Universidad de Sevilla
Área de Psicobiología

Sistemas de memoria relacional y emocional en los peces teleósteos: estudio mediante histoquímica de la citocromo oxidasa y registro óptico con fluorocromos sensibles al voltaje

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Relational and emotional memory systems in teleost fish: a study with cytochrome oxidase histochemistry and voltage sensitive dye imaging

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Trabajo presentado por Sara Uceda Gutiérrez para optar al grado de Doctora por la Universidad de Sevilla

Los directores,

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A mis padres

A toda mi familia

A Leo

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INTRODUCTION

1. The Evolution of the Brain

The traditional theories about forebrain evolution have understood the vertebrate radiation as a linear series of increasing complexity, from “lower” to “higher” groups—fishes, amphibians, reptiles, etc., to finally reach the superior cerebral and cognitive stage of mammals, and specially humans, with the incorporation of new and most complex neural structures, circuits and mechanisms in successive steps or evolutionary stages (Ariëns-Kappers et al., 1936; Crosby and Schnitzlein, 1983; MacLean, 1990; Papez, 1929). According to these traditional theories, the forebrain of fishes was viewed as consisting of a sub-pallium (“paleostriatum”) and a very small pallium (“palleocortex”) both dominated by olfactory inputs, with only relatively simple neural circuits sustaining elemental ‘instinctive' or ‘reflex' forms of behavior, and thus lacking the learning capacities, plasticity and behavioral flexibility that characterize mammals, particularly associated with the expansion of the six-layered neocortex (Ariëns-Kappers et al., 1936; Crosby and Schnitzlein, 1983; Jerison, 1973; MacLean, 1990; Papez, 1929; Romer, 1962). However, this anagenetic, anthropocentristic point of view regarding the evolution of forebrain, behavior and cognition in vertebrates have proven to be largely inadequate in the light of the recent advancements in different fields of evolutionary and developmental biology and comparative neuroscience.

Recent neuroanatomical and functional data show that vertebrate brain and behavior evolution has been far more conservative than previously thought (for revisions, see Broglio et al., 2005; Butler and Hodos, 2005; Nieuwenhuys et al., 1998; Salas et al., 2003; Striedter, 2005) and despite some notable morphological and cytoarchitectural differences, the central nervous system of every vertebrate group is organized in equivalent areas. Multiple evidences indicate that the telencephalon of teleost fish and amniotes share a comparable basic pattern of organization, with pallial and sub-pallial zones, and various main pallial subdivisions, which include regions probably homologous to the hippocampus and the amygdala (Braford, 2009; Broglio et

al., 2005, 2011; Butler and Hodos, 2005; Nieuwenhuys et al., 1998; Nieuwenhuys, 2011; Northcutt, 2008; Salas et al., 2003; Wullimann and Mueller, 2004; Yamamoto et al., 2007). In addition, multiple behavioral and functional data from the last years also challenge the traditional notions on brain and cognition evolution, showing that fishes share complex learning and memory capabilities with land vertebrates, which are also based on equivalent neural mechanisms and brain systems. However, a remarkable variation regarding the vertebrate forebrain exists. This variation is based on the different processes underlying forebrain development during embryogenesis. Thus, whereas the forebrain of actinopterygian fish (for instance, teleost fish) undergoes a process of eversion, the forebrain of the remaining vertebrates (for instance, amniotes) develops by a process of evagination (Nieuwenhuys, 1963, 2011; Northcutt and Braford, 1980; Striedter and Northcutt, 2006). In ray-finned fishes eversion produces two massive telencephalic hemispheres separated by a single ventricular cavity, in which most of its surface is ependymal instead of pial (Butler and Hodos, 2005; Nieuwenhuys, 2011; Nieuwenhuys et al., 1998; see Figure I.1). In addition, this unique developmental and morphological pattern produces the reversal of the pallial topography observed in non actinopterygian which, together with other characteristics of eversion, has traditionally hindered the possibility of identifying homologies among vertebrates.

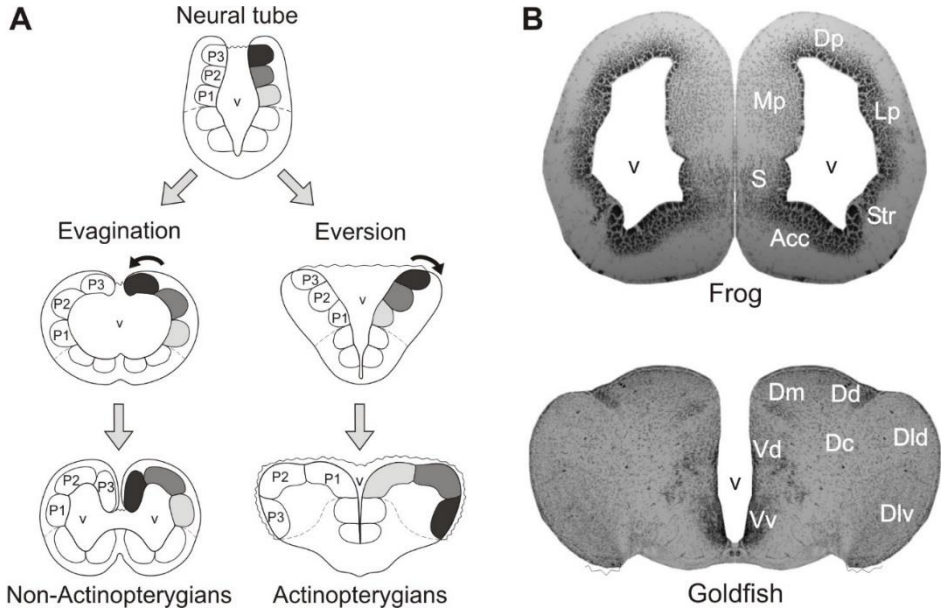

Figure I.1. (A) Schematic representation of the processes involved in the embryonary development of the telencephalon in actinopterygian and non-actinopterygian vertebrates. P1, P2, and P3 correspond to the three main subdivisions of the pallium. (B) Coronal sections of brains representative of the evaginated (frog) and everted (goldfish) patterns of telencephalon development. Note that in actinopterygian fish the inner telencephalic ventricles are not present, and that the position of the main dorsal pallium subdivisions appears inverted relative to the evaginated telencephalon. Abbreviations: Acc, nucleus accumbens; Dc, central division of area dorsalis; Dd, dorsal division of area dorsalis; Dld, dorsal subdivision of lateral division of area dorsalis; Dlv, ventral subdivision of lateral division of area dorsalis; Dm, medial division of area dorsalis; Dp, posterior zone of area dorsalis; Lp, lateral pallium: Mp, medial pallium; S, septal nuclei; Str, striatum; v, ventricle; Vd, dorsal nucleus of area ventralis; Vv, ventral nucleus of area ventralis.

2. Cytoarchitectonics of the Goldfish Telencephalic Pallium

Histological analyses have revealed that the forebrain of actinopterygian fishes consists of two main territories, the area dorsalis telencephali (D) and the area ventralis telencephali (V), that correspond to the pallium and the subpallium, respectively, of inverted forebrains. The morphological pattern of the dorsal area has been found variable between teleost fishes, whereas ventral area remains mostly without anatomical variations (Schroeder, 1980).

The actinopterygian area dorsalis telencephali (D) or telencephalic pallium can be divided into four regions: medial (Dm), dorsal (Dd), lateral (Dl), and posterior (Dp; Braford, 2009; Mueller and Wullimann, 2009). Dm and Dl occupy the rostral pole, Dd lies between Dm and Dl and Dp forms the caudal pole (Figure I.2A-C).

Usually, four zones can be cytologically recognized in Dm (Burmeister et al., 2009; Demski, 2013; Demski and Beaver, 2001; Murakami et al., 1983; Northcutt, 2006; Northcutt and Braford, 1980; Wullimann and Mueller, 2004; Yamamoto and Ito, 2005; Yamamoto et al., 2007), although there is no consensus regarding the topography of these divisions in the teleost pallium. In contrast, an agreement exists concerning the compartmentalization of Dl according to which it can be divided into a dorsal (Dld) and a ventral (Dlv) subdivision. Dp is the main pallial target of the secondary olfactory projections (Folgueira et al., 2004; Levine and Dethier, 1985), together with the nucleus taeniae, a small pallial division located ventral to Dp, which also receives secondary olfactory projections. Finally, two additional pallial divisions are usually recognized in most teleosts: one or more central divisions (Dc) of large cells and a relatively small dorsal division (Dd) composed of small granular cells. It is

probable that Dc is not a primary pallial division; rather, it likely represents the deeper, larger cells of the main pallial divisions (Figure I.2E-F).

According to Northcutt (2006), Dm in the goldfish (Carassius auratus) as in other teleosts, can be divided into two subdivisions (rostral and caudal), although there is little agreement regarding the topography of these divisions. Rostral Dm (Dmr) is characterized by a moderate concentration of calretinin in its neuropil and is gradually replaced medially and at more caudal levels by the caudal subdivision of Dm (Dmc). Although caudal Dm has also a moderate concentration of calretinin in its neuropil, it can be cytologically distinguished from rostral Dm by a superficial layer of cells that are much larger than the superficial granule-like cells of rostral Dm, but like rostral Dm it is also characterized by a core of large cells whose size gradually increases with depth (Figure I.2H-J). Nevertheless, the area where deeper cells are located in caudal Dm (Dmc) has traditionally been designated as a separate, central pallial division. Throughout most of the rostrocaudal extent of the telencephalic pallium, an overlap among the four pallial divisions is noticeable. Thus, rostrally, Dm lies over Dl, and caudally, Dm lies over Dp. At mid-hemispheric telencephalic levels, Dd separates Dm from Dl but also lies over a medial segment of Dl (Figure I.2B, C).

Concerning Dl, this pallial division can be easily separated from Dm by the sulcus ypsiloniformis and citoarchitechtonically by a marked increase in the cell size which characterizes its medial border. More caudally, Dl is distinguished from Dm due to the presence of an area composed by densely packed medium sized cells, which forms Dd (Northcutt, 2006).

Throughout much of their rostrocaudal extent, Dl can be further subdivided into a dorsal and a ventral area. Unlike the ventral subdivision, the dorsal subdivision is characterized by a moderate concentration of calretinin (Northcutt, 2006), as well as by large cells which decrease in size with depth. In contrast to Dm, Dl has bigger cells in a medial region but not in the core. Rostrally, it is hard to distinguish the border between dorsal and ventral subdivisions of Dl (Dld and Dlv, respectively), however, this becomes easier more caudally, since both of them are separated by a shallow sulcus, as well as it is characterized by a moderate concentration of calretinin. Furthermore,

the ventral subdivision of Dl can also be subdivided into a ventral and a dorsal part. The dorsal part of the ventral subdivision of Dl is formed by a large region of scattered cells, whereas the ventral part is formed by a smaller region of densely packed granule-like cells (Figure I.2K).

Perhaps the most difficult border to be distinguished between pallial regions in Carassius auratus is the border between Dlv and Dp. In transverse sections the two parts of the ventral subdivision of Dl, as well as the most prominent dorsal subdivision of Dl, are easily distinguished at the level of the anterior commissure. Not only Dld but also Dlv drop off rapidly in size as they are traced caudally to the anterior commissure. At this level, only the outer layers of the ventral subdivision of Dl can be distinguished. At a caudal telencephalic level it is not fully appreciated any subdivision of Dl, and the ventral caudal pole is dominated by a superficial ring of medium sized cells surrounding a core of densely packed, large neurons that characterize Dp (Fig. I.2G, L). The transition from Dlv to Dp (described as a posterior subdivision of Dl by Northcutt and Braford, 1980) is much more evident in the horizontal plane. In this plane, the caudal pole of Dlv is marked by a different sulcus, and there is actually a narrow, cell-free zone between Dlv and Dp (Northcutt, 2006).

