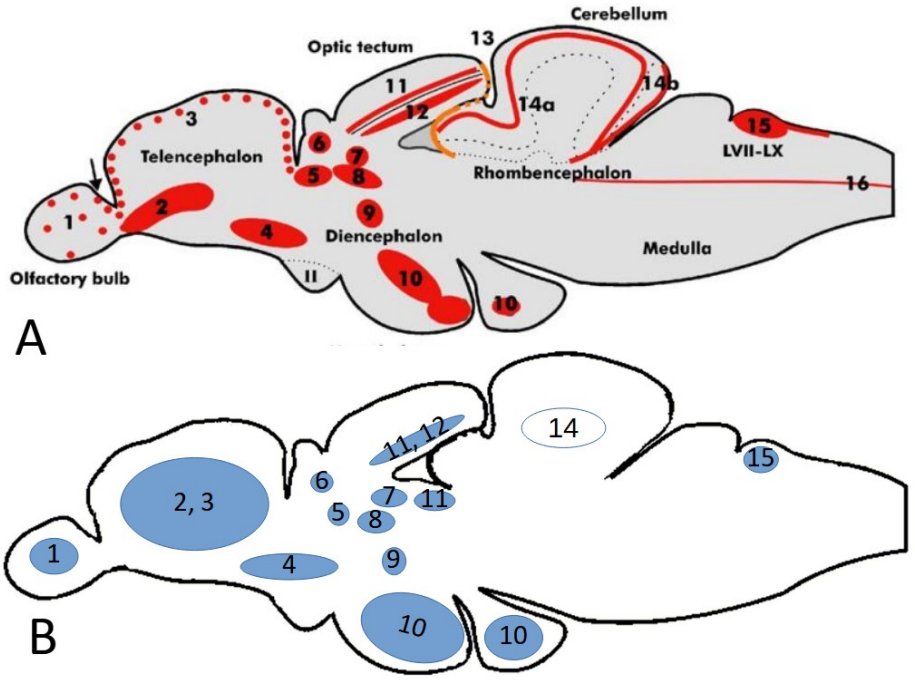
Although there is a positive correlation of proliferation zones with the presence of egr-1+ cells, there are a few exceptions. The most notable omission is the cerebellum. By far, the majority of newborn cells originate along the cerebellar ventricles (Zupanc and Horschke, 1995; Zikopoulos et al., 2000). We found no convincingly stained egr-1 cells in any part of the cerebellum. It is believed that newborn cells migrate primarily into the granular layer and become granular cells. These cells are extremely small and densely packed. It is possible that the antibodies had problems getting access to the nuclear proteins, which would have interfered with staining. We did not use any antigen retrieval procedures or additional steps usually performed with in situ hybridization. We also observed that cells in the torus longitudinalis (TL) and nucleus lateralis valvulae (NLV) were not stained as much as other cells. The torus longitudinalis cells are similar to granular cells in the cerebellum and the same applies to cells in the nucleus lateralis valvulae.

There are reports on scattered BrdU labeled cells in brain areas that did not show egr-1 staining (Ekstrom et al., 2001; Maruska et al., 2012). These BrdU cells are far away from proliferation zones and were detected even after very short survival time after injection. Proliferation occurs only on ventricular surfaces and it would have been unlikely for them to migrate far in the short time

available. One study noted that these scattered cells can be found after BrdU staining, but not with PCNA antibodies (Ekstrom et al., 2001). Most studies on proliferation zones did not consider these scattered cells as signs of active proliferation.

The distribution of egr-1+ cells in our study agreed with other studies that have used egr-1 as activity markers in fish (table 1). These studies have focused on the activation of IEGs in selected areas of interest and did not report the full distribution of egr-1+ cells throughout the brain. However, these studies support current results for the most part. An exception is Mohr et al. (2018), who measured activation of auditory brain stem areas. They found only a few cells, with no significant activation. There are furthermore some studies that found egr-1+ cells in the raphe and PAG, where we did not find any egr-1 staining (table 1).

Figure 5 shows a schematic drawing of the proliferation zones (Grandel et al., 2006) in A, and the distribution of the egr-1 staining found in this study in B. The table in C shows the names of the proliferation zones as found in Grandel et al. (2006), and the corresponding egr-1+ areas found in our study.



C

#	Proliferation zone *	erg-1+ cell groups (this study)
1	olfactory bulb	olfactory bulb
2	ventral telencephalon	ventral telencephalon
3	dorsal telencephalon	dorsal telencephalon
4	preoptic	preoptic area / nucleus supracommissuralis
5	ventral thalamic	nucleus A ?
6	habenular	habenula
7	pretectal	nucleus paracommissuralis
8	dorsal thalamic	dorsal and central posterior thalamic nuclei
9	posterior tubercular	periventricular posterior tuberculum
10	hypothalamic	anterior tuberal nucleus / inferior lobe / torus lateralis / corpus mamaillare - caudal hypothalamus / recessus posterioris
11	tectal	Tectum / torus semicircularis / nucleus lateralis valvulae
12	torus longitudinalis	torus longitudinalis
13	molecular layer	?
14	cerebellar	?
15	facial and vagal lobes	facial / vagal lobes
16	rhombencephalic	?

Figure 5: Comparison of proliferation zones and egr-1+ areas. A: Schematic drawing of the proliferation zones (reproduced with permission from Grandel et al. 2006). B: Distribution of the egr-1 stain found in this study. C: Table showing the names of the proliferation zones as found in Grandel on the left, and the corresponding egr-1+ areas found in our study on the right.

3.2 Comparison to mammals

In mammals, proliferation zones are restricted to the olfactory system and the subventricular zone of the telencephalon. Some of the newborn cells migrate from these areas into the hippocampus and other cortical zones, but proliferation areas are much more restricted than in fishes. However, that does not mean that new cells are added only to these areas in mammals. During embryogenesis, many cells are produced and persist as immature neurons a long time after their proliferation zones have disappeared (Ghibaudi and Bonfanti, 2022). Some of these neurons can be dormant for a long time and the pool of these neurons slowly declines during the life span, as they are continuously maturing and get incorporated into existing networks. It is not easy to detect these neurons, since they are not proliferating anymore and normal proliferation markers obviously do not work. However, other markers for immature neurons have been developed (König et al., 2016). It is now known that immature neurons are present in many brain areas and that cortical layers are waiting to get a signal to 'wake them up' and transform them into functional neurons when needed. They are particularly abundant in areas that show plasticity throughout life (Lopatina et al., 2020).

However, most studies on the activation of immature neurons were performed in the olfactory bulb or hippocampus, areas that receive new neurons from the subventricular proliferation zone (for a review see Kaslin et al. 2015). Those studies show strong evidence that egr-1 is an important signal for immature neurons to get incorporated into existing networks (Veyrac et al., 2014). In fact, egr-1 is found preferentially in new neurons in learning situations indicating that new memories are stored by these new cells (Mak and Weiss, 2010; Magavi et al., 2005). These neurons are required for storing memories and interfering with their maturation leads to memory impairments (Koehl and Abrous, 2011; Aimone et al., 2010).

Therefore, in mammals, immature neurons may not only be restricted to zones that still show proliferation. This is an important difference to fishes, where proliferation zones persist for the entire lifetime of the individual. In fishes, immature neurons are only found close to proliferation zones, which may be the reason why the correlation of egr-1 staining and proliferation zones is so striking. Proliferation, immature neurons, and egr-1 staining are all co-localized in specific areas in fishes, whereas in mammals, proliferation stops in many areas after embryogenesis, while immature neurons (and egr-1 staining) can still be found.

3.3 Distribution of pS6

pS6 is present in all areas where egr-1 is found. The two markers may stain different cell types, but double labeling was not done to show that directly. However, pS6 is present in many more areas void of egr-1 staining and found far from proliferation zones. In some areas, including motor neurons, Mauthner cells and the locus coeruleus, pS6 was present even in controls. Cerebellar Purkinje cells and cells in the reticular formation showed also consistent staining in controls.

Particularly, the permanent presence of pS6 in motor neurons and in cells of the reticular formation is interesting. There are plenty of studies on neuronal plasticity in the context of regeneration after peripheral nerve injury (Matsukawa et al., 2004; Kato et al., 2013). Motor neurons usually do not die, and their axons form new growth cones and try to find their targets again. During axonal growth and plasticity, there is an increased expression of microtubule-associated protein 1B (MAP1B) (Riederer, 2007). This protein, if phosphorylated, destabilizes the microtubules and lead to a rearrangement of the cytoskeleton. In fish (Tench, *Tinca tinca L.)*, MAP1B could be detected during regeneration of the retino-tectal projections after optic nerve crush in adults (Vecino and Avila, 2001). It is also expressed during development and persists in motor neurons and some reticular formation cells in the trout (Alfei et al., 2004). A special kind of reticular formation neuron in fish is the Mauthner cell, which mediates the startle response. Its large dendrites show synaptic plasticity and its axon regenerates after spinal cord injury (Zottoli et al., 2021). The Mauthner cell was also permanently stained with pS6 antibody in our cichlid, but Mauthner cells did not express MAP1B in trout (Alfei et al., 2004). MAP1B is controlled by glycogen synthase kinase (GSK3, Barnat et al., 2016), which in turn is regulated by the mTOR pathway, the same pathway that also leads to an elevation of pS6 (Morgan-Warren et al., 2013). However, whether pS6 positive neurons in *P. zebra* indicate ongoing axonal plasticity needs to be checked by double labeling of cells with MAP1B antibodies.

It may be a general principle that large neuronal pools are not subjected to neuronal turnover but they reorganize or regrowth their axons. For example, the vast reorganization of the nervous system in insects during metamorphosis involves extensive turnover of small interneurons (Truman, 1990), while large motorneurons just reorganize and adapt to their new functions (Levine and Truman, 1985; Knittel and Kent, 2005; Yaniv and Schuldiner, 2016).

The locus coeruleus is also showing permanent pS6 staining. This small group of large cells in the isthmic region is the only source of all noradrenergic fibers within the fish brain. The other catecholaminergic system, the dopamine system, consists of many different cell groups spread

throughout the brain (Yamamoto and Vernier, 2011). Our recent study showed that only the locus coeruleus has permanent pS6 activation even in control animals, whereas cell groups of the dopamine system show no or little ongoing pS6 staining (Calvo et al., 2023a). There is evidence that the modulation of the noradrenergic system is achieved by constant remodeling of the fine terminals in the target regions (Nakamura and Sakaguchi, 1990).

These examples support the idea that a permanent presence of pS6 in neurons indicates that they are constantly reorganizing their terminals. On the other hand, areas that show elevated pS6 staining after specific stimulation may indicate increased synaptic plasticity in response to the event itself.

4. Conclusions

There is a strong correlation of the distribution of egr-1 with proliferation zones in the fish brain and an absence of egr-1 in other areas. This suggests that egr-1 plays some role in the life cycle of new neurons and stimulates them to get incorporated into existing networks. PS6 is more widespread than egr-1 and may indicate increased synaptic plasticity in existing, functional neurons. If so, egr-1 could be used as a marker for areas of memory formation due to the addition of new neurons into existing networks, and pS6 as a marker for neurons that undergo synaptic plasticity. Our results offer the opportunity to use these markers not only to study neuronal activation, but also to localize where specific memory and learning events are taking place.

5. Experimental Procedure

5.1 Behavioral experiments

Animals used in this study (N=20) were *Pseudotropheus zebr*a (also known as *Maylandia zebra*), cichlids from the east African Lake Malawi. They were obtained from a commercial aquarist shop and were between 5.8 cm and 8.5 cm in total length. All fish were bred in captivity and sex was not determined. Fish were kept in isolation for one week in 50-L aquaria (62 cm × 31 cm × 31 cm), filled with aerated and filtered water (25-26 °C). Ten fish were used as control group and did not receive any treatment and were killed after the one-week isolation period. Additional ten fish were moved to a smaller tank (31 cm x 15 cm x 15 cm) and chased with a net for one hour. Then,

they were killed 90 minutes after the chase ended. This procedure introduced chemical, visual, and lateral line/auditory stimulation and caused a strong avoidance reaction together with a stress component. This group is named 'avoidance group'.

To process their brains, fish were anesthetized with Tricaine methanesulfonate (MS-222), the spinal cord was transected, the brain was removed and fixed overnight in 4 % paraformaldehyde (PFA) at 4 °C. The day after, brains were transferred to 30 % sucrose in phosphate puffer saline (PBS), 0.01 M, pH 7.4, and stored overnight at 4 °C. The following day, brains were embedded in O.C.T compound (freezing medium, Leica Biosystem Richmond) and frozen at -20 °C. Thirty-five µm thick sections were cut at - 20 °C with a cryostat (Leica CM1520), collected in three series on slides and then stored until the immunohistochemistry procedure.

5.2 Immunohistochemistry procedure

Immunohistochemistry was performed as described previously (Calvo et al., 2023 a, b). Briefly, a post-fixation procedure was performed with 4 % PFA for 10 min, followed by washes in PBS. Then, the sections were bleached in 1 % H_2O_2 in PBS, washed, and blocked in 10 % normal goat serum (NGS) for 1 hour. Sections were then transferred to a primary egr-1-antibody or pS6-antibody solution (5 % NGS / 1X PBS – 0.3 % Triton X-100, 1:1000 rabbit anti-egr-1 antibody, Santa Cruz Biotechnology Inc.; 1:1000 rabbit anti-pS6 (Ser235/236) antibody, Cell Signaling 2211S) overnight at -4 °C, before being washed several times in PBS. The second antibody reaction (VECTASTAINR biotinylated anti-rabbit IgG secondary antibody, Vector Labs., USA: 1:500) was performed in 5 % NGS / 1X PBS – 0.3 % Triton X-100 for 2 hours at room temperature (RT), followed by repeated washes in PBS. Then, signal amplification was initiated using the ABC method (1X PBS - 0.3 % Triton X-100, VECTASTAINR ABC-Peroxidase kit, Vector Labs., USA) for 1 hour at RT. Following several rinses in PBS, the antibody-Avidin-Biotin complex was visualized using the chromogen-solution (one 3,3'-Diaminobenzidine-Tetrahydrochloride (DAB) buffer tablet (Merck KGaA, Germany) dissolved in 15 ml distilled water, 500 µL 1 % ammonium nickel sulphate, 12 µl 30 % H_2O_2), resulting in a deep greyish reaction product. The reaction was stopped by washes in PBS. Finally, sections were dehydrated in ascending alcohols to xylene before cover slipping with Eukitt (Carl Roth, Germany).

For the present study, the specificity of egr-1 and pS6 antibodies was checked by replacing either the primary or secondary antibodies with PBS, showing no reaction product. No other test of the specificity of the antibody was performed.

The same pS6 antibody from Cell Signaling has been used successfully in several studies on fish (Beckers et al., 2019, 2021; Montesano et al., 2019; Tripp et al., 2019, 2020; Chen et al., 2021; Dunlap et al., 2021; Nunes et al., 2021; Schuppe et al., 2021; Scaia et al., 2022), including cichlids (Butler et al., 2018, 2019, 2020; Maruska et al., 2020; Calvo et al., 2023b). Furthermore, the antibody has been validated in the cichlid *Astatotilapia burtoni* (Butler et al., 2020) by western blot which produced a single band at 32 kDa.

The same egr-1 antibody from Santa Cruz Biotechnology has as well been used in other studies on teleost (Rajan et al., 2011; Cabrera-Álvarez et al., 2017) and sharks (Fuss and Schlüssel, 2018). The specificity of the egr-1 antibody was tested by western blot in rat and turtle brains (Mokin et al., 2006), and in zebra fish (Barbosa et al., 2012). Furthermore, the same antibody was previously validated in birds (Mello et al., 1992, Mello and Clayton, 1994, Ball et al., 1997).

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Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Calvo and Hofmann contributed to the study conception and design. Data collection, processing of brains and sections were performed by Calvo. Calvo and Hofmann analyzed the data. Schlüssel provided financial support. The first draft of the manuscript was written by Calvo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics declarations

Declaration of interest: none

Conflict of interest: The authors have no conflicts of interest to declare

Ethical approval: The research reported herein was performed under the guidelines established by the EU Directive 2010/63/EU for animal experiments and the current German animal protection law and had been approved by the Landesamt für Natur, Umwelt und Verbraucherschutz NRW (approval number 8.87-50.10.37.09.198).

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6. Discussion

With more than 35,000 species, fishes represent more than half of all vertebrates known and their great diversity offers a wide range of species to study cognition in (Kotrschal et al. 1998, Bshary and Brown 2014, Salena et al. 2021). Over the last 50 years, behavioral studies have shown that fish posses cognitive abilities which can no longer be considered inferior to those of many other vertebrates (for reviews see Brown et al. 2011, Schlüssel 2015, Vila Pouca and Brown 2017, Salena et al. 2021). While many behavioral studies have been conducted, only few have focused on the substrates where cognitive information is processed in the brain, i.e. on combining behavioral and neuroanatomical information (Calvo and Schlüssel 2021, Rodriguez et al 2021). A noteworthy exception are spatial memory, emotional memory and social behavior in goldfish, which have been quite extensively studied on both levels (Broglio et al. 2005, Salas et al. 2006, Hurtado-Parrado et al. 2010, Perathoner et al. 2016). Results of these studies showed that some, but not all, cognitive information is processed in the telencephalon. The aim of my project was to uncover any brain areas involved in general visual learning mechanisms like object recognition and object memory, not just in the telencephalon, but throughout the entire brain. In the Malawi cichlid *Pseudotropheus zebra*, I investigated different experimental scenarios in a non-social context with variable degrees of cognitive, sensory and motor components. Cichlids have become a popular model system for studying evolution, genomics and ecology and *P. zebra* has been shown to learn a wide range of visual learning tasks (e.g. Schlüssel et al. 2014, 2018, 2022).

At the beginning of my PhD, I set out to investigate the expression of the IEGs c-fos and egr-1 in the brain of *P. zebra* in response to an object recognition task. In particular, fish were trained to associate a visual object with a food reward. IEGs are markers that can be used to localize brain areas involved in specific behavioral tasks because they get up-regulated in activated neurons, without requiring de novo protein synthesis (Bahrami and Drabløs 2016). Unfortunately, the results I obtained were not as clear as those seen in other studies on fish (e.g. Rajan et al. 2011, Cabrera-Álvarez et al. 2017, Fuss and Schlüssel 2018) due to high background and a non-specific signal. Furthermore, the amount of required antibodies was limited, as the company (Santa Cruz Biotechnology) terminated their production in 2016. Forced by these circumstances, I decided to switch to another neural marker, the phosphorylated ribosome marker pS6. PS6 had already been shown to work in cichlids and provided a popular alternative to IEGs to visualize neural activation in fish (e.g. Butler et al. 2018, Fischer et al. 2018, York et al. 2019, Maruska et al. 2020, Tripp et al. 2020, Chen et al. 2021, Scaia et al. 2022).

For the first chapter, the expression of pS6 was analyzed in 19 different brain areas in four groups of fish, subjected to different behavioral contexts each, i.e. control, avoidance, trained and novelty groups (results have been published in Brain Structure and Function, Calvo et al. 2023). Common to all the three experimental groups, except the control, was the presence of a visual stimulus. In the avoidance group, visual stimulation was (unintentionally) caused by the net used to chase the animal. In the trained group, two visual targets were presented during the behavioral training, and the choice of the correct one was rewarded with food. Finally, in the novelty group, fish were visually habituated to a specific image for five days, then on the sixth day, they were presented with a new, different image. The presence of the visual stimuli in all three experimental groups caused an activation of pS6 in both layers of the optic tectum (TOp and TOs) and the nucleus diffusus of the inferior lobes (IL), but not in the control group.

In cichlids, a visual pathway extends from the optic tectum, via the nucleus corticalis and the nucleus glomerulosus, both located in the diencephalon, to the inferior lobes, which are part of the hypothalamus, also situated in the diencephalon (Wullimann & Meyer 1990, Shimizu et al. 1999, Ahrens & Wullimann 2002, Yang et al. 2007). Studies on the connectivity of the inferior lobes in teleost suggest they also receive somatosensory, auditory and gustatory input (Morita et al. 1980, Lamb and Caprio 1993, Rink and Wullimann 1998) and function as a multisensory integration center (Rink and Wullimann 1998, Shimizu et al. 1999, Ahrens and Wullimann 2002, Yang et al. 2007). The inferior lobes can be very large, in some species like *Synchiropus* (Mandarinfish) or *Eurypegasus* (Little Dragonfish) they are even larger than the entire telencephalon, but their proposed functions in the visual pathway are still unknown (Gebhardt & Hofmann 2023). Estienne and colleagues (2022) found very large inferior lobes in wrasses (*Choerodon anchorago, Labroides dimidiatus, Thalassoma hardwicke*) and suggested that the inferior lobes are involved in higher visual information processing. There is also some controversy about the association of the inferior lobes, while generally accepted that they are part of the hypothalamus (Demski et al. 1975), another recent study in zebrafish found that at least parts of the inferior lobes are derived from mesencephalon and may thus not be related to the hypothalamus (Bloch et al. 2019).

In any case, the inferior lobes are a derived structure in teleosts, not present in other vertebrates, and were anticipated to have some higher multisensory and/or learning functions (Estienne et al. 2022).

Results of this study show that the inferior lobes in fact play a role in visual discrimination learning. Previous studies on visual learning in fish focused mainly on the involvement of the telencephalon in the processing of visual information. For example, Messina et al. (2020) investigated habituation to a complex visual pattern in zebrafish, where a number of objects had to be remembered. Although they noted an up-regulation of c-fos and egr-1 in the tectum in response to object size (Messina et al. 2020), they focused solely on the involvement of the telencephalon in the processing of visual numerosity information in a follow-up study (Messina et al. 2022), since they were interested in the counting aspect of the experimental task than in the size discrimination. Rodriguez-Santiago et al. (2022) investigated visual learning in a social context in the cichlid *Astatotilapia burtoni* and found that the social aspect (but not the learning per se) activated specific telencephalic areas (Dl, Dm and Vs), but they did not assess areas outside the telencephalon. The telencephalon has often been considered the primary center of cognitive information processing (reviewed in Hofmann 2001. Broglio et al. 2003, Yamamoto 2009, Rodríguez et al. 2021), possibly due to the significance the telencephalon, i.e. specifically the cortex, holds in mammals, and despite lesion studies showing early on, that, as opposed to mammals, removal of the teleost telencephalon affects only some (complex) behaviors (for a review see Calvo and Schlüssel 2021), while most basic behaviors (e.g., swimming, feeding, and many learning tasks) are not affected at all (reviewed in Hofmann 2001). In fact, important visual functions, such as object discrimination and courtship behavior, are still intact after damage of the telencephalon. For example, the role of the telencephalon in spatial memory and orientation was tested in a T-maze (Salas et al. 1996a, b). Fish with a lesioned telencephalon were still able to learn the position of a feeding location based on direction, such as right or left, but the same fish failed the test if the starting arm was located in a different position and the feeding location could only be found using place memory (Salas et al. 1996a, b). Their results, together with further ones (reviewed in Broglio et al. 2011) showed that the lateral zone of the dorsal telencephalon plays an important role in allocentric orientation and place memory in goldfish (*Carassius auratus*), but not in egocentric (e.g. direction learning) orientation (Salas et al. 1996a, b, Lopez et al. 2000, Duran et al. 2008, 2010, Costa et al. 2011). Fish with a lesioned telencephalon were still able to discriminate objects (Köhler et al. 1999), to respond to a female and to display courtship behavior (Schönherr 1955), and to take care of the nest building (Aronson 1948). This study showed that the inferior lobes, which are expected to be part of a visual pathway as discussed above, are involved in visual discrimination learning and object recognition.

During the analysis of the results, it became clear that the fish, were exposed to a range of (unintended) additional stimuli during the experiments, unrelated to the perception of the provided visual stimuli and the learning thereof. In fact, it seemed impossible to investigate cognition and/or cognitive behaviors without taking into consideration other sensory and/or motor components of the task that were unrelated to the task as such. As a consequence, activation of brain areas and subsequent increases in pS6 expression originated from these additional stimulations or combinations thereof. Accordingly, I proceeded to analyze the behavioral conditions in their entirety, attempting to consider all possible components, including all unintended ones. For example, fish in the avoidance group showed elevated pS6 expression in the olfactory bulb (OB) and the posterior division of the dorsal telencephalon (Dp) that may have been caused by olfactory stimulation (even in the absence of food), introduced by adding new water and the net to the tank. The movement of the net in the water, and/or any noise made by the experimenter, certainly would have caused activation of the lateral line and the auditory system, as indicated by an increase in activity of pS6 in the torus semicircularis (TS). Elevated pS6 activity was also found in the preoptic area (POA), which appears to be a stress-induced response. In stressful situations, the release of dopamine from the preoptic neurons are known to suppress the production of the gonadotropin releasing hormone (GnRH), which downregulates the release of gonadotropins from the pituitary (Kah et al., 1984; Kah et al., 1986; Kah et al., 1987; Anglade et al., 1993; Linard et al., 1996; Weltzien et al., 2006; Chabbi and Ganesh, 2015; Bhat and Ganesh, 2020). The latter stimulates the synthesis and release of glucocorticoids from the inter-renal tissue, and is downregulated by GnRH during the stress response in fish, including cichlids, compromising normal reproductive activity (Flik et al. 2006, Bryant et al. 2016). In addition, in the avoidance group, the enforced locomotion seemed to have activated the reticular formation, a premotor area. Finally, fish in the training group showed an increase in pS6 activity in the torus lateralis (TLat), a brain area associated with gustatory information processing, that may have been caused by receiving a food reward. Through this detailed comparison of the distinct activation patterns, I determined the inferior lobes to be active in all three experimental groups, and the only one involved in visual learning and object recognition.

After discovering an involvement of the inferior lobes in object recognition and visual learning, I investigated the factors that are modulating the learning process in the brain of three of the four groups (i.e. control, avoidance and trained). As both a stressful stimulus (in the avoidance group) and a rewarded visual stimulus (in the trained group) were used, it was interesting to assess and

compare the activation of the dopaminergic system. Dopamine is a neuromodulator known to be involved in reward, motivation, and memory formation (Kelley and Berridge 2002) but also in stress responses (Salamone et al. 1997, Berridge and Robinson 1998). The activation of dopaminergic cell populations throughout the brain was visualized by fluorescent double-labeling of tyrosine hydroxylase (TH) and pS6 (results published in Journal of Chemical Neuroanatomy, Calvo et al. 2023, chapter 4). Depending on the treatment, different dopaminergic cell groups either in the telencephalon, diencephalon, mesencephalon or in the brain stem were activated. Surprisingly, in both the avoidance and trained group, activation was observed mainly in the periventricular pretectal nucleus (PP) and the nucleus of the posterior tubercle (nTP). The latter projects to the inferior lobes (Ahrens and Wullimann 2002), suggesting a modulation of the lobes by these dopaminergic cell population. Cells in the pretectal nucleus project to the optic tectum, both ipsi- and contra lateral (i.e. on the same side, and on the opposite side) (Striedter 1990, Schlussman et al. 1990, Pérez-Pérez et al. 2003, de Arriba and Pombal 2007, Tay et al. 2011). It seems that PP is the major source of TH+ fibers in the tectum. Studies in mammals show that dopamine modulates visually-evoked reward information processing in the optic tectum (colliculus superior) (Takakuwa et al. 2017, Valdés-Baizabal et al. 2020, Isa et al. 2021, Montardy et al. 2022). A direct connection of the tectal fibers with dopaminergic neurons has been found in lampreys, monkeys, cats and rodents (Comoli et al. 2003, McHaffie et al. 2006, May et al. 2009, Pérez-Fernández et al. 2017), suggesting the presence of a modulation by dopaminergic cells of the periventricular pretectal nucleus directly on the optic tectum.

Dopamine is likely implicated in modulating visual learning, by acting both on the optic tectum and the inferior lobes, but it also plays an important role in modulating other areas in the brain. In the present study, dopamine modulated the activity in the hypothalamic-pituitary axis, that controls the production of stress hormones. In particular, dopaminergic neurons of the preoptic area (POA) were activated in the avoidance group. These dopaminergic cells are known to regulate the production of the gonadotropin releasing hormone (GnRH) (Chabbi and Ganesh 2015, Bhat and Ganesh 2020). In stressful situations, an increased dopaminergic level from the preoptic area suppresses the production of GnRH, which in turn downregulates the release of gonadotropins in the pituitary (Kah et al. 1986, Kah et al. 1987, Anglade et al. 1993, Weltzien et al. 2006, Chabbi and Ganesh 2015, Bhat and Ganesh 2020). Interestingly, the avoidance group showed also a decrease of dopaminergic activation in the nucleus of the posterior recess (nPR) in the hypothalamus. In vertebrates, including fish, dopaminergic cells in the nucleus of the posterior recess project to the

pituitary and control the release of prolactin (Shin 1979; Moore et al 1987). Higher dopamine release suppresses the production of prolactin from the pituitary (Ben-Jonathan 1985, Anthony et al. 1993, Freeman et al. 2000, Torner 2016). Prolactin is known to play an important role in the stress response, causing a stress-induced pathology such as cardiac dysfunction or intestinal dysfunction in restrained mice and rats (Levine and Muneyyirci-Delale 2018), as well as growth suppression and reproductive dysfunction in restrained fish (*Oncorhynchus kisutch,* Avella et al. 1992; *Oncorhynchus mykiss,* Pottinger et al. 1992). In summary, in stressful situations, the dopaminergic activity in the preoptic area increases, suppressing the release of gonadotropins, and causing a decrease in the dopamine production from the tubero-infundibular system to allow for higher prolactin levels. The results in the present study confirmed exactly this for the avoidance group, i.e. a higher activation of the dopaminergic cells of the preoptic area and lower activation of the dopaminergic cells of the nucleus of the posterior recess, when compared to the control group.

In the avoidance and trained group, activation was detected also in the superior raphe (Ras). The superior raphe is a source of serotonergic innervation but a subset of raphe cells in mammals also produces dopamine (e.g. Ochi and Shimizu 1978; Trulson et al. 1985; Stratford and Wirtshafter 1990). In mice, the activity of the dopaminergic cells in the superior raphe is involved in the formation and expression of aversive memory (Groessl et al., 2018; Lin et al., 2020), arousal and response to significant external events (Cho et al, 2017), and can be affected by associative learning of fear (Groessl et al. 2018, Lin et al. 2020, Cho et al. 2021). Furthermore, dopaminergic neurons in the raphe are activated by rewarded stimuli (Lin et al. 2021). Experimental data show that raphe dopaminergic neurons do not encode the valence (either negative or positive) of the stimuli but just the salience (i.e. the motivational component) (Lin et al., 2021). In fish, there have only been two studies which showed the presence of dopaminergic cells in the raphe, i.e. in *Gasterosteus aculeatus (*Three-spined stickleback*) and Dicentrarchus labrax* (European seabass). These studies were investigating the general localization pattern of dopaminergic cell populations in fish brains (Ekström et al. 1990, Batten et al. 1993). In mammals, dopaminergic cells in the raphe respond to social isolation by increases in activity (Matthews et al. 2016). The absence of social isolation, before or during the experimental procedure, is likely the reason why most studies on fish did not detect TH+ cells in the raphe. In the current study, however, dopaminergic cells were detected in the superior raphe of all individuals, possibly in response to being socially isolated for a week before the experimental procedure was initiated. These combined results

suggest that the dopaminergic system modulates learning and memory formation in the areas involved in object recognition and visual learning identified in chapter 1.

