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Divergent Evolution of Brain Structures and Convergence of Cognitive Functions in Vertebrates: the Example of the Teleost Zebrafish

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Divergent evolution of brain structures and convergence of cognitive functions in vertebrates: the example of the teleost zebrafish

Thèse de doctorat de l'Université Paris-Saclay
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Thèse présentée et soutenue à Gif-sur-Yvette, le 2 avril 2019, par

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Introduction

1. General introduction

Human cognitive capacities mainly rely on the cerebral cortex, a 6-layered structure only found in mammals and exceptionally developed in our species. Historically, the evolution of intelligence has therefore been focused on primates, which are our closest relatives and also possess an expanded cerebral cortex compared to other mammals. Accordingly, researchers have been focusing on the structural and computational properties of the cerebral cortex to explain the emergence of higher order cognitive functions.

Numerous behavioral studies on birds, in particular within the groups of corvids and parrots, show that some species possess capacities rivalling those of primates. These include tool use and manufacture, problem-solving, theory of mind, or anticipation. However, birds do not possess a laminated cortex: their pallium (region roughly corresponding to cortex in vertebrates) has a nuclear organization. Although some of the avian pallial circuitry appears to be similar to the mammalian one, this raises the question: how did similar cognitive functions emerge in such different brains that have evolved independently for over 300 million years?

Outside of amniotes, another group of vertebrates contains species with high cognitive performance: teleosts. It is a very diverse vertebrate radiation containing more species than tetrapods all together, and two groups in particular present some complex behaviors such as tool use or logical thinking: cichlids and wrasses. Teleosts make an interesting point of comparison given their evolutionary distance with amniotes. They present a derived brain structure: for instance, their telencephalon develops through evagination instead of invagination as in most vertebrates.

The aim of the research project which encompasses my thesis work is to identify crucial features in the vertebrate brain that could allow the emergence of higher order cognitive functions as observed in some species of mammals, birds, and teleosts. Based on comparisons between mammalian and avian brains, sensory projections terminate to the pallium in a modality specific fashion. They correspond to the primary sensory areas, which are primary sensory cortices in mammals. These primary sensory areas project to secondary sensory areas and undergo further integration into associative areas, bridging modalities together. These are on top regulated by an executive area, which is in the prefrontal cortex in mammals and in an equivalent region in birds, which finally allows the elaboration of a relevant motor output.

Thus, based on the amniote literature, we hypothesized that three factors could be important for evolving “intelligence” in some species.

- an expanded integration center, generally corresponding to the pallium

- sensory-motor integration within the pallium to generate voluntary movements, with modal-specific sensory inputs to specific primary sensory areas, topologically segregated.
- presence of an executive pallium, with dopaminergic inputs being critical for its function

These characteristics exist to some extent in mammalian and bird species. To test whether these are present in a yet more distant vertebrate group, we chose zebrafish as a model for teleosts. Although it does not present higher order cognitive capacities, it is able to perform reversal learning task (Parker et al., 2012), that is to learn reversed rule faster than the initial learning phase. This is typically used to test behavioral flexibility in other species. A related species, goldfish, has been used in operant conditioning paradigm, and is capable of learning matching-to-sample task (Goldman and Shapiro, 1979). Moreover, the availability of transgenic lines in zebrafish makes it an interesting model to study specific neuroanatomical pathway, as well as their developmental origin.

During my PhD project, I addressed two general questions: is the organization of the teleost forebrain comparable to amniotes? Do teleosts possess an executive function?

We brought elements of answer in three articles. Article 1 and 2 address the anatomy of the teleost brain, and article 3 is a behavioral study:

- In article 1, we show a case of divergent evolution in the brain of teleosts: the inferior lobe, a teleost-specific structure which was considered as hypothalamic (forebrain), is actually a midbrain structure. This brings a reassessment of its function: it may be a sensory-motor integration center, thus comparable to the pallium with no equivalent in amniotes.
- In article 2, we show that the main sensory relay nucleus of teleosts, the functional equivalent of dorsal thalamus in mammals, is of midbrain origin. In particular, the main visual pathway to the pallium seems to have evolved independently. This leads to the notion that the sensory relay nucleus does not have to be in the diencephalon to convey the information to the pallium.
- In article 3, we show that zebrafish can perform simultaneous and delayed matching-to-sample tasks. Thus zebrafish possess a working memory and therefore would possess a primordium of executive function. However, there are no dopaminergic cells in the zebrafish midbrain, which suggests that mesencephalic dopamine is not a critical factor to evolve higher order cognition.

Together these results suggest that brain evolution is more plastic than expected, and that a variety of brain architectures can bring the emergence of “smart” species.

The introduction of this work spans over different domains pertaining to evolutionary neurobiology, including comparative neuroanatomy, development, and behavior. Focuses include:

- General principles of vertebrate evolution
- A definition of structural homology
- Early development and regionalization of the vertebrate brains
- Brain morphology and diversity across vertebrates, as a consequence of adaptation

- Behavioral observations related to higher order cognitive functions, and introduction to operant conditioning as a tool to assess cognitive functions in different species
- Comparative neuroanatomy of the amniote and teleost pallia with a focus on sensory and executive areas
- The evolution of dopamine systems in vertebrates, demonstrating molecular and anatomical diversity across vertebrates

2. The vertebrate phylogenetic tree

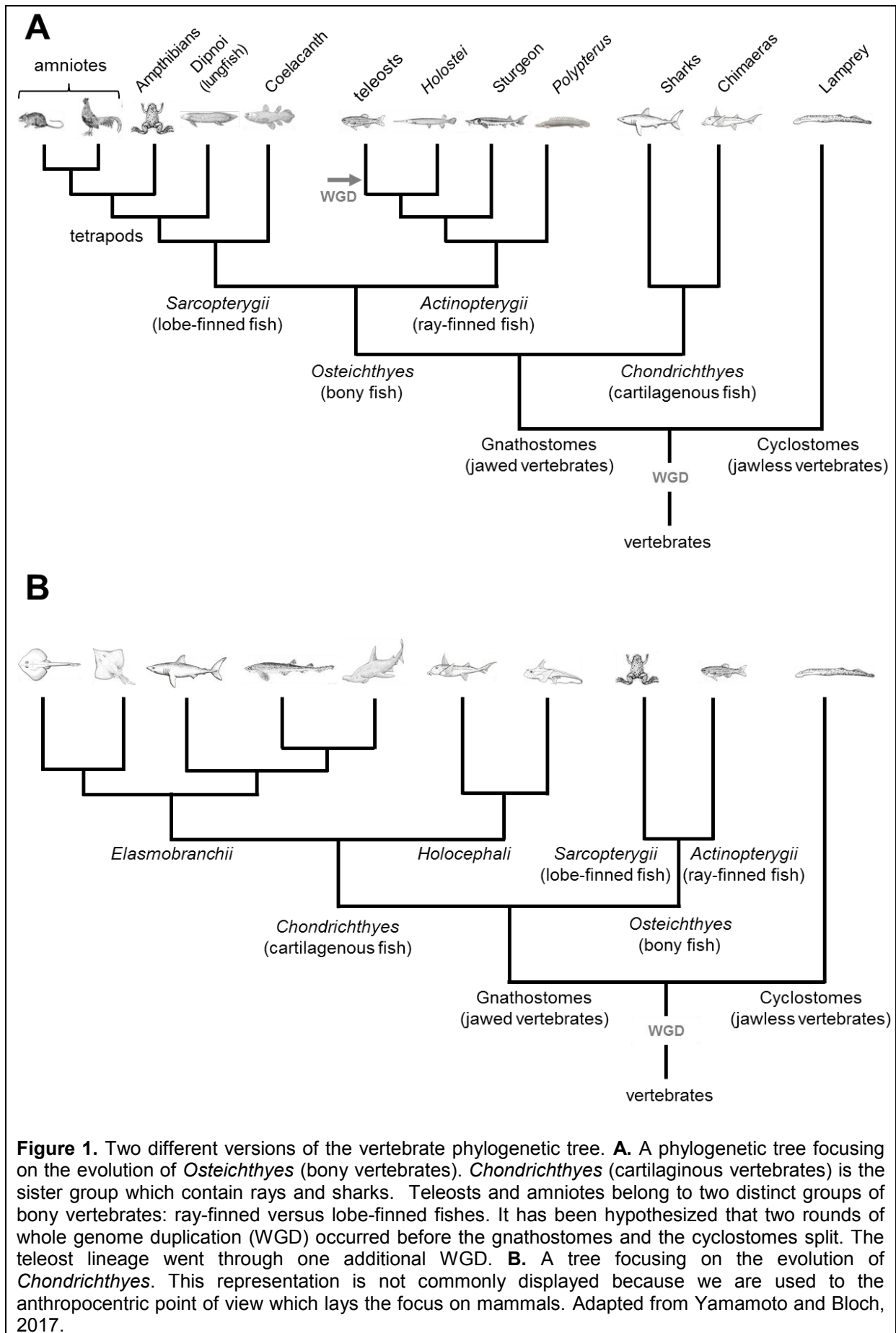
2.1 The phylogenetic position of teleosts in vertebrates

The brain is the enlarged part of the central nervous system (CNS) located in the head of bilaterian animals, which receives and processes information from sensory organs. It has appeared in different animal groups in bilateria such as insects, cephalopods, annelids or vertebrates. My thesis project focuses on the evolution of the vertebrate brain. As such, we rely on phylogeny to interpret neuroanatomical data and reconstruct the evolutionary history of brain structures.

Figure 1A shows a simplified version of the vertebrate phylogenetic tree. Vertebrates are divided into gnathostomes (jawed vertebrates, ~55000 species) and cyclostomes (jawless vertebrates, ~100 species like lamprey and hagfish), the only surviving group of agnathans. Gnathostomes comprise *Osteichthyes* (bony vertebrates, ~54000 species) and *Chondrichthyes* (cartilaginous vertebrates, ~1000 species, like sharks, rays and chimaeras). *Osteichthyes* consists of two large groups, *Sarcopterygii* (lobe-finned fish, ~27000 species) and *Actinopterygii* (ray-finned fish, ~27000 in Nelson et al., 2016). In the last 20 years, many species were discovered: in May 2018, there were actually 33264 confirmed species of ray-finned fish according to the catalog of fishes online (www.calacademy.org/scientists/projects/catalog-of-fishes, W. N. Eschmeyer et al., 2018).

Phylogenetic trees can be biased according to their layout and focus, which can be misleading for the non-specialist. A typical representation of the vertebrate phylogenetic tree is shown in Figure 1A: indeed, in many instances mammals are put on one end of the tree and greater detail is provided about the group of tetrapods. In the context of brain evolution, this can give the false impression that mammals possess a more “derived” or “evolved” brain, which is reinforced by the top to bottom appearance of the tree, with older branches being lower. It is simply because phylogenetic trees tend to be focused on the group of interest, for example, we could draw a phylogenetic tree as presented in Figure 1B if we focus on the evolution of *Chondrichthyes*.

A less misleading representation is shown in Figure 2A: radial representation illustrates that all the current species have evolved for the same time, and prevents the notion of “primitive” or “less evolved” groups of species (instead we use the term “basal” referring to an earlier separation from the rest of the tree). Moreover, these representations do not include extinct branches, in which different scenarios of brain evolution may have occurred.



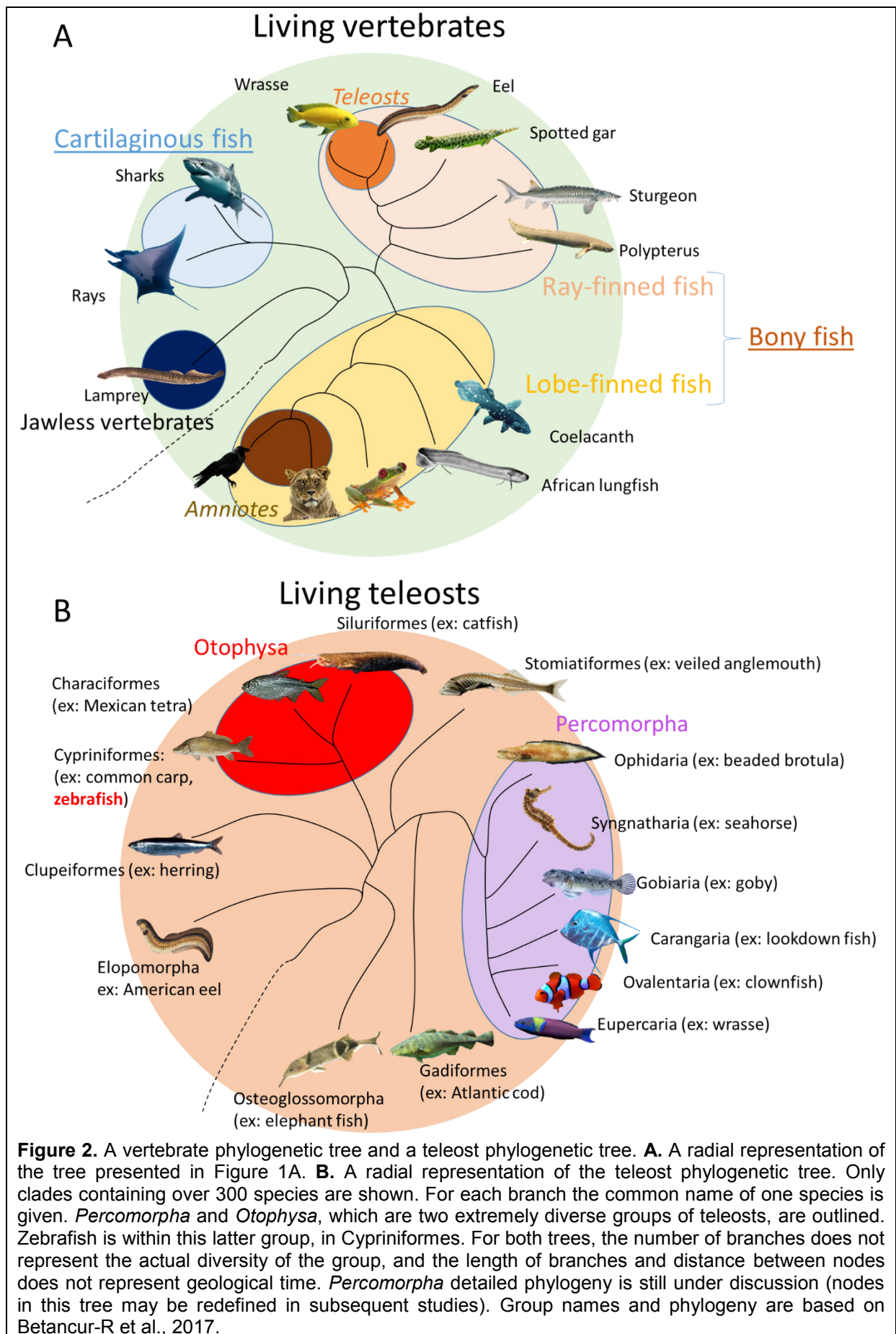
Sarcopterygii are bony vertebrates with fleshy fins. This group comprises lungfish, coelacanth and the whole group of tetrapods. Here it is worth noticing that “fish” is a paraphyletic and often misleading term designating aquatic species without four limbs. For example, lungfish are close relatives of tetrapods. They display rudimentary walk using paired pectoral and pelvic fins. Tetrapods are actually lobe-finned fish adapted to terrestrial life.

Actinopterygii are bony vertebrates with rayed fins. Most of the species are teleosts, only 49 species not being teleosts (W. N. Eschmeyer et al., 2018). These “non-teleosts” include gars and bowfin which are *Holostei* (the closest relatives of teleosts), sturgeons, and *Polypterus*. The latter is the sister group of all the other *Actinopterygii* and retains characters which may have been present in the *Osteichthyes* ancestor, such as lung-like structures permitting air-breathing in hypoxic water conditions (Lechleuthner et al., 1989; Tatsumi et al., 2016). Tatsumi et al. make the hypothesis that *Polypterus* lungs are homologous to tetrapod lungs based on similarities concerning gene expression, development (a primary lung of the foregut bud resembling early lung development in tetrapods), and presence of similar structures in lungfish and coelacanth embryo (Cupello et al., 2015).

Teleosts are a large and extremely diverse group, containing over 32000 species (Malmstrøm et al., 2016), which is as many as the rest of vertebrate species. A general vertebrate tree is shown in Figure 2A, as well as a more detailed tree of teleosts illustrating the diversity of this clade in Figure 2B.

My doctoral work is mainly conducted on one species of teleost, the zebrafish (*Danio rerio*). It belongs to a large and morphologically diverse group of freshwater fishes, Cypriniformes, with 4552 species in May 2018 (W. N. Eschmeyer et al., 2018). Taking into account that new species are discovered every year, some author compare the size of this radiation to the mammalian one in term of species count (Stout et al., 2016). The detailed phylogeny of Cypriniformes is an ongoing debate, since the definition of the families constituting the group and their phylogenetic relationships remain to be accurately determined. Briefly, zebrafish is a part of *Cyprinidae* (cyprinids), a family within Cypriniformes which has been confirmed as a monophyletic by molecular data (Mayden et al., 2009, n.d.; Stout et al., 2016; Tang et al., 2010). However, the resolution of the *Cyprinidae* phylogeny is still under construction (Gaubert et al., 2009; Tao et al., 2013).

Zebrafish belongs to the subfamily *Danioninae*, which has been redefined along with advances in molecular phylogenies (Tang et al., 2010) containing over 300 species (W. N. Eschmeyer et al., 2018). There are at least 17 species currently recognized within the *Danio* genus (Braasch et al., 2015). Interestingly hybrids of *Danio* species can be obtained *in vitro*, with several hybrids able to reach adult stage (Quigley et al., 2005), one (*Danio nigrofasciatus*) producing fertile hybrids (Parichy and Johnson, 2001).



2.2 The teleost genome, a highly divergent vertebrate genome

It is generally accepted that two independent whole genome duplication occurred during early vertebrate evolution (Dehal and Boore, 2005). However other alternatives such as a unique genome duplication going along with major chromosome rearrangements and segmental duplications are still debated, as well as the accurate timing of the duplication(s) (Smith and Keinath, 2015). These major duplication events would account for the origin of the vertebrate genome and serve as a basis for diversification and acquisition of novel functions across the lineage.

Yet this direct involvement of genome duplication in vertebrate diversification is still an ongoing debate. Although it is generally admitted that it can promote evolutionary flexibility, (Comai, 2005), it is difficult to reveal a simple link between genome duplication and consequent radiations or morphological complexification (Donoghue and Purnell, 2005) when fossil species are taken into account. A recent paper provides a hypothesis to explain why the effect of whole genome duplication can take a long time to appear (Robertson et al., 2017). Taking the case of the salmonid-specific whole genome duplication, the authors propose a “Lineage-specific Ohnologue Resolution” model, in which this delay is explained by a progressive rediploidization allowing divergence in lineages with ancestral whole-genome duplication.

One characteristic of the teleost lineage is one additional genome duplication (Amores et al., 1998; Taylor et al., 2003). This teleost-specific genome duplication and the evolutionary distance with tetrapods makes it often difficult to compare genomes from these two groups directly, although recent advances have been made such as with the spotted gar, a non-teleost genome, which is helping to bridge the two distant lineages (Braasch et al., 2016).

In teleosts, the high number of species and the morphological variability is also commonly explained by the third genome duplication, which would have facilitated speciation by gene duplication and decoupling. However, teleost evolutionary history is complex, and a closer look reveals that most of the biodiversity is concentrated in two groups containing over 10000 species each: *Ostariophysii* and *Percomorpha*. *Ostariophysii* roughly corresponds to *Otophysa* in Figure 2B, almost exclusively freshwater species like carps, danios and catfish. *Percomorpha* (perch-like, perciforms) diverged over 150 million years (Ma), after the teleost genome duplication (Hurley et al., 2007; Near et al., 2012). DNA sequence analysis suggests that although genome duplication has played a role in the initial teleost diversification, the secondary diversification events that occurred in these two teleosts groups account for approximately 88% of teleost diversity (Santini et al., 2009). Likewise, the degree of morphological diversification in *Holostei* and teleosts was similar at the Mesozoic period (before teleost main diversification event), challenging the simple explanation of a causal link between genome duplication and phenotypic variability (Clarke et al., 2016). The accurate influence of genome duplication on radiation and morphological diversity thus remains to be elucidated, although they roughly correlate. Nevertheless teleosts display high molecular evolutionary rates and their genome has been qualified as “highly divergent as compared with genomes of other jawed vertebrates” (Ravi and Venkatesh, 2018).

Contrary to other large vertebrate radiations such as mammals or birds, there has been a lack of consensus concerning the phylogeny within teleosts, as well as their time of

divergence. However recent molecular data has provided more solid resolution for teleost phylogeny (Betancur-R et al., 2017; Near et al., 2012). According to the latter study, ray-finned fish would have diverged over 400 Ma ago, while teleosts around 300 Ma ago.

3. The concept of homology

Richard Owen first defined homology as “the same organ in different animals under every variety of form and function” in 1843, in opposition to analogy “a part or organ in one animal which has the same function as another part or organ in different animal” (Owen and Cooper, 1843). Homology is a central concept of comparative anatomy (Kleisner, 2007; Wagner, 1989), and it is the central question of my thesis, relating it to neuroanatomy and cognitive functions. But what is the meaning of homology applied to biology, in other words, when can we say that two structures or characters are homologous?

Two structures are defined as homologous by comparison between two species; it is thus a notion more abstract than just a part of the phenotype. G. Wagner gives three properties that define a “homolog”, or homologous structure: conservation, individuality and uniqueness (Wagner, 1989). That is to say, two structures are homologous if they retain certain characteristics in spite of changes in shape and function, if they are clearly individualized during development, and only if they characterize one monophyletic group (that is, inherited from one common ancestor). The latter point relies on the principle of parsimony, with which homology is inferred according to the relations of the species studied in the phylogenetic tree: for instance, if two sister groups of species possess a derived character, it is more likely that the trait evolved in the common ancestor (one step) rather than independently in the two lineages (two steps).

Homology is therefore generally used as a synonym of synapomorphy, a character, trait or state (“morphe”, shape) shared (“syn”) in a monophyletic group and admitted to be also shared with common ancestor (Patterson, 1988, 1982). Definitions of “character”, “trait” or “state” vary according to the author regarding the level of observation. The latter author adds a probabilistic approach to homology: in addition to similarity and conjunction, congruence with other homologies should be the strongest test for homology. If two sets of homology are in conflict, the most probable scenario is the one encompassing the most important number of species, groups and homologies.

The notion that two structures have to “share certain features” (Wagner, 1989) is not always valid (Striedter and Northcutt, 1991): the degree of similarity between two characters is not a sufficient criterion to determine homology. Indeed, there are cases of parallel evolution in which characters evolve independently a high degree of similarity. This introduces the notion of homoplasy as used first by Lankester: “Homoplasy includes all cases of close resemblance which are not traceable to homogeny” (Lankester, 1870; “homogeny” is an ancient term for homology). This implies different scenarios for homology or homoplasy, summarized in Figure 3.

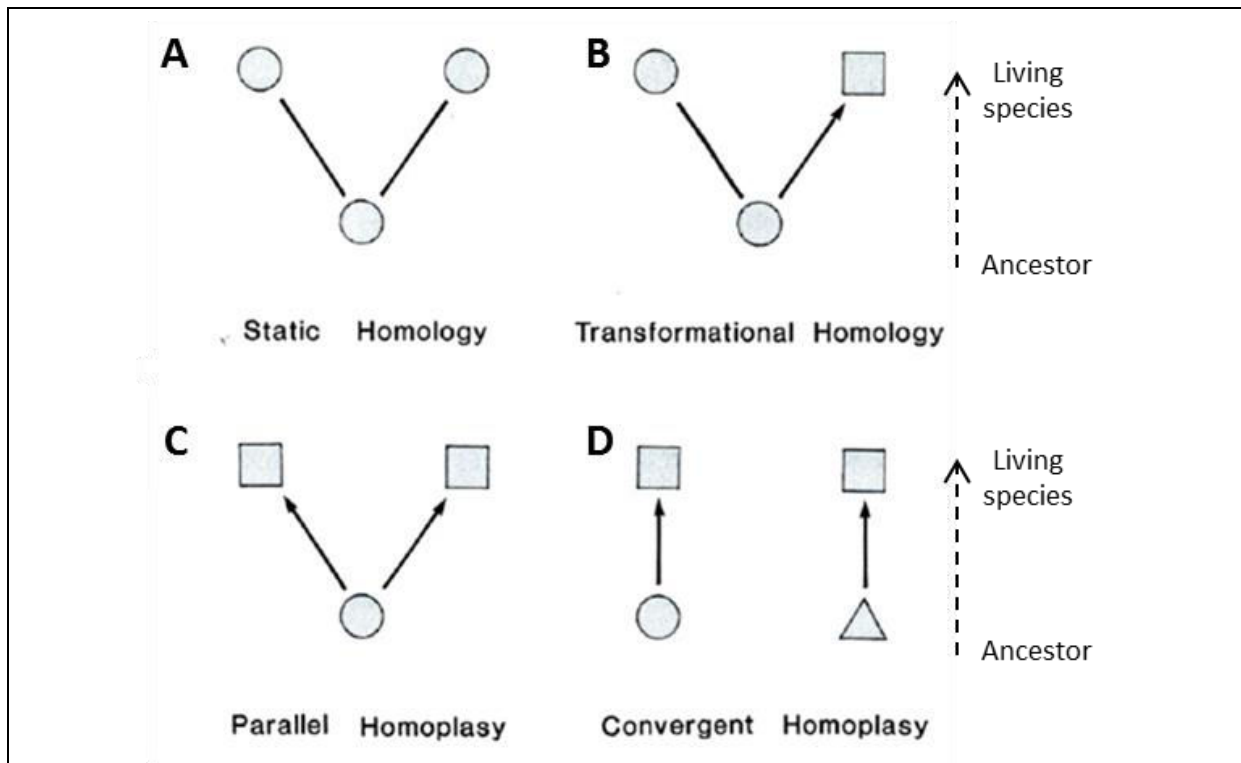


Figure 3. Illustration of the definitions for different cases of homology and homoplasy. In each situation, the trait in the ancestor is presented below and two living species are presented above. Evolutionary events are shown either by a line (no modification) or a full arrow (modification or “transformation” as used by the authors). Dashed arrows represent time passing from the ancestral until the living species. **A.** is the simplest case in which the two traits inherited from the common ancestor remain the same. **B.** the trait has been “transformed” in one of the two living species and thus is different from the ancestral trait although it is still homologous between the two living species (referred as “transformational homology”). **C.** is the case in which the trait has been independently transformed in the two species resulting in the same trait (homoplasy): it is homologous as an inherited ancestral structure (e.g. tetrapod forelimb), but the transformed state is not homologous between the two species (e.g. wing in bats and birds). This is defined as parallel homoplasy by the authors. It is a case of independent evolution of similar characters from the same ancestral character. **D.** The two characters have a different origin in the two species (e.g. tetrapod forelimb in birds and exoskeleton in insects), but are transformed to look alike (e.g. wing in birds and insects). This is defined as convergent homoplasy. Parallel and convergent evolution are two forms of independent evolution. These schemas also highlight a similarity of shape and function does not imply homology. Adapted from Striedter and Northcutt, 1991.

Importantly, homology cannot be reduced to a strict transmission of genes or developmental processes, but rather as a broader “continuity of information” (Van Valen, 1982). “Supracellular building blocks” (Wagner, 1989) such as tissues, organs, or structures are complex characters with multiple components like different cell types, proteins, and signaling networks. Since the parts may not share the same evolutionary history as the whole, it can be sometime difficult to establish homology. For instance this is discussed by Liebeskind et al. (2016) with the origin of the nervous system (Liebeskind et al., 2016).

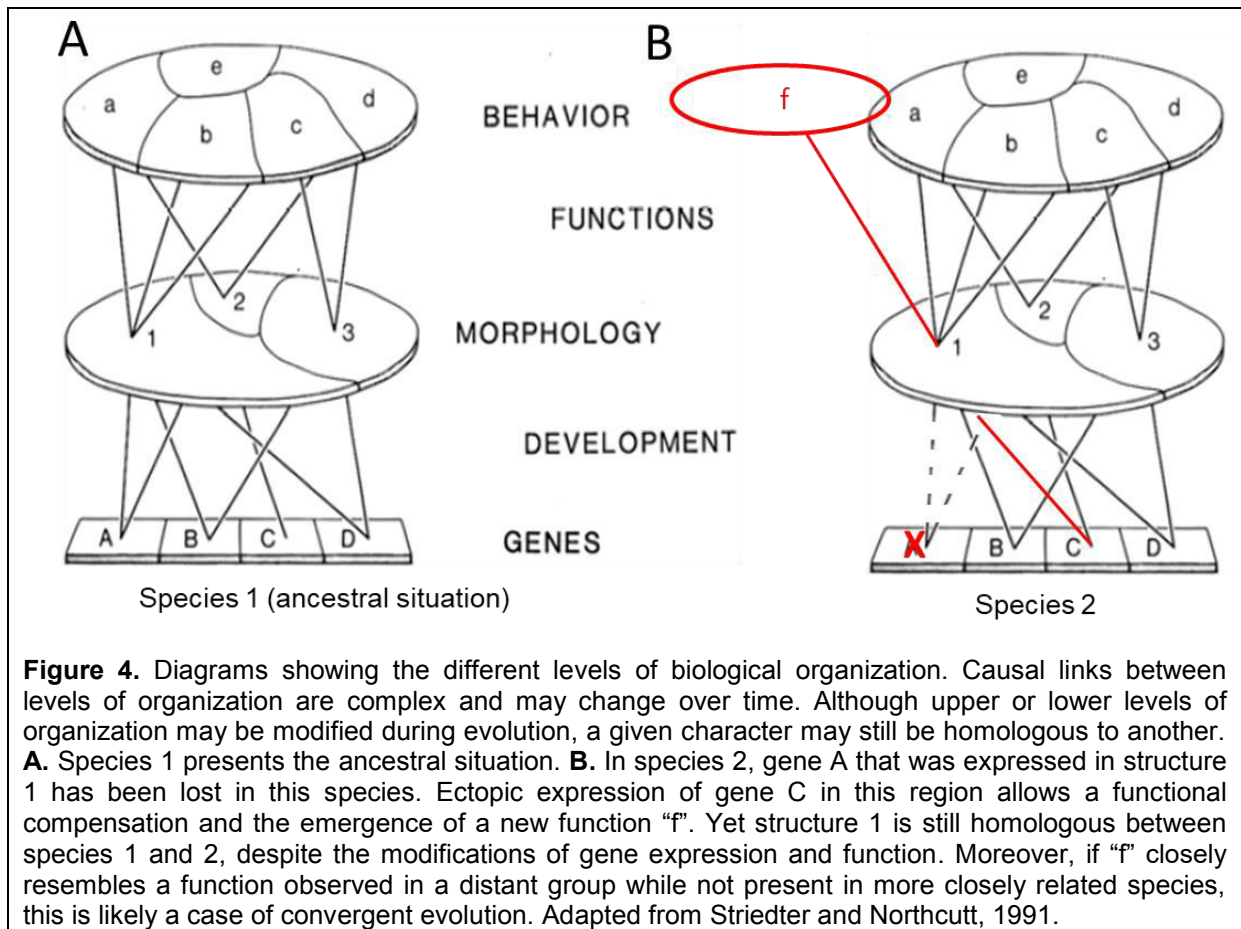
In Striedter and Northcutt, 1991, the authors propose a hierarchical concept of homology to answer this issue. This takes into account that a character can be situated at different levels of biological organization. One can compare genes, developmental processes, organs and their substructures, or behaviors. However, there is no simple one-to-one correspondence between characters at different levels of organization (illustrated in Figure 4).

For instance, a behavior may involve many different morphological structures and conversely, a structure can be involved in many different behaviors. Behavior is an interesting instance since many behaviors are hard to correlate to anatomical structures. Striedter and Northcutt (1991) review different examples of behaviors involving non-homologous structures: one of them is the generation of a weak electric field by gymnotoids (now more commonly called gymnotiformes, but I will keep their term), a group of teleosts. Gymnotoids possess an adult electrical organ derived from muscle tissue innervated by spinal motor neurons, and a larval derived from a different muscle mass but also innervated by spinal motor neurons. The apteronotids, a family within gymnotoids, possess a different electric organ derived from the axons of spinal motor neurons. A muscle-derived electric organ is present in larval apteronotids (similar to other gymnotoids), but it degenerates during development. The authors conclude that “it is more parsimonious [...] to conclude that the ability to generate weak electric fields evolved in the common ancestor of all gymnotoids and was retained with a continuous history in its descendants, i.e. that the behavior is homologous among all gymnotoids, and that the adult myogenic electric organ was [...] lost in the common ancestor of apteronotids and was functionally replaced by the neurogenic organ”.

Likewise, a single gene can participate in different developmental processes of various structures while the development of one structure may implicate many genes. Moreover, relations between those levels of organization may change along evolution (Wagner, 2007).

A simple example of independent evolution is the wing. It has independently evolved in some groups of metazoans. For instance, bats and bees have evolved convergently wings for flight (next I use “wing” as a short cut for “a bilateral pair of wings” since examples given are bilaterian animals). In this case, the components are non-homologous: the insect wing arises from the exoskeleton, while the bat wing derived from the tetrapod forelimb. However, the bat wing and the bird wing are non-homologous as well, but both arise from a tetrapod forelimb. As such, they are homologous as forelimbs, with homologous bone elements, but not as a wing.

This vision of homology implies that these levels of observation should not be mixed. When comparing two characters at a given level of observation, a character is homologous to another character, or not. This excludes the notion of partially homologous or independently evolved characters. The issue is summarized by Striedter and Northcutt as “if characters could be partially homologous, then any characters that involve the action of even a single pair of homologous genes would have to be considered partially homologous” (Striedter and Northcutt, 1991). Homologies also allow the reconstruction of monophyletic groups and the relations between each group according to their distribution (Patterson, 1982). For the sake of clarity, I will use the definition of homology as a synonym of synapomorphy, that is a trait at a given biological level of organization inherited from a common ancestor but which may have undergone drastic changes during evolution. These potential changes are the reason why one requires observing the evolutionary history carefully, preferably on a relevant variety of species and groups, before concluding to homology. Taking the wing example again, one could not have the whole history of the structure without looking at the whole vertebrate tree: looking at non-tetrapods completes the information about the evolution of the tetrapod forelimb which is derived from a pectoral fin. The pectoral fin has also independently evolved the function to fly (or at least to glide) in flying fish (Davenport, 1994).



4. General morphology of the vertebrate brains

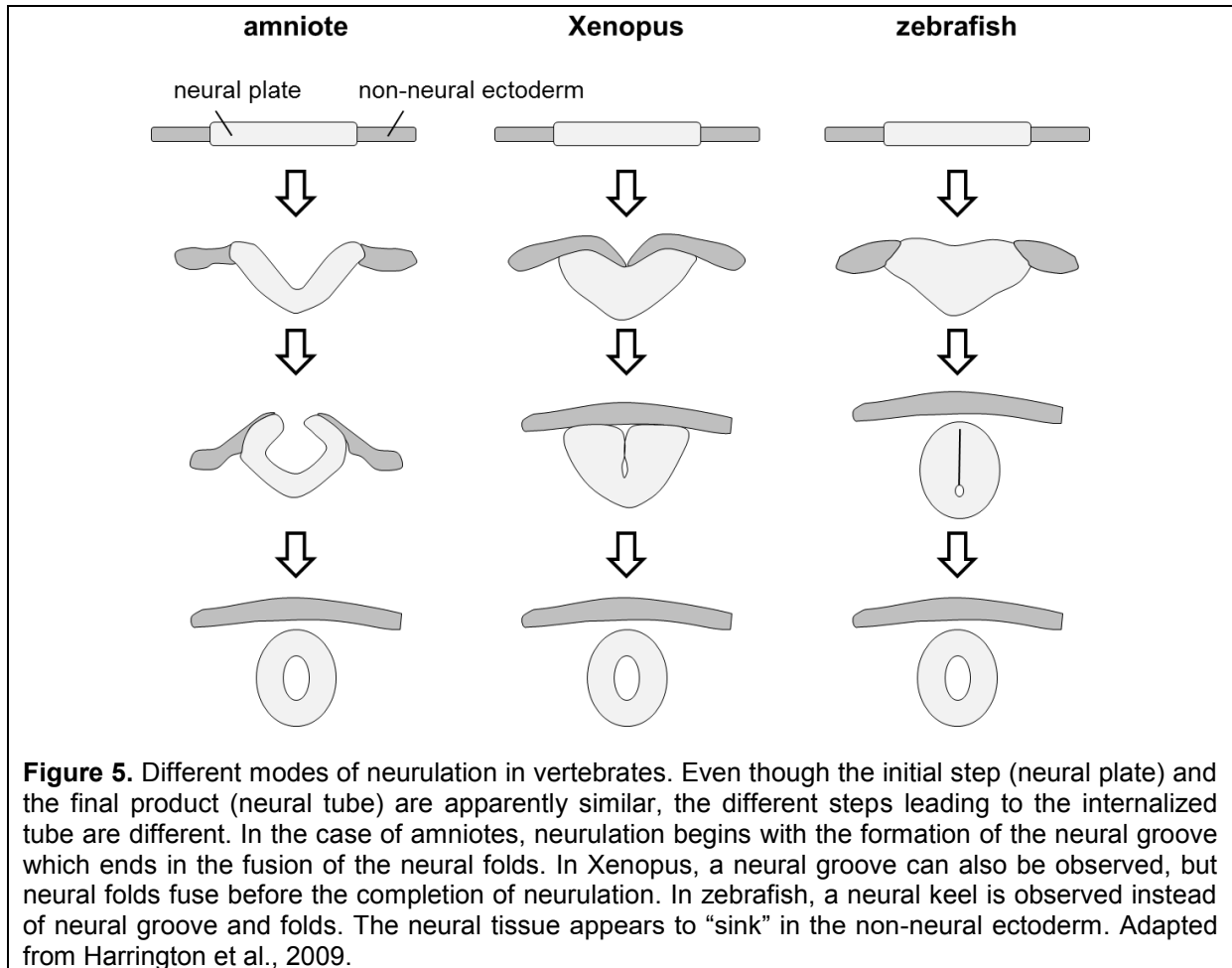
4.1 Early morphogenesis

4.1.1 Formation of the central nervous system

The vertebrate CNS originates in the dorsal epiblast of the gastrula. It develops from an epithelium that surrounds a central lumen: the neural tube. Its formation occurs through the process of neurulation, during which a flat sheet of cells, the neural plate, bends and folds to ultimately form the hollowed neural tube. It will give rise to the brain anteriorly and to the spinal cord posteriorly.

Morphological diversity is observed across vertebrates regarding the way to make a neural tube, based on the comparison between *Xenopus*, zebrafish and amniotes (Harrington et al., 2009). In amniotes, two types of neurulation have been described. Primary neurulation occurs anteriorly with the formation a neural groove surrounded by neural folds, which fuse in the midline to form the neural tube. Secondary neurulation takes place posteriorly at lumbar, sacral and tail levels with condensation of mesenchymal cells forming a transient solid rod, with the formation of a tube by cavitation (Colas and Schoenwolf, 2001). Amphibian neural tube closure looks similar to the amniote situation. Amphibians also undergo neural fold bending and fusion relatively rapidly at all along the neural tube, while amniotes display a sequential closure of the neural tube (“zippering”) with the appearance of closure points of the neural tube, varying in number according to the species (Nikolopoulou et al., 2017). The mode of neurulation in the teleost model zebrafish looks different (cf Figure 5). The lateral edges of the neural plate thicken and appear as a triangular shape with one

point towards the lumen on the tube (Lowery and Sive, 2004), forming the neural keel. In teleosts, the process of neurulation seems to be similar anteriorly and posteriorly, unlike amniotes (Harrington et al., 2010, 2009). Neurulation process in teleosts has similarities with both primary and secondary neurulation in amniotes (Araya et al., 2016). In spite of the morphological variability of the neurulation process across vertebrates, underlying cellular and molecular mechanisms display important similarities (Nikolopoulou et al., 2017).



As such, formation and regionalization of the neural tube is induced by signaling molecules called “morphogens” that are secreted from cell clusters called “signaling centers” or “organizers”. Different organizers appear during development. The Spemann-Mangold organizer (primary organizer) was defined in amphibians (Spemann and Mangold, 1924) through graft experiments of the dorsal lip of the blastopore in newts. They showed that the dorsal lip of the blastopore is essential both for neural ectoderm formation through planar and vertical signalizations and neural induction that consists in the transformation of the neural plate into a neural tube. In other vertebrate groups, the Spemann-Mangold organizer has been compared to Hensen’s node in mammals, and Kupffer vesicle in teleosts, although they do not recapitulate all the properties and functions of the amphibian organizer, some of these activities being taken by other groups of cells (Arias and Steventon, 2018). Moreover the inducing signals emanating from the organizer come both from the mesoderm under the neural plate but also from the ectodermal part of the organizer, thus suggesting that the properties of the organizer are distributed across different cell populations (Stern, 2001).

Different families of signaling molecules are involved in neural plate formation. A first proposition originating in amphibians stated that neural tissue originates from the inhibition of bone morphogenetic proteins (BMP) secreted from the non-neural ectoderm by antagonists like chordin, follistatin and noggin. This idea that neural tissue is induced by inhibition of pre-existing signaling pathway has been named the “default model” (Hemmati-Brivanlou and Melton, 1997). Ulterior studies have shown that it is not as simple. They show the involvement and complex interplay of other morphogens, such as fibroblast growth factors FGF (Linker and Stern, 2004; Rentzsch et al., 2004), sonic hedgehog (Shh) from the notochord (and other hedgehog proteins), and possibly other factors such as Wnt planar signaling, calcium, retinoic acid, and protein kinase C and cAMP (Pera et al., 2014; Stern, 2005). Neural tube closure involves various signaling pathways (BMP, Shh Wnt...), cellular movements and changes in morphology (Fournier-Thibault et al., 2009; Nikolopoulou et al., 2017). Once the neural tube is formed, the dorsal half influenced by BMP is defined as the alar plate, the ventral half influenced by Shh the floor plate (Nitzan et al., 2016; Ribes et al., 2010).

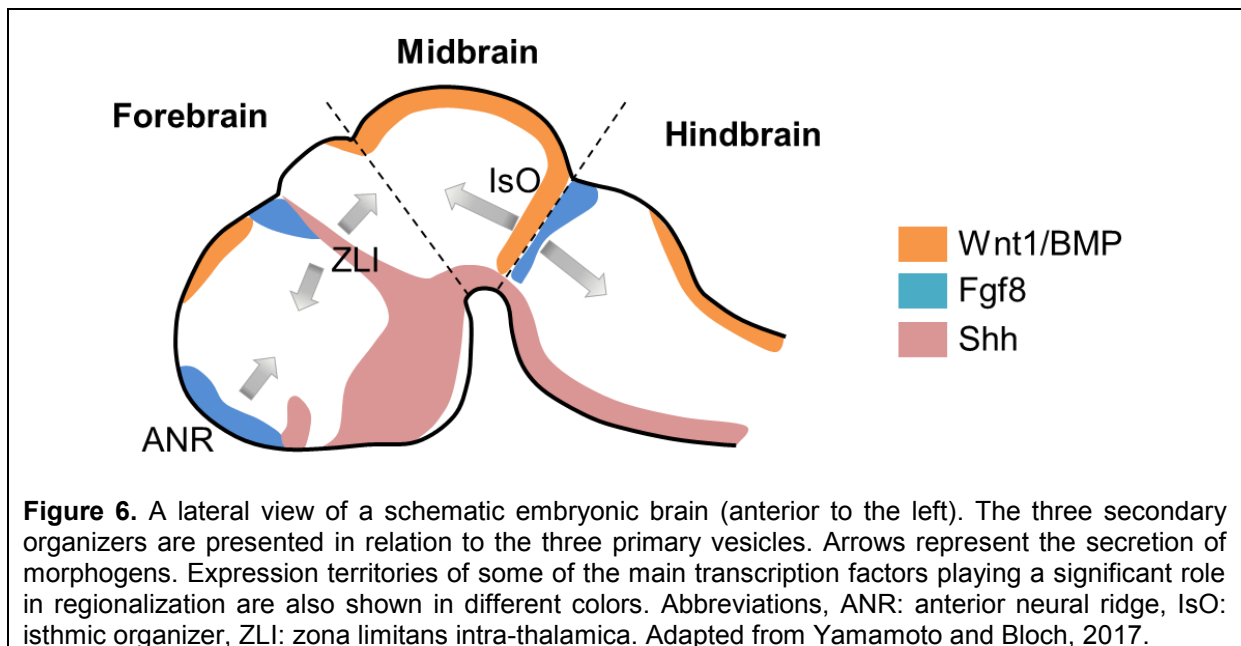
It is generally accepted that the neural tube gives rise to three primary vesicles anteriorly delimited by constrictions: the forebrain (prosencephalon), the midbrain (mesencephalon) and the hindbrain (rhombencephalon) which is continuous with the spinal cord posteriorly. They were first described by von Baer in the 1820's and used formally for comparison across vertebrates by von Kupffer in 1885 (Swanson, 2014).

However, this classical trichotomy can be questioned (Albuixech-Crespo et al., 2017; Ishikawa et al., 2012). Important morphological variations exist across vertebrates in terms of the nature of the accurate location and form of the constrictions delimiting those primary vesicles (Ishikawa et al., 2012). In addition gene expression patterns do not necessarily correlate with the morphological divisions (Kage et al., 2004). Thus, as for the neurulation process, it is important to consider that the subdivisions of the neural tube may be more variable than expected. We also have to bear in mind that these theories are mostly based on studies in tetrapods, therefore these models have to be confirmed encompassing different vertebrate groups.

4.1.2 Patterning of the neural tube: the secondary organizers

“Secondary” organizers come later to shape neural tube development, as opposed to “primary” ones which are non-neural sources of signal capable of inducing undifferentiated ectodermal tissue into a neural destiny (Puelles, 2016). Morphogens secreted from secondary organizers further refine the antero-posterior and dorso-ventral patterning of the neural tube. These secondary organizers have been described as transverse domains along the antero-posterior axis of the neural tube located at “genetic borders”, therefore juxtaposing transcription factors from both sides (Figdor and Stern, 1993; Joyner et al., 2000). They are local signaling centers and are considered to give rise to the three primary vesicles (Vieira et al., 2010). These organizers are defined as small populations of cells which ablation leads to the lack of surrounding structures, while their graft or the ectopic expression of their inducing signals brings the appearance of ectopic structures (Scholpp and Lumsden, 2010). Moreover, the spatio-temporal context of appearance and action of these structures is crucial to elicit the specific responses and differentiation of the target sites. Three secondary organizers have been identified so far in the brain (Figure 6): from rostral to caudal, the anterior neural ridge (ANR) at the most anterior part of the neural plate,

the zona limitans intra-thalamica (ZLI) within the diencephalon, and the isthmus organizer (IsO) at the midbrain-hindbrain boundary (MHB).



4.1.2.1 The Anterior Neural Ridge (ANR)

The ANR is considered as fundamental for the development of the anterior forebrain. Initial hypotheses about the existence of the ANR come from grafts in quail-chick chimeras (Couly and Le Douarin, 1987, 1985), in which it was observed that the most anterior part of the neural tube, especially the “ridges” (corresponding to the elevation of the neural folds) participate in the formation of the telencephalon. Later, in zebrafish, ANR has been defined as a structure at the junction between the most rostral part of the neural plate, the anlage of the anterior commissure and the non-neural ectoderm (Houart et al., 1998; Vieira et al., 2010). Its equivalent in other vertebrate group is still under investigation. In the current literature, it is admitted that the anterior ectoderm of vertebrate embryos appears to have organizing properties, but the accurate location and nature of the ANR remain to be determined. Indeed, the definition of the ANR varies with the model and the developmental stage. It seems to be distributed across the anterior ectoderm in mouse (Cajal et al., 2012) while being localized more anteriorly in zebrafish (Houart et al., 1998).

ANR organizing properties were first identified experimentally in zebrafish through ablation at mid-gastrula stage of the most anterior row of cells in the neural ectoderm. The ablation brought ectopic expression of *Shh* in the anterior forebrain, as well as altered expression of *dlx2* and abolished *emx1* expression (Houart et al., 1998). The same study shows that ectopic transplantation of this “first cell row” induced ectopic expression of *emx1* and *dlx2*, and that this inducing activity appears to be complete by late gastrulation, since ablation at this stage no longer modifies forebrain development. Secreted factors that have been shown to mediate anterior forebrain regionalization are *Fgf8* (for *Fgf8* there are data from mouse, chicken, *Xenopus* and zebrafish) with several other members of the Fgf family which influence the development of the rostral telencephalon (Creuzet et al., 2004; Eagleson and Dempewolf, 2002; Hoch et al., 2015; Storm et al., 2006). Studies in mouse and chicken suggest that *Fgf8* inhibits *Otx2* and *Emx2* expression in interaction with *Bmp4* (Crossley et al., 2001; Martinez-Barbera et al., 2001; Ohkubo et al., 2002).

Another important factor secreted around the anterior neuroectoderm close to ANR (data from mouse, chicken and teleosts) is *Shh* which plays a role in ventral specification throughout the brain including forebrain (Ericson et al., 1995; Miyake et al., 2005; Pottin et al., 2011; Shimamura and Rubenstein, 1997).

4.1.2.2 The Zona Limitans Intra-thalamica (ZLI)

The ZLI (also called mid-diencephalic organizer) is a transverse neuroepithelial domain located in the middle of the diencephalon. Located between the prethalamus and thalamus corresponding to prosomeres 2 and 3 (Puelles and Martinez, 2013), it is a thin strip of cells with a ridge on its ventricular surface with cell-lineage restricted boundaries (Larsen et al., 2001).

The formation mechanisms of the ZLI are still under investigation: it has been proposed that its initiation is due to the abutment of *Fez* and *Otx* transcription factors in mouse, chicken and zebrafish (Scholpp and Lumsden, 2010), while another set of studies argues that *Otx2* and *Barhl2* confer competence for the ZLI formation (Sena et al., 2016).

The ZLI has the peculiarity to express *Shh* both ventrally and dorsally, while this gene is generally expressed ventrally in the rest of the neural tube (Figure 6, red). In non-amniote species, the expression of *Shh* is used to define the ZLI. *Shh* has an important organizing function at the ZLI as promoting the survival, proliferation and patterning of neural progenitors in the diencephalon. Ectopic expression of *Shh* in chick (Kiecker and Lumsden, 2004) or *Shh* expressing grafts (Vieira et al., 2005) induce a local reorganization of gene expression that resembles the one found around the native ZLI in a correct antero-posterior order. In addition longer-term observation of *Shh* positive ZLI grafts at the level of future tectum (Vieira et al., 2005) modify the layered tectal organization to resemble the one observed more anteriorly around the ZLI (that are considered to give rise to the thalamus posterior to ZLI and prethalamus anteriorly). Graft and gain of function experiments in zebrafish have also shown that *shh*, along with *twhh* another member of the hedgehog family, are able to reorganize gene expression around ZLI (e.g. *dbx1a* and *dlx2a*) when ectopically expressed (Scholpp et al., 2006). Other families of secreted factors collaborating with *Shh* for proper diencephalic development in mouse, chicken, zebrafish and amphibians around ZLI are Wnts and FGFs (reviewed in Sena et al., 2016).

Scholpp and Lumsden propose three *Shh*-dependent steps for the patterning of the middle of the diencephalic region. First, in this model, the prethalamus and thalamus acquire different identities through *Shh* exposure, these two diencephalic regions having differential identities and responsiveness to inducing signals, possibly due to specific factors such as Iroquois genes (*Ir*) in the thalamic anlage and *Fez* in the prethalamus. Then, a posterior to anterior wave of *Neurogenin-1* and *Asc-1* expression leads to the differentiation of glutamatergic projection neurons and GABAergic interneurons respectively. Finally, a “finer” *Shh*-dependent specification takes place through a rostro-caudal gradient of this morphogen: the gradient is important for differentiation and establishing the nuclear organization of the thalamus (Szabó et al., 2009).

Results in mouse show that in addition to *Shh*, the differentiation of the thalamic nuclei is dependent on *Gbx2* (which has been showed to be controlled by *Shh* in a study in chick; Hashimoto-Torii et al., 2003). By analyzing the severity of a *Gbx2* or a *Shh* mutation on thalamic development, Szabó et al. (2009) show a differential involvement of the two genes:

Shh is critical for the lateral and medial geniculate nuclei while *Gbx2* is critical for medial nuclei. Expression of the two genes are necessary for central and medial nuclei.

4.1.2.3 The Ishtmic Organizer (IsO) at the midbrain hindbrain boundary (MHB)

The IsO is located at the level of a constriction of the neural tube between midbrain and hindbrain. It is within the midbrain hindbrain domain defined morphologically as the mesencephalic vesicle and the first rhombencephalic vesicle, the latter being subdivided in rhombomere 1 and 2 (Tallafuss and Bally-Cuif, 2003). It has been described as necessary and sufficient for the development of mesencephalic and rhombencephalic structures since it is able to induce them after ectopic transplantation, as shown initially in quail-chick chimeras (Martinez et al., 1991; Martinez and Alvarado-Mallart, 1990; Nakamura et al., 1988). Another important point made by graft experiments is that the tissue induction is polarized: upon reversal of the IsO, rhombencephalic (in particular cerebellar) structures were observed rostrally and mesencephalic (in particular tectal) ones caudally (Marin and Puelles, 1994).

Two genes in particular have initially been identified as involved in IsO positioning at the end of gastrulation: *Otx2* expressed in the forebrain and midbrain, and *Gbx2* in the hindbrain. They are expressed in exclusive domains anterior and posterior to the mesencephalic and rhombencephalic junction respectively (Hidalgo-Sánchez et al., 1999). *Otx2* and *Gbx2* repress each other and are involved in IsO induction and positioning (Katahira et al., 2000). Furthermore *Fgf8* inhibits *Otx2* expression and activates *Gbx2* expression while being regulated by the *Otx2/Gbx2* border, thus forming a feedback loop (Joyner et al., 2000; Liu et al., 1999). A number of other genes have been involved in IsO positioning, induction, and maintenance, depending on the species (Dworkin and Jane, 2013; Gibbs et al., 2017; Lee et al., 1997; Rhinn et al., 2005; Tossell et al., 2011b, 2011a). Also, several genes in Wnt and Fgf families (e.g. *Fgf8* and *Wnt1*) have been identified as important factors for the formation of tectum and cerebellum in mouse, chicken and zebrafish (Gibbs et al., 2017; Martinez et al., 1999; Matsunaga et al., 2002; Rhinn et al., 2009).

Below I will introduce the two genes involved the formation of the mesencephalic region, that we used in our tracing studies in zebrafish: *her5* and *meis2a*.

In zebrafish, the MHB contains a transverse stripe devoid of neurons called the intervening zone in which neurogenesis occurs through the inhibition of differentiation. The bHLH Hairy/E(spl)-related *her5* transcription factor is expressed at the rear of the midbrain vesicle at the MHB (Müller et al., 1996), delineating the intervening zone from the beginning of neurogenesis at late gastrulation. Its expression precedes *pax2.1* and *wnt1* in zebrafish (Wurst and Bally-Cuif, 2001), which makes it the earliest specific marker of the IsO (the time of IsO establishment is not known). During embryogenesis, *her5* is first expressed transiently in endodermal precursors, and then is maintained in the midbrain hindbrain domain (expression starts there during gastrulation as soon as 70% epiboly) to be progressively restricted during somitogenesis (Tallafuss and Bally-Cuif, 2003). *her5* expression later coincides with MHB markers but does not influence regional patterning and identity (Geling et al., 2003). Instead, this study shows *her5* is crucial in the intervening zone formation and upregulates cell proliferation within the midbrain hindbrain domain and inhibits neurogenesis at the intervening zone. In mouse, a comparable role for neurogenesis maintenance is ensured by other bHLH factors *Hes1* and *Hes3* (Hirata et al., 2001), that are closer to zebrafish *her6* and *her3* in sequence (Geling et al., 2003). However, they do not seem to

play a role in the intervening zone formation since the effects of the double knock-out in the previous study on neurogenesis are observable only from late somitogenesis.

All the factors mentioned above are relatively early players of midbrain hindbrain determination, given that they are expressed before or shortly after the establishment of IsO. However later factors are also crucial for regional identity. According to experiments performed in chicken, *MEIS2* is both necessary and sufficient for tectal development, without participating in IsO function. It is able to induce an ectopic tectum when expressed in the diencephalon through interaction with *OTX2* (Agoston and Schulte, 2009). *Meis2* expression starts during late somitogenesis. It acts downstream of *Fgf8* and regulates itself to maintain the tectal fate (Agoston et al., 2012). In zebrafish there are two *Meis2* homologs due to the additional teleost whole genome duplication, *meis2a* (formerly *meis2.2*) which is closer to the mammalian gene in protein sequence (Melvin et al., 2013) and *meis2b* (formerly *meis2.1*). At the moment their function in midbrain development has not been assessed. The only data available is a gene expression study (Waskiewicz et al., 2001): *meis2a* is initially expressed at 2 somites in the forebrain, hindbrain and spinal cord. From 10 somites it is expressed strongly in the dorsal midbrain, and at 24hpf expression is restricted to two symmetric patches in the ventral telencephalon and expression is maintained in the future tectum, hindbrain and spinal cord. Likewise another study shows (Zerucha and Prince, 2001) *Meis2b* has a dynamic expression pattern and is present in different parts of the brain at 24hpf, being present in two small domains in the forebrain (one ventral, one dorsal) and importantly in midbrain, presumptive cerebellum and the rest of the hindbrain.