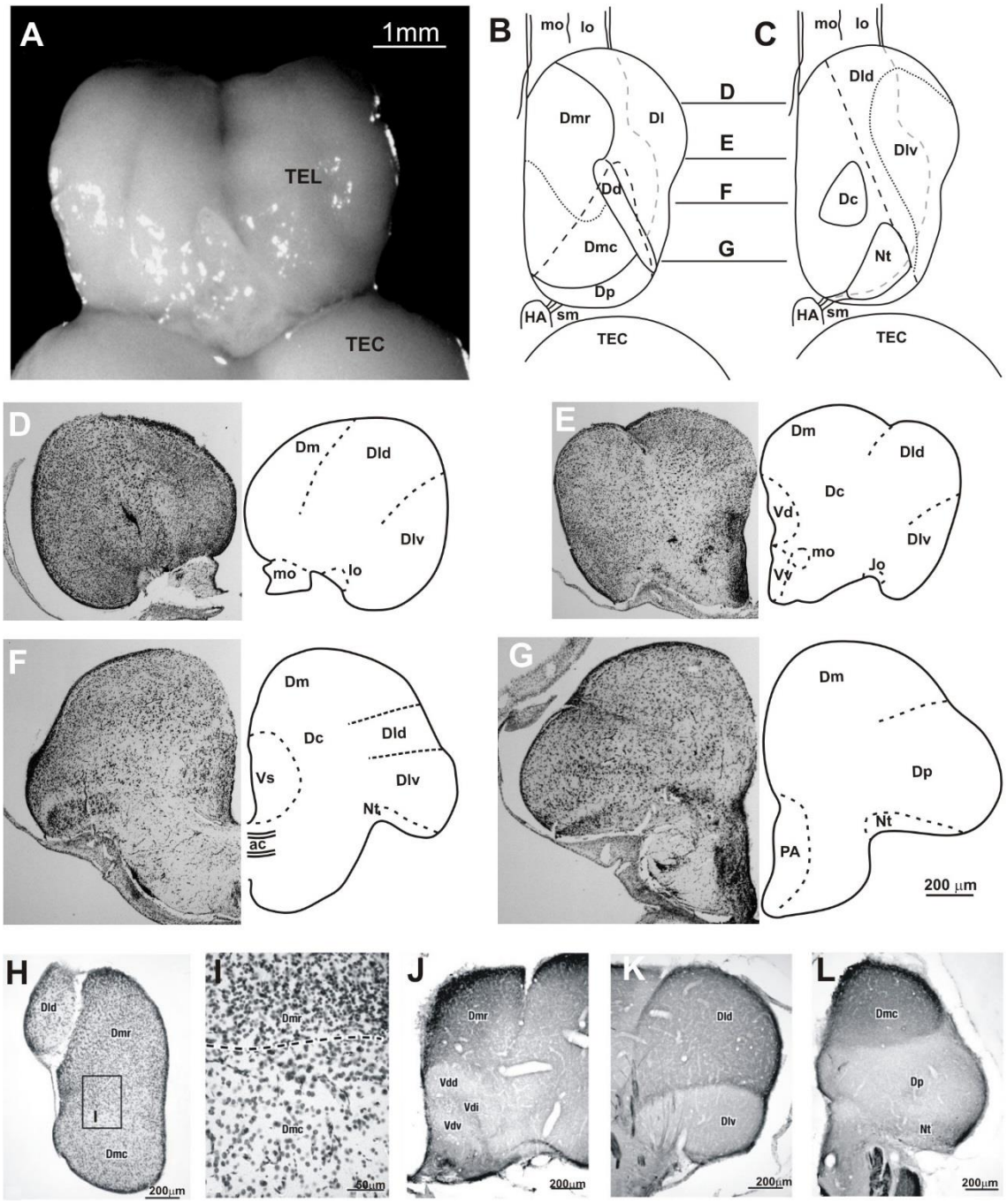

Figure I.2. Cytoarchitectonics of the goldfish telencephalic pallium. (A) Dorsal view of the goldfish telencephalon. (B-C) Reconstruction of the goldfish pallium in a dorsal view. All pallial divisions can be seen from the surface (B), but only rostral medial (Dmr) and caudal medial (Dmc) divisions can be seen in their entirety. The curved, dotted line marks the boundary between these two divisions of Dm. Only the caudal pole of the posterior pallial division (Dp) can be seen from the surface, as much of this pallial division, whose borders are indicated by the dashed line, lies beneath Dm. The dorsal pallial division (Dd) separates Dm from the lateral pallial division (Dl) along much of their rostrocaudal extent, and the medial and lateral attachments of the tela choroidea are indicated by dashed the gray lines. Most of the lateral attachment occurs along the ventrolateral edge of Dl, which cannot be seen in dorsal view and is indicated by the dashed gray line. The full extent of the other pallial divisions can be visualized in a reconstruction that does not include Dm, Dd, or Dp (C). Here the borders of the large-celled subdivision (Dc) of caudal Dm can be seen medially, and the dorsomedial border of Dl is indicated by the dashed oblique line. The lateral pallial division (Dl) can be divided into dorsal (Dld) and ventral (Dlv) subdivisions, and the border between these two subdivisions is indicated by the dotted line. As before,

the lateral attachment of the tela choroidea is indicated by the dashed gray line, which is continued caudally and medially to indicate its site of attachment on the ventrolateral surface of nucleus taeniae (Nt) and the lateral surface of the stria medullaris (sm). The relative positions of the transverse sections of Figures D–G are indicated by labeled lines between the two reconstructions. (D-G) Photomicrographs of cresyl violet-stained transverse sections and corresponding line drawings of the rostro-caudal levels signaled in B and C. (H-L) Photomicrographs of horizontal (H-I) and transverse (J-L) sections illustrating various cytological and immunohistochemical details of the goldfish telencephalon. *H,I:* Cresyl violet-stained sections through the telencephalon showing differences in cell size and density of rostral and caudal Dm. *J:* Reacted section showing the distribution of calretinin in the rostral telencephalon. Note the absence of calretinin in the dorsal subnucleus of Vd, in contrast to rostral Dm. *K:* Reacted section showing the distribution of calretinin in Dl. Note the absence of calretinin in the ventral subdivision of Dl. *L:* Reacted section showing the distribution of calretinin in the caudal telencephalon. Note the strong calretinin immunoreactivity in caudal Dm. Abbreviations: Dc, large-celled subdivision of Dm; Dd, dorsal division of area dorsalis; Dld, dorsal subdivision of lateral division of area dorsalis; Dlv, ventral subdivision of lateral division of area dorsalis; Dmc, caudal part of medial subdivision of area dorsalis; Dmr, rostral part of medial subdivision of area dorsalis; Dp, posterior division of area dorsalis; HA, habenula; lo, lateral olfactory tract; mo, medial olfactory tract; Nt, nucleus taeniae; PA, preoptic area; sm, stria medullaris; TEC, optic tectum; TEL, telencephalon; Vd, dorsal nucleus of area ventralis; Vs, supracommissural nucleus of area ventralis, ac, anterior commissure; vv, ventral nucleus of area ventralis. Modified from Northcutt, 2006.

3. Recent Hypothesis Regarding Teleost Pallium Homologies with Land Vertebrates

Teleosts represent an extremely species-rich and diversified clade within the actinopterygian radiation, and given the large number of species and their long phylogenetic history, it is not surprising that the brains of bony fishes exhibit a remarkable range of variation.

In all vertebrates, the pallium can be divided into four areas (medial pallium, dorsal pallium, lateral pallium and ventral pallium; Puelles, 2001; Puelles et al., 2000; 2004). Several hypotheses of pallial homologies in teleosts and, by extension, in other ray-finned fishes have been proposed over the past years. Clearly, there are a number of differences in interpretation concerning pallial development, but there are also a number of agreements. Although a considerable portion of the pallial teleostean telencephalon is laterally everted, there is a general consensus that at least certain structures are homologous in the pallium of actinopterygians and tetrapods (Figure I.3A-F). Thus, a part of the lateral pallium subdivision (Dl), generally the ventral part (Dlv), has been considered homologous to the medial pallium in amphibians and other tetrapods (Northcutt, 2006; Portavella et al., 2002; Rodríguez et al., 2002; Yamamoto et al., 2007).

Moreover, with the exception of Yamamoto and coworkers, there is a general consensus that at least part, if not all, of Dm in teleosts is derived from the ventral pallium and that it is homologous to the pallial amygdala in amphibians and other tetrapods. This assumption has been based primarily on topological, genetic, and connectional data (Braford, 1995; Northcutt and Braford, 1980; Puelles and Rubenstein, 1993; Wullimann and Rink, 2002). On the other hand, Yamamoto and coworkers (Ito and Yamamoto, 2009; Yamamoto and Ito, 2008; Yamamoto et al., 2007; see Figure I.3G-J) propose that Dm and the dorsal part of Dl must be homologous to the dorsal pallium in amphibians and the isocortex in tetrapods. This assumption is based on the evidence that Dm and Dl receive their major inputs from the preglomerular complex, which has been proposed as homologous to part of the dorsal thalamus in tetrapods (Ishikawa et al., 2007). Nevertheless, other authors suggest that the preglomerular complex has multiple embryonic origins and are skeptics about that homology (Northcutt, 2008). Furthermore, Mueller and co-workers (2011) have hypothesized that Dc is homologous to the dorsal pallium in tetrapods.

There is also an agreement that the posterior region of the pallium (Dp), the area where a great majority of secondary olfactory projections come together, is homologous to the lateral pallium of amphibians and other tetrapods. However, Butler (2000) and Nieuwenhuys (2011) disagree with this hypothesis; based on the assumption that pallial eversion in ray-finned fishes is simple and complete.

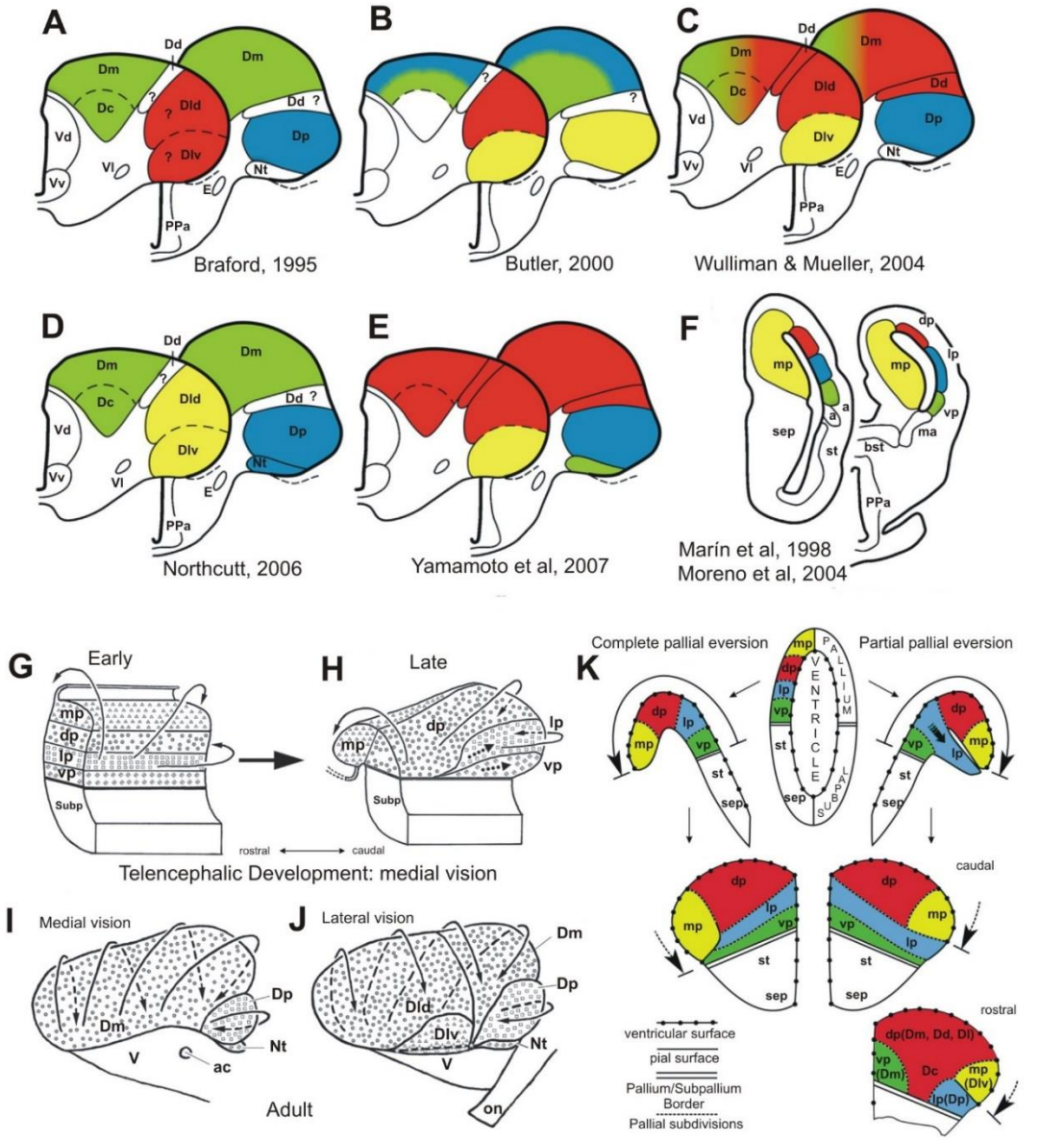

Figure I.3. Recent hypotheses concerning pallial homologies in teleosts. Each hypothesis is illustrated by a rostral and a caudal section of the right cerebral hemisphere of Carassius auratus (A-E) by using a color coding representing the pallial regions supposedly homologous to the pallial regions of an amphibian represented by Rana (F). Question marks indicate uncertainty regarding a specific homology. (G-J) Schematic drawings showing telencephalic development through eversion in actinopterygians, according to Yamamoto and coworkers' hypothesis (2007). (G) Early stage of developing telencephalon maintaining pallial organization similar to other vertebrates. (H) Later stage of telencephalic organization expected if the eversion takes place in direction indicated by arrows. (I-J) Medial (I) and lateral (J) views of adult goldfish telencephalon. (K) Representation of complete vs. incomplete pallial eversion model in teleosts suggested by Wullimann and Mueller (2004). Abbreviations: aa, anterior amygdaloid area; ac, anterior commissure; bst, bed nucleus of the stria medullaris; Dc, central division of area dorsalis; Dd, dorsal subdivision of area dorsalis; Dld, dorsal part of lateral subdivision of area dorsalis; Dlv, ventral part of lateral subdivision of area dorsalis; Dm, medial subdivision of area dorsalis; dp, dorsal pallium; Dp, posterior subdivision of area dorsalis; E, entopeduncular nucleus; lp, lateral pallium; ma, medial amygdala; mp, medial pallium; Nt, nucleus taeniae; on, optic nerve; PPa, anterior parvocellular preoptic nucleus; Subp, subpallial region; sep, septum; st, striatum; V, area ventralis telencephali; Vd, dorsal nucleus of area ventralis; vp,ventral pallium (lateral amygdala); Vv, ventral nucleus of area ventralis. Modified from Northcutt (2008), Yamamoto et al. (2007), and Wullimann and Mueller (2004).