A completely different analytical approach consists of multidimensional structure analyses, such as a principle component analysis (PCA) or hierarchical cluster analysis (HCA). The Principal Component Analysis (PCA) was conducted here in order to identify those dopaminergic cell populations, that most strongly separated the experimental groups, i.e. that differed maximally between groups. The PCA confirmed that differences were mostly driven by co-activation of a combination of the nucleus of the posterior tubercle and the superior raphe, suggesting that these dopaminergic cells might encode aspects of (positive or negative) valence and possibly high salience associated with the stimuli of both the avoidance stimulus and trained task. The second analysis performed was a hierarchical cluster analysis (HCA), i.e. grouping the animals by similarity in their activation pattern. Individuals are grouped based on the pattern of activation of their dopaminergic cell groups without any a prior knowledge of their group membership. The reconstruction of the HCA almost perfectly agreed with the real group memberships, with just two animals misplaced into the wrong group (i.e. one trained specimen grouped together with the avoidance group and one isolation specimen grouped with the trained group). Thus, the PCA and the HCA confirmed the result of the classical analysis discussed above, showing robust differences across the three experimental groups and across brain areas.

However, it remained unclear, how the 'activation' of the inferior lobes leads to learning and memory formation. Some insights into potential mechanisms arose by comparing the pS6 with the egr-1 staining pattern that was performed during the original/preliminary experiments. The activity patterns of the two neural markers were quite different throughout the brain of *P. zebra*. In particular, the egr-1 staining was restricted to specific areas which turned out to be closely associated with proliferation zones found throughout the brain, such as olfactory bulb, telencephalon, habenula, dorsal thalamus, posterior tubercle, torus lateralis, optic tectum, torus longitudinalis and semicircularis, nucleus lateralis valvulae and vagal lobe, while pS6 staining was present in the same areas but also in many other areas far away from proliferation zone (e.g. motorneurons, interpeduncular nucleus, reticular formation, lateral line, facial lobe). One of the main discoveries of the present study was that almost all areas showing egr-1 expression were close to proliferation zones. These are areas, where new cells are generated, even in adult animals. The new cells from the proliferation zones are maturing and eventually make synaptic contacts and functional connections important for the consolidation of new memories (Zupanc et

al. 2005). Proliferation zones are well investigated in several fish species (e.g. Zupanc and Horschke 1995 (*Apteronotus*), Ekstrom et al. 2001 (*Gasterosteus*), Zikopoulos et al. 2000 (*Sparus*), Zupanc et al. 2005 (*Danio*), Grandel et al. 2006 (*Danio*), Fernandez et al. 2011 (*Austrolebias*), Maruska et al. 2012 (*Astatotilapia*)). From studies in mammals, it is known that egr-1 is active in learning and memory processes (Bozon et al. 2002). The increased expression of egr-1 in the experimental groups could therefore be a sign for memory formation due to recruitment of new neurons into existing neural networks.

Unlike egr-1, pS6 staining was identified in areas far away from proliferation zones. PS6 is an important downstream target of the mTOR pathway, which is known to be involved in the reorganization of synaptic contacts in neurons (Biever et al. 2015, Ruvinsky and Meyuhas 2006, Knight et al. 2012). A direct sign of synaptic or axonal plasticity is the presence of the microtubule-associated protein 1B (MAP1B), controlled by glycogen synthase kinase (GSK3, Barnat et al., 2016). The latter in turn is regulated by the mTOR pathway, the same pathway that also leads to an elevation of pS6 (Morgan-Warren et al. 2013). It has been demonstrated that in the tench (*Tinca tinca L.*), the expression of MAP1B increases during regeneration of the optic nerve (Vecino and Avila, 2001). Interestingly, MAP1B is also permanently present in motor neurons throughout the entire brain and in reticular formation cells, which are constantly adjusting their axonal processes as was shown in trout (Alfei et al. 2004). In the current study, high levels of pS6 were observed in motor neurons and cells of the reticular formation in controls, i.e. unstimulated fish, suggesting that higher pS6 levels are also associated with ongoing synaptic plasticity in existing neurons.

The results of my work give some important insights into the understanding of the neuronal substrates involved in visual learning in teleost, yet some questions still remain unanswered and require further investigation. First, more information is needed in relation to how dopamine acts onto the inferior lobes in order to better understand how the dopaminergic modulation controls and influences the visual learning and object recognition in the inferior lobes. The investigation of dopaminergic projections to the inferior lobes by tracer injections could provide additional information about the dopaminergic modulation of visual information processing and visual learning. Furthermore, it would be important to show that egr-1 is indeed acting on immature neurons. This could be done by double labeling of cells, i.e. by tagging proliferating cells with BrdU and testing whether those cells are labeled with egr-1.

Finally, it would be interesting to investigate the direct correlation between pS6 and the microtubule-associated protein 1B (MAP1B), to deeply associate the expression of pS6 and synaptic plasticity. This could be done by administrating inhibitors or antagonist of the mTOR-pS6 pathway (e.g. rapamycin), to examine how the presence of MAP1B and ps6 is influenced by the administration of. Moreover, double labeling for pS6-MAP1B could be carried out in the established 'optic nerve crush model' to study their coexpression in regenerating optic nerve axons, to directly correlate pS6 and synaptic plasticity-regeneration.

7. Conclusion

My results contribute to the understanding of the neural substrates involved in visual learning mechanisms, in particular object recognition and object memory, in teleost, describing the crucial role of dopamine in modulating neural activity during visual learning processes, and found evidence that learning and memory are effects of two different mechanisms, the recruitment of new neurons and plasticity in existing neurons. The inferior lobes are likely a key neural substrate involved in visual learning and object recognition. This challenges the general notion that higher cognitive functions in fish are mostly processed in the telencephalon, as they are in mammals. Studying cognition in fish is important for several reasons. By understanding fish cognition, we gain valuable insights into the evolution of cognition and on fundamental mechanisms that underlie higher-order cognitive functions. By comparing the cognitive abilities of fish with those of other animals, such as primates or birds, that are already well-researched, we can study shared cognitive processes and identify unique adaptations specific to each group.

Results of this study also point to the importance of dopamine in modulating the activity of the inferior lobes during visual learning. Dopamine has long been recognized for its critical role in reward-based learning and motivation, both in mammals and fish, but its involvement in visual learning processes has remained relatively unexplored. My results indicate that dopamine plays an important role in modulating the activity within these regions, thus influencing visual learning outcomes.

Furthermore, my work compared the expression of two of the most used neural activity markers (egr-1 and pS6), suggesting their involvement in memory formation and synaptic plasticity, providing an opportunity to investigate their expression not just to study neural activation, but also to localize where specific memory and learning events are taking place.

Last, the results of this study emphasize again the importance of considering any behavior in its entirety. Studying cognition and cognitive behavior without examining all of its components, including cognition-unrelated ones, such as movement and sensory information as well as other potential stimuli introduced during the experiment along with it, may lead to loss or misinterpretation of data. The context in which cognitive information is received by an animal is important and shapes its response to it. The animal not only receives the incoming cognitive information but combines it with past experiences and information about its internal state, which in turn influence its perception, further information processing and eventually the animal's reaction or behavioral output.

This study contributes significantly to our understanding of the underlying neural substrates of fish cognition. Additional research is needed to further elucidate the precise mechanisms by which the assessed processes take place, as well as to explore potential interactions with other neurotransmitters and brain regions. Nonetheless, my findings provide grounds for future investigations and offer promising directions for understanding fish cognition and learning mechanisms.

8. References

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9. Protocols

9.1 PBS 10X pH 7.4

1L H2Od

- 2 g KCl
- 2 g KH2PO4
- 80 g NaCl
- 11.5 g Na2HPO4

PBS 1X: 900 ml H20d + 100 ml PBS 10X

9.2 PFA 4% in PB

• Solution A 0.2M NaH2PO4:

1L H20d

- 24 g NaH2PO4

• Solution B 0.2M Na2HPO4:

1L H2Od

- 28.4 g Na2HPO4

- 100 ml solution A + 400 ml solution B + 300 ml H2Od
- Add 40 g PFA
- Heat up to 58°C max 60°C till PFA is completely dissolved
- Let cool down at room temperature
- Fill up to 1L with H2Od
- Check pH 7.3 - adjust if necessary
- Filter before perfusion
- Store at 4°C for up to 1 week or aliquot and store at -20°C

9.3 Nissl Staining

1. Scouring and dehydrating

- 70% EtOH	2 min
- 80% EtOH	2 min
- 96% EtOH	2 min
- 3X 100% Isopropanol	2 min

- 3X 100% Xylene	5 min
2. Rehydrating	
- 3X 100% Isopropanol	2 min
- 96% EtOH	2 min
- 80% EtOH	2 min
- 70% EtOH	2 min
- H2Od	10 min
3. Staining	
- Nissl stain	6-7 min
(1 g Cresyl violet acetate + 500 ml H2Od)	
4. De-stain	
- H2Od	2 min
- 70% EtOH	2 min
- 80% EtOH	2 min
- 96% EtOH	2 min
5. Dehydrating	
- 3X 100% Isopropanol	2 min
- 3X 100% Xylene	5 min
6. Mounting on slides	

9.4 Immunohistochemistry

1. Pap-pen around tissue	
2. 4% PFA drop	10 min
3. 3X PBS drop	5 min
4. Quenching (300uL slides):	
- Add 1.5% H2O2 solution	10 min
(0.43ml 35% H2O2 in 9.57ml H2Od)	
5. 3X PBS drop	5 min
6. Blocking solution (300uL slides)	1 H
10% NGS-PBS/Triton 0.3%	
7. Incubate the sections with primary Ab (300uL slides)	ON, 4°C
8. 3X PBS drop	5 min

9. Incubate the sections with secondary Ab (300uL slides)	2 Hrs
- After 1h prepare AB complex	
10. 3X PBS drop	5 min
11. AB complex: 15uL A + 15uL B per ml PBS-Triton	1 H
12. 3X PBS drop	5 min
- After the first one: prepare the 30% H2O2 (8.57ml 35% H2O2 in 1.43ml H2Od)	
13. DAB (500uL slides)	3 min
- Allow tablet to reach RT	
- Dissolve 1 DAB tablet in 15ml TBS on the shaker	
- Add 500uL 1% Nickel	
- Immediately before use: add 12uL 30% H2O2 (8.57ml 35% H2O2 in 1.43ml H2Od)	
14. 2X PBS drop	5 min
15. Rinse in H2O	
16. Dehydration	
- 30% EtOH	3 min
- 50% EtOH	3 min
- 70% EtOH	3 min
- 95% EtOH	3 min
- 100% EtOH	3 min
- Xylol	3 min
17. Coverslip	

9.5 Immunofluorescence

1. Pap-pen around tissue	
2. 4% PFA drop	10 min
2. 3X PBS drop	5 min
3. Blocking endogenous peroxidase	
- Add 30% H2O2 solution (200-300uL slides)	20 min
4. 3X PBS/Triton drop	5 min
5. Blocking non-specific binding	1H
- 10% animal serum in PBS/Triton (after 50mins prepare primary Ab)	

6. Incubate the sections with primary Ab (200-300uL slides)	ON, 4°C
nb: use 5% animal serum in PBS/Triton	
7. 3X PBS/Triton	5 min
8. Incubate the sections with secondary Ab (200-300uL slides)	2 Hrs
nb: use 5% animal serum in PBS/Triton	
9. 3X PBS/Triton	5 min
10. DAPI + coverslip	

9.6 Clearing

1.Dehydrate on shaker	
- 20% MeOH	1 H
- 40% MeOH	1 H
- 60% MeOH	1 H
- 80% MeOH	1 H
- 100% MeOH	1 H
2. Wash in 100% MeOH on the shaker	1 H
3. 66% DCM – 33% MeOH on the shaker	ON
4. 2X Wash in 100% MeOH on the shaker	1H
5. Bleach in 5% H2O2 in 100% MeOH	ON, 4°C
1 Volume 30% H2O2 in 5 Volume MeOH	
6. Rehydrate on the shaker	
- 60% MeOH	1 H
- 40% MeOH	1 H
- 20% MeOH	1 H
- PBS	1 H
7. 2X Wash in PTx.2 on the shaker	1 H
8. Permeabilization solution on the shaker	ON, 37°C
9. Blocking solution on the shaker	ON, 37°C
10. Incubate on the shaker with Ab' in PTWH / 5% DMSO / 3% donkey sermu	2 days, 37°C
Dilution 1:350	
11. 5X Wash in PTWH on the shaker	1H each + ON
12. Incubate on the shaker with	

Ab'' in PTWH / 3% donkey serum	2 days, 37°C
Dilution 1:500	
13. 5X Wash in PTWG on the shaker	1H each + ON
14. Dehydrate on the shaker	
- 20% MeOH	1H
- 40% MeOH	1H
- 60% MeOH	1H
- 80% MeOH	1H
- 100% MeOH	1H
- 100% MeOH	ON
15. Incubate in 66%DCM – 33% MeOH on the shaker	1H
16. Incubate in 66% DCM – 33% MeOH on the shaker	ON
17. Incubate in 100% DCM	1H
18. Incubate in 100% DCM	ON
19. Incubate in DBE	

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11. Appendix

11.1 Publication:

“Neural substrates involved in the cognitive information processing in teleost fish”

11.2 Publication:

“Brain areas activated during visual learning in the cichlid fish *Pseudotropheus zebra*”

11.3 Publication:

“Activation patterns of dopaminergic cell populations reflect different learning scenarios in a cichlid fish, *Pseudotropheus zebra*”

Publication:

“Neural substrates involved in the cognitive information processing in teleost fish”

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REVIEW

Neural substrates involved in the cognitive information processing in teleost fish

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Abstract

Over the last few decades, it has been shown that fish, comprising the largest group of vertebrates and in many respects one of the least well studied, possess many cognitive abilities comparable to those of birds and mammals. Despite a plethora of behavioural studies assessing cognition abilities and an abundance of neuroanatomical studies, only few studies have aimed to or in fact identified the neural substrates involved in the processing of cognitive information. In this review, an overview of the currently available studies addressing the joint research topics of cognitive behaviour and neuroscience in teleosts (and elasmobranchs wherever possible) is provided, primarily focusing on two fundamentally different but complementary approaches, i.e. ablation studies and Immediate Early Gene (IEG) analyses. More recently, the latter technique has become one of the most promising methods to visualize neuronal populations activated in specific brain areas, both during a variety of cognitive as well as non-cognition-related tasks. While IEG studies may be more elegant and potentially easier to conduct, only lesion studies can help researchers find out what information animals can learn or recall prior to and following ablation of a particular brain area.

Keywords Cognition · Behaviour · Neuroanatomy · Learning · Neuroethology · IEG · Lesion studies

Introduction

Behavioural studies over the last few decades have shown that fish possess cognitive abilities greatly exceeding those originally suggested by Tinbergen, who stated that cognitve skills in fish are confined to fixed action patterns (Tinbergen 1951). Instead, there is ample evidence that fish possess cognitive abilities rivalling those of mammals and birds (for reviews see Brown et al. 2011; Schluessel 2015). 'Cognition' hereby refers to higher order mental functions (Brown et al. 2011; Marchetti 2018), that include four different processes: perception, attention, memory formation and learning (Brown et al. 2011; Shettleworth 2010; Schluessel 2015). Together, these four processes provide animals with the ability to make decisions (Shettleworth 2010; Ebbesson and Braithwaite 2012). Animal cognition is a rather modern field of research, aiming to comprehend animals' mental abilities, as well as examining their underlying neural processes and mechanisms. There are several reasons that make fish a particularly interesting group to study this topic in. The group holds some of the most ancient forms of vertebrates, giving them a key position in the vertebrate phylogenetic tree. Compared to other vertebrates, there is also an unparalleled diversity featuring many exciting radiations which allow researchers to study influences of phylogeny versus ecology.

While cognition studies on fish are still less abundant than on mammals or birds—specifically in regards to the number of species studied—there really is a plethora of behavioural cognition studies available (see for example Kotrschal et al. 1998; Bshary et al. 2002; Brown et al. 2011). Furthermore, many studies over the last century have investigated fish, and in particular teleost neuroanatomy, assessing brain structures and their functions as well as neural connections and pathways both on a gross and molecular level (e.g. Nieuwenhuys 1963; Northcutt 1978,

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2011; Northcutt and Braford 1980; Northcutt and Davis 1983; Nieuwenhuys and Pouwels 1983; Nieuwenhuys and Meek 1990; Wullimann 1997; Hofmann 2001; Rodríguez et al. 2005; Salas et al. 2006; Ito et al. 2007; Ito and Yamamoto 2009; Hurtado-Parrado 2010; Rupp et al. 1996; Vernier 2017; Yamamoto and Bloch 2017). Information about fish neuroanatomy is crucial to a deeper understanding of fish cognition as a whole, as cognitive input is processed in various regions throughout the brain. It has been suggested that fish neural architecture involved in cognitive information processing represents both analogous and potentially homologous structures to those found in mammals (Broglio et al. 2003, 2011), thereby supporting the behavioural findings that fish possess higher cognitive capabilities comparable to those of mammals including those of non-human primates (Brown et al. 2011). Unfortunately, only few studies have combined the two fields, i.e. behaviour and neuroanatomy, and identified the neural substrates involved in the specific processing of cognitive information in fish (e.g. Rodriguez et al. 2006; Kotrschal et al. 2013a, b; for reviews see Wullimann and Mueller 2004; Broglio et al. 2011; Ebbesson and Braithwaite 2012; Demski 2013; Maruska and Fernald 2018). In the following review, two approaches (lesions and IEG studies) that can be used to elucidate specific structure–function relationships involved in processing cognitive information will be presented. A third method, i.e. volumetric studies, will only be mentioned briefly, despite being frequently used to determine functionality from structure. Volumetric studies look for correlations between the presence or the extend of a particular ability and the size of a particular brain structure, which from our perspective, provides a worthy first step in identifying potential areas of interest but generally leaves more room for error than alternative methods. In-vivo imaging studies and optogenetic studies are also mentioned briefly at the end of the review, both offering exciting new possibilities. Lesions studies determine specific impairments in cognitive abilities following the removal of a particular brain region or target nuclei, while IEG studies look for differences in gene expression patterns in response to varying treatments, such as learning, stress or recalling of cognitive information compared to untreated controls. Both methods have advantages and disadvantages that will be discussed. To familiarize the reader with the teleost brain, a short overview of potentially relevant brain structures is provided first.

The teleost brain

In the following, the cichlid brain will be introduced briefly for reference purposes. Obviously, fish brains vary in size and structure, and this section is only meant to provide a short overview of the major brain regions and nuclei that will be mentioned in later sections, i.e. in the lesion and IEG study descriptions, several of which have been conducted on cichlids. For this reason, any areas/nuclei mentioned throughout the paper (in any species) will be crosslinked to the figures provided in this section on the structure of the cichlid brain.

Generally, the fish brain follows the common vertebrate Bauplan. The neural tube gives rise to three primary morphological vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon), which is continuous with the spinal cord. The three vesicle stage develops into the five-vesicle stage. As described in the "columnar model", the forebrain— which in fish lacks the mammalian neocortex—is subdivided into the diencephalon caudally and the telencephalon rostrally. The diencephalon is then further divided into the thalamus dorsally and the hypothalamus ventrally, while the telencephalon is further subdivided into the pallium dorsally and the subpallium ventrally. The midbrain connects the forebrain to the hindbrain (rhombencephalon). Finally, the hindbrain is divided into the myelencephalon (containing the medulla oblongata) caudally and the metencephalon (containing the cerebellum and pons) rostrally (Herrick 1910; Wullimann 1997; Simões et al. 2012; Yamamoto and Bloch 2017).

A second model, the "prosomeric model", was proposed by Puelles and Rubenstein in the early 1990s and attributes morphological meaning to gene expression patterns (Puelles and Rubestain 2003). Here, the forebrain is subdivided into the posterior diencephalon and the anterior secondary prosencephalon. The diencephalon is then further subdivided into the pretectum, thalamus, and prethalamus, while the secondary prosencephalon at the anterior end of the forebrain contains the telencephalon dorsally and the hypothalamus ventrally.

The third and newest model, proposed by Affaticati et al. (2015), divides the secondary prosencephalon into three parts: the telencephalon, hypothalamus, and optic recess region (Affaticati et al. 2015). Figure 1 presents the three available models featuring the different subdivisions of the forebrain (Yamamoto et al. 2017 modified), Fig. 2 shows the major brain regions of a teleost fish from a dorsal and lateral perspective.

The telencephalon of Actinopterygians (which represent the largest group within the fishes) undergoes a different embryological development than all other craniates (Fig. 3), a so-called 'eversion' process (Gage 1893) that produces two telencephalic hemispheres separated by a single ventricle (Broglio et al. 2005) and a proliferative zone that lies at the dorsal part of the telencephalon (Mueller and Wullimann 2009). All other craniates undergo an 'evagination' process that produces two telencephalic hemispheres, each one with its own ventricular cavity, and a proliferative zone

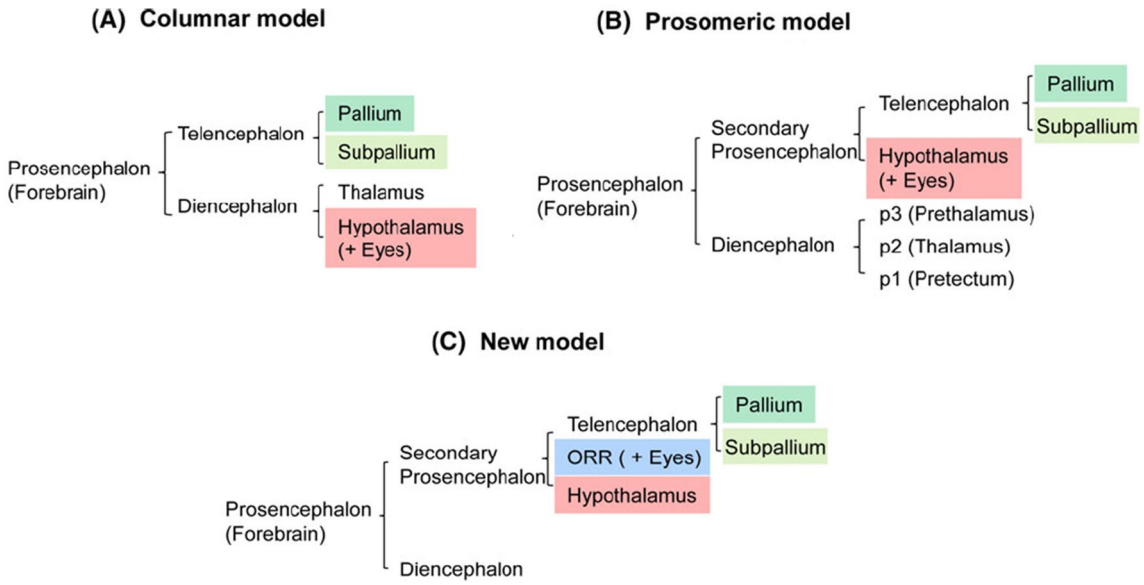


Fig. 1 Models featuring the different subdivisions of the forebrain. **a** The columnar model in which the hypothalamus is considered to be the ventral half of the diencephalon. **b** The prosomeric model originally proposed by Puelles and Rubenstein in which the hypothalamus is proposed to be the ventral half of the most anterior part of the forebrain, and the telencephalon and hypothalamus consist of the secondary prosencephalon. **c** A new model proposed by Affaticati et al. in which the secondary prosencephalon is divided into three parts, the telencephalon, hypothalamus, and optic recess region (ORR) (modified from Yamamoto et al. 2017)

that is oriented towards the ventricles (Muller and Wullimann 2009).

Due to the different embryological development of Actinopterygians, potentially homologous or functionally equivalent structures are found in different locations than in other fish (such as in the chondrichthyans) or other vertebrate groups (Wullimann 1997). The forebrain of ray-finned fishes contains a number of areas common to all species (Northcutt 2002) while other areas are only seen in some highly derived groups, such as cichlids. This can include the presence or absence of nuclei or different structuring of particulate regions.

The neural correlates for most cognitive functions in fish are still largely unknown, with the well-known exception of the lateral and medial divisions of the dorsal telencephalon (see Fig. 4b, section D). These two areas have been investigated in several studies and are by many considered to be potential homologues of the mammalian hippocampus and amygdala, respectively (for reviews see Rodriguez et al. 2006; Broglio et al. 2011; Ebbesson and Braithwaite 2012; but see also Saito and Watanabe 2004; 2006). However, these areas may also be involved in additional, so far unstudied, cognitive processes. Moreover, the functions of other areas within the telencephalon, such as the dorsal or central divisions of the dorsal telencephalic area (see Fig. 4b, sections B, C), are still unknown, as are the functions of most of its ventral regions.

Even less information is available on the diencephalon or the cognitive involvement of brain regions outside of the forebrain. While the connectivity between and within some brain areas may be known (e.g. Ahrens and Wullimann 2002; Folgueira et al. 2004a, b), there is not a single nucleus or area in the diencephalon whose function or contribution to possessing specific cognitive abilities has been studied and identified in detail. This includes the habenula (see Fig. 4b, section D), the thalamus (see Fig. 4b, section D) and the hypothalamus, which is highly derived in many teleost groups (Ahrens and Wullimann 2002). There is a prominent visual pathway extending from the tectum over the nucleus corticalis and the nucleus glomerulosus (see Fig. 4b, sections E, F) to the inferior lobes of the hypothalamus (see Fig. 4b, sections F–H) (Wullimann and Meyer 1990; Butler et al. 1991; Ahrens and Wullimann 2002). There are also some other highly derived areas, such as the mammillary body (see Fig. 4b, section F) and the nucleus of the posterior tuberculum. The latter has extensive projections to the medial part of the dorsal telencephalon (Murakami et al. 1983). In some fish groups, other areas are also markedly elaborated, e.g. the lateral torus (see Fig. 4b, sections D, E), which is related to gustatory functions (Ahrens and Wullimann 2002), and the anterior tuberal nucleus, which is particularly enlarged in catfish, and possibly related to acoustic communication. A range of forebrain areas has been electrically stimulated and behavioural responses have been described for several fish species (e.g. Demski 1973, 1977, 1983; Demski and Knigge 1971; Demski and Picker 1973). Stimulations in a number

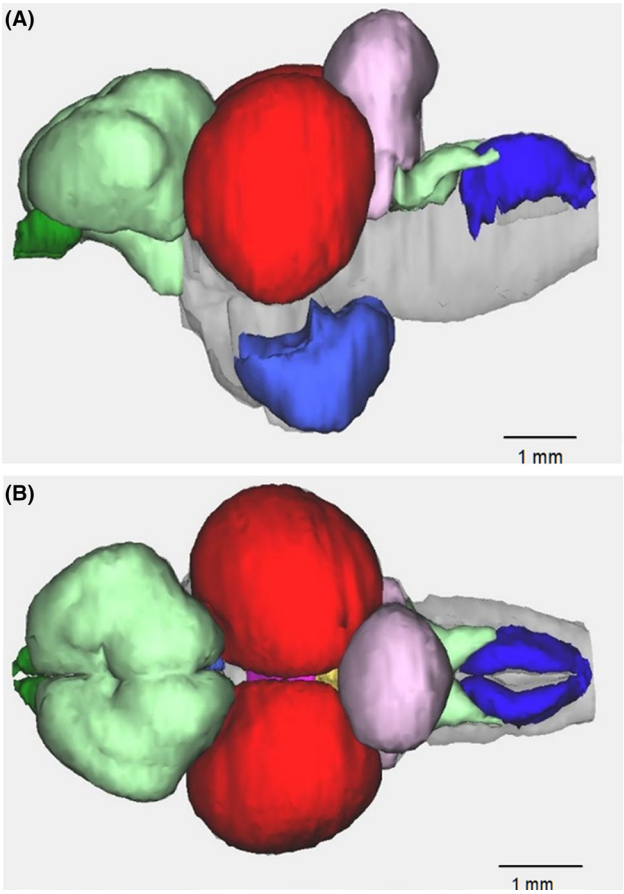


Fig. 2 Brain of a cichlid fish, *Thorichtys meeki*. **a** Lateral view; **b** dorsal view. Six major areas can be identified: olfactory bulbs (dark green), telencephalon (light green), optic tectum (red), cerebellum (pink) + crista cerebellari (light green), inferior lobe of hypothalamus (purple), vagal lobe (blue) (courtesy of Michael Hofmann) (colour figure online)

Fig. 4 **a** Shown is the lateral view of the *Pseudotropheus zebra* brain (rostral to caudal); the oblique lines (A–L) give the locations of the transverse sections through the brain. **b** Cryostat sections of the P. zebra brain (A–L). The right side shows the microphotos of the original sections after Nissl staining, the left side shows a schematic drawing of identifiable areas and nuclei. *CC* crista cerebellaris, *CCe* corpus cerebelli, *Chr* commissura horizontalis, *CIL* central nucleus of inferior lobe, *CM* mamillary body, *CN* nucleus corticalis, *CP* central posterior thalamic nucleus, *D* dorsal telencephalic region, *Dc* central area of D, *Dc-d* dorsal subdivision of Dc, *Dc-r* rostral area of Dc, *Dc-v* ventral division of Dc, *Dd* dorsal area of D, *DIL* diffuse nucleus of the inferior lobe, *Dl* lateral area of D, *Dl-d* dorsal subdivision of Dl, *Dl-g* granular area from Dl, *Dl-v* ventral subdivision of Dl, *Dl-v1,2* parts of Dl-v, *Dm* medial area of D, *Dm-1,2,3* subdivisions of Dm, *DP* dorsal posterior thalamic nucleus, *Dp* posterior area of D, *dpca* decussation of anterior cerebellar peduncle, *EG* eminentia granularis, *EN* entopeduncular nucleus, *FR* fasciculus retroflexus, *GN* nucleus glomerulosus, *H* habenula, *ILdl* dorsolateral part of the inferior lobe, *ILvm* ventromedial part of the inferior lobe, *ILdv* ventromedial part of the inferior lob, *IMRF* intermediate reticular formation, *IRF* inferior reticular formation, *LFB* lateral forebrain bundle, *LLF* lateral-longitudinal fasciculu, *MLF* medial-longitudinal fasciculus, *Mo* medulla oblongata, *MS* spinal cord, *Ni* nucleus isthmi, *NIII* nucleus nervi oculomotori, *NIV* trochlear nucleus, *NVm* motor nucleus of trigeminal nerve, *Ni* nucleus isthmi, *NPT* posterior tuberal nucleus, *OB* olfactory bulb, *Obgl* glomerular area of the olfactory bulb, *Obgra* granular area of the olfactory bulb, *OC* optic chiasm, *OTV* ventrolateral optical tract, *PGZ* periventricular gray zones of the TeO, *POA* pre-optic area, *PTN* nucleus posterior tuberis, *RL* lateral recess, *RV* rhombencephalic ventricle, *SRF* superior reticular formation, *TTB* tectobulbaric tract, *Tel* telencephalon, *TeO* optic tectum, *TeV* tectal ventricle, *TL* torus longitudinalis, *TLA* lateral torus, *TOd* dorsal optic tract, *TOv* ventral optic tract, *TS* semicircular torus, *V* ventral telencephalic area, *VIII* vestibulocochlear nerve, *Vam* medial area of the valvula cerebelli, *Val* lateral area of the valvula cerebelli, *Vc* central area of V, *Vd* dorsal area of V, *VL* vagal lobe, *Vl* lateral area of V, *Vs* supracommissural nucleus of V, *Vv* ventral area of V, *WN* Edinger-Westphal Nucleus, *X* vagus nerve (slides taken from Jauch 2015)