Recently a zebrafish transgenic line partially recapitulating *meis2a* expression was generated (Heuzé, 2017). In this line, the expression becomes limited to the midbrain early in development. We took advantage of this line for our lineage study in article 2.

4.2 Current models of brain regionalization

How the neural tube develops into a mature brain containing elaborate structures with highly differentiated cell types has been a major question for neuroanatomists and developmental biologists. Different models have been proposed and modified over the years. Data presented here are on *Osteichthyes*, since most of them comes from studies on tetrapod and teleost models.

4.2.1 Columnar model

In the classical columnar model, the three primary vesicles are further divided into five secondary vesicles. The hindbrain is subdivided into the myelencephalon (containing the medulla oblongata) caudally and the metencephalon (containing the cerebellum and pons) rostrally. The midbrain is considered to remain one division by itself. The forebrain is subdivided into the diencephalon caudally and the telencephalon rostrally. The diencephalon is further divided into the thalamus dorsally and the hypothalamus ventrally, while the telencephalon is divided into the pallium dorsally and the subpallium ventrally (Figure 7A). These assumptions were based on gross ventricular morphology and on the apparent vertical (columnar) functional organization of the hindbrain, a concept which was extended to the forebrain (Herrick, 1910, reviewed in Puelles and Rubenstein, 2015). It also held that the neural tube ends in the middle of the telencephalon (in other words, telencephalon is the anterior end of the neural tube). Although this model has aged and is now admitted to be

inaccurate by most according to developmental data, it is still commonly taught in introductory neuroscience classes and textbooks due to the seeming direct correspondence between the five secondary vesicles and structures' topology in the adult mammalian brain (Swanson, 2012).

4.2.2 Neuromeric model

Since the end of the 1980's, a neuromeric model of the brain development has been elaborated and accepted by many developmental biologists (Figure 7B). Initially observed as repeated swellings of the neural tube by von Baer in 1828, neuromeres are defined as transversal divisions which appear transiently in the developing neural tube. Rhombomeres, neuromeres within the rhombencephalon, were the first to be clearly demonstrated (Keynes and Lumsden, 1990) as lineage-restricted compartments shaped by specific genetic and cellular mechanisms. Among other hallmarks, rhombomeres are characterized by specific expression of *Hox* genes, limited cell proliferation at their boundaries and repeated patterns of axonal projections and neurogenesis (Kiecker and Lumsden, 2005). Each segment is named r1, r2, r3 etc., from anterior to posterior. The cerebellum is a bulge at the roof of r1, and the different cranial nerves (the sensory and motor innervation to the face) are organized along the rhombomeres.

The prosomeric model has applied the same concept to the prosencephalon (Puelles et al., 1987), with neuromeres in this region named "prosomeres": they were proposed based on morphological hallmarks and gene expression patterns (Puelles and Rubenstein, 2003). In this model, the forebrain is subdivided into the "diencephalon" caudally whose development is influenced by the notochord and the "secondary prosencephalon" rostrally (Figure 7B). The diencephalon is further divided into three prosomeres termed p1 (pretectum), p2 (thalamus), and p3 (prethalamus), from caudal to rostral. The dorsal thalamus corresponds to p2, and the ventral thalamus corresponds to p3. In the mature amniote brain, the dorsal thalamus is extremely enlarged and occupies the majority of the diencephalon. Due to this enlargement of p2 and the cephalic flexure along amniote brain development, the rostral p3 is pushed ventrally, thus ultimately resulting in p3 being located ventral to the p2 in the adult brain. Although the hypothalamus was originally defined as a region which resides ventral to the thalamus (as the name "hypo"-thalamus indicates), it is now considered to be the most anterior part of the neural tube, occupying the ventral part of the secondary prosencephalon. In the embryonic brain, the subpallium is located anterior (instead of ventral) to the pallium, and during the course of development, the pallium expands thus engulfing the subpallium.

This prosomeric model was established mostly based on the development of the mouse and chicken brains and it has subsequently been applied to other vertebrate species. The assumption is that the prosencephalon can be subdivided into common longitudinal and transversal segmentations throughout vertebrates (Figure 7B). Gene expression patterns are often used to delineate subregions of the brain (genoarchitecture), and to identify homologous brain regions. For example, the expression of *Dlx* genes was used as a marker of the subpallium, and the expression of *Otp* was used as a marker of the supraoptoparaventricular region (SPV; a part of the "alar hypothalamus"), and these genes were used to delineate the border between telencephalon and hypothalamus in the *Xenopus* brain (Domínguez et al., 2013). Also, in the most recent versions the whole forebrain is considered to be epichordal (influenced by the notochord) according to some gene

expression data (Sánchez-Arrones et al., 2009) whereas the secondary prosencephalon was considered as prechordal (not influenced by the notochord) in previous versions (Puelles and Rubenstein, 2003).

4.2.3 *New model on the forebrain regionalization*

The prosomeric model continues to be updated through the introduction of a grid-like "checkerboard" framework, leading to more and more "microzones" within the vertebrate brains (Puelles, 2018, 2016). However, when the prosomeric model is applied to the teleost brain, borders delineated by gene expression do not always coincide with morphogenetic borders. Indeed, a checkerboard-like regionalization model may not be adapted to the forebrain organization, in which a simple tube-like morphology is significantly modified due to the evagination of the eyes.

A recent study in zebrafish (Affaticati et al., 2015) analyzing the teleost secondary prosencephalon proposed a modification of the model in which the secondary prosencephalon (anterior forebrain) is subdivided into three regions organized around three corresponding ventricular systems: telencephalon, the hypothalamus, and the optic recess region (ORR) continuous with the eyes (Figure 7C). This new framework takes into account the morphogenetic processes occurring radially around the ventricles: the ORR is defined as the region which develops around the optic recess (Figure 7E-F)). Importantly, the regional boundaries in this model are delineated by abutting differentiated neurons (HuC/D positive cells) originating from different ventricular zones that do not necessarily fit the boundaries of gene expressions (Figure 7E; Figure8).

At a morphological level, the ORR can be delimited by the anterior commissure and the postoptic commissure, which are present throughout vertebrates (Suárez et al., 2014). The anterior commissure is therefore at the border between ORR and telencephalon, and posterior commissure at the border between ORR and hypothalamus. In the developing brain, the area corresponding to ORR has been identified as the "optic stalk" (OS) both in mouse and zebrafish. The OS has not been considered to be a distinct brain region (as represented by the blank between the telencephalon and the hypothalamus in Figure 8D), but re-examination of this area suggests that the area that was designated as "OS" in the prosomeric model (Puelles and Rubenstein, 2003; Shimamura et al., 1995) actually corresponds to the ORR. Studies on the development of the eye show that the ORR and the optic vesicles develop in a similar manner around the optic recess (Ivanovitch et al., 2013; Picker et al., 2009), suggesting that the ORR is a part of the eye field (Yamamoto et al., 2017).

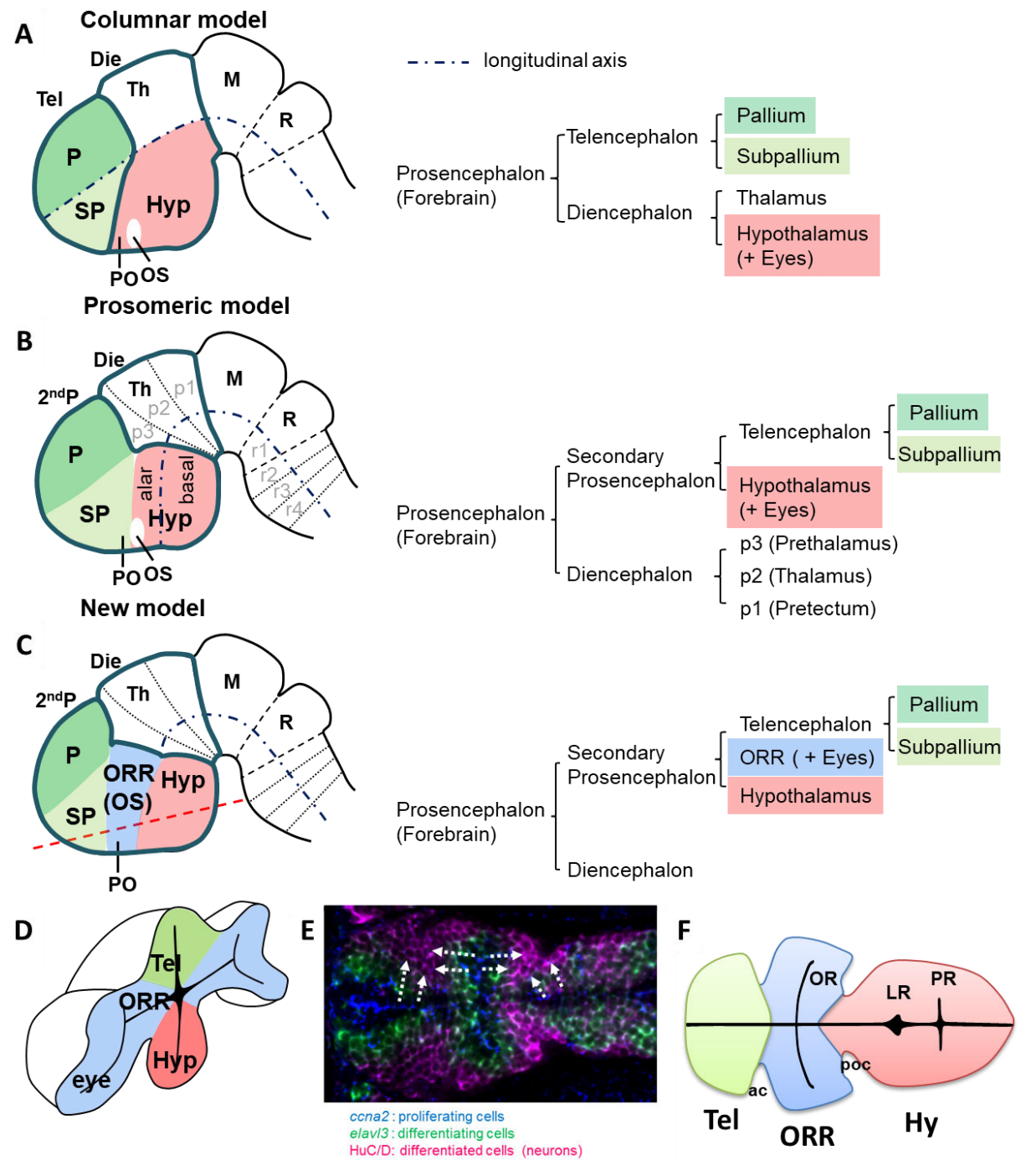
In amniotes, the ORR is hard to identify due to its relatively small size compared to the enlarged telencephalon. However, the identification of the ORR as a third morphogenetic unit between the telencephalon and the hypothalamus solves inconsistencies about regional identification (Yamamoto et al., 2017; Figure 8B).

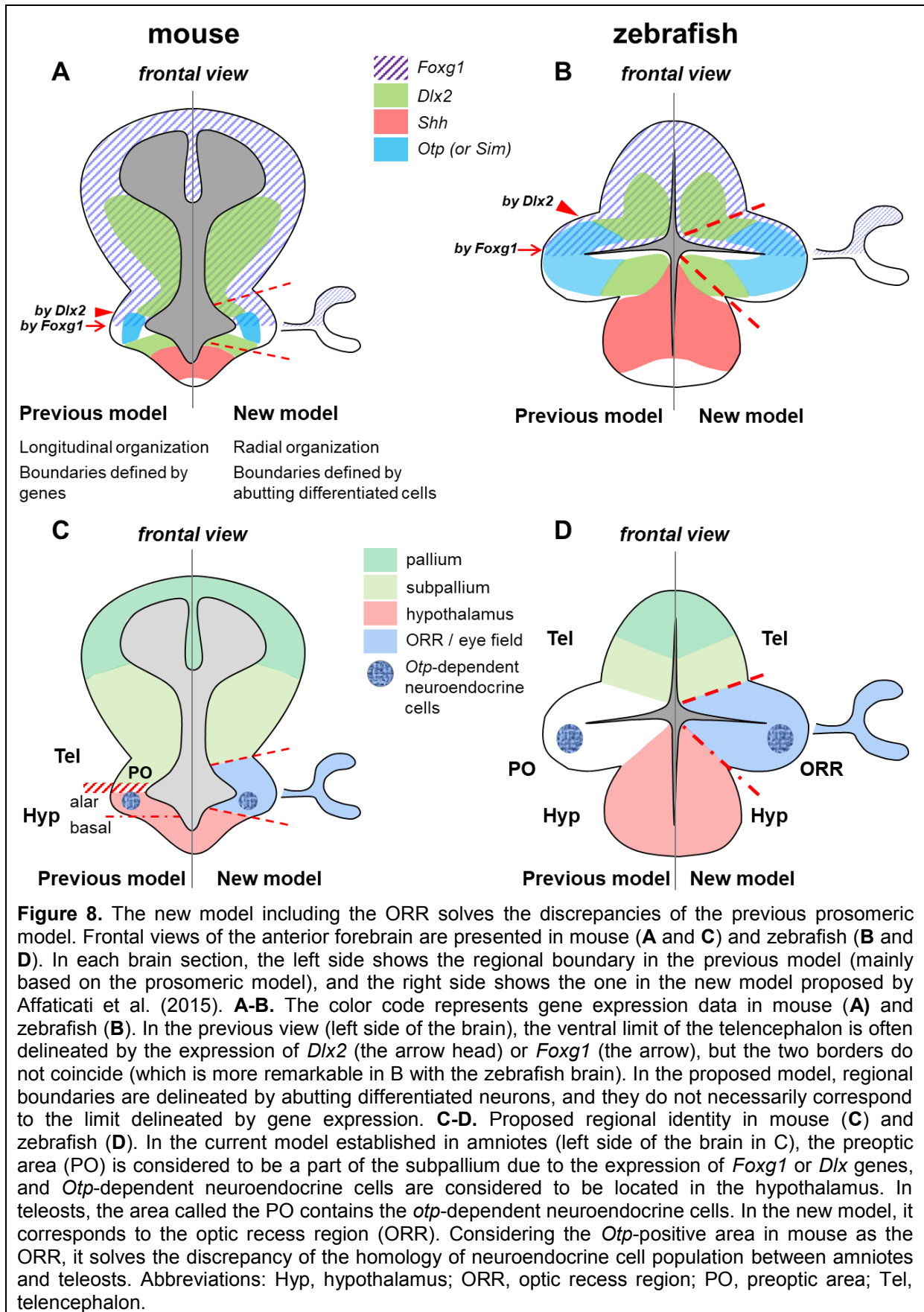
A major impact of this modification is that in the current model, the area containing *Otp*-dependent neuroendocrine cells are considered to be in the ORR instead of in the hypothalamus. Consequently, the area defined as the hypothalamus has been changed according the modification of the regional boundaries. Morphology and functions of the

"hypothalamus" have largely diversified in the different vertebrate groups (Xavier et al., 2017; Yamamoto et al., 2017). A part of my thesis project further modifies the area assigned as the hypothalamus in teleosts (this is discussed below).

Figure 7. Different models of the forebrain regionalization. **A-C** are schemas of the developing vertebrate in lateral view (rostral to the left) illustrating the three different models. **A.** The columnar model considers the hypothalamus as the ventral half of the diencephalon. **B.** The prosomeric model, which was originally proposed by Puelles and Rubenstein in the early 1990s, and has been modified over time. The hypothalamus is proposed to be the ventral half of the most anterior part of the forebrain (secondary prosencephalon). **C.** A new model proposed by Affaticati et al. (2015), in which the secondary prosencephalon is divided into three parts, the telencephalon, hypothalamus and the optic recess region (ORR). The red dotted line indicates the level of the ventral view shown in E and F. **D.** Transversal section and representation in 3D of the ventricular system at the ORR level and its relation to the optic cups. **E.** A ventral view of zebrafish embryonic brains illustrating the morphogenesis along the ventricular organization, based on which the new model (shown in C) is proposed. The white dotted arrows in the left image indicate the direction of cell maturation, from proliferation to differentiation. The abutting Hu-positive mature neurons form the regional boundaries. **F.** A schematic recapitulation in horizontal view. The ORR is the area that develops around the optic recess (OR), whereas the hypothalamus develops the hypothalamic ventricle. Note that the presence of two hypothalamic ventricles (LR and PR in the right diagram) is specific to teleosts, while other

vertebrate groups have only one hypothalamic ventricle. Abbreviations. ac: anterior commissure, Hyp: hypothalamus, LR: lateral recess, M: mesencephalon, OR: optic recess, ORR: optic recess region, OS: optic stalk, P: pallium, p1: prosomere 1, p2: prosomere 2, p3: prosomere 3, PO: preoptic area, poc: postoptic commissure, PR: posterior recess, R: rhombencephalon, SP: subpallium, Tel: telencephalon, Th: thalamus. Adapted from Yamamoto et al., 2017.





4.3 Diversity in mature brains

Although the embryonic vertebrate brain appears similar across species during early development, one can observe a surprising variability of the mature brains regarding their morphology. Depending on the species, structures vary in size, change in shape, cell type or density or some novel structures with no obvious equivalent in other vertebrate species can emerge. Indeed, like the rest of the organs, the brain is shaped by evolution and selection. Bearing that in mind, brain anatomy and function can be seen as an adaptation of species or groups to their specific environments. In this section, I introduce a few examples of diversity in brain structures observed in different vertebrate brains.

Arguably the best-known example is the human species with the extreme enlargement of the cerebral cortex in the forebrain. In Latin, “cerebrum” means “brain” and “cortex” means “bark” or “shell”. “Cortex” or “cerebral cortex” has been historically used in humans because it is the outermost part of the brain and covers almost its entire surface. Neocortex (or isocortex) is a 6-layered cerebral cortex that evolved in mammals with different sensory and motor specializations plus possible associative and higher order areas. These areas vary in size and function according to the ecological niche. Primates have generally expanded visual areas in addition to the primary and secondary visual cortex (V1 and V2, often found in other mammals). They possess higher order visual areas such as V3, V4 and motion-sensitive areas (Kaas, 2011). The star-nosed mole is an example of subterranean mammal relying on touch which displays enlarged somato-sensory cortices in some aspects reminiscent of the visual cortices (Catania, 2011) with a map of the “star-nose” from the primary to the tertiary somato-sensory cortex (S1 to S3). Similarly, in naked mole-rats the frontal-teeth are overrepresented: S1 occupies approximately 31% of the neocortical surface and extends over areas usually dedicated to vision (V1 and V2) in other species (Catania and Remple, 2002). As seen in Figure 9, birds also possess a developed pallium. This will be addressed in more details in the next section of the introduction.

Below the cerebrum is the cerebellum, literally “little brain”, a hindbrain structure which has been classically involved in motor functions: body balance, coordination as well as motor learning and smooth and rapid movement completion. However it is not “little” in terms of cell number (Herculano-Houzel, 2010): in mammals there is an average ratio of 3-4 cerebellar neurons for 1 cortical neuron (4-5 in humans according to the latter study), while cerebellar mass remains relatively small compared to cortical mass. Additional roles for the cerebellum have been identified in mammals, such its involvement in executive function (Dickson et al., 2017), voluntary expression of emotions or social cognition (Schmahmann, 2018), functions traditionally associated with forebrain structures. A study in zebra finch suggests a similar role of cerebellum for birds as lesions impair a spatial working memory task (Spence et al., 2009). Although the basic structure of the cerebellum is conserved in vertebrates, it is an interesting case of diversity in circuit, morphology and function (Hibi et al., 2017). While mammalian cerebella have evolved complex cortico-cerebellar connections, in Mormyrids (weakly electric fishes), the cerebellum is the most prominent structure and covers the rest of the brain (Figure 9). Mormyrids use a specialized structure in the cerebellum for electroreception, the electrosensory lobe, which has evolved independently in another group of fishes, Gymnotids (e.g. the electric eel). This structure has been involved in complex sensory integration from external electric signals as well as the cancellation of the fish’s own movements and electrical field (reviewed in Sawtell, 2017). Other cerebellum-like structures are present in vertebrates and are involved in similar functions (Bell et al., 2008).

In humans, one can observe the two aforementioned protuberances looking at the exterior surface of the brain (cerebrum and cerebellum). The rest of the brain which appears as the “stem” is called the brainstem. This term is broadly used to indicate posterior parts of the brain, including cranial nerves and the medulla oblongata (or medulla in short). The brainstem also contains visceral (autonomic) areas controlling functions critical for survival such as heart-rate, breathing, or reflex centers. In goldfish and carps there is an additional one located caudally to the cerebellum in the dorsal part of the medulla, the vagal lobe, involved in their sophisticated gustatory behavior and occupying about 20% of the brain volume, about as much as the TeO in goldfish. It is a laminated structure containing both a sensory and a motor component related to the palatal organ, specialized in sorting food from detritus (Finger, 2008).

In birds and teleosts, the dorsal part of the midbrain is called the tectum or optic tectum (TeO, tectum meaning “roof” in Latin) because it receives massive input from the eyes. TeO is enlarged compared to the mammalian homologue (Figure 9), superior colliculus. In mammals it is called the superior colliculus (a “colliculus” is a mound in Latin) because of its small size, which is covered by the cerebral cortex. The large TeO is correlated with the high visual capacities of many species of birds and teleosts. This contrasts with many living mammals such as rodents in which the visual system is reduced: ancestral mammals were nocturnal animals and relied on other sensory systems, in particular olfaction with probably developed olfactory bulbs and pyriform cortex with a relatively small neocortex (Molnár et al., 2014). Primates have evolved elaborate visual systems along with specialized visual cortical areas secondarily. The idea has emerged that computation taking place in the visual cortex of mammals, such as a stimulus saliency map in V1 to pay attention to unexpected stimuli in the visual field, may take place in other brain regions such as the TeO of teleosts, although this hypothesis still has to be verified experimentally (Zhaoping, 2016).

In the cases mentioned above, dorsal parts of the neural tube make a protuberance during development. By contrast, in teleosts, two protuberances can be observed in the ventral part of the brain. The hypothalamus is a relatively small structure in the amniote brains, but in teleosts, the hypothalamus is well developed. The organization of the hypothalamus varies among vertebrate groups, which may be correlated to the morphology of the hypothalamic ventricle (Xavier et al., 2017; Yamamoto et al., 2017). Most vertebrates possess one hypothalamic recess, while teleosts have two: the lateral recess (LR) and the posterior recess (PR, Figure 9F). PR is absent in *Chondrichthyes* (sister group of *Osteichthyes*) and in the basal *Actinopterygii* *Polypterus*, and it is present only in teleosts as well as in gars (Parent and Northcutt, 1982). Thus the ancestral jawed vertebrates probably did not have a PR. Taking these large differences of organization between tetrapod and teleost hypothalami into account, the establishment of homology between cell populations requires careful verification.

Teleosts present an additional prominent ventral structure. Posterior to the hypothalamus, there is a paired structure which has been called the inferior lobe of the hypothalamus (IL). IL is present only in teleosts and gars, and its functions remain elusive. In article 1, we show that this structure originates mainly from the midbrain, and is therefore not part of the hypothalamus. We also discuss further about its organization and possible functions.

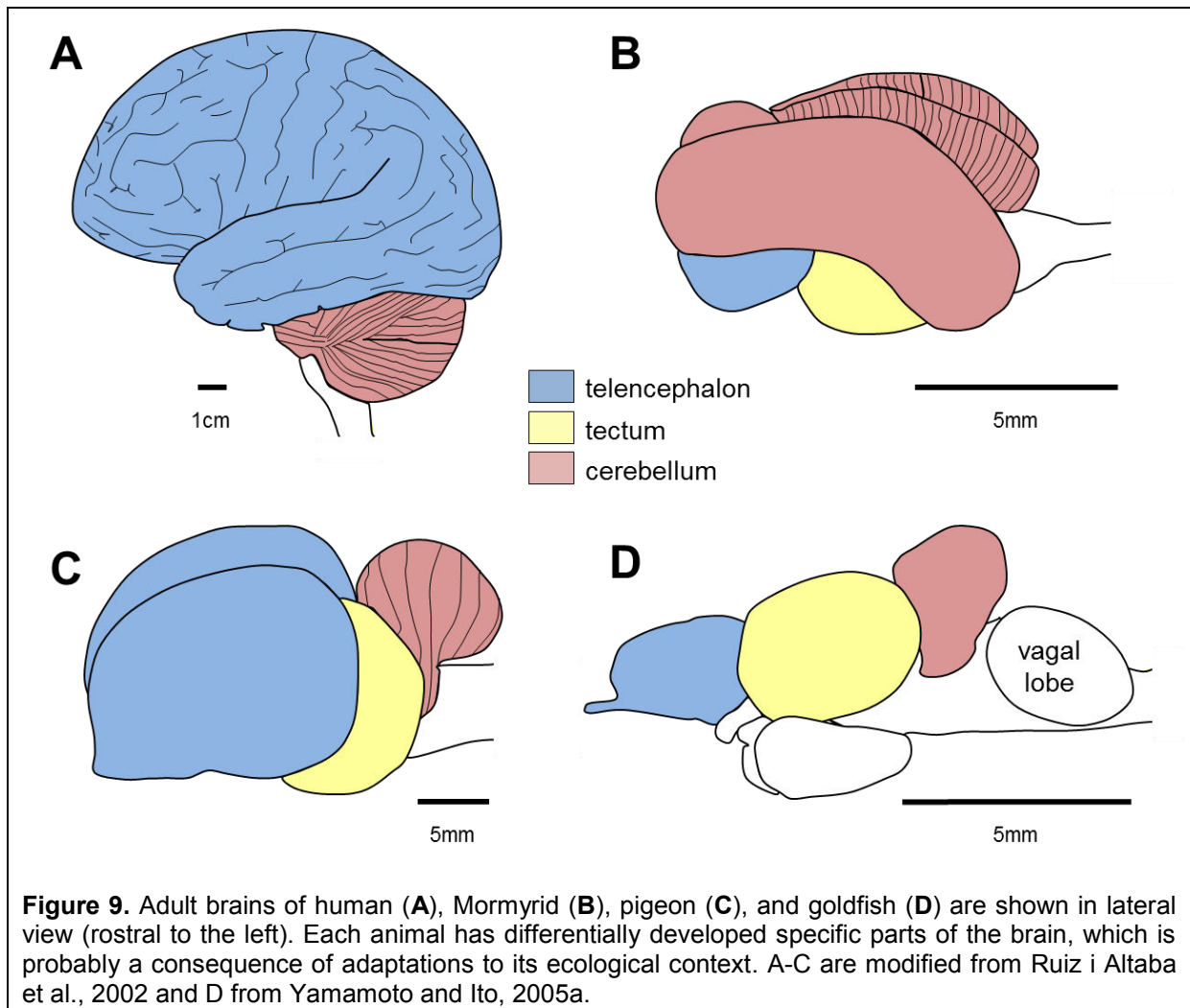


Figure 9. Adult brains of human (A), Mormyrid (B), pigeon (C), and goldfish (D) are shown in lateral view (rostral to the left). Each animal has differentially developed specific parts of the brain, which is probably a consequence of adaptations to its ecological context. A-C are modified from Ruiz i Altaba et al., 2002 and D from Yamamoto and Ito, 2005a.

5. “Intelligence” outside of mammals

5.1 Observations of higher-order cognitive functions in vertebrates

In the previous part, we have observed the diversity of adult brains across vertebrates. Since the main focus of my thesis is the evolution of cognition, I will address below the evolution of the pallium as a major integration center and of the afferent sensory pathways to the pallium.

The evolution of “intelligence” is studied in reference to the human mind and brain, with the commonly admitted view that “primates are, on average, more intelligent than other mammals, with great apes and finally humans on top” (Roth and Dicke, 2012). In humans, individual intelligence is typically measured by IQ (intelligence quotient), testing different cognitive components (verbal, numerical, spatial...). Since tests relying on human language cannot be applied to animals, intelligence or higher order cognitive capacities are evaluated through their behavioral flexibility, in other words their capacity for innovation in front of situations which are not normally part of their behavioral repertoires (Emery and Clayton, 2004; Gould, 2004; Roth and Dicke, 2012, 2005). In human psychology this is also termed “general” or “fluid” intelligence, in contrast to “acquired” or “crystallized”, and has been measured as the general factor “g” (Burkart et al., 2017). The term “general” has been used because it has been observed in humans that there are significant correlations in performance across tasks in different domains (Plomin, 2001). For example, a high

performing individual in a given task will more probably be also performing well in other tasks.

Examples of paradigms commonly used to illustrate a developed behavioral flexibility in non-human animals are tool use and manufacture, imitation, numerosity (representation of numerical quantities), object permanence or theory of mind (Figure 10; Roth and Dicke, 2012). These are considered as “higher order” cognitive functions, in the sense that they are comparable to what is comparable to human cognitive functions regarding general intelligence, and that they were historically thought to be only present in some of our closest relatives, primates.

Neocortical evolution has been historically linked with the evolution of intelligence in mammals. Indeed, neocortex is the most prominent structure in the human brain, occupying around 80% of its size and contains major sensory, motor and associative areas. However, the relative size of the brain or of the neocortex does not seem to be an accurate predictor of cognitive functions, even within primates, although they roughly correlate, since for instance gorillas have a larger neocortex than chimpanzees although the latter are considered to possess higher cognitive capacities. Neocortical cell density, connectivity or diversity (Herculano-Houzel et al., 2007) as well as neocortical functional parcellation are other parameters roughly correlative to cognitive capacities, although none of these parameters is able to predict the cognitive capacities of a given mammalian species.

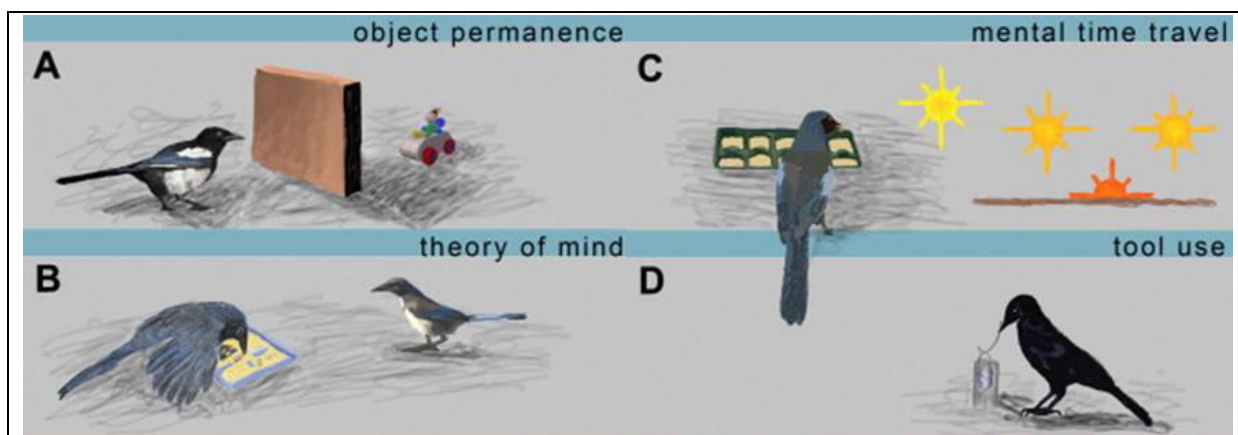


Figure 10. Cognitive capacities reported in corvids considered as insight-related cognition. **A.** Object permanence is the ability to know an object still exists when it is out of sight. **B.** Theory of mind is the attribution of one’s own mental state as well as future actions to another being. **C.** Mental-time travel is the ability to remember past episodes and to plan future needs and motivational state. **D.** Tool use is the ability to manipulate items in the environment to achieve a specific goal. From Kirsch et al., 2008.

Outside of mammals, some bird species display cognitive capacities rivalling those of primates in terms of general intelligence although birds do not have a layered cortex. There are numerous behavioral reports from the group of corvids (and to a lesser extent in parrots) revealing that these animals possess cognitive capacities rivalling those of primates (Emery, 2006; Emery and Clayton, 2004). These include object-permanence, theory of mind, tool use and manufacture, and mental-time travel (Figure 10), together defined as insight-related cognition (Kirsch et al., 2008). Direct comparison between crows and macaques in a working memory task in which the animals had to retain and match a number of items has demonstrated a very similar performance (Balakhonov and Rose, 2017).

Birds have generally higher neuronal densities than mammals, *Passeriformes* (including corvids) and *Psittaciformes* (including parrots) having a proportionally increased neuronal density in the pallium compared to primates (Olkowicz et al., 2016). Moreover, the relative proportion of the telencephalon compared to the rest of the brain is comparable to primates, taking roughly 75% of the brain. This suggests different organizational strategies between mammalian and avian pallia to achieve similar cognitive capacities (e.g. birds have increased cell density without layers).

In teleost fishes, some species have been described as showing some cognitive capacities comparable to birds and mammals, mainly within the groups of wrasses (*Labridae*) and cichlids (*Cichlidae*). These two groups are relatively close within teleosts, belonging to *eupercaria* and *ovalentaria* respectively (Figure 2B, Betancur-R et al., 2017) but rather distant to more classical teleost models such as goldfish or zebrafish (cyprinids). Tool-use has been reported in six different wrasse species using anvils to break open the shell of a prey, either urchins or seashells (Bernardi, 2012; Patton and Braithwaite, 2015). Arguably egg-laying on a loose leaf in two species of South American cichlids could be an example of tool-use: the leaf selection and its displacement upon threat seem flexible (Keenleyside and Prince, 1976; Timms and Keenleyside, 1975), although it is hard to tell if it is a more courtship-related behavior. The cichlid *Astatotilapia burtoni* is capable of transitive inference, that is using known relationships to deduce unknown ones (Grosenick et al., 2007): based on the observation of pair fights, an observer was capable of predicting which individual would be stronger or weaker, even when it had never seen them together (e.g. if A won over B and B won over C, it is able to predict that A will be stronger than C in a new fight). Cleaner wrasses (*Labroides dimidiatus*) present complex foraging strategies integrating future outcomes (Salwiczek et al., 2012), and males are capable of adjusting punishment intensity according to the behavior of a partner female, which in turn will be more or less cooperative (Raihani et al., 2012).

Although the anatomical substrate for these behaviors has not been identified in these teleost species, one can observe a general trend is that species presenting higher order cognitive functions have a larger pallium.

In contrast, there are no reports indicating the presence of higher order cognitive functions in amphibians. Interestingly, amphibian pallia are relatively small and present a tube-like morphology comparable to coelacanth and lungfish, suggesting that this may be the ancestral situation in *Sarcopterygii* (Yamamoto and Bloch, 2017).

5.2 A paradigm to study cognition across vertebrates: operant conditioning

In order to understand the brain organization underlying the cognitive abilities discussed above, behavioral experiments under controlled conditions are critical.

Operant (also Skinnerian or instrumental) conditioning is a powerful tool to study animal perception and cognitive functions. It allows the dissection of these functions into cognitive modules, which in combination with anatomical studies (e.g. tract-tracing or lesion studies) can lead to the identification of the neural substrates of a specific cognitive capacity. Since the second part of my thesis applies the operant conditioning paradigm to zebrafish, I will introduce its principle briefly.

The operant conditioning is based on the association of behavior-contingency: "a frequency of occurrence of a bit of behavior is modified by the consequences of the behavior" (Reynolds, 1975). In other words, operant conditioning is defined by the modification of a given behavior in response to its consequence: the behavior is shaped by a reward or punishment that occurs in consequence.

For example, (Figure 11), when a dog receives food after giving the paw, the dog increases the frequency to give the paw. In this context, the food is called a reinforcer. Now, the food is provided only if the dog gives a paw when a bell rings. Then the dog will learn to give the paw only when the bell rings. In this case, the bell is called the discriminative stimulus. Following the same principles, we can have pigeons peck a key or rat press a lever to obtain food. For example, if the food is provided only when the pigeon pecks a green key, but no food by pecking a red key, the pigeon begins to peck only a green key, but not red (for a typical operant box for pigeon, see Figure 12).

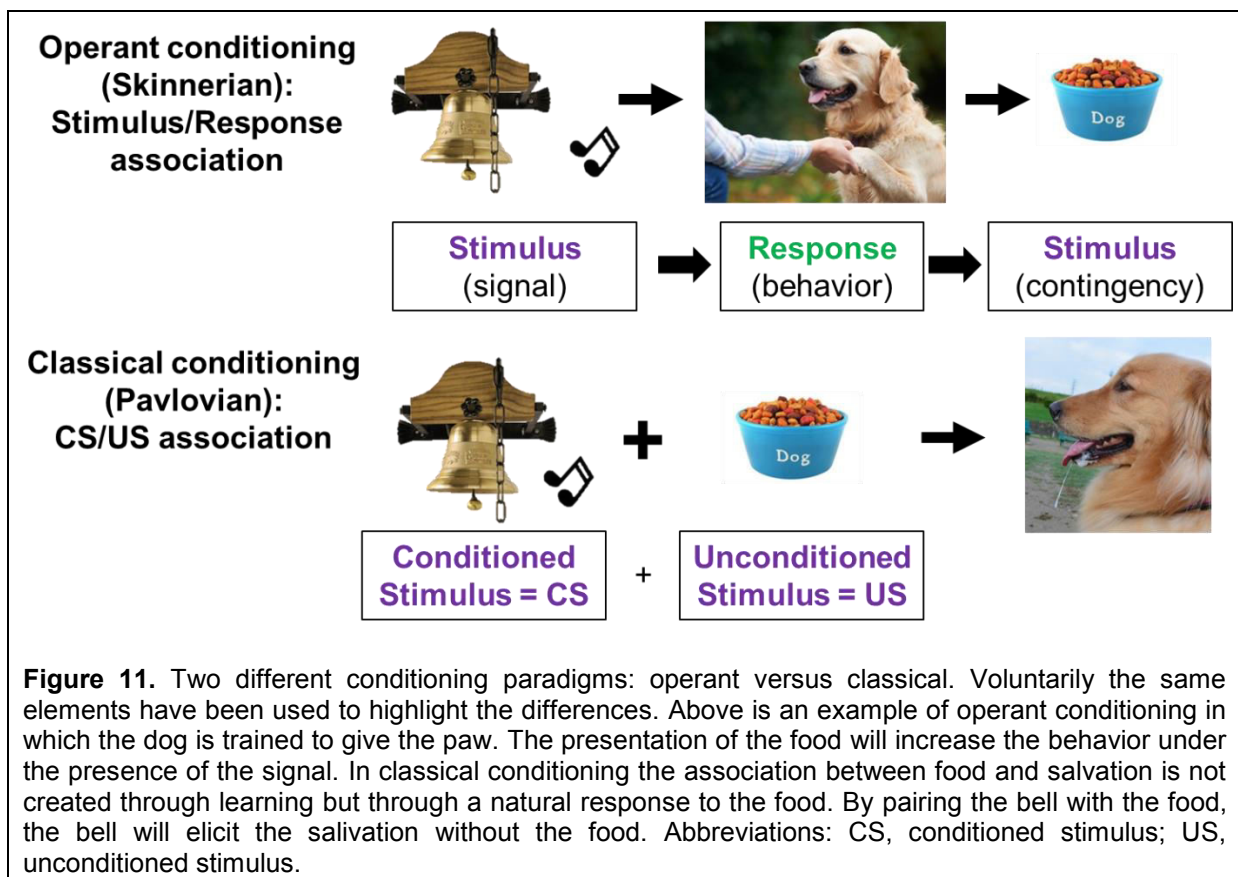


Figure 11. Two different conditioning paradigms: operant versus classical. Voluntarily the same elements have been used to highlight the differences. Above is an example of operant conditioning in which the dog is trained to give the paw. The presentation of the food will increase the behavior under the presence of the signal. In classical conditioning the association between food and salivation is not created through learning but through a natural response to the food. By pairing the bell with the food, the bell will elicit the salivation without the food. Abbreviations: CS, conditioned stimulus; US, unconditioned stimulus.

When this behavior is established, we can tell that the pigeon can distinguish green and red (Figure 12). Thus this paradigm allows asking the animal the question "Is this green or red?". Although animals cannot talk, operant conditioning can have them answer through a behavioral response. As such, there have been many studies about the perceptual abilities of animals, a field which is called psychophysics (Denman et al., 2018; Hahmann and Güntürkün, 1993; Romeskie, 1976).

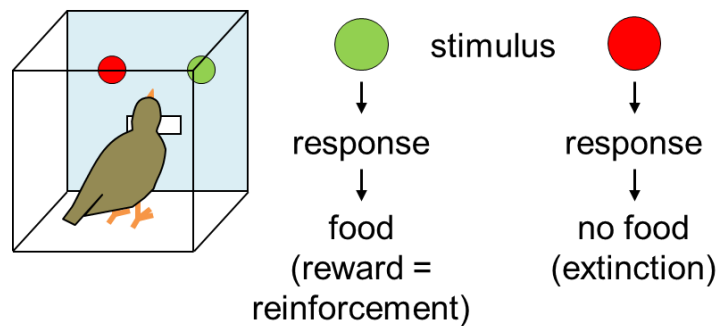


Figure 12. Schema of an operant box for pigeon, with an example of a color discrimination task. Often, in pigeon, color illumination is provided behind the pecking keys. Colors are randomly presented on each side. For example, when the food is provided by pecking the green key, the pigeon learns to peck only the green key. Food is given in the distributor (rectangular shape in the front panel).

There are four general configurations: positive or negative reinforcement, or positive or negative punishment. Reinforcement and punishment indicate that the probability of occurrence of the response will increase or decrease respectively, while positive and negative are in reference to addition or withdrawal of a stimulus (not to be confused with the positive or negative experience of the animal). This is recapitulated in Figure 13. In positive reinforcement, following the stimulus presentation the animal will have to respond correctly in order to receive a reward (reinforcer).

	Positive addition of a stimulus	Negative withdrawal of a stimulus
Reinforcement The behavior frequency increases	If the behavior increases following the presentation of a stimulus, it is called Positive reinforcement	If the behavior increases following the withdrawal of a stimulus, it is called Negative reinforcement
Punishment The behavior frequency decreases	If the behavior decreases following the presentation of a stimulus, it is called Positive punishment	If the behavior decreases following the withdrawal of a stimulus, it is called Negative punishment

Figure 13. Recapitulation of the four configurations for operant conditioning. Each is a combination of either "reinforcement" or "punishment" condition and "positive" or "negative" conditions (see definitions above).

Operant conditioning should not be confused with classical (Pavlovian) conditioning. For comparison of the two paradigms, see above Figure 11. Classical conditioning controls all the events and in particular the relations between the stimuli, while operant conditioning controls the relation between the animal behavior and future stimuli (Rescorla and Solomon, 1967).

Classical conditioning is an association of two stimuli: a conditioned stimulus (CS) and an unconditioned stimulus (US). A typical example is the association of a bell ring and food for a dog (Figure 11). Food (US) naturally triggers a physiological response (UR, salivation). When a neutral stimulus such as bell ring is presented at the same time as food, the bell ends up triggering salivation. After the association occurred, the bell is called conditioned stimulus (CS) which triggers a conditioned response (CR).

For some stimuli, CS and US do not need to be paired repeatedly (some aversive conditioning occurs in one trial), while operant conditioning shapes one response and usually occurs after several trial reiterations (Fanselow and Wassum, 2016).

6. Pallium as an integration center

By using operant conditioning, many studies have shown that the avian pallium is important for specific perceptual abilities and cognitive capacities (Hodos and Karten, 1970; Kalt et al., 1999; Kraemer and Roberts, 1984; Rose and Colombo, 2005; Wagener et al., 2018; Watanabe, 1992), as it is the case for the mammalian cortex.

The term “pallium” means cloak or robe in Latin. It was first introduced by Burdach in 1822 to describe the gray matter of the human neocortex (Swanson, 2014). It later took the broader meaning of the dorsal telencephalon of vertebrates.

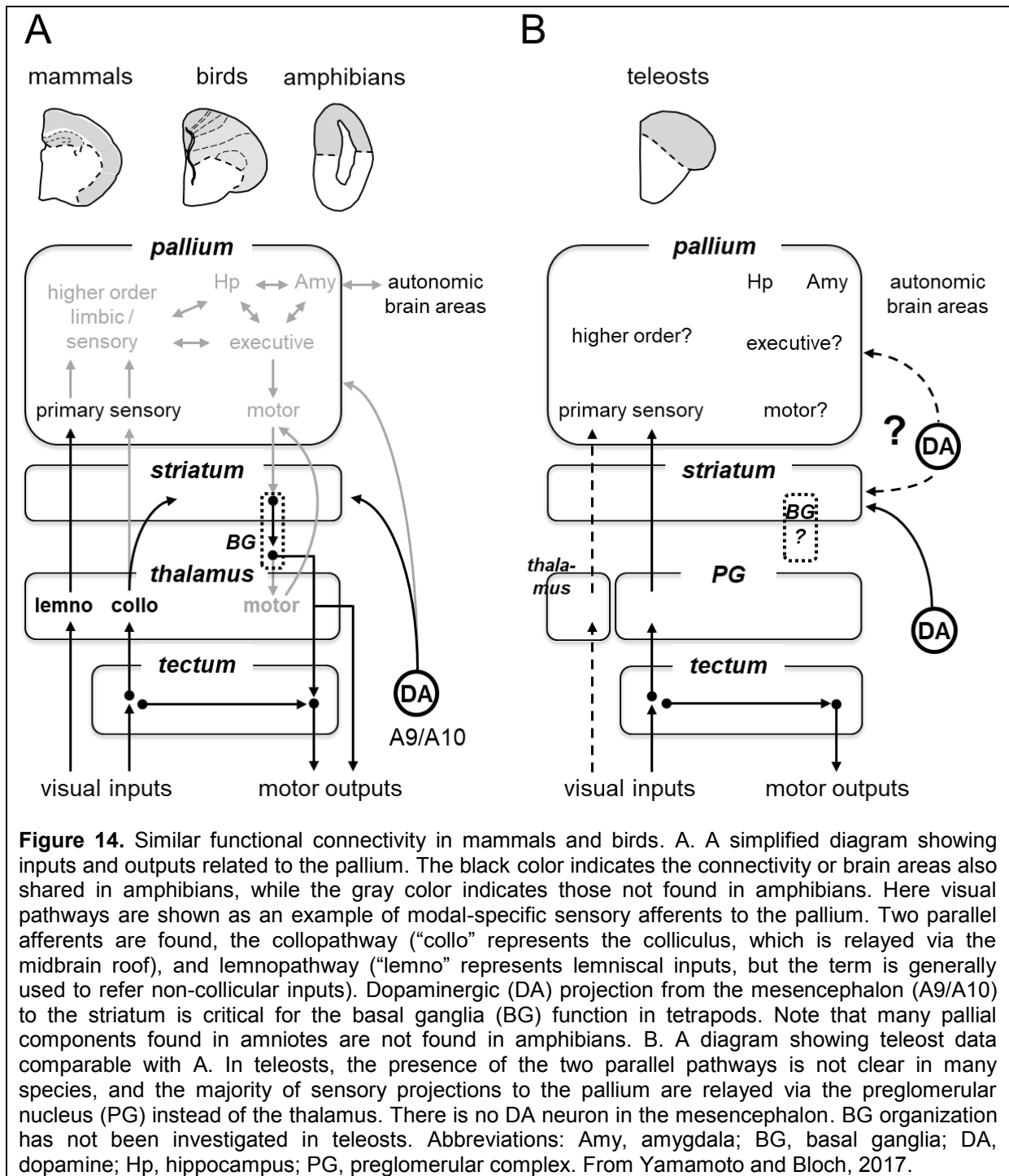
In mammals and birds, pallium is a major center for integrating different sensory modalities and, on the top of it, executive areas coordinate behavioral output based on the context (Güntürkün, 2005a; Shanahan et al., 2013). Thus we think that pallial organization in vertebrates should be critically linked to the evolution of higher order cognitive functions: following this idea, we are interested in the critical features in the organization of the pallium that could allow the emergence of such functions. It is the ultimate goal of the research project I participated in, although I did not address this problem directly in my thesis work.

Concerning this question, very few data are available outside of amniotes. This is why we will first present relevant studies regarding the comparison between mammals and birds. Then, we will summarize what is known on the teleost pallia. Finally, we will discuss the problem of pallial homology. Indeed, vertebrate brains are morphologically diverse although functional similarities have been discovered. This has led to long-lasting controversies about the relations of homology between different parts of the pallium.

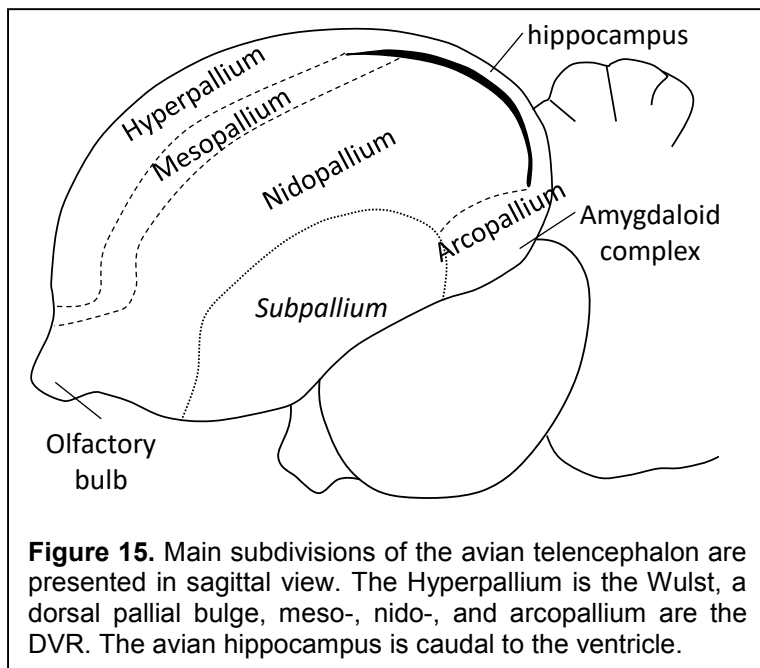
6.1 Similar connectivity in the avian and mammalian pallia

As mentioned above, birds exhibit elaborate cognitive functions, with a few species rivalling with great apes (Emery, 2004; Pepperberg, 2006). Birds have also evolved a pallium displaying striking similarities with the mammalian cortex in the functional organization despite the morphological differences, which will be highlighted in the following subsections.

Historically, the majority of the avian telencephalon was thought to be subpallial, with hypertrophied basal ganglia. Consequently, older studies used the suffixes –striatum to describe the different areas of the avian telencephalon. A combination of numerous behavioral, hodological (study of pathways and connectivity) and gene expression studies has shown that the general organization of the avian pallium is similar to what has been observed in mammals (Figure 14), and that the major part of the avian telencephalon is actually pallium (Jarvis et al., 2005; Reiner et al., 2004).



Yet the mammalian and sauropsid pallia look little alike morphologically. In the mammalian pallium, the 6-layered neocortex spans across the brain surface. In contrast, the avian pallium (Figure 15) presents a nuclear organization with a dorsal ventricular ridge (DVR), a protrusion of the lateral telencephalic wall that grows into the lateral ventricle. The DVR contains mesopallium, nidopallium, entopallium and arcopallium. The dorsal nuclear complex is the hyperpallium, which is often called the Wulst (bulge in German). Non-avian reptiles also have a DVR. The dorsally located 3-layered cortex is considered as ancestral in amniotes, and homologous to the Wulst (Medina and Reiner, 2000). The closest relatives of amniotes are amphibians, which have a simple pallial morphology with neurons distributed close to the ventricle and no DVR. Based on the principle of parsimony, this has led to the



hypothesis that the common amniote ancestor's pallium resembled the extant amphibian's with no DVR or neocortex but already intermingled input, output neurons and intratelencephalic connection neurons (Briscoe and Ragsdale, 2018). Following this view, important architectural variations such as the DVR in sauropsids and neocortex in mammals would have evolved independently, while major cell types would have already been present in ancestral amniotes.

6.1.1. Sensory afferents to the pallium

Similarities in the connectivity between the 6 layers of the mammalian neocortex and between nuclei of the avian pallium have led to the hypothesis that cell populations within cortical layers in mammals are homologous to corresponding cell populations within nuclei in the avian brain (Karten and Shimizu, 1989). In mammals and birds, all sensory modalities have a specific dorsal thalamic relay nucleus to a primary sensory area except for olfaction (Butler, 1994a, 1994b): olfactory bulbs directly project to the pyriform cortex. Sensory information is then progressively integrated in secondary and higher order sensory pallial areas. The different modalities will be reviewed with a focus on the visual system, since this system was studied in my thesis.

The Wulst contains two primary sensory areas, one somatosensory and one visual, while nidopallium contains the primary auditory area, the main primary visual area and another somatosensory area; in turn major outputs are located in the arcopallium and upper hyperpallium (Güntürkün, 2005b; Jarvis, 2009; Reiner et al., 2005).

There are two major visual pathways terminating in the pallium described in mammals and birds (Karten et al., 1973; Schneider, 1969): a thalamofugal and a tectofugal pathway.

The thalamofugal (or lemnothalamic, or geniculate) pathway projects from retina to thalamus, which projects to a primary visual area (Figure 17). In mammals, the thalamic relay is the dorsal lateral geniculate nucleus (dLGN) and the pallial primary visual area is V1. In birds these regions correspond to the opticus principali thalami (OPT, or sometimes called dLGN as in mammals) and the visual hyperpallium (Wulst) respectively. Based on topological, hodological and gene expression data these thalamofugal pathways are generally considered as homologous between mammals and birds (Medina and Reiner, 2000; Reiner et al., 2005). It is the major pathway in the mammalian visual system while it is not the case in many avian species (see below).

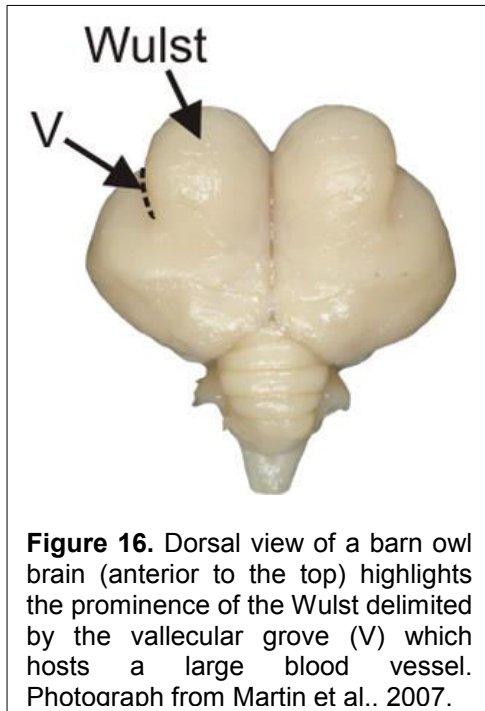


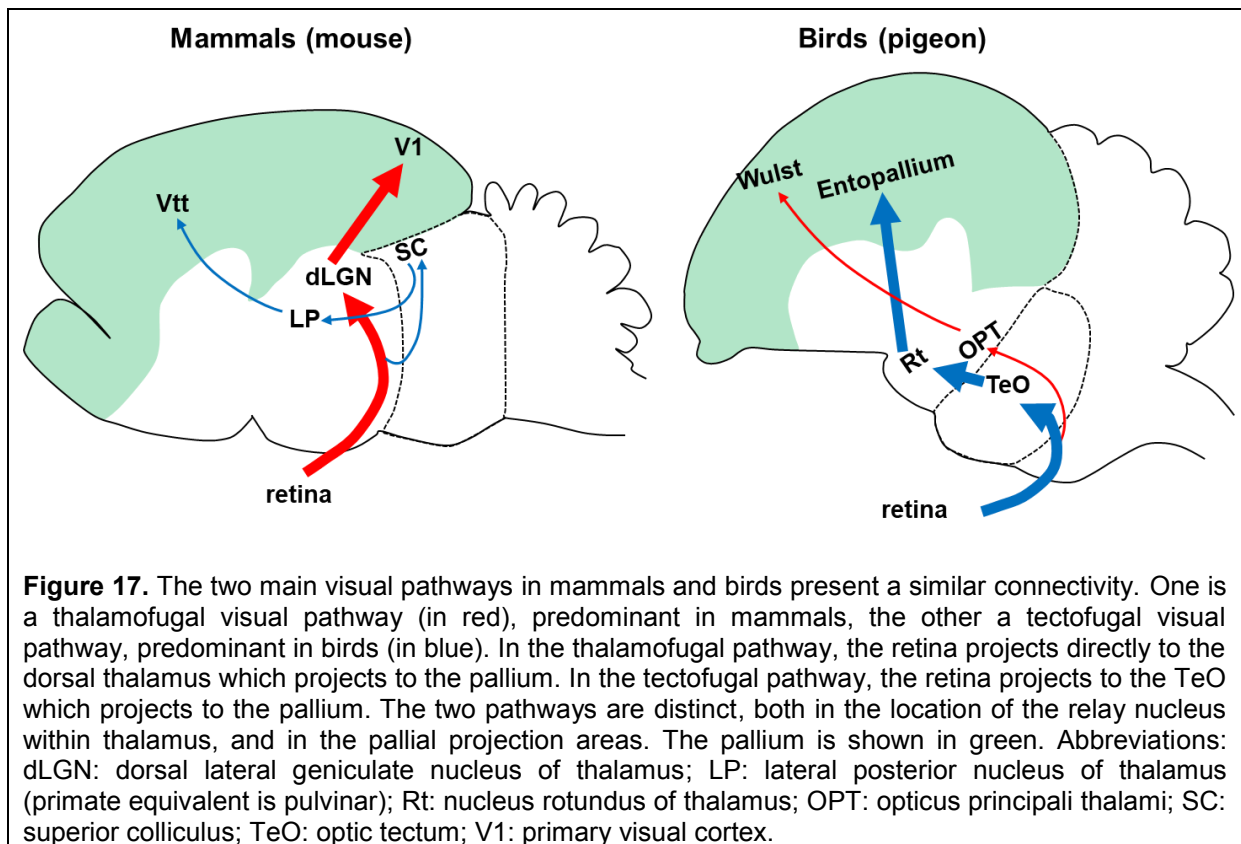
Figure 16. Dorsal view of a barn owl brain (anterior to the top) highlights the prominence of the Wulst delimited by the vallicular groove (V) which hosts a large blood vessel. Photograph from Martin et al., 2007.