4. Functional Organization of the Teleost Telencephalic Pallium

4.1 Amygdaline Pallium

It has long been known that the telencephalon of teleost fishes is involved in emotional and reproductive behavior (de Bruin, 1980; Segaar and Nieuwenhuys, 1963; Shapiro et al., 1974; Overmier and Gross, 1974). In particular, the medial pallium is a telencephalic structure that seems to play an important role in those aspects of behavior in which the motivational and emotional factors must be taken into account. Studies on fish suggest that lesions to the teleost medial pallium disrupt or disorganize aggressive, sexual and parental behavior (de Bruin, 1983; Segaar and Nieuwenhuys, 1963). In addition, electrical stimulation in the medial pallium in free-swimming fishes evokes arousal, defensive behavior and escape responses (Quick and Laming, 1988; Savage, 1971). According to developmental evidences and similarities in the pattern of gene expression, neurochemical distribution and neuroanatomical comparative evidence as well as behavioral data, the medial telencephalic pallium of the actinopterygian fishes has been considered homologous to the pallial amygdala of amphibians and land vertebrates (Braford, 2009; Desjardins and Fernald, 2010; Mueller and Wullimann, 2009; Northcutt, 2006, 2008; Portavella et al., 2004; Wullimann and Mueller, 2004). The pallial amygdala in mammals is an essential component of the neural circuits responsible for emotional learning and memory (LeDoux, 2000; Maren, 2001; McGaugh, 2004).

The initial evidence concerning the neural basis of emotional conditioning in teleost fishes showed that complete telencephalic ablation produces devastating effects on the acquisition and maintenance of conditioned avoidance (Overmier and Hollis, 1990; Overmier and Papini, 1986), suggesting that the teleost telencephalon is involved in the use of emotional states as conditioned reinforcers to produce instrumental responses (Flood et al., 1976; Mowrer, 1960). In the avoidance conditioning paradigm, the animals learn to prevent the presentation of an unpleasant unconditioned stimulus (US; usually a mild electric shock) by producing a particular conditioned response (CR; such as jumping to a safe area) at the presentation of a

conditioned stimulus (CS; usually a light), which signals the presentation of the US. There is good evidence that avoidance learning is based on the acquisition of a mediational state of fear in goldfish (Gallon, 1972; Overmier and Starkman, 1974).

A recent series of lesion studies have addressed the possibility that the medial pallium of teleost fishes, proposed as homologous to the pallial amygdala of mammals, may play an important role in emotional learning and memory. In this line of reasoning, some of these experiments have showed that, for example, the medial but not the lateral pallium lesions impaired the acquisition and the retention of a conditioned avoidance response in goldfish (Portavella et al., 2003, 2004). The impairment observed in medial pallium lesioned goldfish was similar to that found in mammals with amygdalar lesions (Aggleton, 1992; Davis et al., 1992).

In addition, a recent study have revealed that taste aversion learning, a behavioral paradigm in which values and motivational signals need to be codified, seems to be also dependent of the dorsomedial pallium of teleost (Martín et al., 2011). Goldfish learned to avoid the ingestion of a flavor paired with visceral discomfort, when trained in a delayed procedure consisting of the presentation of two flavors on different days, one followed by lithium chloride and the other by saline, both after a 10-minute delay. Dorsomedial pallium lesions impaired the acquisition of taste aversion, whereas damage to the dorsolateral pallium, the most likely homologue of the hippocampus, did not produce significant changes in this learning. Recent neuroanatomical data show that gustatory and general visceral inputs converge in the dorsomedial pallium supporting the notion of its critical role in taste aversion learning (Folgueira et al., 2003, 2004; Northcutt, 2006; Yoshimoto and Yamamoto, 2010), and suggesting that this region, like the amygdala of mammals, could be a site for the taste-malaise integration necessary for the formation of taste aversion memory in teleosts.

Interestingly, another recent study (Rodríguez-Expósito, 2014) have indicated that a medial and precommissural area of Dm (easily discernible as a bulge in the dorsal aspect of Dm) has an important role in the acquisition of fear classical conditioning, and likely serves functions similar to those of the amygdala in mammals

(Blanchard and Blanchard, 1972; Cousens and Otto, 1998; Iwata and otros, 1986; Kim and otros, 1993; LeDoux, 1993; Maren and otros, 1996a; Sananes and Davis, 1992) as its inactivation by the GABAA receptor agonist muscimol abolished the acquisition of a bradycardia conditioned response (Figure I.4A). Additionally, because several studies in mammals have reported that immediate post-training inactivations of the amygdala impairs memory consolidation processes in other brain-memory systems (McGaugh, 2002), it was also examined whether muscimol infusions immediately after fear training sessions can influence the development of conditioned bradycardia. Dm infusions of muscimol before acquisition testing significantly attenuated conditioned bradycardia (Figure I.4A). It is important to note that no deficit was observed either in the reflex response to the US or in the autonomic orientation response to the CS, indicating that the sensorial and motor neural circuits underlying the expression of the unconditioned heart responses were spared in these goldfish. These data suggest that a specialized region in Dm is involved in the acquisition of fear conditioned learning, and suggest a similar functionality between the teleostean Dm and the mammalian amygdala. As a whole, these functional data demonstrate that the dorsomedial pallium in teleosts is, like the amygdala, an essential component of the telencephalon-dependent emotional learning and memory system and provide further support concerning the homology between both structures (Davis, 1997; Davis and Whalen, 2001; Fanselow and Ledoux, 1999; Kapp et al., 1992; Kim and Jung, 2006; LaBar et al., 1998; Lavond et al., 1993; LeDoux, 1993; 2000; Maren, 2001; Medina et al., 2002; Parkes and Westbrook, 2010; Phelps et al., 2004).

4.2. Hippocampal Pallium

In mammals, birds and reptiles, the hippocampal formation is critical for encoding the features of the environment in map-like or relational memory representations (Bingman et al., 1998; Burgess et al., 1999; Eichenbaum et al., 1999; López et al., 2003a, 2003b; O'Keefe and Nadel, 1978; Rodríguez et al., 2002; Sherry and Duff, 1996; Squire et al., 2004). The telencephalon of ray-finned fishes (e.g. teleosts) presents a medial-to-lateral inversion in the topological position of the main pallial subdivisions. Therefore, the lateral pallium of ray-finned fishes, and in particular the

ventral subdivision (Dlv), is the most likely homologue of the hippocampus or medial pallium of land vertebrates, as it occupies the most distal topological position in the pallium. This homology is confirmed by data regarding its extensive interconnections with the likely homologues of the septal nuclei and preoptic area (Butler and Hodos, 2005; Northcutt, 2006), by the distribution of histochemical and molecular markers (Kapsimali et al., 2000), and by the pattern of neurogenesis and migration of interneurons (Grandel et al., 2006; Zupanc et al., 2005). In addition, results from lesion and functional studies agree with the embryological and anatomical data, showing that the lateral telencephalic pallium of teleost fishes, like the hippocampus of land vertebrates, is involved in spatial cognition.

Fishes trained in a variety of spatial procedures display behavioral abilities probably based on multiple learning and memory systems, and their similarity to those described in mammals and birds outlines the central issue of whether these cognitive capabilities are supported by neural centres and circuits corresponding to those that underlie spatial cognition in land vertebrates. A series of experiments analyzing the effects of selective pallial lesions on the performance of goldfish trained in a variety of spatial learning and memory tasks provided clear evidence concerning the role of the teleost dorsolateral pallium (Dl) in spatial cognition.

Several lesion studies indicate that Dl lesions (mainly Dlv lesions) produce dramatic memory impairments in goldfish trained in a place task (Durán et al., 2010; Rodríguez et al., 2002). In fact, the place memory deficit observed after selective lesions to the ventral dorsolateral pallium in goldfish is as severe as that those produced by the complete ablation of both telencephalic hemispheres (López et al., 2000a; Rodríguez et al., 2002; Salas et al., 1996a). In contrast, dorsomedial (Dm) or dorsal (Dd) pallium lesions does not produce any observable impairment in place memory (Rodríguez et al., 2002). Furthermore, the involvement of goldfish Dlv of goldfish in spatial cognition seems to be selective to the allocentric memory system, as damage to this area does not impair the use of cue or other egocentric strategies (Durán et al., 2010; López et al., 2000a; Salas et al., 1996a, 1996b; Rodríguez et al., 2002).

The first clear-cut data concerning the capacity of teleost fishes to use cognitive mapping, in addition to egocentric orientation strategies, were provided by Rodríguez and colleagues (1994). In this study, goldfish were trained to locate a baited feeder in a four-arm maze surrounded by an array of distal visual cues using three different procedures: (1) a procedure that promoted the use of a strategy based on a particular turn response (egocentric strategy), (2) a procedure that promoted the use of a strategy based on the information provided by the distal visual cues (allocentric strategy), and (3) a procedure mix of both sources of information, i.e., both types of strategies, simultaneously. Interestingly, although the fish in every group achieved similar levels of performance, the transfer and probe tests revealed that they used different strategies. Goldfish trained in the allocentric procedure navigated directly to the rewarded place from previously unvisited start locations, spontaneously adopting the most direct routes to the goal although these new paths involved navigating in different or even opposite directions (Rodríguez et al., 1994). The use of appropriate trajectories without a history of previous training, even when these imply new (never experienced before) egocentric relations to landmarks and local views, provides distinct evidence for the capacity of these animals to represent the environment independently of a body-centered reference system. In addition, the goldfish could use orientation (egocentric) strategies, as indicated by their ability to reach the goal by using a fixed body turn (i.e. turn right or left) disregarding environmental information, or could implement simultaneously both body-centered and allocentric strategies and use one or the other according to experimental conditions. Recently, Schluessel and Bleckmann (2005) obtained experimental results that suggest that also elasmobranchs, which are characterised, as land vertebrates, by the evaginated pattern of telencephalon morphology, can use allocentric strategies for navigation. Like goldfish, rays (Potamotrygon motoro) are able to reach the goal using novel routes starting from unfamiliar locations.