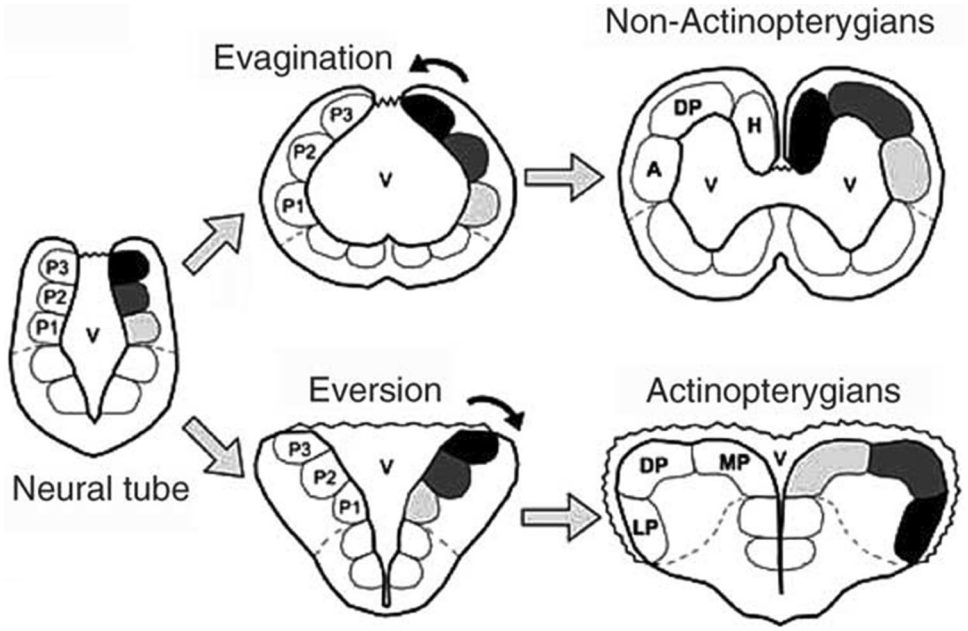
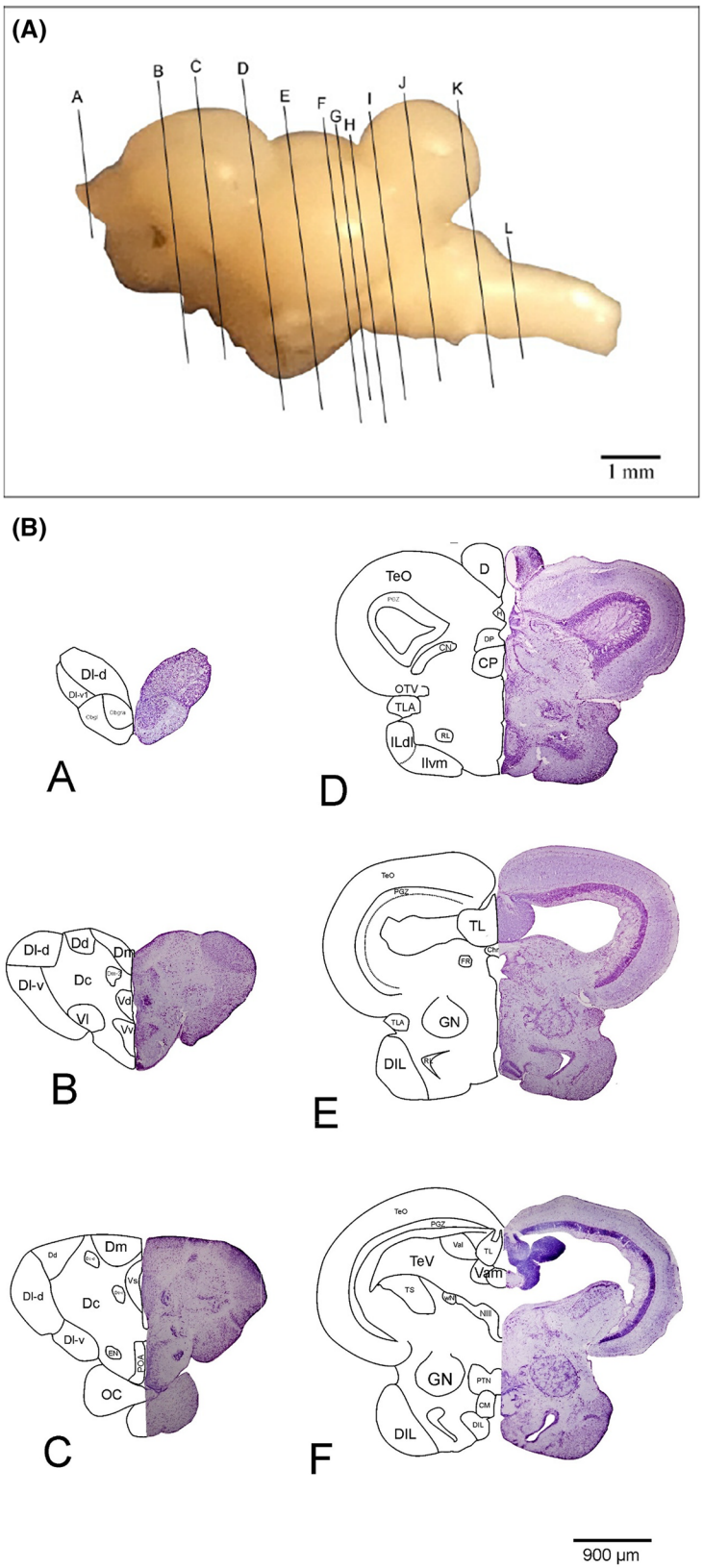


Fig. 3 Schematic representation of the process of evagination in Non-Actinopterygians and eversion in Actinopterygians (modified from Broglio et al. 2005)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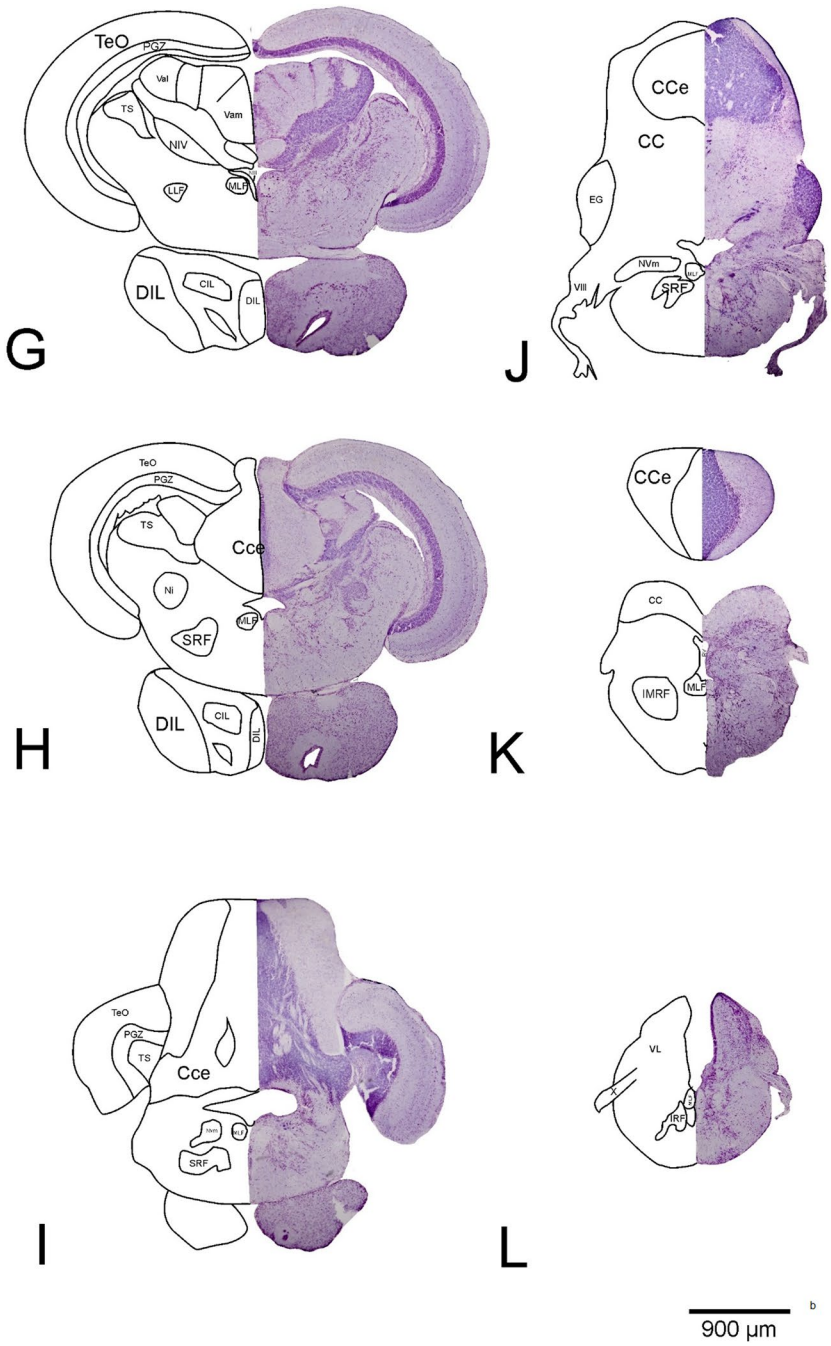

Fig. 4 (continued)

of hypothalamic areas for example changed specific types of social behaviours, suggesting that some social functions are located or mediated by the hypothalamus. Particularly the inferior lobes (see Fig. 4b, sections G, H) can be quite large and receive indirect visual input through the nucleus glomerulosus (see Fig. 4b, sections E, F). In some species, the inferior lobes can be even larger than the entire telencephalon (Hofmann, pers. comm.). In summary, only a few detailed studies, focusing on a few potential roles of specific forebrain areas, exist, while it seems highly likely that at least some of these regions are also involved in the processing of (other) cognitive tasks.

Volumetric and lesion studies

There are many correlative studies linking brain size to cognitive ability, environment and/or ecology (e.g. Pike et al. 2018). Studies across mammals and birds have shown that overall brain size and cognitive abilities usually correlate positively (e.g. Reader and Laland 2002; Sol et al. 2005; Deaner et al. 2007). Also, closely related species occupying different niches may feature distinct differences in the size of major brain areas, related to differences in cognitive abilities (Tebbich and Bshary 2004). For example, higher cognitive abilities are required to find food in complex habitats compared to more simple or unstructured habitats. As a result, associated brain regions, e.g. the hippocampus (or its homologue) are enlarged in species that live and forage in complex environments (e.g. Sherry et al. 1989, 1992; Lucas et al. 2004). Similarly, brain size in fish may correlate with habitat, lifestyle or cognitive capabilities (e.g. Pollen et al. 2007; Salvanes et al. 2013; Northcutt 1977; Yopak et al. 2007; Yopak 2012; Pike et al. 2018). In cichlids, studies have linked brain complexity, size and volume with ecology, lifestyle or behaviour (e.g. Anken and Bourrat 1998; Pollen et al. 2007; Pollen and Hofmann 2008; Burmeister et al. 2009; Shumway 2010; Gutiérrez-Ibáñez et al. 2011). Environmental factors may permanently enhance learning abilities of fish (Kotrschal and Taborsky 2010). In guppies, a larger overall brain size was positively linked to cognitive differences (Kotrschal et al. 2013a,b,2014) and large-brained guppy females outperformed small-brained females in a reversal task but not in a colour discrimination test (Buechel et al. 2018). While these studies provide an important first step towards identifying relevant brain regions involved in cognitive information processing, correlative evidence is prone to errors and usually limited to assessing major brain areas as opposed to determining the detailed functioning of selected nuclei or smaller areas. Lesion studies, despite their own shortcomings (Lomber 1999), provide a more comprehensive method of determining structure–function relationships.

Pioneering lesion studies have focused on the involvement of the telencephalon but no other structures in cognitive processing (reviewed in Hofmann 2001; see also Savage 1980; Overmier and Curnow 1969; Overmier and Savage 1974; Laming and McKinney 1990) and detected impairments following ablation in some learning and memory functions (e.g. avoidance and spatial learning; e.g. Flood et al. 1976; Davis and Kassel 1983; Overmier and Hollis 1983; Rodriguez et al. 2006). Removal of the telencephalon (see Fig. 4b, section E) in teleosts does not seem to be as deleterious as in mammals (e.g. Kaas 1987; Hofmann 2001) and it is likely that cognitive functions are not all situated within the telencephalon. However, the only other brain structure that has been looked at in some detail in this context is the cerebellum (see Fig. 4b, sections J, K). It has been implicated in various conditioning tasks (Karamian 1963; Aronson and Herberman 1960; Álvarez et al. 2002; Gómez 2003) as well as avoidance or emotional learning (e.g. Kaplan and Aronson 1969; Álvarez et al. 2003; Gómez 2003, Yoshida et al. 2004; Rodríguez et al. 2005) and spatial cognition (Durán et al. 2004; Rodríguez et al. 2005).

A cognitive ability, relevant to most species and quite thoroughly investigated in many vertebrate and invertebrate species, is spatial cognition, which includes spatial learning and memory. Behaviours, such as orientation, navigation, migration or homing, depend on spatial cognition (Dodson 1988; Rajan et al. 2011). Spatial learning is directly connected to spatial memory as it allows an individual to record and recall information about its environment and its orientation, for example feeding or nesting locations. For this reason, spatial memory and spatial learning are essential both for animals that do not change habitat, as well as for animals migrating (Wood et al. 2011). In its most complex form, spatial cognition entails the ability of an organism to acquire a mental representation of the environment, i.e. to construct a cognitive spatial map (Glikmann-Johnston et al. 2015). In a series of studies on the goldfish (*Carassius auratus*), behavioral and detailed neuroanatomical approaches were used complementary to elucidate spatial abilities and neuroanatomical correlates (reviewed in Broglio et al. 2011). Results indicated that not the telencephalon as a whole, but specifically the lateral zone of the dorsal telencephalon (see Fig. 4b, sections A–C), considered to be a hippocampus homologue, plays a crucial role in complex place learning (allocentric orientation) and spatial memory in goldfish (e.g. Salas et al. 1996a, b; López et al. 2000; Durán et al. 2008, 2010; Costa et al. 2011). It was also established that egocentric spatial strategies are unlikely to be processed—at least exclusively—in the telencephalon (e.g. Salas et al. 1996a, b; López et al. 2000; Rodríguez et al. 2002). Results were contradicted by findings from Saito and Watanabe (2004,2006), who claimed impairments in the dorsomedial telencephalon (see Fig. 4b, sections B, C), instead of the dorsolateral part, to be responsible for the disruption of spatial abilities (for a critical discussion see Rodriguez et al. 2006). More recently, similar results to those obtained on the goldfish were observed in studies on the spatial abilities of sharks and stingrays (Schluessel and Bleckmann 2005, 2012; Fuss et al. 2014a, b). In sharks, the dorsomedial pallium, like the lateral pallium in teleosts, seems to play a crucial role in processing more complex place learning information (Fuss et al. 2014a) while not being implicated in the processing of egocentric information, i.e. turn procedures (Fuss et al. 2014b). Accordingly, as in the goldfish, different neural substrates seem to be responsible for different spatial functions and mechanisms in sharks. As suggested by Rodriguez et al (2006), results indicate that the dorsomedial portion of the

pallium in sharks may be comparable to the hippocampus of land vertebrates and the lateral pallium of teleosts (Fuss et al. 2014a, b). In two blenniid species, sex-specific differences in regards to spatial demands were found to exist both behaviourally and neuronally (Costa et al. 2011). White and Brown (2015) tried to correlate volume measurements of various brain regions with spatial ability in two species of guppies with different ecological needs. Differences were found in the size of the telencephalon, the optic tectum and the hypothalamus as well as different spatial abilities between the two species.

Very few studies are available assessing the involvement of neural substrates in cognitive behaviours other than spatial orientation. In goldfish, avoidance learning paradigms were investigated and the medial zone of the dorsal telencephalon in teleosts found to perform similar functions to the pallial amygdala of land vertebrates (reviewed in Rodriguez et al. 2006; Portavella and Vargas 2005; Portavella et al. 2002; Broglio et al. 2011). Similar results were observed in juvenile bamboo sharks (Schwarze et al. 2013).

Table 1 gives an overview of the most relevant lesion studies assessing neural structures in fish in a cognitive context.

Lesion studies, where parts of the brain are ablated, can help researchers to identify the neural substrates involved in cognitive information processing by testing what animals can do prior to and following surgery. The most obvious shortcoming of this technique is that lesions are hard, if not impossible to place without damaging non-target tissue 'on route' to the target destination. While electrodes of micromanipulators are extremely fine, the extent of damage created is still difficult to estimate. Additional problems are to actually 'find' the correct target area, finding the same area repetitively in different individuals (also of varying sizes),

Table 1 Overview of studies combining behavioral and neuroanatomical studies in fish

Brain region	Behavior	Author
Lateral zone of pallium	Spatial orientation	Salas et al. (1996a, b)
Dorsolateral telencephalon	Spatial orientation	Vargas et al. (2000)
Lateral zone of pallium	Spatial orientation	López et al. (2000), Durán (2004), Broglio et al. (2005)
Dorsomedial telencephalon	Spatial orientation	Saito and Watanabe (2004,2006)
Lateral pallium	Encoding spatial information	Vargas et al. 2006
Entire telencephalon	Spatial orientation	Durán et al. (2008)
Dorsolateral ventral telencephalic nuclei	Spatial orientation	Costa et al. (2011)
Pallium[a]	Spatial orientation	Fuss et al. (2014c)
Pallium[a]	Spatial orientation	Fuss et al. (2014d)
Telencephalon,hypothalamus, optic tectum	Spatial orientation	White and Brown (2015)
Cerebellum	Spatial orientation	Durán et al. (2004), Rodríguez et al. (2005)
Medial zone of pallium	Emotional memory/avoidance learning	Portavella and Vargas (2005), Portavella et al. (2002, 2004), Durán (2004)
Telencephalon	Avoidance learning	Schwarze et al. (2013), Overmier and Flood (1969), Overmier and Hollis (1990)
Telencephalon	Conditioning tasks and habituation	Savage (1980), Overmier and Curnow (1969), Overmier and Savage (1974), Laming and McKinney (1990)
Telencephalon, optic tectum, cerebellum, dorsal medulla, hypothalamus and olfactory bulb	Reversal learning/spatial learning	Fong et al. (2019)
Optic tectum, olfactory bulbs	Discrimination tasks	Pike et al. (2018)
Cerebellum	Conditioning tasks	Karamian (1963), Aronson and Herberman (1960), Álvarez et al. (2002), Gómez (2003)
Cerebellum	Emotional learning	Kaplan and Aronson (1967), Aovarez et al. (2003), Gómez (2003), Yoshida et al. (2004), Rodríguez et al. (2005)
'Nuclei of the conserved social behavior network'	Social hierarchy*	Maruska et al. (2013)

Note that most studies have tried to look for spatial orientation correlates

*Not cognition studies per se, but studies of behaviors that include some cognitive aspects

[a]The medial pallium in elasmobranchs corresponds to the lateral zone of the pallium in teleosts due to different folding events during embryogenesis. We attempted to remove the medial pallium; however, as it was difficult to target almost the entire pallium was removed in most individuals

destroying a significant portion of a relevant area and rendering it unfunctional in the process, and making sure that the lesioned area is in fact responsible for the processing of a particular information as opposed to simply being part of a relevant pathway in the information transfer. Last but not least, one always has to consider further surgery effects, such as causing motivational, sensory or motor impairments that keep animals from performing at the same level as prior to surgery. For example, damaging the target area could cause a hyperactivation or hyperinactivation of other brain regions that, under normal conditions, are activated or inhibited by the damaged region (Fuster 1989; Damasceno 2010). Some of these shortcomings can be overcome using sham-operated and control animals, as well as lesioning a larger number of individuals (also to overcome intra-specific variation). Some, however, cannot be controlled for. Due to this circumstance, it seems ideal to combine lesion studies with an additional method, that has widely been used in recent years and from our perspective can make up for some of the problems encountered, immediate early gene analyses.

Immediate early gene studies

The study of the expression of immediate early genes (IEGs) is a more recent but very promising method to investigate and visualize neuronal activity in the brain when investigating substrates underlying synaptic plasticity processes, such as long-term potentiation (LTP), long-term depression (LTD) and cognitive functions (Minatohara et al. 2016). IEGs are cellular genes that are responsive to extracellular stimuli, more precisely, they are first response genes whose expression is regulated immediately after stimulation. Transcriptional activation of RNA occurs in the nucleus within five minutes of stimulation and continues for 15–20 min, after which the transcripts are transferred to the cytoplasm (Greenberg and Ziff 1984; Greenberg et al. 1985; Guzowski et al. 1999). Induction occurs within minutes and is short-lived; typically, IEG mRNA levels reach their maximum 30–60 min after stimulation and decline after 2–5 hrs to baseline. The protein concentration reaches its maximum about 60–90 min after stimulation and disappears within four hours of treatment (Curran and Morgan 1995).

Different studies conducted on PC 12 pheochromocytoma cells highlighted the involvement of IEGs in neuronal function (Sheng and Greenberg 1990; Morgan and Curran 1991; Curran and Morgan 1995). In conjunction with more recent research, these studies demonstrate that IEGs are expressed throughout the nervous system and that various types of stimulation (such as pharmacological agents, behavioral tests, seizures, etc.) can increase their expression (Curran and Morgan 1995). In fact, IEG expression is a crucial part of a neuron's response to behaviourally relevant stimuli and codes for several classes of proteins displaying different functions, such as signalling molecules, postsynaptic proteins, metabolic enzymes, cytoskeletal proteins, growth factors or transcription factors (Lanahan and Worley 1998). There is also a correlation between a local increase in IEG expression and neuronal activity, i.e. IEG expression can serve as a marker for neuronal activity. This indicates which types of neurons were activated and, above all, in which area of the brain the activation took place (Long and Salbaum 1998). It is estimated that there are about 30–40 different IEGs that can be expressed in neurons. Of these, 10–15 could serve as regulatory genes, i.e. function as transcription factors (Lanahan and Worley 1998) capable of regulating the expression of target genes (named late-response genes) and influencing neuronal physiology (Curran and Morgan 1987; Curran and Franza 1988; Herdegen and Leah 1998; O'Donovan et al. 1999; Tischmeyer and Grimm 1999; Pinaud 2004; Pinaud et al. 2005; Gallo et al 2018). Among those IEGs that function as transcription factors, the most investigated genes for mapping activity in the brain are *c-fos* and *egr-1*. Both are involved in cell differentiation and proliferation and, most importantly, they serve a crucial function in cognitive processes, particularly in learning and memory, but also in synaptic plasticity in general (Okuno 2011).

C-FOS

C-fos, whose induction was the first one among IEGs to be shown as activity-dependent (Morgan and Curran 1988; Sagar et al. 1988; Gallo et al. 2018) belongs to the Fos family and is a protooncogene (Morgan and Curran 1989). It encodes the nuclear C-FOS protein, a 62-kDa product which undergoes post-translational modifications that mainly consist of serine and threonine phosphorylation (Curran et al. 1984; Barber and Verma 1987). Furthermore, *c-fos* can negatively regulate its own expression and this characteristic is required for a rapid decline in its expression (Morgan and Curran 1991). In neurons, the first detailed studies assessing the regulatory mechanisms of IEGs were performed on *c-fos* (Schilling et al. 1991; Sheng et al. 1990; Okuno 2011) and, under baseline conditions, there is little or no expression of this gene in most neurons (Morgan and Curran 1989; Hoffman et al. 1993). The expression of various late-response genes involved in different neuronal processes (for example growth control or plastic changes) is induced by the activation of *c-fos* gene (Sukhatme et al. 1988; Williams et al. 2000; Bozon et al. 2003; Maddox et al. 2011; Gallo et al. 2018).

EGR-1

The gene *egr-1* is also known as *zif/268*, *krox-24*, *TIS8*, *NGFI-A* or *zenk*; it codes for a transcription factor (*Egr-1*) that is a member of a four-gene family of *Egr* and also plays

an important role in neural plasticity during neuronal activation through sensory stimulation. *Egr-1* is a phosphorylated protein and it is synthesized in the nucleus, where it remains thereafter (Cao et al. 1990). Furthermore, this protein has the ability to autoregulate its own expression (D. Gius, X. Cao, and V. P. Sukhatme, unpublished, referenced in Cao et al. 1990). The *Egr-1* expression in the brain is specific to neurons and its activity is strongly (but not exclusively) regulated by synaptic activity (Worley et al 1991). *Egr-1* expression is continually induced by ongoing synaptic activity (Burmeister and Fernald 2005) as a consequence of the basal physiological synaptic activity (Worley et al. 1991). This is unlike other, similar inducible transcription factors (such as *C-fos*), whose expression declines after the initial stimulation (Herdegen et al. 1995; Kaczmarek and Chaudhuri 1997). It is still unclear which are the targets that *egr-1* regulates under physiological stimulation in vivo, but synapsins (Petersohn et al. 1995; Thiel et al. 1994; Burmeister and Fernald 2005) and neurofilaments (Mello 2004; Burmeister and Fernald 2005) are two likely candidates (Burmeister and Fernald 2005).

To investigate the evolutionary conservation of *egr-1*, cichlid *egr-1* (*Astatotilapia burtoni*) was cloned by Burmeister and Fernald (2005) and its protein sequence compared to available representatives of other vertebrate groups. They demonstrated that *A. burtoni egr-1* shares 81% sequence similarity with zebrafish (*Danio rerio*) and 66% with mouse (*Mus musculus*) *egr-1*. This has been the only characterization of *egr-1* in a vertebrate other than a mammal or bird (Burmeister and Fernald 2005). Another important discovery coming out of this study showed that the *egr-1* expression kinetics is similar to the one of mammals (Zangenehpour and Chaudhuri 2002) and birds (Mello and Clayton 1994) by reaching its highest expression levels 30 min after stimulation (Burmeister and Fernald 2005).

The following provides a summary of previous research and advances that have successfully used IEGs as markers of cognitive processing in fish (see Table 2).

Choice behaviour, i.e. the ability to make choices and perform actions and behaviours as a result of these choices, is critical for the survival of all individuals (Lau et al. 2011). To test this behaviour in fish, light avoidance, an innate choice behaviour, was examined in zebrafish (Lau et al. 2011). First, fish were introduced to a light/dark choice chamber. After giving them time to explore the environment, researchers found two distinct groups of animals: one composed of animals showing light-avoidance behaviour, and a second one that did not. These two different behaviours occurred regardless of whether the animals were initially placed on the dark or the bright side of the chamber. In situ hybridization analyses of the *c-fos* expression were performed and compared between the two groups. In the animals exhibiting light-avoidance behaviour, *c-fos* expression was detected in the medial zone of the dorsal telencephalic region (see Fig. 4b, sections B, C), potentially homologous to the mammalian amygdala. Another increase in *c-fos* expression was found in the dorsal nucleus of the ventral telencephalic area, possibly the teleost homologue of the mammalian striatum (Rink and Wullimann 2002). In the diencephalon, *c-fos* was detected in the hypothalamus and in different nuclei of the dorsal thalamus [anterior nucleus, dorsal posterior thalamic nucleus, central posterior thalamic nucleus (see Fig. 4b, section E)] and in the preglomerular nucleus [the last four nuclei are visually activated in the teleost brain (Wullimann 1997)]. Furthermore, *c-fos* expression was detected in the optic tectum (see Fig. 4b, sections G–L), in the periventricular grey zone (see Fig. 4b, sections E–K) and in the cerebellum (see Fig. 4b, sections J, K). In animals that exhibited low or no light-avoidance behaviour, the *c-fos* expression detected in the hypothalamus and in the visually related nuclei was similar to that of the 'avoidance group' but little *c-fos* was detected in the medial zone of the dorsal telencephalic region (see Fig. 4b, sections A–C) and in the dorsal nucleus of the ventral telencephalic area. The differential *c-fos* expression in the medial zone of the dorsal telencephalon and in the dorsal nucleus of the ventral telencephalon, and consequently the divergent activation of these regions, within the two groups of fish, led researchers to believe that these two regions may be involved in a circuitry that determines the performance of the light-avoidance behaviour. Furthermore, since the dorsal nucleus of the ventral telencephalon is "downstream" of the dorsal telencephalic region, it seems that the latter could play the role of a "choice centre" in this behaviour (Lau et al. 2011).