The functions of the avian Wulst are still debated in birds: it seems to be involved in binocular vision (Pettigrew and Konishi, 1976), motion perception (Baron et al., 2007) and spatial orientation (Michael et al., 2015). Lesion studies have led to the proposition that Wulst may be involved in an avian “where” stream, as it is the only source of visual information for the avian hippocampus (Atoji and Wild, 2006; Watanabe et al., 2011) while it has little involvement in simple discrimination tasks (Parker and Delius, 1980). Owls are an interesting case since their Wulst is larger than in any other avian species (Figure 16). This may be linked with their developed binocular vision and stereopsis, reaching 45 to 50° of binocular overlap in some species (Iwaniuk and Wylie, 2006): as an illustration, over 80% of the neurons are in the Wulst of owls (Pettigrew, 1979), and a general correlation across birds has been found between Wulst size and orbit orientation but not with other visual areas such as TeO (Iwaniuk et al., 2008).

The tectofugal (or collothamic, or extra-geniculate) pathway presents an additional relay in TeO. The TeO corresponds to the superior colliculus (SC) in mammals, literally “upper hill” in Latin referring to two anterior bumps they make on the top of the mammalian midbrain. In mammals, retinal inputs go to the SC which projects to the lateral posterior nucleus of thalamus (called pulvinar in more visual species such as primates), which projects in the visual area of the temporal cortex (Vtt). In birds, this “SC→LP→Vtt” pathway corresponds to layer 13 of TeO, which projects to the nucleus rotundus (Rt), which projects to the entopallium (E), a part of the DVR (Reiner et al., 2005). This “TeO→Rt→E” tectofugal pathway is well developed in birds (Figure 17), as 90% of the retinal inputs are allocated to this pathway in pigeon (Remy and Güntürkün, 1991) versus less than 5% in primates. As the main visual pathway in birds (at least in laterally eyed birds), it conveys different properties of visual information, such as color, pattern, brightness or movement discrimination (Nguyen et al., 2004; Sun and Frost, 1998; Wang et al., 1993; Watanabe et al., 2008). The information treated in the tectofugal pathway in birds has been compared to those treated in the “what stream” in the primate visual cortices (Watanabe et al., 2011). In most avian species, the tectofugal pathway is dominant, except in a few nocturnal avian species (Kakapo and Kiwi) in which it is reduced (Corfield et al., 2011; Craigie, 1930; Martin et al., 2007). This may be associated with less reliance on the visual information from this pathway.

Early mammals would have reduced the tectofugal pathway initially involved in color vision in parallel with loss of cone, and developed the thalamofugal involved in light-sensitivity. Later, diurnal mammals would have developed the capacity for color vision in the thalamofugal pathway (reviewed in Heesy and Hall, 2010). In mammals the tectofugal pathway encodes gaze direction through coordinated eye and head movements by sensori-motor coupling in SC (Hall and Moschovakis, 2003). Vtt appears to correspond to the middle-temporal area of visual cortex (MT) in primates (Berman and Wurtz, 2008; Glendenning et al., 1975), which

has been involved in movement perception. The functions of this pathway in mammals are still under discussion and appear to vary across species (Chomsung et al., 2010).



In addition to these two visual pathways to the pallium (recapitulation in Figure 17) proposed by Schneider (1969), there are reports of other visual pathways to the pallium in mammals and birds, although their functions and respective homology remain unclear. A second tectofugal visual pathway has been described in pigeon and songbirds, ending in a region of the nidopallium bordering the entopallium dorsomedially (Gamlin and Cohen, 1986; Wild and Gaede, 2016). A study reports still another visual pathway ending in the caudal telencephalon of pigeons, with latencies to reach the telencephalon shorter than the two “classical” visual pathways (Güntürkün, 1984), which may have an equivalent in mammals to a second thalamofugal pathway projecting to the limbic system as identified in some rodents (Itaya et al., 1981; Kuljis and Fernández, 1981).

Primates studies report an even more different situation: in addition to the “classical” tectofugal route through SC, a direct retinal input to the pulvinar which also projects to MT has been shown in neonates. This pathway rapidly regresses and appears to have a minor role in adults. However this pathway may play a major role in newborn primates, and may functionally compensate early V1 lesions (Bridge et al., 2016; Liao and Ghazanfar, 2018; Warner et al., 2015, 2012). This is an intriguing case of functional compensation (Gross et al., 2004) given the difference in topology of the cortical target (occipital for V1 versus temporal for MT).

The primary auditory area (A1) in the avian pallium is located in the DVR and is called field L. It receives input from a thalamic nucleus called nucleus ovoidalis (Ov) due to its ovoid shape, which receives input from the central nucleus of the nucleus mesencephalicus

lateralis, pars dorsalis (MLd), the auditory relay nucleus beneath TeO (Karten, 1968, 1967). MLd is considered to be homologous to the inferior colliculus (IC) of mammals. Field L has been divided into L1, L2 and L3 based on cytoarchitecture, L2 mainly receiving thalamic inputs from Ov (Wild et al., 1993). This pathway resembles the mammalian projection to the primary auditory cortex in the temporal lobe receiving thalamic inputs from the ventral part of the medial geniculate nucleus (MGNv), which receives inputs from the central nucleus of IC (Clerici and Coleman, 1990; Morest, 1964). This auditory pathway in birds is particularly studied in the context of the independent emergence of complex vocalizations and vocal learning in parrots, songbirds, hummingbirds and humans (Petkov and Jarvis, 2012).

There are two somato-sensory areas in the avian pallium (Figure 18). One is located in the rostral Wulst which is relayed by thalamus, and the other in the rostral DVR which receives direct inputs from the principal trigeminal nucleus (Delius and Bennetto, 1972; Wild, 1987). The somatosensory Wulst is similar to mammalian S1 since both receive inputs from the dorsal thalamus (called DIVA in birds, nucleus dorsalis intermedialis ventralis anterior) which receives inputs from the dorsal column nuclei (Medina and Reiner, 2000). In both mammals and birds S1 presents a somatotopic representation of the body (Funke, 1989).

In summary, both in mammals and birds, the primary sensory areas receiving modal-specific sensory information (such as V1, A1 or S1) project to other pallial areas located at their periphery. In birds, areas receiving projections from primary sensory areas are considered to be secondary or higher order sensory areas (Atoji and Wild, 2012; Güntürkün, 2005b). The higher order sensory areas interconnect with the nidopallium caudolaterale (NCL), which is considered as the executive area (see below). NCL sends projections to the arcopallium (Kröner and Güntürkün, 1999), which gives rise to distal projections to the subtelencephalic areas (Zeier and Karten, 1971), and these distal projections are considered as motor. Thus in this view, this general connectivity observed in birds is compared to the mammalian cortex organization in sensory, motor and associative areas (Figure 18).

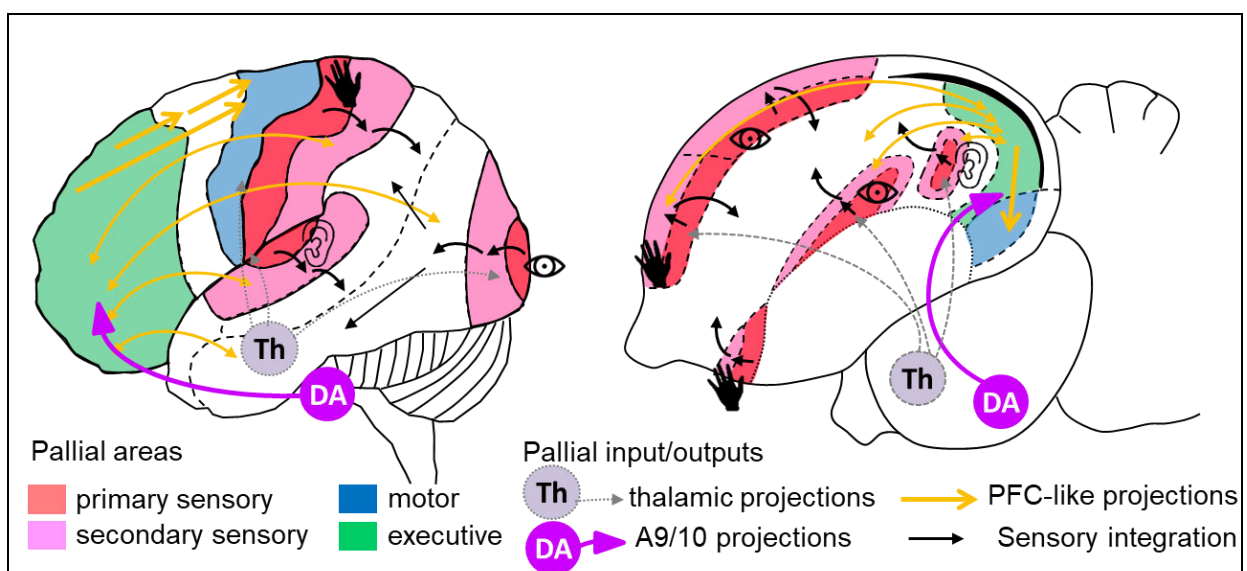


Figure 18. A similar connectivity underlies cognitive function in mammals and birds. Modality-specific projections from the dorsal thalamus (Th) are integrated in primary sensory areas in the pallium. Sensory modality is indicated by a symbol (eye for visual, ear for audition, and hand for somatosensory). Note that birds have two visual areas: visual Wulst (dorsally) and entopallium (more medially). Primary sensory areas in turn project to secondary sensory areas that project to higher

order sensory and associative areas. The latter make the different sensory modalities communicate as represented by converging black arrows. They are reciprocally connected with executive areas (PFC and NCL in mammals and birds respectively) which are the center for goal-directed behaviors. Executive areas project to motor areas which generate the motor outputs. DA is critical for executive function and is provided by midbrain cell groups A9 and A10 (especially A10, VTA). The avian brain schema is adapted from Güntürkün, 2005b.

Another interpretation of this connectivity pattern in birds is that it is similar to the connectivity between cortical layer in mammals (Jarvis et al., 2013; Karten and Shimizu, 1989). Although there are alternative interpretations regarding homology (see below), similarity in the microcircuitry within the sensory pallium has been highlighted (Jarvis et al., 2013, 2005; Karten, 1991). In mammals, layer IV neurons receive sensory information from thalamus and afferent superficial layers II-III. The latter project to deep layers V-VI, which contain descending projection neurons to the brainstem. In the avian pallium, a comparable organization is observed, but segregated in nuclei (Figure 19).

In addition to functional connectivity, molecular data show that thalamo-recipient area in the avian pallium express layer IV markers such as *Rorb* and *Eag2* (Dugas-Ford et al., 2012; Suzuki et al., 2012). Pallial local projection neurons (Layer II-III) and deep layer neurons (layer V-VI), respectively, also express the same set of genes in both groups (Figure 19). In this view, the pallial organization is rather considered to be conserved in terms of cell types.

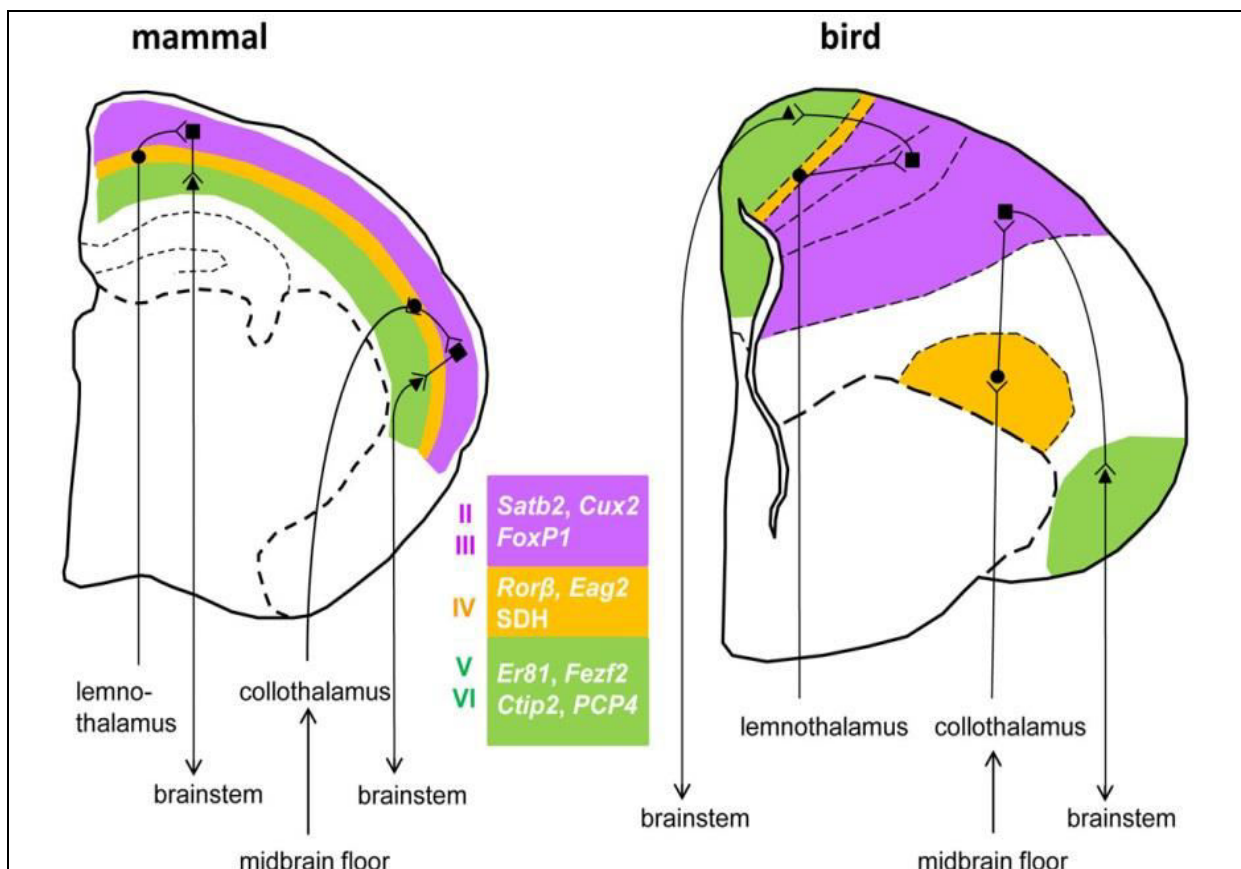


Figure 19. Sensory information exemplified with the visual modality is processed in a similar manner in the mammalian and avian pallia (frontal sections). Cell populations within the mammalian cortical layers and avian pallial nuclei are comparable in terms of connectivity and gene expression. Thalamo-recipient areas (layer IV in mammals) are colored in yellow, and they express a similar set of genes. Similarly, areas containing intra-telencephalic projection neurons are in purple (layer II-III in mammals), while areas sending extra-telencephalic projections (layer V-VI in mammals) are in green.

In mammals, this microcircuitry is organized in cortical layers, while in birds it is organized in different pallial clusters.

6.1.2. Executive pallium

Even though little is known about the associative areas in the avian pallium, the executive area has been identified (Divac et al., 1985; Mogensen and Divac, 1982): NCL, which is connected with higher order sensory areas as well as with motor areas as the prefrontal cortex (PFC) in mammals (Figure 18).

The avian NCL was proposed to be equivalent to the mammalian PFC initially based on behavioral effects of lesion studies. As for the mammalian PFC, the lesion of NCL impaired performance in a delayed alternation task without affecting visual discrimination (Mogensen and Divac, 1993, 1982). Another common feature between them is a massive DA innervation (Divac et al., 1985). Later studies further confirmed the similar functional properties of NCL and PFC serving executive functions (Güntürkün and Bugnyar, 2016; Hartmann and Güntürkün, 1998; Lissek et al., 2002; Rose et al., 2010; Rose and Colombo, 2005).

Executive functions comprehend different inter-related modules which can be tested by different tasks. Based on human psychology, different authors have theorized that there are three “core” executive functions on which rely “higher order executive functions”, such as reasoning, planning or problem solving (Diamond, 2013; Lehto et al., 2003; Miyake et al., 2000).

The three “core” executive functions (Diamond, 2013) are:

- 1) cognitive flexibility, also termed set-shifting
- 2) inhibition, including inhibitory control and selective attention (interference control)
- 3) working memory

PFC is critically involved in these “core” and higher order functions, and allows the selection and coordination between sensory, motor and mnemonic information (Miller and Wallis, 2009).

The same “core” executive functions have been described in avian studies, and it has been shown that pigeons possess the cognitive flexibility, selective attention, and working memory (Güntürkün, 2005b). They can be tested using operant conditioning paradigm in animals.

As we have seen above, operant conditioning tasks are convenient tools to ask questions to animals. Instead of simple tasks such as forced-choice color discrimination (evaluating the animal perception), tasks can be more elaborate to test if an animal possesses specific cognitive capacities. Below are given examples of tasks that have been used in birds, and that can be used to test the three different modules of executive functions evoked in the previous paragraph.

Cognitive flexibility is the ability to change perspectives and adjust to changed demands (Diamond, 2013). It can be tested in a reversal learning task, that consists in being able to switch to a new rule. For instance, a study in pigeon (Hartmann and Güntürkün, 1998) has tested if NCL is involved in this task by lesioning this region. First, pigeons were able to learn a pattern discrimination task in an operant conditioning paradigm: when presented a pair of stimuli, the animal was rewarded when pecking the positive stimulus. Then, when the

animals had learnt the task and when a learning criterion was reached, they were lesioned in NCL or other brain areas and re-trained to reach the criterion. Finally, the rule was reversed: the non-rewarded stimulus became the rewarded stimulus and vice-versa. This revealed that only NCL lesions correlated with a deficit in reversal learning, which is comparable with the impairment of reversal learning following PFC lesions in mammals (Irle and Markowitsch, 1984; Iversen and Mishkin, 1970; Kolb et al., 1974). Similar results were obtained in pigeon through NMDA receptor blockade, which impaired reversal learning (Lissek et al., 2002).

Selective attention is related to inhibitory control, and allows to selectively attend to one stimulus while suppressing attention to other stimuli (Diamond, 2013). It can be tested in a moving-dot paradigm. For instance an experiment in pigeon inspired by the shell-game (Rose et al., 2010) tested if dopamine (DA) is involved in maintaining attention. First, the animals were trained to peck on a target square. Then distractors of the same aspect were introduced, and all the squares including the target one started moving. Pigeons had to maintain attention to the target square during a few seconds and peck at the target square after this duration to be rewarded. Finally, a D1 class DA receptor blocker was infused in NCL, which impacted performance of the pigeons. Similarly, D1 blockade in PFC elicits attention deficits in mammals (Chudasama and Robbins, 2004; Granon et al., 2000).

Working memory represents the ability to hold information currently attended online for later use, and mentally working with this information (Diamond, 2013). It can be tested by behavioral task such as delayed matching-to-sample (DMTS). For instance, in a study in pigeons (Diekamp et al., 2002), the animals are first exposed to a central key illuminated in one color (e.g. green). Then, after a delay period during which no illumination of the keys is provided, two illuminated keys are presented (e.g. one green, one red). Finally, the pigeon will be rewarded only if it pecks at the matching color. DMTS performance was impaired after NCL lesion, which is similar working memory deficits observed after PFC lesions in mammals (Granon et al., 1994).

In avian studies, working memory was initially investigated in pigeon (Blough, 1959; Diekamp et al., 2002) and later also in crows. Jungle crows have been shown succeed even with a long delay, up to 64 seconds (Goto and Watanabe, 2009). Importantly, working memory capacity has been correlated to general cognitive abilities, both developing in parallel with age in humans (Cowan, 2016). Mammals and birds also present similar working memory characteristics, and thus could be used as an approximation for general cognitive function (Lind et al., 2015; Matzel and Kolata, 2010; Sawaguchi and Goldman-Rakic, 1991). Additionally, DA in PFC and NCL is important for working memory both in mammals and birds, and its effects are mainly mediated through the D1 receptor family (Güntürkün, 2005a; Herold et al., 2012, 2008; Karakuyu et al., 2007; Phillips et al., 2004).

Despite such similarities, PFC and NCL are generally considered to be the result of convergent evolution (Güntürkün, 2005b), since PFC is located at the anterior end of the pallium while NCL is located at the posterior end.

It has been proposed that an important DA innervation to a specific pallial area could be critical to evolve PFC-like functions (Güntürkün, 2005b, 2005a). Both PFC and NCL receive massive inputs from the mesencephalic DA neurons (A9/A10). Even though the pallial targets are not homologous, the mesencephalic DA nuclei in mammals and birds are

accepted to be homologous. The evolution of DA neurons will be discussed in the later section.

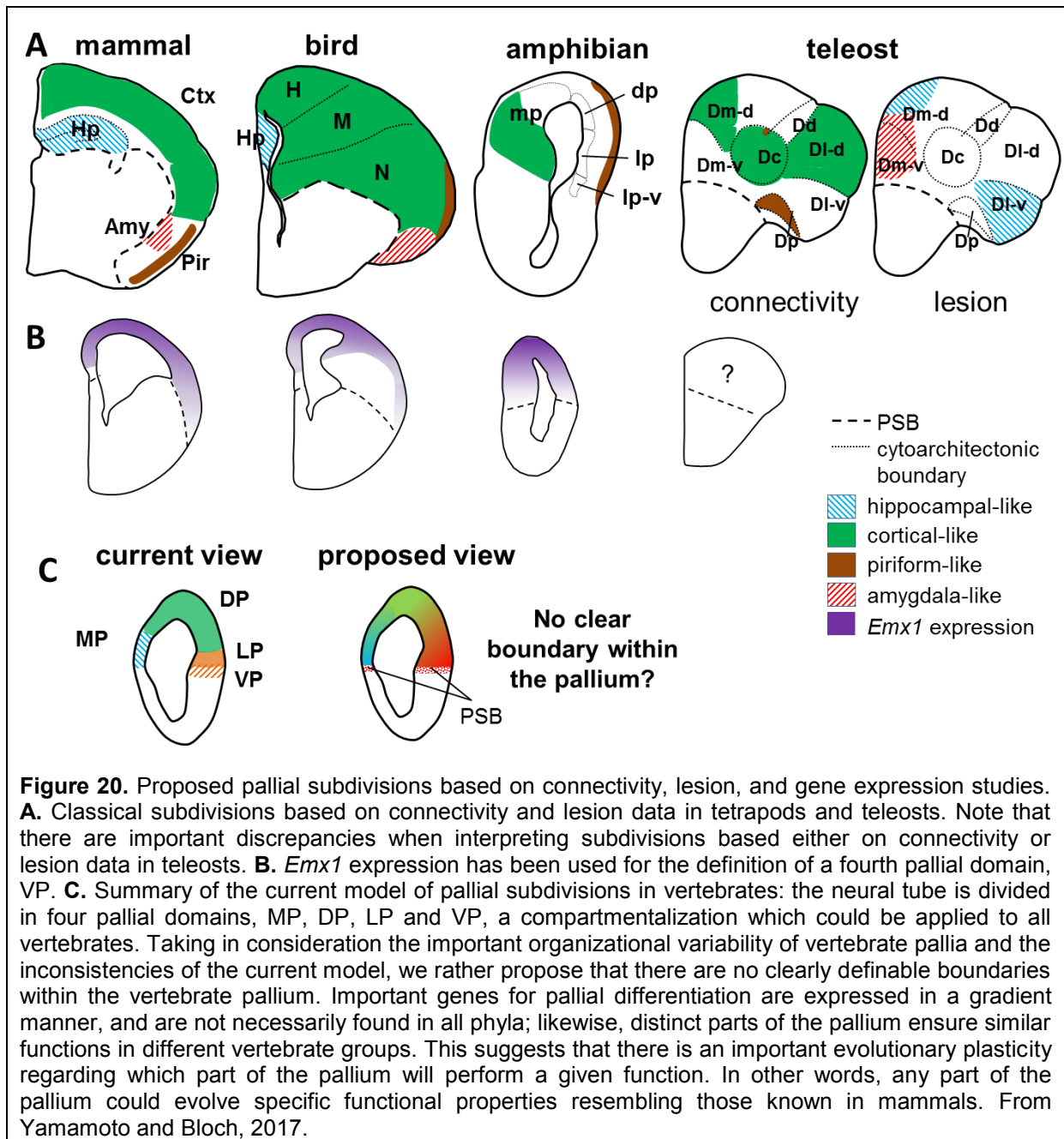
6.1.3. Problem of homology

Despite the functional similarities between mammalian and avian pallia described above, homology of pallial areas serving the same functions is not clear. More specifically, there has been a controversy whether the avian DVR containing the sensory recipient areas and executive functions are homologous to the mammalian cerebral cortex.

Some authors claim that they are homologous due to the high degree of similarity (Figure 20A, Butler et al., 2011; Karten, 1991; Reiner et al., 2005). Another hypothesis is that the avian DVR is homologous to the non-layered structure corresponding to the claustrum-amygdaloid complex in mammals (Fernandez et al., 1998; Puelles et al., 2000). The latter claim comes from data showing that *Emx1*, a transcription factor which is expressed throughout the mammalian cortex during development, is not expressed in the avian DVR. This *Emx1* negative domain was named as the ventral pallium (VP, see below, Figure 20B). In this view, the visual inputs to the avian nidopallium would be homologous to the visual inputs to the claustrum or lateral amygdala, which do not have retinotopic organization. Thus the similar retinotopic organization in the avian primary visual areas and the mammalian visual cortex would be a consequence of convergent evolution (homoplasy).

The third hypothesis is somewhat in between the two previous propositions, suggesting that there is no one to one correlation as a region. Instead, the avian nidopallium is homologous as a field to both the temporal cortex and the claustrum-amygdaloid complex (Butler and Molnár, 2002; Jarvis et al., 2013; Molnár and Butler, 2002). Indeed, in terms of molecular features, all the genes are expressed in gradient and many of the genes expressed in the cortical layer V-VI (deep layers) are also expressed in the amygdala (Jarvis et al., 2013). Thus, the nature of the amygdala may not be so different from that of the cortical areas, although it is organized in layers.

Discussing each hypothesis is out of the scope of my thesis, but it illustrates that regional homology is difficult to demonstrate even within amniotes. Accordingly, it is even harder to identify homologues in other vertebrate groups. Nonetheless, many interpretations of data on non-mammalian models rely on prevailing hypotheses about regional homology (Figure 20). As you may see below, it is the case for teleosts.



6.2. Teleost pallial organization

6.2.1. New model of morphogenesis of the teleost pallium

In comparison with amniote pallia, teleosts present a radically different organization. A major difference exists between *Sarcopterygii* and *Actinopterygii* concerning the development of the telencephalon (Holmgren, 1922). In most vertebrates including *Sarcopterygii* the telencephalon develops through a process called evagination, in which the roof of the neural tube converges to divide the central lumen of the neural tube in two lateral ventricles. In *Actinopterygii* the roof follows an opposite movement of elongation which forms one telencephalic ventricle which covers the pallium. This process is called eversion (Figure 21A).

Based on the idea that stem vertebrates should have possessed pallial subdivisions comparable to current mammals, comparative neuroanatomists have been trying to fit the subdivisions of mammalian cortex to those of teleosts (or other *Actinopterygii*).

Originally Holmgren postulated the existence of three subdivisions (Holmgren, 1925, 1922): the hippocampal pallium (hp), the general pallium (gp) and the piriform pallium (pp). In current literature, they are more commonly designated as the medial pallium (MP=hp), dorsal pallium (DP=gp) and lateral pallium (LP=pp) as an indication of their topology in the pallium of *Sarcopterygii* (current view in Figure 20C, 21A). It has generally been accepted that this organization was present in the common ancestor of vertebrates, and these three subdivisions thus defining the “morphotype” of the vertebrate pallium (Northcutt, 1995). However, these subdivisions were based on the position of mammalian structures: the hippocampus within the MP, the 6-layered neocortex for the DP and the 3-layered pyriform cortex plus pyriform lobe containing the claustral and amygdaloid complexes for LP. Later, a fourth subdivision was introduced based on *Emx1* negativity in amniotes, which was named the ventral pallium (VP; Fernandez et al., 1998; Puelles et al., 2000).

Correspondence with non-mammalian pallia is not simple. We just discussed the problem in the case of the avian brain. Even in amphibians (which have a simple tube-like pallium), connectivity studies show that pallial inputs from thalamus and output projections are rather found in mp than dp, while olfactory projection extend to dp (Figure 20A). This has led to the proposition that amphibian dl and lp are homologous to the mammalian LP, while mp is homologous to MP/DP (Bruce and Braford, 2010).

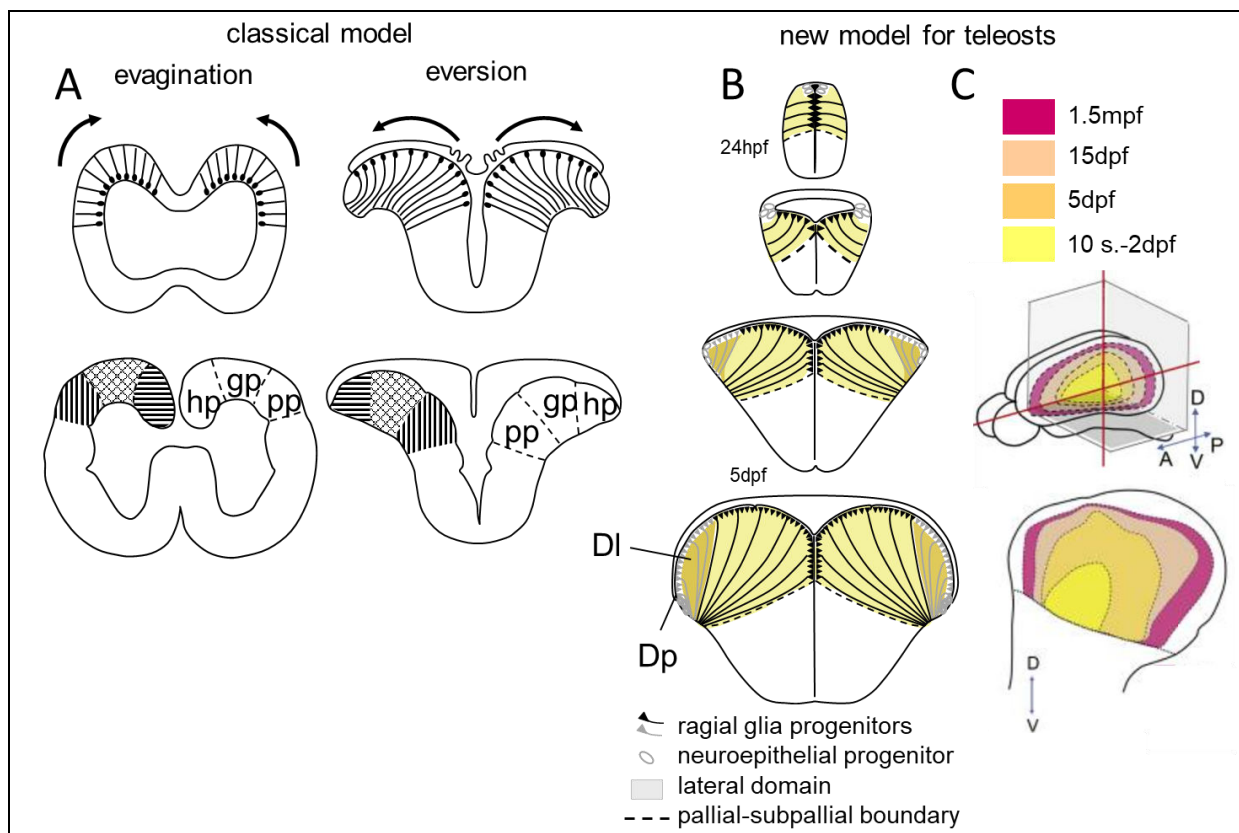


Figure 21. Development of the telencephalon and pallium in *Sarcopterygii* and *Actinopterygii*. *Sarcopterygian* telencephalon undergoes evagination, while *Actinopterygian* telencephalon undergoes eversion. **A.** Historically, pallial regions in *Actinopterygii* have been considered as a simply

reversed version of the Sarcopterygian pallium. **B.** However, lineage tracing data indicate that the construction of the Actinopterygian pallium is not the reversed version of the Sarcopterygian pallium, and that the DI and Dp are built by a delayed neurogenesis, later in development. Thus Dp does not correspond to pp. **C.** Later in development, the pallium is built by progressive “stacking-up”. Abbreviations: dpf, days post-fertilization; DI, lateral part of the dorsal telencephalic area; Dp posterior part of the dorsal telencephalic area; gp, general pallium; hp, hippocampal pallium; mpf, months post-fertilization; pp, pyriform pallium; s., somites. A is adapted from Holmgren, 1925, 1922, B from Dirian et al., 2014, and C from Furlan et al., 2017.

In teleosts, a topological nomenclature of pallial subdivisions has been adopted to describe the mature brain (Wulliman et al., 1996). “D” standing for dorsal telencephalon (pallium), it is divided into different parts (Figure 22): central (Dc), dorsal (Dd), dorso-medial (Dm), dorso-lateral (DI) and posterior-lateral (Dp). Based on the eversion theory (basically assuming that the teleost pallium is a reversed version of the tetrapod pallium), DI was considered to be homologous to the mammalian hippocampus (MP), while Dm was considered to be homologous to the mammalian amygdala (LP or VP; see below). Many results obtained from teleost brains have been interpreted with these assumptions (Lal et al., 2018; Mueller, 2012; Mueller et al., 2011; von Trotha et al., 2014). However, this is not obvious taking a closer look at the adult teleost pallium: Dp is the primary olfactory area in teleosts. Accordingly, following the eversion theory, it should correspond to LP/pp and thus should originate from the ventral part of the pallium near subpallium, closer to the midline (Figure 21), while it is actually located at the lateral edge of the pallium.

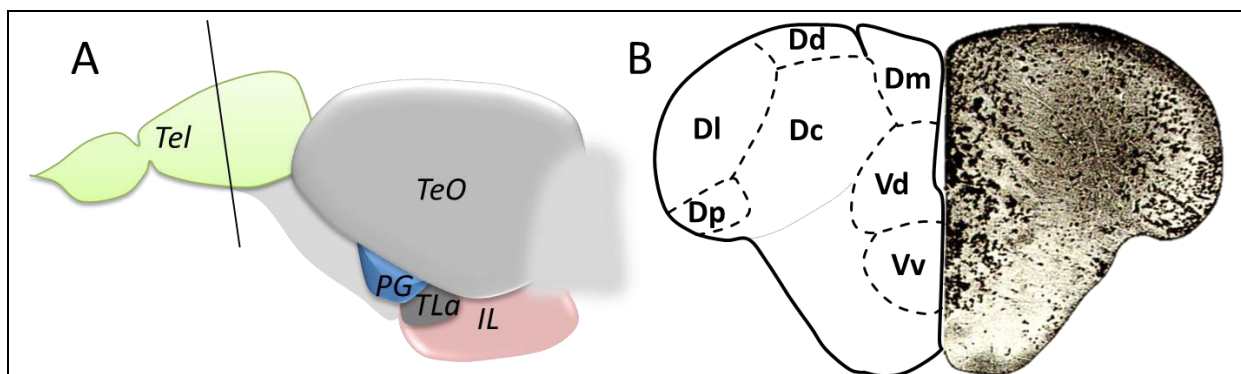


Figure 22. General view of the teleost brain (**A**) and major pallial parts following the topological nomenclature (**B**). **A.** The teleost brain in lateral view, with visible midbrain and forebrain structures displayed (caudal levels including cerebellum are cut). **B.** Frontal section in the middle of the telencephalon showing the major pallial subdivisions on the left and the corresponding cytoarchitecture on the right (picture from the atlas). The pallial-subpallial boundary is shown in light grey. Abbreviations: D, dorsal telencephalic area; Dd, dorsal zone of D; Dc, central zone of D; DI, lateral zone of D; Dm, medial zone of D; Dp, posterior zone of D; IL, inferior lobe; PG, preglomerular nucleus; Tel, telencephalon; TeO, optic tectum; TLa, torus lateralis; V, ventral telencephalic area; Vd, ventral nucleus of D; Vv, ventral nucleus of V. Adapted from the zebrafish atlas of Wulliman et al., 1996.

Recently, data in zebrafish has disproven the view that the teleost pallium is a simple reversed version of the Sarcopterygian pallia due to its eversion development (Figure 20B). Cell lineage experiments show that the lateral parts of the pallium (corresponding DI and Dp) originate from the dorsal tip of the pallium later than the medial part (Dirian et al., 2014). Thus the DI and Dp are not pushed laterally, but added by proliferation at later stage during development (after 5 days post-fertilization in zebrafish).

A yet more recent study (Furlan et al., 2017) has further shown that the construction of the whole adult zebrafish pallium is obtained by “sequential stacking” along development (Figure 20C). Dc is the first part to be formed between 10 somites and 24hpf. The other pallial domains cannot be defined by a specific temporal window or a clonal unit, as they are progressively added around Dc by stacking in an inside-out fashion (Figure 20C).

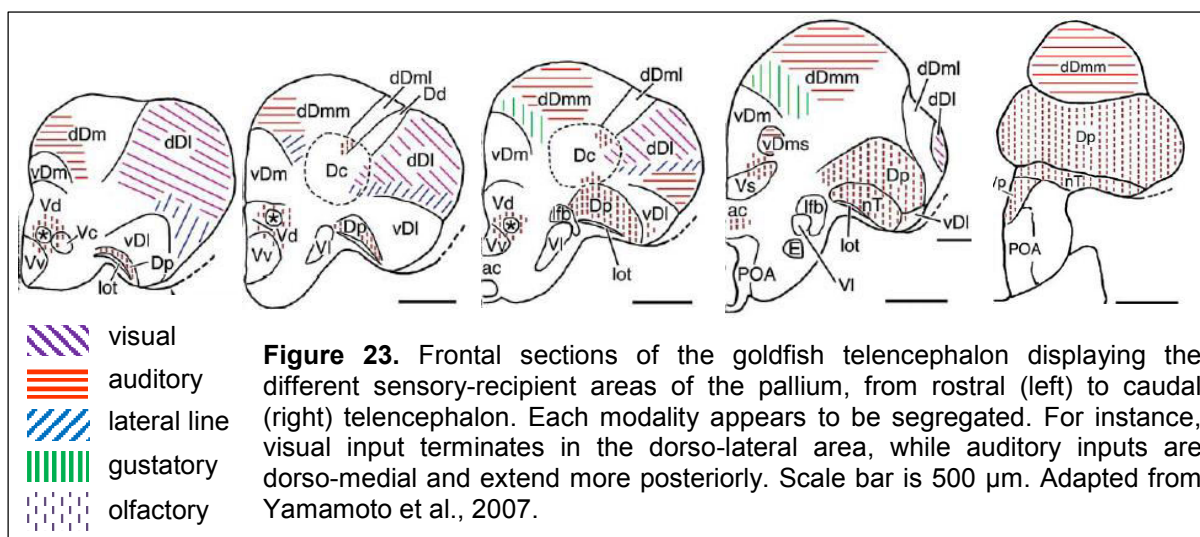
In conclusion, these lineage tracing data revoke the classical eversion theory. Thus the current homology of pallial subdivisions between teleosts and amniotes need to be reconsidered. The teleostean pallium may not be comparable to the amniote pallium by assuming a simple one-to-one correspondence (cf Figure 20 and 21).

It is also worth mentioning that regarding gene expression in the pallium, there is no data showing an *Emx1*-poor and *Tbr1*-positive domain outside of tetrapods (Figure 20B). Data actually suggest a differential implication of *Emx* genes in mammals and teleosts. *Emx1* and *Emx2* play an important role in mammalian pallial development (Hamasaki et al., 2004; Hong et al., 2007). In contrast knocking down these genes has no significant effect in zebrafish, while in this species *Emx3* plays an important role in pallial development (Viktorin et al., 2009). *Emx3* gene has been lost in most tetrapods except a few species like *Xenopus* or opossum. Thus one *Emx* gene may not be the best way to define a pallial region.

6.2.2. Sensory afferents to the pallium

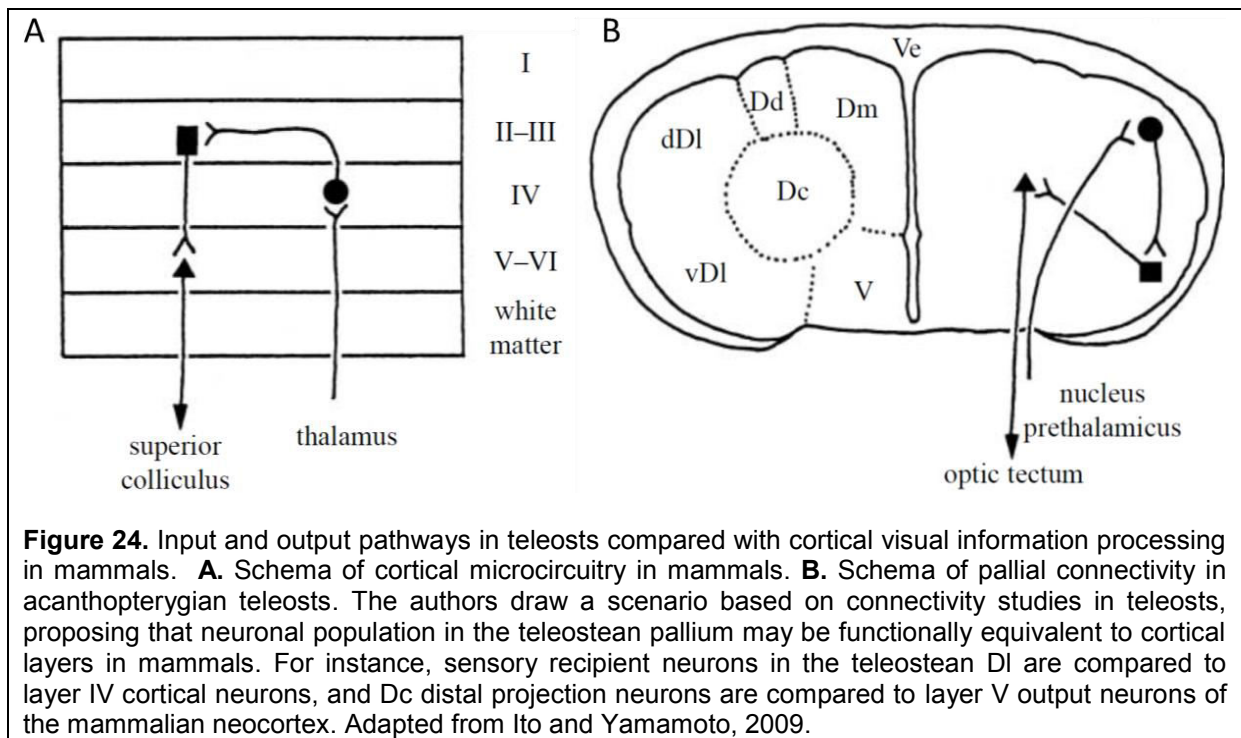
Although no consensus about regional homology has been reached, some connectivity studies show there are some similarities between teleosts and amniotes.

Studies in several teleost species including goldfish have demonstrated that the teleost pallium receives sensory inputs of different modalities: visual, auditory, olfactory, gustatory, and lateral line. This has been compared to the primary sensory areas in mammals (Ito and Yamamoto, 2009). The sensory-recipient areas present a wide distribution throughout the goldfish pallium, visual inputs terminating in the DI, auditory to the Dm, and olfactory to the Dp (Figure 23).



Based on hodological studies focusing on sensory inputs and outputs of the teleostean pallium, the neuronal populations within the different subdivisions of the teleost pallium have been compared to the layers of the mammalian neocortex (Ito and Yamamoto, 2009). The

authors formulate the hypothesis that teleosts may have a “non-laminar cortex” (Figure 24), as it has been proposed for birds (Figure 19).



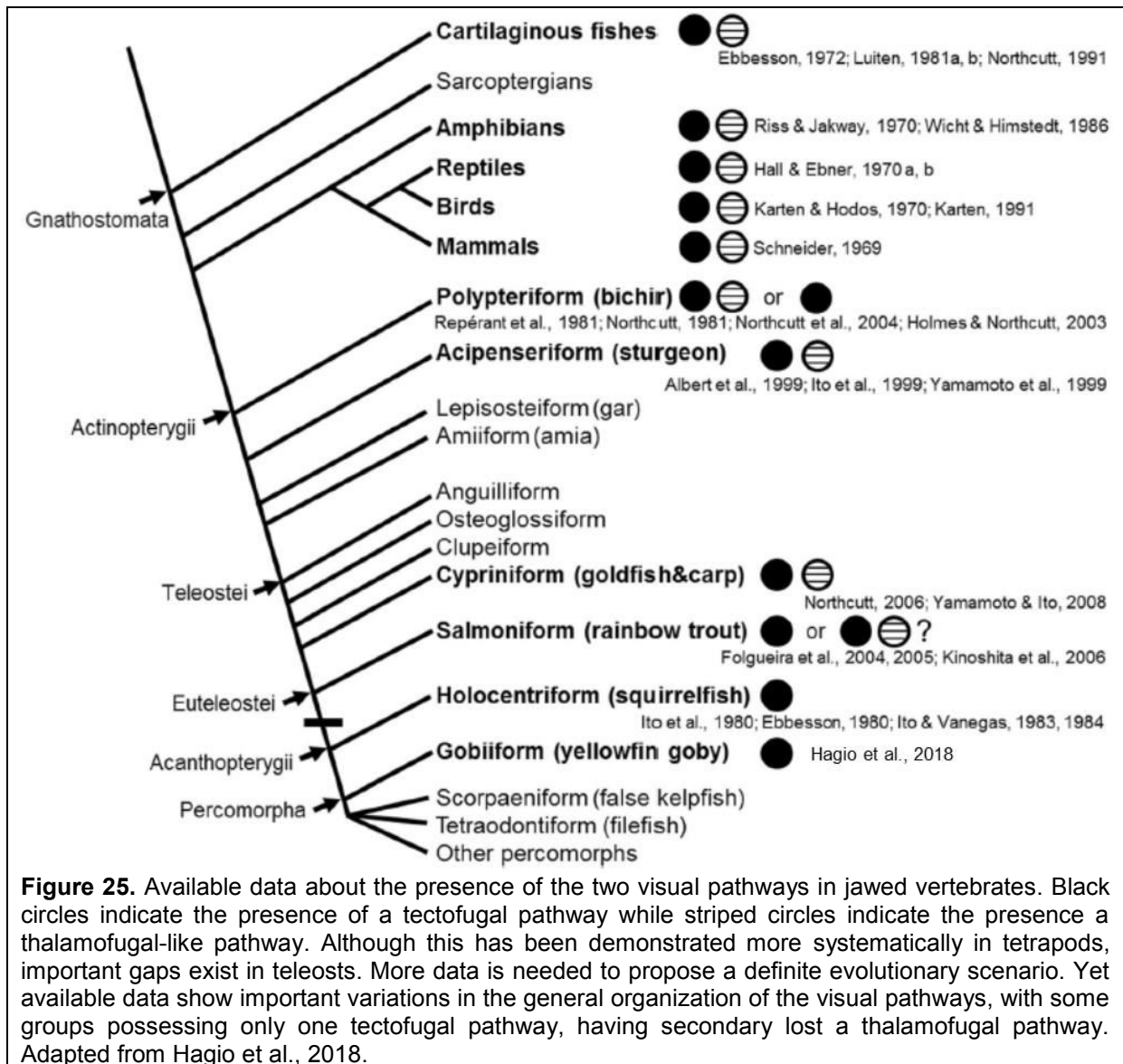
A major difference between teleost and tetrapod sensory pathways is that the major sensory relay nuclei to the pallium are not located in the dorsal thalamus (dorsal part of the diencephalon) but in the preglomerular nucleus, or preglomerular complex (PG), a structure located in the posterior tuberculum (ventral part of the diencephalon) (Butler and Hodos, 2005).

Sensory information is also relayed to the telencephalon by nuclei defined as thalamic based on topology (Wulliman et al., 1996), but in a minor way (Mueller, 2012). The main target actually appears to be the subpallium (Northcutt, 2006). Based on connectivity, the only modality which has been consistently reported to reach the pallium in goldfish and two species of vocalizing teleosts (*Batrachoididae*, *Percomorpha*) is auditory (to the Dm), with auditory torus semicircularis (TS) inputs in the central posterior thalamic nucleus (Goodson and Bass, 2002; Northcutt, 2006). One study has reported that visual stimulation elicits recordable responses in this thalamic nucleus, but surprising not in PG (Kirsch et al., 2002).

Based on studies in goldfish and carp, PG has a similar connectivity as the dorsal thalamus in mammals and birds, since it relays various sensory afferents to the pallium in a topologically segregated manner. For visual information, PG receives visual inputs from TeO and retina in dorsal zone of the rostromedial region the lateral PG (PGlr-d). In turn, PGlr-d project to DI (Yamamoto and Ito, 2008). In the case of auditory information, PG receives auditory inputs from the central nucleus of TS (compared to mammalian IC; Nieuwenhuys et al., 2014) in different parts of the PG: anterior PG (PGa), the caudomedial region of lateral PG (PGlc), and a medial zone of medial PG (PGm) (Yamamoto and Ito, 2005a, 2005b). In turn, these PGa, PGlc and medial PGm project mainly to Dm (Figure 23).

However, there are no functional studies assessing which kind of information is processed in these primary sensory pallial areas and their relays. Moreover, there are little data about the development of these pathways. In particular PG origin has been debated, although always considered as diencephalon. It was initially proposed to be migrated from the periventricular posterior tuberculum (basal plate; Nieuwenhuys et al., 2014). Then based on topology and migrating cells expressing *Pax6* from both basal and alar diencephalon, it has been proposed that PG may have a composite origin (Wullimann and Rink, 2002). Finally another study in medaka has suggested that PG originates mainly from the alar plate, based on the observation of strips *Pax6* or *Dlx2* positive cells which appeared to migrate caudolaterally (Ishikawa et al., 2007). Surprisingly, our results clearly demonstrate that PG originates mainly from the midbrain, probably from two different progenitor pools (see article 2 and discussion).

Regarding the ascending visual pathways, it is generally admitted that jawed vertebrates generally possess two visual pathways to the pallium: a thalamofugal-like and a tectofugal-like. However, there is some variation in organization within *Actinopterygii*. Indeed, some species seem to have only one visual pathway to the pallium (reviewed in Hagio et al., 2018, Figure 25). PG has been described in cyprinids (goldfish and carp), which are thus relatively close to zebrafish. In holocentrids and gobies, which are related to *Percomorpha* and thus relatively distant phylogenetically from cyprinids, visual information is relayed by a different set of nuclei in the nucleus prethalamicus (PTh), which has not been identified in cyprinids. As its name indicates, it located in a more rostral and dorsal position than PG, and it receives afferents from the optic tectum; injections in different parts of the pallium leads to the labeling of different parts of the PTh, as seen for PG (Hagio et al., 2018; Xue et al., 2003).



Another area receiving different sensory inputs is the teleostean IL (Figure 22A, in red). Based on connectivity data, IL was first described as a gustatory center (Morita et al., 1983), an important sensory modality for cyprinids, it also receives other sensory modalities such as visual (Ahrens and Wullimann, 2002), viscerosensory (Finger and Kanwal, 1992), or electrical in gymnotids (Keller et al., 1990), which led to the hypothesis it may be a multi-sensory integration center (Rink and Wullimann, 1998).

6.2.3. Executive pallium in teleosts?

Until this day, there is no anatomical data that could point at an executive area in teleosts. Secondary and higher order sensory areas have not been described yet. However, some behavioral studies suggest that teleosts may have some learning flexibility.

Parker et al. (2012) have shown that zebrafish are capable of performing a reversal learning task, which suggests the existence of learning flexibility in this species. In this study, zebrafish learn a forced-choice color discrimination task: they learn to go to the compartment harboring the color that is rewarded and not to the non-rewarded color. When the rule is reversed (e.g. they learn first that green is rewarded and not red then red is rewarded and

not green), the animals learn faster than in a naive condition, suggesting that there are capable of generalizing the rule (Parker et al., 2012).

It has been shown that goldfish are capable to perform a simultaneous matching-to-sample task (SMTS), although the experiments were performed with a small number of subjects (Goldman and Shapiro, 1979).

In the second part of my thesis, we have further investigated the cognitive abilities of zebrafish. More specifically we wanted to know if this species is able to perform other tasks that could reflect the existence of executive functions. For this purpose, based on the set-up of Parker, we developed a delayed matching-to-sample task (DMTS) for zebrafish.

Our results show that zebrafish are capable of performing a DMTS task successfully, revealing the existence of working memory. Since working memory is considered as one of the “core” executive functions, our results corroborate the idea that teleost species possess executive functions.

7. Diversity of dopamine systems in vertebrates

As mentioned above, the mesencephalic DA cell population (also known as A9/A10) plays a critical role in executive functions both in mammals and birds. One of the striking differences in teleost brains is the absence of this mesencephalic DA cells. It is intriguing that teleosts can perform a reversal learning task (Parker et al., 2012) without a DA innervation from the mesencephalon.

It is important to note that the basic knowledge on the DA system was established from mammalian studies. Data on non-mammalian models have been interpreted based on the assumption that there would be a one-to-one correspondence among different vertebrate groups. However, an accumulation of data on the DA systems in vertebrates suggests that the DA system is much more diversified than it had been thought. When put into the context of vertebrate evolution, one can see important molecular and neuroanatomical variations of the systems, especially between teleosts and mammals.