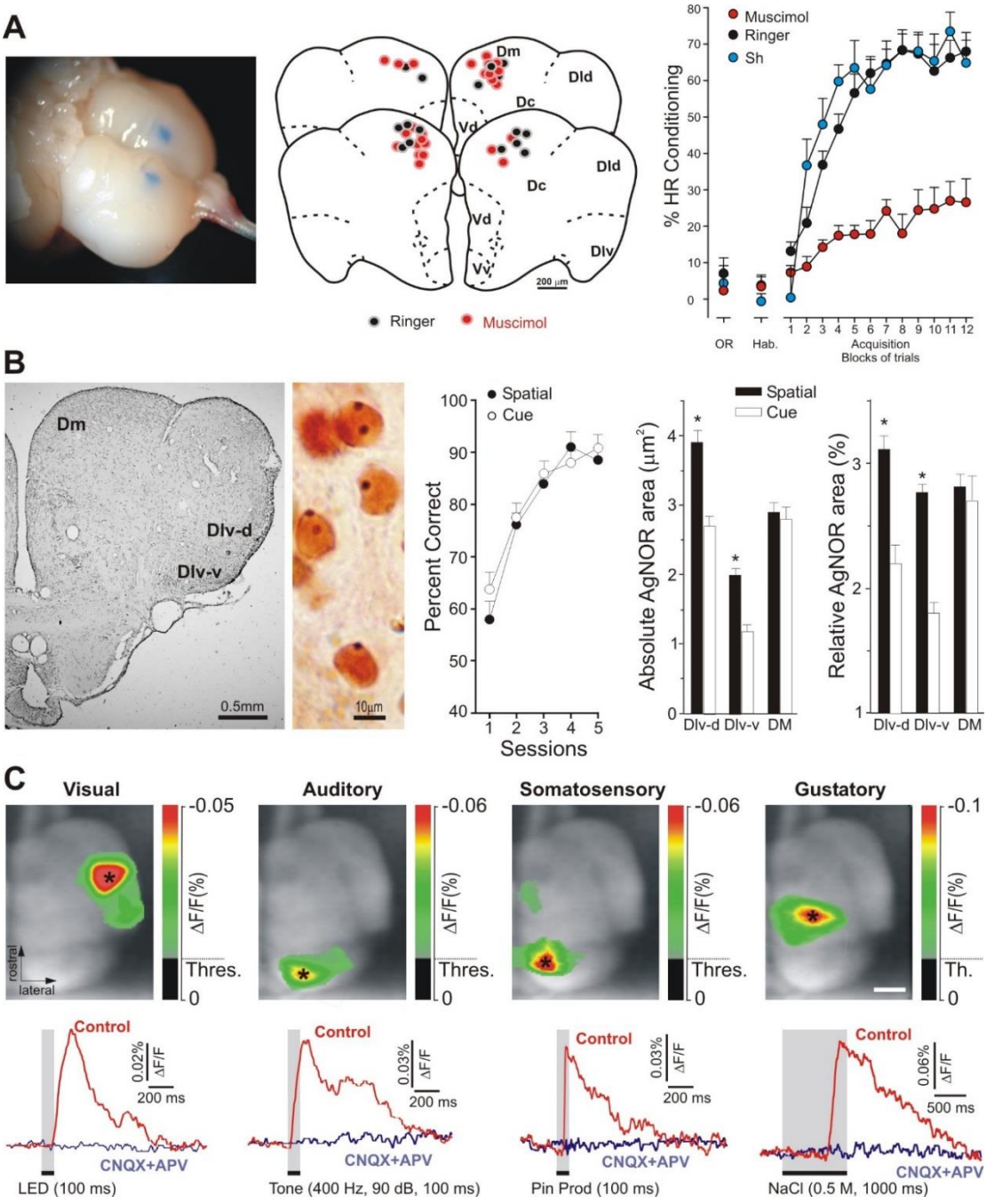

Figure I.4. Functional organization of the teleost pallium. (A) specialized region in goldfish Dm is involved in the acquisition of fear heart rate conditioning. Inactivation by the GABAA receptor agonist muscimol in Dm abolished the acquisition of a bradycardiac conditioned response. Sh sham operated group; OR orientation response; Hab habituation; for more abbreviations see Figure I.1. Modified from Rodríguez-Expósito, 2014. (B) Spatial learning induces morphological changes in Dlv neurons of goldfish. Training in a spatial constancy task produced an increment in the transcriptive activity which was selective to the neurons of Dlv, as indicated by increases in the size of the nucleolar organizing region (NOR), the nucleolar organelles associated with the synthesis of ribosomal proteins. In contrast, training in a cue version of the same task did not produced observable changes. Dlv-d and Dlv-v, dorsal and ventral subdivisions of Dlv. Modified from Broglio et al., 2010. (C) In vivo voltage-sensitive dye imaging reveals segregated visual, auditory, somatosensory and gustatory areas in the goldfish pallium. The optical images show the response evoked by a stimulus of each modality in a representative animal. The curves represent the time course of the VSD signals before (red) and after (blue) pharmacological blockade with CNQX+APV, measured at the region with the maximal activation in the control situation (black asterisk). The grey boxes mark the stimulus duration. Modified from Ocaña et al., 2015.

A distinctive piece of evidence concerning cognitive mapping in goldfish is provided by their efficiency to locate a goal in the absence of some familiar conspicuous environmental cues (Durán et al., 2008; López et al., 1999; Rodríguez et al., 1994). As cognitive maps store redundant environmental information, when a subset of spatial cues becomes unavailable, accurate navigation is still possible on the basis of those that remain (O'Keefe and Nadel, 1978; Thinus-Blanc, 1996). The study by Rodríguez and colleagues (1994) provided interesting evidence in this regard: although the performance of the fishes trained in the allocentric task became as poor as that of the control fishes when all the cues were simultaneously excluded, indicating that they used the information provided by those cues to solve the task, it did not deteriorate when the most salient ones were individually removed or hidden. Similar results were provided by a study performed by Durán and coworkers (2008) in which goldfish were trained in a spatial procedure analogous to the hole-board task commonly used with rodents, and in other study performed by López and coworkers (1999) in which goldfish were trained in a spatial constancy task or in a cued version of the same procedure. All of these results indicate that the performance of goldfish trained in allocentric, relational spatial tasks is based on the knowledge of the relationships among the goal location and many environmental cues, such that when some of them are missing, the remaining ones are sufficient to locate the goal. Moreover, fishes can simultaneously encode the spatial relationships among landmarks and the shape (geometry) of the environmental boundaries (Broglio et al., 2000; Sovrano et al., 2002, 2003, 2007; Vargas et al., 2004). These results reveal that fishes, like mammals and birds, are able to integrate spatial information of different nature and from various sources, for allocentric navigation (Gallistel, 1990; Cheng, 1986; Cheng and Gallistel, 1984; Cheng and Newcombe, 2005; Chiesa et al., 2006).

Consistent results were obtained when studying telencephalon ablation during reversal learning. The use of allocentric or relational frames of reference for navigation endows spatial behavior with remarkable flexibility, evident also when the goal location is changed after the mastering of the task (reversal learning). Thus, goldfish trained in allocentric or relational spatial tasks show faster reversal learning than fishes

trained in egocentric procedures, and relative to their own learning rate in acquisition (López et al., 1999, 2000b; Rodríguez et al., 1994). In both cases, the ablated goldfish needed more trials to learn the new goal location relative to their own initial learning and to the sham group (Salas et al., 1996a, 1996b). Indeed, the reversal performance of the ablated fishes trained in the place and the spatial constancy task did not differ from that of the animals trained in the turn procedure (Salas et al., 1996a) or in a cue version of the spatial constancy task (Salas et al., 1996b), indicating that they had lost the behavioral flexibility that characterizes the use of allocentric frames of reference. These reversal learning deficits are similar to those observed in mammals and birds with hippocampal lesions (Good, 1987; Good and MacPhail, 1994; Hampton et al., 2004; Hirsch and Segal, 1972; Nonneman et al., 1974).

Additional evidence has been obtained from a recent experiment aimed to analyze possible learning-related changes in the transcriptive activity of the pallial neurons in goldfish trained in a spatial task or in a cue version of the same procedure (Broglio et al., 2010; Figure I.4B). The results showed that training in the spatial constancy task produced an increment in the transcriptive activity in the neurons of the ventral part of the dorsolateral pallium, as indicated by increases in the size of the nucleolar organizing region (NOR), i.e. the nucleolar organelles associated with the synthesis of ribosomal proteins (Derenzini, 2000). Moreover, these changes were selective to training in the spatial constancy task as training in the cue version of the same procedure did not produce observable changes, although the tasks used in this experiment were characterized by identical visual complexity and response requirements, and only differed on the type of spatial representations necessary to succeed (López et al., 1999; Salas et al., 1996b).

In summary, the data presented here clearly show that the ventral subdivision of the dorsolateral telencephalic pallium of teleost fishes, like the medial cortex or hippocampus of amniotes, underlies the ability of fishes to navigate on the basis of allocentric representations of the environment. These results are consistent with developmental and neuroanatomical data and provide additional evidence regarding the homology of the teleost lateral pallium with the hippocampal pallium in vertebrates with evaginated telencephala.

4.3. Sensory Pallium

The teleost telencephalic pallium receives inputs from all sensory modalities, similar to the tetrapod cortex (Nieuwenhuys et al., 1998; Wullimann and Vernier, 2009; Yamamoto et al., 2007). The main olfacto-recipient area is located in a limited portion of the teleost pallium, the posterior region of area dorsalis (Dp; Levine and Dethier, 1985; Northcutt, 2006); in contrast, visual and gustatory inputs reach mainly the lateral (Dl) and medial (Dm) regions of the area dorsalis, respectively; and mechanosensory and auditory ascending pathways reach both Dl and Dm (Folgueira et al., 2003, 2004; Kato et al., 2012; Northcutt, 2006; Rink and Wullimann, 1998; Striedter, 1991; Yamamoto and Ito, 2005, 2008). Although hodological data indicate that Dm and Dl are the main recipients of diencephalic sensory inputs, functional studies are still scarce and critically needed (Echteler, 1984, 1985a; Kanwal et al., 1988; Prechtl et al., 1998; Rakic et al., 1979; Saidel et al., 2001). In fact, little is known about the kind of sensory processing performed by the teleost fish pallium and how non-olfactory sensory modalities are represented in this structure, and whether the functional properties of teleost fish pallium sensory areas are comparable to those of the tetrapod isocortex.

In a recent study Ocaña and colleagues (2015) used in vivo voltage-sensitive dye imaging, which allows the simultaneous recording of activity in different neuron populations with high spatio-temporal resolution (Chemla and Chavane, 2010; Grinvald and Hildesheim, 2004), to functionally localize Dm and Dl sensory areas in goldfish and to elucidate whether they are segregated and topographically organized. In an acute in vivo goldfish preparation the optical activity evoked by visual, auditory, somatosensory, and gustatory stimuli was recorded to functionally locate the pallial areas involved in sensory processing and to determine their temporal dynamics. This study demonstrated that the teleost pallium contains segregated sensory areas with functional properties that resemble those of the tetrapod isocortex and may also be responsible for complex representation and sensory stimuli processing (Fig 4Cxx).

The results showed that stimuli of different modalities activated distinct and well-localized pallial domains in Dm and Dl, which were highly consistent across animals. Data revealed that the visual area is located in the medio-caudal region of Dld, whereas auditory, somatosensory and gustatory information are represented in circumscribed zones within Dm (i.e. the gustatory area) immediately rostral to the caudal sulcus, and the somatosensory and auditory areas in partially overlapped zones of the caudal Dm (Ocaña et al., 2015). These results are consistent with physiological studies that describe that Dld is involved in visual processing (Prechtl et al., 1998; Rakic et al., 1979; Saidel et al., 2001), and they extend those findings by demonstrating that this area is a unimodal region —i.e. it is activated exclusively by visual stimuli. Previous works also reported gustatory-, auditory- and somatosensory-related activity in Dm (Echteler, 1985; Kanwal et al., 1988; Prechtl et al., 1998). Nevertheless, most of these studies focused on recording the activity evoked by few modalities (two at most) within a particular region, rather than on mapping the entire pallium. Prechtl and colleagues (1998), analyzed the responses evoked by four different modalities and suggested the existence of segregated sensory areas in the mormyrid fish pallium; however, their electrophysiological recording approach did not allow them to determine how stimuli of different modalities are represented in the pallium or precisely describe the location and topological relationships of the different sensory areas. The optical imaging data of Ocaña and colleagues (2015) show that the gustatory area is located immediately rostral to the anterior commissure and caudal sulcus, and that the auditory area is located immediately caudal to it. Somatosensory stimulation activated two different regions of Dm (caudal, Dmc and ventral, Dmv). Based on the higher depolarization, longer duration, and shorter latency of the responses a primary role of Dmc for processing somatosensory information it has been proposed. In contrast, Dmv activation could reflect the processing of fearful or unpleasant attributes of touch on the body surface. As reviewed in the previous sections, Dmv is critically involved in behaviors with significant emotional components (Broglio et al., 2005; Desjardins and Fernald, 2010; Lau et al., 2011; Martín et al., 2011; Portavella et al., 2004) and has been proposed to be the homologue of the mammalian pallial amygdala (Braford, 1995; Wullimann and Mueller, 2004; Wullimann and Rink, 2002).

5. References

Aggleton, J.P. (1992). The Amygdala: Neurobiological Aspects of Emotion, Memory, and Mental Dysfunction. New York. Wiley-Liss.

Ariëns-Kappers, C.U., Huber, G.C. and Crosby, E.C. (1936). The Comparative Anatomy of the Nervous System of Vertebrates, Including Man. New York. Macmillan Publishing Company.

Bingman, V.P., Riters, L.V., Strasser, R. and Gagliardo, A. (1998) Neuroethology of avian navigation. In: R. Balda, I. Pepperberg and A. Kamil (eds) Animal Cognition in Nature, pp. 201–226. Academic Press, New York.

Blanchard, D.C. and Blanchard, R.J. (1972). Innate and conditioned reactions to threat in rats with amygdaloid lesions. Journal of Comparative and Physiological Psychology, 81, 281–290.

Braford, M.R. Jr. (1995). Comparative aspects of forebrain organization in the ray-finned fishes: touchstones or not? Brain, Behavior and Evolution, 46, 259–274.

Braford, M.R. Jr. (2009). Stalking the everted telencephalon: comparisons of forebrain organization in basal ray-finned fishes and teleosts. Brain, Behavior and Evolution, 74, 56–76.

Blanchard, D.C. and Blanchard, R.J. (1972). Innate and conditioned reactions to threat in rats with amygdaloid lesions. Journal of Comparative and Physiological Psychology, 81, 281–290.