One of the most important decisions is choosing suitable mates or partners. The choice of a male partner by females, based on the information about male–male social interactions, was analysed by Desjardins et al. (2010) in *A. burtoni*. The study aimed to investigate specifically, which brain regions respond to visual information when choosing a mate. The expression of the two IEGs *c-fos* and *egr-1* was analysed in the proposed fish homologue of the brain nuclei of SBN (social behaviour network) in mammals (which includes the medial amygdala, the lateral septum, the preoptic area, the anterior hypothalamus and the ventromedial hypothalamus, the periaqueductal grey, the dorsolateral telencephalon, the cerebellum and the raphe nucleus) (Newman 1999). IEGs expression was compared in the brains of gravid females' that, after having chosen a mate, witnessed a fight between the males of their choice. Females who had seen their respective preferred male win or lose a fight showed differences in IEG expression in all SBN brain nuclei. Additionally, differences in the level of *egr-1* and *c-fos* expression were found in other brain areas. More precisely, females who saw their preferred male win, had higher IEG expression in the ventromedial hypothalamus and in the preoptic area

Table 2 Overview of studies combining behavioral and IEGs studies in fish

Brain region	IEGs	Behavior	Author
Pre-optic area, medial zone of the dorsal telencephalon, ventral subdivision of the lateral zone of the dorsal telencephalon	c-fos egr-1	Social habituation	Weitekamp et al. (2017)
Telencephalon, hypothalamus, cerebellum	egr-1	Alloparental-care opportunity and submissive behavior	Kasper et al. (2018)
Nuclei of social decision-making network (SDMN): ventral nucleus of ventral telencephalon, rostral portion of the ventral nucleus of the telencephalon, supracomissural nucleus of the ventral telencephalon, dorsal part of the ventral telencephalon, anterior tuberal nucleus, anterior part of the periventricular preoptic nucleus, parvocellular division of the magnocellular preoptic nucleus, magnocellular division of the magnocellular preoptic nucleus, lateral nucleus of dorsal telencephalon, granular region, the posterior nucleus of the dorsal telencephalon	c-fos	Social behavior	Field and Maruska (2017)
Telencephalon, optic tectum, cerebellum	egr-1 c-fos	Social behavior	Sadangi (2012)
Nuclei of social behavior network (SBN): lateral part of the dorsal telencephalon, medial part of the dorsal telencephalon, ventral nucleus of the ventral telencephalon, supracommissural nucleus of the ventral telencephalon, preoptic area, ventral tuberal nucleus, anterior tuberal nucleus, corpus cerebellum	egr-1 c-fos	Social behavior—social hierarchy	Maruska et al. (2012)
Telencephalon and hypothalamus	egr-1 bdnf	Early social behavior	Nyman et al. (2017)
SDMN	egr-1 c-fos	Social behavior—aggressive behavior	Almeida et al. (2019)
Dorsolateral telencephalon, dorsomedial telencephalon, ventral telencephalon, ventral hypothalamus, central gray area, pituitary gland, nucleus preopticus, anterior tuberal nucleus, ventral sopracommissural telencephalon, cerebellum	egr-1	Mating behavior	Wong et al. (2012)
Telencephalon, optic tectum, hypothalamus, pituitary gland, cerebellum, medulla oblongata and the anterior part of the spinal cord	c-fos	Mating behavior	Okuyama et al. (2011)
Whole brain	egr-1	Mating behavior	Cummings et al. (2008)
Medial part of the dorsal telencephalon, the supracommissural nucleus of the ventral telencephalon, the ventral nucleus of the ventral telencephalon, the preoptic area	c-fos	Social buffering	Faustino et al. (2017)
Medial octavo lateralis nucleus, ventro lateral portion of the torus semicircularis, central portion of the torus semicircularis, central posterior thalamic nucleus, posterior part of the dorsal telencephalon	c-fos	Social behavior—social interaction	Butler and Maruska (2016)
SDMN regions: anterior tuberal nucleus, central part of the dorsal telencephalon, granular zone of the lateral zone of the dorsal telencephalon, magnocellular preoptic nucleus magnocellular division, magnocellular preoptic nuclus parvocellular division, periventricular nucleus of the posterior tuberculum, caudal subdivision of the dorsal part of the ventral telencephalon, supracomissural nucleus of the ventral telencephalon, ventral portion of the ventral telencephalon			

Table 2 (continued)

Brain region	IEGs	Behavior	Author
Dorsal telencephalon (lateral, medial, central)	egr-1	Social behavior—individual recognition	Harvey-Girard et al. (2010)
Anterior preoptic area	egr-1	Social behavior—social hierarchy	Burmeister et al. (2005)
Nuclei of SBN: lateral part of the dorsal telencephalon, medial part of the dorsal telencephalon, ventral nucleus of the ventral telencephalon, supracommissural nucleus of the ventral telencephalon, preoptic area, ventral tuberal nucleus, anterior tuberal nucleus, and corpus cerebellum	egr-1 c-fos	Social behavior—social hierarchy	Maruska et al. (2013)
Forebrain	c-fos	Social behavior—paternal care	O'Connell et al. (2012)
Telencephalon	egr-1	Visual discrimination	Fuss and Schluessel (2018)
Olfactory bulb	c-fos	Behaviors evoked by odorants	deCarvalho et al. (2013)
Medial zone of the dorsal telencephalic region, dorsal nucleus of the ventral telencephalic area dorsal thalamus (anterior nucleus, dorsal posterior thalamic nucleus, central posterior thalamic nucleus), hypothalamus, preglomerular nucleus, optic tectum, periventricular gray zone and in cerebellum	c-fos	Choice behavior	Lau et al (2011)
Social behavior network nuclei: medial amygdala, lateral septum, preoptic area, anterior and ventromedial hypothalamus, periaqueductal gray, dorsolateral telencephalon, cerebellum and raphe nucleus	egr-1 c-fos	Choice behavior	Desjardins et al. (2010)
Pre-optic area, lateral septum, anterior and ventromedial hypothalamus, periaqueductal gray, dorsomedial and dorsolateral telencephalon, cerebellum, raphe nucleus	egr-1 c-fos bdnf	Spatial cognition	Wood et al. (2011)
Telencephalon	egr-1	Spatial cognition	Rajan et al. (2011)
Dorsomedial telencephalon, dorsolateral telencephalon, preoptic area and cerebellum	egr-1 c-fos	Mirror image fighting	Desjardins and Fernald (2010)
SDM network nuclei: three subregions of the medial part of the dorsal telencephalon, one subregion of the supracommissural nucleus of the ventral pallium, the lateral subdivision of the lateral part of the dorsal telencephalon, parvocellular as well as magnocellular and gigantocellular cell groups of the preoptic area	c-fos	Social behavior—cooperative behavior	Weitekamp and Hofmann (2017)
Medial zone of the dorsal telencephalon, lateral zone of the dorsal telencephalon, ventral nucleus of the ventral telencephalon	egr-1 c-fos bdnf npas4	Emotion-like states	Cerqueira et al. (2017)
Central part of the dorsal telencephalon, ventral zone of the ventral region of the lateral part of the dorsal telencephalon, posterior part of the dorsal telencephalon, central part of the ventral telencephalon, ventral part of the ventral telencephalon, parvocellular and magnocellular subnuclei of the preoptic area	c-fos	Sensory integration of social signals	O'Connell et al. (2013)
Pre-optic area	egr-1	Aggressive and reproduction-related behaviours	Loveland and Fernald (2017)

(see Fig. 4b, section C), known to be involved in reproductive behaviour. In the lateral septum, the expression of *c-fos* and *egr-1* was higher in females seeing their preferred male lose. The lateral septum is implicated in the modulation of anxiety-like behaviour, indicating that females seeing their respective male lose, could have experienced anxiety. In all other brain areas, there were no detectable differences in *egr-1* or *c-fos* between the two groups of females (Desjardins et al. 2010).

Wood et al. (2011) tested if *A. burtoni* could be trained in a spatial task and assessed if successful execution of the task was related to the expression of *c-fos*, *bdnf* and *egr-1* in the pre-optic area (see Fig. 4b, section C), lateral septum, anterior hypothalamus, ventromedial hypothalamus, periaqueductal grey, dorsomedial telencephalon, cerebellum (see Fig. 4b, sections J, K), raphe nucleus and the dorsolateral telencephalon (see Fig. 4b, sections A–C) (some of these nuclei were also included in the SBN, see above). Fish were divided in three groups (learners, non-learners and non-attempting) based on their performance in the task (finding a hole in a clear barrier that separated the tank in two compartments). In the dorsolateral telencephalon of learners, mRNA levels of both *bdnf* and *egr-1* were expressed at significantly higher levels than in non-attempting and non-learner fish, suggesting that the dorsolateral telencephalon may play a key role in spatial cognition. The lower activity of IEGs in the periaqueductal grey suggests lower stress levels in the learners than in non-learners and non-attempting fish. The preoptic area, playing a role in the reward and motivation pathway, also showed an increase in IEG expression, indicating increased motivation in learners over the training period. Overall, higher levels of IEG activity, a decreased stress response, and an increased motivation in learners suggest a heightened ability to learning a spatial task. In the brains of non-attempting fish, expression of *bdnf* and *egr-1* was increased in both the periaqueductal grey and in the dorsomedial telencephalon, indicating an activation within brain areas associated with anxiety and stress. Non-learners also exhibited lower levels of *bdnf* and *egr-1* within the dorsolateral telencephalon than learners. Furthermore, non-learners also exhibited lower levels of *egr-1* in the dorsolateral telencephalon, while featuring "intermediate" expression levels in the periaqueductal grey. In conclusion, learner fish showed high levels of activity within the area associated with learning and memory (dorsolateral telencephalon), no activity in areas associated with fear and stress (dorsomedial telencephalon and periaqueductal grey) and some activity in the preoptic area, indicative of high motivation (Wood et al. 2011).

In the same year, Rajan and colleagues examined whether spatial learning induces *egr-1* expression in the telencephalon (see Fig. 4b, sections A–C) of goldfish. Researchers divided a tank in four different compartments using three vertical transparent acrylic barriers and trained fish to pass through the barriers one by one. When fish had successfully accomplished the task, the third barrier was replaced by a modified one. Analysis showed that fish attempted more often to pass through the first barrier than the others, as the task was novel and the solution unknown, but already familiar when encountering the second and third barrier. *Egr-1* expression levels in the telencephalon were higher in a fish having mastered to cross the first barrier than in a resting control. However, the level of *egr-1* expression decreased again, when fish had learned to pass through barriers two and three. When the modified gate three was introduced, researchers observed an increased number of attempts correlating with an increased level of *egr-1* expression in the telencephalon. In conclusion, the study highlighted an increase in *egr-1* expression in the telencephalon of *C. auratus* while exploring a novel environment and when learning a new task. As already demonstrated in several other studies (Burgess et al. 2001; Vargas et al. 2004, 2006), goldfish can encode both non-geometric and geometric information and encode the goal location using geometrical clues (Bingman and Mench 1990; Salas et al. 1996a, b; Durán et al. 2008). In conclusion, fish needed to encode new geometric information due to the introduction of the modified third gate (Rajan et al. 2011).

An interesting question is whether animals possess self-awareness, such as recognizing themselves in a mirror (for a recent behavioural study see Kohda et al. 2019). In particular, since fish cannot self-recognize, Desjardins and Fernald (2010) asked whether fish could distinguish between fighting a mirror image and fighting a real fish. They used qRT-PCR (Quantitative Reverse Transcription Polymerase Chain Reaction) to measure mRNA expression of *egr-1* and *c-fos* in four brain regions of *A. burtoni*, i.e. the dorsomedial telencephalon, the dorsolateral telencephalon (see Fig. 4b, sections A–C), the preoptic area (see Fig. 4b, section C) and the cerebellum (see Fig. 4b, sections J, K). Fish were divided in three groups: (1) fish subjected to fighting with a conspecific male across a clear barrier (opponent group), (2) fish subjected to fighting with a mirror image (mirror group) or (3) fish without an opponent (control). No differences in aggression levels were found between 'opponent' and 'mirror' males, but 'mirror fights' and 'opponent fights' had different effects on the brain. 'Mirror' males had higher levels of *egr-1* expression in the dorsolateral telencephalon than 'opponent' males or controls, while *c-fos* expression was significantly higher in 'opponent' males, than in 'mirror' or control males. Furthermore, 'mirror' males had much higher *egr-1* and *c-fos* expression levels in the dorsomedial telencephalon (a potential amygdala homologue) than 'opponent' males or controls. This suggests that fish may experience fear when fighting their mirror image. In the cerebellum, there were no differences in *egr-1* or *c-fos* expression among any of the males. Overall, males fighting an opponent through a clear barrier or fighting their mirror image showed similar behaviour and similar gene expression in the pre-optic area and in the cerebellum but different gene expression in the dorsolateral and dorsomedial telencephalon. To explain the increase of *egr-1* activity in the dorsolateral telencephalon in 'mirror' males, two hypotheses were formulated. The first one assumes that in the dorsolateral

telencephalon *egr-1* may operate as a transcription factor for genes involved in stress responses (for example, glucocorticoid) (Bannerman et al. 1995), indicating the encoding of "stress-related spatial information" (Desjardins and Fernald 2010). It was rejected though, as there was a simultaneous increase of *c-fos* expression in the dorsolateral telencephalon in 'opponent' males rather than in 'mirror' males. The second, more likely hypothesis assumes that the mirror image represents "a perfectly size matched opponent", possibly inducing fear in *A. burtoni* males by not reacting in familiar ways (Desjardins and Fernald 2010).

Both inter- and intra-specific cooperative behaviours are common among animals (Dugatkin 1997; Sachs et al. 2004), i.e. two or more individuals may act together to achieve a goal that each individual cannot achieve independently (Taborsky 2007; Brosnan and de Waal 2002). Weitekamp and Hofmann (2017) examined the immunohistochemical expression of *c-fos* in the social decision-making (SDM) network, known to be involved in reward processing and in the integration of salient stimuli across vertebrates (O'Connell and Hofmann 2011, 2012; Weitekamp and Hofmann 2017), in *A. burtoni*, during cooperative territory defence behaviour (Hofmann 2003). This behaviour refers to a territorial male cooperating with another male to defend his territory from an intruder. This confers an advantage, as renegotiating boundaries usually is more expensive than cooperating with a neighbour (Getty 1987). The aim of the study was to determine how neural activation of the SDM network causes variation in cooperation with neighbours and residents and to examine whether the neural activation in specific nodes of SDM network is associated with the specific role individuals play in a cooperative context. *C-fos* expression was analysed in three subregions of the medial part of the dorsal telencephalon (potentially homologous to the mammalian basolateral amygdala), one subregion of the supracommissural nucleus of the ventral pallium (see Fig. 4b, section C) (potentially homologous to the medial amygdala/bed nucleus of the stria terminalis of mammals), in the lateral subdivision of the lateral part of the dorsal telencephalon (potentially homologous to the hippocampus) (see Fig. 4b, sections A–C), in the parvocellular (potentially homologous to the paraventricular nucleus) as well as magnocellular and gigantocellular cell groups (potentially homologous to the supraoptic nucleus (Moore and Lowry 1998; O'Connell and Hofmann 2011) of the preoptic area (see Fig. 4b, section C). Furthermore, the role of dopamine was assessed by co-labeling *c-fos* with tyrosine hydroxylase (TH), a marker of dopaminergic cells (O'Connell et al. 2011) to determine if there was an increase in activity in reward-related regions and if this increase was led by cooperative behaviour. To analyse how cooperative behaviour is correlated with neural activity in SDM networks, the researchers calculated the Engagement Index (EI), a measure of "how likely an individual is to engage in cooperative defence independent of its own size or the size of the intruder" (Weitekamp and Hofmann 2017). Results indicate that in neighbours, EI is associated with aggressive displays towards the intruder and, with the increase of EI, *c-fos* expression decreased in one subregion of the medial part of the dorsal telencephalon and in a magnocellular cell group. EI was also correlated with *c-fos* induction in dopaminergic neurons of both magnocellular and parvocellular cells groups. The magnocellular cell group is considered a potentially homologous structure to the supraotic nucleus which, in mammals, produces oxytocin (OT) involved in behaviour and social cognition (Ross and Young 2009). In the same way, the magnocellular cell group of *A. burtoni* contains isotocin (OT homolog) neurons (Huffman et al. 2012) that can mediate cooperative behaviour and can cause the increase of neural activity measured (Weitekamp and Hofmann 2017). In residents, EI was associated with an aggressive display towards the intruder; there was no up-regulation of any IEGs in any brain region assessed. Furthermore, having demonstrated that the resident modulates his aggression towards the intruder based on the behaviour of the neighbour, researchers also demonstrated that there was a negative association between the *c-fos* induction in the lateral part of the dorsal telencephalon of the resident and the neighbour's aggression directed to the intruder. The lateral part of the dorsal telencephalon is assumed to be involved in context-dependent decision-making and social cognition (Rubin et al. 2014). Since there is a negative correlation between the aggression from the neighbour directed towards the intruder and the neural activity in the lateral part of the dorsal telencephalon of the resident, and since the resident modulates its behaviour based on its neighbour's decision, these results suggest that the lateral part of the dorsal telencephalon plays a role in this modulation of behaviour. In conclusion, partaking of the resident male in territorial defence behaviour is based on the behaviour and size of its neighbour. Additionally, neighbour behaviour is associated with neural activity in the lateral part of the dorsal telencephalon in the resident (Weitekamp and Hofmann 2017). The neighbour also participates in territorial defence based on the perceived threat of the intruder, with a correlated activity in the preoptic area as well as in preoptic dopaminergic neurons. These results suggest that, during cooperative territory defence, neighbour and resident evaluate the presence of an intruder depending on the behavioural role they play, and this role would be associated with distinct neural activity in key nodes of the SDM network. Furthermore, the reward system may mediate the cooperation in this context (Weitekamp and Hofmann 2017).

The ability of an organism to assess numerical information and compare quantities represents an advantage for many behaviours, such as foraging, reproduction and socializing (Hager and Helfman 1991; Botham and Krause

2005; Beran et al. 2013). Although much information about numerical abilities has been collected in fish (e.g. Agrillo and Bisazza 2018), information about the neural bases underling these processes was limited to non-human primates and corvids (Nieder 2013; Ditz and Nieder 2016). Very recently though, brain regions involved in quantity discrimination processes in zebrafish were identified (Messina et al. 2020). IEGs expressions of *c-fos* and *egr-1* were analysed using RT-qPCR in different areas, i.e. the retina, the optic tectum (see Fig. 4b, sections G–L), the thalamus (see Fig. 4b, section D), the telencephalon (see Fig. 4b, sections A–C), the cerebellum (see Fig. 4b, sections J, K) and the medulla oblongata. In behavioural tests (please refer to original paper for details on testing), it was found that zebrafish preferentially chose a novel stimulus when the latter changed in numerousness but not in shape or size (Messina et al. 2020), a finding that agrees with results found for macaques (Cantlon and Brannon 2007). However, on a molecular level, IEG expression was either influenced by changes in stimulus surface area, i.e. stimulus size (retina and optic tectum) or numerousness (thalamus and telencephalon). *C-fos* expression in the zebrafish retina was positively correlated with stimulus surface area, while *egr-1* expression in the retina was not affected by an increase in surface area but increased with a decrease in surface area instead. In the optic tectum (see Fig. 4b, sections G–L), *c-fos* expression decreased with an increase, and increased with a decrease in surface area. *Egr-1* expression in the tectum increased with a decrease in stimulus surface area while an increase had no effect. In the thalamus, *c-fos* expression decreased in fish that were habituated to 3 dots and tested with 9 dots, but increased in fish habituated to 9 dots and tested with 3 dots. The results for *egr-1* expression were similar but not statistically significant. In the telencephalon, *egr-1* and *c-fos* expressions decreased with an increase in numerosity and vice versa. In the cerebellum, only a change in surface area affected *egr-1* expression, while no changes in *c-fos* expression were observed regardless of testing scenario. In the medulla oblongata, there was no clear pattern of IEG expression (Messina et al. 2020).

The impulse of organisms to socialize and approach individuals of their own species, the so-called 'social preference behaviour' has been found in a variety of species, including humans and zebrafish (Sloan Wilson et al. 1994). However, as in other social species, a small part of a normally raised zebrafish population will have fewer social preferences than most other individuals or may even be aversive to social cues, i.e. exhibit a type of 'loner' behaviour (Sloan Wilson et al. 1994; Dreosti et al. 2015). Recently, it was tested how brain activity and behaviour are affected by social isolation (Tunbak et al. 2020) and compared between such 'loner' fish (anti-social fish found in the normal population) and fish that were simply deprived of social contact, termed 'lonely' fish. Fish ('loners' and 'lonely' fish and controls) were subdivided in additional experimental and control groups [please refer to original paper by Tunbak et al. (2020)]. Whole-brain two-photon imaging of *c-fos* expression was performed, focusing on brain structures implicated in the SBN (Social Behaviour Network), (O'Connell and Hofmann 2011). The average activity map for each rearing/sociality group was then compared to the average activity map of a sibling fish, raised under similar conditions and tested for 30 min without social cues. There were two areas where significant differences were found, i.e. the caudal hypothalamus and the preoptic area (see Fig. 4b, section C), highlighting their roles in social preference behaviour. Furthermore, *c-fos* brain maps of control and isolated fish not exposed to social clues during the experiment were compared. There was an increase in activity in the optic tectum (see Fig. 4b, sections G–L) as well as in the posterior tuberal nucleus. These structures are known to be involved in visual processing and in stress responses, respectively (McDowell et al. 2004; Ziv et al. 2013; Wee et al. 2019). Results found suggest that isolation increases visual sensitivity (activity changes in the optic tectum), as well as increased activity in the posterior tuberal nucleus (Tunbak et al. 2020). Overall, there were significant differences regarding neural activity in brain areas linked to social behaviour, social cue processing, and anxiety or stress between the groups. Short isolation increases the sensitivity to social stimuli, but the increased sensitivity to social stimuli leads to an increase of anxiety and stress levels if the isolation is prolonged. Social preference in 'lonely' fish could be restored by an anxiolytic drug that acts on the monoaminergic system, i.e. by reducing serotonin levels.

The expression of IEGs can be induced not only by cognitive processes, but also by a variety of other factors including pharmacological stimulation. For example, the exposure of zebrafish larvae (*Danio rerio*) to pentylenetetrazole (PTZ, a common convulsant agent) induced the expression of *c-fos* in the optic tectum (see Fig. 4b, sections D–I) and cerebellum (see Fig. 4b, sections J, K) as well as behavioural changes ending up in clonus-like convulsions (Baraban et al. 2005). Similarly, the injection of kainic acid (a glutamate receptor agonist) in *A. burtoni* altered *egr-1* expression in different regions of the diencephalon (including the anterior part of parvocellular preoptic nucleus, magnocellular preoptic nuclei, and the anterior nucleus of the thalamus) in the olfactory bulbs (see Fig. 4b, section A), the ventral nucleus of the ventral telencephalon, the central and lateral zone of the dorsal telencephalon (see Fig. 4b, sections A–C), and in the optic tectum (see Fig. 4b, sections D–I) (Burmeister et al. 2005). An interesting study combining pharmacological stimulation, IEGs expression and motivational behaviour showed that the administration of D-amphetamine increased the expression of *c-fos* in the lateral zone and the medial zone of the adult zebrafish telencephalon and that the lateral

zone of the telencephalon is involved during drug-seeking behaviour (von Trotha et al. 2014). Other studies combining pharmacological stimulation and IEGs expression were conducted on rainbow trout (*Oncorhynchus mykiss*) (Matsuoka et al. 1998) and zebrafish (*Danio rerio*) (Ruhl et al. 2017).

Similar to lesion studies, Immediate Early Gene Analyses have their own shortcomings. First, one has to identify appropriate genes for the species and task in question and establish exact protocols, which can vary considerably between species. Depending on baseline gene expression levels and intra-specific variation, it may be very difficult to quantify training (learning) effects and clearly separate these effects from other, potentially confounding factors not closely related to the treatment/stimulus of interest, such as changes in motivation or stress between experimental groups and controls. A solution to this problem may be found in in vivo imaging or optogenetics studies. Another field for future research may be provided using knock-out mutants. In mice, it has already been shown that specific knock-out mutants may lack memory for socially relevant odors, while retaining spatial memory and the ability to smell in general (Wersinger et al. 2004).

In vivo imaging techniques allow behaviour to be linked to neural substrates activated in live animals. In the last few years, zebrafish and its larvae have proven to be a suitable model system to perform such in vivo imaging studies, especially for analysing whole-brain activity (Portugues et al. 2014; Preuss et al. 2014). For instance, Preuss et al. (2014) used calcium imaging to analyse how the visual system of zebrafish detects and categorizes moving objects. To select appropriate responses, such as approach or escape behaviour, knowing the size of an object is critical. Results show, that the tectum categorizes visual targets on the basis of retinally computed size information. Calcium imaging was also used by Temizer et al. (2015) to analyse how the visual system extracts information about a looming-stimulus feature, triggering escape behaviour in zebrafish. There are three areas, including the optic tectum (see Fig. 4b, sections G–L), that respond selectively to features of looming stimuli. Furthermore, through targeted laser ablations, researches also demonstrated that, to trigger the looming-escape behaviour, the optic tectum plays a critic role (Temizer et al. 2015). Using whole-brain functional imaging, it was analysed and identified how the zebrafish larvae brain collects and implements sensory information over different time scales to select appropriate behaviours (Bahl and Engert 2020; Dragomir et al. 2020). Through random dot motion stimuli, an 'optomotor response' was invoked, an innate behaviour to follow the direction of the perceived motion. During the decision-making process, neuronal clusters in the midbrain and hindbrain were activated. In the midbrain, including the pretectum, and in the medial parts of the reticular formation in the anterior hindbrain, there was a concentration of neurons encoding momentary sensory information, whereas in the lateral parts of the reticular formation, the dorsal raphe nucleus and the caudal interpeduncular nucleus, the dorsal part of the pretectum, the dorsal thalamus (see Fig. 4b, section D), the torus longitudinalis (see Fig. 4b, sections E, F) and the habenula (see Fig. 4b, section D) were neurons that integrated sensory evidence (Dragomir et al. 2020; Bahl and Engert 2020).

Last but not least, another technique worth mentioning is optogenetics, which refers to the ability to control and observe cellular activity through the use of light-sensitive proteins (Rost et al. 2017). It was first used to describe genetically targeted photoreceptor expression in neurons for their selective activation or inhibition with light (Deisseroth et al. 2006) and later extended to include other photosensitive proteins (Dugué et al. 2012; Miesenböck 2009). It is not difficult to understand how this technique, over the last few decades, has revolutionized the study of neuronal activity (Scanziani and Häusser 2009). It allows to perform an experiment within a specific time window and to control neuronal activity with a very high spatial–temporal resolution (Rost et al. 2017). One can manipulate neurons to verify how the manipulation alters brain circuits and changes behaviour accordingly (Rost et al. 2017). For example, the optogenetic activation of a class of interneurons in zebrafish spinal cord is sufficient to produce a coordinated swimming pattern without sensory stimuli or input from higher brain structures, thus representing the excitatory unit of the locomotor circuitry in the fish (Ljunggren et al. 2014). Optogenetic activation of the left-dorsal habenula (see Fig. 4b, section D) in eye-removed zebrafish larvae triggers innate light-preference behaviour in zebrafish larvae (Zhang et al. 2017)., This behaviour is critical for survival and highly conserved (Crozier and Pincus 1927; Gong et al. 2010; Steenbergen et al. 2011; Wang et al. 2014; Ward et al. 2008; Yamanaka et al. 2013; Zhang et al. 2017).

Conclusion

In this review, the currently available information about the neural substrates involved in cognitive information processing in fish is summarized, giving a roadmap for future research. IEG and lesion studies have proven to be powerful and potentially complementary methods, allowing identification of brain areas underlying several cognitive aspects, specifically if used in combination. Optogenetics and in vivo studies may further complement these techniques. Scientific research in this field is still in the early stages and many interesting questions remain unanswered. While a lot is known about the fish brain in general, specific functions of many brain regions are still unknown or have only partially

been exposed, specifically in regards to cognitive abilities. Lastly, both the neuroanatomy and the behaviour involved in cognitive processes have only been studied in a few representatives of the more than 33,000 extant fish species. In the future, hopefully more scientific endeavours will aim to address cognition in fish using a more doverse range of species and a more holistic approach, i.e. by not only asking whether or not an animal can perform a cognitive task, but also by trying to discover what neural substrates are involved in the processing of such a task using several if not all of the methods currently available.

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ORIGINAL ARTICLE

Brain areas activated during visual learning in the cichlid fish *Pseudotropheus zebra*

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Abstract

The neural correlates of most cognitive functions in fish are unknown. This project aimed to identify brain regions involved in visual learning in the cichlid fish *Pseudotropheus zebra*. The expression of the protein pS6 was measured in 19 brain areas and compared between groups of individuals subjected to four different behavioral contexts (control, avoidance, trained, and novelty groups). Control group individuals were sacrificed with minimal interactions. Fish in the avoidance group were chased with a net for an hour, after which they were sacrificed. Individuals in the trained group received daily training sessions to associate a visual object with a food reward. They were sacrificed the day they reached learning criterion. Fish in the novelty group were habituated to one set of visual stimuli, then faced a change in stimulus type (novelty stimulus) before they were sacrificed. Fish in the three treatment groups showed the largest activation of pS6 in the inferior lobes and the tectum opticum compared to the control group. The avoidance group showed additional activation in the preoptic area, several telencephalic regions, the torus semicircularis, and the reticular formation. The trained group that received a food reward, showed additional activation of the torus lateralis, a tertiary gustatory center. The only area that showed strong activation in all three treatment groups was the nucleus diffusus situated within the inferior lobe. The inferior lobe receives prominent visual input from the tectum via the nucleus glomerulosus but so far, nothing is known about the functional details of this pathway. Our study showed for the first time that the inferior lobes play an important role in visual learning and object recognition.

Keywords Cichlid · pS6 · Brain · Visual learning · Behavior · Neural activity

Abbreviations

A	Anterior thalamic nucleus
ATN	Anterior tuberal nucleus
CC	Crista cerebellaris
CM	Corpus mammilare
Cor	Corpus cerebelli
CP	Central posterior thalamic nucleus
Dc	Central division of the dorsal telencephalon
Dcd	Dorsal subdivision of the central division of the dorsal telencephalon
Dcv	Ventral subdivision of the central division of the dorsal telencephalon
Dd	Dorsal division of the dorsal telencephalon
Dl	Lateral division of the dorsal telencephalon
Dld	Dorsal subdivision of the lateral division of the dorsal telencephalon
Dlv	Ventral subdivision of the lateral division of the dorsal telencephalon
Dm	Medial division of the dorsal telencephalon
Dmd	Dorsal subdivision of the medial division of the dorsal telencephalon
Dmv	Ventral subdivision of the medial division of the dorsal telencephalon
DON	Descending octaval nucleus
Dp	Posterior division of the dorsal telencephalon
DP	Dorsal posterior thalamic nucleus
E	Entopeduncular nucleus
EG	Eminentia granularis
fr	Fasciculus retroflexus
G	Granular layer of the cerebellum
Gl	Granular layer of the olfactory bulb
Hab	Habenula
IL	Inferior lobe
ILc	Inferior lobe, central nucleus
ILrec	Inferior lobe, nucleus of the lateral recess

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IP Nucleus interpeduncularis
ll Lateral lemniscus
M Molecular layer of the cerebellum
Ma Mauthner cell
Mg Magnocellular octaval nucleus
ME Median eminence of the hypothalamus
mlf Medial longitudinal fascicle
mIII Oculomotor nucleus
MON Medial octavolateral nucleus
mV Trigeminal motor nucleus
mX Vagal motor nucleus
ND Nucleus diffuses
NDl Nucleus diffusus pars lateralis
NDm Nucleus diffusus pars medialis
NG Nucleus glomerulosus
NGT Tertiary gustatory nucleus
NI Nucleus isthmi
NLT Nucleus lateralis tuberis
nlVal Nucleus lateralis valvulae
NSC Suprachiasmatic nucleus
OB Olfactory bulb/granular layer
OT Optic tract
PGc Commissural preglomerular nucleus
PGm Medial preglomerular nucleus
POA Preoptic area
PTc Pretectal area, centralis
PTco Pretectal area, corticalis
PTpo Pretectal area, nucleus of the posterior commissure
PTsm Pretectal area, superficialis magnocellularis
PTsp Pretectal area, superficialis parvocellularis
pV Principal trigeminal nucleus
Ras Raphe superior
RFi Inferior reticular formation
RFm Medial reticular formation
RFs Superior reticular formation
SPV Stratum periventriculare of the tectum
sVII Sensory root of the facial nerve
TL Torus longitudinalis
TLat Torus lateralis
TO Tectum opticum
TOp Periventricular layer of the tectum opticum
TOs Superficial layer of the tectum opticum
TS Torus semicircularis
Val Valvula cerebelli
Vd Dorsal nucleus of the ventral division of the telencephalon
VIII Octaval nerve
Vl Lateral nucleus of the ventral division of the telencephalon
VL Vagal lobe
VM Ventromedial thalamic nucleus
Vv Ventral nucleus of the ventral division of the telencephalon
X Vagal nerve

Introduction

Fish have been the subject of a large number of visual discrimination experiments, which have shown that fish possess many of the same cognitive abilities as birds and mammals (for reviews see Brown et al. 2011; Schluessel 2015). Such experiments range from object recognition and categorization (e.g. Neri 2012; Schluessel and Bleckmann 2012), recognition of symmetrical symbols (Schluessel et al. 2014), size, shapes, and form constancy (Douglas et al. 1988; Schuster et al. 2004; Frech et al. 2012; Schluessel et al. 2014; DeLong et al. 2018), to numerical competency (e.g. Agrillo et al. 2017; Mehlis et al. 2015; Schluessel et al. 2022) as well as optical illusions (e.g., Wyzisk and Neumeyer 2007; Fuss et al. 2014; Agrillo et al. 2020). Unfortunately, only a few studies so far have described where and how cognitive information is processed in the fish brain. With the exception of the lateral and medial division of the dorsal telencephalon, which are supposed to be homologs of the hippocampus and the pallial amygdala of land vertebrates, respectively, the neural correlates for most cognitive functions in fish are still unknown (for reviews see Rodríguez et al. 2006; Broglio et al. 2011; Ebbesson and Braithwaite 2012; Kotrschal et al. 2014; Calvo and Schluessel 2021). This includes functions and cognitive involvement of other areas within the telencephalon, as well as the diencephalon and regions outside the forebrain. The diencephalon includes the habenula, the thalamus, the hypothalamus, and the posterior tubercular region, which is highly derived in many teleost groups (Ahrens and Wullimann 2002). In addition, teleosts have a prominent visual pathway extending from the tectum over the nucleus corticalis and the nucleus glomerulosus to the inferior lobes (Wullimann and Meyer 1990; Butler et al. 1991; Shimizu et al. 1999; Ahrens and Wullimann 2002; Yang et al. 2007). Other parts of the inferior lobes and the corpus mammilaris project back to the tectum (Hagio et al. 2018; Sawai et al. 2000). In addition, the corpus mammilare and the commissural preglomerular nucleus have extensive projections to the dorsal telencephalon (Murakami et al. 1983; Sawai et al. 2000). This shows that a large part of visual information processing and integration with other senses is taking place in the posterior tubercle/inferior lobe region in teleosts. However, these regions have not been included in studies that try to localize cognitive functions in fishes.