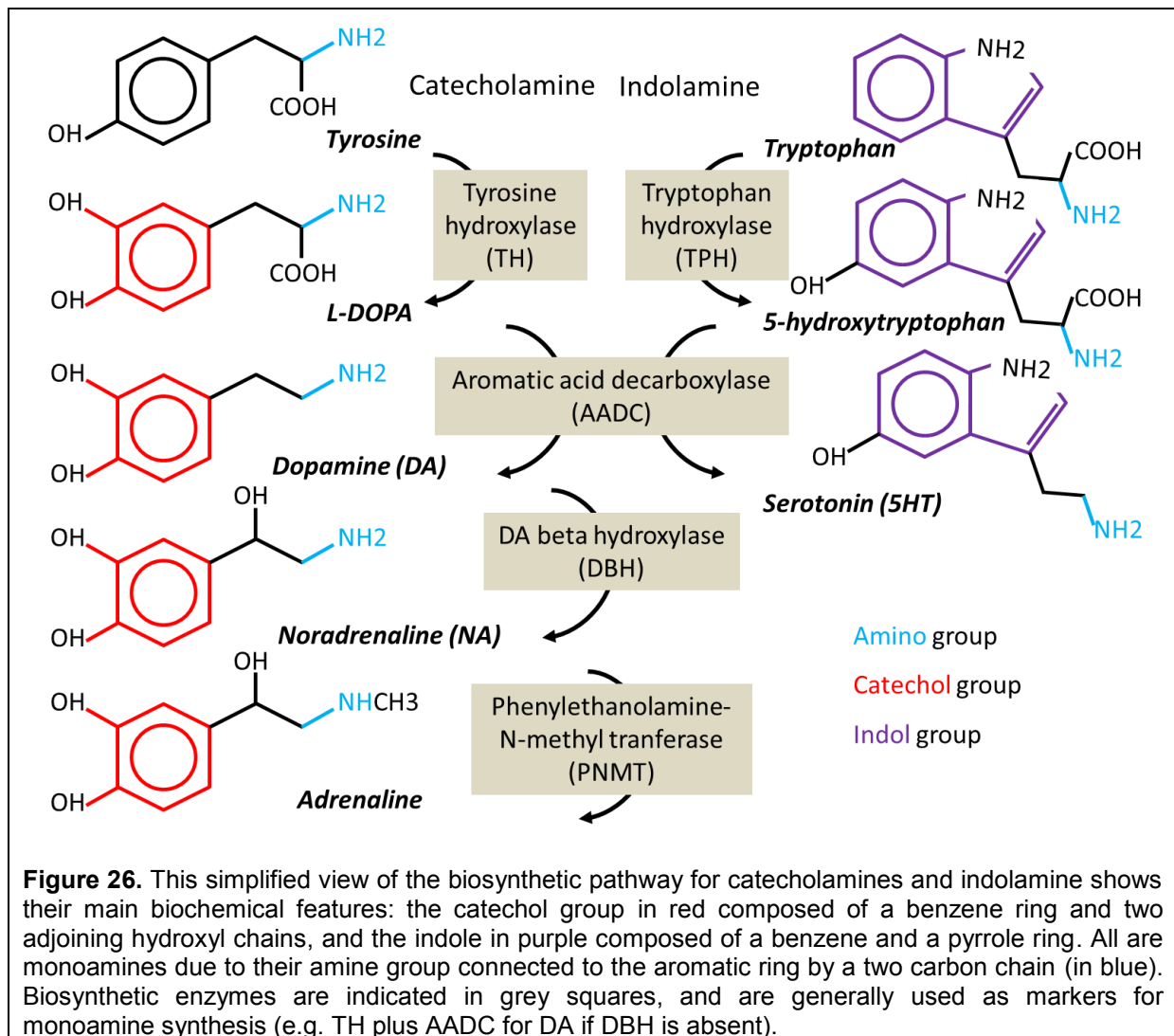
7.1. Molecular diversity

7.1.1. Metabolic and catabolic components of DA

DA belongs to the monoamines, which are compounds with one amine group connected to an aromatic ring: these include catecholamines such as noradrenaline (synonymous with norepinephrine, NA) or adrenaline (epinephrine), indolamines (compounds with a pyrrole ring) such as serotonin (5HT) or melatonin, and trace amines which are present in low amounts in vertebrates such as octopamine.

DA is synthesized in two steps from L-tyrosine. The first limiting step is performed by a tyrosine-hydroxylase (TH) which converts tyrosine into L-DOPA. While there is only one *TH* gene in mammals, a second TH gene was first discovered in teleosts (Candy and Collet, 2005). A later study has revealed that most vertebrates possess two *TH* genes (*TH1* and *TH2*) in the genome. It is likely that two *TH* genes emerged as a consequence of a whole-genome duplication before the divergence of jawed vertebrates, and that *TH2* was secondarily lost in placental mammals (Yamamoto et al., 2010). Placental mammals are the only group to have secondarily lost a second TH enzyme, and non-placental mammals such as platypus (monotreme) and opossum (marsupial) possess an ortholog of the *TH2* gene.

In the second non-limiting step, an aromatic amino-acid decarboxylase (AADC) converts L-DOPA into DA; AADC is not specific for the catecholamine metabolic pathway since it is shared with indolamine such as 5-HT (Figure 26). Additional steps can occur to convert DA into NA with DA beta hydroxylase (DBH), then NA into adrenaline with phenylethanolamine-N-methyl transferase (PNMT). Upon synthesis, monoamines are packed into synaptic vesicles through the vesicular monoamine transporter VMAT2, which is expressed in CNS neurons.



Following neurotransmitter release, the DA transporter (DAT) re-uptakes DA into neurons. Although DAT is the “DA transporter”, it is not specific to DA. Conversely, DA can be transported through the other monoamine transporters such as the serotonin transporter (SERT) and norepinephrine transporter (NET). Indeed, DAT, SERT and NET present some affinity for all the three molecules, DA, 5HT and NA (Daws, 2009).

In vertebrates, there are actually two SERT genes, SERT1 and SERT2: as for TH2, SERT2 was secondarily lost in placental mammals (Caveney et al., 2006; Yamamoto and Vernier, 2011). DAT has secondarily lost in sauropsids (the sister group of mammals including birds and reptiles). It has been shown that function of DAT is replaced by NET expression in the two major midbrain nuclei: substantia nigra pars compacta (SNc) and ventral tegmental area

(VTA), both in a bird and a lizard species (Lovell et al., 2015). Moreover, regulatory elements of the mammalian DAT promoter are common with the sauropsid NET, suggesting that NET is directly expressed in DA neurons in sauropsids. Interestingly, NET is expressed at high levels in the PFC in mammals (Morón et al., 2002; Valentini et al., 2004). It is possible that the function of NET might play an important role in the similar executive functions found in mammals and birds.

DA is degraded by the extracellular catechol-O-methyl transferase (COMT) and intracellular monoamine oxidase (MAO). In most vertebrates there are two MAO genes, MAO-A preferentially degrades DA and MAO-B 5HT, with the exception of some teleost species including zebrafish which only have one MAO (Anichtchik et al., 2006).

7.1.2. Dopamine receptor genes

Dopamine (DA) is a neuromodulator with a wide range of functions in vertebrate brains. These include reward, olfaction, motivation, mood, reproduction, sleep, learning and memory, and voluntary movements (Gerfen et al., 1987; Goldman-Rakic, 1999; Hornykiewicz, 1966; Kehagia et al., 2010; Matsumoto et al., 1999; Oishi and Lazarus, 2017; Schultz, 1998; Tuomisto and Männistö, 1985; Vogt, 1973). Such different roles of DA largely depend on the projection targets, where DA is transmitted to post-synaptic neurons via DA receptors.

Two DA receptors were initially identified based on their pharmacological characteristics: D1 agonists activate adenylate cyclase through G_s heterotrimeric G proteins, while D2 activation modulates different intracellular signaling pathways through G_{i/o} G proteins (Kebabian and Calne, 1979). In mammals, five DA receptors have been identified and classified based on these pharmacological characteristics: in the D1 family are D1 and D5 (sometimes called D1A and D1B in non-humans), in the D2 family are D2, D3 and D4. Molecular phylogeny of monoamine receptor families shows that they are not grouped according to their ligand. Therefore they evolved the capacity to bind a neurotransmitter independently: it is the case for D1 and D2 families, which are actually more related to 5HT₆ and 5HT₂ respectively (Yamamoto et al., 2015, 2013).

It has been proposed that the common ancestor of *Osteichthyes* should have possessed nine DA receptor subtypes (Yamamoto et al., 2015). Four are in the D1 family (D1, D5, D6 and D7), five are in the D2 family (D2, D3, D4, D8 and D9). This situation could be explained by the two whole genome duplications (or at least large scale genome duplications) that occurred at the origin of vertebrates. Secondary losses occurred in many species, for example there are seven DA receptor genes in avian species such as chicken, and five in placental mammals. In contrast zebrafish possesses fourteen DA receptor genes, as a result of additional whole genome duplication (Figure 27).

7.2. Diversity of DA cell populations

Historically DA cell populations were described in the rat brain, with the formaldehyde-induced fluorescence technique (FIF), which permitted to visualize catecholamines, in particular DA and NA, thanks to a peak of fluorescence around 430 nm (Falck et al., 1962). 12 catecholamine cell groups were thus identified, named A1 to A12 from caudal to rostral. In the 80's this technique was replaced by immunohistochemistry using specific antibodies for the different components of the monoamine catabolism tested in a wide range of vertebrates, recognizing in particular the synthetic enzymes, such as TH for DA and DBH for

NA (or adrenaline), which permitted a better identification of the neurotransmitter expressed in one cell group. Immunocytochemistry led to the identification of additional cell groups, leading to a nomenclature still widely used, with 17 NA or DA cell clusters (A1-A17) and 3 adrenergic (C1-C3) in the rat brain (Smeets and Reiner, 1994). A1-A7 are NA cell groups (with the exception of A3 which has not been confirmed), A1-A3 being in the medulla while A4-A7 are in the pons, A6 being the locus coeruleus. Since no DBH or PNMT expression was observed rostral to the MHB, all TH-immunoreactive cells of A8-A17 were classified as dopaminergic (general review in Smeets and González, 2000).

This classification of DA cells has been well accepted and many comparative neuroanatomists have been trying to correlate non-mammalian DA cell populations to one of these cell populations. Even though it was found that mammalian DA system is not necessarily a representative case of vertebrate DA systems (Xavier et al., 2017; Yamamoto et al., 2017), the majority of the knowledge on DA functions comes from mammalian studies, and non-mammalian data have been interpreted by comparison with mammalian data. Thus in the following sections, I introduce how the DA cell populations have originally been classified, and try to compare with non-mammalian data when possible.

7.2.1. Midbrain DA cell populations

A8-A10 are classified as the mesencephalic cell groups, even though A9 and A10 actually extend into the ventral diencephalon (Verney et al., 2001). A8 is the retrorubral field, A9 corresponds to the substantia nigra pars compacta (SNc), and A10 to the ventral tegmental area (VTA).

SNc and VTA are the most studied DA populations, containing the majority of DA neurons in the human brain, and have been involved in major motor and cognitive functions. DA cell loss in SNc has long been described as the major cause for Parkinson disease symptoms (Obeso et al., 2010). VTA has been described as a major integrator for reward, motivation, emotion and PFC-related cognition. VTA has been implicated in a variety of neuropsychiatric disorders such as addiction, depression (Russo and Nestler, 2013) or schizophrenia (Rice et al., 2016). In mammals, VTA mainly projects to PFC (called the mesocortical pathway), and to the limbic system (called the mesolimbic pathway), including amygdala and ventral/visceral basal ganglia structures such as nucleus accumbens. An important heterogeneity exists within VTA DA neurons regarding their projections to other brain regions, coexpression of other neurotransmitters or neuropeptides and their electrophysiological properties: their specific roles are just starting to be unraveled (Morales and Margolis, 2017). SNc mainly projects to the dorsal/somatic basal ganglia such as the dorsal striatum (called the nigro-striatal pathway).

Although these projection patterns are not strictly segregated (for instance VTA also projects to the caudate putamen and SNc to the PFC), this connectivity has been used as a base for the identification of the homologous A9 and A10 in other vertebrates. Birds present a very similar organization concerning these cell groups: avian SNc has been named based on its relative dorsolateral location and its massive inputs to the dorsal striatum, while VTA is ventromedial and projects mainly to the ventral striatum (Reiner et al., 2004). The mesencephalic DA cells are mostly *TH1*-expressing cells in birds, but weak expression of *TH2* was also found in chicken (Xavier et al., 2017). A similar configuration exists in non-avian reptiles, suggesting a common organization in all amniotes (Medina and Reiner, 1995). In amphibians, nigro-striatal and meso-limbic pathways have been identified as well,

but the DA cells are located more rostrally, mostly in the diencephalon. Moreover DA cells are not segregated according to their projection target (Marín et al., 1997).

In teleosts and in lampreys (Pierre et al., 1997), there are no DA cells in the midbrain. In contrast, *Chondrichthyes* (cartilaginous fish) possess midbrain DA cells (Smeets and Reiner, 1994). For this reason, it is difficult to assess the situation in the last common ancestor of vertebrates.

7.2.2. Forebrain TH1 cell populations

A11-17 cell groups are located in the forebrain. Apart from those in the olfactory bulb (A16) and in the retina (A17), the forebrain cell groups are loosely defined, because they are not necessarily segregated in a specific cell nucleus. Roughly, A11 is located in the caudal diencephalic area (or some authors consider in the posterior hypothalamic area), which are known to project to the spinal cord. A13 is the ventral thalamus (prethalamic) cell group, which is known with incerto-hypothalamic projections in mammals. A12 is located in the arcuate nucleus of the hypothalamus (known as the tuberal cell group). A12 projects to the median eminence, and controls the anterior pituitary (adenohypophysis) via the portal blood system. A14 is the rostral periventricular group which projects along the pituitary stalk to the intermediate and posterior pituitary (neurohypophysis). A15 cells are even more dispersed (from the hypothalamus to the preoptic area). In some species such as in sheep, A15 (retrochiasmatic nucleus) DA neurons plays an important inhibitory role on pituitary gonadotropic functions by counteracting the stimulatory effects of GnRH (Gayrard et al., 1995; Vigié et al., 1998).

This inhibitory control of DA on reproduction can be found in the group of birds, amphibians, and teleosts (Dufour et al., 2005; Fontaine et al., 2013), but only in some species within each group. Comparisons between sheep (a well-studied mammalian species) and zebrafish suggest that the DA cell populations involved in this function are not homologous, as the sheep retrochiasmatic nucleus is in the hypothalamus while the zebrafish DA is located in the anterior end of the ORR close to the telencephalon (Fontaine et al., 2015). In addition, the mode of DA transmission is different. In amniotes, DA is released at the level of the median eminence and transmitted via the portal blood system to the pituitary, while in teleosts DA neurons directly innervate the pituitary (Ball, 1981). Thus it is likely that the similar DA functions on reproduction have evolved independently in different vertebrate groups.

In teleosts, prominent DA cell populations are located in the ventral diencephalic area called the posterior tuberculum. The posterior tuberculum is not well identified in amniotes (because the dorsal diencephalon is extremely enlarged). As they project to the subpallium the posterior tubercular DA cells had been a candidate for an equivalent of A9 cells (Rink and Wullimann, 2001). Later studies by Driever and his colleagues proposed that the teleost posterior tubercular DA cells are rather homologous to the mammalian A11. They both require *Otp* and *Nkx2.1* for their specification (Löhr et al., 2009). Moreover cells located in the posterior tuberculum have been shown to project to the spinal cord in zebrafish (Tay et al., 2011).

There are DA cells in the telencephalon of many vertebrate species including mammals, which were not initially included in the A1 to 17 clusters because they were not detected in the adult rat and mouse brain. TH immunoreactivity was later observed in the adult rat brain

(Kosaka et al., 1987) but without AADC colocalization: thus L-DOPA may be their final product. In rodents the number of these TH positive cells peaks during development and falls in adulthood. A proposed function for a subset of these cells is blood flow regulation (Asmus et al., 2011). These “TH-only” cells, initially thought to be dopaminergic because of the lack of DBH expression, have also been observed in primates including humans (Gaspar et al., 1987; Smeets and Reiner, 1994) and cetaceans (Hof et al., 1995) in a scattered manner in the telencephalon.

Outside of mammals, many vertebrate species actually have dense DA cell clusters within the telencephalon. They are remarkably different according to the species observed. Lungfishes, the closest living relatives of tetrapods, present a large distribution of DA cells within their pallium, in particular in the dorsal part (López and González, 2017), while the subpallium presents a dense DA innervation but no DA cell bodies. In the latter study variation was observed between the two species of lungfishes since one species had numerous DA cell bodies in the lateral pallium while the other had only scarce labeling. In contrast, Cladistians (including *Polypterus*, the most basal group of *Actinopterygii*) present at least two large DA cell populations in the subpallium, and no DA cell body in the pallium although there is dense innervation in the pallium (López et al., 2018). This may be an ancestral situation in jawed vertebrates since DA cells at this location were observed in *Chondrichthyes* (Carrera et al., 2012), teleosts such as zebrafish (Yamamoto et al., 2010) and archerfish (Karoubi et al., 2016). The function of these telencephalic DA cells is not known.

7.2.3. Forebrain TH2 cell populations

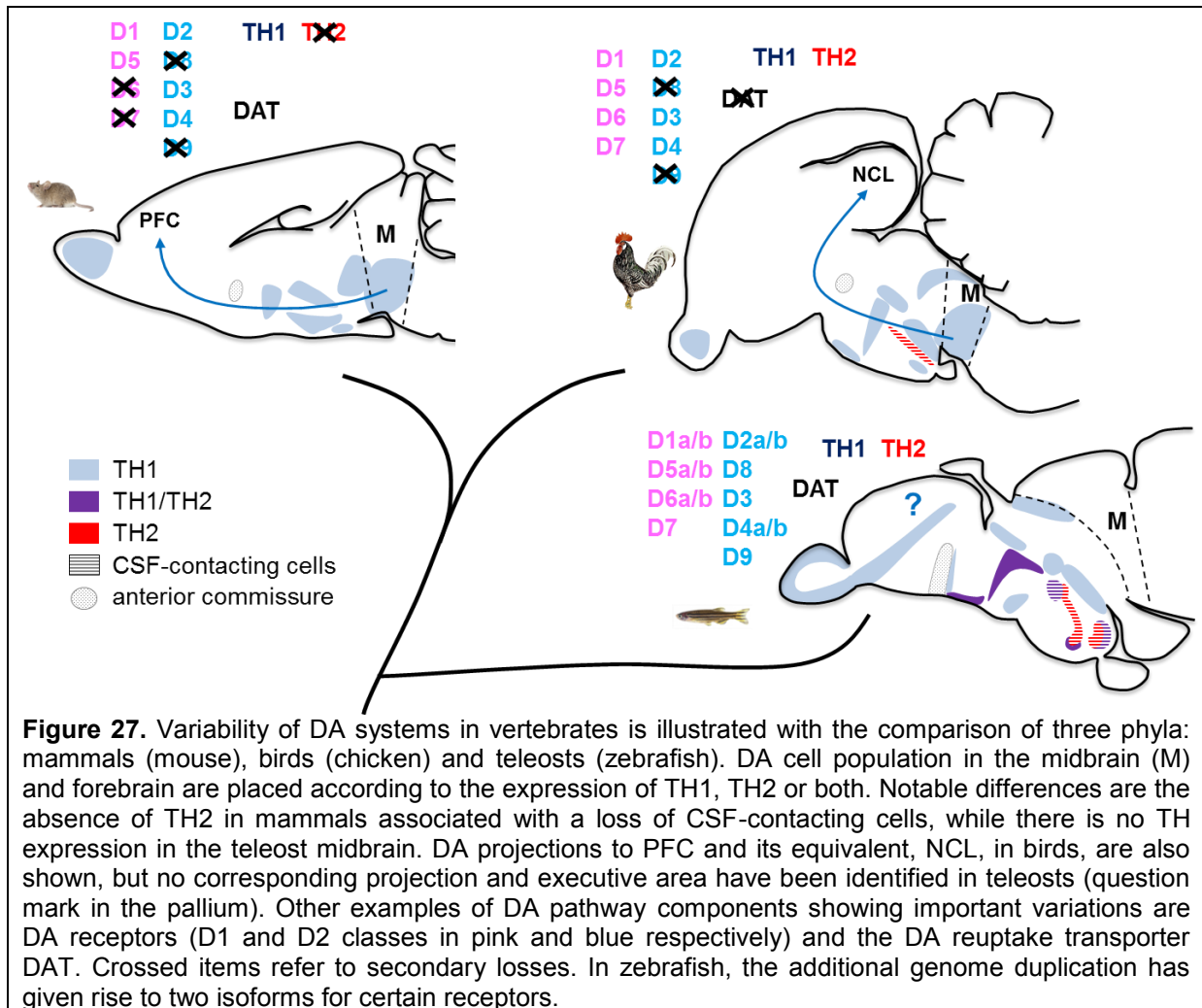
TH2 is abundantly expressed in cerebrospinal fluid (CSF)-contacting cells in the periventricular organ (PVO) in the hypothalamus in non-mammals (Smeets and Reiner, 1994; Xavier et al., 2017; Yamamoto et al., 2010; Yamamoto and Vernier, 2011). These cells present a dual neurotransmitter phenotype, containing both DA and 5HT and expressing *TH2* and *TPH1* (Xavier et al., 2017). A study in the quail PVO shows the PVO 5HT cells express a UV sensitive opsin 5, suggesting that PVO may be involved in seasonal reproduction (Nakane et al., 2010). The TH2 CSF-contacting cells are present throughout the vertebrate except placental mammals, and this fits a hypothesis on the existence of deep brain photoreception in non-mammals (Vigh and Vigh-Teichmann, 1998).

The *TH2*-expressing CSF-contacting cells are particularly abundant in teleosts (Figure 27). Their CSF-contacting cells are organized in three distinct cell populations in relation to their derived ventricular morphology. In zebrafish, the term PVO is used only for the anterior CSF-contacting cell population (or sometimes called PVOa). CSF-contacting cells around the lateral recess (LR) are located in the intermediate nucleus (IN) (or called PVOi), and the most posterior CSF-contacting cells are located in the caudal zone of periventricular hypothalamus (Hc) around the posterior recess (PR) (or called PVOp). A recent study (McPherson et al., 2016) showed that *TH2*-positive cells positively regulate the frequency of spontaneous swimming. Since the PR and the surrounding hypothalamic area exist only in teleosts, it is possible that teleost-specific hypothalamic functions which had never investigated may be present.

In teleosts, abundant *TH2* expression is also found in non-CSF-contacting cells in the teleost ORR, majority of which co-expressing *TH1*. The functions of these cells are not yet

investigated, but the abundance of TH2 cell populations suggests its importance in the teleost system.

Thus, considering the absence of mesencephalic DA cells and increase of TH2 cells in teleosts, the mammalian and teleost DA systems represent two extreme cases in *Osteichthyes*.



Although I did not study DA systems directly during my thesis, our results show that zebrafish possess a working memory, an executive function for which DA inputs from mesencephalic DA cells have been shown to be critical in mammals and birds. Given the apparent differences of teleost DA systems compared to amniotes, one can wonder which teleostean DA cell group (or groups) would be involved in executive functions.

8. The thesis project

My thesis is integrated in a broader project, which is the evolution of higher-order cognitive functions and the anatomo-functional requirements to allow the emergence of such functions across vertebrates. Data available in mammals and birds allow making general assumptions about neural prerequisites of higher order cognition as observed in the groups of primates and corvids. NCL has been identified as the equivalent of the PFC, both regions yielding similar cognitive capacities underlying general intelligence and having common traits,

probably convergently evolved. Briefly, here is a recapitulation of these common features between mammals and birds which we addressed in the introduction:

1. There are modality specific sensory areas in the pallium
2. These primary sensory areas receive sensory inputs from thalamus
3. PFC and NCL are both involved in executive function within the pallium, although they are probably not homologous
4. PFC and NCL functions are modulated by midbrain DA inputs, in particular from VTA
5. PFC and NCL receive various sensory inputs from associative and higher order sensory areas

Our interest was to study a vertebrate group phylogenetically further, such as teleosts. Some teleost species present higher order cognitive capacities, which makes them an interesting group to study the evolution of the brain in relation to cognition. However, less information is available about this group, as indicated in the introduction. We chose adult zebrafish as a teleostean model. Little is known about its cognitive capacities and neuroanatomy.

1. Primary sensory pallial areas appear to be modality specific based on connectivity studies in some species such as goldfish, but no data is available in zebrafish.
2. Primary sensory areas receive sensory inputs from a relay nucleus, primarily PG in cyprinids, but no connectivity data is available in adult zebrafish.
3. No executive area equivalent to the PFC and NCL has been identified.
4. In teleosts there are no DA cells in the midbrain, and there is no obvious candidate for an equivalent.
5. No information is available concerning associative or higher order sensory areas.

During the course of my PhD work, I investigated the organization of the teleost brain by taking advantage of the developmental and genetic tools available in zebrafish. This yielded two different articles. Article 1 demonstrates that IL, a structure which was considered as hypothalamic, mainly originated from the midbrain. In article 2 I focus on the visual pathway to the pallium and demonstrate that the major relay nucleus in teleosts, PG, is also of midbrain origin. This has intriguing implication for the evolution of the vertebrate brain.

In parallel, I demonstrated that zebrafish are indeed capable of performing a working memory task, thus implying that it has a primordium of executive area but without input from midbrain DA cells. This is presented in article 3.

Although I could not locate the executive area, I showed that teleost brain organization is divergent, thus suggesting that comparable cognitive capacities can be achieved with different anatomical substrates.


Article 1

RESEARCH ARTICLE

Open Access



Mesencephalic origin of the inferior lobe in zebrafish

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Abstract

Background: Although the overall brain organization is shared in vertebrates, there are significant differences within subregions among different groups, notably between Sarcopterygii (lobe-finned fish) and Actinopterygii (ray-finned fish). Recent comparative studies focusing on the ventricular morphology have revealed a large diversity of the hypothalamus. Here, we study the development of the inferior lobe (IL), a prominent structure forming a bump on the ventral surface of the teleost brain. Based on its position, IL has been thought to be part of the hypothalamus (therefore forebrain).

Results: Taking advantage of genetic lineage-tracing techniques in zebrafish, we reveal that cells originating from *her5*-expressing progenitors in the midbrain-hindbrain boundary (MHB) participate in the formation of a large part of the IL. 3D visualization demonstrated how IL develops in relation to the ventricular system. We found that IL is constituted by two developmental components: the periventricular zone of hypothalamic origin and the external zone of mesencephalic origin. The mesencephalic external zone grows progressively until adulthood by adding new cells throughout development.

Conclusion: Our results disprove a homology between the IL and the mammalian lateral hypothalamus. We suggest that the IL is likely to be involved in multimodal sensory integration rather than feeding motivation. The teleost brain is not a simpler version of the mammalian brain, and our study highlights the evolutionary plasticity of the brain which gives rise to novel structures.

Keywords: Teleost, Midbrain, Forebrain, Evolution, Homology, Vertebrate, Comparative neuroanatomy, Development, Cell lineage, Ventricle

Background

The vertebrate brain is considered to be divided into three main domains: the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon). Brain morphogenesis at early embryonic stages is controlled by local signaling centers called the “organizers.” Different organizers are set up successively over time during early development, with the primary organizer being fundamental for the primary neural induction. By interacting with transcription factors, the secondary organizers such as the anterior neural ridge (ANR) and the *zona limitans intrathalamica* (ZLI)

control the morphogenesis of the forebrain, while the isthmic organizer (IsO) located at the midbrain-hindbrain boundary (MHB) controls the morphogenesis of the midbrain and the anterior hindbrain (reviewed in [1]).

Compared to the large morphological diversity in adulthood, embryonic brains appear relatively similar among different vertebrate groups. For this reason, anatomical comparison at this stage is helpful to understand the basic arrangement of the brain morphology. It is accepted that the brain regionalization depends on the establishment of subdivisions along the anterior-posterior (A-P) and dorsal-ventral (D-V) axes of the neural tube. Furthermore, the neuromeric model has refined this view by introducing the notion of segmentation unit along these axes [2, 3].

Regarding regional subdivisions of the most anterior part of the forebrain (secondary prosencephalon),

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modification of the current prosomeric model has been proposed [4–6]: based on the morphological analysis focusing on the ventricular organization, the secondary prosencephalon is divided into three subdivisions (Fig. 1), the telencephalon, the hypothalamus, and the optic recess region (ORR) that extends laterally to form the primordium of the retina [4, 5]. This new view redefines the boundary of the “hypothalamus.” For example, the “alar hypothalamus” of the prosomeric model in tetrapods containing neuroendocrine cells appears to be part of the ORR.

The analysis focusing on the ventricular organization also sheds light on the diversity of the hypothalamic morphology in *Osteichthyes* (bony vertebrates) [7]. In mammals and birds (amniotes), the hypothalamic ventricle is thin and indistinguishable from the diencephalic part of the third ventricle (3V, Fig. 1a, amniotes). In amphibians, the 3V is extended laterally and thus it is named “the lateral recess (LR) of the infundibulum” [8] (LR; Fig. 1a, amphibians). In teleosts, the hypothalamic ventricle is larger and more complex. In addition to the lateral recess (LR), which corresponds to the tetrapod 3V, teleosts have another recess named the posterior recess (PR; Fig. 1a, b, teleosts). Furthermore, the teleost LR is extremely elongated in the mature brain surrounding the PR, and it forms an additional structure named the “inferior lobe of the hypothalamus” (IL; Fig. 1b, teleosts, red dotted area) [9, 10]. The IL is a structure, which

is not present in tetrapods, likely to have evolved specifically in a certain group of Actinopterygii that contains teleosts and holosteans (including gars; Lepisosteidae).

In this study, we examined the developmental origin of the IL in the zebrafish brain, by taking advantage of a cell lineage method based on tamoxifen inducible Cre-lox recombination. It revealed that the external zone of the IL is composed by the progeny of *her5*-expressing MHB cells, suggesting that a large part of the IL is actually of mesencephalic origin. Inductions at different time points during development provided further information on how this structure forms.

Results

Distribution of cells originating from the 24 hpf MHB in the adult brain

The *her5* transcription factor is known to be an early marker of the midbrain-hindbrain domain in zebrafish [11, 12]. Indeed, the zebrafish MHB is well established at 24 hours post-fertilization (hpf), and at this stage, *her5* expression is restricted to the MHB ([13]; see also Fig. 2a, b, Additional file 1: Movie S1.). In sagittal sections, the *her5* expression territory appears as a thin band, extending from dorsal to ventral. To follow the fate of these *her5*-expressing progenitors, we crossed two transgenic lines: *Tg(her5:ERT2-CreERT2)* and *Tg(βact:lox-stop-lox-hmgb1:mCherry)* (Fig. 2d).

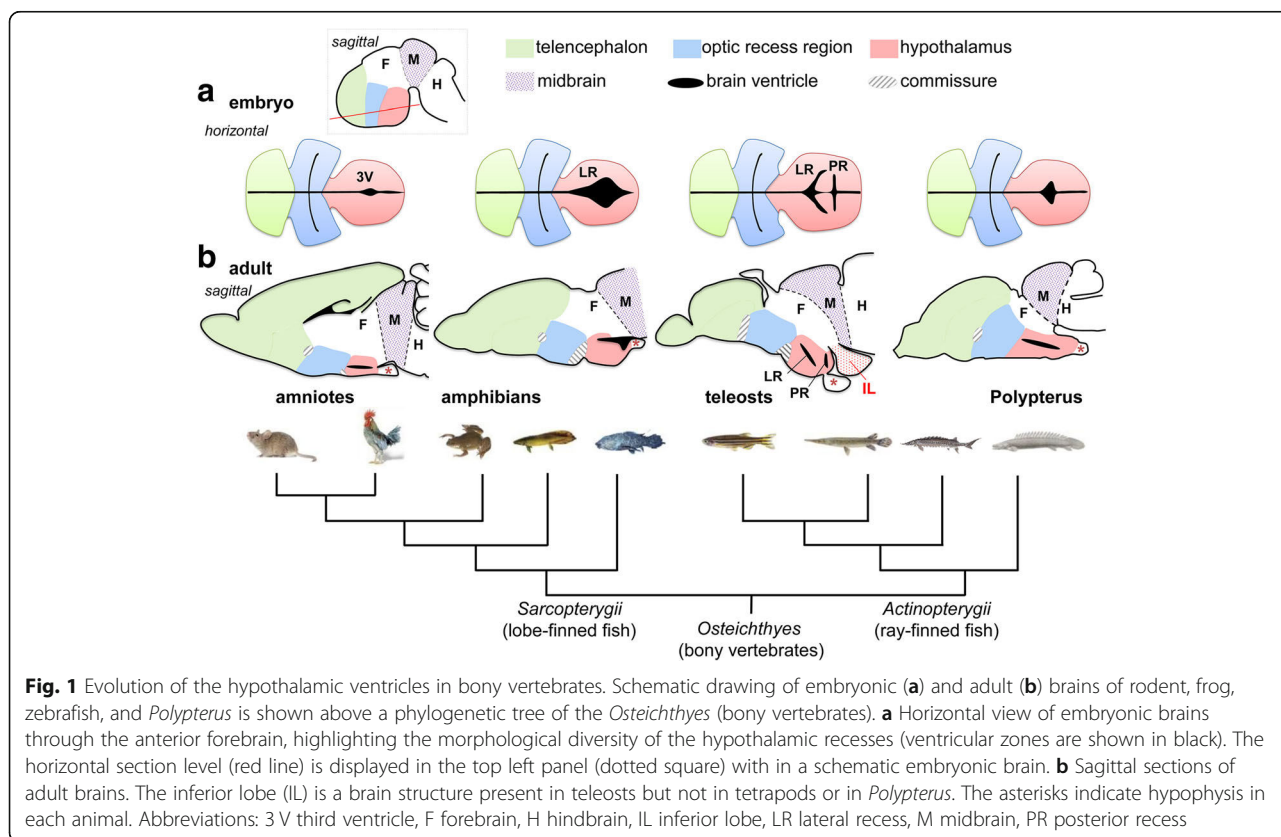


Fig. 1 Evolution of the hypothalamic ventricles in bony vertebrates. Schematic drawing of embryonic (a) and adult (b) brains of rodent, zebrafish, and *Polypterus* is shown above a phylogenetic tree of the *Osteichthyes* (bony vertebrates). **a** Horizontal view of embryonic brains through the anterior forebrain, highlighting the morphological diversity of the hypothalamic recesses (ventricular zones are shown in black). The horizontal section level (red line) is displayed in the top left panel (dotted square) with in a schematic embryonic brain. **b** Sagittal sections of adult brains. The inferior lobe (IL) is a brain structure present in teleosts but not in tetrapods or in *Polypterus*. The asterisks indicate hypophysis in each animal. Abbreviations: 3V third ventricle, F forebrain, H hindbrain, IL inferior lobe, LR lateral recess, M midbrain, PR posterior recess

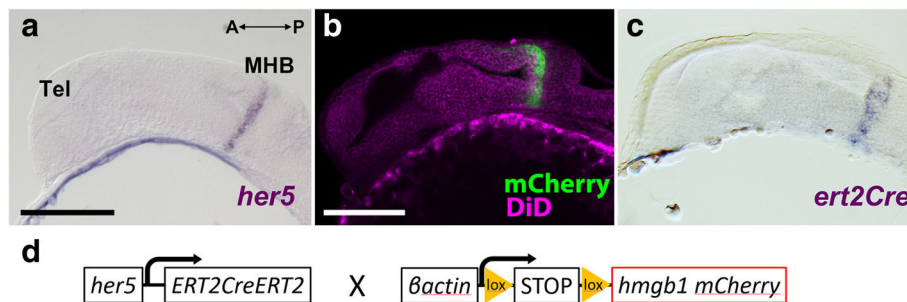


Fig. 2 Verification of the expression profile of the zebrafish transgenic line used in this study. **a–c** Sagittal sections of 24 hpf embryos showing that expression of *her5* and *ert2Cre* is restricted to the MHB. The anterior part of the embryo is on the left. **a** In situ hybridization (ISH) of *her5* confirms its specific expression in the MHB (purple). **b** Expression of mCherry (green) in a transgenic line *Tg(her5:mCherry)*, which is identical to the *her5* ISH pattern. The morphology is shown with DiD fiber labeling (magenta). **c** The expression pattern of *ert2Cre* is also identical to the *her5* ISH (**a**) and mCherry in *Tg(her5:mCherry)* (**b**). **d** A simplified schema of the constructs of *Tg(her5:ERT2CreERT2)* and *Tg(betaactin:lox-stop-lox-hmgb1:mCherry)* which were used in this cell lineage study. Scale bars, 100 μm. Abbreviations: MHB midbrain-hindbrain boundary, Tel telencephalon

We verified that the Cre expression is limited to the MHB at 24 hpf, recapitulating the *her5* expression (Fig. 2c). Thus, after tamoxifen treatment at 24 hpf, cells expressing *her5* and their progenies express mCherry, and we can interpret that all the mCherry-positive cells observed at later stages (after induction) are derived from the MHB.

As expected, in the brains of adult fish (3 months or older), mesencephalic structures such as the tectum (TeO), the torus semicircularis (TS), and the tegmental area were massively mCherry positive after induction with tamoxifen at 24 hpf (Fig. 3a–c). There was no labeling in forebrain structures such as the hypothalamus or the pretectum (Hy and PT respectively; Fig. 3a, b).

However, some unexpected structures exhibited mCherry-positive cells. The preglomerular nucleus (PG; Fig. 3a') is a sensory relay nuclear complex that is considered to be part of the posterior tuberculum of the diencephalon. The PG is continuous with a nucleus named the torus lateralis (TLa; Fig. 3b). TLa is classified as part of the tegmentum (thus midbrain) by some authors [14, 15], while as a diencephalic (thus forebrain) by others [10, 16]. The cluster of mCherry-positive cells is continuous from PG to IL through TLa.

The IL is usually considered as part of the hypothalamus [14] because of its location posterior to the hypothalamus proper. Indeed, this structure develops as a lateral elongation of the teleost LR (which corresponds to the amniote 3V in the hypothalamus). However, our data showing a massive of mCherry-positive cells in IL (Fig. 3b, c, Additional file 2: Figure S1) suggest that a large part of this structure is formed by cells originating from the MHB. The diffuse nucleus of the inferior lobe [10] (DIL; Fig. 3b', c') is the most labeled area. Numerous mCherry-positive cells are also found in the area corresponding to the central nucleus of the inferior lobe (CIL; Fig. 3c'). Interestingly, most of the outer region of the IL

is mCherry positive, but the inner part, close to the LR, is mCherry-negative (Fig. 3b', c'). The dorso-medial structure called the corpus mamillare (CM) is also mCherry-negative (Fig. 3c').

Migration of the cells originating from the MHB during development

Our data on the adult brain show that IL has a mesencephalic component. In order to decipher the development and anatomy of this structure in further details, we followed the mCherry-positive cells in whole brains at different stages after induction at 24 hpf.

At 3 days post-fertilization (dpf), all the mCherry-positive cells are still located around the MHB, and there is no labeling at the level of the forebrain (Fig. 4a–g, Additional file 3: Movie S2). This confirms the specificity of the molecular cell tracing method and the absence of leaky expression during the induction process. It further supports that the anterior structures, in which we observe mCherry expression at later stages, are composed of the progeny of cells originating from the MHB exclusively.

At 3 dpf, we could not identify the IL. In the lateral view (Fig. 4e), the MHB progenies (cells which were expressing *her5* at 24 hpf) extend in a triangular cluster, wider in the dorsal part. The IL was first observable at 5 dpf (Fig. 4h, i; arrows), being more remarkable at 7 dpf (Fig. 4j, k; arrows). MHB progenies extend ventrally in the outer surface of IL (Fig. 4i, k).

From late larval to juvenile stages, we could clearly identify the IL as a ventral protuberance with mCherry cells. Observation of global mCherry expression in the whole brain confirms a continuity of the mCherry labeling between the IL and more dorsal midbrain structures known to be part of the tectum and tegmentum (Fig. 5a–d, Additional file 4: Movie S3). This is also

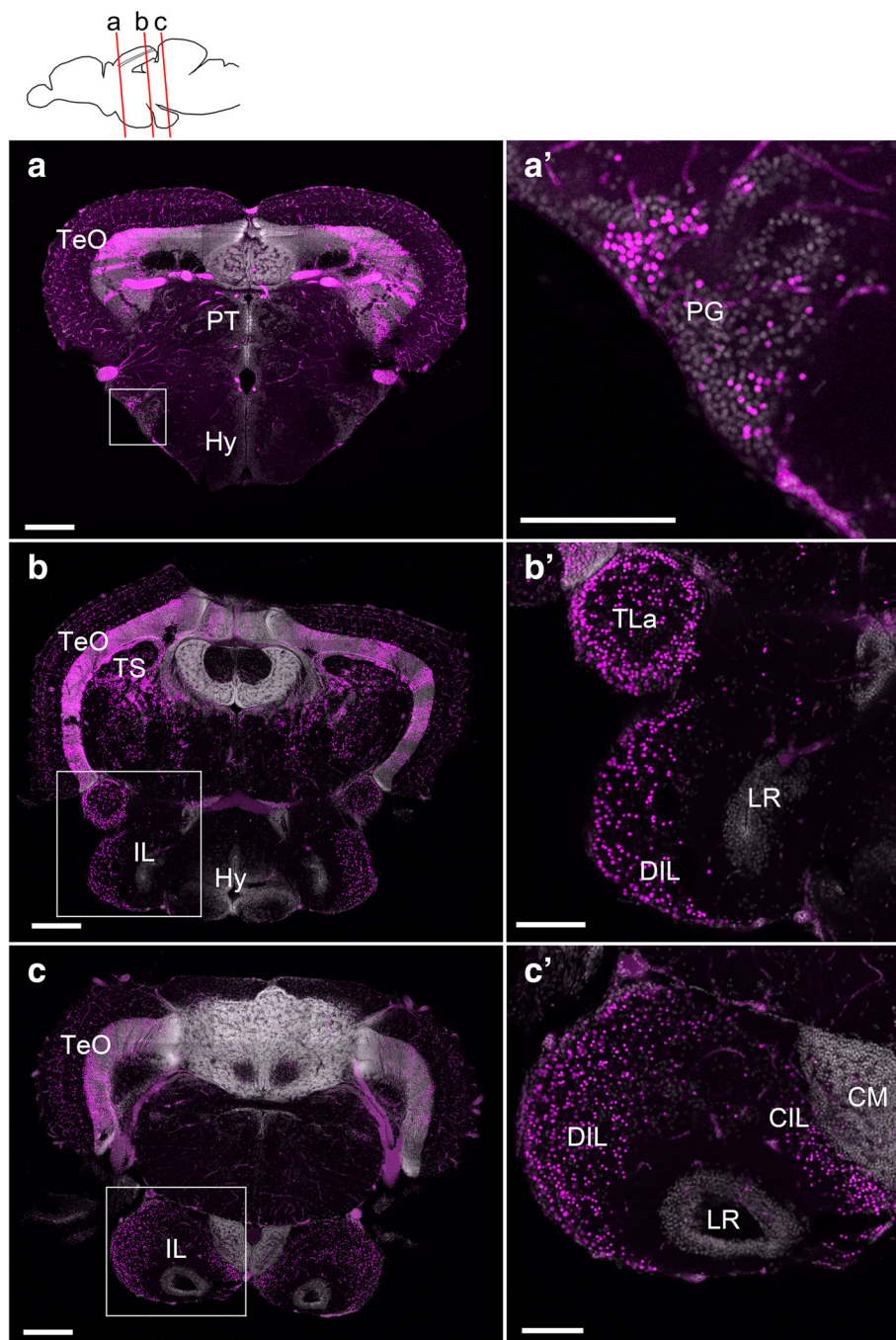


Fig. 3 Localization of the mCherry-positive cells in the adult brain of *Tg(her5:ERT2CreERT2;βact:lox-stop-lox-hmgb1:mCherry)* zebrafish treated with tamoxifen at 24 hpf. **a–c** Confocal images of frontal sections showing global views of the mCherry distribution (Z-projection, 5 μm for **a** and 10 μm for **b** and **c**). The mCherry-positive cells are shown in magenta, and DAPI nuclear labeling is shown in gray. The plane of each section is indicated in the schematic drawing on the top. **a'–c'** Higher magnifications of the areas squared in **a–c**, showing the preglomerular nucleus (PG; **a'**) and the inferior lobe (IL; **b', c'**). Abbreviations: CM corpus mamillare, CIL central nucleus of the inferior lobe, DIL diffuse nucleus of the inferior lobe, Hy hypothalamus, IL inferior lobe, LR lateral recess, PG preglomerular nucleus, PT pretectum, TeO optic tectum, TLa torus lateralis, TS torus semicircularis. Scale bars: **a–c**, 200 μm; **a'–c'**, 100 μm

visible in frontal sections at 19 dpf, in which mCherry-positive cells form a continuous strip from the dorsal tectum to the ventral IL (Fig. 5f). At this stage,

frontal sections of the IL already resemble those in adult, both anteriorly (Fig. 5e–g) and posteriorly (Fig. 5h–j). At 5 weeks post-fertilization (wpf), the IL continues to grow

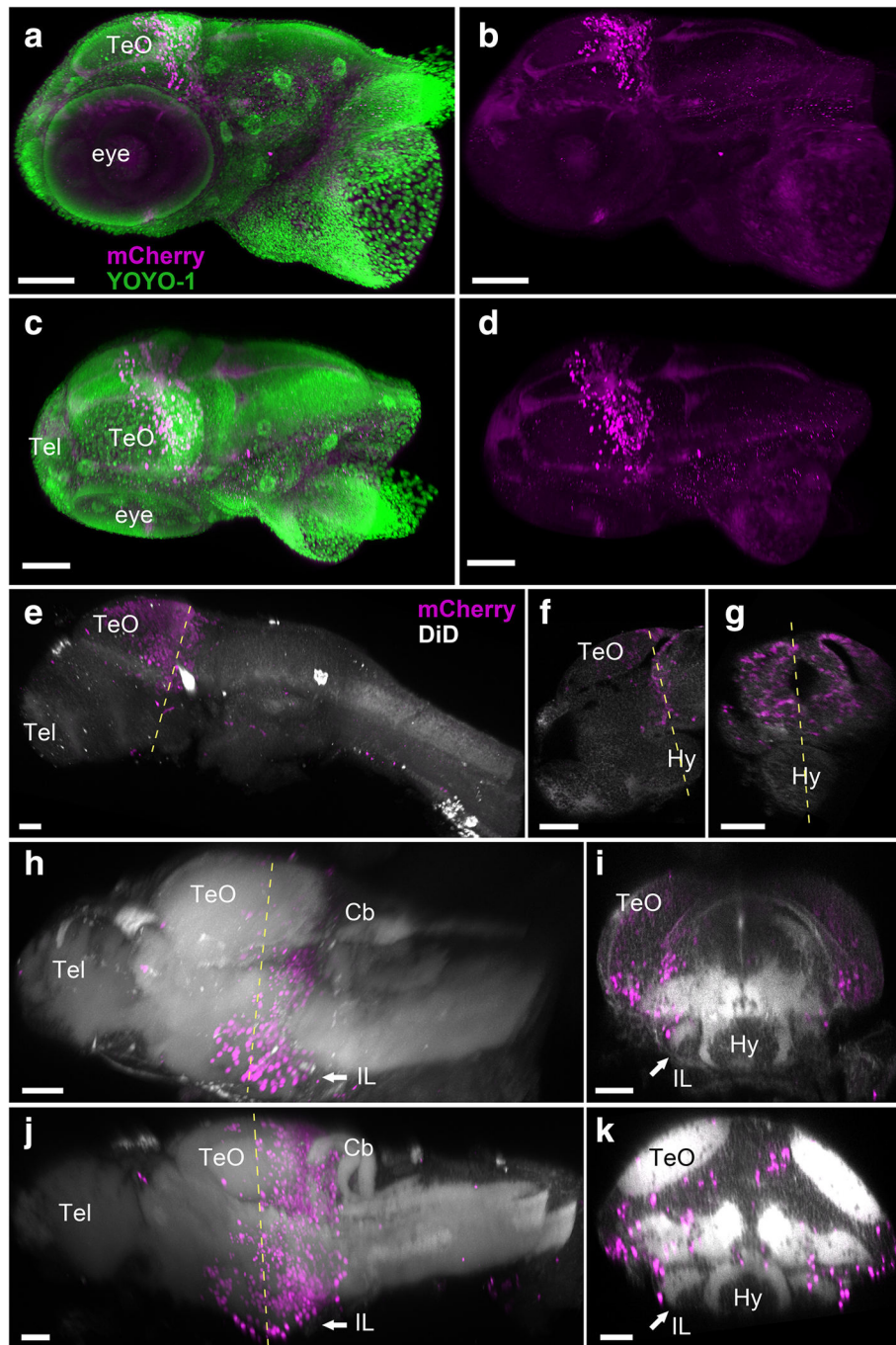


Fig. 4 Localization of the mCherry-positive cells in young larval brains of *Tg(her5:ERT2CreERT2;βact:lox-stop-lox-hmgb1:mCherry)* zebrafish treated with tamoxifen at 24 hpf. Anterior to the left for **a–d, e, h, and j**. **a–d** 3D reconstruction from confocal images of a whole head of 3 dpf larva. mCherry-positive cells are shown in magenta, and YOYO-1, a nuclear marker, is shown in green. **a, b** Side view of the larval head with **(a)** and without **(b)** YOYO-1 labeling. **c, d** Top view of the larval head with **(c)** and without **(d)** YOYO-1 labeling. The mCherry-positive cells are still close to the MHB at this stage. Some cells are starting to migrate anteriorly, but there are no mCherry-positive cells in the forebrain or in other brain areas. **e–k** 3D reconstruction from confocal images of dissected brains of 3 dpf **(e–g)**, 5 dpf **(h, i)**, and 7 dpf **(j, k)** larvae. mCherry-positive cells are shown in magenta, and DiD fiber labeling is shown in gray. **e** A whole brain at 3 dpf is shown in lateral view. **f** A sagittal section through the same specimen. **g** A frontal section. The hypothalamus (Hy) is extending in ventral position below the midbrain and is devoid of mCherry-positive cells. **h** A whole brain at 5 dpf is shown in lateral view. **i** A frontal section from the same brain showing the first appearance of the inferior lobe (IL; arrow), with a few mCherry-positive cells at the periphery of the structure. **j** A whole brain at 7 dpf is shown in lateral view. **k** A frontal section from the same brain showing the growing IL (arrow), with more mCherry-positive cells added laterally. Abbreviations: Cb cerebellum, Hy hypothalamus, IL inferior lobe, TeO optic tectum, Tel telencephalon. Scale bars: **a–d**, 100 μm. **e–k**, 50 μm

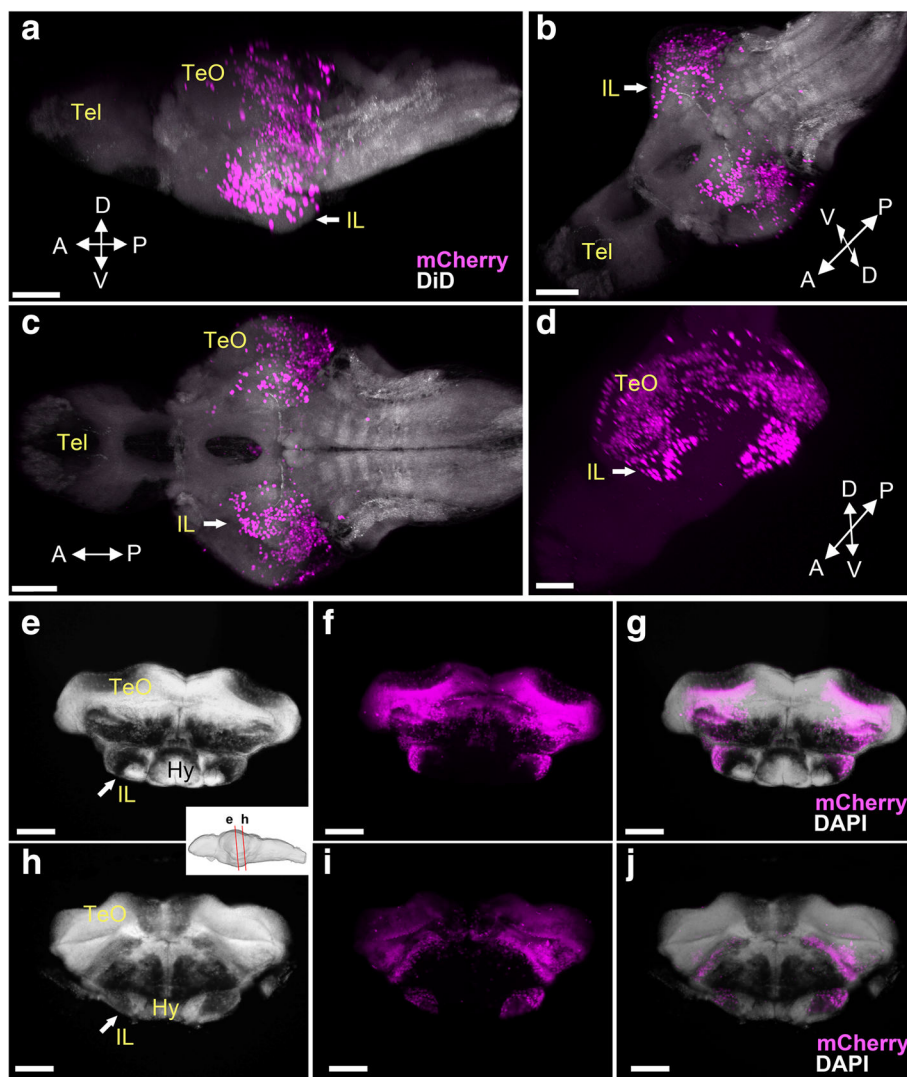


Fig. 5 Localization of the mCherry-positive cells in late larval brains of *Tg(her5:ERT2CreERT2;βact:lox-stop-lox-hmgb1:mCherry)* zebrafish treated with tamoxifen at 24 hpf. **a–d** 3D reconstruction from confocal images of a 14 dpf brain, showing mCherry-positive cells in magenta (**a–d**) and DiD fiber labeling in gray (**a–c**). Four different views are presented: lateral (**a**), ventral (**c**), and two different obliques (**b**, **d**). Arrows point at the IL on one hemisphere. The IL bulging can be seen in **a** and **b**, while **d** displays the continuity of the mCherry-positive cells in the IL with other midbrain structures. **e–j** Frontal sections of a 19 dpf brain, showing mCherry-positive cells in magenta and DAPI nuclear labeling in gray. IL is clearly visible in frontal sections (arrows), at two different antero-posterior levels (indicated in sagittal view in the white box). Anteriorly (**e–g**) the mCherry-positive cells are on the lateral part of IL that appears continuous with more dorsal midbrain structures, while posteriorly (**h–j**) a cluster of the mCherry-positive cells is seemingly detached from the dorsal midbrain structures. At the posterior IL, most of the IL is mCherry positive. Scale bars, 80 μm. Abbreviations: Hy hypothalamus, IL inferior lobe, TeO optic tectum, Tel telencephalon

and appears as a ventral protuberance (Additional file 5: Figure S2).

Formation of the IL in relation to the lateral recess

In order to better understand how the IL is formed around the LR, we performed a 3D reconstruction of the mCherry-positive cells in relation to the ventricular morphology (Fig. 6a–d, <https://zenodo.org/record/2556246>). ZO-1 immunostaining labels tight junctions of neuroepithelial

cells [17, 18]; thus, it can be used to visualize the ventricular zones of the brain.

Teleosts possess two distinct hypothalamic recesses, LR and PR, which are already observable at 48 hpf [4, 7]. A vast extension of the LR is found at later stages of development, and in the 14 dpf brain, the LR elongates in a posterior direction close to the PR (Fig. 6c, <https://zenodo.org/record/2556246>).

3D visualization of mCherry-positive cells in relation to the ventricular zone clearly shows that the

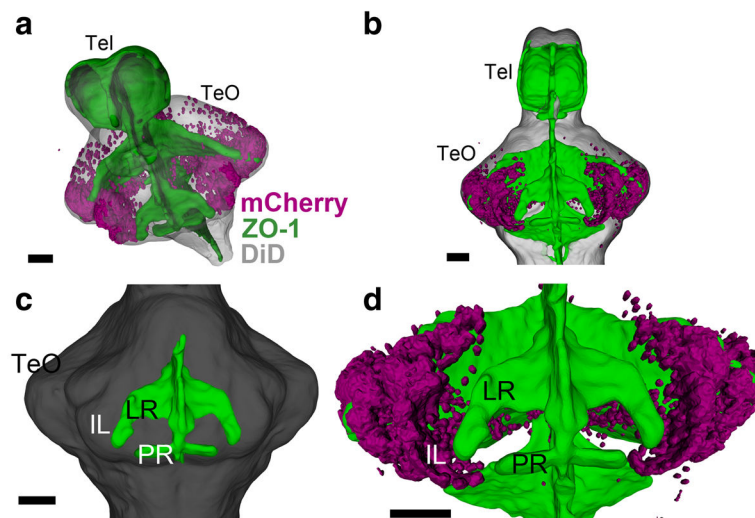


Fig. 6 Developing IL in relation to the ventricular morphology. 3D reconstruction of image segmentation from confocal images of a 14 dpf zebrafish brain. ZO-1 (ventricular labeling) is shown in green (**a–d**), mCherry-positive cells are shown in magenta (**a, b, d**), and DiD fiber labeling is shown in gray (**a–c**). **a, b** Oblique (**a**) and ventral (**b**) views of the brain, showing the general distribution of the mCherry-positive cells in relation to the ventricular organization. **c** A ventral view highlighting the lateral recess (LR) and the posterior recess (PR) (anterior of the brain to the top). **d** A higher magnification of **b** focusing on the mCherry cells in relation to the LR. The mCherry cells are continuous from the tectal region, but they are devoid of proximity of the ventricular zone. Scale bars, 50 μm . Abbreviations: IL inferior lobe, LR lateral recess, PR posterior recess, Tel telencephalon, TeO optic tectum. The interactive version of this figure can be found at <https://zenodo.org/record/2556246>

mCherry-positive cell cluster, which is continuous with the tectal region, covers the external part of the IL (Fig. 6a). In contrast, the ventricular zone of the LR is devoid of mCherry-positive cells (Fig. 6d, <https://zenodo.org/record/2556246>). In situ hybridization for *ccna2*, a cell proliferation marker, demonstrates that ventricular cells around the LR are in proliferation, whereas there is no proliferating cell in the external zone where mCherry-positive cells are found (Fig. 7a, a'). A closer look at the IL shows that the LR ventricular zone and the mCherry-positive external zone are separated by a cell-free fiber-rich zone (Fig. 7a, b, c; asterisks). Thus, IL is constituted of two anatomically distinct areas: the mCherry-negative ventricular zone is likely to be formed by the cells originating from the LR wall, while the mCherry-positive external zone is formed by the progeny of cells originating from the MHB.