Broglio, C., Gómez, Y., López, J.C., Rodríguez, F., Salas, C. and Vargas, J.P. (2000) Encoding of geometric and featural properties of a spatial environment in teleostean fish (Carassius auratus). XXVII International Congress of Psychology, Stockholm.

Broglio, C., Gómez, A., Durán, E., Ocaña, F.M., Jiménez-Moya, F., Rodríguez, F. and Salas, C. (2005). Hallmarks of a common forebrain vertebrate plan: specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Research Bulletin, 66, 277–281.

Broglio, C., Rodríguez, F., Gómez, A., Arias, J.L. and Salas, C. (2010). Selective involvement of the goldfish lateral pallium in spatial memory. Behavioural Brain Research, 210, 191–201.

Broglio, C., Gómez, A., Durán, Salas, C., and F., Rodríguez, F. (2011) Brain and cognition in teleost fish. In Culum Brown, Kevin Laland and Jens Krause. Blackwell Publishing Ltd. 66, 277-281.

Burgess, N., Jeffery, K.J. and O'Keefe, J. (1999). The Hippocampal and Parietal Foundations of Spatial Cognition. Oxford University Press, London.

Burmeister, S.S., Munshi, R.G. and Fernald, R.D. (2009). Cytoarchitecture of a cichlid fish telencephalon. Brain, Behavior and Evolution, 74, 110–120.

Butler, A.B. (2000). Topography and topology of the teleost telencephalon: a paradox resolved, Neuroscience Letters, 293, 95–98.

Butler, A.B. and Hodos, H. (2005). Comparative Vertebrate Neuroanatomy: Evolution and Adaptation, 2nd Edition. New York. Wiley-Liss.

Chemla, S. and Chavane, F. (2010) Voltage-sensitive dye imaging: technique review and models. J. Physiol. (Paris), 104, 40-50.

Cheng, K. (1986) A purely geometric module in the rat's spatial representation. Cognition, 23, 149–178.

Cheng, K. and Gallistel, C.R. (1984) Testing the geometric power of an animal's spatial representation. In: H.L. Roitblat, T.G. Bever and H.S. Terrace (eds) Animal Cognition, pp. 409–423. Erlbaum, New Jersey.

Cheng, K. and Newcombe, N.S. (2005) Is there a geometric module for spatial orientation? Squaring theory and evidence. Psychonomic Bulletin & Review, 12, 1–23.

Chiesa, A.D., Speranza, M., Tommasi, L. and Vallortigara, G. (2006) Spatial cognition based on geometry and landmarks in the domestic chick (Gallus gallus). Behavioural Brain Research, 175, 119–127.

Cousens, G and Otto, T. (1998). Both pre- and posttraining excitotoxic lesions of the basolateral amygdala abolish the expression of olfactory and contextual fear conditioning. Behavioral Neuroscience, 112, 1092–103.

Crosby, E.C. and Schnitzlein, H.N. (1983). Comparative Correlative Neuroanatomy of the Vertebrate Telencephalon. New York. Macmillan Publishing Co.

Davis, M. (1992). The role of the amygdala in fear and anxiety. Annual Review of Neuroscience, 15, 353–375.

Davis, M. (1997). Neurobiology of fear responses: the role of the amygdala. The Journal of Neuropsychiatry and Clinical Neurosciences, 9, 382–402.

Davis, M. and Whalen, P.J. (2001). The amygdala: vigilance and emotion. Molecular Psychiatry, 6, 13–34.

de Bruin, J.P.C. (1980). Telencephalon and behavior in teleost fish. A neuroethological approach. En: Ebbesson, S.O.E. (Ed.). Comparative Neurology of the Telencephalon. New York. Plenum Press.

de Bruin, J.P.C. (1983). Neural correlates of motivated behavior in teleost fish. En: Ewert, J.P., Capranica, R.R. e Ingle, D.J. (Eds.). Advances in Vertebrate Neuroethology. 969–995. New York, Plenum Press.

Demski, L.S. (2013). The Pallium and mind Behavior Relationships in Teleost Fishes. Brain, Behavior and Evolution, 82, 31–44.

Demski, L.S and Beaver, J.A. (2001). Brain and cognitive function in teleost fishes. En: Roth, G. y Wullimann, M.F. (Eds.). Brain, Evolution and Cognition. 297–332. New York, Wiley.

Derenzini, M. (2000). The AgNORs. Micron, 31, 117–120.

Desjardins, J.K. and Fernald, R.D. (2010). What do fish make of mirror images? Biology Letters, 6, 744–747.

Durán, E., Ocaña, F.M., Broglio, C., Rodríguez, F. and Salas, C. (2010). Lateral but not medial telencephalic pallium ablation impairs the use of goldfish spatial allocentric strategies in a "hole-board" task. Behavioural Brain Research, 214, 480–487.

Echteler, S.M. (1984) Connections of the auditory midbrain in a teleost fish, Cyprinus carpio. J. Comp. Neurol., 230, 536-551.

Echteler, S.M. (1985a) Organization of central auditory pathways in a teleost fish, Cyprinus carpio. J. Comp. Neurol., 156, 267-280.

Eichenbaum, H., Dudchenko, P., Wood, E., Shapiro, M. and Tanila, H. (1999) The hippocampus, memory, and place cells: is it spatial memory or memory space? Neuron, 23, 1–20.

Fanselow, M.S. and LeDoux, J.E. (1999). Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. Neuron, 23, 229–232.

Flood, N.B., Overmier, J.B. and Savage, G.E. (1976). The teleost telencephalon and learning: an interpretative review of data and hypotheses. Physiology & Behavior, 16, 783–798.

Folgueira, M., Anadón, R. and Yánez, J. (2003). Experimental study of the connections of the gustatory system in the rainbow trout, Oncorhynchus mykiss. Journal of Comparative Neurology, 465, 604–619.

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

Gallon, R.L. (1972). Effects of pre-trainingwith fear and escape conditioning on shuttle-box avoidance acquisition by goldfish. Psychological Reports, 31, 919–924.

Good, M. (1987). The effects of hippocampal-area parahippocampalis lesions on discrimination learning in the pigeon. Behavioural Brain Research, 31, 207–220.

Good, M. and Macphail, E.M. (1994). The avian hippocampus and short-term memory for spatial and non-spatial information. The Quarterly Journal of Experimental Psychology, 47, 293–317.

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I. and Brand, M. (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. Developmental Biology, 295, 263–277.

Grinvald, A. and Hildesheim, R. (2004) VSDI, a new era in functional brain imaging of cortical dynamics. Nat. Rev. Neurosci., 5, 873-884.

Hampton, R.R., Sherry, D.F., Shettleworth, S.J., Khurgel, M. and Ivy, G. (1995). Hippocampal volume and food-storing behavior are related in parids. Brain, Behavior and Evolution, 45, 54–61.

Hirsch, R. and Segal, M. (1972). Complete transection of the fornix and reversal of position in the rat. Physiology & Behavior, 8, 1051–1054.

Ishikawa, Y., Yamamoto, N., Yoshimoto, M., Yasuda, T., Maruyama, K., Kage, T., Takeda, H. and Ito, H. (2007). Developmental origin of diencephalic sensory relay nuclei in teleosts, Brain Behavior and Evolution, 69, 87–95.

Ito, H. and Yamamoto ,N. (2009). Non-laminar cerebral cortex in teleost fishes? Biology Letters, 5(1), 117-21.

Iwata, J., LeDoux, J.E. and Reis, D.J., (1986). Destruction of intrinsic neurons in the lateral hypothalamus disrupts the classical conditioning of autonomic but not behavioral emotional responses in the rat. Brain Research, 368, 161–166

Jerison, H. (1973) Evolution of the Brain and Intelligence. Academic Press, New York.

Kanwal, J.S., Finger, T.E. and Caprio, J. (1988) Forebrain connections of the gustatory system in ictalurid catfishes. J. Comp. Neurol., 278, 353-376.

Kapp, B.S., Whalen, P.J., Supple, W.F. and Pascoe, J.P. (1992). Amygdaloid contributions to conditioned arousal and sensory information processing. En: Aggleton, J.P. (Ed.). The Amygdala: Neurobiological Aspects of Emotion, Memory, and Mental Dysfunction. 229–254. New York. Wiley-Liss.

Kapsimali, M., Vidal, B., Gonzalez, A., Dufour, S. and Vernier, P. (2000). Distribution of the mRNA encoding the four dopamine D(1) receptor subtypes in the brain of the European eel (Anguilla anguilla): comparative approach to the function of D(1) receptors in vertebrates. Journal of Comparative Neurology, 419, 20–43.

Kato, T., Yamada, Y. and Yamamoto, N. (2012) Ascending gustatory pathways to the telencephalon in goldfish. J. Comp. Neurol., 520, 2475-2499.

Kim, J.J., Rison, R.A. and Fanselow, M.S. (1993). Effects of amygdala, hippocampus, and periaqueductal gray lesions on short- and long-term contextual fear. Behavioral Neuroscience, 107, 1093–1098.

Kim, M. and Jung, M.W. (2006). Neural circuits and mechanisms involved in Pavlovian fear conditioning: A critical review. Neuroscience Biobehavioural Review, 30(2), 188–202.

LaBar, K.S., Gatenby, J.C., Gore, J. C., LeDoux, J.E. and Phelps, E.A. (1998). Human amygdala activation during conditioned fear acquisition and extinction: A mixed-trial fMRI study. Neuron, 20(5), 937–945.

Lau, B.Y., Mathur, P., Gould, G.G. and Guo, S. (2011) Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc. Natl. Acad. Sci., 108, 2581-2586.

Lavond, D.G., Kim, J.J. and Thompson ,R.F. (1993). Mammalian brain substrates of aversive classical conditioning. Annual Review of Psychology, 44, 317–342.

LeDoux, J.E. (1993). Emotional memory systems in the brain. Behavioural Brain Research, 58(1–2), 69–79.

LeDoux, J.E. (2000). Emotion circuits in the brain. Annual Review of Neuroscience, 23, 155–184.

Levine, R.L. and Dethier, S. (1985). The connections between the olfactory bulb and the brain in the goldfish, The Journal of Comparative Neurology, 237, 427–444.

López, J.C., Broglio, C., Rodríguez, F., Thinus-Blanc, C. and Salas, C. (1999). Multiple spatial learning strategies in goldfish (Carassius auratus). Animal Cognition, 2, 109–120.

López, J.C., Bingman, V.P., Rodríguez, F., Gómez, Y. and Salas, C. (2000). Dissociation of place and cue learning by telencephalic ablation in goldfish. Behavioral Neuroscience, 114, 687–699.

López, J.C., Gomez, Y., Vargas, J.P. and Salas, C. (2003a) Spatial reversal learning deficit after medial cortex lesion in turtles. Neuroscience Letters, 341, 197–200.

López, J.C., Vargas, J.P., Gómez, Y. and Salas, C. (2003b) Spatial and non-spatial learning in turtles: the role of medial cortex. Behavioural Brain Research, 143, 109–120.

MacLean, P. (1990) The Triune Brain in Evolution. New York. Plenum Press.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. Annual Review of Neuroscience, 24, 897–931.

Maren, S., Aharonov, G., Stote, D.L. and Fanselow, M.S. (1996a). Retrograde abolition of conditional fear after excitotoxic lesions in the basolateral amygdala of rats: absence of a temporal gradient. Behavioral Neuroscience, 110, 718–26.

Martín, I., Gómez, A., Salas, C., Puerto, A. and Rodríguez, F. (2011). Dorsomedial pallium lesions impair taste aversion learning in goldfish. Neurobiology of Learning and Memory, 96, 297–305.

McGaugh, J.L. (2002). Memory consolidation and the amygdala: a systems perspective. Trends in Neurosciences, 25, 456–461.

McGaugh, J.L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annual Review of Neuroscience, 27, 1–28

Medina, J.F., Christopher Repa, J., Mauk, M.D. and LeDoux, J.E. (2002). Parallels between cerebellum- and amygdala-dependent conditioning. Nature Reviews Neuroscience, 3, 122–131.

Mowrer, O.H. (1960). Learning Theory and Behavior. John Wiley & Sons. New York.

Mueller, T. and Wullimann, M.F. (2009). An evolutionary interpretation of teleostean forebrain anatomy. Brain, Behavior and Evolution, 74, 30–42.

Mueller, T., Dong, Z., Berberoglu, M.A. and Guo, S. (2011). The dorsal pallium in zebrafish, Danio rerio (Cyprinidae, Teleostei). Brain Research, 138, 95–105.

Murakami, T., Morita, Y. and Ito, H. (1983). Extrinsic and intrinsic fiber connections of the telencephalon in a teleost, Sebastiscus marmoratus, The Journal of Comparative Neurology, 216, 115–131.

Nieuwenhuys, R. (1963) The comparative anatomy of the actinopterygian forebrain. J Hirnforsch 13:171–192

Nieuwenhuys, R., ten Donkelaar, H.J. and Nicholson, C. (1998). The Central Nervous System of Vertebrates. Berlin. Springer-Verlag.