Markers for immediate early gene (IEG) expression have been used during the last two decades to identify brain areas involved in different cognitive functions in fishes. C-fos and

egr-1 are generally the two main proteins assessed, which can be detected by immunocytochemistry inside the nucleus of activated neurons. However, the availability of antibodies for fishes is problematic and many studies used in situ hybridization and PCR on micro dissected brain parts to localize the expression of genes correlated with the activation of neurons. More recently, the phosphorylated ribosome marker pS6 has become a popular alternative to visualize neural activation in fish (e.g. Benítez-Santana et al. 2017; Travanca dos Santos 2017; Butler et al. 2018; Fischer et al. 2018; York et al. 2018; Montesano et al. 2019; Tripp et al. 2019; York et al. 2019; Baran and Streelman 2020; Butler et al. 2020; Maruska et al. 2020; Tripp et al. 2020; Chen et al. 2021; Dunlap et al. 2021; Nunes et al. 2021; Schuppe et al. 2021; Suzuki et al. 2021; Scaia et al. 2022). S6 protein is a component of the 40S ribosomal subunit. Its inducible phosphorylation, which occurs in response to a large variety of stimuli, was the first post-translational modification described in ribosomal proteins (Gressner and Wool 1974; Meyuhas 2008) and has attracted attention since its discovery in 1974 in rat liver regeneration (Gressner and Wool 1974). The phosphorylation of S6 is supposed to be a more sensitive method than IEG markers since it does not require gene activation and antibodies are readily available due to the highly conserved S6 protein sequence.

To investigate brain areas involved in different visual learning tasks, we used pS6 antibodies in the cichlid *Pseudotropheus zebra*. To facilitate the measurement of as many brain areas as possible, we developed an automated image analysis procedure and analyzed 19 brain areas in 40 individuals from four groups yielding more than 3000 individual areas extracted from the stained image stacks. The activation of these areas ranging from sensory to motor centers was then compared with three different learning situations that each involved different sensory, cognitive, and locomotor components.

Materials and methods

Animals

Animals used in this study ($n = 40$) belong to the species *Pseudotropheus zebra,* also known as *Zebra mbuna* (Konings and Stauffer 1997), *Metriaclima zebra* or *Maylandia zebra* (Boulenger 1899). This species belongs to the family Cichlidae (www.fishbase.org). Cichlids are teleosts and represent one of the most varied extant vertebrate radiations (Seehausen 2006), showing a high degree of variability in terms of trophic morphology, (including specialist algal scrapers, planktivorous, insectivores, piscivores, paedophages, snail crushers, and fin biters (Albertson et al. 1999), color pattern (Konings 1995; Albertson et al. 1999), as well as polyandrous mating systems (Kellogg et al. 1995; Albertson et al. 1999). For this reason, African cichlids offer an unequaled system of animals to study cognition, molecular evolution, speciation, and ecological plasticity (van Staaden et al. 1995; Salzburger et al. 2005).

Experimental procedures—behavioral experiments

Each fish was kept individually, without any social contact, in a tank that served both as a holding as well as an experimental tank. All fish were kept in isolation for at least one week prior to being sacrificed (control group) or to being used in behavioral experiments (avoidance, trained and novelty groups). The isolation was necessary to avoid the activation of brain areas involved in social interactions. Walls and floor of each tank consisted of light grey PVC, while the front was made of white frosted plexiglass. A grey partition was inserted into the middle of the tank separating a back from a front compartment; the partition was fitted with a passage for the fish, that could be closed with the help of a transparent guillotine door during the training of the fish. In the back compartment of the tank, a fish shelter, a heating element, a filter system as well as a pump were placed. The water temperature was kept at about 25 °C.

Figure 1 shows a schematic representation of the four different experimental groups. For the trained and the novelty stimulus group the stimuli presented during the behavioral experiments are shown on the right-hand side of the figure.

Control group

Fish in the control group ($N = 10$) did not receive any treatment. They were fed once daily, except on the day of sacrifice to prevent any activation of sensory brain areas involved in olfactory and gustatory pathways. After being kept in isolation without any social contact for seven days, fish were taken out of the tank and sacrificed.

Avoidance group

Fish in the avoidance group ($N = 10$), were fed once daily, except on the day of the behavioral procedure. After being kept for seven days in isolation and immediately prior to the behavioral procedure, they were moved to a new compartment. Fish were then chased with a net for one hour before being sacrificed 90 min after the chase ended.

Trained group

Fish in the trained group ($N = 10$) were kept in isolation for one week before the training started. On the inside of each plexiglass front wall of the tank, two food holders were installed, consisting of two small pieces of plastic pipe

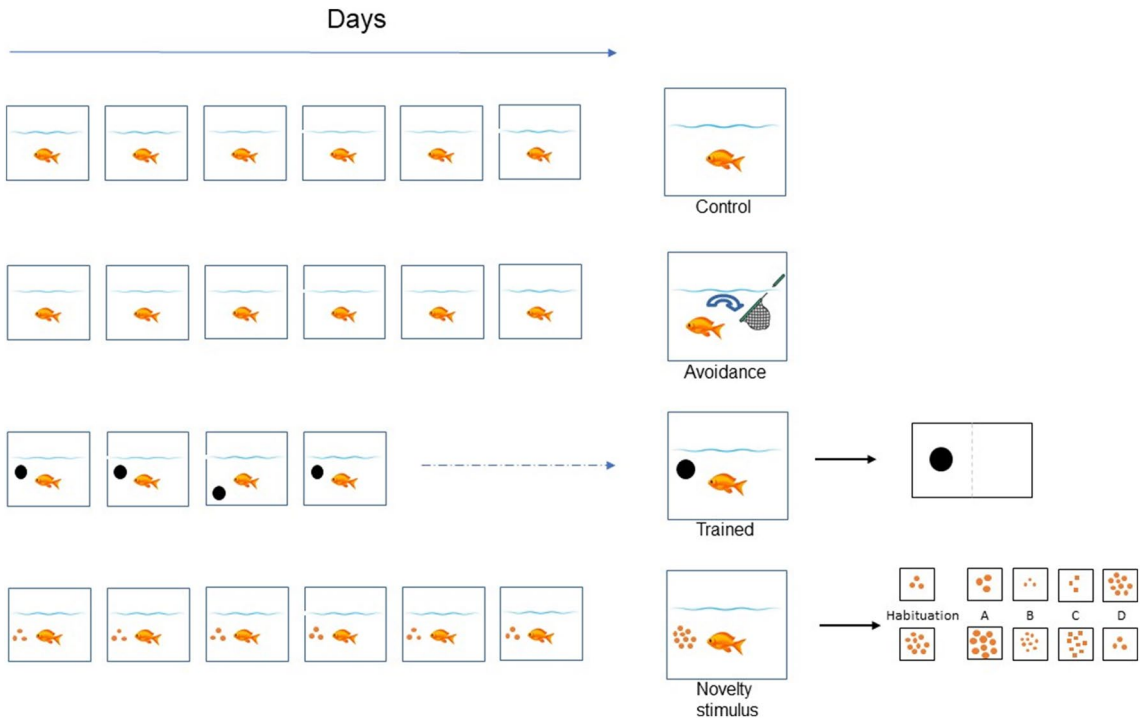


Fig. 1 Schematic representation of the four different experimental groups, highlighting the different learning conditions. For trained and novelty stimulus groups, relevant stimuli are presented on the right

which were attached to the wall with suckers; in each pipe, a hose filled with food was inserted. On the other end, the hose was connected to a syringe through which the food delivery was controlled remotely. With the help of a plastic divider, the front compartment was divided into a right and left compartment. Parallel and 3 cm away from the frontal wall a line indicated the start of the decision-making area into which the fish had to swim in order for its choice to be valid. Please refer to Schluessel et al. (2018) for a picture of the experimental setup.

Before the actual training commenced, the experimentally naive animals were given time to get used to the experimental setup and to feeding from the food holders in the decision area. The guillotine door was open so that the fish could freely swim throughout both compartments of the aquarium. After the fish had learned to swim through the open guillotine door and collect food from the dispenser at the front of the tank while the projector (no stimulus presentation) was on, training started. Experiments were conducted daily, twice a day always at the same time, during daylight hours. At the beginning of the experiment, the two training symbols (a black dot over a white background (positive stimulus) and a white background without a symbol (alternative, negative stimulus), Fig. 1) were projected onto the plexiglass front with the help of an LCD projector (ES 521 Optoma, DLP®, China) which was located in front of the tank and connected to a notebook. The stimuli were projected on the right and left side, just below the respective food holders and at the same height as the guillotine door to allow the fish to see them, even from the posterior area of the tank. For each of the ten trials within a session, the position of the positive stimulus was randomly determined prior to the experiments (creating a rotational scheme) during which the positive stimulus was shown five times on each side of the tank in total; however, it was never shown more than twice in a row on the same side. In total, four rotational schemes were used consecutively over every four sessions. Before each trial, the door was closed, restricting the fish to the back compartment. With the help of a webcam, positioned above the experimental tank, it was possible to observe the behavior of the animal in the tank, without affecting its decision. The guillotine door was then remotely opened and the fish allowed to enter the front compartment. Once the door was opened, the fish had to make a choice within 2 min, otherwise the trial was terminated. To prevent olfactory cues, both feeders were baited in each trial and always simultaneously re-baited to prevent unintentional cuing by the experimenter.

Trial time was taken from the time the individual passed through the guillotine door with the tip of its mouth, until crossing the decision line in front of the projector (indicating that the choice was made, see Schluessel et al. 2018 for details).

A correct choice was rewarded with food. Immediately following an incorrect choice or after consuming the food, the fish was ushered back into the posterior part of the tank.

The learning criterion was established to be seven or more correct decisions out of ten trials in three consecutive sessions. During the experiment, the pumps and heating rods in the back compartment of the respective tanks were turned off. Immediately after the learning criterion was achieved, fish went through as many trials as possible for the duration of 1 h, called a "supersession". The animals were then sacrificed 90 min after the supersession was finished.

Novelty stimulus group

Fish in the novelty stimulus group ($N = 10$) were kept in isolation for one week before starting the experiment. For this group, a modification of the habituation-dishabituation experiment elaborated by Messina and colleagues was used (Messina et al. 2022). The setup was the same as for the trained fish. The stimuli used for the habituation and the novel stimuli are shown in Fig. 1. During habituation, one set of either three or nine dots on a white background was projected onto the plexiglass with the LCD projector. After a delay of 30 s, food was released near the stimulus. The stimulus was turned off two min after the food delivery. Five min later, a new trial was started. One session consisted of four trials and three sessions were done each day. On the last day, the fish received only one session. After that, fish were left in their respective tanks for 5 h before one of the novelty stimuli was shown for 30 s (dishabituation phase). Fish were randomly assigned to the five novelty stimuli. The novel stimulus differed from the habituated one in size, shape, or number of dots (see Fig. 1). No food was provided during these test trials. The animals were sacrificed 90 min after the dishabituation phase was terminated.

Immunohistochemistry

All fish were anesthetized with Tricaine methanesulfonate (MS-222). The brain was removed and fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, then cryoprotected overnight in 30% sucrose at 4 °C. The following day, the brain was embedded in O.C.T. compound (freezing medium, Leica Biosystem Richmond) and frozen at − 20 °C. Thirty-five µm thick sections were cut at − 20 °C with a cryostat (Leica CM1520) and mounted on gelatin-coated slides in three series, then stored until immunohistochemistry (IHC).

To minimize differences in stain intensity, IHC was performed twice within three days, each time including 5 brains for each group (i.e. 5 control, 5 avoidance, 5 trained and 5 novelty).

During the IHC, sections were rehydrated by washes in PBS drops; then a post-fixation procedure was performed with 4% PFA drops for 10 min, followed by several washes in PBS. The sections were incubated for 30 min in distilled water (H_2O_d) containing 1.5% H_2O_2 to deactivate endogenous peroxidase, followed by several washes in PBS. Slides were then blocked in 10% normal goat serum (NGS) for 1 h. The sections were transferred to a primary pS6-antibody solution (5% NGS / 1X PBS—0.3% Triton X-100, rabbit anti-pS6 (Ser235/236) antibody, Cell Signalling 2211S: 1:1000) overnight at 4 °C, before being washed several times in PBS.

The following day, the second antibody reaction (VECTASTAIN biotinylated anti-rabbit IgG secondary antibody, Vector Labs., USA:1:500) was performed in 5% NGS/1X PBS—0.3% Triton X-100, followed by repeated washes in PBS. Then, signal amplification was initiated using the ABC method (1:1500, 1X PBS—0.3% Triton X-100, VECTASTAIN ABC-Peroxidasekit (PK6100 elite), Vector Labs., USA) for 1 h at RT. Following several rinses in PBS, the antibody-Avidin–Biotin complex was visualized using the chromogen-solution (one 3,3′-Diaminobenzidine-Tetrahydrochloride (DAB) buffer tablet (Merck KGaA, Germany) dissolved in 10 ml H_2O, 500 µL 1% ammonium nickel sulfate, 12 µL 30% H_2O_2) for ~ 15 min, resulting in a deep greyish reaction product confined to the cell bodies of activated neurons. The reaction was stopped by several washes in PBS. Subsequently, sections were dehydrated in ascending alcohols before coverslipping from xylene with Eukitt (Carl Roth, Germany).

For the current study, the specificity of pS6 antibody was checked by replacing either the primary or secondary antibodies with PBS, showing no reaction product. No other test of the specificity of the antibody was performed.

The same pS6 antibody from Cell Signaling has been used successfully in several studies on fish (Beckers et al. 2019, 2021; Montesano et al. 2019; Tripp et al. 2019, 2020; Chen et al. 2021; Dunlap et al. 2021; Nunes et al. 2021; Schuppe et al. 2021; Scaia et al. 2022), including cichlids (Butler et al. 2018, 2019, 2020; Maruska et al. 2020). In cichlid, the antibody has been validated in *Astatotilapia burtoni* (Butler et al. 2020) by western blot which produced a single band at 32 kDa. The same result has been obtained in midshipman (*Porichthys notatus,* Tripp et al. 2019; Schuppe et al. 2021). Furthermore, the antibody detects endogenous levels of ribosomal protein S6 only when phosphorylated at serine 235 and 236, which are among the phosphorylation sites on S6 that are evolutionarily conserved (Meyuhas 2008). In sections, the antibody binds to ribosomes in the endoplasmatic reticulum (Nissl substance of neurons). The nucleus and the dendrites and axons are not stained. This staining pattern was also observed in our material.

Data analysis

To measure the activation of pS6, slides were scanned with a custom build scanning stage attached to a Zeiss microscope with a resolution of 1.6 µm. Individual sections were extracted from the scanned slides and an image stack was created for each brain. In these image stacks, the boundaries of different brain areas were marked. Figure 2 shows the location of the different brain areas analyzed, listed in Table 1. Even areas without pS6 stained cells could be identified by a slight unspecific background staining due to the ABC kit. Details about how the areas were identified and other image analysis procedures can be found in the supplementary materials.

The segmentation of the areas was the only step with user intervention and was performed blind, i.e. the user selected an area to be marked and the computer presented an image stack randomly from the four groups without showing any labels that could identify the group. The user then had to identify the area of interest in the image stack and define an area for the subsequent analysis. The user was also instructed to exclude possible artefacts due to the cutting and staining procedures. For a certain brain part, several areas were segmented from both sides of the brain or from different sections. This resulted in more than 3000 individual areas defined for the 19 brain regions in the 40 animals belonging to the four groups. An automated image analysis workflow was used to measure the segmented areas. The details are described in the Supplementary methods. The results of these measurements are shown in Fig. 4, 'Group means'. Each value is the ratio of the area stained by the pS6 antibody divided by the total area segmented for each brain part, averaged for each group.

Next, a *U*-test (alglib software package) was performed for each brain part comparing the learning groups with the control group to check for significant differences (Fig. 4, *U*-Test Group/Control). Lastly, the activation in each brain area was calculated by dividing the pS6 stain level into the three learning groups by the staining level in the control group and log-transformed (Fig. 4 Relative log(Group/Control)).

Results

Behavioral situations require input from different sets of sensory, cognitive, and motor components. To correlate the activation of different brain parts with the four experimental groups, the behavioral components that characterized the last hour before the fish were sacrificed were analyzed. Table 2 lists the four experimental groups and the components that may play a role for the brain areas activation pattern.

In the control group, no specific or intentional behavior was elicited before the fish were taken out of the tank and sacrificed (Table 2, 'Control'). Individuals in the avoidance group were transferred to a new environment and chased with a net. These two modifications introduced different sets of behavioral responses (Table 2, 'Avoidance'). First of all, the presence of a net chasing the fish created a stressful situation; additionally, there were visual/auditory/hydrodynamic stimuli due to the movement of the net. Furthermore, new olfactory, as well as new spatial cues were introduced due to the presence of the new environment. Lastly, the forced movement caused by the chasing treatment induced a strong motor component.

In the trained group, the primary stimulus was a visual target, but auditory and hydrodynamic inputs cannot be completely excluded due to handling procedures (Table 2, 'Trained'). Since the fish were rewarded with food, gustatory and olfactory stimulation was also present. The training itself involved locomotion and possibly a small spatial component because the fish had to pass the guillotine door and to swim to the target to get a reward.

The novelty group was more comparable to the control group than the avoidance or the trained groups. No sensory stimulation was introduced other than the visual image. Fish were not actively trained and did not receive food before being sacrificed hence avoiding the presence of gustatory-olfactory stimulation. Furthermore, the fish did not enter a new environment, thus limiting the introduction of a spatial component. The only new stimulus introduced on the last day was the visual stimulus (Table 2, 'Novelty').

After the analysis of the potentially induced behavioral components, pS6 staining was analyzed in 19 brain areas (see Table 1). Figure 3 shows examples of the staining in some selected areas in the four groups. For each group and brain region, the total area of stained cells was measured and divided by the total area selected. Then, we determined the activation in each brain area for each experimental group relative to the control group and log-transformed the data (Fig. 4). The stars indicate differences that are significant ($p < 0.05$) according to a *U*-test.

As anticipated, the avoidance group showed activation in many brain areas (see Figs. 3 and 4). The visual areas (TO and NDl) as well as the TS (lateral line/auditory centers) were activated. The reticular formation was also activated in this group. Several telencephalic areas were also positively stained, especially the olfactory-related OB and Dp. The avoidance group was also the only one that showed activation in the POA. In the other groups, fewer brain parts were activated. The only area that is activated in all learning groups is the nucleus diffusus of the inferior lobe.

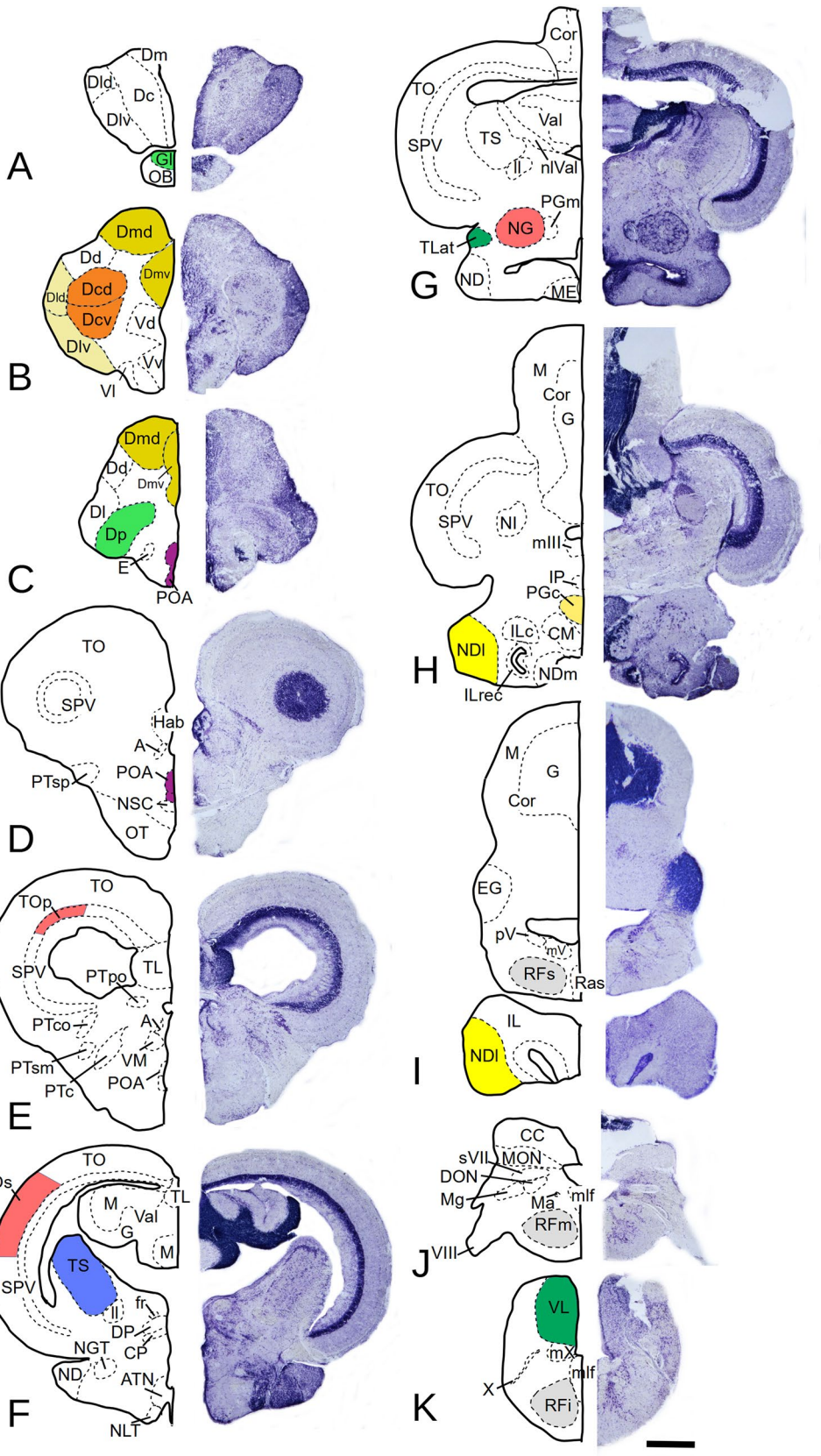


Fig. 2 Cryostat sections of the *P. zebra* brain. The right side shows the micro photos of the original sections after Nissl staining, and the left side shows a schematic drawing of identifiable areas and nuclei (see list of abbreviations). Areas analyzed are highlighted in different colors. Scale bar: 500 µm for each section

Table 1 List of brain areas analyzed

Telencephalon
OB olfactory bulb/granular layer
Dmd dorsal subdivision of the medial division of the dorsal telencephalon
Dmv ventral subdivision of the medial division of the dorsal telencephalon
Dldm dorsal subdivision of the lateral division of the dorsal telencephalon, pars magnocellularis
Dldp dorsal subdivision of the lateral division of the dorsal telencephalon, pars parvocellularis
Dlv ventral subdivision of the lateral division of the dorsal telencephalon
Dcd dorsal subdivision of the central division of the dorsal telencephalon
Dcv ventral subdivision of the central division of the dorsal telencephalon
Dp posterior division of the dorsal telencephalon
POA preoptic area
Diencephalon
NG nucleus glomerulosus
NDI nucleus diffusus lobi inferioris
PGc commissural preglomerular nucleus
Mesencephalon
TOp periventricular layer of the tectum opticum
TOs superficial layer of the tectum opticum
TS torus semicircularis
TLat torus lateralis
Brainstem
RF reticular formation
VL vagal lobe

Table 2 List of behavioral components that may correlate with the brain areas activation pattern

	Olfactory	Spatial cognition	Stress	Vision	LL/auditory	Taste	Locomotion
Control	–	–	–	–	–	–	–
Avoidance	+ + + +	+	+ + + +	+ + + +	+ +	–	+ + + +
Trained	+	+ +	+	+ + + +	+	+ + +	+ + +
Novelty	–	–	–	+ + + +	–	–	+

Discussion

The immediate early gene markers *c-fos* and *egr-1* and, more recently, the phosphorylation of the ribosomal protein S6, have been used as tools to study neuronal activation in diverse behavioral situations in fishes. Activation can occur at different levels, from sensory information processing to decision-making circuits to centers controlling emotional and hormonal states, premotor and motor areas. In fact, the first description of *c-fos* expression in fish was obtained by electrically stimulating motor neurons in the spinal cord and behaviorally by eliciting startle responses, which activate Mauthner cells and other reticular neurons in the rainbow trout (Bosch et al. 1995). The only other motor center that has been investigated with these activity markers in fish is the vocal motor nucleus in the sound-producing midshipman (Mohr et al. 2018; Schuppe et al. 2021). The majority of studies involving IEGs have focused on social behavior and decision-making circuits in the telencephalon and the diencephalon, e.g. the preoptic area, and the hypothalamus. The periaqueductal gray has also been investigated because of its role in emotional and motivational behaviors in mammals (Schuppe et al. 2021; Wood et al. 2011; Wong et al. 2012; Desjardins et al. 2010; Maruska et al. 2013), similar to the raphe superior (Desjardins et al. 2010; Wood et al. 2011; Butler et al. 2018). The only areas outside of the forebrain, that have been assessed in any detail are the tectum and the cerebellum.

In most studies, only a specific sensory modality and its relevant sensory pathways were investigated. For example, Butler and Maruska (2016) studied the role of the lateral line in social communication in cichlids and included in their analysis all major areas involved in the lateral line pathway, leading from the medulla to the diencephalon. Acoustic pathways were investigated in the midshipman (Mohr et al.