Comparison between 14 dpf and adult IL (after induction at 24 hpf) shows that the relative size of the mCherry-positive external zone is significantly increased in the adult IL. At 14 dpf, there is only a thin layer of mCherry-positive cells (Fig. 7b'). In the adult (Fig. 7c), the mCherry-positive area is enlarged, forming a thicker mass laterally (which corresponds to the DIL). It is also worth noticing that the relative size of the whole IL is larger in adult and that the increase of the number of mCherry-positive cells largely contributes to the growth of IL mainly through its external portion.

Later maturation of IL

The *her5* expression domain decreases in size during development but remains specific to the midbrain (Fig. 8a–c, Additional file 6: Movie S4 for 2 wpf; Fig. 8d, e, Additional file 7: Figure S3C for 4 wpf; Fig. 8f, g, Additional file 7: Figure S3F for 6 wpf; and Fig. 8h, i for 8 wpf). In the juvenile brain, *her5* is expressed only in two cell clusters along the tectal ventricular zone: one more anteriorly (Fig. 8; yellow arrowheads) and another more posteriorly (Fig. 8; blue arrowheads), which are considered to be the alar part of the mesencephalon. A short-term tracing experiment showed that a few days after the tamoxifen induction, a few induced mCherry cells were observed at the two mesencephalic locations, but not in the forebrain (Additional file 8: Figure S4).

Observation of their progeny at 3 months post-fertilization (mpf) confirms that they contribute to the formation of IL and that IL continues to grow until late juvenile stages. The *her5* progenies are restricted to the outer zone close to the surface of the IL (Fig. 8j–o). This suggests that cells are inserted at the periphery of the IL, all along development. Comparison of 3 mpf brains induced at 4 wpf (Fig. 8j, k), 6 wpf (Fig. 8l, m), and 8 wpf (Fig. 8n, o) clearly shows that induction at later stages results in less mCherry labeling. Thus, the growth of IL is slowing down over time, and IL is nearly mature around 8 wpf.

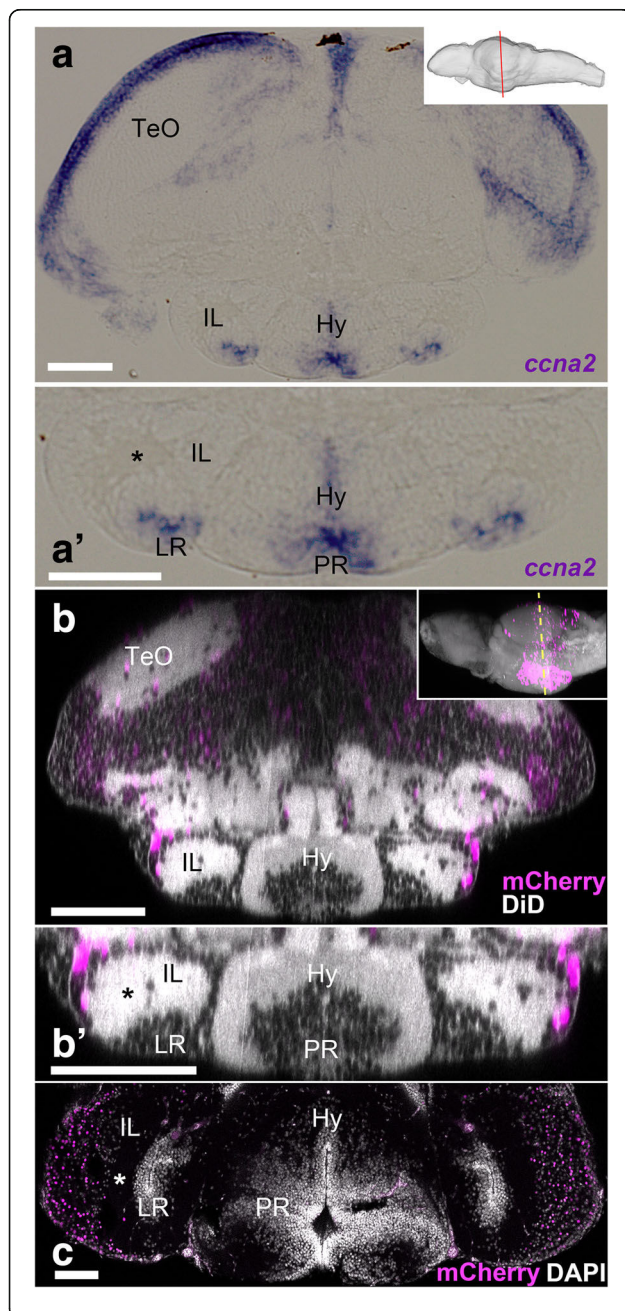


Fig. 7 Comparison of juvenile and adult IL in zebrafish. **a, a'** Frontal section of the 14 dpf brain showing the transcripts of *ccna2*. The plane of the section is indicated in sagittal view in the right upper corner. **a'** A higher magnification of the ventral part of **a** containing IL. The expression of *ccna2* is found around LR. **b, c** Brains of *Tg(her5:ERT2CreERT2;βact:lox-stop-lox-hmgb1:mCherry)* zebrafish treated with tamoxifen at 24 hpf. **b, b'** Frontal view of the 14 dpf brain which was obtained from 3D reconstruction of confocal images, showing mCherry-positive cells in magenta and DiD fiber labeling in gray. The plane of the section is indicated in sagittal view in the right upper corner. **b'** A higher magnification of the ventral part of **b** containing IL. **c** Frontal section of the adult IL showing mCherry-positive cells in magenta and DAPI nuclear labeling in gray. Note that the gray represents DiD fiber labeling in **b** and **b'**, while it corresponds to DAPI nuclear labeling in **c**. The asterisks indicate the cell-free fiber zone that separates the mCherry-positive external zone and the ventricular zone (around LR). The mCherry-positive cells are much abundant in the adult IL than in the larval IL. Scale bar, 90 μm. Abbreviations: Hy hypothalamus, IL inferior lobe, LR lateral recess, PR posterior recess, TeO optic tectum

Comparison between zebrafish and cichlid IL

The IL is a brain structure observed in all teleost species investigated so far. We compared the general organization of the IL of zebrafish with another teleost species, a Malawian cichlid (*Maylandia zebra*). As it is the case for the zebrafish, cichlid IL is also divided into a cell-dense ventricular zone along the LR and a cell-sparse external zone, which are separated by a cell-free fiber zone (Fig. 9; asterisks).

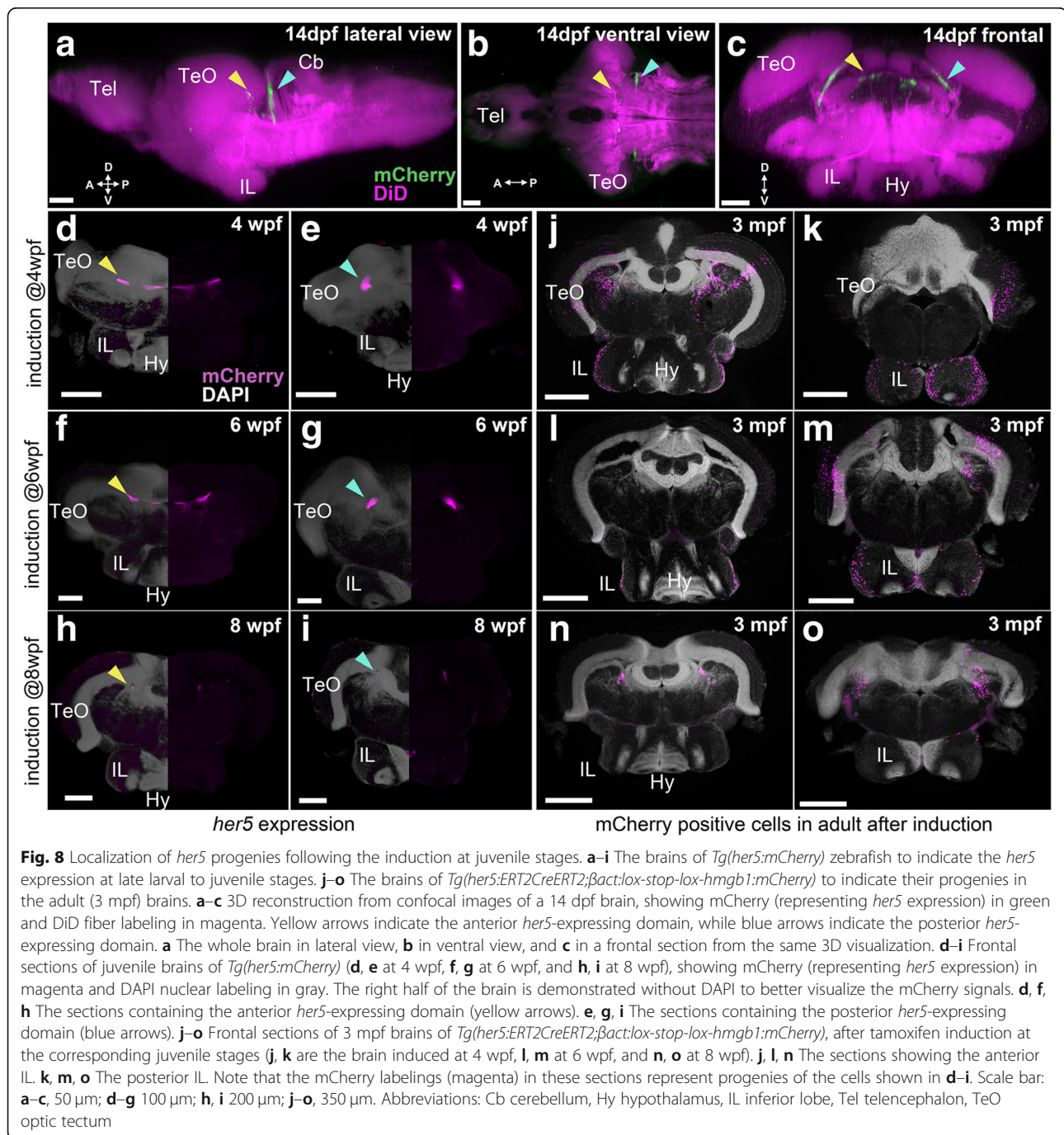
Nonetheless, there are significant differences between zebrafish and cichlid IL. The relative size of the IL is much larger in cichlid. This is obvious by comparing the IL (the size indicated in green arrows in Fig. 9a, d) with the caudal zone of the hypothalamus (Hy; the size indicated in red arrows in Fig. 9a, d) that is located medially. It is also clear that the proportion of the external zone (mesencephalic part; the size indicated in green arrows in Fig. 9b, e) in comparison with the ventricular zone (hypothalamic part; the size indicated in red arrows in Fig. 9b, e) is much larger in the cichlid IL.

Thus, the organization of the IL changes significantly between young and mature zebrafish brains and also between different species of teleosts. Note that in the cichlid brain, the relative size of the pallium (dorsal telencephalon) is also much larger than in the zebrafish brain (compare Fig. 9c, f, also see the “Discussion” section).

Discussion

New hypothesis on the developmental origin of IL

We took advantage of an inducible Cre transgenic line under the control of *her5* promoter to trace the progenies of cells originating from the MHB in zebrafish [13]. The *her5* transcription factor is known to be expressed in the MHB, corresponding to the midbrain primordium



including both tectum and tegmentum [11, 12]. We followed the ontogeny of the induced mCherry-positive cells, from the earliest time point when the mCherry expression is visible (3 dpf). We confirmed that all the mCherry-positive cells are derived strictly from the MHB area, in other words, none is derived from the forebrain primordium. Thus, based on the cell lineage, we could conclude that all mCherry-positive cells found anterior to the MHB are of mesencephalic origin.

We have shown that PG and IL that were previously classified as forebrain are mainly composed of MHB progenies and therefore are actually mesencephalic. Our data showing a mesencephalic component for the PG shed a new light on the evolutionary scenario of ascending sensory pathways. The PG relays ascending sensory inputs to the pallium, and it has been compared to the dorsal thalamus (which is in the forebrain) in mammals [19]. Thus, our finding suggests that the teleost relay nucleus may not be

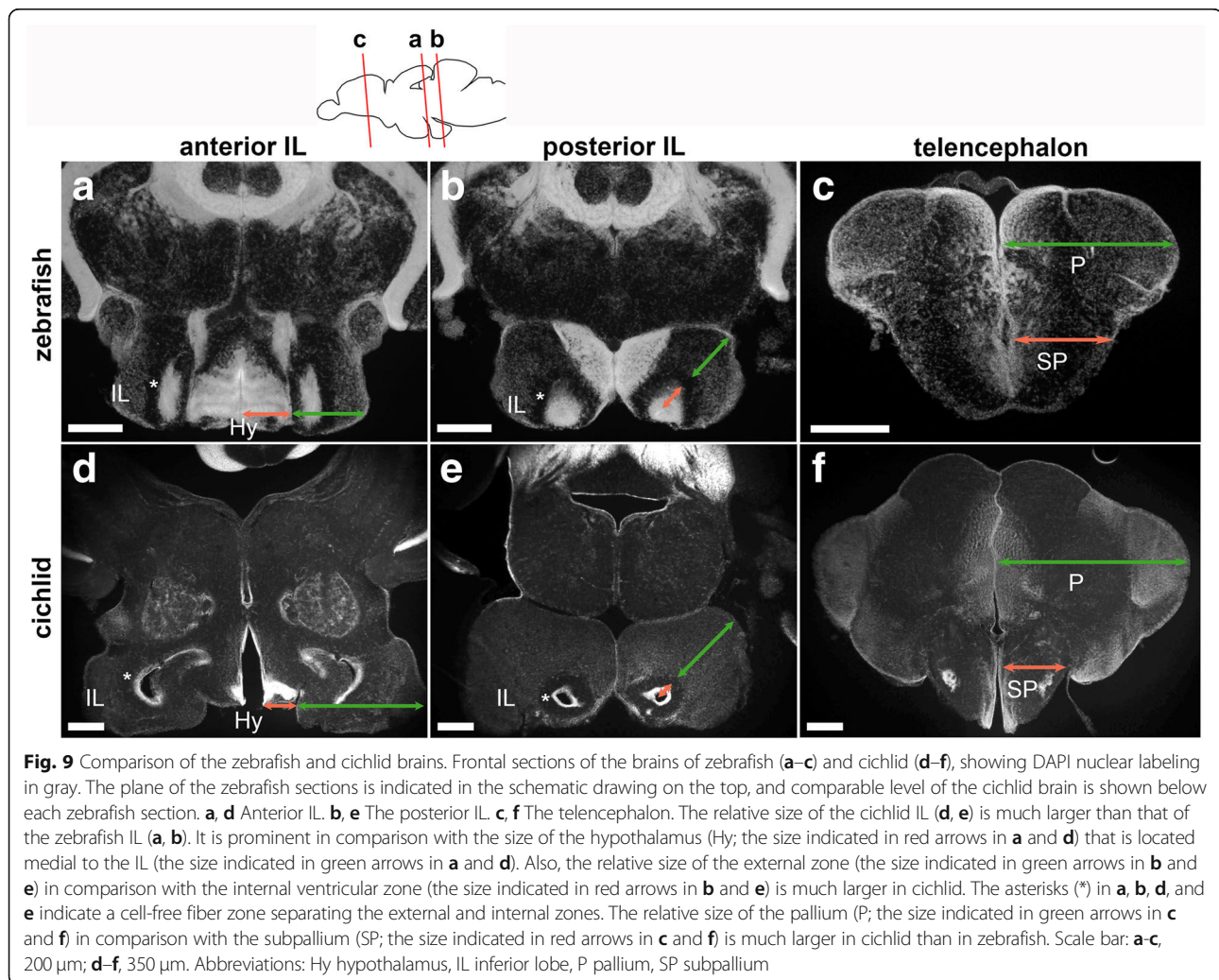


Fig. 9 Comparison of the zebrafish and cichlid brains. Frontal sections of the brains of zebrafish (a–c) and cichlid (d–f), showing DAPI nuclear labeling in gray. The plane of the zebrafish sections is indicated in the schematic drawing on the top, and comparable level of the cichlid brain is shown below each zebrafish section. **a, d** Anterior IL. **b, e** The posterior IL. **c, f** The telencephalon. The relative size of the cichlid IL (d, e) is much larger than that of the zebrafish IL (a, b). It is prominent in comparison with the size of the hypothalamus (Hy; the size indicated in red arrows in a and d) that is located medial to the IL (the size indicated in green arrows in a and d). Also, the relative size of the external zone (the size indicated in green arrows in b and e) in comparison with the internal ventricular zone (the size indicated in red arrows in b and e) is much larger in cichlid. The asterisks (*) in a, b, d, and e indicate a cell-free fiber zone separating the external and internal zones. The relative size of the pallium (P; the size indicated in green arrows in c and f) in comparison with the subpallium (SP; the size indicated in red arrows in c and f) is much larger in cichlid than in zebrafish. Scale bar: a–c, 200 µm; d–f, 350 µm. Abbreviations: Hy hypothalamus, IL inferior lobe, P pallium, SP subpallium

homologous to the mammalian thalamus. The sensory pathways to the pallium would have evolved independently in mammals and teleosts.

The IL has been considered to be a part of the teleostean hypothalamus (thus forebrain). This structure appears as a caudal continuation of the hypothalamus forming an additional “lobe.” There is no equivalent structure in tetrapods or in ray-finned fishes such as *Polypterus* and sturgeon; thus, IL would have evolved only in the teleostean and holostean fishes (*Neopterygii*). Our data suggest IL is formed by cell populations that have different developmental origin: the LR ventricular zone, which is hypothalamic (mCherry-negative), and the external zone, which is mesencephalic (mCherry-positive). The presence of proliferating cells around the LR suggests that the internal ventricular zone is generated by progenitors along the LR. Considering that the LR is an elongation of the hypothalamic ventricle, we can conclude that the mCherry-negative ventricular zone is hypothalamic (Fig. 10a). By contrast, we postulate that the external zone

of IL is generated by migrating cells from the tectal ventricular zones, since there is no sign of proliferation in the external zone of IL. The continuous stream of mCherry-positive cells from the dorsal tectal area to the ventral IL suggests that the mesencephalic cells invade a brain structure that grows from the anterior “hypothalamic” LR towards posterior.

Interestingly, IL matures progressively until the late juvenile stage (summarized in Fig. 10b). Our cell counting of mCherry-positive cells (Additional file 2: Figure S1) indicates that at least half of the progenitors of external IL cells would be generated around 24 hpf (since not all the cells would recombine), and the rest of IL progenitors would be progressively generated during the larval-juvenile stages.

The cells are added at the periphery all along development, with less cells added the older the fish is. The newer cells do not seem to form new layers stacked upon the previous ones, but intercalate with preexisting cells. If successive layers were apposed on each other,

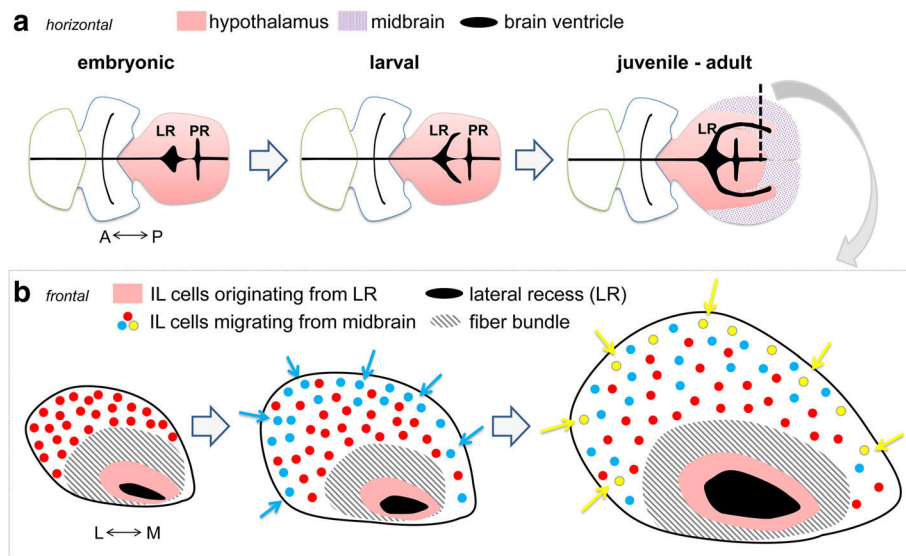


Fig. 10 Schematic drawing summarizing development of IL. **a** Horizontal views of the zebrafish anterior brain, highlighting the development of the lateral recess (LR). LR elongates caudolaterally and covers the posterior recess (PR), forming IL. The ventricular zone of IL around the LR is hypothalamic (pink), while the external zone of IL is formed by cells migrating from the midbrain (purple dots). **b** Frontal views of IL showing maturation of the external zone of IL during juvenile stages. The external zone grows by insertion of cells originated from the midbrain. The red, blue, and yellow circles represent mesencephalic cells added at different time points during development

mCherry-positive cells induced at 4 wpf should be located in a deeper layer than those induced at 6 wpf (compare Fig. 8k, m). Instead, we always observe the mCherry-positive cells at the surface. Thus, IL seems to grow by adding newer cells inserted between the existing cells on the surface.

Functional implication of IL

Considering that a large part of IL is not hypothalamus, the interpretation of IL functions needs also to be reconsidered. Even though the periventricular part is hypothalamic, our previous studies have already demonstrated that the “hypothalamus” of teleosts and mammals is very different and that careful comparative analyses are required before concluding a simple one to one homology [4, 5, 7]. For example, we have previously shown that a large part of the teleost hypothalamus is mainly composed of a cell type (CSF-contacting neurons) that has been secondarily lost in placental mammals [7]. The hypothalamic CSF-contacting populations are particularly increased in teleosts, around the additional hypothalamic recess (posterior recess, PR) that evolved specifically in teleosts. Thus, the teleost hypothalamic functions cannot be inferred in a simple manner based on available mammalian data. IL would be another example showing the particularity of the teleost brain.

Functions of IL were initially suggested by a set of studies of electrical stimulation of the brain in freely moving fish. Demski and his colleagues showed that

electrical stimulation of IL evoked movements such as biting at mirror or snapping objects [20, 21]. IL is also known to receive gustatory sensory inputs [22–24]. These data were interpreted with the assumption that IL is hypothalamic, and this is why IL has been proposed to be involved in feeding behaviors [14, 25].

A recent study using zebrafish larvae has shown that IL is activated by visual detection of moving objects [26]. The activation of IL and chasing behavior were evoked by presenting moving objects other than food, such as a moving spot on a screen. Nonetheless, the interpretation was that IL is involved in feeding motivation, because IL was assumed to be homologous to the lateral hypothalamus of mammals. Without this preconception of homology, the data by Muto et al. (2017) simply suggest the involvement of IL in visual detection, and there is no data concerning the motivational state. Indeed, their results rather favor another hypothesis proposing that IL is involved in sensory integration [24, 27–29].

Based on connectivity data in several teleost species, IL receives various sensory inputs in addition to gustatory: visual, somatosensory, auditory, and probably lateral line. Thus, IL has also been proposed to be a multisensory integration center [24, 27–29]. Taking the electrostimulation data into consideration, IL may integrate multimodal sensory information and evoke motor responses. In the zebrafish larval brain, neuronal connectivity is not fully established, and stimulus-response association is rather simple: visual detection of small

moving objects evokes chasing and biting, which is synonymous with feeding behavior.

In the mature brain, notably in teleost species like cichlids, the sensory-motor integration should be more complex. Some species of cichlids and wrasses demonstrate behavioral repertoires such as nest construction or tool use [30–32]. Based on available anatomical data, these families have a large IL as well as a large pallium [33, 34], being consistent with our observations (Fig. 9d–f). IL is known to receive efferent projections from the pallium in various species [24, 27, 29, 35, 36]. In this context, anatomical and functional correlation between the pallium and IL is an interesting issue to be examined.

Conclusion

Our findings have revealed that some structures which have been considered as part of the forebrain are actually mesencephalic in the zebrafish brain. This refines the current model of the brain regionalization. In addition, the revision on the regional identity of IL modifies the interpretation of previous studies concerning its function. Zebrafish has become an important model in neuroscience. In order to interpret data correctly, we have to be careful with the interpretation of the homology between structures in teleosts and mammals.

Methods

Animals

Zebrafish (*Danio rerio*) used in this study were raised in our own colony. Zebrafish embryos and larvae cannot be categorized as male or female. For adult zebrafish, both sexes were used and there was no significant difference between male and female. For *her5* and *ccna2* in situ hybridization, wild-type (AB) embryos ($n \sim 30$) were used. For studying the lineage tracing of *her5*-expressing progenitors ($n \sim 100$ for 24 hpf and ~ 10 for each experiment of 3–8 wpf), we crossed two transgenic lines previously used in a recent publication [13]: *Tg(her5:ERT2CreERT2)* and *Tg(βact:lox-stop-lox-hmgb1-mCherry)* (see tamoxifen treatment for details). For visualization of *her5* expression, we also used another transgenic line ($n \sim 30$) *Tg(her5BAC:nls-mCherry^{gy3})* that is simply referred to as *Tg(her5:mCherry)* [13]. Embryos/larvae up to 5 days post-fertilization (dpf) were maintained and staged as described previously [37]. After larval stages, zebrafish were raised in our fish facility. Three months post-fertilization (3 mpf) or older zebrafish is considered as adult.

Juvenile Malawian cichlids (*Maylandia zebra*) were kindly provided by Dr. Joël Attia (Neuro-PSI, Université de Lyon/Saint-Etienne). Three brains (2 females and 1 male) were used in this study, and there was no

difference in the general cytoarchitecture of IL between male and female.

Tamoxifen treatment

Tamoxifen treatments were performed in double transgenic fish generated by crossing *Tg(her5:ERT2CreERT2)* and *Tg(βact:lox-stop-lox-hmgb1:mCherry)* as described previously [13, 38]. 4-Hydroxytamoxifen (Sigma-Aldrich T176) was dissolved in ethanol at a concentration of 10 mg/ml and stored at -20°C until use. The working solution was freshly prepared before the treatment, further diluted with embryo medium (for 24 hpf) or fish water (for 4–8 wpf). The animals were incubated in the tamoxifen working solution at 28°C in the dark.

24 hpf embryos were dechorionated with Pronase (Sigma-Aldrich P5147; 1 mg/ml) prior to the tamoxifen treatment. Embryos were placed into the six-well culture plate (Thermo Fisher Scientific) and were incubated with $10\ \mu\text{g/ml}$ tamoxifen for 6 h. After the incubation, the fish were rinsed three times with embryo medium, then put back to the incubator.

For juvenile stages (4–8 wpf), fish were placed in a beaker (100–200 ml fish water depending on the number of fish) with an air pump and incubated with $2\ \mu\text{g/ml}$ tamoxifen on four consecutive days. The incubation time per day was 4 h, but the treatment was interrupted whenever the fish looked sick. At the end of each incubation, the fish were gently rinsed three times with fish water, placed back to a clean fish tank, and fed.

The tamoxifen-induced mCherry expression was observed at 3 mpf, except for the experiment of short-term tracing. In case of the short-term tracing (Additional file 8: Figure S5), the fish was sacrificed 4 days after the end of the tamoxifen treatment (that is 1 week after the beginning), and immunofluorescence anti-dsRed (see below) was performed to observe the mCherry expression.

Tissue preparations

Zebrafish embryos at 24 hpf and 3 dpf were fixed in ice-cold 4% paraformaldehyde (PFA; Electron Microscopy Sciences) in 0.01 M phosphate-buffered saline (PBS; Fisher Scientific) containing 0.1% Tween20 (PBST) overnight at 4°C . Zebrafish older than 5 dpf were deeply anesthetized using 0.2% tricaine methanesulfonate (MS222; Sigma-Aldrich) diluted in fish water. The fish were fixed in 4% PFA in PBST overnight at 4°C , then brains were dissected out. Zebrafish embryos used for in situ hybridization (ISH) were dehydrated in ethanol gradient series and kept at -20°C in methanol at least for a couple of days. They were rehydrated prior to ISH. For immunolabeling, samples were conserved in a stocking solution containing 0.5% PFA and 0.025% sodium azide. Brains were sectioned in a frontal plane

(80 µm) with a vibratome (Leica VT 1000 S). Tissue clearing was performed for whole-mount imaging of zebrafish embryos or larvae (see below).

Cichlid brains were obtained from juvenile individuals (body size around 6–8 cm). The animals were deeply anesthetized in 0.2% MS222 diluted in water and transcardially perfused with cold 4% PFA in PBS. Brains were dissected, post-fixed with 4% PFA overnight at 4 °C, then conserved in the stocking solution until use. Brains were sectioned in a frontal plane (80 µm) with a vibratome.

Tissue clearing

Considering the relatively small thickness of zebrafish brains at larval stages, a simplified clearing protocol was applied as described in Affaticati et al. (2018) [39]. Depigmentation step was applied as follows: up to 15 larvae were incubated in 10 mL of pre-incubation solution in a petri dish (0.5× saline sodium citrate buffer (SSC), 0.1% Tween20) for 1 h at room temperature without stirring. Then, samples were bleached by incubation in depigmentation solution (0.5× SSC, 5% formamide, 3% H₂O₂). Samples were left in the solution until pigments were completely degraded. Samples were then washed three times in PBST and left overnight in PBST.

Immunofluorescence

Immunofluorescence on larval zebrafish brains (14 dpf and earlier) was performed in 2 mL glass vials. Thorough PBST washes were performed between each antibody incubation step. The samples were incubated at room temperature for 5 h in a blocking solution containing 10% normal goat serum (NGS), 10% dimethyl sulfoxide (DMSO), 5% PBS-glycine 1 M, 0.5% Triton X-100, 0.1% deoxycholate, and 0.1% NP-40 in PBST. Samples were then incubated in staining solution (2% NGS, 20% DMSO, 0.05% sodium azide, 0.2% Triton X-100, 1× PBS, 0.1% Tween20, 10 µg/ml heparin) with anti-dsRed (1:600; Clontech Laboratories 632496, RRID: AB_10013483, Lot# 1612022) at room temperature for 3–4 days with gentle shaking, on a 3D rocker.

Double immunolabeling for dsRed and ZO-1 (1:100; Invitrogen 33-9100, RRID: AB_87181, Lot# SA241427) was performed in 14 dpf brains. Samples were incubated with secondary antibodies conjugated to fluorophores (1:600) in staining solution at room temperature for 3–4 days with gentle shaking. Alexa Fluor 546 goat anti-rabbit (Thermo Fisher Scientific A-11010, RRID: AB_143156, Lot# 1733163) was used for dsRed, and Alexa Fluor 488 goat anti-mouse (Thermo Fisher Scientific A-11001, RRID: AB_2534069, Lot# 1572559) was used for ZO-1. At the end of the second day of the secondary antibody incubation, DiD (Invitrogen/Thermo Fisher Scientific L7781; 1 µg/ml) was added for membrane labeling, while YOYO-1 (Molecular Probes/

Thermo Fisher Scientific Y-3601; 200 nM) was added for nuclear labeling. Finally, samples were incubated in a fructose-based high-refractive index (RI) solution that is adjusted to 1.457. This solution was obtained as described in Affaticati et al. [40].

For brain sections of the adult individuals induced at 24 hpf, mCherry endogenous fluorescence was bright enough to allow direct imaging. For other brain sections, anti-dsRed immunofluorescence (1:600 in PBST containing 4% NGS and 0.3% Triton X-100) was performed. The sections were incubated with the primary antibody at 4 °C overnight and then with secondary antibody Alexa Fluor 546 (1:1000 in PBST) at 4 °C overnight. In order to visualize the brain morphology, the sections were counterstained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Sigma-Aldrich; 5 µg/ml) at room temperature for 15 min.

In situ hybridization (ISH)

cRNA probes of zebrafish *her5* [13, 41], *ccna2* [4, 42], and *ert2Cre* [13, 43] were provided by Dr. L. Bally-Cuif (Pasteur Institute, Paris, France). ISH were performed in toto for 24 hpf zebrafish embryos, while on brain sections for animals older than 14 dpf. Detailed ISH procedures have been described in our previous publications [4, 7].

After rehydration, the 24 hpf embryos or brain sections were first incubated in hybridization buffer at 65 °C for 4 h and then hybridized with 2 ng/ml of cRNA probe in hybridization buffer at 65 °C for at least 18 h. Samples were then washed in gradient series of formamide/2× SSC mixture at 65 °C: 75% formamide/25% 2× SSC, 50% formamide/50% 2× SSC, 25% formamide/75% 2× SSC, then washed in 2× SSC and finally in 0.2× SSC. After being rinsed with PBST at room temperature, the samples were incubated with anti-digoxigenin conjugated with alkaline phosphatase (1:2500; sheep anti-DIG-AP Fab fragments; Roche Diagnostics 11093274910, RRID: AB_514497, Lot# 12486522) at 4 °C overnight. After PBST washes, the signal was visualized by incubation with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) solution (Roche Diagnostics 11681451001) in 0.1 M Tris-HCl (pH 9.5)/0.1 M NaCl in H₂O (TN buffer). The 24 hpf embryos were embedded into 3% agarose, sectioned with a vibratome in a sagittal plane (40 µm), and slide mounted.

Image acquisition

A Leica TCS SP8 laser scanning confocal microscope was used to image adult sections with a ×25 water immersion objective. For tissue-cleared in toto specimens, the same microscope was used with a Leica HC Fluotar L ×25/1.00 IMM motCorr objective. For all these acquisitions, fluorescence signal was detected

through laser excitation of fluorophores at 405, 488, 552, or 638 nm and detection was performed by two internal photomultipliers. Steps in the Z-axis were fixed at 1 μ m. Epifluorescence images were acquired using a Multi-zoom AZ100 (Nikon). Bright-field images were either acquired with an upright microscope BX43 (Olympus) or the Multizoom. Acquired images were adjusted for brightness and contrast using ImageJ/FIJI software.

Quantification of mCherry-positive cells in IL

The mCherry-positive cells in the adult external IL were counted from confocal images using the ImageJ cell counter module. We used stacks of 10 μ m frontal sections at anterior and posterior levels, obtained from two specimens of independent experiments of inductions at 24 hpf. The total number of cells was determined with DAPI nuclear labeling of the external IL, that is excluding the ventricular (inner) portion of and corpus mamillare (CM).

3D image reconstruction

Whole specimens (3 dpf entire head; 3, 5, 7, and 14 dpf entire brain) imaged in confocal microscopy were reconstructed in 3D using Imaris 8.0.1 software (Oxford Instrument Company) using the “3D view” visualization tool on a Dell T3610 workstation.

Image segmentation was performed interactively in the segmentation editor of Amira 6.0.1 (FEI Company) on a Dell T630 running Ubuntu 16.04. In preparation of the segmentation of the ventricle volumes, the raw data of the ZO1 signal was cleaned up by subtracting the mCherry signal: [ZO1]-[mCherry]. Similarly, the raw data of the mCherry signal were cleaned up before segmentation by weighted subtraction of the reference channel (DiD): [mCherry]-([DiD]/10). These steps remove low-level contamination (DiD signal bleed-through) with the subtracted signal (mCherry or DiD respectively) from the signal of interest (ZO1 or mCherry), and by this facilitate thresholding and reconstruction. The ZO1 signal was isolated using a combination of local thresholding (Magic Wand) and manual segmentation (Brush) along all three cardinal axes of the data’s coordinate system. For coping with the thin, sheet-like shape of the ventricles, we dilated the segmented volume by two voxels and smoothed it in 3D (three times with a mask size of 6). The mCherry signal was segmented from the cleaned-up data by global thresholding and smoothed in 3D with a mask size of 3. The resulting surfaces were generated with Amira’s “Generate Surface” module (Smoothing: Existing Weights), simplified to about 180K faces each, using Amira’s Simplification Editor and exported to the “Polygon File Format” (ply) for the visualization in other software tools.

Additional files

Additional file 1: Movie S1. Expression of *her5* in the 24 hpf zebrafish embryo. Expression of mCherry in a transgenic line *Tg(her5:mCherry)* is shown in magenta, and DiD fiber labeling indicating the morphology of the embryo is shown in gray. (MP4 1999 kb)

Additional file 2: Figure S1. Quantification of mCherry-positive cells in the external IL. Proportion of mCherry labeled cells (gray bar) in relation to the total number of DAPI-labeled cells was calculated from frontal sections of adult zebrafish brains (tamoxifen induction at 24 hpf). The section level of “anterior” corresponds to the level of Fig. 3b, and the level of “posterior” corresponds to the level of Fig. 3c. The “total” represents sum of them. (PDF 51 kb)

Additional file 3: Movie S2. Localization of the mCherry-positive cells in the 3 dpf larval head of *Tg(her5:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry)* zebrafish treated with tamoxifen at 24 hpf (3D visualization of Fig. 4a–d). mCherry-positive cells are shown in magenta, and YOYO-1, a nuclear marker, is shown in green. (MP4 5759 kb)

Additional file 4: Movie S3. Localization of the mCherry-positive cells in the 14 dpf larval brain of *Tg(her5:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry)* zebrafish treated with tamoxifen at 24 hpf (3D visualization of Fig. 5a–d). mCherry-positive cells are shown in magenta, and DiD fiber labeling is shown in gray. (MP4 2602 kb)

Additional file 5: Figure S2. Localization of the mCherry-positive cells in the 5 wpf juvenile brain of *Tg(her5:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry)* zebrafish treated with tamoxifen at 24 hpf. Frontal sections showing mCherry-positive cells in magenta and DAPI nuclear labeling in gray. A–C show the anterior IL and D–E show more posterior IL. Scale bars: 100 μ m. Abbreviations, Hy: hypothalamus, IL: inferior lobe, TeO: optic tectum. (TIF 10750 kb)

Additional file 6: Movie S4. 14 dpf brain of *Tg(her5:mCherry)* zebrafish, with a focus on the cells expressing *her5* endogenously (3D visualization of Fig. 8a–c). For better visualization, the brain is truncated anteriorly in the middle of the optic tectum (TeO) and posteriorly in the cerebellum (Cb). The section includes about half of IL ventrally. mCherry-positive cells are shown in green, and DiD fiber labeling is shown in magenta. The movie starts in frontal view. When switching to lateral/dorsal views, anterior is on the left, posterior is on the right. (MP4 2500 kb)

Additional file 7: Figure S3. Endogenous expression of *her5* in juvenile zebrafish brains. In situ hybridization of *her5* on frontal sections of 4 wpf (A–C) and 6 wpf (D–F) brains. The plane of each section is indicated in the schematic drawing on the top. There is no *her5* expression in the anterior sections containing forebrain regions (A, B, D, E). In the brain sections containing the mesencephalic region, *her5* expression is found along the tectal ventricular zone (C, F; arrows). Scale bar: 100 μ m. Abbreviation, Di: diencephalon, IL: inferior lobe, Tel: telencephalon, TeO: optic tectum, Tg: tegmentum. (TIF 17072 kb)

Additional file 8: Figure S4. Short-term tracing of tamoxifen-induced mCherry-positive cells in the *Tg(her5:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry)* juvenile zebrafish brain. Frontal sections of a 4 wpf brain, showing mCherry-positive cells in magenta and DAPI nuclear labeling in gray. The plane of each section is indicated in the schematic drawing on the top. A, B Anterior brain sections containing forebrain regions where there is no mCherry-positive cell. C, D More posterior brain sections containing mesencephalic regions where a few mCherry-positive cells are found close to the tectal ventricular zone. C' and D' show the area squared in C and D at a higher magnification. Scale bar: 60 μ m for A and B, 100 μ m for C and D, and 10 μ m for C' and D'. Abbreviation, Hy: hypothalamus, IL: inferior lobe, PG: preglomerular nucleus, Tel: telencephalon, TeO: optic tectum, V: ventricle. (TIF 18788 kb)

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Availability of data and materials

All data generated in this study are included in this published article and its additional files. The interactive 3D visualization of image segmentation illustrating the 14 dpf IL in relation to the ventricular morphology (Fig. 6-interactive) can be found at <https://zenodo.org/record/2556246>.

Authors' contributions

SB and KY designed the experiments and wrote the manuscripts. SB, MT, IC, EM, PA, and KY performed the experiments. SB, MT, and KY analyzed the data. SG generated the *Tg(her5:ERT2CreERT2)* and *Tg(her5BAC:nls-mCherry^{pv3})* transgenic lines. AJ performed the image segmentation and 3D reconstruction. All authors read and approved the manuscript.

Ethics approval

All experimental protocols and care of laboratory animals were conducted in compliance with the official regulatory standards and approval of the French Government (reference document n°APAFIS#1286- 2015062616102603 v5).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Article 2

Non-thalamic origin of zebrafish sensory relay nucleus indicates convergent evolution of the visual pathways in amniotes and teleosts

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Running head: Mesencephalic origin of the teleost PG

Keywords: sensory afferent, visual pathway, thalamus, diencephalon, homology

SUMMARY

The thalamocortical ascending projections are critical for sensory perception and cognition in the mammalian brains. For the visual system, a tectofugal pathway is present in various species of vertebrates. This pathway conveys retinal inputs through the tectum to the telencephalon, relayed via a thalamic-like nucleus. Thus, the thalamic relay has been considered as a conserved feature in the vertebrate visual systems. In this study, we demonstrate that zebrafish have a tectofugal pathway to the pallium (dorsal telencephalon), which is relayed via the preglomerular nucleus (PG). However, this relay nucleus is derived from the midbrain, while the thalamus is derived from the forebrain. Based on cell lineage tracing using the Cre-lox system, we show that the majority of the PG cells originate from the alar portion of the midbrain. We also demonstrate that the zebrafish PG develops gradually until juvenile stage, unlike the thalamus in amniotes (mammals and birds). Our finding strongly suggests that the teleost PG is not homologous to the amniote thalamus. Thus, the similar connectivity of the ascending visual pathways is likely to have evolved independently in amniotes and teleosts, highlighting the evolutionary plasticity of vertebrate brains. This also implies that, regardless of the developmental origin, the presence of relay nuclei might be an important characteristic in animal groups such as mammals, birds, and teleosts, which exhibit elaborated visual perception.

INTRODUCTION

In the classical view influenced by *scala naturae*, evolution is progressive and unilinear [1], - from fish, to amphibians, to reptiles, to birds and mammals, and finally to primates including humans. The terms "lower vertebrates" or "higher vertebrates" reflect this theory. However, most biologists have abandoned this way of thinking. The current consensus is that each animal has evolved differently, as a consequence of adaptation to the environment.

Concerning brain evolution, it is accepted that the basic organization is inherited from the common ancestor. For example, all vertebrate brains possess the regions recognizable as the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). Brain morphogenesis at early embryonic stages depends on regionalization processes established along the anterior-posterior and dorsal-ventral axes of the neural tube [2–6]. Functional organization of the brain (e.g. neuronal connectivity) is established at later developmental stages, and it is a fundamental question to which extent the nervous system is conserved or diversified [7,8].

Sensory ascending pathways have been investigated in a wide range of vertebrate groups, and findings of similar connectivity patterns among different vertebrate groups have led to the idea that the brain organization may be relatively conserved [9–11]. The visual system is one of the most intensively studied. In mammals, there are two major visual pathways terminating to the cortex [12]: one is called the thalamofugal pathway, or geniculate pathway, in which retinal inputs terminate to the striate visual cortex (V1) via a thalamic relay nucleus (lateral geniculate nucleus in the case of mammals). The other is called the tectofugal pathway, or extra-geniculate pathway, in which the retinal inputs terminate to the extrastriate visual cortex

via two relays, the tectum (superior colliculus in mammals) and another thalamic nucleus (pulvinar in primates and lateral posterior nucleus in other mammals like rodents). Studies examining visual afferents in non-mammals have often been compared to these mammalian pathways [13–17]. In non-mammalian tetrapods, two different thalamic nuclei relay two separate visual pathways to the telencephalon: one receiving directly the retinal inputs, the other receiving them via an additional relay in the tectum. Thus the presence of thalamo- and tecto-fugal pathways has been considered to predate the divergence of tetrapod lineage [18].

Nonetheless, the evolutionary scenario of the tectofugal pathway within amniotes is under debate [9,19–22]. This is largely due to the lack of sister groups which demonstrate an intermediate situation between mammals and birds. Unlike amniotes, in which the major projection target is the pallium (dorsal telencephalon), amphibians' sensory projections mainly terminate to the striatum (ventral telencephalon) [18]. Thus it is hard to conclude what was the ancestral situation in tetrapods.

Outside of tetrapods, a connectivity pattern similar to the tectofugal pathway terminating to the pallium has also been found in the group of teleosts [23–28]. Note that tetrapods and teleosts belong to two different groups of *Osteichthyes* (bony vertebrates): tetrapods are *Sarcopterygii* (lobe-finned fish) whereas teleosts are *Actinopterygii* (ray-finned fish). In cyprinids (e.g. goldfish and carp), the visual inputs from the optic tectum (TeO) are relayed via a relay nucleus called the preglomerular nucleus (PG), and terminate in the lateral part of the dorsal telencephalic area (DI) of the pallium. The PG in cyprinids relay also other sensory modalities such as auditory, lateral line, and possibly somatosensory. Thus the PG-pallium projection has been compared to the "thalamocortical pathway" in mammals [11,26,27] (Figure 1).

However, our recent study has indicated that many PG cells are of midbrain origin, instead of forebrain [29]. This result suggests that the PG is not homologous to the tetrapod thalamus (which is a part of the diencephalon in the forebrain). In this case, the similarities of the cyprinid PG and the tetrapod thalamus would have evolved independently, and would not be inherited from the common ancestor.

To test this hypothesis, we have examined the developmental origin of PG by taking advantage of zebrafish transgenic lines. Our results reveal that the PG cells projecting to DI in the pallium originate from the mesencephalic region. Thus the teleost PG is not homologous to the amniote thalamus, and their similar connectivity pattern has evolved independently in each animal group.

RESULTS

Zebrafish transgenic line labeling the visual afferent projection to the pallium

Numerous zebrafish transgenic lines expressing the engineered Gal4 transcription factor have been created by *To12* transposon-based gene trap and enhancer trap [30,31]. By crossing with a reporter line expressing EGFP under the control of UAS (*Tg(UAS:GFP)*), the offspring express GFP only in a specific set of cells. We screened a zebrafish line expressing GFP in projection neurons to the pallium.

We found that one of the Gal4 fish lines, *Tg(gSAGFF279A)*, crossed with *Tg(UAS: GFP)*, demonstrated GFP-positive (GFP+) cells projecting to a part of the pallium. In this study, we always used the offspring screened with GFP expression. Thus hereafter we simply refer to this double transgenic line *Tg(gSAGFF279A;UAS:GFP)* as *Tg(279A-GFP)*.

In the *Tg(279A-GFP)*, abundant GFP+ cell bodies are found in the preglomerular nucleus (PG; Figure 2A), and GFP+ fibers are present in the lateral zone

of dorsal telencephalic area (DI; Figure 2B). 3D reconstruction of confocal images of the entire brain allowed us to follow the projection from the PG to the pallium (Figures 2C and 2D; Movie S1). We confirmed that axonal projections originating from the PG terminate to the ipsilateral DI (Figure 2D; Movie S1).

This projection from PG to DI is very similar to the afferent visual projection of other cyprinid species such as goldfish [26]. In goldfish, most of the retinal projections terminate to the optic tectum (TeO), and TeO neurons project to the lateral PG (PGI), which in turn projects to the DI in the pallium. We thus decided to take advantage of the *Tg(279A-GFP)* line to visualize the visual afferents in zebrafish.

The expression of GFP in PG starts to be observable around 6 weeks post-fertilization (wpf; Figure S1A and B). The GFP+ fiber labeling in the DI becomes visible only around 8 wpf (Figure S1C). At 3 months post-fertilization (mpf), there are around 200 GFP+ cells found in PG.

Validation of the tectofugal visual pathway in zebrafish

In order to verify whether the GFP+ projections recapitulate the afferent visual pathway in zebrafish, we performed a tract tracing study using Dil and biocytin. We initially tried Dil labeling on the *Tg(279A-GFP)* to visualize both GFP and Dil in fluorescence, but the Dil labeling was not sensitive enough to visualize the nerve terminals accurately. Here we show the results from biocytin injections except for a retrogradely labeled TeO cell.

It has been known that retinal projections terminate in the upper layers of TeO in a wide range of species of ray-finned fish [32], and it is also the case in zebrafish (data not shown). In order to confirm whether the zebrafish PG receives the visual input from TeO, we injected Dil in the PGI (Figures 3A and 3C, asterisks) of the *Tg(279A-*

GFP) line, in which the PGI could be clearly visible as a GFP+ cell cluster. After 2-3 weeks of incubation, we observed cell bodies retrogradely labeled in the TeO (one is shown in Figure 3D). The cell extends its dendrites up to the retino-recipient upper layer of TeO, and this morphology is identical to the neuron receiving the retinal input in carp and goldfish [26]. Biocytin injection in TeO (Figure 3E, arrowheads) labeled nerve terminals in the PGI (Figure 3F, arrowheads), confirming that PGI receives tectal inputs.

Following the biocytin injection in PGI (Figure 3G, asterisk), we observed abundant fiber labeling in DI (Figure 3H). This DI labeling pattern is identical to the GFP+ fiber labeling of the *Tg(279A-GFP)* (Figures 2B and 3I). Conversely biocytin injection in DI (Figure 3J, asterisk) labeled the perikarya of PGI neurons (Figure 3K, arrows), as the GFP+ cells in *Tg(279A-GFP)* (Figure 3L, arrows). These data confirm that PGI neurons project to DI of the pallium.

Thus we conclude that the PGI relays visual projections from TeO to DI, and that the GFP+ projection from PGI to DI in *Tg(279A-GFP)* recapitulates this visual pathway.

Mesencephalic progenitors give rise to the GFP+ PG cells

Based on a cell lineage method using tamoxifen inducible Cre-lox recombination, we have recently revealed that some brain structures that have been considered to be of prosencephalic origin (forebrain) are indeed of mesencephalic origin (midbrain) [29]. PG is one of them, thus we further investigated the development of PG cells. Previously we used a double transgenic line generated by crossing *Tg(her5:ERT2CreERT2)* and *Tg(β act:lox-stop-lox-hmgb1:mCherry)*, in which inactive Cre is expressed under the control of *her5* promoter. Temporal control of mCherry expression is achieved by applying tamoxifen, which activates Cre. The transcription factor *her5* is exclusively

expressed in the midbrain-hindbrain boundary (MHB) at 24 hours post-fertilization (hpf). Since we observed mCherry-positive (mCherry+) cells in PG after tamoxifen induction at 24 hpf, we can conclude that these PG cells originate from the MHB.

In order to confirm whether the PG afferent neurons projecting to the pallium express the mCherry, we generated a quadruple transgenic line *Tg(her5:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry;279A-GFP)*, and performed tamoxifen induction at 24 hpf. Observing the PG in the adult stage, we found that there are cells co-expressing both GFP and mCherry (Figure 4, arrows). This suggests that at least some of the PG cells composing the visual afferents originate from the MHB.

We have confirmed that the quadruple transgenic line is identical to the double transgenic lines in terms of the expression of GFP and mCherry, and their brain development is unaltered. However, the fertility and survival rate of young larva after tamoxifen treatment was relatively low in this quadruple transgenic line. Thus, in order to repeat the experiments adequately, we decided to use an additional transgenic line.

As an alternative to the *Tg(her5:ERT2CreERT2)*, we used *Tg(Dr830:ERT2CreERT2)* [33]. In this line, the expression of Cre recombinase is larger than *Tg(her5:ERT2CreERT2)* at 24 hpf, but the expression is limited to the tectal area after 30-48 hpf (Figures S2 and S3). We first generated the double transgenic line *Tg(Dr830:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry)* and then crossed with *Tg(279A-GFP)*, in order to verify whether GFP+ afferent projection neurons express mCherry.

We performed tamoxifen treatments at different developmental stages from 24 hpf up to 8 wpf (Table S1). We found GFP/mCherry co-expressing cells consistently in all the developmental stages, although the co-labelled cells are not abundant in one

particular stage (Figure 5). mCherry+ cells were less and less numerous along development. At 6 wpf, there were very few mCherry+ cells and there was only one cell co-localized with GFP among all the specimens examined.

Thus our results suggest that the GFP+ PG cells are gradually developing throughout the larval/juvenile stages around up to 6 wpf. Considering the number of PG cells, the short-term tamoxifen induction time, and the distribution of mCherry+ cell distribution within PG, it would be reasonable to conclude that the GFP+ afferent projection neurons are progenies of cells derived from the tectal region.

DISCUSSION

Ontogeny of the teleost PG

Together with our previous publication [29], our results show that zebrafish PG cells derive from the mesencephalic region. Thus, PG is not homologous to the tetrapod thalamus (or the dorsal thalamus that derives from the alar p2 based on the prosomeric model; Figure 6).

The PG has been often compared to the tetrapod thalamus mostly because it relays afferent sensory projections to the pallium. Topologically, however, PG has been considered to be situated in the posterior tuberculum, the ventral (basal) portion of the diencephalon [34]. The anatomical term "posterior tuberculum" is not used in the adult brain of amniotes, because their dorsal (alar) diencephalon is very enlarged and there is no prominent diencephalic structure derived from the ventral (basal) diencephalon.

The difficulty of comparison between amniote and teleost brain is partially due to this difference: in teleost brains, ventral brain structures are relatively large in comparison to the amniote brains. However, our results show that the PG is not derived

from the ventral (basal) portion of the neural tube, but migrating from the dorsal (alar) part of the mesencephalon. Based on the tamoxifen treatment at early embryonic stages, we could not conclude whether PG migrate from the alar or basal part of the MHB. However, at later stages, the expression of Cre in the *Tg(Dr830:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry;279A-GFP)* line is limited to the tectal area. Thus at least at later stages, all the PG cells are generated from the tectal region, the alar part of the mesencephalic region.

The cells giving rise to PG start to be generated at early embryonic stages and are continuously added until around 6 wpf. However, at least based on the GFP expression in the *Tg(279A-GFP)* line, the fibers terminating to the DI are not visible at 6 wpf (Figure S1A). The projections from PG to DI start to be established only at around 8 wpf (Figure S1C). Thus unlike the thalamocortical projections that are already abundant at late embryonic stages, the afferents to the pallium in zebrafish are not mature until a late juvenile stage.

It has been known that zebrafish larvae can coordinate body orientation against the current, capture food, or escape from predators using relatively simple tectal circuitry (retina \rightarrow TeO \rightarrow motor outputs) [35–37]. Such visuo-motor processing at the level of tectum (without reaching the forebrain) is comparable to the circuitry involved in saccade (unconscious adjustment of eye movement following the detection of motion) in mammals [8]. It is possible that larvae and early juvenile zebrafish behaviors are largely dependent on this tectal circuitry, and the visual circuitry involving telencephalic circuitry becomes more important at later stages.

Evolution of visual pathways in *Osteichthyes*

In teleosts, most of the retinal inputs project to TeO, and there are few studies reporting the presence of thalamofugal-like visual pathway [25,26]. Since two visual pathways are found in sturgeons (non-teleost *Actynopterygii*) [38,39] and also in sharks (cartilaginous vertebrates; *Chondrichthyes*) [40,41], the interpretation has been that the ancestral jawed vertebrate possessed two visual pathways and that the thalamofugal pathway may have been secondarily lost in teleost lineage [25].

The teleost visual system must have undergone a large diversification, since significant differences exist even in the tectofugal pathway. Indeed, PG is not the only sensory afferent relaying the tectofugal projection in teleost species. In other teleosts species such as squirrelfish and goby (belonging to the group of *Acanthopterygii*), the tectal inputs are relayed to the pallium via another nucleus called the nucleus prethalamicus (PTh) [23–25] that is located more anterodorsal to PG. The PG is also present in *Acanthopterygii*, but it does not seem to receive visual inputs [25]. In contrast, the nucleus corresponding to the PTh is not identified in cyprinids. Cyprinids and *Acanthopterygii* form two large clades within teleosts, and they may have evolved different tectofugal pathways independently.

It would be premature to draw evolutionary scenarios in each clades of vertebrates, but at least, our study demonstrates that the similar functional property as a relay nucleus in cyprinid PG and in tetrapod thalamus would not be inherited from the common ancestor of *Osteichthyes*.