Nieuwenhuys, R. (2011). The development and general morphology of the telencephalon of actinopterygian fishes: synopsis, documentation and commentary. Brain Structure & Function, 215, 141–157.

Nonneman, A.J., Voigt, J. and Kolb, B.E. (1974). Comparisons of behavioural effects of hippocampal and prefrontal cortex lesions in the rat. Journal of Comparative and Physiological Psychology, 87, 249–260.

Northcutt, R.G. (2006). Connections of the lateral and medial divisions of the goldfish telencephalic pallium. The Journal of Comparative Neurology, 494, 903–943.

Northcutt, R.G. (2008). Forebrain evolution in bony fishes. Brain Research Bulletin, 75, 191–205.

Northcutt, R.G. and Braford, M.R. Jr. (1980). New observation on the organization and evolution of the telencephalon of the actinopterygian fishes. En: Ebbeson, S.O.E. (Ed.) Comparative neurology of the telencephalon. 41–98. New York. Plenum Press.

O'Keefe ,J. and Nadel, L. (1978). The hippocampus as a cognitive map. London: Oxford UP.

Overmier, J.B. and Gross, D. (1974). Effects of telencephalic ablation upon nest-building and avoidance behaviors in East African mouth breeding fish, Tilapia mossambica. Behavioral Biology, 12, 211–222.

Overmier, J.B. and Starkman, N. (1974). Transfer of control of avoidance behavior in normal and telencephalon ablated goldfish (Carassius auratus). Physiology & Behavior, 12, 605–608.

Overmier, J.B. and Papini, M.R. (1986). Factors modulating the effects of teleost telencephalon ablation on retention, relearning, and extinction of instrumental avoidance behavior. Behavioral Neuroscience, 100, 190–199.

Overmier, J.B. and Hollis,K.L. (1990). Fish in the think tank: learning, memory and integrated behavior. En: Kesner, R.P. y Olton, D.S. (Eds.). Neurobiology of Comparative Cognition. 204–236. Hillsdale. Lawrence Erlbaum Associates.

Papez, J. (1929). Comparative Neurology. New York. Crowell.

Parkes, S.L. and Westbrook, R.F. (2010). The basolateral amygdala is critical for the acquisition and extinction of associations between a neutral stimulus and a learned danger signal but not between two neutral stimuli. Journal of Neuroscience, 30(38), 12608–12618.

Phelps, E.A., Delgado, M.R., Nearing, K.I. and LeDoux, J.E. (2004). Extinction learning in humans: Role of the amygdala and vmPFC. Neuron, 43(6), 897–905.

Portavella, M., Vargas, J.P., Torres, B. and Salas, C. (2002). The effects of telencephalic pallial lesions on spatial, temporal, and emotional learning in goldfish. Brain Research Bulletin, 57, 397–399.

Portavella, M., Vargas, J.P., Salas, C. and Papini, M. (2003). Involvement of the telencephalon in spaced-trial avoidance learning in the goldfish (Carassius auratus). Physiology & Behavior, 80, 49–56.

Portavella, M., Torres, B. and Salas, C. (2004). Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. Journal of Neuroscience, 24, 2335–2342.

Prechtl, J.C., von der Emde, G., Wolfart, J., Karamürsel, S., Akoev, G.N., Andrianov, Y.N. and Bullock, T.H. (1998) Sensory processing in the pallium of a mormyrid fish. J. Neurosci., 18, 7381-7393.

Puelles, L. (2001). Brain segmentation and forebrain development inamniotes. Brain Research Bulletin, 55, 695–710.

Puelles, L. and Rubenstein, J.L.R. (1993). Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggests a neuromeric organization. Trends in Neuroscience, 16, 472–479.

Puelles, L., Kuwana, E., Puelles, E., Bulfone, A., Shimamura, K., Keleher, J., Smiga, S. and Rubenstein, J.L.R. (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. Journal of Comparative Neurology, 424, 409–438.

Puelles, L., Martínez, S., Martínez-de-la-Torre, M. and Rubenstein, J.L.R. (2004). Gene maps and related histogenetic domains in the forebrain and midbrain. En: Paxinos, G. (Ed.). The Rat Nervous System. 3–25. New York. Elsevier.

Quick, I.A. and Laming, P.R. (1988). Cardiac, ventilatory and behavioural arousal responses evoked by electrical stimulation in the goldfish (Carassius auratus). Physiology & Behavior, 43, 715–727.

Rakic, L., Belekhova, M.G. and Konevi, D. (1979) Visual projections in the telencephalon and diencephalon of the teleost Serranus scriva. Zh. Evol. Biokhim. Fiziol., 15, 357-367.

Rink, E. and Wullimann, M.F. (1998) Some forebrain connections of the gustatory system in the goldfish Carassius auratus visualized by separate DiI application to the hypothalamic inferior lobe and the torus lateralis. J. Comp. Neurol., 394, 152-170.

Rodríguez, F., Durán, E., Vargas, J.P., Torres, B. and Salas, C. (1994) Performance of goldfish trained in allocentric and egocentric maze procedures suggests the presence of a cognitive mapping system in fishes. Animal Learning and Behavior, 22, 409–420.

Rodríguez, F., López, J.C., Vargas, J.P., Gómez, Y., Broglio, C. and Salas, C. (2002). Conservation of spatial memory function in the pallial forebrain of amniotes and ray-finned fishes. Journal of Neuroscience, 22, 2894–2903.

Romer, A.S. (1962) The Vertebrate Body. Saunders, Philadelphia.

Saidel, W.M., Marquez-Houston, K. and Butler, A.B. (2001) Identification of visual pallial telencephalon in the goldfish, Carassius auratus: a combined cytochrome oxidase and electrophysiological study. Brain Res., 919, 82-93.

Salas, C., Broglio, C., Rodríguez, F., López, J.C., Portavella, M. and Torres, B. (1996a). Telencephalic ablation in goldfish impairs performance in a spatial constancy problem but not in a cued one. Behavioural Brain Research, 79, 193–200.

Salas, C., Rodríguez, F., Vargas, J.P., Durán, E. and Torres, B. (1996b). Spatial learning and memory deficits after telencepalic ablation in goldfish trained in place and turn maze procedures. Behavioral Neuroscience, 110, 965–980.

Salas, C., Broglio, C. and Rodríguez, F. (2003). Evolution of forebrain and spatial cognition in vertebrates: conservation across diversity. Brain, Behavior and Evolution, 62, 72–82.

Sananes, C.B. and Davis, M. (1992). N-methyl-D-aspartate lesions of the lateral and basolateral nuclei of the amygdala block fear-potentiated startle and shock sensitization of startle. Behavioral Neuroscience, 106, 72–80.

Savage, G.E. (1971). Behavioural effects of electrical stimulation of the telencephalon of the goldfish, Carassius auratus. Animal Behaviour, 19, 661–668.

Schluessel, V. and Bleckmann, H. (2005) Spatialmemory and orientation strategies in the elasmobranch Potamotrygon motoro. Journal of Comparative Physiology A, 191, 695–706.

Schroeder, D.M. (1980). The telencephalon of teleosts. En: Ebbesson, S.O.E. (Ed.). Comparative Neurology of the Telencephalon. 99–115. New York. Plenum Press.

Shapiro, S.M., Schuckman, H., Sussman, D. and Tucker, A.M. (1974). Effect of telencephalic lesions on the gill cover response of Siamese fighting fish. Physiology & Behavior, 13, 749–755.

Sherry, D.F. and Duff, S.J. (1996) Behavioral and neural bases of orientation in food storing birds. Journal of Experimental Biology, 199, 165–172.

Segaar, J. and Nieywenhuys, R. (1963). New etho-physiological experiments with male Gasterosteus aculeatus, with anatomical comment. Animal Behaviour, 11, 331–344.

Sovrano, V.A., Bisazza, A. and Vallortigara, G. (2002) Modularity and spatial reorientation in a simple mind: encoding of geometric and nongeometric properties of a spatial environment by fish.iCognition, 85, 51–59.

Sovrano, V.A., Bisazza, A. and Vallortigara, G. (2003) Modularity as a fish (Xenotoca eiseni) views it: conjoining geometric and nongeometric information for spatial reorientation. Journal of Experimental Psychology. Animal Behavior Processes, 29, 199–210.

Sovrano, V.A., Bisazza, A. and Vallortigara, G. (2007) How fish do geometry in large and in small spaces. Animal Cognition, 10, 47–54.

Squire, L.R., Stark, C.E. and Clark RE. (2004) The medial temporal lobe. Annual Review of Neuroscience, 27, 279–306.

Striedter, G.F. (1991) Auditory, electrosensory and mechanosensory lateral line pathways through the forebrain of channel catfishes. J. Comp. Neurol., 312, 311-331.

Striedter, G.F. (2005). Principles of Brain Evolution. Sunderland. Sinauer Associates.

Striedter, G.F. and Northcutt, R.G. (2006). Head size constrains forebrain development and evolution in ray-finned fishes. Evolution & Development, 8, 215–222.

Thinus-Blanc, C. (1996) Animal Spatial Cognition. Behavioral and Neural Approaches. World Scientific Publishing Co., Singapore.

Vargas, J.P., Lopez, J.C., Salas, C. and Thinus-Blanc, C. (2004) Encoding of geometric and featural spatial information by goldfish (Carassius auratus). Journal of Comparative Psychology, 118, 206–216.

Wullimann, M.F. and Rink, E. (2002). The teleostean forebrain: a comparative and developmental view based on early proliferation, Pax6 activity and catecholaminergic organization. Brain Research Bulletin, 57, 363–370.

Wullimann, M.F. and Mueller, T. (2004). Teleostean and mammalian forebrains contrasted: evidence from genes to behavior. The Journal of Comparative Neurology, 475, 143–162.

Wullimann, M.F. and Vernier, P. (2009) Evolution of the telencephalon in anamniotes. In Binder, M.D., Hirokawa, N. and Windhorst, U. (eds), Encyclopedia of Neuroscience. Springer-Verlag, Berlin, Vol 2, pp. 1424-1431.

Yamamoto, N. and Ito, H. (2005). Fiber connections of the anterior preglomerular nucleus in cyprinids with notes on telencephalic connections of the preglomerular complex. The Journal of Comparative Neurology, 491, 212–233.

Yamamoto, N., Ishikawa, Y., Yoshimoto, M., Xue, H.G., Bahaxar, N., Sawai, N., Yang, C.Y., Ozawa, H. and Ito, H. (2007). A new interpretation on the homology of the teleostean telencephalon based on hodology and a new eversion model. Brain, Behavior and Evolution, 69, 96–104.

Yamamoto, N. and Ito, H. (2008). Visual, lateral line, and auditory ascending pathways to the dorsal telencephalic area through the rostrolateral region of the lateral preglomerular nucleus in cyprinids. The Journal of Comparative Neurology, 508(4), 615-47.

Yoshimoto, M. and Yamamoto, N. (2010). Ascending general visceral sensory pathways from the brainstem to the forebrain in a cichlid fish, Oreochromis niloticus (Tilapia). Journal of Comparative Neurology, 518, 3570–3603.

Zupanc, G.K.H., Hinsch, K. and Gage, F.H. (2005). Proliferation, migration, neuronal differentiation, and long-term survival of new cells in the adult zebrafish brain. Journal of Comparative Neurology, 488, 290–319.

AIMS

The data reviewed above indicate that the telencephalic pallium of teleost fish shows a complex organization and that it could contain specialized areas involved in sensorial and cognitive processing, including several forms of learning and memory. These results also indicate that such different memory systems have separate neural basis. More specifically, the areas of the teleost fish telencephalic pallium that have been proposed as homologous to the hippocampus of land vertebrates seems to be an essential part of a specialized memory system involved in spatial and relational information processing. On the contrary, the areas of the teleost pallium considered homologous to the amygdala seem to be part of a memory system specialized in the processing of the emotional aspects of the experience.

The general aim of the present research is to analyze the functional organization of the teleost fish telencephalic pallium in regard to the spatial and emotional learning and memory processes. In particular, to mapping the neural activity of the goldfish pallial areas involved in spatial learning by means of quantitative cytochrome oxidase histochemistry (Experiments 1 and 2), and to analyze the spatio-temporal activity patterns produced in the goldfish telencephalic pallium during emotional learning and memory by means of optical recording using voltage sensitive dyes (Experiment 3).