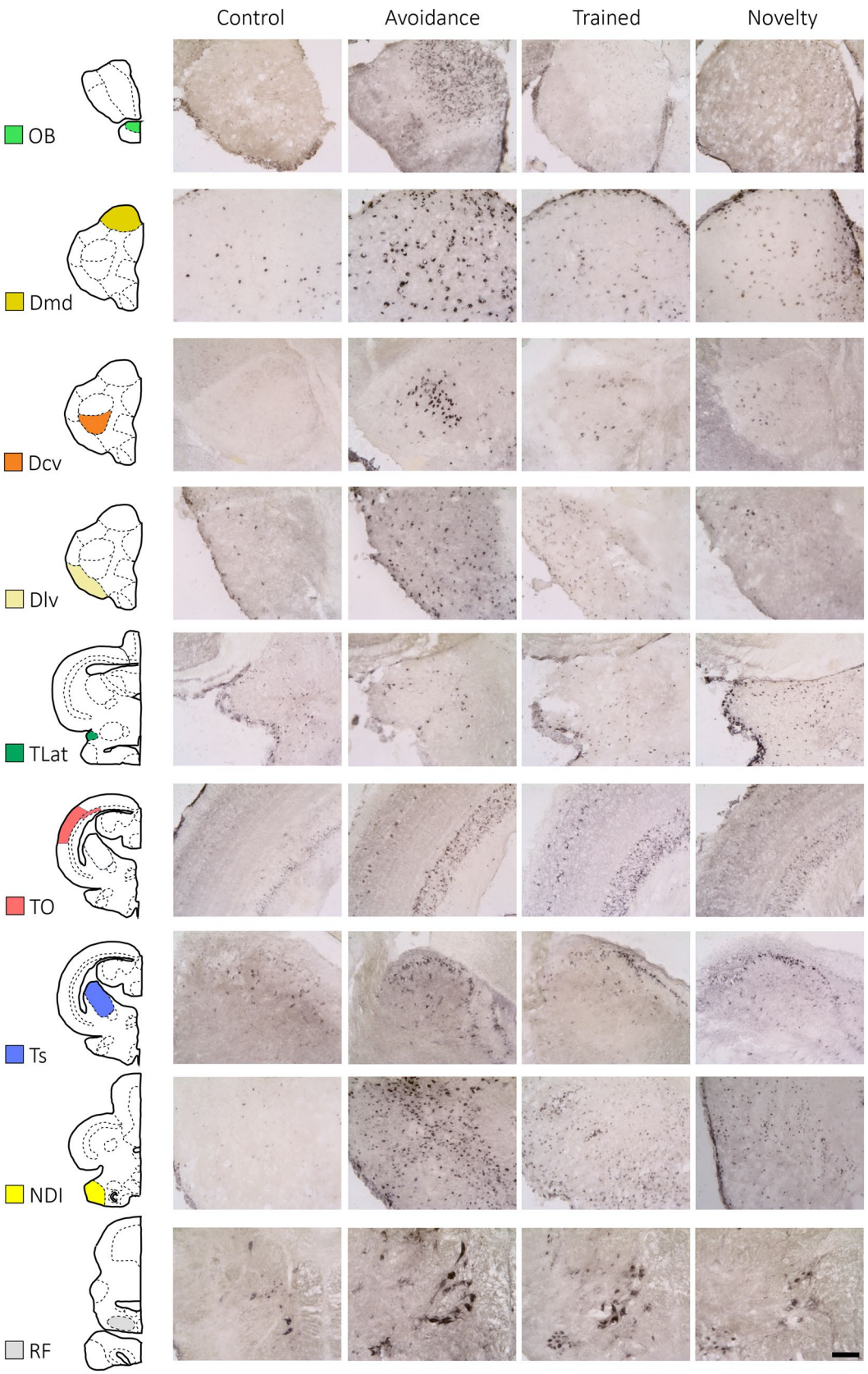


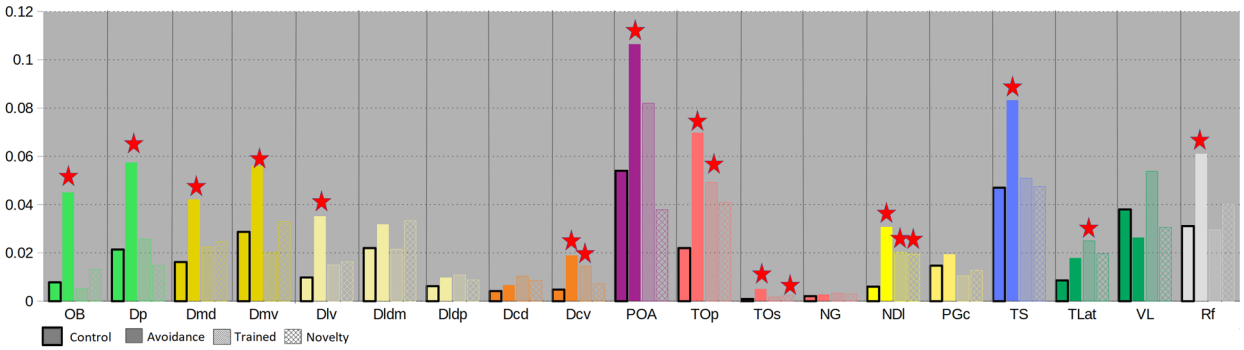
Fig. 3 Staining for pS6 in 9 different brain areas (OB, Dmd, Dcv, Dlv, TLat, TO, TS, IL, and RF) in the four groups. Scale bar on the bottom right: 100 µm for each picture

Olfaction		Dm		Dl			Dc		POA	Vision			NDl	PGc	LL/aud	Taste		Motor
OB	Dp	Dmd	Dmv	Dlv	Dldm	Dldp	Dcd	Dcv	POA	TOp	TOs	NG	NDl	PGc	TS	TLat	VL	Rf

Group Means

Control	0.0078	0.0214	0.0162	0.0287	0.0098	0.022	0.0062	0.0042	0.0048	0.054	0.022	0.0009	0.0021	0.006	0.0147	0.047	0.0086	0.038	0.0311
Avoidance	0.045	0.0574	0.0421	0.0559	0.0351	0.0318	0.0097	0.0066	0.0189	0.1064	0.0697	0.005	0.0027	0.0307	0.0194	0.0832	0.0178	0.0263	0.061
Trained	0.0052	0.0257	0.0225	0.0203	0.015	0.0215	0.0109	0.0103	0.0145	0.082	0.0492	0.0017	0.0033	0.0203	0.0105	0.0509	0.025	0.0538	0.0295
Novelty	0.0131	0.0147	0.0247	0.033	0.0163	0.0333	0.0088	0.0086	0.0072	0.0379	0.0409	0.0022	0.0029	0.0195	0.0128	0.0475	0.0197	0.0306	0.0401

A



U-test: Group/Control

Avoidance	0.0007	0.0005	0.0001	0.0031	0.0001	0.0831	0.743	0.1331	0.0002	0.0001	0.0003	0.0097	0.8632	0.001	1.0407	0.0006	0.0535	0.36	0.0044
Trained	0.6202	0.6456	0.0649	0.2952	0.1417	0.8663	0.1891	0.094	0.0093	0.1048	0.0359	0.5348	0.1903	0.0142	0.4141	0.8632	0.0359	0.2809	1.0374
Novelty	0.3175	0.2765	0.0927	0.6806	0.4634	0.1604	0.5054	0.1141	0.4079	0.1048	0.0545	0.0229	0.6034	0.0001	0.8663	1.0315	0.211	0.2369	0.3152

Relative: log(Group/Control)

Avoidance	0.7617	0.4286	0.4151	0.2889	0.5551	0.1598	0.1969	0.2007	0.5971	0.2946	0.5002	0.7455	0.119	0.7118	0.1192	0.2475	0.3167	-0.1601	0.2923
Trained	-0.1782	0.0792	0.1427	-0.1505	0.1868	-0.0099	0.2474	0.3942	0.4837	0.1817	0.3493	0.2762	0.2048	0.5333	-0.1469	0.0338	0.4651	0.1511	-0.0237
Novelty	0.2254	-0.1632	0.1825	0.06	0.2228	0.1799	0.1531	0.3161	0.1784	-0.1532	0.2686	0.3959	0.135	0.516	-0.0595	0.0038	0.3609	-0.0934	0.1105

B

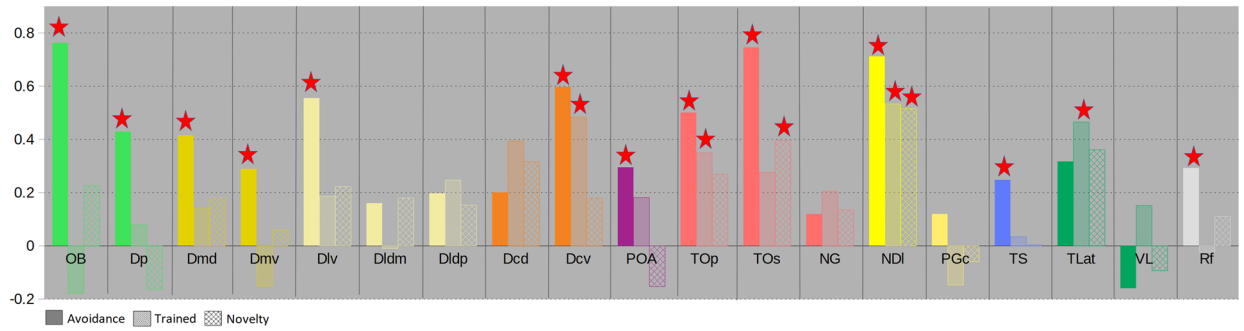


Fig. 4 Results of brain areas analysis. Color code refers to Figs. 2 and 3. Graph A shows the mean pS6 staining intensity for all four groups in the 19 brain areas investigated. Graph B shows the values of the three learning groups relative to the control group (log ratios). The green highlighted values in the *U*-test table indicate *p* values below 0.05 and correspond to the asterisks in Graph B

2018, Tripp et al. 2019, 2020). Finally, the gustatory system has been investigated in species that show bower or castle building during courtship (York et al. 2018, 2019). In most other studies on social behavior, the relevant sensory modality was not specified, and accordingly, sensory centers were not investigated (with the exception of the tectum opticum).

Few studies have focused on non-social behaviors. Lau et al. (2011) studied the innate avoidance of light, to describe the decision-making circuits. Messina et al. (2020, 2022) were investigating the habituation to a complex visual pattern in zebrafish, where the number of objects had to be remembered. Although they noted an upregulation of *c-fos* and *egr-1* in the tectum in response to object size (Messina et al. 2020), they focused on the telencephalon in a follow-up study (Messina et al. 2022), since they were more interested in the counting aspect of the task than in the size discrimination. Rodriguez-Santiago et al. (2021) investigated visual learning in a social context and found that the social aspect (but not the learning per se) was activating various telencephalic areas, but they did not investigate specific visual areas outside the telencephalon.

The aim of this project was to investigate some of the neural substrates involved in visual learning in the cichlid *Pseudotropheus zebra*. We excluded social contacts, but other behavioral components are certainly present to a variable degree and can hardly be excluded. Accordingly, we included in our analysis sensory areas, decision-making circuits in the telencephalon, the preoptic area, and the reticular formation, which is involved in locomotion. In contrast to many other studies, we were not focusing on one aspect

of the behavioral task but tried to analyze and describe the entire behavioral situation with all of its components and correlate it with the activity in a large number of brain areas. Briefly summarized our results show the following:

1. The control group did not receive any sensory-motor or learning treatment. The fish were held in the tank for a week before being sacrificed. They were fed once a day, except the last day. This group shows baseline activity of pS6 in all areas. The activation of pS6 in the other groups (2–4) was measured relative to the baseline activity in the control group.
2. In the stress/avoidance group, fish were moved to a different tank and were chased with a net for one hour. Many sensory areas were activated. Olfactory stimulation correlated with the activation of OB and Dp, and was likely induced by the presence of new water and the net. The latter also seemed to have caused activation of the visual system, reflected by the strong activity of pS6 in both TOp and TOs. The lateral line/hearing system also showed activation (indicated by the activity of pS6 in TS) possibly due to the movement of the net in the water or noises made by the experimenter. There was also activity of pS6 in the preoptic area, which appears to be a stress-related response. In addition, the enforced locomotion activated the reticular formation, a premotor area. The avoidance learning component likely caused activation of the dorsomedial part of the telencephalon and the inferior lobes.
3. In the active training group, fish were trained every day to choose the correct target to get food. Once the fish reached the learning criterion ($\geq 70\%$ correct choices three times in a row), it was sacrificed. Compared to the control group, there was a little additional signal, possibly due to the continuous repetition of training. However, there was an increase in pS6 activity in the torus lateralis, a brain area associated with a taste that was likely activated by receiving a food reward. In addition, the inferior lobes together with both layers of the tectum opticum (TOp and TOs) were activated—possibly due to visual learning.
4. In the habituation/novelty group, staining was also similar to the control group. During the habituation phase, the stimulus was shown to the fish for 5 days, followed by a food release. On the last day, the novelty stimulus was shown. The presentation of a novel stimulus appears to be associated with a small increase in the activity of pS6 in the tectum (vision) and the inferior lobes.

The changes in activity in the different brain areas correlate with the different behavioral responses observed in the four groups. Common to all experimental groups is a strong activation of the inferior lobes. This may be explained by the fact that a visual learning component is present in all groups, except the control group. Higher cognitive functions are often thought to be located in the telencephalon, as is the case in mammals. However, lesion studies and further anatomical evidence have shown that the telencephalon of teleosts is less important for many sensory, motoric and cognitive tasks. Lesion studies have shown that the lesion/ablation of the telencephalon in teleosts has little effect on many behaviors (for a review see Calvo and Schluessel 2021), in particular, basic behaviors—such as swimming, feeding, and reproduction—are not affected by lesions at all (Steiner 1888; Bethe 1899; Rizzolo 1929). However, more intricate behaviors such as the reproductive behavior in sticklebacks (Schonherr 1955) may be heavily impacted, e.g. male sticklebacks show severe deficits in place memory without a telencephalon (e.g. the male does not show the female the correct entrance of the nest or does not find the nest itself). Similar results were found for Tilapia (Aronson 1948). A few studies investigating the role of the fish telencephalon in learning demonstrated impairments in avoidance behavior (e.g. Flood et al. 1976; Davis and Kassel 1983; Overmier and Hollis 1983). Other learning experiments showed no involvement of the telencephalon in simple conditioning and object recognition tasks (Froloff 1925, 1928; Bull 1928; Nolte 1932), pointing to other non-telencephalic areas that may be responsible for the processing of such information.

In 1996, Salas et al. (1996a, b) stimulated new interest in investigating telencephalic functions by discovering that allocentric place memory is located in the telencephalon, which was confirmed in a number of studies. Subsequent studies found the place memory to be restricted to lateral parts of the telencephalon (see Rodríguez et al. 2021 for a review). Further studies showed that the medial parts of the telencephalon play an important part for avoidance learning similar to the amygdala of mammals (see Broglio et al. 2005). Although these studies have shown that some functions of the telencephalon may be conserved across vertebrates, there are still some important differences in the organization of sensory pathways.

Anatomical studies on visual pathways in fish suggest that the major target of retinal fibers is the tectum in the midbrain (Northcutt and Wullimann 1988; Nieuwenhuys et al. 1998). There appears to be no prominent direct thalamic relay of retinal information to the telencephalon. The telencephalon receives visual and other sensory information, but through indirect routes via the preglomerular nuclei, which are part of a posterior tuberal area that is highly derived and elaborated in teleosts (Nieuwenhuys et al. 1998; Rodríguez et al. 2021). These ascending projections may serve the special functions that reside in the telencephalon like allocentric place memory and emotional learning, but skills such as general object recognition and egocentric spatial memory are probably organized in other di-and mesencephalic areas

(Rodríguez et al. 2021). Several accessory areas are reciprocally connected with the tectum like the nucleus isthmi (Xue et al. 2001; Northmore and Gallagher 2003), torus longitudinalis (Wullimann 1994; Xue et al. 2003), and the pretectal areas (Fernald and Shelton 1985; Striedter and Northcutt 1989). A more complex pathway is reaching the inferior lobes via the nucleus corticalis and nucleus glomerulosus (Wullimann and Meyer 1990; Butler et al. 1991; Shimizu et al. 1999; Ahrens and Wullimann 2002; Yang et al. 2007). This system is especially prominent in spiny ray-finned fishes (acanthopterygian). The inferior lobes are located lateral to the traditional hypothalamus, a structure that is shared by all vertebrates. The inferior lobes, in contrast, are present only in teleosts and not found in any other vertebrate group. A recent study suggests that the inferior lobes are not derived from the forebrain like the hypothalamus, but are of mesencephalic origin (Bloch et al. 2019). These anatomical data indicate that the inferior lobes are involved in functions different from the 'traditional' hypothalamus. Our study showed for the first time that the inferior lobes, particularly the nucleus diffusus, are activated in all three visual learning situations. This is the first physiological evidence of the role of this structure in visual discrimination and memory formation, which is a common component of the behavior experienced in all treatment groups.

Conclusion

The activation of ribosomal proteins can be detected in many brain areas and corresponds well with specific behavioral responses present in the four different control and learning situations investigated in this study. The only area consistently activated in all three treatment groups was the nucleus diffusus. It is located in the inferior lobes and the target of a prominent visual pathway originating in the tectum via the nucleus corticalis and the nucleus glomerulosus. Our study shows for the first time that this pathway may be involved in visual object recognition and memory formation. The inferior lobes may thus be one of the most important structures for higher cognitive functions outside of the telencephalon.

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Author contributions RC and VS contributed to the study's conception and design. Data collection, processing of brains and sections were performed by RC. MHH designed the data analysis procedure, and RC and MHH analyzed the data. The first draft of the manuscript was written by RC and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

Ethics approval The research reported herein was performed under the guidelines established by the EU Directive 2010/63/EU for animal experiments and the current German animal protection law and had been approved by the Landesamt für Natur, Umwelt und Verbraucherschutz NRW (approval number 81-02.04.2020.A432).

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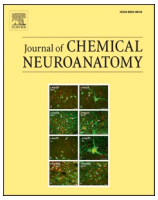

Activation patterns of dopaminergic cell populations reflect different learning scenarios in a cichlid fish, *Pseudotropheus zebra*

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ABSTRACT

Dopamine is present in all vertebrates and the functional roles of the subsystems are assumed to be similar. Whereas the effect of dopaminergic modulation is well investigated in different target systems, less is known about the factors that are causing the modulation of dopaminergic cells. Using the zebra mbuna, *Pseudotropheus zebra*, a cichlid fish from Lake Malawi as a model system, we investigated the activation of specific dopaminergic cell populations detected by double-labeling with TH and pS6 antibodies while the animals were solving different learning tasks. Specifically, we compared an intense avoidance learning situation, an instrumental learning task, and a non-learning isolated group and found strong activation of different dopaminergic cell populations. Preoptic-hypothalamic cell populations respond to the stress component in the avoidance task, and the forced movement/locomotion may be responsible for activation in the posterior tubercle. The instrumental learning task had little stress component, but the activation of the raphe superior in this group may be correlated with attention or arousal during the training sessions. At the same time, the weaker activation of the nucleus of the posterior commissure may be related to positive reward acting onto tectal circuits. Finally, we examined the co-activation patterns across all dopaminergic cell populations and recovered robust differences across experimental groups, largely driven by hypothalamic, posterior tubercle, and brain stem regions possibly encoding the valence and salience associated with stressful stimuli. Taken together, our results offer some insights into the different functions of the dopaminergic cell populations in the brain of a non-mammalian vertebrate in correlation with different behavioral conditions, extending our knowledge for a more comprehensive view of the mechanisms of dopaminergic modulation in vertebrates.

1. Introduction

The biogenic amine dopamine (DA) is a potent neuromodulator in all bilaterian animals (Wintle and Van Tol, 2001; Callier et al., 2003; Moroz et al., 2021) and plays a fundamental role in the regulation of approach and avoidance behaviors (O'Connell and Hofmann, 2011). In humans, dysregulation of the dopaminergic system underlies numerous neurological and psychiatric disorders [e.g. Parkinson's disease,

Abbreviations: ATN, anterior tuberal nucleus; *Cer*, cerebellum; *Dc*, central division of the dorsal telencephalon; *Dd*, dorsal division of the dorsal telencephalon; *Dl*, lateral division of the dorsal telencephalon; *Dld*, dorsal subdivision of the lateral division of the dorsal telencephalon; *Dlv*, ventral subdivision of the lateral division of the dorsal telencephalon; *Dmd*, dorsal subdivision of the medial division of the dorsal telencephalon; *Dmv*, ventral subdivision of the medial division of the dorsal telencephalon; *Dp*, posterior division of the dorsal telencephalon; *E*, entopeduncular nucleus; *fr*, fasciculus retroflexus; *Ha*, habenula; *Hc*, caudal hypothalamus; *Hyp*, hypothalamus; *IGL*, internal granular layer of the olfactory bulb; *IL*, inferior lobes of the hypothalamus; *ILr*, inferior lobe, nucleus of the lateral recess; *LC*, locus coeruleus; *NDI*, nucleus diffusus pars lateralis; *NG*, nucleus glomerulosus; *NLT*, nucleus lateralis tuberis; *nPR*, nucleus of the posterior recess; *nTP*, nucleus of the posterior tubercle; *OB*, olfactory bulb; *pc*, posterior commissure; *PP*, periventricular pretectal nucleus; *POAa*, preoptic area, anterior part; *POAp*, preoptic area, posterior part; *PTc*, pretectal area, centralis; *PTco*, pretectal area, corticalis; *pTP*, periventricular posterior tuberculum; *PTsm*, pretectal area, superficialis magnocellularis; *Ras*, raphe superior; *RFs*, superior reticular formation; *SCN*, suprachiasmatic nucleus; *SGN*, secondary gustatory nucleus; *Tel*, telencephalon; *TGN*, tertiary gustatory nucleus; *TL*, torus longitudinalis; *TLat*, torus lateralis; *TO*, tectum opticum; *Vc*, central nucleus of the ventral division of the telencephalon; *Vd*, dorsal nucleus of the ventral division of the telencephalon; *Vl*, lateral nucleus of the ventral division of the telencephalon; *VM*, ventromedial thalamic nucleus; *Vv*, ventral nucleus of the ventral division of the telencephalon.

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schizophrenia, attention deficit hyperactivity disorder (ADHD)] (Callier et al., 2003; O'Connell et al., 2011). Due to its critical role in regulating neural and behavioral processes, the dopaminergic system has therefore been studied in great detail, especially in mammals. The first evidence of the presence of dopamine-producing neurons came in the early 1960 s by Annica Dahlström and Kjell Fuxe, who also introduced the conventional numbering of the catecholaminergic cell populations from A1 to A12 in the rat brain (Dahlström and Fuxe, 1964). Later on, additional cell populations were added (A13 - A17) (Hökfelt, 1984; Lindvall and Björklund, 1984). This nomenclature of dopaminergic cell populations is still used today because dopaminergic cells are not located in a specific nucleus and furthermore their locations and distributions vary between vertebrates and even between different species of mammals (Yamamoto and Vernier, 2011). In general, dopaminergic cells (A8 to A17) are divided in different populations: retinal (A17), olfactory bulb (A16), diencephalic (A11–A15) and diencephalo-midbrain (A8–A10) populations (Smeets and González, 2000), while A1 to A7 cells are noradrenergic neurons localized in the medulla oblongata and pons (Nagatsu, 2007). In the central nervous system (CNS) of mammals, dopaminergic cell populations distribute their fibers in five major pathways (mesocortical, mesolimbic, nigrostriatal, tuberoinfundibular, and spinal tract systems). The mesocortical and mesolimbic pathways originate from the A10 neurons of the ventral tegmental area (VTA) and project respectively to the cortex and the nucleus accumbens (Horvitz, 2000; Wise, 2009). Together, they form the mesocorticolimbic system, which plays a central role in reward and motivation (Kelley and Berridge, 2002).

There is ample evidence that stressful and aversive stimuli can also result in dopamine release from the mesolimbic system (Ikemoto and Panksepp, 1999), suggesting the involvement of the dopaminergic system in the stress response (Salamone et al., 1997; Berridge and Robinson, 1998). The nigrostriatal pathway is involved both in learning and motor function and is formed by the A9 dopaminergic neurons in the substantia nigra projecting to the striatum (Hikosaka et al., 2002). Dopaminergic neurons from the arcuate and periventricular nuclei of the hypothalamus project to the pituitary gland, resulting in the tuberoinfundibular pathway, which is involved in the regulation of the secretion of prolactin from the pituitary (Demarest et al., 1984). Finally, the spinal projecting dopaminergic system originates from A10 and A11 cell populations (Qu et al., 2006). It is thought to modulate locomotion, but is also involved in sensory processing as it modulates nociception in the spinal cord (Piña-Leyva et al., 2022).

The dopaminergic system of non-mammalian vertebrates, especially that of teleost fishes, has received much less attention. Several studies have described dopaminergic neuron populations and, in some cases, fiber projections in a handful of species [e.g. goldfish *Carassius auratus* (Hornby et al., 1987), the brown ghost knifefish (*Apteronotus leptorhynchus*; Sas et al., 1990), the zebrafish (*Danio rerio*; Rink and Wullimann, 2001; Kaslin and Panula, 2001), and Burton's mouthbrooder cichlid (*Astatotilapia burtoni*, O'Connell et al., 2011; O'Connell et al., 2013a)]. Although some differences exist to the situation in mammals, there is broad consensus that the major dopaminergic components found in mammals are conserved in teleosts. However, to which extent the teleost dopaminergic system is functionally similar to its mammalian counterpart is much less clear.

DA plays an important role in learning in many vertebrates. Many areas in the telencephalon are rich of dopamine receptors like the striatum, amygdala, and hippocampus and their involvement in learning is well established (El-Ghundi et al., 2007; Puig et al., 2014). There is also evidence for the role of DA in learning in motor and sensory areas (Macedo-Lima and Remage-Healey, 2021). Although homologies of telencephalic areas between mammals and teleosts are uncertain, DA seems to play a role in learning also in fishes (e.g. Naderi et al., 2016).

Several studies have assessed the activation of different brain areas during different behavioral contexts using neural marker (i.e. immediate early genes – IEGs – for a review see Calvo and Schluessel, 2021). To get a more detailed understanding of the function of the dopaminergic system in teleost, the IEGs have been used to selectively mark the activation of dopaminergic cell populations. O'Connell et al., (2013b) investigated whether social stimuli would induce c-fos expression in dopaminergic populations in the brain of *A. burtoni*. They observed an increase of c-fos in the Vc of both intruder and reproductive opportunity contexts compared to the control group (O'Connell et al., 2013b). The same animal model was used by Weitekamp and Hofmann (2017) to assess whether cooperation would increase the activity of specific dopaminergic cell populations (Weitekamp and Hofmann, 2017). In larvae of zebrafish, handling stress, chemical stressor and pH change were reported to induce c-fos expression in the dopaminergic cell populations of the posterior tuberculum and hypothalamus (Semenova et al., 2014) and olfactory deprivation has an effect of the dopamine system within the olfactory bulb (Kress and Wullimann, 2012). These studies focused on specific dopaminergic subsystems.

Whereas zebrafish offer several advantages to study basic functions of the nervous system, other fish groups have also been subjects of different behavioral and anatomical studies. Cichlids are well studied and the organization of their brains and their complex behavior well documented (e.g. Pollen et al., 2007; Shumway, 2008, 2010). The distribution of tyrosine hydroxylase positive (TH+) cell populations and dopaminergic receptors has been investigated in detail (O'Connell et al., 2011). In addition, activity markers have been applied to study the activation of many different brain areas during different learning situations (Calvo et al., 2023).

In the present study, we examined the activation of the dopaminergic systems in the zebra mbuna cichlid, *P. zebra,* by co-labeling TH with the phosphorylated ribosome marker pS6, now commonly used to visualize neural activation in fish (Calvo et al., 2023 and references therein). In particular, we investigated the activation of thirteen different dopaminergic cell populations in fish subjected to two learning tasks that differed in the level of stress, locomotion, motivation, and reward. We hypothesized that different behavioral components would activate different dopaminergic populations.

2. Material and methods

2.1. Behavioral experiments

Animals used in this study (N = 30) were zebra mbuna cichlids, *Pseudotropheus zebra*, from the east African Lake Malawi. Fish were obtained from a commercial aquarist shop and were between 4.0 cm and 11.0 cm in total length. All individuals were maintained in captivity and sex could not be determined phenotypically. The same individuals were assessed in a previous study concerning activation of different brain areas by investigating the activity of the ribosomal marker pS6 (Calvo et al., 2023). For a detailed explanation of the experimental procedures and setups, we refer the reader to Calvo et al., 2023. Briefly, fish were kept in isolation for one week prior to being stressed (Avoidance group), trained (Trained group) and/or killed (Isolation group). Fish were housed in aerated and filtered 50-L aquaria (62 cm × 31 cm × 31 cm) at a temperature of 25–26 °C. Fish in the Isolation group (N = 10) did not receive any treatment and were killed after seven days of isolation. Fish in the Avoidance group (N = 10) were moved to a new smaller tank (31 cm × 15 cm x 15 cm) after seven days of isolation and chased with a net for one hour to simulate a strong stressor. Fish were killed 90 min after the chase ended. Fish in the Trained group (N = 10) underwent a daily visual training after seven days of isolation. During the training, the fish had to make a choice between the two different training symbols [a black dot over a white background (positive stimulus) and a white background without a symbol (alternative, negative stimulus)] projected onto the plexiglass of the experimental tank (see Schluessel et al., 2018 for details of the experimental setup). A correct choice was rewarded with food. After the learning criterion was achieved (seven or more correct decisions out of ten trials in three consecutive sessions), fish went through a so called "supersession" (1-hour non-stop training).

Then, the fish were killed 90 min after the supersession was finished. To process the brains, fish were anesthetized with tricaine methanesulfonate (MS-222). The spinal cord was transected and the brain was removed and fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, then cryo-protected overnight in 30% sucrose at 4 °C. The following day, the brains were embedded in O.C.T compound (freezing medium, Leica Biosystem Richmond) and frozen at − 20 °C. Thirtyfive μm thick sections were cut at − 20 °C with a cryostat (Leica CM1520) and collected in three series on slides. One series was used for tyrosine hydroxylase immunohistochemistry (IHC) alone, one series for double-labeling with TH and pS6 antibodies and one series for cresyl violet staining for a cytoarchitecture reference. To minimize differences in stain intensity, IHC was performed twice within three days, each time including five brain per group (i.e. 5 control, 5 avoidance, 5 trained), starting from the day after the cutting.

2.2. Immunohistochemistry for tyrosine hydroxylase

Immunohistochemistry was performed as described previously (Calvo et al., 2023). Briefly, frozen sections were bleached in 1% H_2O_2 in phosphate-buffered saline (PBS), 0.01 M, pH 7.4, washed, and blocked in 10% normal goat serum (NGS) for 1 h then transferred to a primary antibody solution [5% NGS / 1X PBS - 0.3% Triton X-100, rabbit anti-tyrosine hydroxylase antibody - Neuronal Marker ab112 (abcam, Cambridge MA): 1:500] overnight at 4 °C, before being washed several times in PBS. The second antibody reaction (VECTASTAIN biotinylated anti-rabbit IgG secondary antibody, Vector Labs., USA: 1:500) was performed in 5% NGS / 1X PBS - 0.3% Triton X-100, followed by repeated washes in PBS. Then, signal amplification was initiated using the ABC method (1:1500, 1X PBS - 0.3% Triton X-100, VECTASTAIN ABC-Peroxidase kit, Vector Labs., USA) and visualized using the chromogen-solution [one 3,3'-Diaminobenzidine-Tetrahydrochloride (DAB) buffer tablet (Merck KGaA, Germany) dissolved in 15 ml distilled water, 500 μL 1% ammonium nickel sulphate, 12 μL 30% H_2O_2] for ~30 min. Sections were then dehydrated in ascending alcohols to xylene before cover slipping with Eukitt (Carl Roth, Germany).

2.3. Fluorescent co-labeling of pS6 and TH

To quantify the activation of dopaminergic neurons, pS6 and TH were co-localized by fluorescent double-labeling immunohistochemistry using a mix of 1:500 rabbit anti-tyrosine hydroxylase antibody (Neuronal Marker ab112 abcam, Cambridge MA) and 1:500 mouse anti-pS6 (Ser235/236 antibody, Cell Signaling E2R1O). To our knowledge, there are no published reports on the mouse anti-pS6 antibody used in this study. A rabbit anti-pS6 antibody from Cell Signaling ((Ser235/236) antibody, Cell Signaling 2211 S) has been used successfully in several studies on fish, including cichlids (for references see Calvo et al., 2023). Dr. Ross DeAngelis, from Hans Hofmann's laboratory group in Austin (Texas, United States), performed a double-labeling rabbit anti-pS6 and mouse anti-pS6 in the cichlid *Astatotilapia burtoni* showing no differences in the staining, that is rabbit anti-pS6 and mouse anti-pS6 stained the exact same cells (results not shown here). After incubation overnight in primary antibody, slides were washed twice in PBS and then incubated in a mix of 1:500 goat anti-rabbit IgG (H+L) cross-absorbed secondary antibody Alexa Fluor 594 and 1:500 goat anti-mouse IgG (H+L) cross adsorbed secondary antibody Alexa Fluor 488 (ThermoFisher scientific, Dallas, Texas, United States). Slides were then rinsed twice in PBS and cover-slipped with 4', 6-diamidino-2-phenylindole (DAPI) hardset fluorescent mounting media (Vector Laboratories, Burlingame, CA, USA).

The anti-tyrosine hydroxylase antibody was the same as in the single DAB immunohistochemistry protocol. The staining pattern in the double-labeling and single-labeling material was identical. Furthermore, the staining was very similar to the one reported by O'Connell et al. (2011). They noted that their antibody recognized both forms of TH (TH1 and TH2), but with a weaker staining of TH2.

2.4. Cell count

Thirteen dopaminergic cell populations were analyzed in each individual. The locations of the cell populations throughout the cichlid brain are shown in Fig. 1 and listed in Table 1.

Each of the 30 fish brains used in this study was screened for dopaminergic cell populations from rostral to caudal using a Zeiss Axioscope microscope with three filter set. First, the cytoarchitecture was visualized and identified with the DAPI stain, then the TH+ cell populations were visualized and photographed with the rhodamine filter set. For visualizing pS6 stained cells, the filter cube was changed to a FITC filter set and the same population photographed again. Both photographs were merged to an RGB image with the TH+ cells in the red channel and the pS6 stain in the green channel.

The images contained all TH+ cells that were found for a given area and animal. Since only every third section was stained with the double-labeling procedure and some sections were damaged, only a subset of TH+ cells could be tested for co-localization with pS6. Because some TH+ cell populations are very small, sometimes no TH+ cells could be found for a given area in an animal. However, this is not critical because the statistical test used is based on the sum of all cells per group (see below).

After all necessary images were obtained, counting was performed with the help of a custom-made program (Hofmann, MH). The user selected a brain area to be analyzed and the computer generated a random list of images to be used for counting. The counting was done blind, i.e. the user did not know from which animal group the image was selected from by the computer. The images were viewed as RGB images, but the user could also view each channel independently by pushing a key on the keyboard. This allowed for a better evaluation of double-labeling compared to the yellowish color of red and green channels combined. Frequently, the brightness of the different cells could vary in the red and green channels, and the combination would give all kinds of yellowish shades. Focusing on a cell with the red channel and switching back and forth between the red and green channels, proved to be a way more reliable to detect double-labeled cells, independently of their brightness.