Furthermore, focusing on the projections to the pallium, we do not find any evolutionary continuity in the group of *Osteichthyes*. In amphibians, the major sensory relay nucleus is the dorsal thalamus, but unlike amniotes, there are very few projections to the pallium, and the majority terminates in the subpallium (ventral telencephalon) [18]. Similarly, sturgeons also have a poorly developed pallium and

the afferent projections seem to terminate in the ventral portion of the telencephalon [38,39]. It is also worth noticing that modal-specific sensory afferents are well developed only in amniotes and teleosts. Thus it may be that the common ancestor of *Osteichthyes* possessed rather simple afferent systems, and they have been developed independently in amniotes and teleosts. The ancestors of amniotes and teleosts would have faced similar selective pressures that lead to evolve sensory relay nuclei, notwithstanding the developmental origin. This ancestral situation might have been an important property to evolve the elaborated visual perception as found in these animal groups.

To conclude, our study suggests that the cyprinid PG and tetrapod thalamus are not homologous. Their similar functional properties (relaying the sensory afferents to the pallium) would have evolved independently in each lineage. In our previous publications, we have already demonstrated the unexpected diversity of dopamine systems despite the similarities in phenotype [8,42–44]. Taking all these data into consideration, the nervous system may be more plastic during the evolution than it has been thought.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish Lines

For tract-tracing study, wild-type zebrafish (*Danio rerio*) with the Oregon AB genetic background of both sexes were used.

The *Tg(gSAGFF279A)* and *Tg(UAS:GFP)* transgenic lines were generated in the National Institute of Genetics (Mishima, Japan) [30,31,46], and their offspring *Tg(gSAGFF279A;UAS:GFP)*, abbreviated *Tg(279A-GFP)*, were used in this study. This zebrafish line was maintained either by incross or by crossing with AB.

The *Tg(279A-GFP)* fish line was crossed with other transgenic lines to perform cell lineage studies. They were crossed either with the *Tg(her5:ERT2CreERT2)* [47] or *Tg(Dr830:ERT2CreERT2)* [33], plus with the *Tg(β act:lox-stop-lox-hmgb1-mCherry)*, thus obtaining the quadruple *Tg(her5:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry;279A-GFP)* or *Tg(Dr830:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry;279A-GFP)*.

For *in situ* hybridization of *ert2Cre*, double transgenic lines *Tg(her5:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry)* and *Tg(Dr830:ERT2CreERT2; β act:lox-stop-lox-hmgb1-mCherry)* were used.

Fish maintenance and staging

Zebrafish used for the biocytin tract tracing were maintained at Nagoya University (Japan) in aquaria at 22-26°C. For the rest of the experiments, zebrafish were raised in the animal facility in Neuro-PSI (Gif-sur-Yvette, France). Embryos/larvae up to 5 days post-fertilization (dpf) were maintained and staged as described [45]. After larval stages, zebrafish were raised in a fish facility (maintained at 26-28°C). 3 mpf or older zebrafish is considered as adult.

Ethical approval

The experimental protocols and care of laboratory animals were conducted in compliance with the official regulatory standards and approval of the French Government (reference document n°APAFIS#1286- 2015062616102603 v5), the official Japanese regulations for research on animal and the regulations on Animal Experiments in Nagoya University.

METHOD DETAILS

Dil tract-tracing

To examine brain connectivity in the adult zebrafish, we embedded crystals of Dil (1,1'-Dilinoleyl-3,3,3',3'-Tetramethylindocarbocyanine, 4-Chlorobenzenesulfonate; FAST Dil™ solid, Invitrogen-Molecular Probes, D7756) in the telencephalon and the optic tectum. Dil is a fluorescent lipophilic tracer that diffuses in lipid membranes, allowing both antero- and retrograde labeling of neural processes. Adult zebrafish (n = 40) were fixed with 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) overnight at 4°C.

The brains were dissected out and a small crystal was inserted in the brain using a glass pipette. The crystal was left in the brain for dye migration during 10 days - 2 weeks at 37°C in PBS, or PBS containing 0.05% sodium azide to avoid fungal contamination. The brains were embedded in 3% agarose, and sectioned at 80µm (in frontal and sagittal) using a vibratome (Leica VT 1000 S).

Biocytin tract-tracing

Biocytin (Sigma-Aldrich, B4261) was injected in adult zebrafish brains (n = 39), both *in vivo* and *in vitro*.

For *in vivo* tract-tracing, fish were anesthetized by immersing in fresh water containing 200 mg/L tricaine methanesulfonate (MS222; Sigma- Aldrich, A5040) and set in a device for physical restraint. A small amount of fresh water containing 150-200 mg/L MS222 were poured on the fish for aeration and also to maintain the anesthetic condition. A dorsal portion of the cranium was opened with forceps to expose the brain. For injections into TeO and DI, we injected crystals of biocytin with a minute insect pin. After the injection, the cranial opening was closed by a flap made of a paraffin sheet

(Parafilm, Bemis Company) that was affixed to the cranium with an acrylic adhesive (Aron alpha, Toagosei, JIS S 6040). Postoperative fish were maintained in aquaria for 3-5 hours. After the survival period, the fish were deeply anesthetized with MS222 (over 200 mg/L) and perfused through the heart with 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH7.4 (PB). The brains were removed from the skull and post-fixed in fresh solution of the same fixative at 4°C for 1 to 2 days.

We also injected biocytin into the TeO, PGI, and DI *in vitro* because it was difficult to maintain postoperative fish in aquaria for hours following injections *in vivo*. A detailed *in vitro* tract-tracing method has been reported previously [26]. Fish were deeply anesthetized with MS222 (over 200 mg/L). We quickly dissected the brain from the skull and then injected crystals of biocytin into TeO, PGI, and DI with a minute insect pin. The brain was kept in a container filled with 50 mL normal artificial cerebrospinal fluid solution for marine teleosts (126mM NaCl, 4.0mM KCl, 1.0 mM MgSO₄, 1.7mM CaCl₂ mM, 26mM NaHCO₃, 1.0mM NaH₂PO₄, and 10mM glucose; [48]) at room temperature. The solution was aerated and changed every 30 minutes. After 3-4.5 hours, we fixed the brain by immersion in 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M PB for 1-3 days at 4°C.

Tissue processing following the biocytin injection

The fixed brains were cryo-protected by immersion in 0.1 M PB containing 20% sucrose at 4°C overnight. Cryo-protected brains were embedded in 5% agarose (type IX, ultra-low gelling temperature; Sigma-Aldrich, A2576) containing 20% sucrose and frozen in n-hexane at -60°C. Then, transverse sections were cut at a thickness of 40 µm on a cryostat and mounted on gelatin-coated glass slides. The sections were dried for one hour at room temperature and washed once with 0.05 M TBS containing 0.1%

Tween 20 (TBST) and twice with TBS each for 10 minutes. To quench non-specific peroxidase activities, sections were steeped in methanol containing 0.3% H₂O₂ for 10 minutes and washed three times with TBS and once with 0.03% TBST each for 10 minutes. Sections were incubated with a solution of avidin-biotin-peroxidase complex (1:100; VECTASTAIN Elite ABC Standard Kit, Vector Laboratories, PK-6100) overnight at room temperature. After a wash with TBST and three washes with TBS each for 10 minutes, sections were reacted for one hour with 0.5% 3,3'-diaminobenzidine (Sigma-Aldrich, D5637) solution in 0.1MPB containing 0.04% nickel ammonium sulfate and 0.01% H₂O₂. The reaction was stopped by four times washes with TBS, and the sections were counterstained with 0.05-0.1% cresyl violet, dehydrated, and coverslipped.

Tamoxifen treatment

Tamoxifen treatments were performed in quadruple transgenic fish (see above) as described previously [29,47,49]. 4-Hydroxytamoxifen (Sigma-Aldrich, T176) was dissolved in ethanol at a concentration of 10mg/ml and stored at -20°C until use. The working solution was freshly prepared before the treatment, then further diluted with embryo medium (for 24 hpf, 30 hpf, and 7 dpf) or fish water (for 2-6 wpf). The animals were incubated in the tamoxifen working solution at 28°C in the dark.

Embryos at 24 hpf and 30 hpf were dechorionated with Pronase (1mg/ml; Sigma-Aldrich, P5147) prior to the tamoxifen treatment. Embryos were placed into the six-well culture plate (Thermo Fisher Scientific) and were incubated with embryo medium containing tamoxifen. 24 hpf embryos were treated with 10 µg/ml tamoxifen for 6 hours, and 30 hpf embryos were treated with 5 µg/ml tamoxifen for 24 hours. After the incubation, the fish were washed 3 times with embryo medium, then put back to

the incubator. 7 dpf larvae were treated in a large petri dish (around 100 ml embryo medium) with 5 µg/ml tamoxifen on 2 consecutive days, with an incubation time for 4 hours each.

For juveniles (2-6 wpf), fish were placed in a beaker (100-200 ml fish water depending on the number of fish) with an air pump, and incubated with 2 µg/ml tamoxifen on 4 consecutive days. The incubation time per day was 2-4 hours, and the treatment was interrupted whenever the fish looked sick. At the end of each incubation, the fish were gently washed 3 times with fish water, placed back to a clean fish tank and fed.

The tamoxifen-induced mCherry expression was observed at 3 mpf. The fish were sacrificed and double-immunofluorescence anti-GFP and anti-dsRed were performed (see below).

Tissue preparations for immunofluorescence or *in situ* hybridization

Zebrafish embryos up to 48 hpf were fixed in ice-cold 4% paraformaldehyde (PFA; Electron Microscopy Sciences) in 0.01M PBS containing 0.1% Tween 20 (PBST) overnight at 4°C. Zebrafish older than 5 dpf were deeply anesthetized using 0.2% tricaine methanesulfonate (MS222; Sigma-Aldrich) diluted in fish water. The fish were fixed in 4% PFA in PBST overnight at 4°C, then brains were dissected out.

Samples used for *in situ* hybridization (ISH) were dehydrated in ethanol gradient series, and kept at -20°C in methanol at least for a couple of days. They were rehydrated prior to ISH. For immunolabeling, samples were conserved in a stocking solution containing 0.5% PFA and 0.025% sodium azide. Adult brains were sectioned in a frontal plane (80 µm) with a vibratome.

Tissue clearing of zebrafish brain by passive CLARITY technique (zPACT) was performed as described in Affaticati et al. (2017) [50]. Dissected brains were fixed in freshly prepared ice-cold methanol-free 4% PFA in PBS (pH 7.4) at 4°C overnight. Samples were then soaked in a precooled solution of hydrogel (0.01 M PBS, 0.25% VA-044 initiator, 5% dimethyl sulfoxide, 1% PFA, 4% acrylamide, and 0.0025% bis-acrylamide) at 4°C for 2 days. The hydrogel polymerization was triggered by replacing atmospheric oxygen with nitrogen in a desiccation chamber at 37°C for 3 hours. Passive tissue clearing was performed at 37°C for 5 days in the clearing solution (8% SDS, 0.2 M boric acid, pH adjusted to 8.5) under circular rotation in a hybridization oven. After clearing, brains were washed in PBST at room temperature with gentle shaking for 2 days. Brains were incubated in a depigmentation solution (0.5X SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.2), 5% formamide, 0.5X SSC, 3% H₂O₂, 0.1% Tween 20) for 40 min under light until all remaining pigments were bleached. After washing in PBST brains were post-fixed in PFA 4% in PBS (pH 7.4) at 4°C overnight.

Immunofluorescence

Double immunolabeling for GFP (1:1000; Aves Labs, GFP-1020; RRID: AB_10000240) and dsRed (1:600, Clontech Laboratories, 632496; RRID: AB_10013483) was performed on adult brain sections of quadruple transgenic zebrafish. Primary antibodies were incubated in PBST containing 4% NGS and 0.3% Triton X-100 at 4°C overnight. Then samples were incubated with secondary antibodies conjugated to fluorophores (1:1000; Alexa Fluor® 488 and 546, Molecular Probes/Thermo Fisher Scientific) in PBST at 4°C overnight. Alexa Fluor 488 goat anti-chicken (A-11039; RRID: AB_142924) were used for anti-GFP, and Alexa Fluor 546 goat anti-rabbit (A-11010; RRID: AB_143156) was used for dsRed. In order to visualize

the brain morphology, the sections were counterstained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; 5µg/ml, Sigma-Aldrich) at room temperature for 20 minutes.

CLARITY-processed brains were incubated in blocking solution (0.01 M PBS, 0.1% Tween 20, 1% Triton X-100, 10% dimethyl sulfoxide, 10% normal goat serum, 0.05 M glycine) at 20°C for 3 hours. Subsequently samples were incubated in staining solution (0.01 M PBS, 0.1% Tween 20, 0.1% Triton X-100, 9% dimethyl sulfoxide, 2% normal goat serum, 0.05% azide) with the chicken anti-GFP (1:400; Avès Labs, GFP-1020; RRID: AB_10000240) for 7 days at room temperature under gentle agitation. After four washing steps in PBST, samples were incubated in staining solution with secondary antibody (1:400; goat anti-chicken Alexa Fluor 488, Invitrogen, A-11039; RRID: AB_142924) at room temperature for 7 days. Samples were washed for 2 days in PBST and mounted in a fructose-based high refractive index solution (fHRI); 70% fructose, 20% DMSO in 0.002 M PBS, 0.005% sodium azide. The refractive index of the solution was adjusted to 1.457 using a refractometer (Kruss). The clarified samples were incubated in 50% fHRI for 6 hours and further incubated in fHRI for 1 day. For imaging, samples were mounted in 1% low melting point agarose and covered with fHRI.

***In situ* hybridization (ISH)**

ISH for *ert2Cre* [33,47,51] were performed in zebrafish brains of different developmental stages, in order to verify the expression of Cre recombinase. Detailed ISH procedures have been described in our previous publications [52,53].

After rehydration, the samples were permeabilized with proteinase K (1 µg/ml; Sigma-Aldrich, P6556) at 37°C for 5-10 minutes. The proteinase K reaction was

stopped by incubation with 2 µg/µl glycine. After PBST washes, the samples were incubated in hybridization buffer at 65°C for 4 hours, then hybridized with 2 ng/ml of cRNA probe in hybridization buffer at 65°C for at least 18 hours. Samples were then washed in gradient series of formamide/2X SSC mixture at 65°C: 75% formamide/25% 2X SSC, 50% formamide/50% 2X SSC, 25% formamide/75% 2X SSC, then washed in 2X SSC and finally in 0.2X SSC. After being rinsed with PBST at room temperature, the samples were incubated with anti-digoxigenin conjugated with alkaline phosphatase (1:2500; sheep anti-DIG-AP Fab fragments, Roche Diagnostics, 11093274910; RRID: AB_514497) at 4°C overnight. After PBST washes, the signal was visualized by incubation with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) solution (Roche Diagnostics, 11681451001) in 0.1 M Tris-HCl (pH9.5) / 0.1 M NaCl in H₂O (TN buffer).

For embryos, the whole ISH procedures were performed *in toto*. After revelation, the embryos were embedded into 3% agarose and sectioned with a vibratome in a sagittal plane (40 µm), and slide mounted for imaging. For juvenile brains, the hybridization was performed *in toto*, and the brains were sectioned with a vibratome in a frontal plane (40 µm) before incubating with anti-DIG-AP.

Image acquisition

A Leica TCS SP8 laser scanning confocal microscope was used to image adult sections with a 25x or 40x water immersion objective. For clarified brain the same microscope was used with a Leica HC Fluotar L 25x/1.00 IMM motCorr objective. For all these acquisitions, fluorescence signal was detected through laser excitation of fluorophores at 405, 488, 552 nm sequentially and detection was performed by PMTs

(photomultipliers). Steps in the Z-axis were fixed at 1 μm . Epifluorescence images were acquired using a Multizoom AZ100 (Nikon).

Bright-field images were either acquired with an upright microscope BX43 or BX60 (Olympus). Acquired images were adjusted for brightness and contrast using ImageJ/FIJI software.

Selective visualization of arborization pattern of *Tg(279A-GFP)*

The signal of the *Tg(279A-GFP)* was selectively visualized by manual segmentation of the arborization pattern in Amira (Thermo Fisher Scientific, FEI). For the classification of the staining of the specimen, we broke down the staining pattern into four categories: background, specimen background, specimen signal and specimen auxiliary signal, which contains a widely spread population of unknown cells. The process of manual segmentation is an iterative succession of initial freehand (Brush) segmentation and subsequent refinement with interactive thresholding tools (Magic wand, Threshold). The segmentation was initialized by a coarse manual segmentation of the pattern of interest and was refined region by region in up to seven iterations. The rather high number of manual and threshold-based segmentation iterations is due to the auxiliary signal, which was also embedded within the pattern of interest. The manual segmentation before computational removal of this signal needed its own empiric calibration procedure. Local thresholds for the (negative) segmentation of the auxiliary signal were selected with respect to the voxel values of the surrounding region to a lower boundary value of 150-180 (higher boundary always was 255). The resulting regions of exceptionally bright voxels were dilated in all three dimension by two pixels for removing the auxiliary signal in their entirety from the region of interest.

For the final visualization we multiplied the original grey value dataset with the binary 3D mask of the segmentation. Since in this mask the region of interest is encoded as one (1) and the background as zero (0) the multiplication of the image with its mask will result in the separation of its original grey values within the mask from the background signal because the latter is multiplied by zero. This isolated signal was visualized using the orange-yellow colormap (look-up-table, LUT) `volrenRed.col` with a truncated dynamic range (0-200) for better visibility of the signal of interest in a Volren-module of Amira.

For the context of the signal of interest, we visualized in parallel the entire dataset in a separate Volren-module using a grey colormap with slightly truncated dynamic range (10-255) as a means of global contrast enhancement.

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AUTHOR CONTRIBUTIONS

SB, HH, MT, EL, JMH, IC, and KY performed experiments. The tract-tracing study was directed by NY and the other studies were directed by KY. AH created the *Tg(Dr830:ERT2CreERT2)* transgenic line and KK created the *Tg(gSAGFF279A;UAS:GFP)* transgenic line. PA performed tissue clearing and whole-brain image acquisition, and AJ performed 3D reconstruction of the images. The data was analyzed by SB, HH, MT, NY and KY, and the manuscript was written by SB, HH, NY and KY.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Figure legends

Figure 1. Tectofugal pathways in mammals, birds, and teleosts (cyprinids) and their phylogenetic relationships

Schematic drawing of a sagittal section of the brain of rodent, bird, and cyprinid are shown above a phylogenetic tree of the *Osteichthyes* (bony vertebrates). The tectofugal visual pathways are shown with red arrows. The relay nucleus giving rise to

the telencephalic projections is the thalamus (Th) in mammals and birds (amniotes), while it is the preglomerular nucleus (PG) in cyprinids.

Abbreviations, PG: preglomerular nucleus, SC: superior colliculus, TeO: optic tectum, Th: thalamus.

Figure 2. GFP+ afferents in *Tg(279A-GFP)* zebrafish transgenic line

(A and B) Frontal sections of *Tg(279A-GFP)* adult brain showing the GFP+ cell bodies in PG (A) and GFP+ fibers in DI (B). The plane of the sections is indicated in the schematic drawing on the upper left. (A) The brain section contains different levels of PG, the right side being slightly more anterior than the left side. Higher magnification of the right side of PG is shown in the inset. (B) The GFP+ fibers terminate to the DI of the pallium, as is the case for the visual terminals in the goldfish pallium (upper right schematic drawing from Yamamoto 2009).

(C and D) Selected visualization of the GFP+ projections from PG to DI. After 3D reconstruction of the whole brain imaging of *Tg(279A-GFP)*, the GFP+ signal of the PG cells was selectively visualized in order to follow their projections. (C) shows the lateral view of the brain (anterior to the left) and (D) shows the dorsal view (anterior to the top). The original movie is shown in the Movie S1.

Abbreviations, DI: lateral part of dorsal telencephalic area, PG: preglomerular nucleus, Pall: pallium, TeO: optic tectum. Scale bars, 100 μm (A and B); 500 μm (C).

Figure 3. Cytoarchitecture and connections of the lateral preglomerular nucleus (PGI) presented in the frontal sections of zebrafish

(A and B) Cresyl violet-staining of PG showing lateral (PGI) and anterior (PGa) subdivisions. The nomenclature for the zebrafish subdivisions is adapted from goldfish.

(A) shows PG at a high magnification and (B) shows a global view of the brain section.

The asterisk indicates the neuropil of PGI and the arrows indicate cells of PGI.

(C and D) Tectal neurons projecting to PGI. (C) Injection site of Dil in PGI (asterisk).

(D) Retrogradely labeled tectal neurons extending their dendrites (arrowheads) in the superficial layer of the optic tectum (TeO).

(E and F) Projection target of TeO neurons. (E) Injection site of biocytin in TeO (white

asterisk). (F) Anterogradely labeled terminals (arrowheads) in the ipsilateral PGI.

(G-I) Projection target of PGI neurons. (G) Injection site of biocytin in PGI (white asterisk). Dendrites of PGI neurons extend to the superficial neuropil zone. (H)

Anterogradely labeled terminals in the ipsilateral DI. The right top inset shows a higher magnification of the squared area, showing numerous labeled terminals. (I) GFP+ fiber labeling in DI of *Tg(279A-GFP)* zebrafish line, demonstrating the identical arborization pattern with (H).

(J-L) PGI neurons projecting to DI of the pallium. (J) Injection site of biocytin in DI (white

asterisk). (K) Retrogradely labeled neurons in the ipsilateral PGI. Labeled cells (white arrows) extend apical dendrites that ramify in the neuropil. (L) GFP+ perikarya labeling

in PGI of *Tg(279A-GFP)* zebrafish line (white arrows), demonstrating the identical cell localization with K.

Abbreviations, Dc: central part of dorsal telencephalic area, Dd: dorsal part of dorsal telencephalic area, Dl: lateral part of dorsal telencephalic area, Dm: medial part of dorsal telencephalic area, Dp: posterior part of dorsal telencephalic area, Pall: pallium, pc: posterior commissure, PG: preglomerular nucleus, PGa: anterior preglomerular nucleus, PGI: lateral preglomerular nucleus, TeO: optic tectum, TL: torus longitudinalis.

Figure 4. Co-localization of GFP and mCherry in the adult PG cells of *Tg(her5:ERT2CreERT2;279A-GFP; β act:lox-stop-lox-hmgb1:mCherry)* following the tamoxifen induction at 24 hpf

(A) *In situ* hybridization of *ert2Cre* showing that the expression of Cre in this line is limited to the midbrain-hindbrain boundary (MHB) at 24 hpf. Anterior of the brain to the right.

(B) Schematic drawing of the adult zebrafish brain indicating the frontal plane shown in C-G containing the PG.

(C) A single plane confocal image showing a global view of the frontal section of a 3 months post-fertilization (3 mpf) zebrafish brain. The white square indicates the PG shown in D-G.

(D-G) A confocal image (5 μ m projection) showing the co-localization of GFP and mCherry in PG (arrowheads). Inset of F shows the double-labelled cell at a higher magnification.

Figure 5. Co-localization of GFP and mCherry in the adult PG cells of *Tg(Dr830:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry;279A-GFP)* following the tamoxifen induction at different developmental stages

(A-G) A confocal image (5 μ m projection) showing the co-localization of GFP and mCherry in PG (arrowheads). Arrowheads indicate double-labelled cells. The brain structure and section plane shown here are identical to that in Figure 4. Scale bar, 30 μ m.

Figure 6. Non-homologous relationship of the tectofugal relay nuclei in amniotes and teleosts

Schematic drawing of representative embryonic brains of amniote and cyprinid are shown above a phylogenetic tree of the *Osteichthyes* (bony vertebrates). The tectofugal visual pathways are shown with red arrows. The relay nucleus in amniotes, the thalamus (Th) is a diencephalic (forebrain) structure, while the preglomerular nucleus (PG) in cyprinids is a mesencephalic structure.

Abbreviations, IL: inferior lobe, P: pallium, PG: preglomerular nucleus, SC: superior colliculus, TeO: optic tectum, Th: thalamus.

Figure S1. GFP expression of the *Tg(279A-GFP)* transgenic line at juvenile stages

Frontal sections of 6 wpf (A) and 8 wpf (B and C). There are very few GFP+ cells in PG at 6 wpf (A), while they become obvious at 8 wpf (B). In 8 wpf, their projections in DI are visible but weak (C).

Abbreviations, DI: lateral part of dorsal telencephalic area, PG: preglomerular nucleus, TeO: optic tectum.

Figure S2. Expression of *ert2Cre* in embryonic brains

(A-C) Sagittal sections of embryonic brains. At 24 hpf (A), *ert2Cre* is highly expressed in the mesencephalic domain, but it is also found in the anterior part of the brain. Later, at 30 hpf (B), the expression becomes limited to the mesencephalon. At 48 hpf (C), the expression is found exclusively in the tectal area. In C, the section plane is slightly tilted showing a more dorsal view of the embryo.

Abbreviations, Mes: mesencephalic area, Tel: telencephalic area. Scale bars, 100 μ m.

Figure S3. Expression of *ert2Cre* in juvenile brains

(A-F) Frontal sections of juvenile brains showing the telencephalic (Tel) and mesencephalic (Mes) areas. The plane of the sections is indicated in the schematic drawing on the top. In the 2 wpf (A and B), 3 wpf (C and D), and 5 wpf (E and F) juvenile brains, *ert2Cre* is expressed exclusively in the tectal area (B, D, and F).

Abbreviations, IL: inferior lobe, Mes: mesencephalic area, Tel: telencephalic area, TeO: optic tectum. Scale bars, 100 μm (A-E); 200 μm (F).

Figure 1

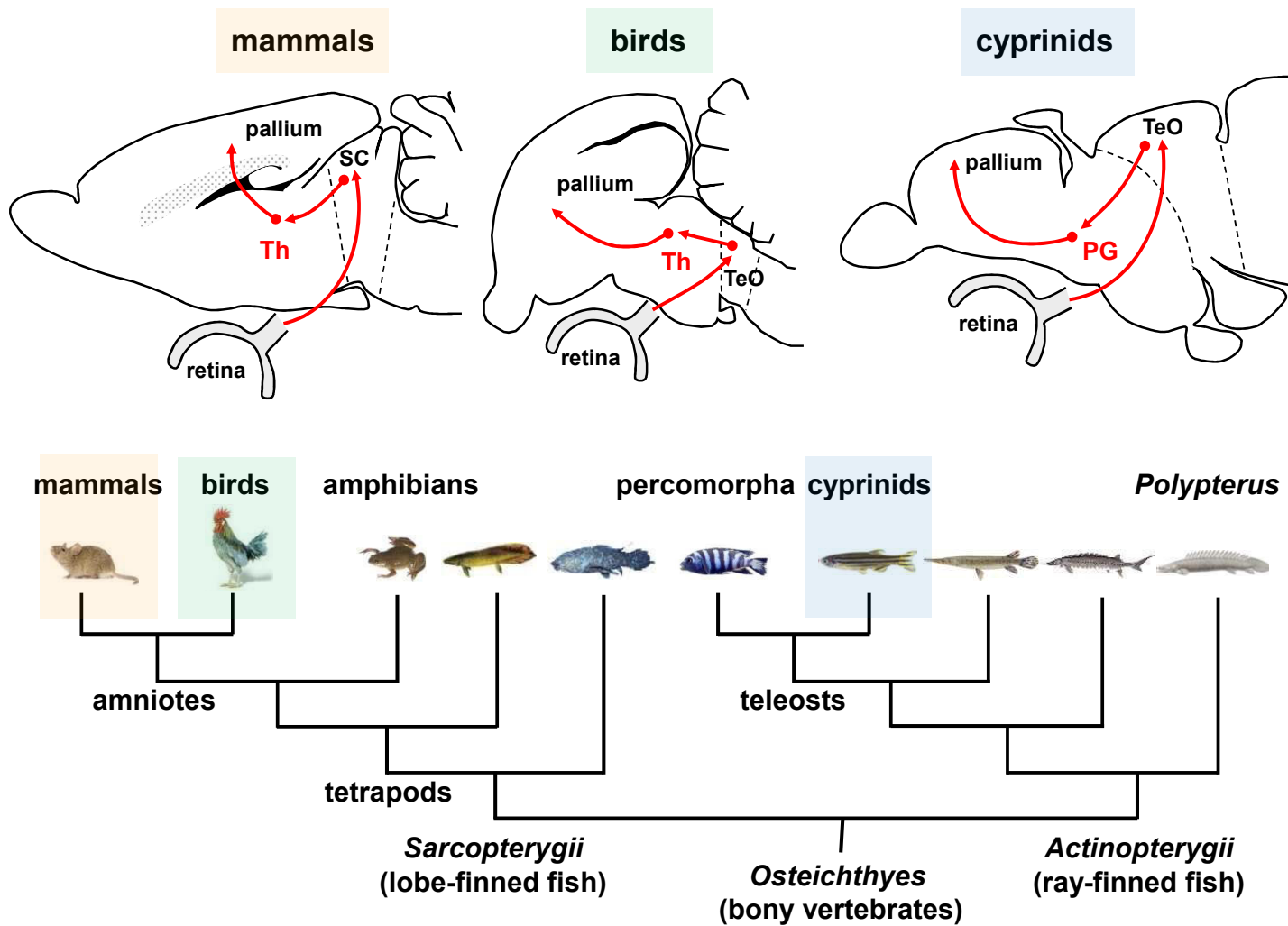


Figure 2

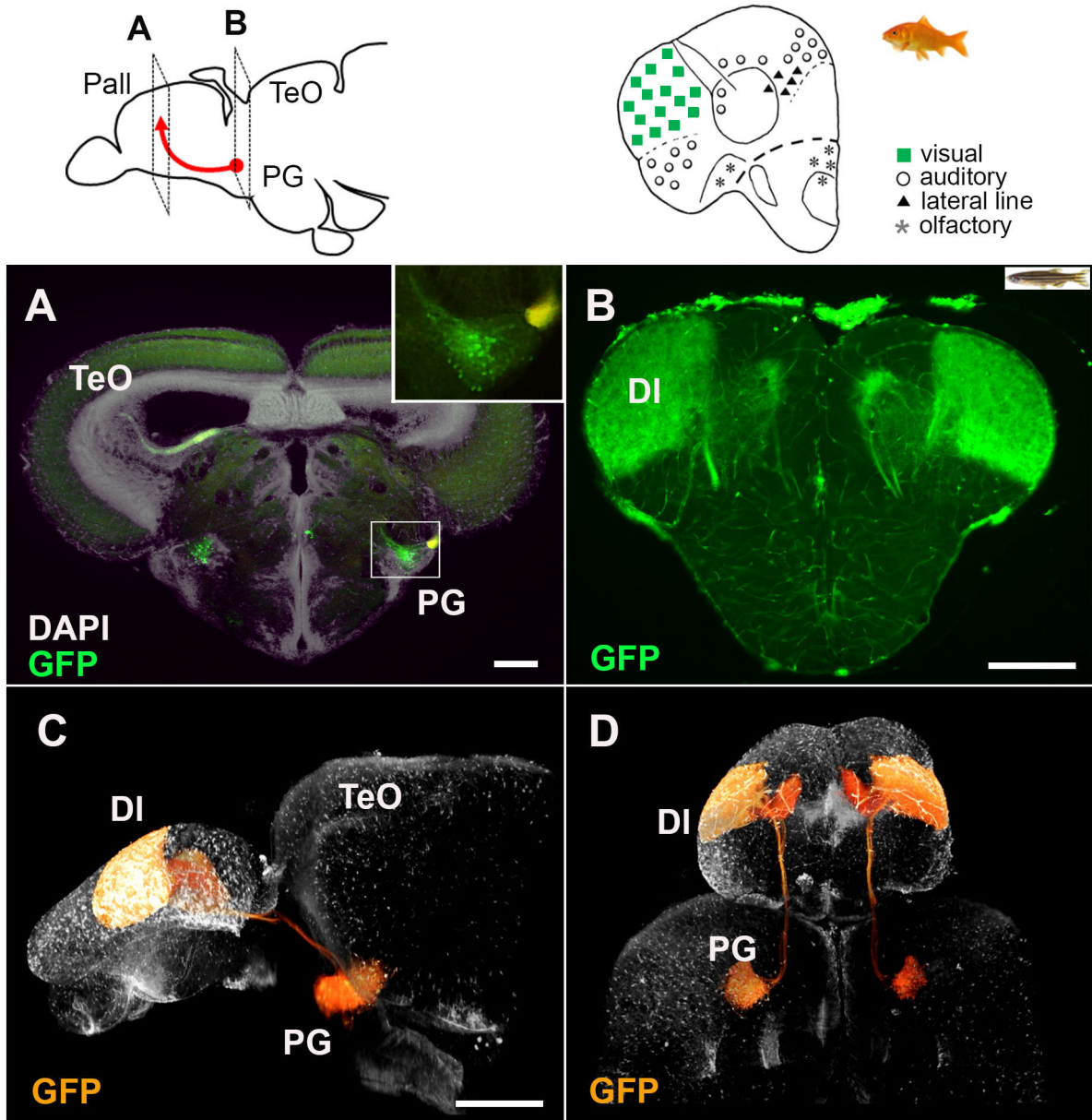


Figure 3

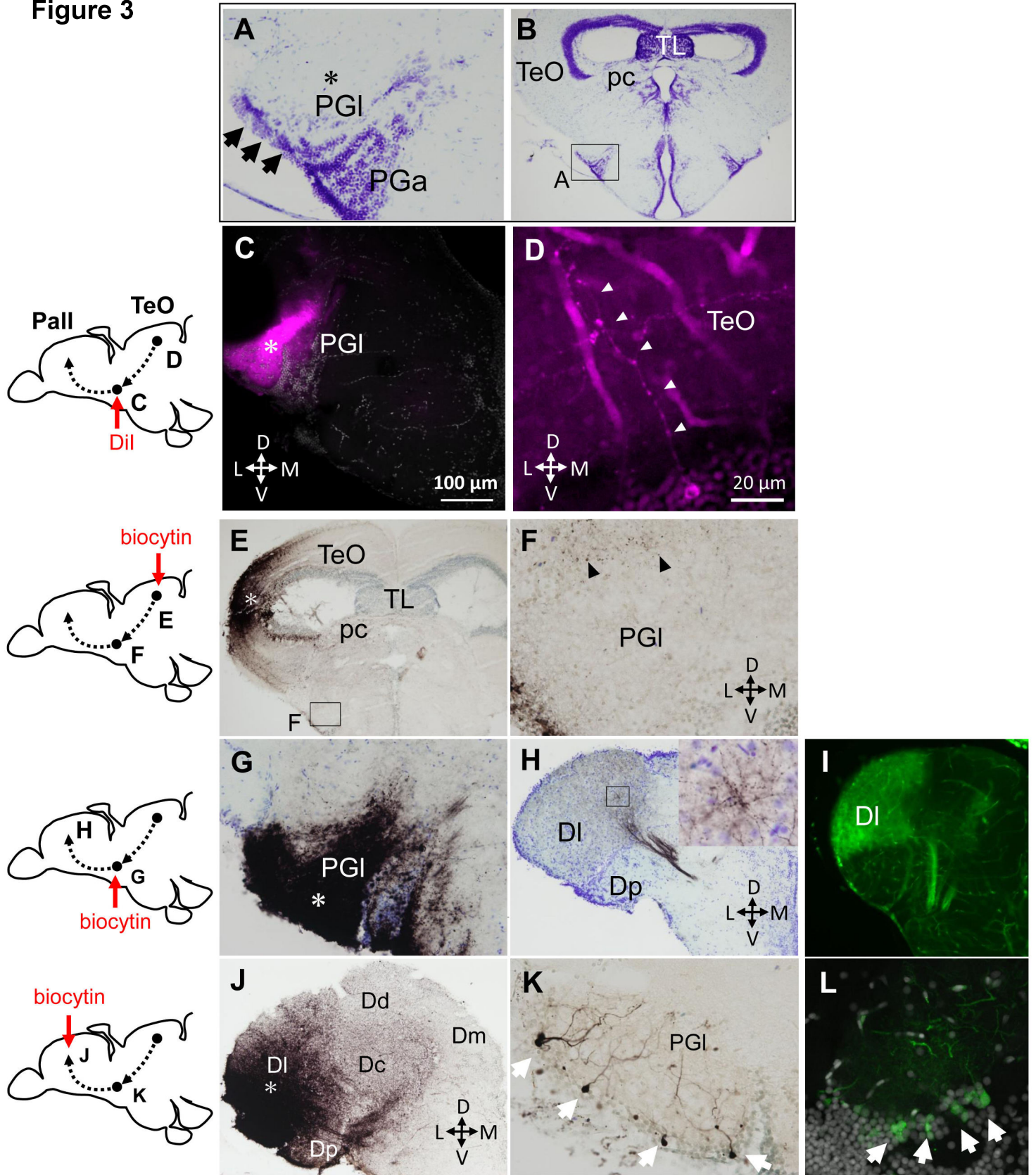


Figure 4

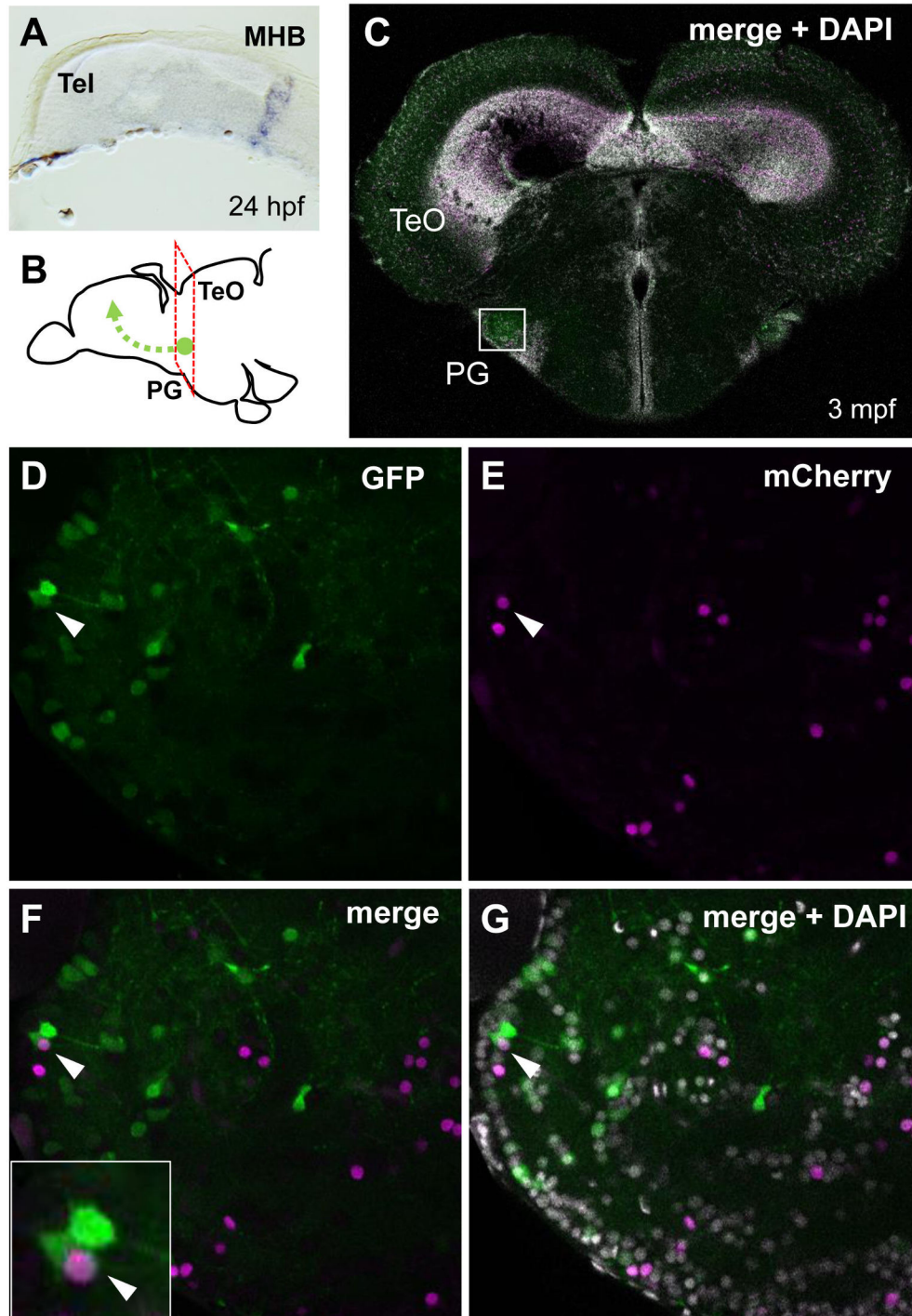


Figure 5

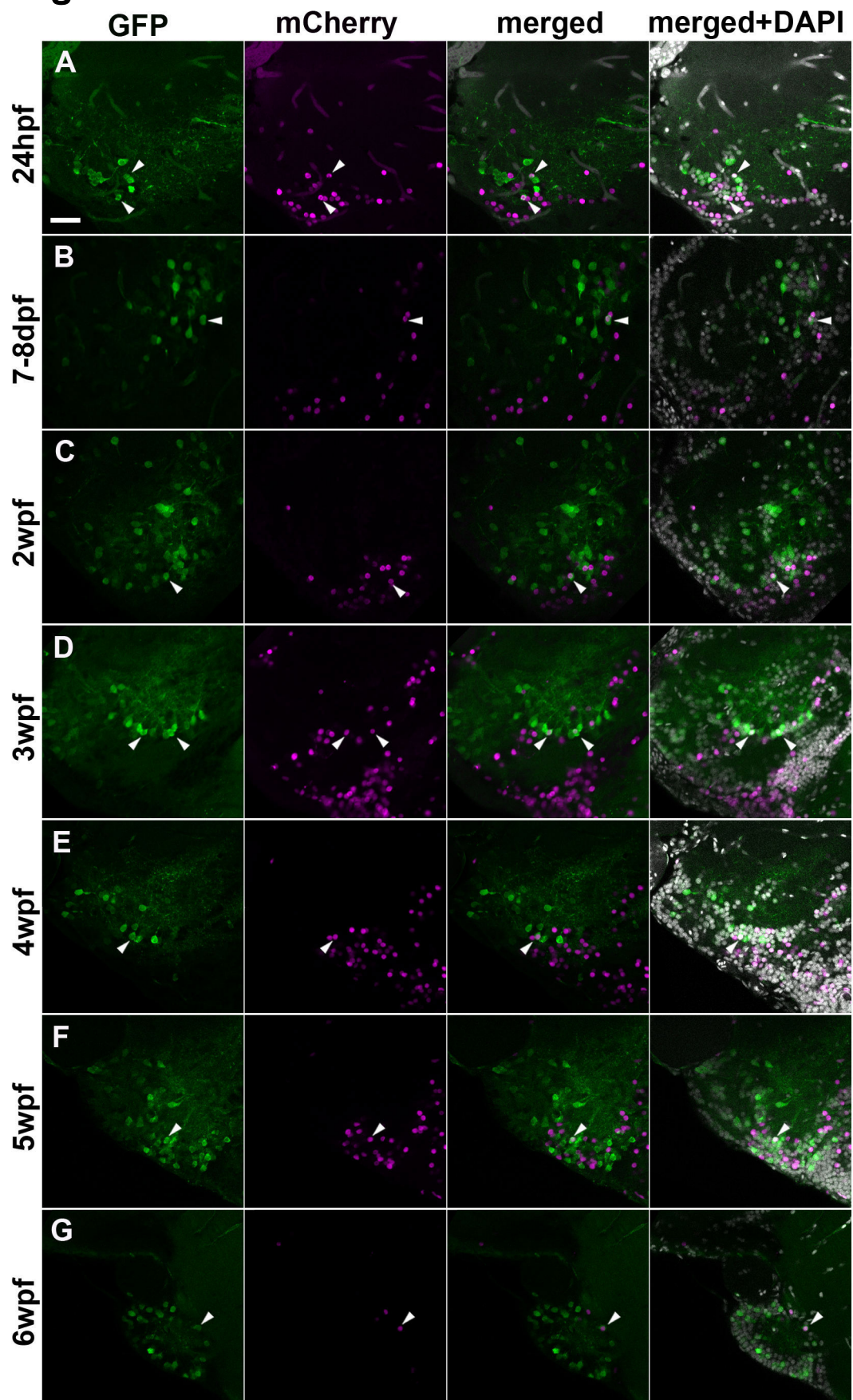
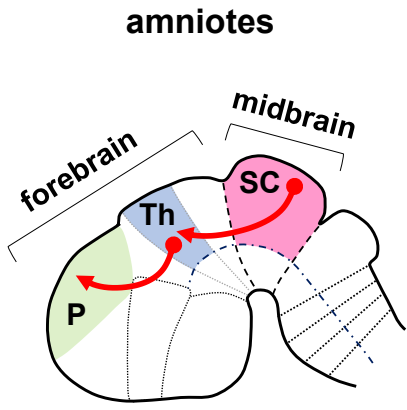
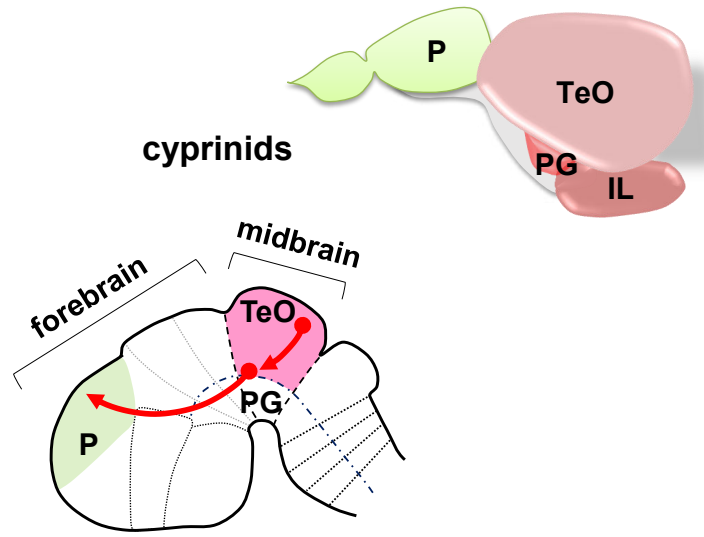


Figure 6



dorsal thalamus = forebrain



PG = midbrain

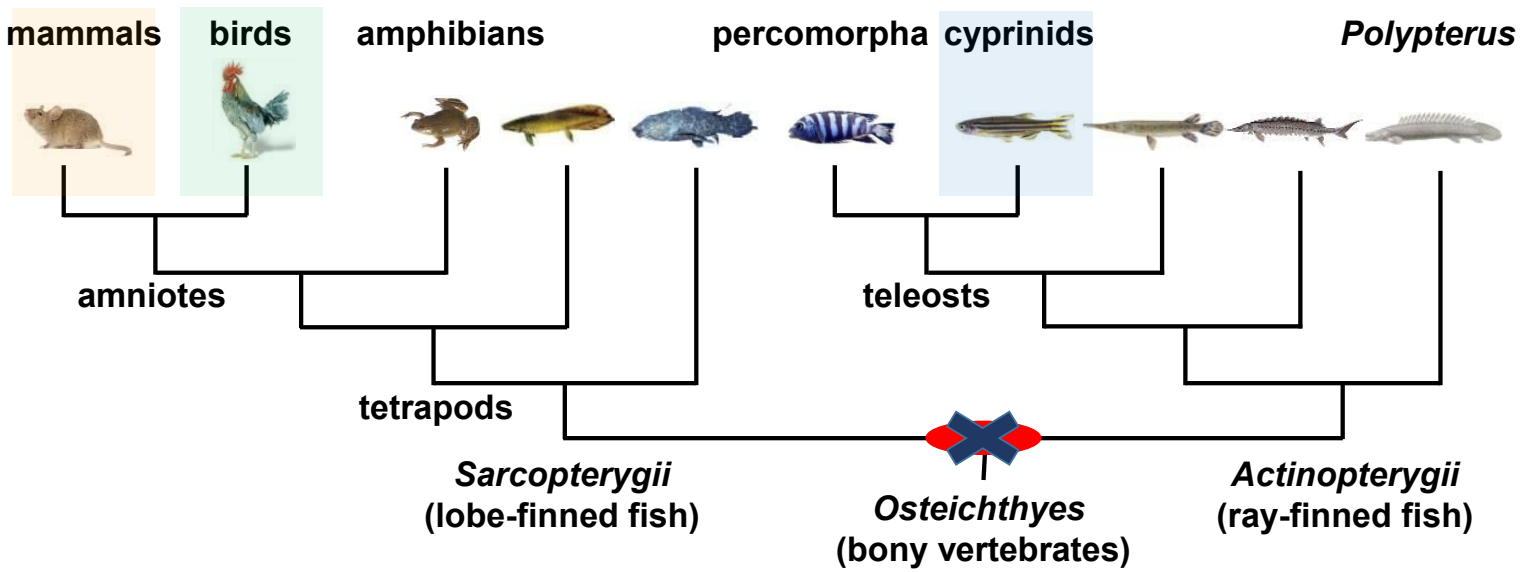


Figure S1

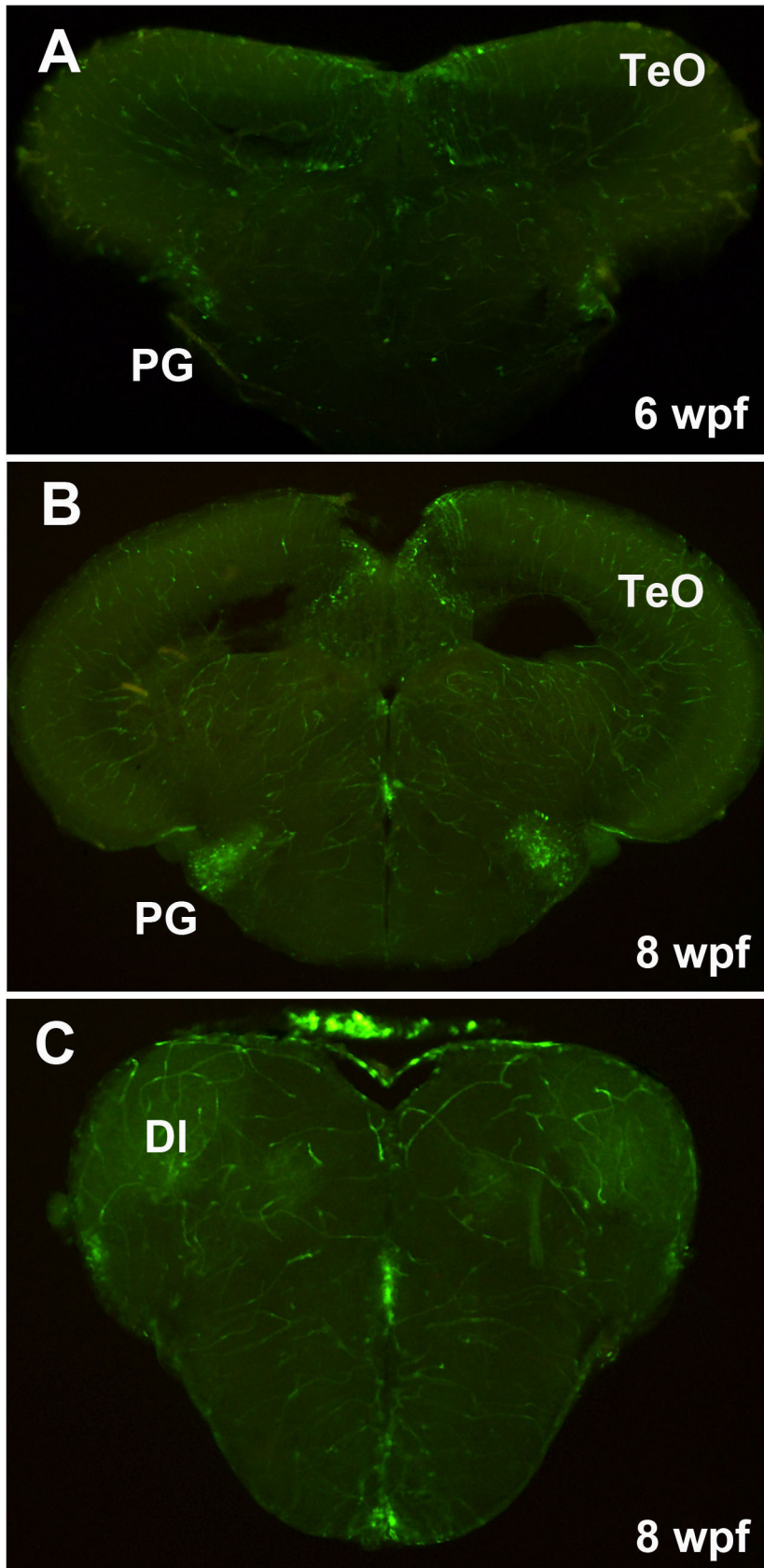


Figure S2

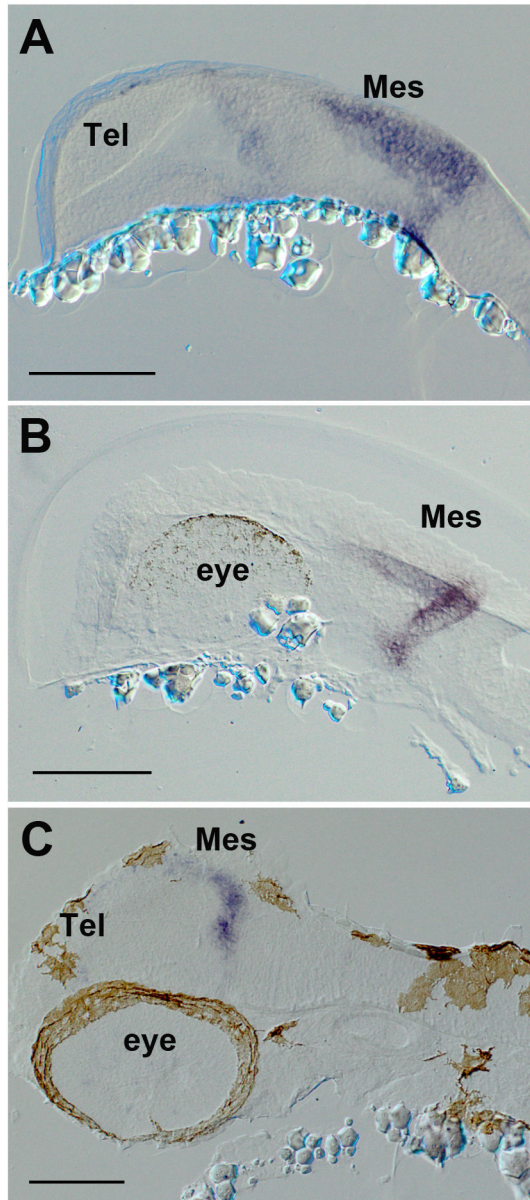


Figure S3

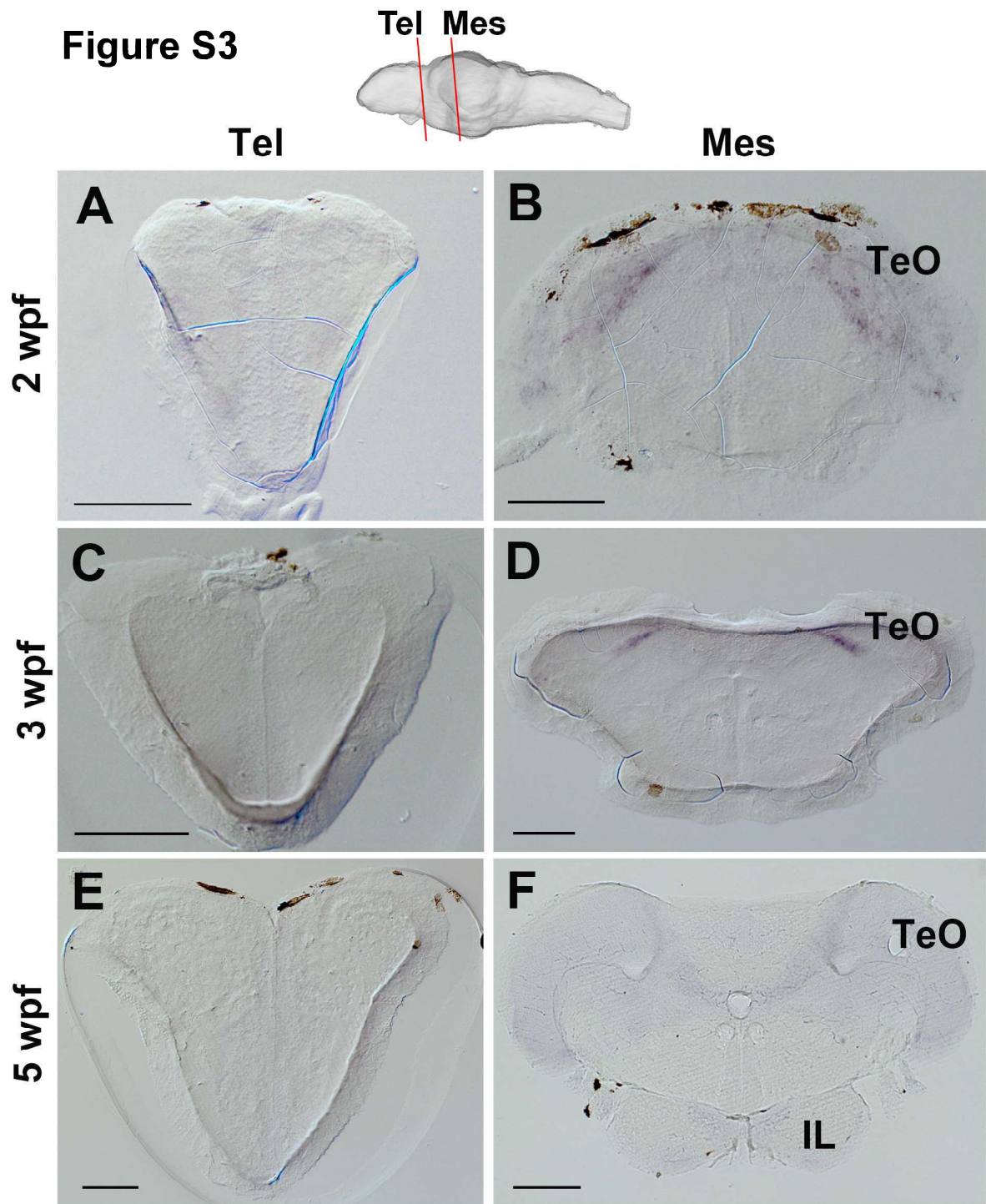


Table S1. Conditions of tamoxifen treatment

age		tamoxifen treatment			tamoxifen	age of analysis	# fish analyzed
start	end	duration/day	# days	total duration	concentration		
Tg(her5:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry;279A-GFP)							
24 hpf	30 hpf	6 h	1	6 h	10 μ g/mL	3 mpf	14
Tg(Dr830:ERT2CreERT2; β act:lox-stop-lox-hmgb1:mCherry;279A-GFP)							
24 hpf	30 hpf	6 h	1	6 h	10 μ g/mL	3 mpf	3
24 hpf	30 hpf	6 h	1	6 h	10 μ g/mL	6 mpf	6
30 hpf	54 hpf	24 h	1	24 h	5 μ g/mL	3 mpf	6
48 hpf	54 hpf	6 h	1	6 h	10 μ g/mL	3 mpf	6
3 dpf	4 dpf	24 h	1	24 h	5 μ g/mL	3 mpf	6
7 dpf	9 dpf	4 h	2	8 h	5 μ g/mL	3 mpf	6
2 wpf	2 wpf, 4 dpf	2 h	4	8 h	2 μ g/mL	3 mpf	6
3 wpf	3 wpf, 4 dpf	2 h	4	8 h	2 μ g/mL	3 mpf	6
4 wpf	4 wpf, 4 dpf	2 h	4	8 h	2 μ g/mL	3 mpf	6
5 wpf	5 wpf, 4 dpf	2 h	4	8 h	2 μ g/mL	3 mpf	6
6 wpf	6 wpf, 4 dpf	4 h	4	16 h	2 μ g/mL	3 mpf	6
8 wpf	8 wpf, 4 dpf	4 h	4	16 h	2 μ g/mL	3 mpf	6

Article 3

Existence of working memory in teleosts: establishment of the delayed matching-to-sample task in adult zebrafish

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ABSTRACT

Operant conditioning is a powerful tool to study animal perception and cognition. Compared to mammals and birds, there are very few behavioral studies using operant conditioning paradigm in teleosts. Here we aim to establish matching-to-sample task (MTS) in adult zebrafish, using visual cues (colors) as discriminative stimuli. Unlike simple one-to-one color-reward association learning, MTS requires ability for context integration. In this study, zebrafish learned to perform the simultaneous-matching-to-sample (SMTS) within 15 sessions. After the SMTS training, working memory was tested by inserting a delay period (delayed matching-to-sample; DMTS). Zebrafish could perform the DMTS with a delay of at least 3-4 seconds. They could also learn to perform the DMTS even with a delay period from the beginning of the training session. These results strongly suggest that adult zebrafish possess working memory. However, our study also indicates limitations of zebrafish in cognitive flexibility or attention: they could perform SMTS/DMTS only in a certain set-up. The presence of working memory without the mesencephalic dopamine neurons indicates the convergent evolution of this function in amniotes and teleosts.