EXPERIMENT 1. SPATIAL LEARNING-RELATED CHANGES IN METABOLIC BRAIN ACTIVITY CONTRIBUTES TO THE DELIMITATION OF THE HIPPOCAMPAL PALLIUM IN GOLDFISH

1. Introduction

Among vertebrates, the actinopterygian fish, e.g. teleosts, represent a separate case because of the particular anatomical organization of their forebrain. The telencephalic hemispheres of this vertebrate group undergo a unique embryonic development (i.e. the eversion or outward bending of the distal walls of the prosencephalic vesicle) that produces solid telencephalic hemispheres separated by a single ventricle, as well as the reversal of the pallial topography observed in non-actinopterygians, e.g. land vertebrates (Braford, 1995; Nieuwenhuys, 1963, 2011; Northcutt, 1995, 2006; Northcutt and Braford, 1980; Striedter and Northcutt, 2006). As a consequence, the teleostean area homologous to the hippocampal or medial pallium is hypothesized to occupy a lateral position in the telencephalic pallium, whereas the region comparable to the pallial amygdala would occupy a medial position (Butler, 2000; Nieuwenhuys and Meek, 1990; Northcutt, 1995; Northcutt and Braford, 1980). In fact, on the basis of topological, hodological, neuroanatomical, histochemical, developmental, and functional comparative evidence, a broad consensus has been obtained in considering that at least part of the lateral division of area dorsalis telencephali (Dl) is homologous to the mammalian hippocampus, and that at least part of the medial division of area dorsalis telencephali (Dm) might be considered homologue to the amygdala (Kapsimali et al., 2000; Northcutt, 2006; Salas et al., 2008; Wullimann and Mueller, 2004; Yamamoto and Ito, 2008). In agreement with this view, lesions of the ventral part of Dl (Dlv) in goldfish produce severe spatial learning and memory impairments, comparable to those observed after hippocampus damage in land vertebrates (Broglio et al., 2005, 2010; Durán et al., 2010; Rodríguez et al., 2002; Salas et al., 2006). Furthermore, higher spatial behavior demands in natural environments have been associated with increases in the relative size of Dlv (Costa et

al., 2011), and morphofunctional studies have shown changes in the transcriptional activity of the neurons in Dlv, related to spatial learning (Broglio et al., 2010; Vargas et al., 2000). Regarding the Dm region, it has been shown that its ventromedial area, similar to the mammalian amygdala, plays a critical role in the emotional conditioning as well as in encoding values and motivational signals (Broglio et al., 2005; Desjardins and Fernald, 2010; Lau et al., 2011; LeDoux, 2000; Maren, 2001; Martín et al., 2011; Portavella et al., 2004; von Trotha et al., 2014).

However, as the precise mechanisms of eversion have not been thoroughly identified and the functional organization of the teleost telencephalon is not completely understood, several contending hypotheses about the homologies of the teleost pallium subdivisions have been proposed (Mueller and Wullimann, 2009; Nieuwenhuys, 2011; Northcutt, 2008; Yamamoto et al., 2007). For example, it is currently assumed that the Dl can be cytoarquitectonically divided into a dorsal (Dld) and a ventral subdivision (Dlv) throughout most of its rostrocaudal extent (Northcutt, 2006), but is still under discussion whether the entire Dl, i.e. Dld plus Dlv, or by contrast Dlv exclusively, are comparable to the hippocampal pallium. Thus, some hypotheses propose Dlv as the homologue to the mammalian hippocampus and Dld as a part of the dorsal pallium or isocortex (Ito and Yamamoto, 2009; Wullimann and Mueller, 2004; Yamamoto et al., 2007). In particular, it has been proposed that Dld is homologue to the primary, geniculate recipient visual pallial region in the dorsal pallium or isocortex of other vertebrates (Demski, 2003, 2013; Saidel et al., 2001). The topological position of this region, its visual ascending inputs, and its visually related activity argue in favor of this hypothesis (Ito and Yamamoto, 2009; Wullimann and Mueller, 2004; Yamamoto and Ito, 2008). In contrast, other hypotheses, based on a topological criterion and the presumable similarity in the pattern of connections of the Dld and the Dlv, propose that the whole Dl should be viewed as a single primary pallial division, homologous to the mammalian hippocampus (Harvey-Girard et al., 2012; Northcutt, 2006).

In the present work we investigated further the regions of the teleost fish pallium involved in spatial learning. With this objective, we trained goldfish in a spatial task and measured the learning-related neural activity in Dld, Dlv and Dm regions using

quantitative cytochrome oxidase (CO) histochemistry. Since CO is a mitochondrial enzyme involved in the generation of ATP associated with cell metabolic demands, and therefore in neuronal activation, CO histochemistry is a useful tool to index regional functional activity in the brain (Wong-Riley, 1989; Gonzalez-Lima and Cada, 1994) and, in particular, to identify sustained activity changes induced by learning situations (Conejo et al., 2010; Poremba et al., 1998). Thus, the present experiment could contribute to clarify the identity of Dlv, Dld, and Dm by means of assessing their possible involvement in spatial learning, one of the most unambiguous functions of the hippocampus.

2. Methods

2.1. Subjects

Goldfish (*Carassius auratus*) 11 to 13 cm in length were obtained from a local supplier and were kept in large tanks with aerated and filtered water at 19±1ºC on a 14/10-light/dark cycle for several weeks prior to the experiments. A week before the experiments, animals were distributed into small groups of four subjects and kept in the same conditions. At the end of each experimental session, the fish were returned to their home aquaria and fed with Tetrapond Pondsticks (Ulrich Baensch GmbH, Germany). Fish were identified individually based on phenotypic features. All animal procedures were performed in accordance with Directive 86/609/CEE of the European Community Council and Spanish legislation (R.D. 53/2013).

2.2. Apparatus

The apparatus and training procedure have been described previously (Broglio et al., 2005; Salas et al., 1996). Briefly, the maze consisted of a diamond-shaped enclosure (choice compartment) and two identical, removable circular start compartments that were placed diagonally in the middle of an 85x85 cm aquarium. The aquarium was filled with aerated and filtered water at 19±1ºC to a depth of 30 cm. The experimental apparatus was made of dark grey PVC. The choice compartment,

which was 324 cm^2 and 24 cm high, had four openings that were 6.5 cm wide and 20 cm high. One opening was placed in each corner. Two of the diagonally opposite openings led to the start boxes, and the two remaining openings served as doors (see Fig. 1A). One of the doors remained open and provided the only exit (goal), and the other door was blocked with a transparent glass barrier. In each trial, only one start compartment was used, and the access to the opposite compartment was blocked with a sliding dark grey PVC barrier. The visual cues consisted of two removable grey PVC panels with five 2-cm-wide white vertical stripes on each that entirely covered two walls of the choice compartment. The other two remaining walls were dark grey. To ensure that no other cues were available except those provided by the experimental setting, the walls of the experimental room were homogeneously covered with dark grey curtains. Illumination was provided by an 18-W fluorescent light placed at a height of 25 cm and oriented along the axis between the two start compartments. For each trial, the observer, who was behind the start compartment, recorded the animal's behavior. Sessions were conducted with groups of four fish, but the animals were trained individually. Two glass enclosures placed in the aquarium behind the start compartments served as waiting areas during inter-trial intervals. The subject within the maze could not see the others until it completed the trial by exiting the choice compartment through the exit.

2.3. Behavioral Procedure

Fish were randomly distributed into two groups: naive (n=8), and a group trained for 3 sessions in a spatial constancy task (n=10). Fish in the naive group were not exposed to the apparatus. The trained animals where first individually allowed to explore the experimental apparatus by swimming out of each start box five times (i.e., 10 total trials). The order of the beginning start box was random. Because the glass barrier was not used in these trials, the animals made spontaneous exits through either door. After this habituation session, the fish were trained for three 25-trial sessions on consecutive days. During training, the exit door (goal) was located in a constant spatial position relative to the entire experimental setup that was formed by the choice compartment and the cues (striped panels); thus, the spatial position of the

goal was independent of the entrance point. The other door was blocked with a transparent glass barrier (see Fig. 1A). To make egocentric (turn) responses irrelevant, the start boxes were selected in a pseudorandom order. In addition, to ensure that the striped panels were the only features that were relevant and spatially invariant, the choice compartment and the cues were rotated in a pseudorandom order within the aquarium between trials, but the relative position of the cues and the goal remained constant throughout the experiment. To begin a trial, the animal was carefully placed in one of the start boxes with free access to the choice compartment. A correct trial was registered when the first choice was the exit (goal) and the animal passed its head through the exit without previously bumping against the glass barrier. An error was scored when the first choice was the blocked door and the fish bumped against the glass barrier before reaching the exit. The fish was allowed to remain in the choice compartment until it passed through the exit (correction procedure).

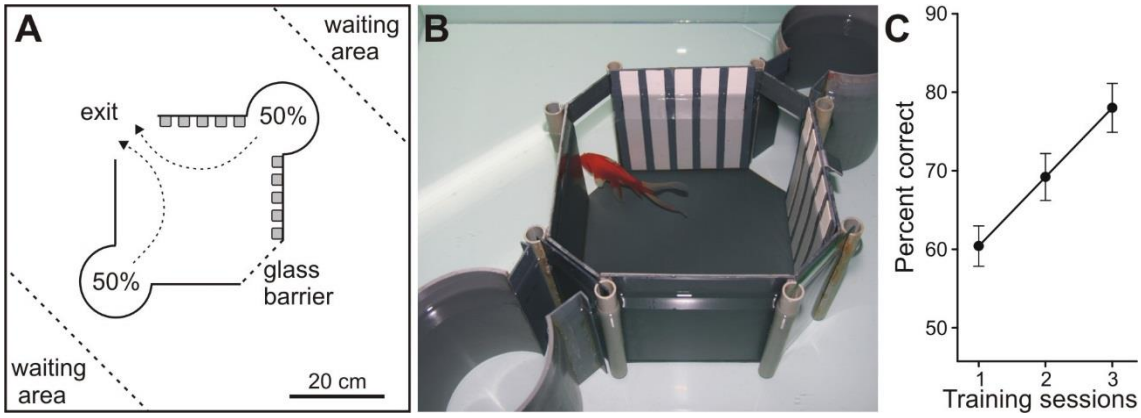

Figure 1.1. (A) Schematic representation of the spatial constancy task showing the choice compartment, the location of the cues (grey boxes), the start compartments, the exit door (goal), the glass barrier, and the waiting areas. The numbers represent the percentage of trials on which each start compartment was used. The arrows represent the most appropriate trajectory to the exit during the training sessions. The glass barrier was not used during habituation. (B) The percentages of correct choices during the training sessions. Data are represented as mean±SEM.

2.4. Cytochrome Oxidase Histochemistry

Immediately after the end of the behavioral procedures, animals from the trained group were deeply anesthetized (ethyl 3-aminobenzoate methanesulphonate,

MS-222, Sigma) and perfused transcardially with cold PBS 0.1 M (pH 7.4) followed by cold fixative solution (2% paraformaldehyde and 0.5% glutaraldehyde in PBS 0.1 M, pH 7.4). Naive fish were taken from the home aquaria and processed as above. Following perfusion, the brains were removed from the skull and placed in a sucrose solution (30% in PBS 0.1 M, pH 7.4) overnight. Then, the brains were embedded in a cryoprotective gel (OCT Compound, Sakura Finetek) and stored at -70ºC. Two series of cryostat adjacent coronal sections (30 µm, Leica CM 1850) were collected on clean slides and stored at -70 ºC. One series was processed for quantitative cytochrome oxidase (CO) histochemistry using previously described procedures (Gonzalez-Lima and Jones, 1994), and the second series was Nissl-stained to delimitate the areas to be measured. Cryostat slides containing sections of goldfish brain homogenate of different thickness (10, 30, 40, 50 and 60 µm) were also obtained to be used as calibration standards. Previously, CO activity of the homogenate was spectrophotometrically assessed. The complete set of brain slices of the naive and the trained animals and the brain homogenate standards of known CO activity, were simultaneously processed in a single CO staining bath. The slides were lightly fixed for 5 min with a 1.5% glutaraldehyde solution and then rinsed three times in PBS and incubated at 37 ºC for 1 h in the dark with continuous stirring in a solution containing 0.05 g diaminobenzidine, 4 g sucrose and 0.015 g cytochrome c (type III, from horse heart, Sigma) dissolved in 100 ml PBS 0.1 M (pH 7.4). The slides were rinsed three times with PBS, dehydrated and coverslipped with DPX (Sigma).

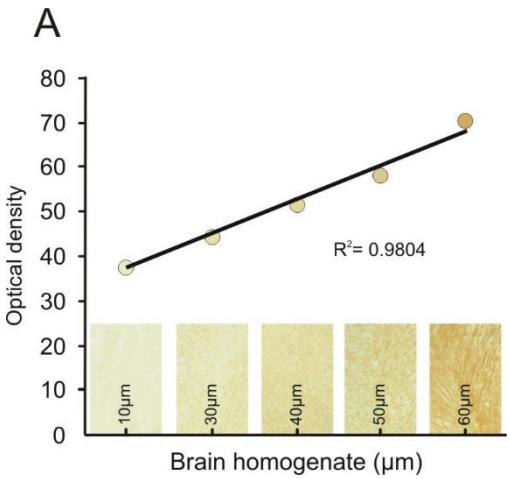

Figure 1.2. Linear correlation between optical density and brain homogenate sections. Microphotographies show a representative brain homogenate tissue corresponding to the micrometres above

Photographs of each telencephalic hemisphere section were taken with a digital camera mounted on a Zeiss Axioskop 2 microscope and captured using AxioVision 4 (Carl Zeiss Imaging Solutions, GmbH). Staining intensities were measured and converted to 256 grey scale values with the public domain program ImageJ 1.44p (NIH, USA). Regression curves between section thickness and known CO activity measured in each set of standards were calculated. Finally, relative optical density readings were converted to CO activity units (µmol of cytochrome c oxidised/min/g tissue wet weight) using the calculated regression curve for the homogenate standards.