First, the user marked all TH+ cells in the area of interest and then counted the number of double-labeled cells. The computer kept track of all counting and stored the value under the correct animal name, unknown to the person counting. The data were exported to a spreadsheet program and tables and bar graphs generated from there.

If the same dopaminergic cell population was detected in several different sections in the same animal, the counts were combined computationally. The entire procedure was repeated until all animals were counted. This resulted in a list with all individual TH+ cell numbers for each area and the number of double-labeled cells for each dopaminergic area and behavioral group (Isolation, Avoidance, Trained). For the micrographs shown in Fig. 3, the RGB images were split into the individual red and green channels with ImageJ to separately auto-adjust each image for brightness and contrast. The channels were subsequently combined again into a single RGB image.

2.5. Statistics and data analysis

The primary data set contained binary data, a given TH+ cell can be co-labeled with pS6 or not. Furthermore, some areas within a group were never double-labeled (0%) or always double-labeled (100%). For these kinds of data, a parametric test is not appropriate and we used the Fisher's exact test, which is designed for such cases. A matrix was created in the spreadsheet program, featuring the numbers of both double-labeled and single-labeled TH+ cells as well as the sums of the column and rows. Fisher's exact test was used to calculate whether there were significant differences in the expression pattern found among

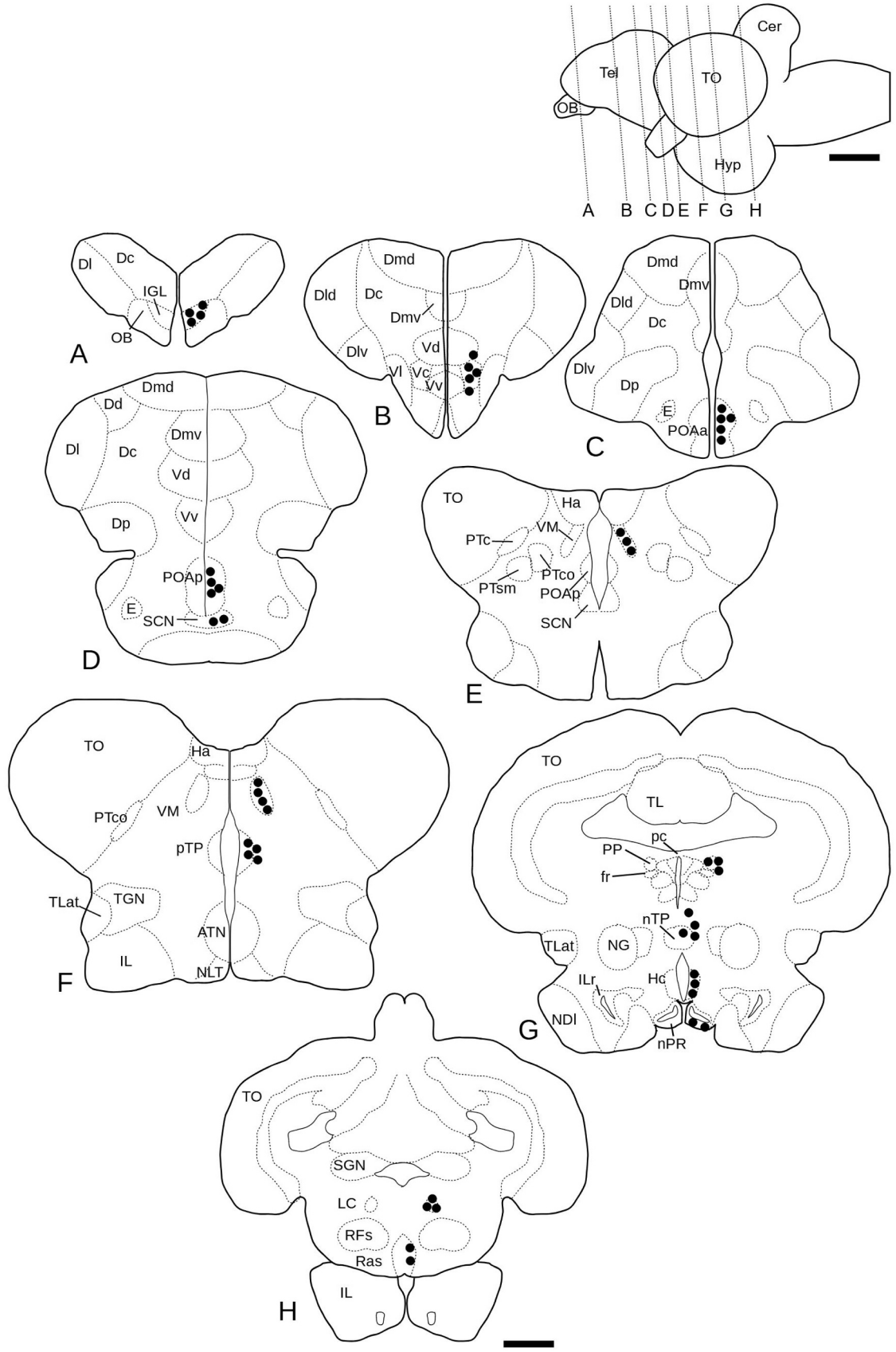


Fig. 1. Dopaminergic cell populations. Schematic drawing of the thirteen dopaminergic cell populations analyzed (see list of abbreviations). Scale bar in H equals 500 μm and applies to all cross sections. Scale bar of the sagittal view equals 1 mm. The schematic sagittal diagram of *P. zebra* brain shows the approximate locations of each section.

Tablee 1
List of brain areas analyzed.

IGL	internal granular layer of the olfactory bulb
Vc	central nucleus of the ventral division of the telencephalon
POAa	preoptic area, anterior part
POAp	preoptic area, posterior part
SCN	suprachiasmatic nucleus
VM	ventromedial thalamic nucleus
pTP	periventricular posterior tuberculum
PP	periventricular pretectal nucleus
nTP	nucleus of the posterior tubercle
Hc	caudal hypothalamus
nPR	nucleus of the posterior recess
LC	locus coeruleus
Ras	raphe superior

Avoidance, Trained, and Isolation groups. A significant level of $p < 0.05$ was chosen to reject the null hypothesis. Details on the Fisher test can be found in the supplementary materials. In addition, the non-parametric Mann-Whitney-test was calculated to test for group differences.

We used the R package pheatmap (Kolde, 2012) to perform hierarchical clustering analyses of the dataset, clustering both individuals and double-labeled TH+ cell populations, using average linkage as agglomeration method and correlation as distance metric. Using the R package pvclust (Suzuki and Shimodaira, 2006), we then estimated the robustness of any resulting clusters by multiscale bootstrap resampling. Clusters for which $p < 0.05$ are indicated with bootstrap values ≥ 95. Because of the multivariate nature of the dataset, we conducted a Principal Components Analysis (PCA) in R. For the PCA and cluster analysis, it was necessary to replace missing values. We calculated the mean and standard deviation within each brain area and group and replaced missing values with a Gaussian random number created from the mean and standard deviation. This would preserve the mean and also the variance in the data set. Since there is a high number of missing values in some areas, we tested the procedure by creating 1000 data sets of random numbers and checked for variability in the first principle component of the PCA. There were little variations in the loadings of the components and the PCA was very robust and reproducible (see Fig. S2).

3. Results

3.1. TH immunoreactive cell populations

The distribution of dopaminergic cell bodies in the brain of *P. zebra* was investigated with an antibody against TH and DAB as a chromogen. All major cell populations identified (Figs. 1, 2) were then used to study dopamine activation in three behavioral contexts, i.e. isolation, avoidance and visual discrimination learning. Differences in cell morphology and cell size were not measured systematically, but Fig. 2 shows high magnification photographs of all areas.

The first population of TH immunoreactivity was found in the olfactory bulb, internal granular layer (IGL, Figs. 1A, 2A). While TH+ cell bodies were found in the cellular (granular) layer, fibers were localized in the mitral layer. A population of large TH+ cell bodies extended along the medial olfactory tract and many more were located in the central nucleus of the ventral division of the telencephalon (Vc, Figs. 1B, 2B).

Two prominent TH+ cell populations were located in the preoptic area. An anterior population between the anterior commissure and the beginning of the optic chiasm (POAa, Figs. 1C, 2C) was distinguished from a more posterior population (POAp, Figs. 1D, 2D). At the level of the rostral POAp population, the suprachiasmatic nucleus contained also some TH+ cells (SCN, Figs. 1D, 2E).

A large number of small TH+ cell bodies was located in the ventromedial thalamic nucleus (VM, Figs. 1E-F, 2F). Their fibers seemed to be orientated dorsolateral, towards the tectum opticum. More caudally, another cell population was present in the periventricular pretectal nucleus just below the posterior commissure, dorsal to the fasciculus retroflexus (PP, Figs. 1G, 2H). It extended caudally along the posterior commissure. More caudally, TH+ cell populations were observed above the tract of the posterior commissure, but those cells seemed to be continuous with the ventral cell population and are thus also part of PP. Additional TH immunoreactivity was found in the cells of the periventricular posterior tuberculum (pTP, Figs. 1F, 2G). This population showed large cells with fibers oriented lateral and seemed to extend into a small tract visible between the lateral end of the tectum opticum and the torus lateralis. This tract could be followed into the brain stem (data not shown).

Scattered big TH+ cells were found more caudally and ventrally to the pTP. They showed fibers larger than the ones of the pTP, with extensions into the brain stem tract (Figs. 1G, 2I). However, many of the fibers followed a more dorsal route. Although these cells are located in the area of the nucleus of the posterior tubercle (nTP), they constitute only a subset of cells of the nTP.

Ventral to the nTP, two further populations of TH+ cells were found. Small cells were found along the ventricle in the periventricular caudal hypothalamus (Hc, Figs. 1G, 2J). A small number of weakly stained cells were also present around the posterior recess (nPR, Figs. 1G, 2K).

At the isthmic region, a small number of very large cells were TH+ and identified as the locus coeruleus (LC, Figs. 1H, 2L). TH is present in all cells that produce catecholamines. Dopamine is produced in all forebrain areas; the locus coeruleus is the only cell population that produces noradrenaline. Although our study focuses on the dopaminergic system, we included the locus coeruleus because it is also an important modulatory system and was stained with both TH and pS6 antibodies.

At the same level, smaller TH+ cells were located along the midline, identified as the raphe superior (Ras, Figs. 1H, 2M).

Other TH+ cell populations were present in the vagal region in the brain stem, but were not included in this study. There were also some TH+ cells in the paraventricular organ, but they were very weakly stained and visible in only a few individuals. Therefore, this region was not included in our study.

3.2. Co-localization of TH and pS6

Examples of cell populations analyzed for co-localization of pS6 and TH-+ are shown in Fig. 3. Co-localization of the two proteins was visualized by the overlap of the two secondary antibodies' colors (green for pS6 and red for TH), resulting in a yellowish colored cell. The experimental groups are shown in different columns, i.e. Isolation, Avoidance, Trained, from left to right.

3.2.1. Isolation group

In the cell populations of the Isolation group (Fig. 3 "Isolation" column, A1-M1), there was little co-localization of TH and pS6. A few cells showed co-localization in the nPR (Fig. 3, K1) and all cells in the locus coeruleus were double-labeled (Fig. 3, L1).

3.2.2. Avoidance group

The Avoidance group (Fig. 3 "avoidance" column, A2-M2) showed the biggest number of cell populations with double-labeled cells compared to the Isolation and the Trained group. In particular, co-localization of TH and pS6 was found in Vc (Fig. 3, B2), POAa (Fig. 3, C2), POAp (Fig. 3, D2), SCN (Fig. 3, E2), VM (Fig. 3, F2), pTP (Fig. 3, G2), PP (Fig. 3, H2), nTP (Fig. 3, I2), Hc (Fig. 3, J2), LC (Fig. 3, L2) and Ras (Fig. 3, M2).

3.2.3. Trained group

In the Trained group (Fig. 3 "trained" column, A3-M3) co-localization of TH and pS6 was found in cells of pTP (Fig. 3, G3), PP (Fig. 3, H3), nTP (Fig. 3, I3), LC (Fig. 3, L3) and Ras (Fig. 3, M3).

Besides direct co-localization of TH and pS6, there was a marked increase of pS6-ir cell bodies in some areas, especially in the Avoidance

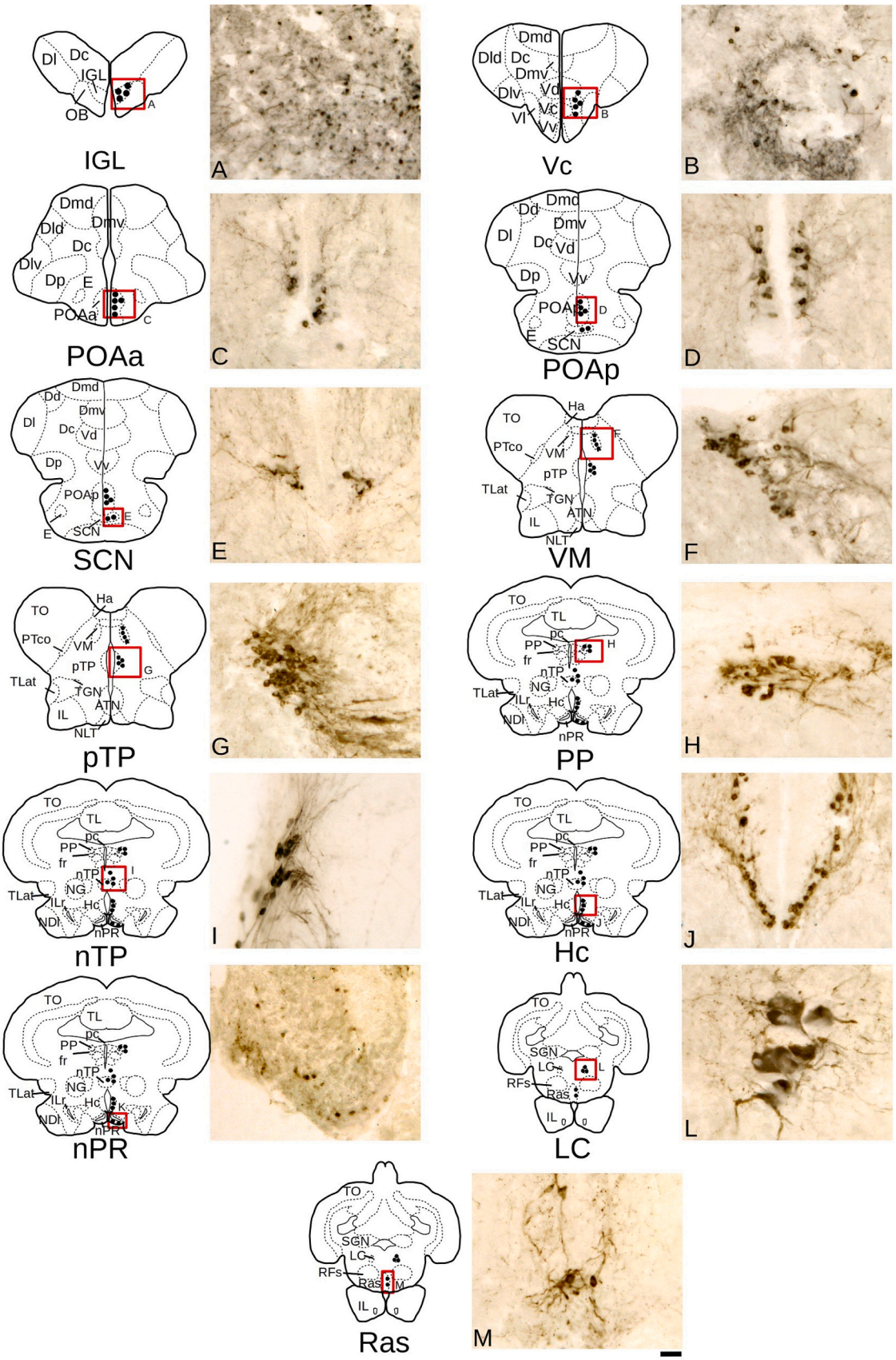


Fig. 2. Representative micrographs (A-M) of the dopaminergic cell populations investigated. On the left of each micrograph, a drawing highlighting the localization in the brain of the respective cell population is shown. Scale bar: 50 μm.

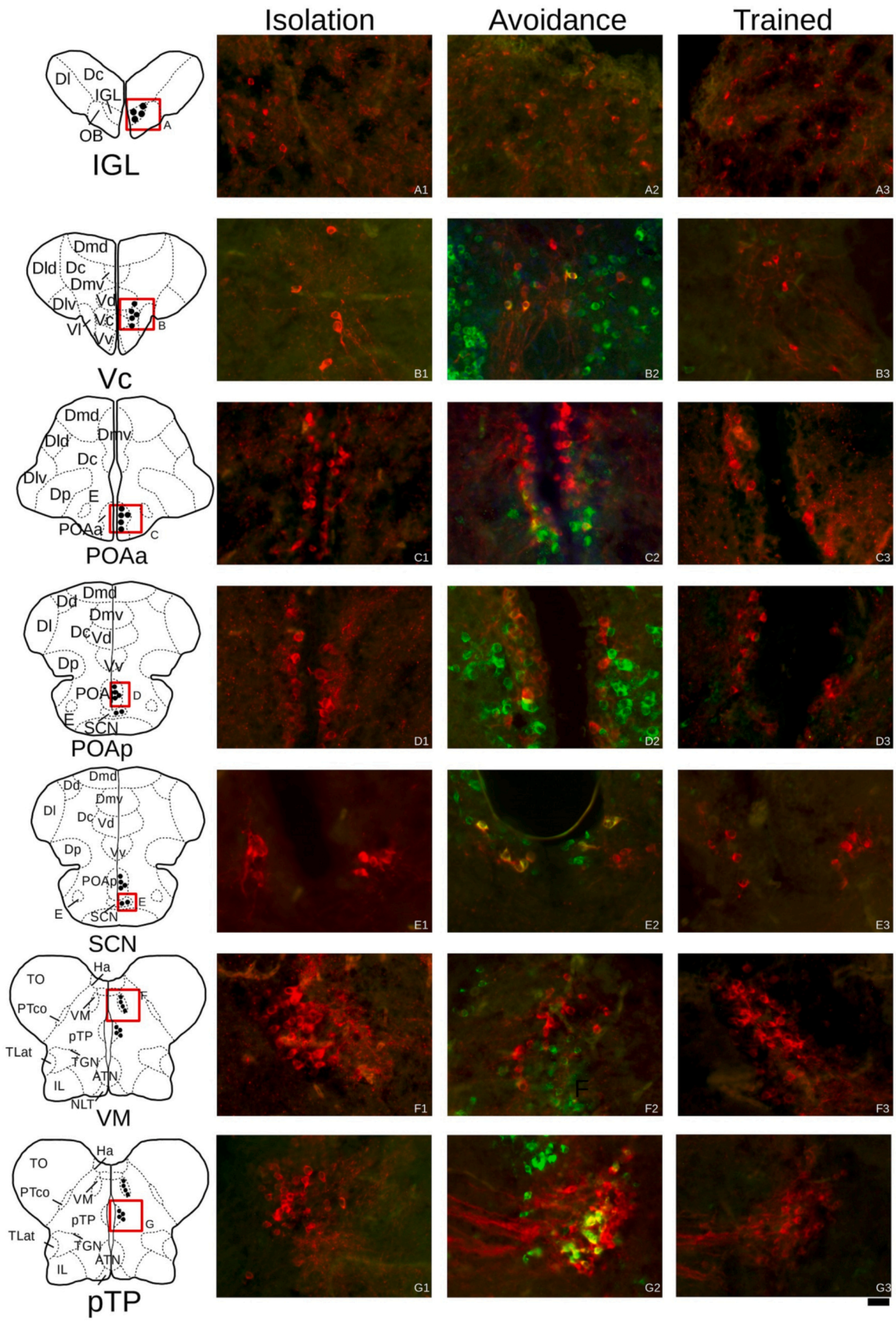


Fig. 3. Double-labeling micrographs. Double-labeling (yellow) of pS6 (green) – TH (red) in the three different groups (Isolation, Avoidance and Trained) in the thirteen dopaminergic cell populations analyzed (IGL, Vc, POAa, POAp, SCN, VM, PP, pTP, nTP, Hc, nPR, LC, Ras – see list of abbreviations). Scale bar: 20 μm.

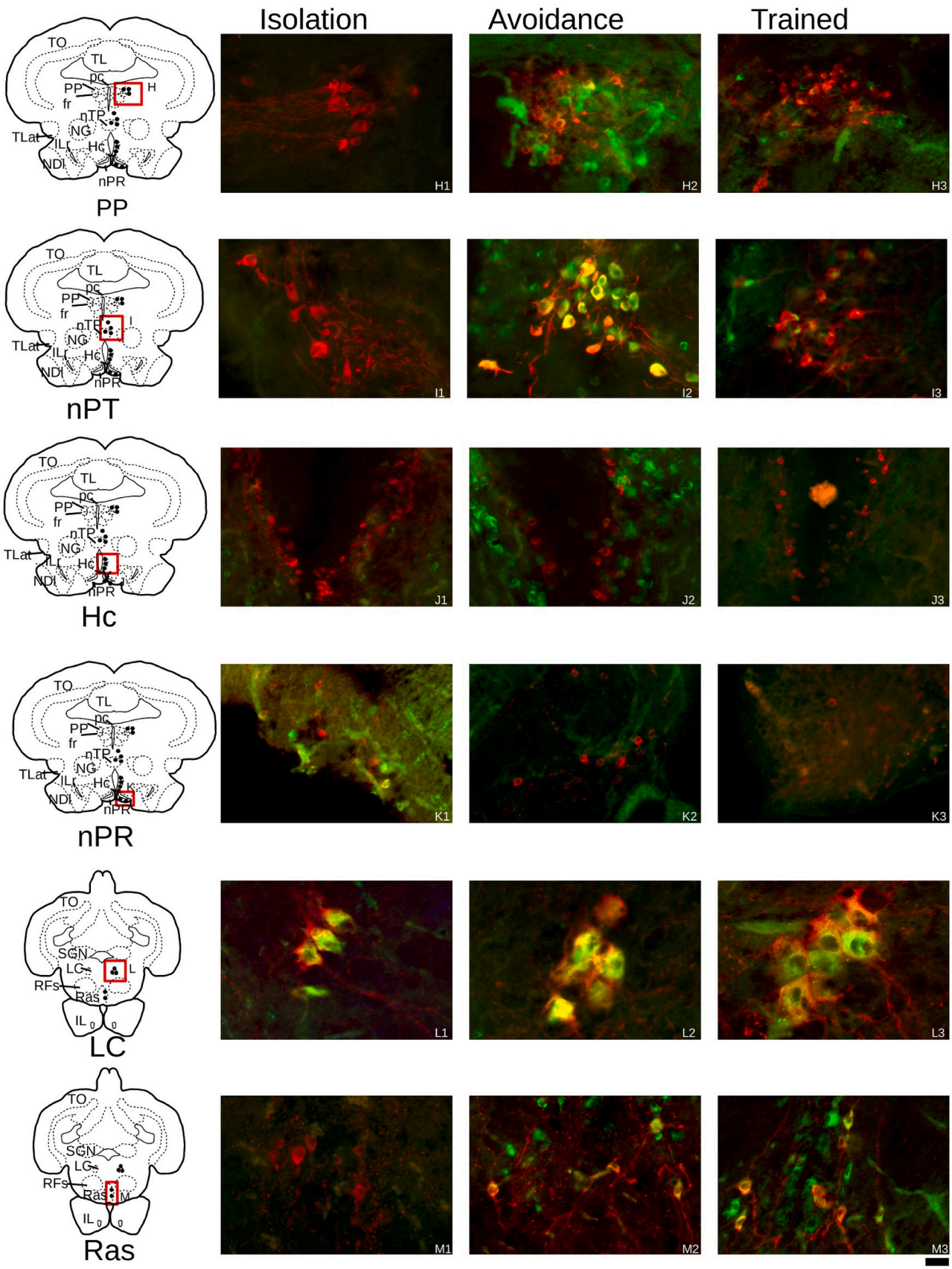


Fig. 3. (*continued*).

group. Sometimes, the additionally labeled pS6 cell bodies were located in between the TH+ cells, often they were found around the TH+ cell population. This could be seen in the POAa (Fig. 3, C) and POAp (Fig. 3, D), the PP (Fig. 3, H), and the Hc (Fig. 3, J) populations. Here, there was an increase in double-labeled cells, but there were many more additionally activated pS6 labeled cells that were not TH+ . In the nTP, almost all cells were double-labeled in the Avoidance group and no cells were exclusively pS6 labeled. In the SCN, half of the TH+ cells were stained for pS6, with many additional cells that only showed pS6 stain.

3.3. Quantification of co-localization

For quantification, the number of TH+ cells in the different areas and the number of TH+ cells that were double-labeled with pS6 antibodies were counted. Fig. 4A shows the percentage of double-labeled cells for each animal and Fig. 4B the averages in each area for the three experimental groups. In Fig. 4C, the differences are visualized in a bubble chart and Fig. 4D lists the p-values calculated according to the Fisher and Mann-Whitney tests. Detailed information about the raw data, the actual cell counts, and statistical tests can be found in the supplementary material. The Avoidance group showed the majority of the double-labeled cell populations. The large cells in the nTP showed the largest extent of activation. A strong activation was found also in the SCN, compared to the Isolation group. The Avoidance group showed an increase in double-labeled cells also in almost all other TH+ areas, except the IGL, where no activation was detected. The nPR cell population was the only area where fewer double-labeled cells were found relative to the Isolation group. In the locus coeruleus almost all big cells were double-labeled, in all groups. This is in contrast to the Ras, which showed a strong activation in both the Avoidance and the Trained group, but not in the Isolation group.

In addition to the Ras, the Trained group showed an increase of activation in the PP and the nTP. In the PP, the activation was not strong, similar to the Avoidance group. In the nTP, there was less activation than in the Avoidance group, but still significantly more than in the Isolation group.

3.4. Co-activation patterns of dopaminergic cell populations

Above, we described the average group values of the activation of TH+ cells in the different brain areas, but the variations within each group was not taken into account. Fig. 4A shows the individual values within each group and area, which shows that the response is sometimes very heterogeneous and nonlinear. This is especially apparent in the SCN in the Avoidance group and the nTP in the Trained group. Some animals showed zero activation whereas others showed a high proportion of double-labeled cells. The Fisher test does not take this into account, but other statistical tests are not compatible because the data are not normal distributed, variances are not equal between groups, and there are many zero values in the data set. The non-parametric Mann-Whitney test shows some agreement with the Fisher test, but it is not applicable in some cases with many zero values (ties in the ranks). However, there are two other methods that we used to investigate the variability of the animals. These are based on the pattern of activation in all brain areas rather than on investigating each area separately.

First, we conducted a Principal Components Analysis (PCA) to identify the double-labeled TH+ cell populations that most strongly separated the experimental groups. We discovered that principal component (PC) 1, which explained 69.0% of the variance (Fig. 5A), significantly clustered the three experimental groups from each other (ANOVA: F27, 2 = 135.4, p = 8.4 * 10–15; Fig. 5D, E). This clustering was largely driven by the nTP cell population as well as, to a lesser extent, by double-labeled TH+ in the SCN and raphe (Fig. 5B). The three groups can be separated by the first component alone with no overlap (Fig. 5E). Double-labeled TH+ in the SCN and raphe loaded even more strongly on PC2 (Fig. 5C), which further separated the Trained group from both the Isolation and Avoidance groups (ANOVA: F27, 2 = 17.95 p = 1.1 * 10–5; Fig. 5F).

The second analysis was a hierarchical clustering that is grouping the animals by similarity in their activation pattern. The cluster analysis itself is not aware of any group memberships, but a post-hoc visualization showed that the cluster analysis reconstructed the groups almost perfectly with just two animals misplaced into the wrong group (Fig. 6).