Key Words: working memory; executive function; operant conditioning; teleost; convergent evolution

1. Introduction

Brain complexity is still commonly believed to increase linearly with the phylogenetical “modernity” of vertebrate species. In this view inherited from the *scala naturae*, so-called “higher” vertebrates have increased their brain size by adding newer brain structures on the older ones. For instance the prefix “neo” referring to the six-layered mammalian “neocortex” reflects this view [1,2]. However, many behavioral studies suggest that birds such as corvids and parrots demonstrate cognitive capacities rivaling those of primates, such as theory of mind, tool use and manufacture, although they do not possess a mammalian-like “neocortex” [3–5]. It is now well accepted that the presence of so-called higher order cognitive functions is not specific to mammals.

In order to analyze specific cognitive components of complex behaviors, behavioral studies under controlled conditions are necessary. Operant conditioning is a powerful tool to decipher animal cognition. For example, studies using operant conditioning tasks based on visual discrimination have revealed that pigeons are capable of “categorizing objects” [6,7]. Furthermore, presence of executive functions in birds (mainly in pigeons) has been demonstrated by operant conditioning: reversal learning task to test learning flexibility [8,9], moving-dot paradigm (shell game) for selective attention [10], and delayed matching-to-sample task (DMTS) for working memory [11–13]. Executive functions are crucial for cognitive processes such as planning, cognitive flexibility, decision-making, and inhibiting inappropriate actions [14]. In mammals, these functions are performed in the prefrontal cortex (PFC). Several studies have demonstrated that the avian pallium (dorsal telencephalon) also contains an area functionally equivalent to the mammalian PFC: the nidopallium caudolaterale (NCL) [14–17].

Outside of amniotes (a group containing mammals and birds), some species of teleosts also demonstrate cognitive capacities such as transitive inference [18] and tool use [19]. Such behavioral repertoires have been observed only in some teleost species in the families of cichlids or wrasses. Interestingly, these teleost species have an enlarged pallium. Mammals and teleosts are phylogenetically much more distant than mammals and birds. Thus comparative studies including teleost species will provide considerable information on evolution of cognitive functions, notably on their convergent evolution [20].

In this study, we developed operant conditioning tasks in the adult zebrafish, a teleost species that is becoming a popular animal model in neuroscience. Although the size of the pallium and behavioral repertoires are not as remarkable as cichlids or wrasses, the accessibility for genetic tools makes it an interesting model to correlate the behavioral studies with brain anatomy and physiology. A previous study has demonstrated that zebrafish can perform reversal learning task [21], suggesting that zebrafish also have some learning flexibility. Thus zebrafish may possess a primordium of executive functions. To test this hypothesis, we examined whether zebrafish can perform matching-to-sample tasks (MTS): simultaneous matching-to-sample (SMTS) and delayed matching-to-sample (DMTS). MTS requires capacity for context integration (the logic of “if... then...”). DMTS is a MTS with a delay period, and it is often used to study working memory in non-human animals.

We specially aimed to establish an operant conditioning paradigm directly comparable to previous studies in terrestrial animals. For this purpose, we tried two different types of set-up. In order to validate the adequacy of our set-up for zebrafish, we also performed simple color discrimination tasks in each set-up, since zebrafish is known to be able to perform color discrimination in operant paradigm.

2. Materials and methods

2.1. Subjects

90 experimentally naive adult zebrafish (*Danio rerio*; between 3-12 months) were used in this study. The zebrafish were all raised in our own colony in the animal facility (maintained at 28°C with a 14-/10-hours light/dark cycle). The animals were maintained with their siblings in a fish tank (generally 10 to 25 individuals) until used for the experiment. All experimental protocols and care of laboratory animals were conducted in compliance with the official regulatory standards and approval of the French Government (reference document n°APAFIS#1286- 2015062616102603 v5, AP 2014-28 V2 mars).

2.2. Apparatus and stimuli

The subjects were tested individually in a home-made operant box. A schematic drawing of the operant box is shown in Fig. 1. A rectangular aquarium (32 cm x 14 cm width, approximately 7 cm height of water) was divided into two areas: the waiting area and the responding area. During the inter-trial interval (ITI), the waiting area and the responding areas were separated by a removable plastic board (middle lid). The middle lid was in opaque white so that the subject cannot see the stimuli before the lid is removed. At the end of the responding area, there were one or two food distribution cylinders (2 cm diameter). A small piece of reinforcer was provided manually after a correct response.

As a reinforcer (reward), Gemma Micro 600 (SKRETTING, Westbrook, Maine, USA) was used in the Experiment 1 (Exp 1) and Exp 3, while a small piece of dried

mosquito larva (NovoFil; JBL, Neuhofen, Germany) was used for the rest of the experiments (see Discussion).

We have tried slightly different set-ups in order to find the most suitable operant box for zebrafish. The main difference is the definition of the response of the fish: one is “Entering the hallway” (Fig. 1A, C), and the other is “Passing through the window” (Fig. 1B, D, E) (see below for more details). In the “Entering the hallway” set-up, the responding area is further divided into right and left responding areas. In the “Passing through the window” set-up, a panel containing square windows (2 cm X 2 cm, 2 cm above the bottom of the box) was placed at the position of the middle lid.

Since zebrafish is known to be able to distinguish colors [21], we used colored panels in green (approximate RGB color code = R: 5, G: 170, B:110) and red (R: 240, G: 40, B:80) as discriminative stimuli. The luminance of the two panels was almost the same (around 18 cd/m²). In the “Entering the hallway” set-up, the two color panels were placed at the end of the responding area behind each food distributor (Fig. 1A, C), and in the “Passing through the window” set-up, the colors were displayed at the position of the middle lid (Fig. 1B, D, E). In SMTS and DMTS, the “sample” (either green or red color depending on the trial) was demonstrated at one end of the box in the waiting area. In SMTS/DMTS with “Passing through the window” response, most of the data shown here were obtained with the set-up shown in Fig. 1E, in which two choice stimuli and two food distributors are placed at the end of the responding area. This is due to the fact that the fish showed a difficulty for performing the task in the set-up with a single distributor shown in Fig. 1D (see Results for details).

2.3. Experimental procedure

2.3.1. Habituation

Naive zebrafish were raised in group in a large tank in the animal facility, but during the experiment, the fish were kept individually in the same tank used for the experiment (operant box). In most cases (except Exp 2), 10 fish were used in each experiment. Each subject was used only for one experiment, and we never used the same subject in different experiments. The fish were separated in the operant box for 5-7 days, and deprived of food during 2 days before starting the pretraining. Until the completion of each experiment, the fish were kept in the same operant box.

2.3.2. Pretraining

The fish were first trained to eat food under the food cylinder. In the pretraining, the color stimuli were removed from the operant box. At the beginning of the trial, the middle lid was closed and the subject is placed in the waiting area. After the middle lid was removed, a small piece of reinforcer was dropped close to the fish and left until the fish ate it (the fish was left freely up to 2 minutes). When the fish consumed the food, the location of food delivery became gradually closer to the food cylinder in the following trials. The pretraining continued until the fish came to eat the food delivered through the cylinder. In the set-up with two food cylinders, the food was delivered equally often on the right and on the left side to avoid developing position preference.

In case of the “Entering the hallway” response, once the fish learnt to eat the food under the cylinder, they were reinforced only when coming close by a cylinder. The distance required to be reinforced was gradually reduced, and in the end, the food was provided only when the head of the fish came just in front of a cylinder, or tapping it.

In case of “Passing through the window” response, entering the responding area through a window was considered to be a response. Thus regardless the distance of

the fish from the cylinder, the food was provided once the fish passed through the window. When the fish did not pass the window after 2 minutes, the experimenter pushed the window panel towards the end of the waiting area (as a consequence, the waiting area narrows) to facilitate the fish passing through the window. The advantage of the “Passing through the window” response was that it was clearer to determine whether the fish responded or not. This behavior occurred rarely for untrained fish, thus we could better validate the intention of the fish to respond (for example, it could serve to reduce the false-positives due to hyperactivity).

The middle lid was closed during the food consumption. After finishing eating, the middle lid was re-opened and the fish came back to the waiting area. When the fish did not come back by itself after 10 seconds, it was manually helped to move to the waiting area.

A session consisted of 10 trials, and one or two sessions were performed per day. That is, the subjects were fed at least 10 pieces of reinforcer every day. We could not perform more than two sessions per day because of the loss of motivation for food which occurred above 10 trials. In the end of the week, Gemma Micro 300 (SKRETTING, Westbrook, Maine, USA), which is the food normally provided in our fish facility, was fed in order to compensate the nutrition.

2.3.3. *Color discrimination*

After the fish established the response-reward (food consumption) association, discrimination stimuli (green/red colors) were displayed. Half of the fish were rewarded when they responded on the green side ($S^+ = \text{green}$), while the other half were rewarded when they responded on the red side ($S^+ = \text{red}$). The right/left position of the

two colors was randomized. We have performed experiments having both colors to be S+, although the data for “S+ = green” is demonstrated in Results.

When the fish chose the S+, a small amount of reward was provided. When the fish chose the S-, reward was not provided and the middle lid is closed and the fish was confined within the responding area for 30 seconds. Since zebrafish do not like to be placed in a small area, this serves as a mild punishment.

The tasks were performed using two different setups, one with “Entering the hallway” (Fig. 1A, Exp 1), and the other with “Passing through the window” (Fig. 1B, Exp 2). In the case of the “Entering the hallway” response (Exp 1), entering the S+ hallway (responding area) and putting the head under the cylinder or poking it was considered to be a correct response. Poking the cylinder of S-, or entering the S- hallway 3 times within a trial (even without poking the wrong cylinder) was considered as an incorrect response (error). If the fish did not choose any color within 30 seconds, it was also recorded as “incorrect response”, thus the middle lid was closed for 30 seconds, and the next trial started.

Each trial was followed by an inter-trial-interval (ITI) of 10 seconds. One session consisted of 10 trials, and one or two sessions were performed per day. The learning criterion was 70% correct response rate or above ($\geq 70\%$) for 3 consecutive sessions.

2.3.4. Matching-to-sample (MTS)

In the matching-to-sample (MTS), subjects were trained to choose a given sample stimulus out of two subsequent choice stimuli. Choosing the choice stimulus that is the same as the sample (S+) was rewarded with food, while choosing that is different from the sample (S-) was not rewarded. The simultaneous matching-to-

sample (SMTS) is a task in which the sample stimulus continues to be presented after the onset of the choice stimuli.

In the SMTS procedure, the sample stimulus (either green or red panel) was first shown alone in the end of the waiting area for 10 seconds. Then the middle lid opened, and choice stimuli (green and red panels) were displayed. Choosing the same color as the sample (S+) within 30 seconds was recorded as a correct response, and the fish was rewarded with food. When the fish chose the different color as the sample (S-), reward was not provided and the middle lid was closed for 30 seconds (mild punishment). If the fish did not choose any color within 30 seconds, the middle lid was closed, and the next trial started after the mild punishment.

When the sample is retrieved before the appearance of the choice stimuli, the task becomes the delayed matching-to-sample (DMTS). The sample color on the wall of the waiting room was first removed, then the middle lid was removed several seconds later, and the choice stimuli were displayed. Thus the only difference between the SMTS and DMTS was the insertion of the “delay” between the removal of the “sample” and the appearance of the “choice”. In the Exp 3, different durations of the delay were tested, starting with 3 seconds.

Green or red “sample” was demonstrated in a random manner within a session. Appearance of each sample color was 50% in average, and the right/left position of the two choice stimuli was also randomized.

Each trial was followed by an inter-trial-interval (ITI) of 10 seconds. One session consisted of 10 trials, and one session was performed per day. The learning criterion was the same as described above: we considered that the fish mastered the task when it could retain the correct response rate $\geq 70\%$ for 3 continuous sessions.

We performed 6 experiments for SMTS and DMTS. In the Exp 3, SMTS training sessions followed by DMTS test sessions were performed with “Entering the hallway” response, and Gemma Micro 600 was used as a reinforcer. In the Exp 4, the same training was performed with a new reinforcer, using dried mosquito larva. Exp 5 was performed with “Passing through the window” response. In the Exp 6-8, the fish were divided into two groups, one was trained for SMTS while the other was training directly for DMTS with a delay of 3 seconds.

2.4. Data analysis

We generally started the experiments with 10-12 subjects, and often there were one or two fish that showed a freezing behavior. When the fish froze throughout the experiment, the subject was removed from the analysis. When a subject responded more than half of the trials within a session, the percentage of correct responses were calculated. Otherwise, the session displayed no data (indicated “-” in the Tables).

We tested our learning criterion of “3 consecutive sessions achieved with a score $\geq 70\%$ ” using a binomial test, yielding a $p = 0.021$ for an expected probability of success for each trial at chance level ($p = 0.5$), meaning that our learning criterion is robust. “Two consecutive sessions achieved with a score $\geq 70\%$ ” using a binomial test yielded a $p = 0.058$, which we did not consider as robust enough to use as our main criterion ($p > 0.05$).

We visualized the results of each experiments in two different manners. The first demonstration is the progression of the mean correct response rate of the group session by session, indicating the standard deviation (Fig. 2A and B, Fig. 3A-C, Fig. 4). The second demonstration is comparison of the performance between the initial phase versus final phase of the training. For the latter analysis, the average correct

response rate of the last 3 sessions (30 trials of the final phase) of each subject was tested against the correct response rate of the first 3 sessions (30 trials of the initial phase) using a paired t-test (Fig. 2C and D, Fig. 3D and E, Fig. 5). Data was considered significant for $p < 0.05$.

For each dataset (initial phase or final phase of each experiment), we performed a Shapiro-Wilk test to verify the normality of our data with a threshold of $p < 0.05$, which confirmed the normal distribution (H_0 accepted for this threshold), except for the final phase of Exp 5. In order to apply the paired t-test that is designed for continuous datasets, we systematically compiled 3 successive sessions (30 trials) for each subject. For this reason, some of the DMTS data could not be used for statistical analysis. For example, the DMTS test in Exp 4 is not demonstrated as the others, because there is only one test session (10 trials).

The data of Exp 6, 7, and 8 were combined for analysis, since they consist in the same test conditions. The performance of SMTS in the Exp 7 and 8 decreased even after reaching the learning criterion, thus it was not clear how to define the final phase. In this particular case, we performed two analyses for the subjects which had reached the learning criterion at least once. For the first analysis, the final phase was defined as the last 3 sessions of the experiments for all the subjects (Fig. 5A and B). For the second analysis, if the subject reached the learning criterion, the data of the last 3 sessions before reaching the criterion (high performance phase) were used for the statistical analysis (Fig. 5C and D). If the subject did not reach criterion, it was defined as the last 3 sessions of the experiments as in the first analysis.

3. Results

3.1. Color discrimination (Exp 1 and 2)

We first performed a two-forced choice color discrimination task. Although a previous study has already shown that zebrafish are capable of performing color discrimination task with a set-up similar to the “Entering hallway” set-up [21], we verified whether our set-up is well-adapted for operant conditioning in zebrafish (Exp 1). We have confirmed that adult zebrafish can learn the visual discrimination task quickly. Table 1 shows the percentage of correct responses in each session of each subject. All the fish reached the correct response rate $\geq 70\%$ in 4 sessions, and the performance was quite stable, 9 out of 10 subjects reaching the learning criterion ($\geq 70\%$ for 3 continuous sessions) in 6 sessions. The mean correct response rate of the 10 subjects in each session is shown in Fig. 2A, demonstrating that the performance of the group improved through the training sessions. We compared the correct response rate of the first 3 sessions (initial phase; Ini) and the last 3 sessions (final phase; Fin), and confirmed that the performance is significantly higher after the training ($p < 0.01$, Fig. 2C).

Zebrafish are also capable of performing the color discrimination with a “Passing through the window” set-up (Exp 2). Although individual variation seemed larger, most of the subjects reached the criteria very quickly and kept a stable performance (Table 2, Fig. 2B). As the correct response rate was already high from the initial sessions, there was no significant different between the initial phase and final phase (Fig. 2D).

3.2. SMTS training followed by DMTS test with “Entering the hallway” response

3.2.1. SMTS training and DMTS test with a nutritious reinforcer (Exp 3)

In the beginning, we used Gemma 600 as a reinforcer. Table 3 shows the correct response rate in each session for each subject. The correct response of all animals was around a chance level (30-60%) in the first session, but most of them

achieved the learning criterion after 14 sessions (some fish have reached the criterion during the DMTS test). The mean correct response rate also demonstrates the general improvement of the group during the SMTS training (Fig. 3A). The paired t-test showed significant difference ($p < 10^{-5}$) in performance between the initial phase (Fig. 3D, Ini) and the final phase of the training (Fig. 3D, Fin).

The DMTS test was performed after the 14 sessions of SMTS training. We found that the high correct response rate was retained during the DMTS test sessions. The duration of the delay was 3 seconds for the first test. 7 out of 9 fish could achieve $\geq 70\%$ correct response. We then increased the duration of the delay, and the delay of 4 seconds was tested in the following session. Still 7 out of 9 fish could achieve $\geq 70\%$ correct response, thus we further tested the delay of 5 seconds. There were only 2 subjects that could reach $\geq 70\%$ correct response. When we reduced the delay (3 seconds) in the following session, all the subjects could achieve again $\geq 70\%$ correct response rate. The correct response rate obtained after SMTS training was maintained during the DMTS tests ($p < 0.01$; Fig. 3D, DMTS). We thus conclude that zebrafish could perform the DMTS, with the delay up to around 4 seconds.

Although all subjects could achieve 70% correct response rate, certain subjects had difficulty to retain the high performance continuously. We considered that this may be due to a lack of motivation (food saturation), thus we reduced the frequency of the experiments at later stages of the Exp 3: after the 6th session, there were at least 2 days of gaps between sessions. This improved the performance of many subjects. For this reason, the number of sessions does not correspond to the number of days. In the Tables showing MTS trainings, the numbers of days are indicated together with the number of sessions. The gap was sometimes long because of practical limitation. Interestingly, we found that zebrafish could retain the high correct response rate even

after 11 days of gap between 2 sessions (between the 8th and 9th sessions, Table 3). This result suggests that zebrafish can learn SMTS regardless of the irregular training schedule.

3.2.2. SMTS training and DMTS test with a new reinforcer (Exp 4)

Since we observed the food saturation in the Exp 3, we changed the reinforcer in this experiment. Dried mosquito larvae were chopped in small pieces, and one piece was provided at a time as a reinforcer.

Among the 9 subjects, 8 of them (except fish #10) had achieved the criterion at least once by the end of the 12th session (23 days) (Table 4). The high performance was relatively well retained: some individual (n°1) kept $\geq 70\%$ correct response for 8 continuous sessions (Table 4). The general high performance is also visible by the mean score (Fig. 3B).

After majority of the subjects reached the learning criterion, a DMTS test with 4 seconds was tested (Table 4, Fig. 3B). 5 subjects out of 9 reached $\geq 70\%$ correct response rate.

3.3. SMTS training with “Passing through the window” response (Exp 5)

We initially tried the “Passing through the window” set-up with a single food distributor (Fig. 1D). In the color discrimination task the same set-up (Fig. 1B) nicely worked and fish can learn the task quickly (Table 2, Fig. 2B). However, fish had difficulty for performing SMTS using this set-up. They could not achieve the criteria, and there were many sessions in which the fish did not even respond (data not shown). We thought that close spatial association of choice and reward may be important for

the fish, thus we further modified the set-up (Fig. 1E) and another experiment was performed using naive fish.

Nonetheless, even after the modification, all fish had difficulty for learning the task (Table 5, Fig. 3C). 8 out of 9 could perform $\geq 70\%$ for 2 consecutive days, but there were only 3 animals achieving for 3 consecutive days (Table 5). These animals could not retain the high performance in a stable manner. A paired t-test with the correct response rate of the last 3 sessions against that of the first 3 sessions did not yield a significant difference in performance (Fig. 3E).

DMTS (delay for 3 seconds) was tested only with one animal, and the correct response rate was 60% (Table 5, n°8 in the 22nd session). This animal went back to SMTS training in the following session, but it could not reach again the criterion.

3.4. Insertion of the delay in training sessions (Exp 6-8)

We tested whether zebrafish are capable of establishing the MTS even with a delay from the beginning of the training. Half of the subjects was trained with SMTS as the previous experiments (positive control), and the other half was trained with DMTS with the delay of 3 seconds. Because of the low performance using the “Passing through the window” response, we used the “Entering the hallway” response (mosquito larva as a reinforcer) for this experiment. As there are only 5 animals for each condition, we repeated the experiments 3 times (n = 15 for each group). The correct response rate of each subject in Exp 6-8 are shown in Tables 6-8, and progression of the mean score of each group is shown in Fig. 4A-F.

In contrast to the SMTS training, in which most subjects reach the criterion within 15 sessions (Tables 6-8 SMTS), there was a larger variation in the DMTS training groups. In Exp 6, 4 fish out of five reached the criterion within 15 sessions (Table 6,

Fig. 4A). However, in Exp 7 and Exp 8, most of them could reach $\geq 70\%$ correct response rate for 2 consecutive sessions, but not for 3 consecutive sessions (Tables 7 and 8). Nonetheless, when we tested the difference between initial phase and the last phase, we found a significant difference ($p < 0.01$; Fig. 5B and D).

As the DMTS training groups did not reach the criterion quickly, we continued the experiments for more than 25 sessions in Exp 7 and Exp 8. Zebrafish were not capable of retaining the high performance after a certain number of sessions. Even in the group of SMTS training, there was no fish which could retain a high correct response rate until the end of the experiment, when it exceeded 20 sessions. This has important implications for the use of zebrafish in operant conditioning tasks.

This is clearly demonstrated by the statistical analysis. When we compared the performance of the final phase (the last 3 sessions in the end of the experiments) with the initial phase, there was no significant improvement (Fig. 5A), although there is a significant improvement when we use the data of 3 sessions reaching the learning criterion, instead of the final phase (Fig. 5C).

To conclude, our results show that zebrafish can perform DMTS with at least 3-4 seconds of delay period. However, they could obtain a high behavioral performance only in a certain set-up. Also, zebrafish could keep a high performance only within a limited number of sessions.

4. Discussion

4.1. Establishment of operant conditioning tasks in adult zebrafish

Although there are some studies investigating spatial learning and memory in some teleosts including zebrafish [22–25], the presence of executive functions remains to be investigated. In this study, we developed an operant conditioning paradigm to

study teleost executive functions using adult zebrafish, which allows to compare the data in teleosts with those in mammals and birds.

We demonstrate that adult zebrafish is able to perform SMTS and DMTS. It has been shown that zebrafish is capable of performing color discrimination task using operant conditioning by Parker et al. (2012) [21], and we also confirmed that zebrafish can perform two-choice color discrimination tasks. The main difference with this previous study is the food delivery system. In Parker et al. (2012), a small amount of water containing artemia (around 10 μ l) was delivered by turning a screw. In our study we delivered solid food, so that we could confirm the reward was completely consumed by the fish before starting the next trial.

After comparing the two different solid foods, Gemma Micro 600 (Exp 3) and dried mosquito larva (Exp 4), we decided to use the dried larva as a reinforcer, because the latter seems to maintain the motivation for food better (less nutritious). The Exp 3 required longer training (39 days) because we could not perform the experiment every day due to the saturation with the food. However, the long-lasting training experiment with a long interval between sessions have revealed an interesting point: the task performance did not decline much despite pauses lasting days. This suggests that the adult zebrafish are capable of retaining the rule at least for several days.

By contrast, our experiments using two different types of set-up (“Entering the hallway” and “Passing through the window”) revealed that zebrafish are not flexible enough to adapt their performance to different set-ups. The interesting point is that they could perform the “Passing through the window” response in the color discrimination task, but not in the MTS. It is probably because the cognitive attention required for MTS is much heavier than that for a simple visual discrimination task. Based on the behavioral observation, passing through the window by itself may require

considerable attention for zebrafish. Thus they can perform correctly when the task is simple (color-food one to one association), but may be distracted when the task requires additional attention.

We also show that zebrafish may not be able to perform a task which requires long training sessions (more than 20 sessions). The decline of the performance could be because of lack of long-term attention and/or motivation, or stress from the long isolation. In any case, correct choice of the task is important for a good use of zebrafish in behavioral studies.

4.2. Working memory in zebrafish

The ability to perform DMTS shows the presence of working memory in zebrafish. Working memory is a kind of active memory, holding ongoing information of any modality online. In this aspect, it can be considered as a kind of short-term memory, but the important point is that working memory involves the ability to manipulate the information according to the contextual needs of the moment [17,26,27]. This internal maintenance of goal states is necessary for goal-directed behaviors.

In pigeons, such internal maintenance has been directly demonstrated by the presence of neurons activated during the delay period of DMTS in NCL (working memory neurons; [11,28]). The authors could nicely correlated the working memory task with brain activity, since the behavioral paradigm is programmed like Russian “Matryoshka”, in which cognitive subcomponents increases in a stepwise manner [13]. The only difference between the SMTS and DMTS is the presence of the delay period. Thus we can consider that cognitive components required for performing DMTS consists of those required for SMTS plus working memory.

Based on behavioral observation of certain species, it is not surprising that teleosts possess working memory. For example, goal-directed behavior such as tool use observed in the sixbar wrasse [19] should require working memory. Nonetheless the presence of working memory has never been demonstrated experimentally. Our study showing that adult zebrafish can perform the DMTS task strongly supports the presence of working memory in teleosts. Adult zebrafish can keep the memory at least for 3-4 seconds. This is similar to the result performed in honey bee [29]. In our experiments, it seemed difficult for zebrafish to keep the information in working memory for more than 5 seconds. However, our experiments were not aiming to assess the maximal timespan of zebrafish working memory and additional experiments would be needed to determine it.

In this study, we tried two types training procedures. 1) Animals are trained with SMTS and then tested with a trial inserted a delay period. 2) Training with delay of 3 seconds from the start. In pigeon, it has been shown that training with delays from the start, results in rather slow acquisition [30]. In our data in zebrafish also, training with delays from the start was obviously more difficult (Exp 7 and 8).

Our results support the presence of working memory in the adult zebrafish, but zebrafish could perform DMTS only under certain conditions. This brings a difficulty for performing behavioral experiments combining with electrophysiological or imaging set-ups, which would be required to identify working memory neurons *in vivo*. For now, imaging techniques on freely moving animals can be applied only in zebrafish larvae, but our preliminary data suggest that the executive functions are not yet developed at this stage. On the other hand, adult zebrafish is too small to perform the classical brain lesion. Indeed, we have tried lesion study in zebrafish, but could not obtain stable

behavioral data after the brain lesion. Further studies and practical considerations are required to pursue the anatomical investigation of the executive areas in teleosts.

4.3. Convergent evolution of the executive functions

Working memory is one of the well-defined cognitive components of executive functions. Our current study together with a previous study showing that zebrafish are capable of performing reversal learning test, it is likely that zebrafish possess at least a primordium of the executive area. The next step would be to identify it.

In birds, based on combination of behavioral, anatomical, pharmacological, and electrophysiological studies, Güntürkün and his colleagues have proposed that NCL is a functional equivalent to the mammalian PFC (reviewed in [14,17]). Although mammalian PFC and avian NCL are functionally similar, they are not considered to be homologous as an executive pallium because of the topological location within the pallium (PFC at the anterior end while NCL at the posterior end of the pallium). Thus the similar functions are considered to have evolved independently in two lineages.

Güntürkün argues that recruitment of dopamine (DA) neurotransmission in the NCL may have been a critical factor for evolving the functional properties similar to the mammalian PFC [17]. Indeed, to perform the DMTS, DA innervation from midbrain DA cells (corresponding A10) to the executive pallium is necessary both in mammals and birds. Nonetheless, there is a significant difference between the DA systems of birds and mammals. For example, D1-family DA receptors play important roles in DA neurotransmission, but the DA receptor composition is different between mammals and birds. The avian pallium express additional DA receptor subtypes that had secondary lost in mammals (previously called D1D and newly proposed to rename as D6; reviewed in [31]). Thus it seems that DA neurotransmission is similarly important,

but detailed mechanisms on how the executive pallia work may be different between mammals and birds [20].

Teleosts are phylogenetically distant from mammals, and the teleost executive area, if any, is unlikely to be homologous to the mammalian PFC. Our comparative studies of DA systems suggest that the teleost DA system is very different from the amniote DA systems, more specifically this difference is extreme when compared with mammals [20,31–33]. For example, teleosts have much more DA receptor subtypes than other vertebrates, with 14 DA receptor genes in case of zebrafish, while only 5 in mammals [31,34]. In addition, teleosts do not have DA cell in the mesencephalon. Thus it is likely that non-homologous DA cells play an equivalent role to the amniote A9/A10 cells. Finding the DA cells involved in the working memory task would help to identify the anatomical requirements playing the same role as A9/A10 cells.

Acknowledgements

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Figure legends

Table 1. Correct response rate (%) of each animal through the color discrimination learning in Exp 1. The sessions with $\geq 70\%$ correct response rate are indicated in italic. The level of the performance retention is indicated with light or dark greys: light grey indicates achieving $\geq 70\%$ for 2 continuous sessions, and dark grey indicates $\geq 70\%$ for 3 or more continuous sessions. The “-” indicates that the subject did not respond more than half of the trials.

Table 2. Correct response rate (%) of each animal through the color discrimination learning in Exp 2. The sessions with $\geq 70\%$ correct response rate are indicated in italic.

The level of the performance retention is indicated with light or dark greys: light grey indicates achieving $\geq 70\%$ for 2 continuous sessions, and dark grey indicates $\geq 70\%$ for 3 or more continuous sessions. The “-” indicates that the subject did not respond more than half of the trials.

Table 3. Correct response rate (%) of each animal through SMTS training sessions and DMTS test sessions in Exp 3. The sessions with $\geq 70\%$ correct response rate are indicated in italic. The level of the performance retention is indicated with light or dark greys: light grey indicates achieving $\geq 70\%$ for 2 continuous sessions, and dark grey indicates $\geq 70\%$ for 3 or more continuous sessions. The DMTS test sessions are indicated in bold. Double lines indicate more than 2-days gap between the sessions.

Table 4. Correct response rate (%) of each animal through SMTS training sessions and DMTS test sessions in Exp 4. The sessions with $\geq 70\%$ correct response rate are indicated in italic. The level of the performance retention is indicated with light or dark greys: light grey indicates achieving $\geq 70\%$ for 2 continuous sessions, and dark grey indicates $\geq 70\%$ for 3 or more continuous sessions. The DMTS test sessions are indicated in bold. Double lines indicate more than 2-days gap between the sessions.

Table 5. Correct response rate (%) of each animal through SMTS training sessions in Exp 5. The sessions with $\geq 70\%$ correct response rate are indicated in italic. The level of the performance retention is indicated with light or dark greys: light grey indicates achieving $\geq 70\%$ for 2 continuous sessions, and dark grey indicates $\geq 70\%$ for 3 or more continuous sessions. There is only one DMTS test session that is indicated in

bold with “d3)” (delay for 3 seconds). Double lines indicate more than 2-days gap between the sessions.

Table 6. Correct response rate (%) of SMTS training animals and DMTS training animals in Exp 6. The sessions with $\geq 70\%$ correct response rate are indicated in italic. The level of the performance retention is indicated with light or dark greys: light grey indicates achieving $\geq 70\%$ for 2 continuous sessions, and dark grey indicates $\geq 70\%$ for 3 or more continuous sessions. The DMTS test sessions are indicated in bold. Double lines indicate more than 2-days gap between the sessions.

Table 7. Correct response rate (%) of SMTS training animals and DMTS training animals in Exp 7. The sessions with $\geq 70\%$ correct response rate are indicated in italic. The level of the performance retention is indicated with light or dark greys: light grey indicates achieving $\geq 70\%$ for 2 continuous sessions, and dark grey indicates $\geq 70\%$ for 3 or more continuous sessions. The DMTS sessions are indicated in bold. For test sessions (with a delay period) in the SMTS training animals, the duration of the delay is indicated: d4) = delay for 4 seconds, and d5) = delay for 5 seconds. Double lines indicate more than 2-days gap between the sessions.

Table 8. Correct response rate (%) of SMTS training animals and DMTS training animals in Exp 8. The sessions with $\geq 70\%$ correct response rate are indicated in italic. The level of the performance retention is indicated with light or dark greys: light grey indicates achieving $\geq 70\%$ for 2 continuous sessions, and dark grey indicates $\geq 70\%$ for 3 or more continuous sessions. The DMTS sessions are indicated in bold. For test sessions (with a delay period) in the SMTS training animals, the duration of the delay

is indicated: d3) = delay for 3 seconds, d4) = delay for 4 seconds, and d5) = delay for 5 seconds. The same indication is used in case a delay period more than 3 seconds was tested in the DMTS training group. Double lines indicate more than 2-days gap between the sessions.

Fig. 1. Schematic drawings of operant boxes used in this study. (A and B) Set-ups used for color discrimination tasks: “Entering the hallway” set-up (A) and “Passing through the window” set-up (B). (C, D, E) Set-ups used for matching-to-sample tasks: “Entering the hallway” set-up (C), “Passing through the window” set-up with a single food distributor (D), and “Passing through the window” set-up with two food distributors (E).

Fig. 2. Progress of the correct response rate in color discrimination tasks. (A and B) Session by session progression of mean percentage of correct responses of the group in Exp 1 (A) in Exp 2 (B). Vertical bars show the standard deviation. The horizontal dotted line demonstrates the 70% correct response rate. (C and D) Comparison of the correct response rate of the initial phase (Ini) and the final phase (Fin). $**p < 0.01$ in Exp 1 (D), not significant (NS) in Exp 2 (D).

Fig. 3. Progress of the correct response rate in SMTS trainings and DMTS tests. (A-C) Session by session progression of mean percentage of correct responses of the group in Exp 3 (A), Exp 4 (B), and Exp 5 (C). Vertical bars show the standard deviation. The horizontal dotted line demonstrates the 70% correct response rate. SMTS trainings are demonstrated with lines while DMTS test sessions are demonstrated with separate dots. (D) Comparison of the correct response rate of the initial phase (Ini) and

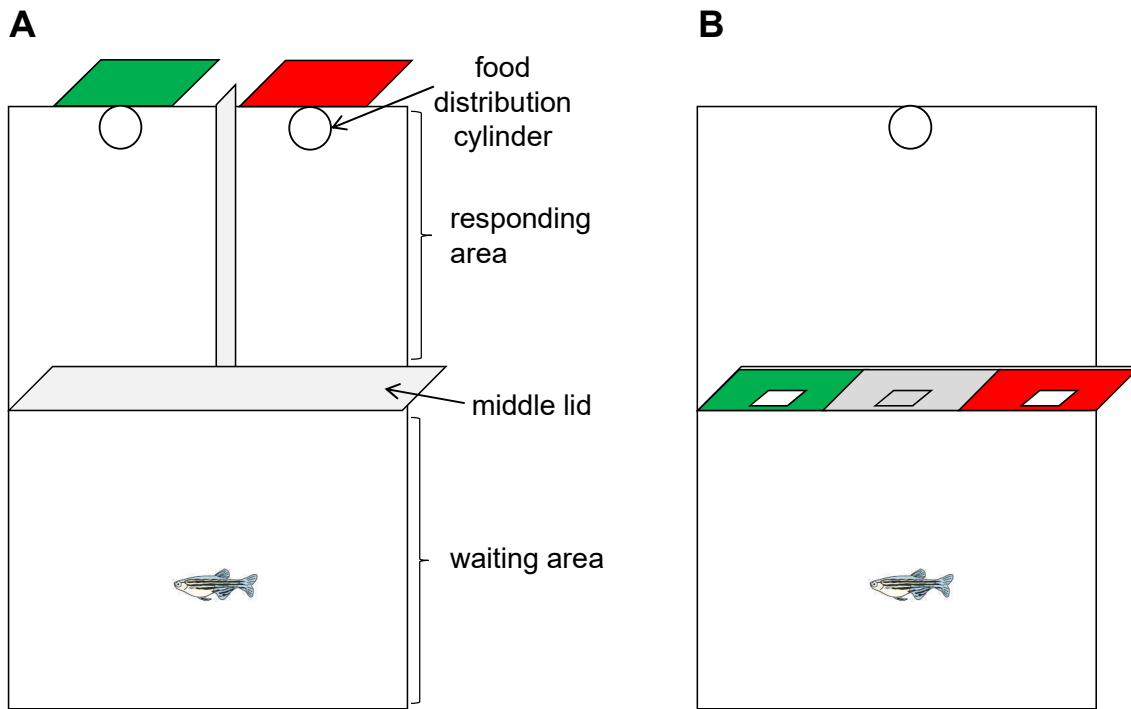
the final phase (Fin) of SMTS training sessions, as well as the first 3 sessions of the DMTS test in Exp 3. $**p < 0.01$, $***p < 10^{-4}$. (E) Comparison of the correct response rate of the initial phase (Ini) and the final phase (Fin) of the SMTS training in Exp 5. There is no significant difference (*NS*).

Fig. 4. Session by session progression of mean percentage of correct responses of the group in Exp 6 (A and B), Exp 7 (C and D), and Exp 8 (E and F). SMTS training groups (A, C, E) and DMTS training groups (B, D, F) are shown separately. Vertical bars show the standard deviation. The horizontal dotted line demonstrates the 70% correct response rate.

Fig. 5. (A and B) Comparison of the correct response rate of the initial phase (Ini) and the final phase (Fin) in Exp 6-8. SMTS training groups (A) and DMTS training groups (B) are shown separately. Not significant (*NS*) in SMTS (A), $**p < 0.01$ in DMTS (B). (C and D) Comparison of the correct response rate of the initial phase (Ini) and the last 3 sessions before reaching the criterion (high performance phase; High) in Exp 6-8. SMTS training groups (C) and DMTS training groups (D) are shown separately. $***p < 10^{-4}$ in SMTS (C), $**p < 0.01$ in DMTS (D).

Figure 1

color discrimination



SMTS/DMTS

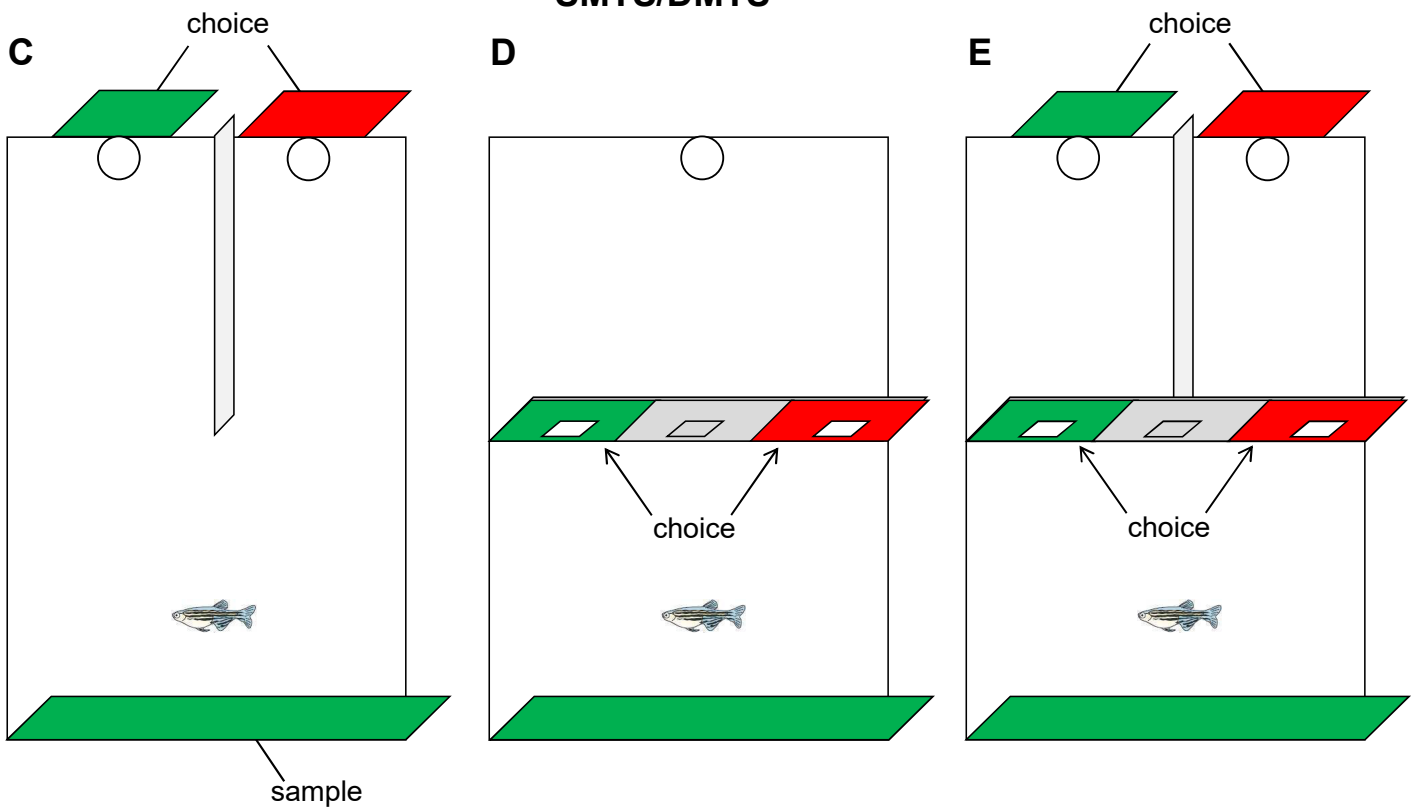


Figure 2

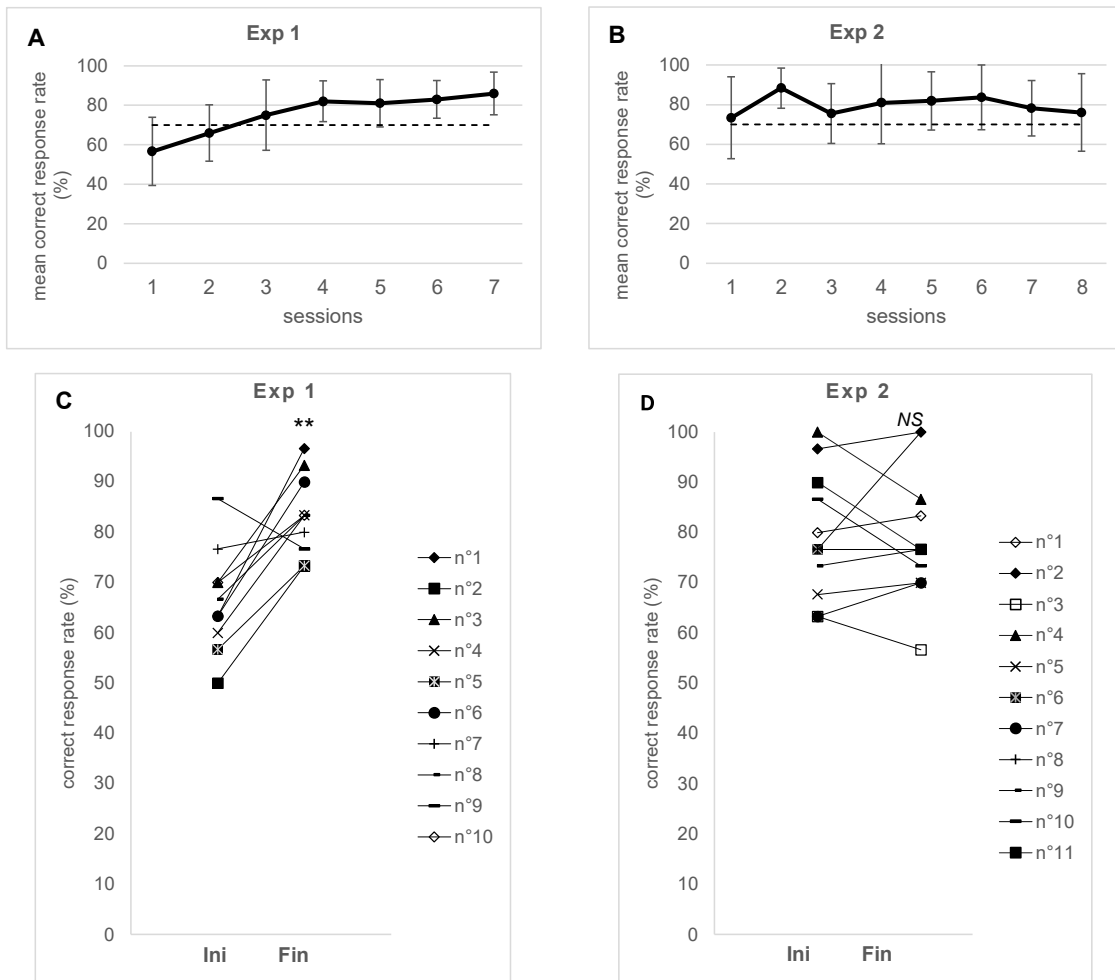


Figure 3

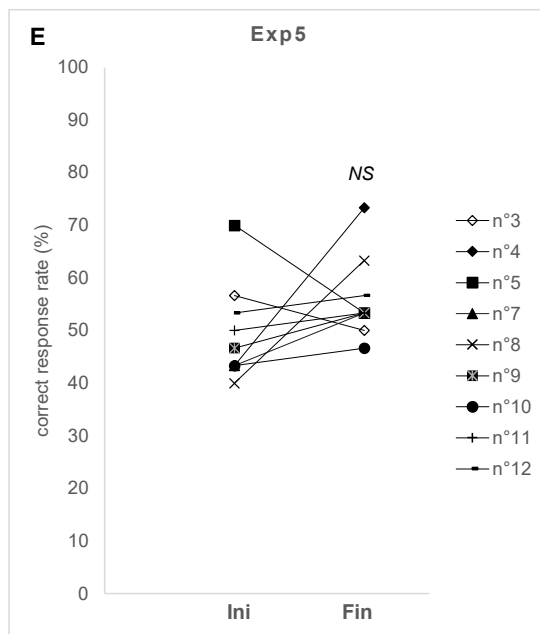
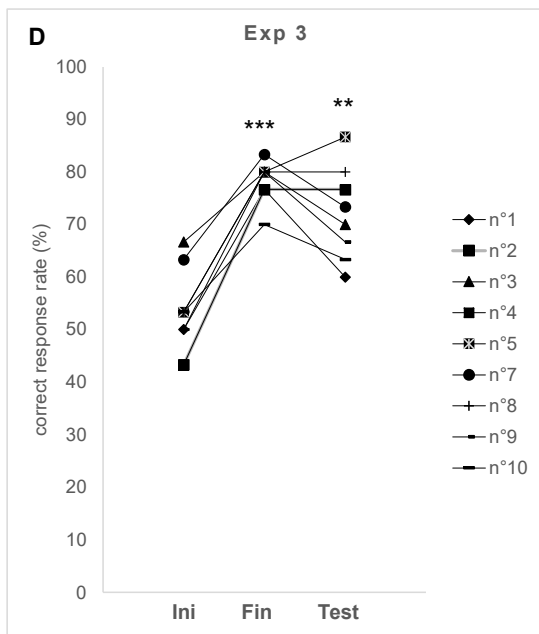
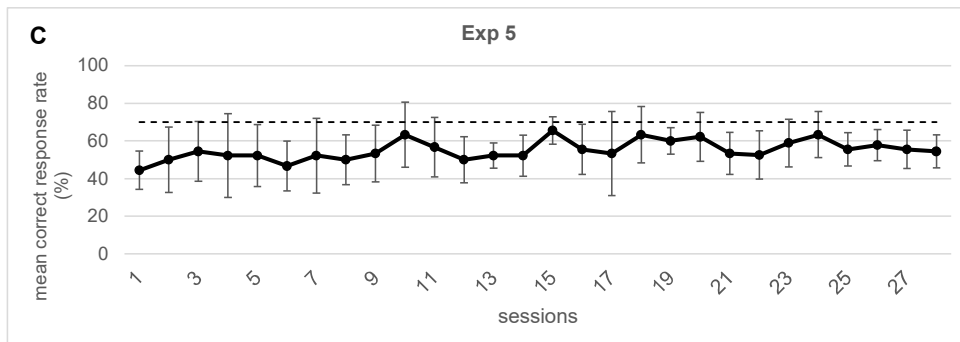
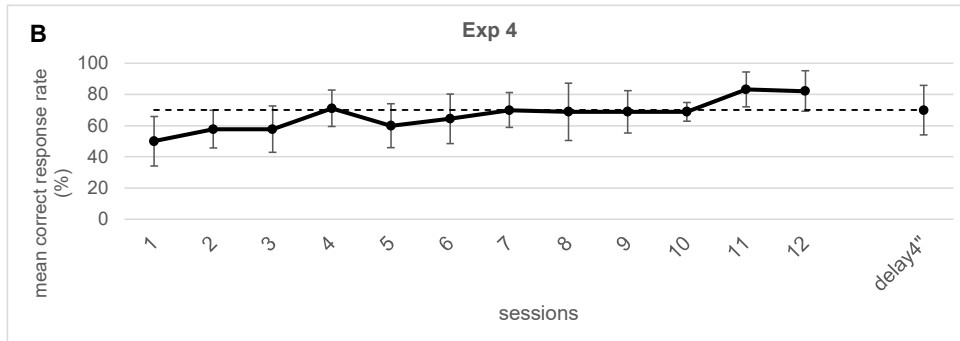
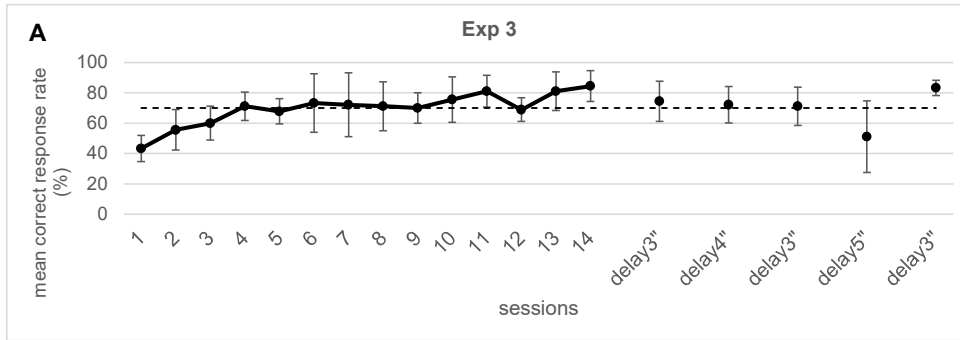