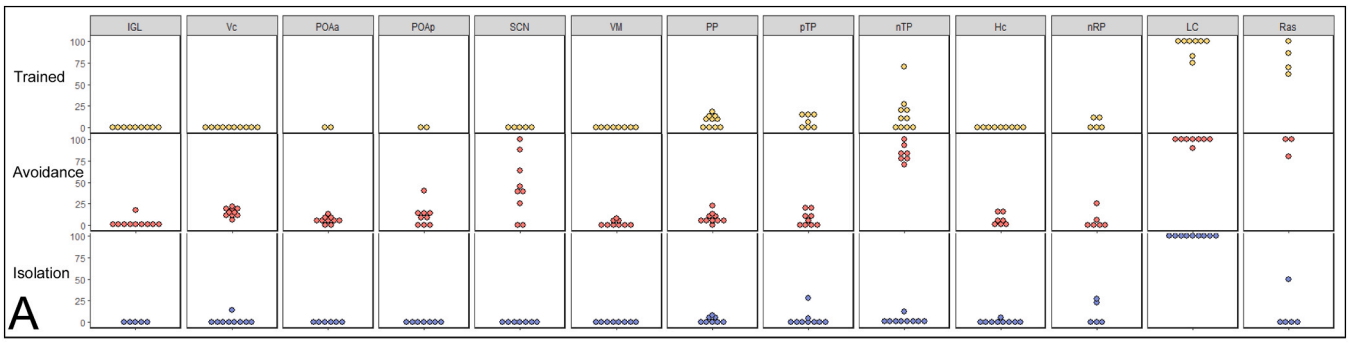
4. Discussion

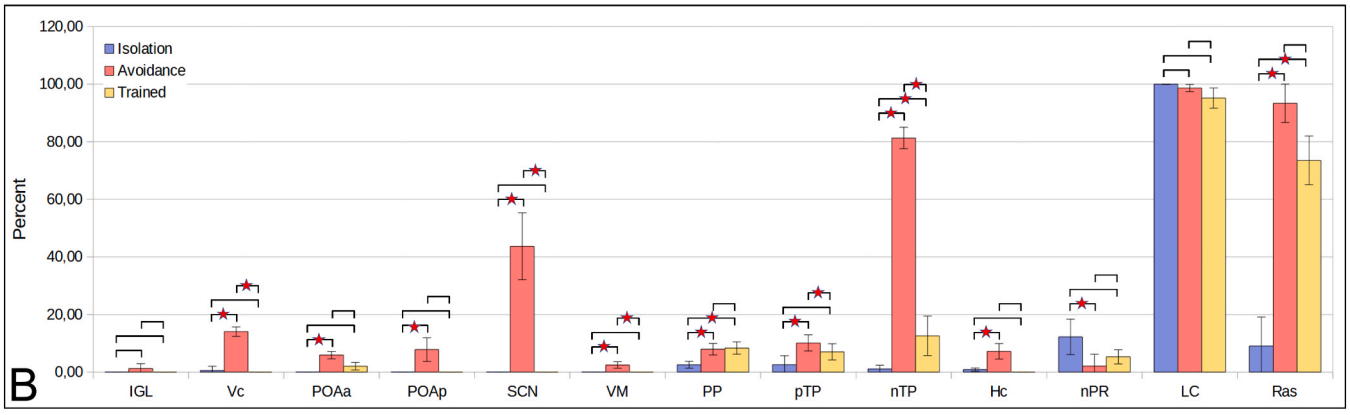
4.1. Dopaminergic cells distribution and double-labeling

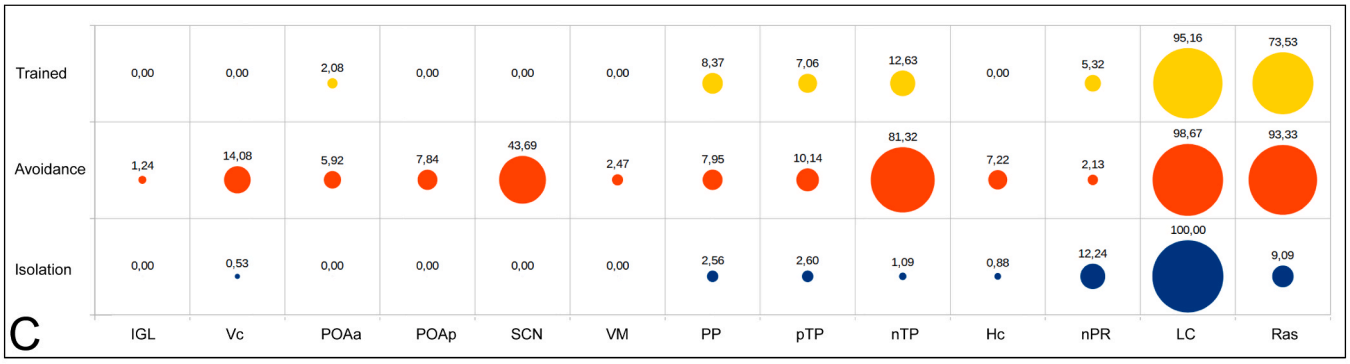
The dopaminergic system is well investigated in many vertebrate groups mainly by using antibodies against tyrosine hydroxylase (TH), the enzyme that catalyzes the rate limiting step in catecholamine synthesis (Fernstrom and Fernstrom, 2007) and thus present in all cells producing catecholamines. In the brain, noradrenaline is found only in the locus coeruleus and all TH+ cell populations rostral to the brain stem are considered to be dopaminergic (Ma, 1994). There are several TH+ cell populations described, extending from the olfactory bulb and preoptic area into the di- and mesencephalon, as well as rhombencephalon and spinal cord. In mammals, an A1-A17 classification scheme has been established (Lindvall and Björklund, 1984; Hökfelt, 1984; Smeets and González, 2000; Nagatsu, 2007). Studies in fish have mainly used a teleost-specific nomenclature (see references below), even though more recent studies in zebrafish have adopted the mammalian classification. To facilitate the comparison with older fish literature, we use here the traditional terminology. The areas we identified in *P. zebra* correspond well to other studies in a variety of fish species (*Carassius*, Hornby et al., 1987; *Anguilla*, Roberts et al., 1989; *Apteronotus*, Sas et al., 1990; *Clarius*, Corio et al., 1991; *Dicentrarchus*, Batten et al., 1993; *Salmo*, Manso et al., 1993; *Gnathonemus*, Meek et al., 1993; *Solea*, Rodríguez-Gómez et al., 2000; *Danio*, Rink and Wullimann, 2001; *Rhodeus*, Pushchina, 2009; *Poecilia*, Parafati et al., 2009; Filippi et al., 2010; *Astatotilapia*, O'Connell et al., 2011; O'Connell et al., 2013a; *Cirrhinus*, Kumar et al., 2014; *Porhichtys*, Goebrecht et al., 2014; *Nothobranchus*, Borgonovo et al., 2021).

Although the location of the cell populations and the distribution of their processes is well known, specific connections of TH+ cells are not known and additional studies are required. Moreover, little is known about the function of the different subsystems in teleost fish. Activation markers have been used to study some subcomponents, but the more commonly used nuclear markers c-fos and egr-1 may not be expressed in all dopaminergic systems. In contrast, the ribosomal marker pS6 shows activation in many more areas (Calvo et al., 2023). We investigated here the activation of TH+ cells in 13 different areas in two experimental groups and the Isolation group. This shows for the first time how different behavioral situations are changing the balance between the different dopaminergic subsystems that all act together to determine the complex emotional, motivational, and hormonal state of the animal.

The activation of TH+ cells shows considerable variations both between but also within groups. In some areas, some animals within a group may show no activation whereas others had a high percentage of double-labeled cells. This may be due to nonlinear response properties. In addition, many areas showed zero activation, particularly the Isolation group. This made it difficult to find an appropriate statistical test. Tests based on normal and equal distributions are not appropriate whereas non-parametric tests based on ranks do not take the magnitude of the activation into account and values with many zeros (ties) are problematic. The Fisher exact test was used to test for significant differences here. In this test, all TH+ cells found in all animals of a group were pooled for each brain area and the number of double-labeled cells compared among groups. The Fisher test can be applied for such data, but it ignores all within-group variations. The non-parametric Mann-Whitney test can compare within-group variations based on ranks, but fails in cases with many zero values (ties in the ranks). As an alternative







Fisher Test

A / I	IGL	Vc	POAa	POAp	SCN	VM	PP	pTP	nTP	Hc	nRP	LC	Ras
p	0.06856336	0.00000001	0.00294038	0.00082442	0.00000000	0.01006037	0.00000635	0.00001009	0.00000000	0.00026908	0.02194826	0.57692308	0.00020475
p (adj)	0.89132368	0.00000010	0.03822488	0.01071742	0.00000000	0.13078476	0.00008256	0.00013112	0.00000000	0.00349798	0.28532736	7.50000000	0.00266169
T / I													
p	1.00000000	0.45563549	0.30000000	1.00000000	1.00000000	1.00000000	0.00083635	0.61285344	0.00000428	0.21573120	0.19406209	0.14538884	0.00000170
p (adj)	13.00000000	5.92326139	3.90000000	13.00000000	13.00000000	13.00000000	0.01087252	7.96709476	0.00005569	2.80450554	2.52280719	1.89005497	0.00002204
A / T													
p	0.15286155	0.00003466	1.23760986	1.04090211	0.00022006	0.04396242	1.64958881	0.00411084	0.00000000	0.99829634	0.28100000	0.56894654	0.11169625
p (adj)	1.98720014	0.00045061	16.08892822	13.53172739	0.00286073	0.57151143	21.44465458	0.05344086	0.00000000	12.97785238	3.65300000	7.39630497	1.45205129

Mann-Whitney

A / I													
p	0.2189117	0.000145369	0.00355367	0.00910504	0.00265082	0.09184881	0.01558781	0.19472538	0.00017482	0.01696521	0.7512481	0.29084962	NA
p (adj)	2.8458521	0.001889797	0.04619771	0.11836552	0.03446066	1.19403453	0.20264153	2.53142994	0.00227266	0.22054773	9.7662253	3.78104506	NA
T / I													
p	NA	0.31885944	NA	NA	NA	NA	0.11840682	0.20507806	0.20507806	0.34798425	0.60096879	0.11517662	NA
p (adj)	NA	4.14517272	NA	NA	NA	NA	1.53928866	2.66601478	2.66601478	4.52379525	7.81259427	1.49729606	NA
A / T													
p	0.9049317	0.0001	NA	NA	0.01083507	0.09184881	0.97011415	0.99822141	0.00017538	0.00222908	1.06277096	0.483576775	NA
p (adj)	11.7641121	0.0013	NA	NA	0.14085591	1.19403453	12.61148395	12.97687833	0.00227994	0.02897804	13.81602248	6.286498075	NA

D

Fig. 4. Charts of the activation of the 13 dopaminergic cell populations. A: Dot plot showing the activation of all animals of the three groups for each brain area in percent on the y axis. Individual animals are spread out in the x axis if they have the same value. B: Mean percent of co-localization of pS6 and TH in all three groups. A star indicates significant ($p < 0.05$) differences according to the Fisher test between the groups. C: Bubble plot of the activation data showing the percent of activated TH+ cells in the three groups as a matrix with the size of the circles reflecting the magnitude. D: p-values according to the Fisher test and the Mann-Whitney test for all brain areas and all possible group combinations. P (adj.) are the Bonferroni adjusted values. Green cells indicate p-values < 0.05. All original values and cell counts can be found in the supplementary materials. The Fischer test has always one degree of freedom.

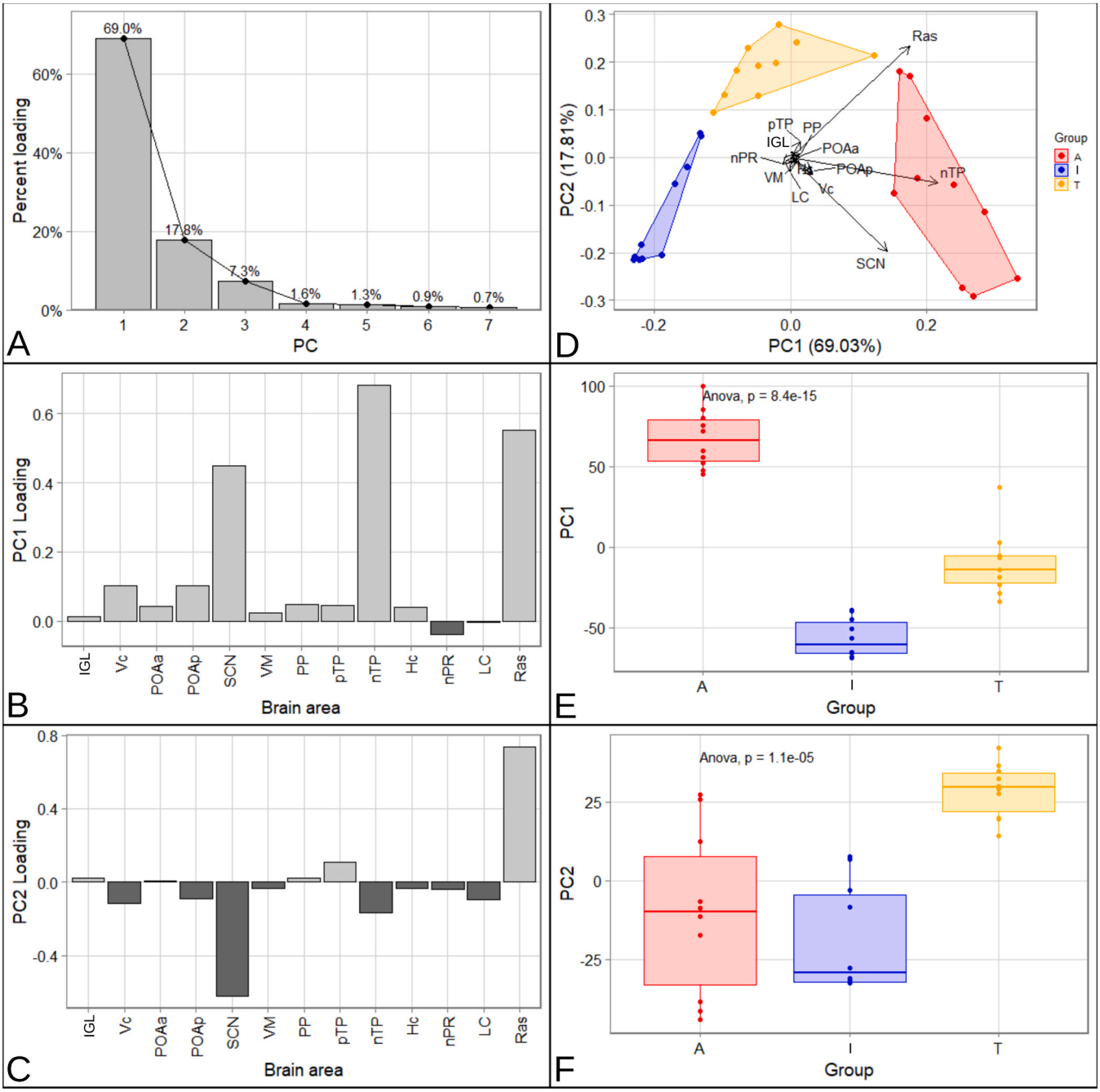


Fig. 5. PCA analysis of the activation in the 13 dopaminergic cell populations. (A) shows that the first component contains most of the variances in the data set. (B) shows the different contributions of the areas to the first component (PC1) and (C) the composition of the second component (PC2). The scatter plot in (D) shows the location of the groups with the PC1 and PC2 space. The differences between groups are mostly caused by SCN, nTP, and Ras in PC1. PC2 can still separate the Trained group from the others. (E) shows the box plots of the groups on the PC1 axis and (F) on the PC2 axis. All three groups can be separated by PC1. The Trained group is different from the others in PC2 due to the activation of Ras and a lack of activation in SCN in contrast to the strong activation in the Avoidance group. Groups are abbreviated as Isolation 'I', Avoidance 'A' and Trained 'T'. See Table 1 for a list of brain part abbreviations. Axes in D are scaled eigenvalues.

to statistical tests, we made a principle component analysis and a cluster analysis that are both based on the pattern of activation in all brain areas.

The data for each of the 30 animals were analyzed with these methods that are initially not aware of any group memberships. The PCA showed in the first two components the combination of areas with the largest variance in the data set. When these data are plotted in a scatter plot and post-hoc color coded for groups, it becomes clear that the largest variations in the data set are due to group differences. This was tested with an ANOVA. An ANOVA is possible now because the values for the ANOVA are the sum of the weighted reconstructions of the principle components. This clearly showed that the different treatments (groups) are dominating the variances seen in the data set and not the within-group variances. In fact, the first principle component is very similar to the actual group differences shown in Fig. 4B.

The second analysis we performed was the cluster analysis. Here, all 30 animals were analyzed and grouped by the similarities based on their activation pattern. A post-hoc color coding shows that the activation pattern is highly characteristic for each group. Only two animals were assigned to the wrong clusters.

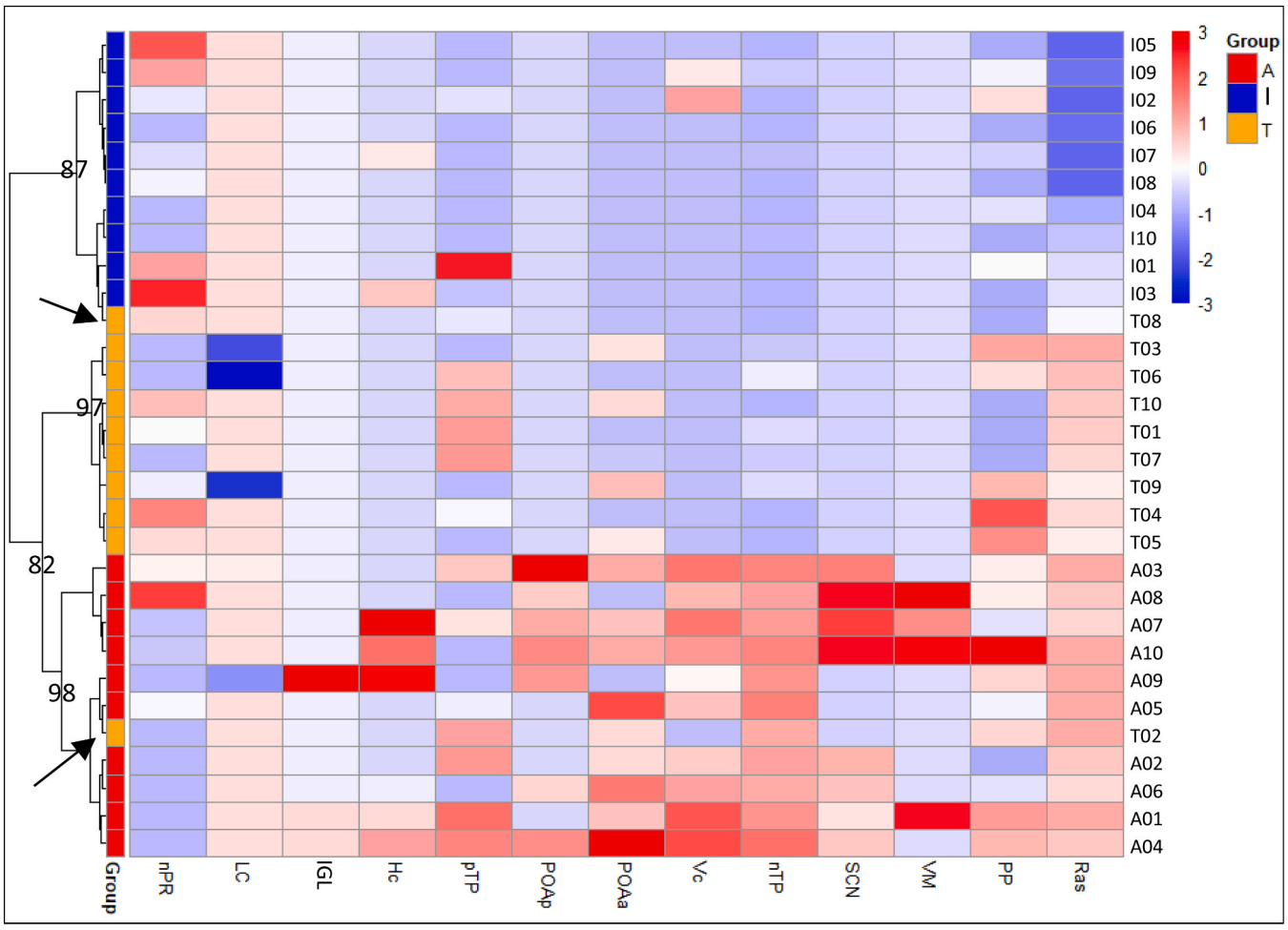


Fig. 6. Hierarchical cluster analysis of the data set. The animals are grouped according to the similarity of the activation pattern of TH+ cell populations. The heatmap of the activity pattern of each animal is scaled by column. Numbers on the dendrogram represent the p-values of the branchings. The color code next to the dendrogram shows the group assignments of the animals. The cluster analysis was reconstructing the correct groups almost perfectly, there were only two animals belonging to the Trained group that were clustered incorrectly (arrows).

Both, the PCA and the cluster analysis showed that there is a distinct pattern of the percentages of double-labeled TH+ cells that is group (treatment) specific. Although the activation of a single area in a given animal can be highly variable, the combined pattern across all areas is highly characteristic for each group.

4.1.1. *Telencephalic cell populations*

A large population of TH+ cells was located in the olfactory bulb as in all other vertebrates investigated so far. Other TH+ cells are located in different ventral telencephalic areas. In our material, most cells were found in the Vc and those were used to quantify double-labeling with pS6. Ventral telencephalic TH+ populations were found in all other studies in fishes, but their assignment to the different ventral areas differs. Some authors located also TH+ cells in Dc (Hornby et al., 1987; Roberts et al., 1989; Piñuela and Northcutt, 2007; O'Connell et al., 2011; O'Connell et al., 2013a), in Dp (Piñuela and Northcutt, 2007; O'Connell et al., 2011), in Dl (O'Connell et al., 2011; O'Connell et al., 2013a), and in Dm (Piñuela and Northcutt, 2007). TH+ cell populations in the telencephalon in addition to the IGL were also found in elasmobranchs (Meredith and Smeets, 1987), lampreys (Pierre et al., 1997; Pombal et al., 1997), and lungfish (Reiner and Northcutt, 1987) and seem to be basal vertebrate characteristic that was lost in tetrapods.

In our study, a specific activation of the TH+ cells was not found in the olfactory bulb although general pS6 activity did increase in the IGL in the Avoidance group (Calvo et al., 2023). Apparently, the pS6 activation did not affect the TH+ cells in the IGL. In contrast, the ventral telencephalic cell population (Vc) showed a specific activation of the TH+ cell population in the Avoidance group. The functional significance of this activation is not clear. The medial olfactory tract sends fibers into the area and the ventral telencephalon responds well to olfactory stimulation. However, there are projections from many other areas to the ventral telencephalon that could also affect the TH+ cells in this region. Most processes of the ventral telencephalic TH+ cells course laterally to innervate heavily Dp (O'Connell et al., 2011). This would indicate a modulation of information processing in the Dp. Interestingly, unilateral olfactory deprivation in zebrafish led to a down regulation of TH+ cells in the olfactory bulb but did not show changes in the ventral telencephalon (Kress and Wullimann, 2012) as indicated with co-localization of TH+ cells with egr-1. However, this study is not directly comparable to ours, as Kress and Wullimann (2012) investigated larval zebrafish that are about to be imprinted to kin odors and they used the nuclear marker egr-1, which is acting at a different level compared to the ribosomal pS6 marker.

4.1.2. *Preoptic-hypothalamic system*

TH+ cells in the preoptic-hypothalamic area are located in four specific populations, the POAa, POAp, Hc, and nPR. These areas are known to be involved in hormonal control and stress response acting on the pituitary (Tuomisto and Männistö, 1985; Semenova et al., 2014; Fontaine et al., 2015). The dopaminergic neurons of the POAa are

known to regulate the production of gonadotropin releasing hormone (GnRH) in an inhibitory way. In stressful situations, an increased dopaminergic level suppresses the production of GnRH, which in turn downregulates the release of gonadotropins in the pituitary (Kah et al., 1984; Kah et al., 1986; Kah et al., 1987; Anglade et al., 1993; Linard et al., 1996; Weltzien et al., 2006; Chabbi and Ganesh, 2015; Bhat and Ganesh, 2020). Dopaminergic cells in the nucleus of the posterior recess project directly to the pituitary and control the release of prolactin. Higher dopamine release suppresses the production of prolactin from the pituitary (Ben-Jonathan, 1985; Anthony et al., 1993; Freeman et al., 2000; Torner, 2016). It has been demonstrated that restraint stress in rats causes a decrease in the tubero-infundibular activity of dopaminergic neurons, with a consequent increase of circulating levels of prolactin (Shin, 1979; Moore et al., 1987). Thus, in stressful situations the dopaminergic activity in the POA would rise to suppress the release of gonadotropins and the dopamine production in the tubero-infundibular system would decrease to allow higher prolactin levels. This is exactly what we found in the Avoidance group. The POA TH+ cells showed higher pS6 co-localization but in the nPR co-localization was lower than in the Isolation group, indicating a deactivation. In the Trained group, no differences were found in both the POA cell populations or nPR.

4.1.3. Suprachiasmatic nucleus

Although we know the suprachiasmatic nucleus should be included in the "preoptic-hypothalamic system", we found it necessary to discuss it as a separate system, as the SCN contains the main pace maker of the circadian clock, which synchronizes other clock oscillators in the brain and throughout the body (Reppert and Weaver, 2002; Lowrey and Takahashi, 2011; Lu and Kim, 2022). The most important signal for the entrainment of circadian rhythms is light. The responses of several brain areas to photic stimulation have been well studied in many animals (e.g. Enger, 1957; Takeuchi et al., 1991; Leard et al., 1994). The effect of non-photic stimulations is mainly studied in mammals (Tahara and Shibata, 2018). Especially arousal stimuli like handling, social interactions, locomotor activity, and stress can change the circadian rhythm in mice and hamster (Antle, Mistlberger, 2000; Mistlberger and Skene, 2004; Mistlberger and Antle, 2011). Some studies in goldfish showed that different feeding times can be remembered (Sánchez-Vázquez et al., 1997; Sunuma et al., 2009). This shows that events can be associated relative to the circadian clock.

In some vertebrates, an important signal to synchronize different clock oscillators in the brain is dopamine (Mendoza and Challet, 2014; Moore and Whitmore, 2014; Korshunov et al., 2017; Grippo et al., 2020). In the present study, there was strong activation of TH+ cells in the SCN in the Avoidance group. Although there was no change in the light regime in our experiments, the timing of a strong event relative to the circadian clock may be an important signal that leads to an activation of the SCN.

4.1.4. Ventral thalamic and pretectal populations

In the diencephalon there are two dorsal cell populations with a large number of TH+ cells. One was located in the ventral thalamus (VM) and the other one in the periventricular pretectum adjacent to the posterior commissure (PP). Dopaminergic cells in the ventral thalamus were found in most other studies in fish. They may correspond to the VM of Ito et al. (1986), who investigated the connections of this nucleus in Rockfish *Sebastiscus marmoratus*. The VM receives inputs from many sources including the retina, TS, TO and telencephalon and it projects back to many of them. The specific connections of the TH+ cells are not known.

TH+ cells in the PP were also reported in many other fishes. Less is known about the connections of this cell population, but tracer injections into the tectum opticum consistently labeled cells in this area (Grover and Sharma, 1981; Luiten, 1981; Fiebig et al., 1983; Striedter, 1990; Schlussman et al., 1990; Perez-Perez et al., 2003; de Arriba and Pombal, 2007). Although double-labeling with TH antibodies was not done in those tracer studies, it seems the VM and PP are the major source of TH+ fibers in the tectum opticum and probably also to the torus semicircularis. The tectum opticum (colliculus superior) receives also dopaminergic projections in mammals. One source of these fibers is TH+ cells in the zona incerta (Bolton et al., 2015), which may not be homologue to the VM or PP in fishes. In reptiles and birds, cell populations were present in a location that could correspond to the PP of fishes (Smeets et al., 1986; López-García et al., 1992; Rodman and Karten, 1995). Although the homologies of the dopaminergic cells projecting to the tectum opticum is uncertain across vertebrate groups, studies in mammals show that information processing in the tectum (colliculus superior) is modulated by dopamine (Takakuwa et al., 2017; Valdés-Baizabal et al., 2020; Montardy et al., 2021). Our study showed an activation of PP in the Avoidance and Trained groups and an additional, although low, activation of VM in the Avoidance group only. This may indicate a specific modulation of tectal and/or toral information processing possibly due to the visual stimulation present in both groups.

4.1.5. Posterior tubercle

There were two TH+ cell populations in the posterior tubercle in our fish. One was a compact population in the pTP and another one with larger cells was located in the nTP. The posterior tubercle dopamine cells project primarily down into the brains stem and spinal cord (Tay et al., 2011), but some ascending projections to the telencephalon were also present (Rink and Wullimann, 2001). In this respect, they are similar to the A11 cell population of mammals (Björklund and Skagerberg, 1979; Takada et al., 1988; Takada, 1993). Traditionally, they are thought to modulate locomotion in the spinal cord, but there is accumulating evidence that descending projections also effect early sensory processing (Reinig et al., 2017; Haehnel-Taguchi et al., 2018). The nTP cells showed the largest activation in the Avoidance group and only weak activation in the Trained group. There was also some activation in the pTP. Individuals in the Avoidance group received very strong stimulation by being chased with a net, which enforced locomotion. But this also stimulated other senses with primary centers in the brain stem and spinal cord (lateral line, cutaneous mechanoreception, hearing/vestibular system). More refined experiments are necessary to discriminate between an activation of locomotion and the modulation of sensory processing.

4.1.6. Superior raphe

The superior raphe is known as a source of most of the serotonergic innervation within the brain. A subset of raphe cells are also known to produce dopamine in mammals (e.g. Ochi and Shimizu, 1978; Trulson et al., 1985; Stratford and Wirtshafter, 1990). It has been demonstrated that in mice their activity can be affected by learning (Groessl et al., 2018; Lin et al., 2020; Cho et al., 2021) and that are also involved in the formation and expression of aversive memory (Groessl et al., 2018; Lin et al., 2020), arousal and response to significant external events (Cho et al., 2017). Furthermore, the activity of dopaminergic neurons of the raphe reflects the incentive salience of the stimulus (Lin et al., 2021). In particular, dopaminergic neurons in the raphe are activated by rewarding stimuli, similar to the VTA neurons. Unlike these, raphe dopaminergic neurons are activated also by aversive stimuli (Matthews et al., 2016; Cho et al., 2017; Groessl et al., 2018). Therefore, experimental data show that raphe dopaminergic neurons encode the salience but not the valence of the stimuli (Lin et al., 2021). In fish, there are only two studies showing TH+ cells in the raphe (Ekström et al., 1990; Batten et al., 1993), but TH+ cells were consistently detected in the superior raphe in our material. The reason why other studies failed to detect TH+ cells in the raphe could be related to the observation that dopaminergic cells in the raphe respond to social isolation or the recovery from it (Matthews et al., 2016). They may have significant amounts of TH only under these conditions or they may have been overlooked since most studies focus on forebrain dopaminergic system in fish. Nevertheless, there was a strong activation of TH+ Ras cells in both the

Avoidance and Trained groups. Whether this is due to general arousal in these groups need to be determined with more refined experiments.

4.1.7. Locus coeruleus

Although the locus coeruleus is not dopaminergic, it is included in our study because it shows TH+ staining due to the production of another catecholamine, noradrenaline (Smeets and González, 2000). It shows 100% co-labeling with pS6 antibodies in the Isolation group, whereas the co-labeling was very low in other TH+ areas. In the Avoidance and Trained groups some non-double-labeled cells were found, but this was not significant. We don't know why the locus coeruleus shows a permanent activation in contrast to the dopaminergic cell populations. The locus coeruleus is interesting as it may play a role in attention and arousal (Ross and Van Bockstaele, 2021; Maness et al., 2022). However, it is unlikely that pS6 is modulating the arousal level because of the 100% labeling with pS6 in the Isolation group and the presence of pS6 in other larger neurons as well.

4.2. Multivariate analyses

Our multivariate analyses revealed complex co-activation patterns of dopaminergic and noradrenergic cell populations. Specifically, hierarchical clustering of the double-labeled TH+ cell populations demonstrated robust group differences in activity patterns. There is only one Trained specimen grouped together with the Avoidance cluster and one Isolation in the Trained cluster (see Fig. S1). PC1 of the PCA we conducted confirmed that differences between groups were largely driven by co-activation of a combination of the SCN, nTP, and superior raphe, suggesting that these activated TH+ cells might encode aspects of (negative) valence and possibly high salience associated with stressful stimuli of both the Avoidance stimulus and Trained task. Interestingly, double-labeled TH+ cells in the SCN and superior raphe loaded even more strongly on PC2, though in opposition to each other, thereby separating the Trained group from the other two experimental groups. This result may suggest that dopaminergic neurons in SCN and superior raphe may also have a learning and memory function in teleosts. Clearly, more detailed studies are required to understand these relationships better, and our results point at potentially profitable avenues of future research.

5. Conclusions

In the present study, we investigated the activation of dopaminergic cell populations by co-labeling TH and pS6 in individuals of the cichlid fish *P. zebra* subjected to different behavioral conditions. The dopaminergic system is composed of different subsystems with different functions ranging from sensory perception, hormonal control, regulating locomotion, to reward driven learning. The nature of each situation and any past experiences can change the balance of these subsystems and determine a complex emotional or motivational state of the animal. It is the first time the dopaminergic system has been investigated so in depth in a teleost, in particular showing how the activity of different dopaminergic cell populations are modulated in different behavioral conditions.

Ethical approval

The research reported herein was performed under the guidelines established by the EU Directive 2010/63/EU for animal experiments and the current German animal protection law and had been approved by the Landesamt für Natur, Umwelt und Verbraucherschutz NRW (approval number 8.87–50.10.37.09.198).

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Author statement

Calvo and Schluessel contributed to the study conception and design. Data collection, processing of brains and sections were performed by Calvo under the supervision of Hofmann HA in Austin, Texas, US. Hofmann MH designed the data analysis procedure, and Calvo analyzed the data. The first draft of the manuscript was written by Calvo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jchemneu.2023.102342.

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