Figure 4

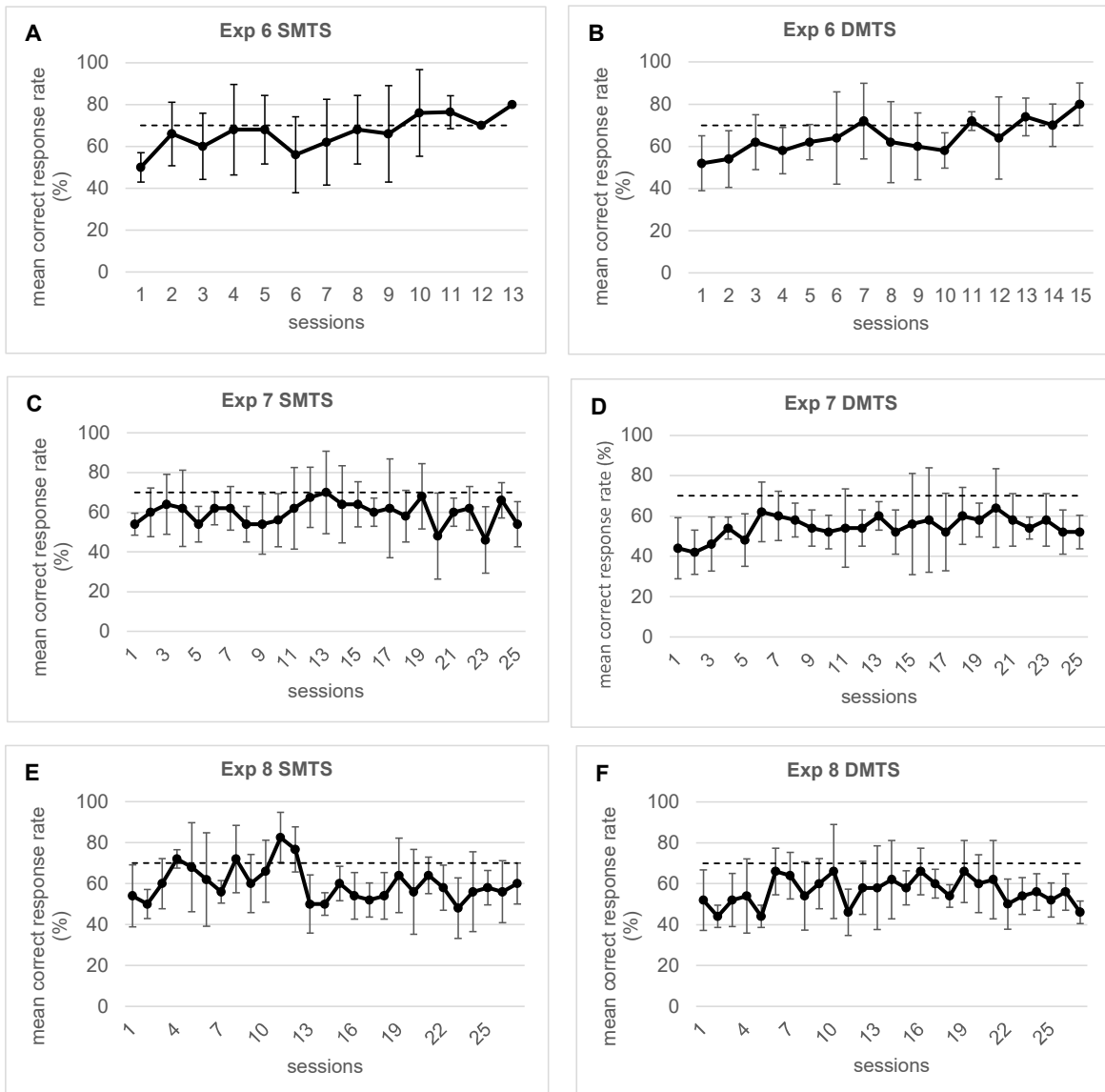


Figure 5

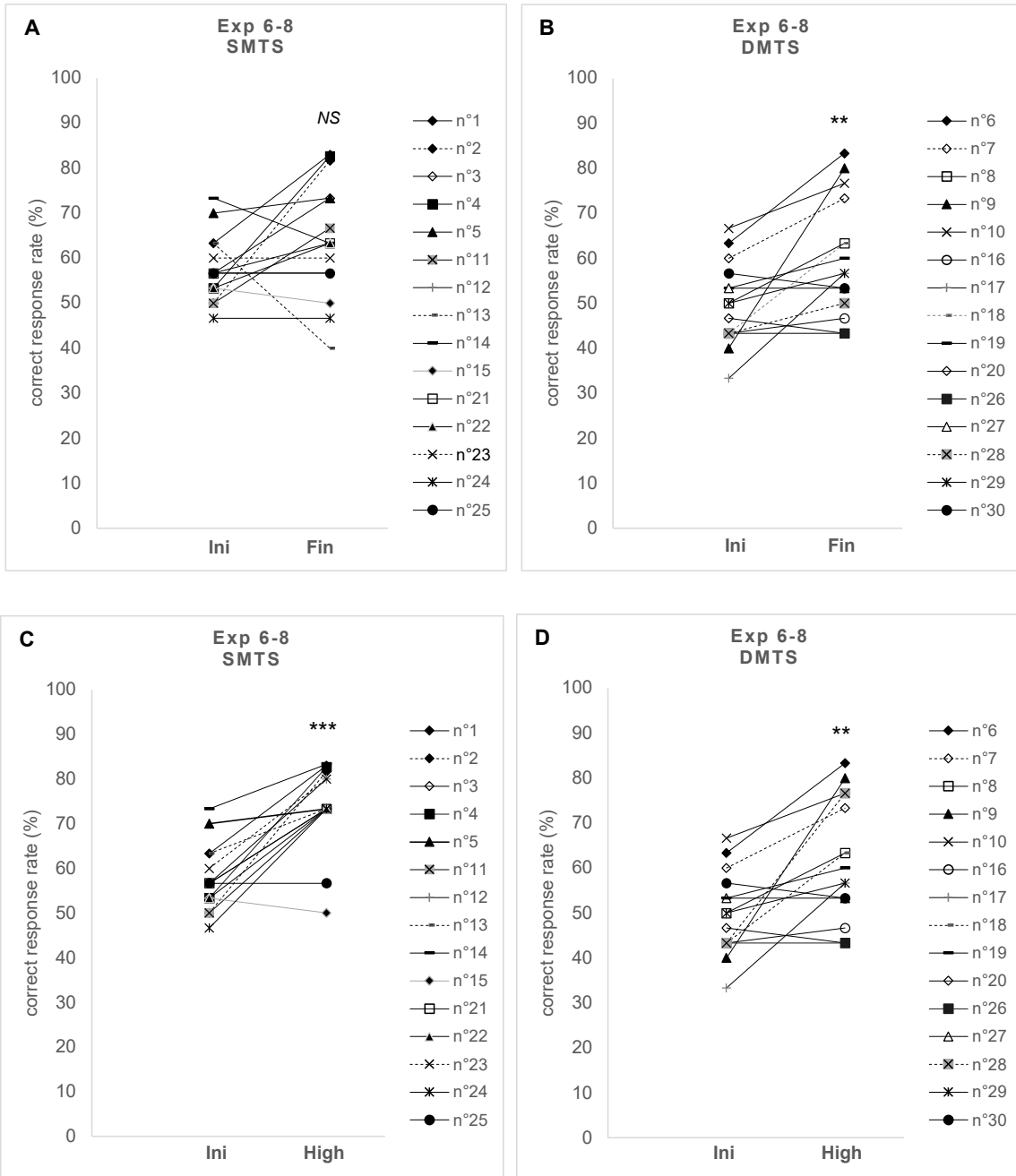


Table 1. Correct response rate (%) of each animal through the color discrimination learning in Exp 1.

sessions	Percentage (%) of correct responses										mean
	n°1	n°2	n°3	n°4	n°5	n°6	n°7	n°8	n°9	n°10	
1	50	50	40	-	50	60	50	60	<i>100</i>	50	56.7
2	70	60	70	50	50	50	80	60	90	80	66.0
3	70	40	100	60	70	80	100	80	70	80	75.0
4	100	80	90	70	80	70	80	70	90	90	82.0
5	100	60	90	90	80	90	80	70	70	80	81.0
6	100	80	90	80	70	80	70	90	90	80	83.0
7	90	80	100	80	70	100	90	90	70	90	86.0

italic sessions with $\geq 70\%$ correct response rate
light grey $\geq 70\%$ for 2 continuous sessions
dark grey $\geq 70\%$ for 3 or more continuous sessions
– not responding more than half of the trials

Table 2. Correct response rate (%) of each animal through the color discrimination learning in Exp 2.

sessions	Percentage (%) of correct responses											mean
	n°1	n°2	n°3	n°4	n°5	n°6	n°7	n°8	n°9	n°10	n°11	
1	80	100	60	100	60	60	40	-	70	-	90	73.3
2	70	100	80	100	83	90	80	-	90	90	100	88.3
3	90	90	50	100	60	80	70	70	60	80	80	75.5
4	100	100	40	100	60	70	60	80	90	90	100	80.9
5	100	100	90	100	70	70	90	80	60	70	70	81.8
6	80	100	50	100	70	80	100	100	70	80	90	83.6
7	90	100	80	80	70	60	60	100	80	70	70	78.2
8	80	100	40	80	70	90	50	100	80	-	70	76.0

italic sessions with $\geq 70\%$ correct response rate
 light grey $\geq 70\%$ for 2 continuous sessions
 dark grey $\geq 70\%$ for 3 or more continuous sessions
 - not responding more than half of the trials

Table 3. Correct response rate (%) of each animal through SMTS training sessions and DMTS test sessions in Exp 3.

sessions	days	Percentage (%) of correct responses									
		n°1	n°2	n°3	n°4	n°5	n°7	n°8	n°9	n°10	mean
1	1	40	30	50	40	40	60	40	50	40	43.3
2	2	60	40	70	30	60	70	50	60	60	55.6
3	3	50	60	80	60	60	60	70	40	60	60.0
4	4	70	80	60	80	80	60	80	70	60	71.1
5	5	80	60	60	60	60	70	80	70	70	67.8
6	8	80	40	70	60	80	60	70	100	100	73.3
7	10	60	60	90	70	40	90	90	50	100	72.2
8	12	80	70	60	50	60	100	70	90	60	71.1
9	23	60	60	80	80	80	80	60	70	60	70.0
10	25	70	90	60	50	80	70	80	100	80	75.6
11	29	60	80	90	80	70	90	90	80	90	81.1
12	32	80	60	70	80	70	70	60	70	60	68.9
13	36	60	90	70	80	100	90	90	80	70	81.1
14	39	90	80	100	70	70	90	90	90	80	84.4
delay3"	43	50	80	60	90	80	80	90	70	70	74.4
delay4"	46	50	70	70	80	90	70	80	80	60	72.2
delay3"	52	80	80	80	60	90	70	70	50	60	71.1
delay5"	59	30	60	70	40	100	60	30	30	40	51.1
delay3"	61	90	80	90	80	80	80	80	90	80	83.3

italic sessions with $\geq 70\%$ correct response rate
light grey $\geq 70\%$ for 2 continuous sessions
dark grey $\geq 70\%$ for 3 or more continuous sessions
– not responding more than half of the trials
bold DMTS test sessions
double line more than 2-days gap between the sessions

Table 4. Correct response rate (%) of each animal through SMTS training sessions and DMTS test sessions in Exp 4.

sessions	days	Percentage (%) of correct reponses									
		n°1	n°2	n°3	n°4	n°5	n°6	n°7	n°8	n°10	mean
1	1	20	50	50	40	60	<i>70</i>	40	<i>70</i>	50	50.0
2	2	50	40	50	80	60	<i>70</i>	60	50	60	57.8
3	3	60	40	50	50	60	<i>70</i>	50	<i>90</i>	50	57.8
4	4	60	60	<i>80</i>	<i>90</i>	70	60	<i>80</i>	<i>80</i>	60	71.1
5	5	<i>70</i>	<i>70</i>	50	60	<i>70</i>	<i>70</i>	<i>70</i>	30	50	60.0
6	8	<i>70</i>	50	60	70	<i>80</i>	<i>80</i>	<i>70</i>	30	70	64.4
7	9	<i>70</i>	70	70	<i>80</i>	50	60	<i>70</i>	70	<i>90</i>	70.0
8	11	<i>80</i>	<i>90</i>	<i>80</i>	<i>70</i>	40	70	50	<i>90</i>	50	68.9
9	12	<i>70</i>	<i>80</i>	60	<i>70</i>	50	<i>90</i>	<i>70</i>	<i>80</i>	50	68.9
10	16	<i>70</i>	<i>70</i>	<i>80</i>	<i>70</i>	60	<i>70</i>	<i>70</i>	<i>70</i>	60	68.9
11	17	<i>70</i>	<i>70</i>	<i>100</i>	<i>80</i>	<i>80</i>	<i>80</i>	<i>90</i>	<i>100</i>	<i>80</i>	83.3
12	23	<i>90</i>	<i>70</i>	<i>90</i>	<i>80</i>	<i>100</i>	<i>90</i>	<i>70</i>	<i>90</i>	60	82.2
delay4"	24	<i>70</i>	50	<i>90</i>	50	60	<i>90</i>	<i>80</i>	<i>80</i>	60	70.0

italic sessions with $\geq 70\%$ correct response rate
light grey $\geq 70\%$ for 2 continuous sessions
dark grey $\geq 70\%$ for 3 or more continuous sessions
– not responding more than half of the trials
bold DMTS test session
double line more than 2-days gap between the sessions

Table 5. Correct response rate (%) of each animal through SMTS training sessions in Exp 5.

sessions	days	Percentage (%) of correct reponses									
		n°3	n°4	n°5	n°7	n°8	n°9	n°10	n°11	n°12	mean
1	1	50	50	60	50	30	50	30	40	40	44.4
2	2	50	30	80	50	30	60	40	40	70	50.0
3	3	70	50	70	30	60	30	60	70	50	54.4
4	5	50	50	20	40	50	90	30	80	60	52.2
5	15	70	30	70	50	50	30	70	40	60	52.2
6	16	40	50	40	50	60	60	60	40	20	46.7
7	18	30	80	40	70	50	20	50	60	70	52.2
8	19	50	40	50	80	40	60	40	40	50	50.0
9	22	60	50	20	60	60	60	60	70	40	53.3
10	23	60	90	60	60	90	50	40	70	50	63.3
11	25	70	60	40	70	60	70	30	40	70	56.7
12	30	50	50	30	60	40	40	70	50	60	50.0
13	37	50	60	40	60	50	60	50	50	50	52.2
14	38	30	60	40	60	60	60	60	50	50	52.2
15	39	70	70	50	70	70	60	70	70	60	65.6
16	40	50	60	30	60	60	50	60	80	50	55.6
17	43	10	70	50	80	50	30	70	70	50	53.3
18	44	80	80	60	60	60	60	70	70	30	63.3
19	45	50	60	70	60	70	60	60	60	50	60.0
20	47	60	70	70	80	70	50	40	50	70	62.2
21	51	50	40	60	40	70	50	70	50	50	53.3
22	52	70	50	60	50	d3)60	70	40	40	40	52.5
23	53	50	70	40	70	60	80	60	50	50	58.9
24	54	80	50	50	60	70	50	80	60	70	63.3
25	58	40	60	60	50	60	50	50	60	70	55.6
26	59	50	70	60	50	70	60	50	50	60	57.8
27	65	50	80	50	50	60	50	50	60	50	55.6
28	66	50	70	50	60	60	50	40	50	60	54.4

italic sessions with $\geq 70\%$ correct response rate
light grey $\geq 70\%$ for 2 continuous sessions
dark grey $\geq 70\%$ for 3 or more continuous sessions
– not responding more than half of the trials
bold DMTS test session (**d3**: delay for 3 seconds)
double line more than 2-days gap between the sessions

Table 6. Correct response rate (%) of SMTS training animals and DMTS training animals in Exp 6.

sessions	days	Percentage (%) of correct reponses											
		SMTS						DMTS					
		n°1	n°2	n°3	n°4	n°5	mean	n°6	n°7	n°8	n°9	n°10	mean
1	1	50	50	50	40	60	50.0	60	60	60	30	50	52.0
2	2	<i>80</i>	50	50	<i>80</i>	<i>70</i>	66.0	60	60	40	40	70	54.0
3	3	60	50	<i>70</i>	40	<i>80</i>	60.0	70	60	50	50	<i>80</i>	62.0
4	4	<i>70</i>	60	<i>70</i>	40	<i>100</i>	68.0	50	50	70	50	<i>70</i>	58.0
5	7	<i>80</i>	<i>70</i>	<i>80</i>	<i>70</i>	40	68.0	70	50	60	60	<i>70</i>	62.0
6	8	60	60	30	50	<i>80</i>	56.0	90	30	70	60	<i>70</i>	64.0
7	9	40	<i>70</i>	<i>80</i>	<i>80</i>	40	62.0	90	50	60	90	<i>70</i>	72.0
8	10	<i>90</i>	50	60	<i>80</i>	60	68.0	60	40	90	50	<i>70</i>	62.0
9	15	<i>70</i>	<i>90</i>	60	<i>80</i>	30	66.0	80	60	<i>70</i>	40	50	60.0
10	16	<i>90</i>	<i>80</i>	<i>80</i>	<i>90</i>	40	76.0	50	70	60	50	60	58.0
11	17	<i>89</i>	<i>75</i>	<i>70</i>	<i>78</i>	<i>70</i>	76.4	80	<i>70</i>	<i>70</i>	<i>70</i>	<i>70</i>	72.0
12	18			<i>70</i>		<i>70</i>		50	50	50	90	80	64.0
13	21					<i>80</i>		80	70	60	80	80	74.0
14	22							80	70	60			
15	23							90	80	70			

italic sessions with $\geq 70\%$ correct response rate
light grey $\geq 70\%$ for 2 continuous sessions
dark grey $\geq 70\%$ for 3 or more continuous sessions
– not responding more than half of the trials
bold DMTS sessions
double line more than 2-days gap between the sessions

Table 7. Correct response rate (%) of SMTS training animals and DMTS training animals in Exp 7.

sessions	days	Percentage (%) of correct responses											
		SMTS						DMTS					
		n°11	n°12	n°13	n°14	n°15	mean	n°16	n°17	n°18	n°19	n°20	mean
1	1	50	60	50	60	50	54.0	40	30	30	60	60	44.0
2	2	50	60	60	<i>80</i>	50	60.0	30	40	60	40	40	42.0
3	3	50	50	<i>80</i>	<i>80</i>	60	64.0	60	30	40	60	40	46.0
4	4	<i>70</i>	40	50	<i>90</i>	60	62.0	50	60	50	60	50	54.0
5	7	60	40	50	60	60	54.0	40	30	60	60	50	48.0
6	8	50	<i>70</i>	<i>70</i>	60	60	62.0	60	<i>70</i>	40	60	<i>80</i>	62.0
7	9	50	50	<i>70</i>	<i>70</i>	<i>70</i>	62.0	60	<i>70</i>	40	<i>70</i>	60	60.0
8	10	50	60	40	60	60	54.0	50	60	50	<i>70</i>	60	58.0
9	11	<i>70</i>	50	40	<i>70</i>	40	54.0	<i>70</i>	50	50	50	50	54.0
10	15	<i>70</i>	50	<i>70</i>	40	50	56.0	60	60	50	40	50	52.0
11	16	<i>80</i>	<i>70</i>	<i>80</i>	40	40	62.0	40	<i>80</i>	60	30	60	54.0
12	17	d5)60	60	<i>70</i>	50	<i>90</i>	67.5	40	50	60	60	60	54.0
13	18	d4)60	<i>90</i>	d5)50	40	<i>80</i>	<i>70.0</i>	50	<i>70</i>	60	60	60	60.0
14	21	50	<i>80</i>	50	<i>90</i>	50	64.0	50	50	<i>70</i>	40	50	52.0
15	22	60	<i>70</i>	<i>80</i>	50	60	64.0	<i>90</i>	60	60	20	50	56.0
16	24	60	d5)60	50	60	<i>70</i>	60.0	<i>90</i>	50	40	<i>80</i>	30	58.0
17	25	<i>80</i>	<i>80</i>	40	<i>80</i>	30	62.0	60	<i>80</i>	30	40	50	52.0
18	28	50	50	60	50	<i>80</i>	58.0	50	<i>80</i>	50	<i>70</i>	50	60.0
19	29	<i>80</i>	50	<i>90</i>	60	60	68.0	50	60	60	<i>70</i>	50	58.0
20	30	60	60	20	30	<i>70</i>	48.0	50	50	<i>90</i>	50	<i>80</i>	64.0
23	31	<i>70</i>	50	60	60	60	60.0	60	50	<i>70</i>	40	<i>70</i>	58.0
22	32	60	60	60	50	<i>80</i>	62.0	50	60	50	50	60	54.0
23	35	60	50	20	60	40	46.0	50	60	<i>70</i>	<i>70</i>	40	58.0
24	36	<i>80</i>	<i>70</i>	60	60	60	66.0	40	50	<i>70</i>	50	50	52.0
25	37	60	50	40	<i>70</i>	50	54.0	50	60	50	60	40	52.0

italic sessions with $\geq 70\%$ correct response rate
light grey $\geq 70\%$ for 2 continuous sessions
dark grey $\geq 70\%$ for 3 or more continuous sessions
– not responding more than half of the trials
bold DMTS sessions (**d4**, **d5**: delay for 4 and 5 seconds respectively)
double line more than 2-days gap between the sessions

Table 8. Correct response rate (%) of SMTS training animals and DMTS training animals in Exp 8.

sessions	days	Percentage (%) of correct reponses											
		SMTS						DMTS					
		n°21	n°22	n°23	n°24	n°25	mean	n°26	n°27	n°28	n°29	n°30	mean
1	1	50	70	60	30	60	54.0	50	70	30	50	60	52.0
2	2	50	50	60	50	40	50.0	50	40	40	40	50	44.0
3	3	70	40	60	60	70	60.0	30	50	60	60	60	52.0
4	13	70	70	70	80	70	72.0	70	40	30	70	60	54.0
5	20	60	80	50	100	50	68.0	40	50	40	40	50	44.0
6	21	60	80	90	40	40	62.0	70	60	80	70	50	66.0
7	22	60	50	60	50	60	56.0	60	80	60	70	50	64.0
8	24	80	50	90	60	80	72.0	60	40	80	40	50	54.0
9	27	70	50	40	70	70	60.0	70	60	70	40	60	60.0
10	28	70	70	80	70	40	66.0	50	90	60	40	90	66.0
11	29	d3)70	80	70	80	100	82.5	50	40	50	60	30	46.0
12	30	d4)60	70	90	d4)70	70	76.7	70	40	60	70	50	58.0
13	31	d3)70	d4)40	d4)70	d5)70	50	50.0	60	40	90	40	60	58.0
14	34	d4)50	d3)50	d4)60	d5)60	50	50.0	90	50	40	70	60	62.0
15	35	d3)50	60	d3)50	d4)40	60	60.0	60	60	50	70	50	58.0
16	41	70	40	50	60	50	54.0	70	70	80	60	50	66.0
17	42	60	40	60	50	50	52.0	60	60	70	50	60	60.0
18	43	50	50	70	60	40	54.0	60	50	50	50	60	54.0
19	44	60	40	60	70	90	64.0	80	70	70	40	70	66.0
20	48	90	40	60	50	40	56.0	70	50	70	70	40	60.0
21	49	60	80	60	60	60	64.0	50	60	d5)90	40	70	55.0
22	50	40	70	60	60	60	58.0	60	60	50	50	30	50.0
23	51	70	40	30	50	50	48.0	50	60	60	60	40	54.0
24	52	50	90	40	50	50	56.0	60	50	50	50	70	56.0
25	55	70	60	60	50	50	58.0	40	50	50	60	60	52.0
26	56	50	80	60	40	50	56.0	50	70	50	60	50	56.0
27	57	70	50	60	50	70	60.0	40	40	50	50	50	46.0

italic sessions with $\geq 70\%$ correct response rate
light grey $\geq 70\%$ for 2 continuous sessions
dark grey $\geq 70\%$ for 3 or more continuous sessions
– not responding more than half of the trials
bold DMTS sessions (**d3**, **d4**, **d5**: delay for 3, 4, 5 seconds respectively)
double line more than 2-days gap between the sessions

General discussion and perspectives

The initial aim of my PhD work was to investigate the prerequisites for higher order cognitive capacities in vertebrates. Although our results do not resolve this interrogation, they provide elements of answer, as well as important evolutionary implications concerning vertebrate brain evolution. Coming back to the points I raised in the thesis project section, below are presented the elements of answer we could obtain:

1. We identified the primary visual area in zebrafish, located in a segregate manner in the dorsolateral pallium (DI). This supports the idea that the teleost pallium possesses modality-specific primary sensory areas, as it has been suggested in goldfish.
2. This sensory information is relayed by a specific relay nucleus (PG in zebrafish). Nevertheless, it originates from midbrain, therefore it is not homologous to thalamus.
3. No executive area equivalent to the PFC and NCL has been identified yet in teleosts. However, our behavioral data suggest the existence of executive function in teleosts, at least in a primordial form.
4. Teleosts do not possess mesencephalic DA cells. Yet the presence of working memory suggests there may be another DA cell population which may have an equivalent function as A9/A10 in amniotes.
5. Since we have not identified the executive area, the presence of association areas in the teleost pallium still needs to be investigated. Nonetheless, our study raises the possibility of an integration center outside of the pallium, IL. Although we did not investigate the motor outputs, our new interpretation of IL data encourages further studies about the potential sensory-motor integration taking place in IL.

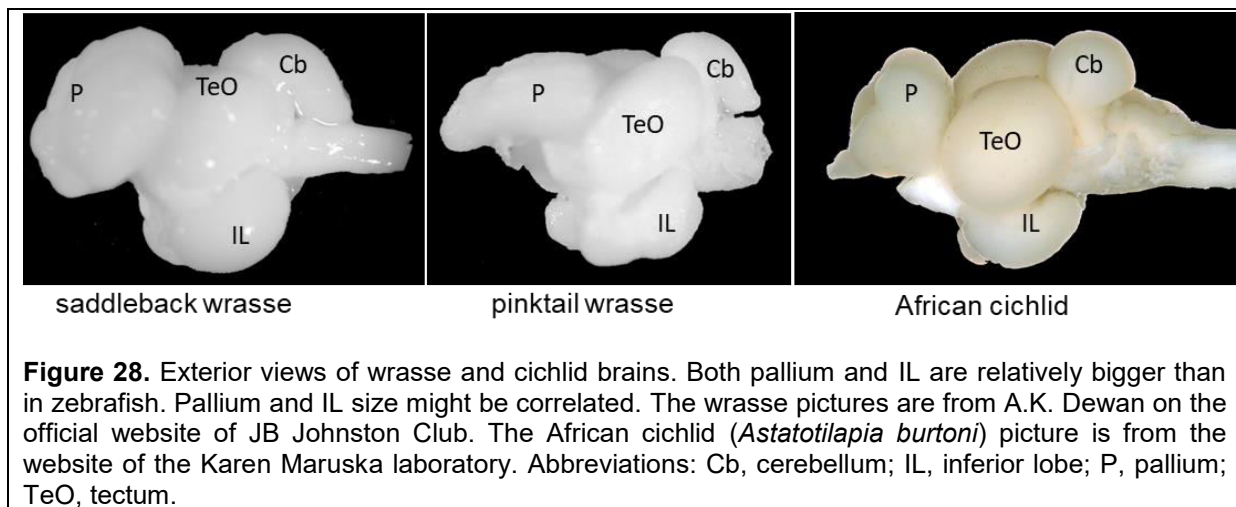
Although the information is relayed by a midbrain nucleus, at least the modal specificity appears to be present in teleosts (Ito and Yamamoto, 2009). This suggests that this property is an important step to evolve executive and higher order cognitive functions. Given that the major sensory pathway to the pallium has appeared independently in teleosts, this pallial organization is likely to be the result of independent evolution.

1. A different strategy for sensory integration in teleosts

In amniotes, sensory information is relayed through thalamus and integrated in a modal-specific manner in the pallial sensory areas. The amniote pallium has a major involvement in the integration of sensory information and is enlarged compared to other vertebrate groups. Amniote thalamus is the major sensory relay nucleus to the pallium. In contrast, our data demonstrate that the main sensory relay nucleus to the pallium in teleosts has a different embryonic origin: PG comes from the midbrain. This shows that similar functions and connectivity patterns can be achieved without a forebrain sensory relay to the pallium.

IL is another sensory structure in teleosts which functions remain elusive. Connectivity studies show that it receives diverse sensory inputs, which has led to the hypothesis that it could be a multi-sensory integration center (Ahrens and Wullimann, 2002; Rink and Wullimann, 1998). By contrast, other studies indicate that IL is capable of eliciting motor responses (Demski and Knigge, 1971; Muto et al., 2017). Thus IL can integrate sensory inputs and generate motor responses, which is similar to pallial functions. This would be in

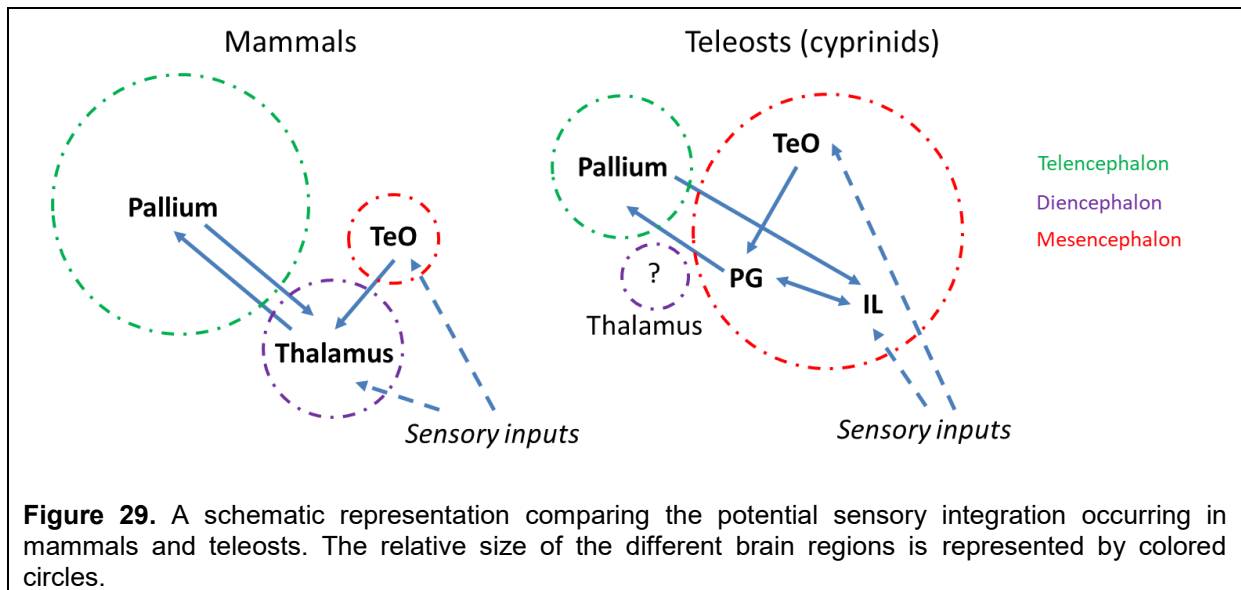
agreement with the observation that IL size appears to be correlated to pallial size in teleosts (Article 2, Yamamoto, unpublished, Figure 28).



PG and IL have been shown to be reciprocally connected, and IL receives descending projections from the pallial area receiving PG inputs, in a cyprinid (goldfish, Rink and Wullimann, 1998), a *Percomorpha* (Shimizu et al., 1999) and a Siluriforme (Lamb and Caprio, 1993). Given IL and PG common mesencephalic origin, it would be interesting to study how sensory information is distributed and processed between IL, PG, and pallium.

Actually, the telencephalon may not be the only structure involved in sensory motor processing in teleosts. One study in goldfish shows that a forced choice color discrimination task is only slightly impaired when the whole telencephalon is ablated, and it does not impair feeding or locomotion (Ohnishi, 1989). The author assumes that telencephalon may facilitate learning, although its involvement is not crucial. It would be interesting to compare the effects of telencephalic impairment in a forced choice color discrimination task versus in a more complex one such as DMTS: would telencephalon be essential for task acquisition in the latter case?

In this line of thought, it would also be interesting to investigate the accurate role of TeO in teleosts, compared with amniotes. In mammals, the homologous structure, superior colliculus (SC) is involved in saccade control and orientation towards a stimulus (Barker and Baier, 2015). We can observe the equivalent visuo-motor function in zebrafish larvae, with a direct control of the whole body. The projection to the telencephalon is not well-developed in larvae (article 2), and TeO plays a critical role in perception in larvae. The detailed function of the TeO is not well studied in adult, but its relatively large size indicates its importance even at the adult stage. It would be interesting to directly compare the capacities of mammalian, avian, and teleostean adult specimens, for example regarding a discrimination task classically involving a primary sensory area in the cortex of mammals.



If the connection patterns as illustrated in Figure 29 are consistently found in teleosts, one can imagine that sensory information in teleosts are integrated in a more distributed manner than in amniotes, with at least three distinct brain regions able to process sensory information and generate motor outputs: pallium, TeO and IL.

In order to assess this hypothesis, more functional studies are needed to determine the specific roles of these three regions, as well as more information about the sensory modalities involved (IL has been studied mainly in the context of the gustatory system so far). This might yield surprising results about how teleosts process sensory information to generate motor outputs. Very little is known about the motor circuitry in teleosts, and many questions remain to be addressed: what is the equivalent of the basal ganglia? Does PG contain motor nuclei, as thalamus?

In summary, our data about IL and PG development suggest that any part of the brain may give rise to novel structures, with convergent functional properties. Vertebrate brain evolution is more plastic than expected.

Moreover, a recent study in amphioxus, a non-vertebrate chordate, suggests that midbrain and diencephalon would be more closely related than expected (Albuixech-Crespo et al., 2017). In the light of these results, it is not very surprising that parts of the teleost midbrain are able to achieve similar connectivity as the diencephalic region. The molecular events leading to such a “functional programming” allowing the emergence of convergent functions for PG, compared to amniote dorsal thalamus, would be a potential area of study.

Given the organizational differences we observed in subtelencephalic structures, it would be surprising that a similar layer-like connectivity would have been conserved from the common ancestor of mammals, birds, and teleosts. In addition, the recent study of Furlan et al. (2017) has tested some of the layer-specific markers used to identify mammalian cortical layers and their equivalent in birds. This did not reveal a comparable organization, neither in layers nor in nuclei: some markers were not expressed (e.g. *Rorβ* or *ER81*) or expressed in a broad parenchymal pattern (e.g. *cux2a* or *fezf2*). Thus, if an input/output pattern similar to amniotes exists in the teleost pallium, it is likely to be the result of convergent evolution.

2. A “roof” from the top to the bottom in the teleost midbrain: continuity between PG, IL and TeO

From a developmental standpoint, our results also suggest that PG, IL, as well as TLa (the structure medial to PG and IL) share a common origin with TeO: they appear to originate from the dorsal (alar) part of the mesencephalon (supplementary figure in article 1). Thus it would be interesting to know how PG and IL form during development and if they arise from a common cell mass. Are they really different structures or do they form together a “sensory complex”?

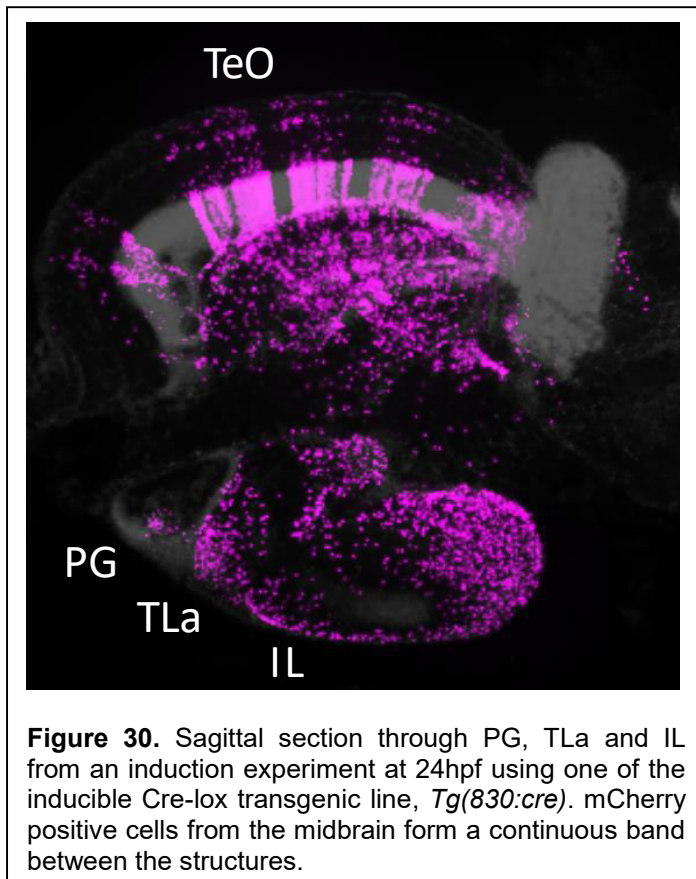


Figure 30. Sagittal section through PG, TLa and IL from an induction experiment at 24hpf using one of the inducible Cre-lox transgenic line, *Tg(830:cre)*. mCherry positive cells from the midbrain form a continuous band between the structures.

Here I will talk about the observations of the two lines that we used in article 1 and article 2: *Tg(her5:ERT2CreERT2)* and *Tg(Dr830:ERT2CreERT2)* crossed with *Tg(βact:lox-stop-lox-hmgb1:mCherry)*. Here I will abbreviate the F1 *Tg(her5:cre)* and *Tg(830:cre)*. Indeed, based on observations in the adult after induction at 24 hpf, PG, TLa and IL appear to be continuous, when going through confocal images in the frontal plane at the limit of PG and TLa, or TLa and IL. Also, in sagittal plane, there is an apparent continuity of these structures (Figure 30). Taking a close look younger at 2 wpf, the PG is not formed yet, but the most anterior ventral cells may be the ones giving rise to PG, and they are continuous until IL. Both transgenic lines yield very similar

distribution patterns in PG and IL, thus indicating that different progenitor pools populate these structures. Interestingly, observation of the *Tg(830:cre)* induction experiments (e.g. Figure 30) shows a similar distribution at different time points of induction, reducing with time, as seen for *Tg(her5:cre)* experiments. This suggests that both progenitor pools populate PG, IL and TLa in a similar timeframe and pattern.

3. The question of DA involvement in executive function in teleosts

At the behavioral level, our data show that zebrafish possess a primordium of executive functions: they can successfully learn SMTS and DMTS tasks using visual cues. The former shows some context integration in this species, while the latter shows the existence of a working memory of a few seconds. Together with previous work in reversal learning, we can consider that zebrafish is capable of performing tasks involving executive functions.

3.1 Functional implications of the DA receptor diversity?

In mammals and birds, DA is critically involved in such functions through modulation of the executive area, in particular through D1-like DA receptors. A study in birds (Herold et al.,

2012) has investigated the more detailed implication of the different receptor in the different cognitive subcomponents of a task. Intriguingly, it shows that D6 (D1C/D), which does not exist in mammals, is dynamically regulated in NCL according to the nature of the task. In particular, D6 is upregulated in NCL in DMTS compared to SMTS. In birds, D6 is specifically expressed in the pallium within the telencephalon (Kubikova et al., 2010; Yamamoto et al., 2013). Thus one could imagine that D6 has a specific role in avian cognition.

If we assume that DA is also important for executive function in the teleost pallium, a similar approach could be used in teleosts to dissect the involvement of the different receptors in specific cognitive modules, and could be compared to the avian and mammalian situations.

In teleosts, we have to take into consideration that their DA receptor repertoire is very different from amniotes. As mentioned in the introduction, some teleosts such as zebrafish have a greater number of DA receptor subtypes than amniotes. This is both due to less secondary loss and the additional whole genome duplication specific to teleosts. This complicates their study due to the closeness in sequence between paralogs, which makes them hard to distinguish both in terms of expression and pharmacology. In addition, our preliminary data suggest that receptor subtypes are widely expressed, and do not seem to segregate in a simple manner (e.g. the expression level in the subpallium is not as remarkable as in amniotes). Nonetheless, it would be surprising that the teleost specific paralogs are completely redundant in function. Thus, more studies are needed to evaluate the DA receptor function in teleosts.

3.2 DA innervation to the pallium in teleosts

In teleosts, the most abundant DA fibers are in Dp. This was known previously in zebrafish (Yamamoto et al., 2011), and our preliminary data suggest it is also the case in cichlids (Figure 31). However, Dp main function appears to be related to olfaction. It is known that Dp receives massive projections from the olfactory bulbs (Levine and Dethier, 1985), and recent works in zebrafish have shown the involvement of Dp in olfactory information processing (Rupprecht and Friedrich, 2018; Yaksi et al., 2009). The latter authors were also able to modulate the activity of Dp neurons by applying DA. Another study has shown that Dp actually receives DA inputs from three different forebrain areas: the telencephalic population, the preoptic area and the posterior tuberculum (poster tuberal nucleus, PTN), considered as diencephalic (Schärer et al., 2012). This demonstrates that different DA population within the forebrain reach the pallium.

Looking at Figure 31C, DA fibers in the cichlid pallium are mainly located in Dp, but are not distinguishable in other pallial areas. More studies are needed to identify more accurately the cell populations projecting to the pallium. This may help to identify the executive area in teleosts, which would be innervated by DA terminals, as it is the case for PFC and NCL in amniotes.

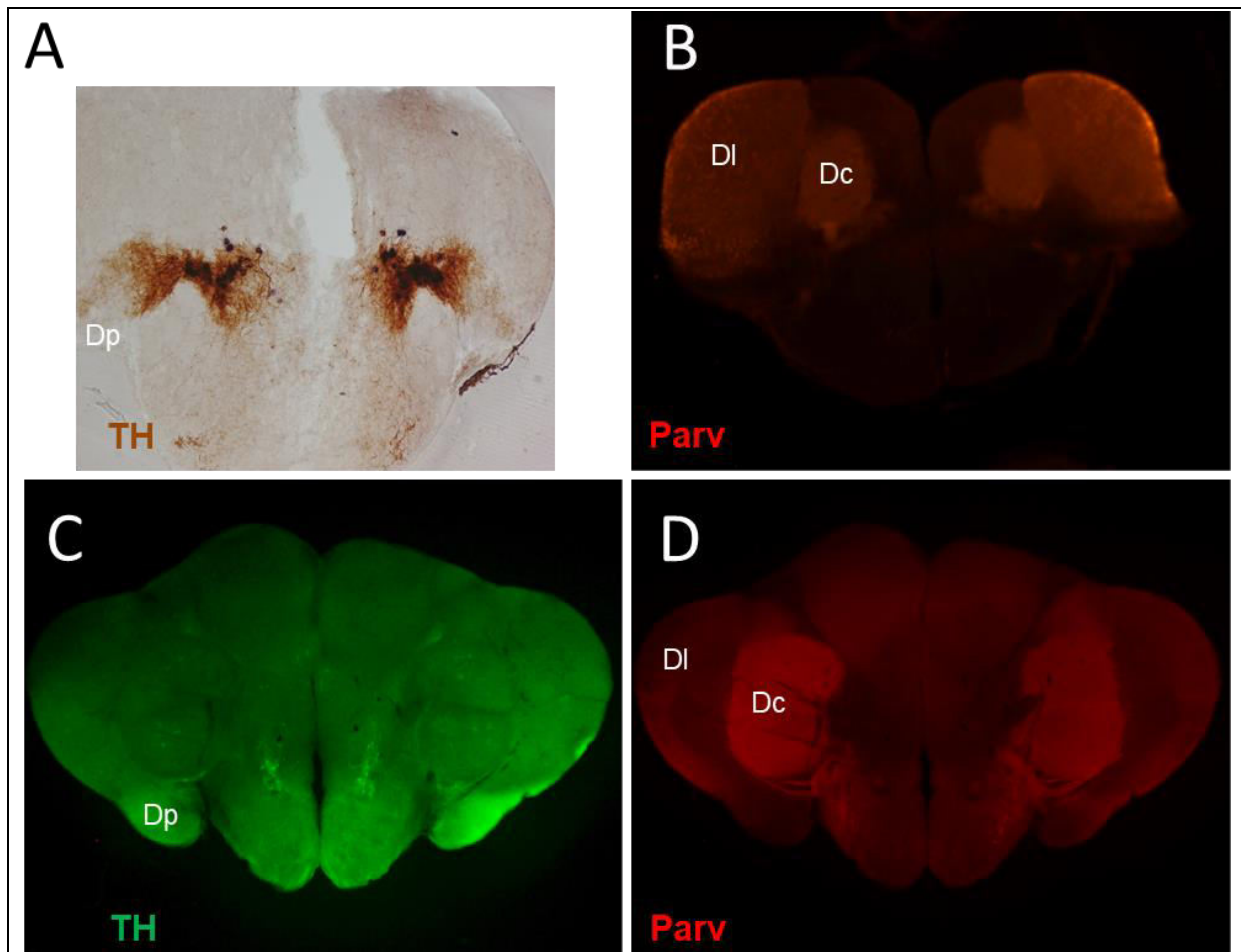


Figure 31. Comparison of the zebrafish pallium and the cichlid pallium (*Maylandia zebra*). **A.** Zebrafish frontal section of the telencephalon with a TH immunolabeling. **B.** Zebrafish telencephalon at a comparable level with a paravalbumin immunofluorescence. **C.** Cichlid brain in frontal section at a comparable level with TH immunofluorescence. **D.** Same section as C with paravalbumin immunofluorescence. Both pallia receive DA inputs in Dp (A, C). In the cichlid (B), Dc is much larger in proportion to the rest of the pallium than in zebrafish (D). Abbreviations: Dc: central part of the dorsal telencephalic area; Dl: lateral part of the dorsal telencephalic area; Parv: paravalbumin; TH, tyrosine hydroxylase.

4. Limits of the adult zebrafish as a model to study cognition

My thesis work suggests that teleosts possess executive functions and encourages more research in order to locate the executive area. However, my thesis work also highlights the limits of the zebrafish as a model for studying such questions, that I will explain below.

Although zebrafish are capable of performing SMTS and DMTS in our hands, it still shows a limited behavioral flexibility. When we changed the set-up (passing through a window located downwards), animals were able to learn a forced choice color discrimination task, but were not able to perform SMTS or DMTS. Additional difficulties come from their generally irregular performance in operant tasks (an individual is rarely capable to maintain a criterion for long) and their high basal locomotor activity. Indeed, they move constantly, which makes it difficult to differentiate a behavioral response from locomotion. Moreover, their constant movement may disrupt their attention to the task.

I have tried different approaches to link brain structure and functions, which turned out to be very challenging in adult zebrafish and did not yield interpretable results.

In the initial form of the project, after establishing operant conditioning tasks in adult zebrafish, we wanted to locate the pallial areas involved. For this, we already had the UAS/Gal4 line (*Tg(279A-GFP)* in article 2) targeting the visual PG cells projecting to DI. Our first aim was to destroy this projection and investigate its consequences on a color discrimination task. If this had worked, we would have applied the same technique to study the executive area in zebrafish.

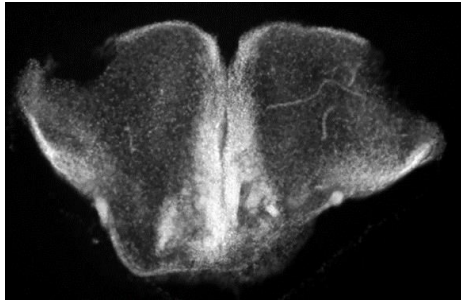


Figure 32. Example of a successful bilateral lesion on a frontal section of the telencephalon, possibly in anterior DI. An additional issue we were confronted with was that the lesion provoked autofluorescence, which made it hard to locate DI in the *Tg(279A-GFP)* transgenic line.

First, the genetic ablation using nitroreductase or tetanus toxin did not work in adult zebrafish (although studies in larvae used it successfully). Then we tried physical ablation (such as mechanical and electrolytic). However, this was challenging due to the relatively small size of zebrafish and its vulnerability. We could not have the adequate number of subjects to perform the behavioral tests.

Finally, we tried to relate neuronal activity to task performance. We rapidly abandoned electrophysiological recordings, both for the same reasons as the lesions and because this has not been well established for adult specimens.

Moreover, it would have been impossible to have

the fish perform the operant task under the set-up. A last promising possibility was to sacrifice the fish after operant conditioning, and to relate performance to a neuronal activity marker. We tried several, at the mRNA or protein level. These included pERK and cfos. Although specific labeling was obtained in some cases, this was difficult to interpret, and we could not see any consistent differences between control groups and experimental groups.

In the future, it would therefore be interesting to consider other teleostean models to study the evolution of higher order cognitive function. Although zebrafish can be interesting to address general developmental and neuroanatomical questions about the teleost brain, it is limited in terms of cognitive capacities and functional studies in adult specimens. Cichlids could be good candidates for comparative studies with mammals and birds. They live in fresh water (unlike wrasses), and may present developed cognitive capacities (Grosenick et al., 2007). Practically, adults are generally larger than zebrafish which can facilitate functional approaches such as lesions or electrophysiological recordings. Moreover, their pallium is well-developed, and the relative enlargement of some pallial areas compared to the zebrafish pallium may hint at associative and executive areas. For instance, in Figure 31, you can see that the cichlid species has a significantly enlarged Dc compared to zebrafish, so it might be a good candidate for a higher order area.

Further studies in cichlids may provide a new perspective on how higher order cognitive functions have evolved. For instance, if an executive area is found in teleosts, which characteristics would it have in common with PFC and NCL? Given the differences in brain organization between amniotes and teleosts, yet more surprising discoveries may be obtained: one could imagine that executive functions do not reside in a pallial area, but for instance, in IL. An intermediate scenario could be that it might be distributed in pallium and subtelencephalic areas such as IL.

In conclusion, comparative studies including teleosts may inform us on a different way to build intelligence.

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Annex

Synthèse en français / Summary in French

Le projet de recherche dans lequel s'est inscrit mon travail de thèse avait pour but d'identifier les conditions et les mécanismes de l'évolution de la cognition de haut niveau chez les vertébrés. Cette cognition de haut niveau est illustrée par l'émergence indépendante de certains comportements tels que l'utilisation d'outil ou des formes de raisonnement logique chez certaines espèces dans différents groupes de vertébrés, en particulier les primates au sein des mammifères, les corvidés chez les oiseaux, et certains poissons téléostéens. Historiquement, ces capacités étaient considérées comme l'apanage de l'homme et éventuellement de ses proches cousins. Leur présence au sein d'autres groupes d'animaux possédant une organisation cérébrale différente interroge sur les caractéristiques cérébrales permettant de générer ces comportements flexibles, parfois décrits en psychologie humaine comme une « intelligence générale », permettant de générer de nouveaux comportements qui ne font pas partie du répertoire comportemental ordinaire.

L'objectif de ma thèse a été d'identifier des composants anatomiques essentiels qui sous-tendent les fonctions exécutives, considérées comme les briques de base pour construire les fonctions de plus haut niveau évoquées ci-dessus. Les fonctions exécutives permettent de prendre en compte le contexte et d'avoir une certaine flexibilité.

Les hypothèses actuelles sur les prérequis anatomo-fonctionnels permettant l'apparition des fonctions exécutives et fonctions cognitives de haut niveau sont basées sur la comparaison des oiseaux et des mammifères. Chez les mammifères, le cortex préfrontal joue un rôle prépondérant dans la génération de ces capacités. Chez les oiseaux, une partie du cerveau joue un rôle équivalent : le nidopallium caudolatéral. Ces deux régions ont des propriétés communes, probablement acquises indépendamment au cours de l'évolution. La situation chez les poissons téléostéens est peu connue, mais elle est susceptible de fournir des informations supplémentaires sur l'évolution de la cognition chez les vertébrés car ce groupe possède une organisation cérébrale encore plus différente que celle des amniotes (oiseaux et mammifères).

Dans ce but, nous avons utilisé une approche fondée sur la comparaison de capacités cognitives élaborées et déjà bien étudiées chez certains amniotes avec celles que l'on peut analyser chez le poisson zèbre, un modèle animal bien établi en biologie du développement et en génétique. Ce modèle présente un double intérêt. D'une part, il permet d'utiliser des tests comportementaux semblables à ceux testés chez d'autres espèces afin d'identifier les composants essentiels de la cognition. D'autre part, certains outils génétiques permettent d'analyser l'organisation neuratomique.

Un premier aspect de mon travail de thèse a été de développer des tests de conditionnement opérant chez le poisson zèbre adulte, semblables à ceux utilisés chez les rongeurs, les primates, et les oiseaux. Nous avons commencé par le développement de tests de discrimination de couleur, puis d'autres plus complexes couramment utilisés pour tester différents modules des fonctions exécutives chez les mammifères et oiseaux, tels que le « delayed matching-to-sample ». La nature du test détermine la nature des modules cognitifs étudiés, et permet donc une comparaison directe avec les mammifères. Ainsi nous

avons démontré que le poisson zèbre possède une mémoire de travail, un des composants essentiels des fonctions exécutives.

Cependant la neuroanatomie du poisson zèbre adulte reste encore mal connue. Une seconde partie de mon travail a donc été d'étudier plus en détail les structures cérébrales adultes potentiellement impliquées dans les fonctions cognitives. Nous avons utilisé des lignées transgéniques qui permettent de connaître l'origine développementale des structures adultes en suivant leur lignage cellulaire. Cette méthode a permis de redéfinir l'origine des certaines parties du cerveau du poisson, dont l'organisation générale s'est avérée, de façon inattendue, très divergente de celle des autres vertébrés. En particulier, nous avons découvert que certaines structures considérées jusqu'ici comme faisant partie du cerveau antérieur (prosencephale) font en fait partie du cerveau médian (mésencéphale) chez le poisson zèbre : le lobe inférieur, précédemment considérée comme faisant partie de l'hypothalamus, ainsi que le noyau préglomérulaire, le relai sensoriel majeur vers le pallium, précédemment considéré comme diencephalique et comparable fonctionnellement au thalamus des amniotes.

Ces études anatomiques ont des implications importantes pour la connaissance de l'évolution du cerveau des vertébrés en général, mais aussi pour l'utilisation du poisson zèbre comme modèle en neurosciences.

Pour revenir au but initial du projet, ce travail apporte quelques éléments de réponse sur les conditions anatomo-fonctionnelles nécessaires à l'émergence de fonctions exécutives :

- L'aire visuelle primaire dans le pallium (équivalent du cortex) chez le poisson zèbre est bien délimitée. Cela soutient l'idée que l'information sensorielle est intégrée de manière ségréguée dans le pallium des téléostéens.
- Cette information sensorielle est relayée par un relais spécifique, mais d'origine mésencéphalique : il n'est donc pas homologue au thalamus des mammifères et des oiseaux (qui fait partie du prosencephale).
- L'équivalent du cortex préfrontal n'a pas été identifié, mais la présence de mémoire de travail suggère l'existence de fonctions exécutives chez les téléostéens
- Les téléostéens ne possèdent pas de neurones dopaminergiques dans le mésencéphale, qui ont un rôle crucial dans les fonctions exécutives chez les mammifères et les oiseaux. Nos données comportementales suggèrent qu'une autre population dopaminergique pourrait avoir une fonction similaire.
- Par ailleurs, nos données suggèrent l'existence d'un centre d'intégration en dehors du pallium : le lobe inférieur.

Mon travail s'inscrit dans une perspective comparative, qui cherche à comprendre comment des cerveaux différents dans leur structure et leur fonctionnement tels que ceux des poissons téléostéens et des mammifères peuvent générer des fonctions cognitives similaires. Isoler les éléments indispensables à l'existence de ces capacités exécutives chez un animal modèle relativement simple nous permettrait de mieux comprendre leur origine et la logique de leur organisation chez l'homme. Nos résultats révèlent que, même si certaines structures cérébrales ont la même fonction chez un téléostéen tel que le poisson zèbre et chez les mammifères, elles peuvent avoir une origine évolutive et développementale différente. Ces structures ont ainsi convergé vers une même fonction.

Title: Divergent evolution of brain structures and convergence of cognitive functions in vertebrates: the example of the teleost zebrafish

Summary: The aim of my research project was to link brain structures and functions, to better understand the fundamental bases of cognition. The first part of my thesis consisted in the development of behavioral tests to analyze the essential principles of cognition. The results strongly suggest the existence of executive functions in teleosts similar to those of mammals. Then I looked for the anatomical structures responsible for these cognitive capacities, in particular in the pallium (equivalent of the mammalian cerebral cortex). However, little is known about adult zebrafish neuroanatomy. Indeed, zebrafish is often studied at larval stage. A second part of my work aimed at studying adult structures in more detail through their developmental origin. This has redefined some parts of the brain. We have discovered that some of the structures that were considered as part of the forebrain (prosencephalon) are actually part of the midbrain (mesencephalon) in zebrafish. This includes the inferior lobe, previously classified as hypothalamus. Another structure is the major sensory relay nucleus, the preglomerular nucleus, functional analogue of the thalamus (part of the forebrain) in amniotes. This sensory pathway contains the main visual pathway to the pallium. Thus, even if some structures have the same function, they may have an evolutionary and developmental origin different from structures known in mammals. In summary, similar functions have independently evolved in amniotes and teleosts. This comparative work adds new perspectives for neuroscience research. It also allows us to approach the fundamentals of cognition in a new way: what are the essential building blocks for a higher level of cognition like the human one?

Keywords: cognition, brain evolution, forebrain, zebrafish, behavior, vertebrates

Titre : Évolution divergente des structures cérébrales et convergence des fonctions cognitives chez les vertébrés : l'exemple d'un téléostéen, le poisson zèbre

Résumé : L'objectif de mon projet de recherche était de faire le lien entre structures cérébrales et fonctions, pour mieux comprendre les bases de la cognition. La première partie de ma thèse a été de développer des tests comportementaux pour analyser la cognition et ses fondamentaux. Les résultats suggèrent fortement que les téléostéens possèdent des fonctions exécutives semblables à celles des mammifères. J'ai par la suite cherché le substrat anatomique de ces capacités cognitives nouvellement mises à jour chez cette espèce, notamment dans le pallium (équivalent du cortex cérébral des mammifères). Cependant la neuroanatomie du poisson zèbre adulte est mal connue, car il est souvent utilisé au stade larvaire. Une seconde partie de mon travail a cherché à analyser et identifier l'origine développementale des structures cérébrales adultes. Nous avons découvert que certaines structures considérées jusqu'ici comme faisant partie du cerveau antérieur (prosencephale) font en fait partie du cerveau médian (mésencéphale) chez le poisson zèbre. L'une de ces structures est le lobe inférieur, précédemment considéré comme hypothalamique. Une autre structure est le noyau préglomérulaire, le noyau sensoriel relais majeur et analogue fonctionnel du thalamus. Cette voie sensorielle contient la principale voie visuelle vers le pallium. Ainsi, même si certaines structures ont la même fonction, elles peuvent avoir une origine évolutive et développementale différente des structures connues chez les mammifères. En résumé, des fonctions similaires ont évolué indépendamment chez les amniotes et les téléostéens. Ce travail élargit ainsi les champs d'application pour la recherche en neurosciences, et permet d'approcher les fondamentaux de la cognition de manière nouvelle par l'identification des structures essentielles à l'émergence d'une cognition de haut niveau telle qu'elle est observée dans l'espèce humaine.

Mots clefs : cognition, évolution du cerveau, prosencéphale, poisson zèbre, comportement, vertébrés.