In the present work, we measured the CO activity in Dld and Dlv at a precommissural level (see Fig. 2). Besides, we also measured the CO activity in Dm as a control region since several studies have consistently showed that this area is not involved in spatial cognition (Broglio et al., 2005, 2010; Durán et al., 2010; Rodríguez et al., 2002). Dm, Dld, and Dlv were delimitated according to Northcutt (Northcutt, 2006) and the CO activity of each of these regions was bilaterally measured in twelve adjacent sections. These twelve values were averaged to a single measure for each region per hemisphere.

2.5. Data Analysis

The percentage of correct responses measured daily for the trained group were analyzed using a repeated-measures ANOVA. Student's t-tests were used to compare the percentages of door choices during habituation and the CO unit differences between hemispheres per region.

A repeated-measures ANOVA with group as the between-subject factor and pallial region as the repeated measures was used to analyze the differences in CO activity. Post hoc tests with Bonferroni correction were used when ANOVA indicated significant overall differences between means. All group data are reported as the mean ±the SEM. A probability threshold of $p<0.05$ was used to determine statistical significance. All statistical computations were performed with the SPSS 17.00 statistical software.

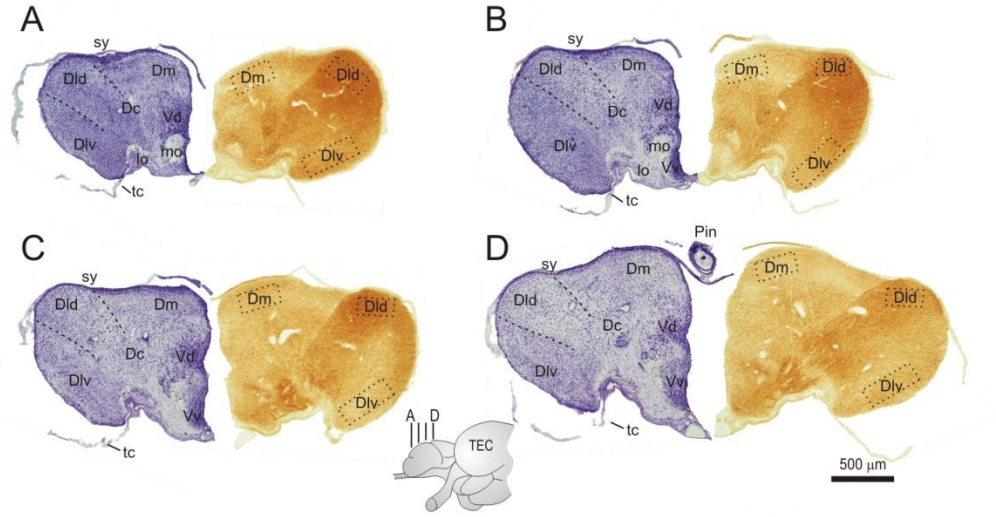

Figure 1.3. Images of Nissl (left) and CO (right) stained sections of four representative equidistant transversal levels (A-D) through the telencephalic rostro-caudal extent in which CO activity was measured. The dotted boxes in the CO stained sections show the sampled areas. The drawing at the bottom depicts a lateral view of a goldfish forebrain, in which the relative position of the transverse sections are indicated by the vertical lines.Abbreviations: Dc, central division of area dorsalis; Dd, dorsal division of area dorsalis; Dld, dorsal subdivision of lateral division of area dorsalis; Dlv, ventral subdivision of lateral division of area dorsalis; Dm, medial subdivision of area dorsalis; lo, lateral olfactory tract; mo, medial olfactory tract; Pin, pineal organ; sy, sulcus ypsiloniformis; tc, tela chroroidea; TEC, optic tectum; Vd, dorsal nucleus of area ventralis; Vv, ventral nucleus of area ventralis.

3. Results

3.1. Behavioral Results

The performance of the animals trained in the spatial task is shown in Fig. 1B. No significant preferences for either door were observed during the pretraining habituation session (paired Student's t-test, p=0.865). A one-way repeated measures ANOVA revealed that the accuracy in solving the task improved along training, as demonstrated by the significant increase in the percentage of correct responses ($F_{2,18}$=14.552; p=0.0001). In fact, the animals reached a high percentage of correct responses on session 3 (78%±3.11).

3.2. Cytochrome Oxidase Activity

After having averaged the twelve original measures into a single one for each region per hemisphere, we first compared the CO activity values of the right and left

hemispheres in each region. No significant differences were found between the CO activity of either hemisphere for either region (all ps>0.435 for the naive group and all ps>0.436 for the trained group). Therefore, measures from left and right hemispheres were averaged to obtain three single CO activity values, one for each region of interest (Dm, Dld and Dlv). Figure 3 shows the CO units of these three different regions in the two groups. An overall analysis of the CO values with a 2x3 (group x pallial region) global ANOVA revealed a significant main effect of pallial region ($F_{2,32}$=186.789, p=0.0001) and group by pallial region interaction ($F_{2,32}$=3.513, p=0.042) but not significant effects for group ($F_{1,16}$=2.692, p=0.120). Thus, in order to interpret the interaction, post-hoc multiple comparisons adjusted by Bonferroni were conducted.

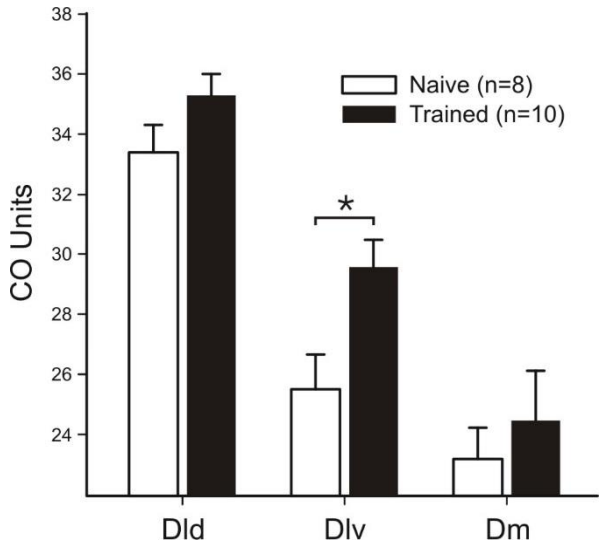

Figure 1.4. CO activity values (µmol of cytochrome c oxidised/min/g tissue wet weight) obtained from the three regions of interest. Asterisk denotes the only significant group by pallial region interaction. Data are represented as mean±SEM.

Table 1 shows the mean CO activity values measured in the three regions of interest (Dm, Dld, and Dlv) for the experimental animals, and the naive control group for baseline comparisons. The statistical analysis of pair-wise differences between groups showed that CO activity in Dlv increased significantly in trained animals compared to animals in the naive group (p=0.013). In contrast, no significant between group differences were found regarding Dld or Dm (p=0.116 and p=0.545 for Dld and Dm, respectively). These results indicate that training animals in the spatial constancy

task exclusively engaged Dlv, and that Dld and Dm were not critically involved in spatial learning.

Additionally, the analysis of the pallial region effect showed significant differences in the basal CO activity. In this regard, Dld presents significantly higher CO values than Dm and Dlv independently of the experimental treatment (all ps<0.0001; see Table 1).

	Naive	Trained
Dm	23.19±1.04$^{\&}$	24.47±1.65$^{\&}$
Dld	33.17±0.90	35.26±0.71
Dlv	25.51±1.15$^{\&}$	29.56±0.90$^{*\&\#}$

Table 1. Cytochrome oxidase activity units (µmol/min/g tissue wet weight) measured in the selected brain regions of the two groups. Data represent mean±S.E.M. Symbols denote significant differences: * significant effect of group by pallial region interaction, & significant differences between Dld and Dm as well as Dld and Dlv, and # significant differences between Dlv and Dm. Data are represented as mean±SEM.

4. Discussion

In the present work we used quantitave cytochrome oxidase (CO) histochemistry mapping to identify the regions of the goldfish telencephalic pallium involved in spatial learning. Our results reveal that goldfish trained in a spatial constancy task exhibited a selective increase of the CO activity in the ventral part of the lateral division of area dorsalis telencephali (Dlv) compared to the naive animals, but not in the dorsal part of the lateral division of area dorsalis telencephali (Dld), nor in the medial division of area dorsalis telencephali (Dm).

Histochemistry of CO, an enzyme involved in oxidative phosphorylation associated to the metabolic activity of cells, is a widely used approach to index regional functional activity in the brain (Gonzalez-Lima and Cada, 1994; Wong-Riley, 1989). Here we used quantitative CO histochemistry, which allows determining the amount of

CO present in brain tissue sections by directly relating optical density measurements to CO concentration, to assess the regional differences in brain activity among animals in different training groups (Gonzalez-Lima and Garrosa, 1991; Melendez-Ferro et al., 2013). It is important to emphasize that this approach enables the identification of brain regions undergoing long-term training-dependent changes. Brain changes in CO histochemistry after prolonged training seem to reflect chronic and sustained changes in the baseline neural metabolic capacity associated with the oxidative energy demands over hours or days, instead of short-term cellular energy requirements in response to stimulus-evoked neuronal activity during an acute testing period (Gonzalez-Lima, 1992; Gonzalez-Lima and Cada, 1998; Poremba et al., 1998). Therefore, the results in this experiment showing an increased CO activity in the goldfish Dlv after three consecutive training-days in a spatial constancy task likely reflect the cumulative effects of training in this region, which suggests an important role of Dlv in spatial navigation.

Previous studies showed that goldfish solve the spatial constancy task using cognitive-mapping strategies based on the codification of the whole experimental environment in a unique representation, which includes the spatial relationships among its geometrical features, the goal and the array of visual cues (Broglio et al., 2010; López et al., 1999; Salas et al., 1996). This map-like representation provides the goldfish with a stable spatial reference frame for navigation, which enables them to reach readily the goal even when required to navigate from novel start locations or despite the removal of whichever of the individual visual cues (López et al., 1999; Salas et al., 1996, 2008). In mammals, birds, and reptiles the hippocampus is the critical brain region for encoding environmental information in map-like or relational spatial memory representations (Bingman et al., 1998; Burgess et al., 1999; López et al., 2003; Morris, 2006; O'Keefe and Nadel, 1978; Rodríguez et al., 2002; Sherry and Duff, 1996). In teleost fish, a broad consensus has been achieved concerning the homology of at least part of the lateral division of area dorsalis telecephali (Dl) with the amniote hippocampus (Northcutt, 2008). This conclusion is based on developmental, topological, hodological, and histochemical evidence (Kapsimali et al., 2000; Northcutt, 2006; Wullimann and Mueller, 2004; Yamamoto and Ito, 2008), and it is also supported

by functional comparative data (Broglio et al., 2005; Salas et al., 2003, 2008). Actually, lesion and morphofunctional studies show that the teleost Dlv is comparable to the amniote hippocampus regarding spatial cognition. For example, damage selective to Dlv, but not to Dld or Dm, causes a severe impairment in the performance of goldfish trained in a variety of allocentric or relational spatial memory procedures, including the spatial constancy task used in the experiment presented here (Broglio et al., 2010; Durán et al., 2010; Rodríguez et al., 2002; Salas et al., 2006). Other experiments have shown that training goldfish in the spatial constancy task produces an increment in the transcriptive activity in the neurons of Dlv, but not in those of Dm, as indicated by the increases in the size of the nucleolar organizing region (NOR), the nucleolar organelles associated with the synthesis of ribosomal proteins (Broglio et al., 2010; Vargas et al., 2000). Interestingly, the present results showing an increased CO activity in the goldfish Dlv after long-term training in a spatial task, parallel those obtained in mammals in which training in spatial tasks produces learning-dependent morphofunctional changes in the hippocampus, including CO increased activity (Conejo et al., 2007; Middei et al., 2014; Ros et al., 2006). All together, the present findings add further support to the hypothesis of homology between the teleostean Dlv and the mammalian hippocampus.

In relation to Dm, the results in this experiment showing that training goldfish in the spatial constancy task did not increase the CO activity in this area agree with previous reports indicating that Dm has not an important role in solving spatial tasks (Broglio et al., 2010; Durán et al., 2010; Rodríguez et al., 2002). Instead, our results are consistent with the hypotheses based on neuroanatomical and developmental data proposing that at least part of Dm is homologous to the pallial amygdala of mammals (Braford, 1995; Northcutt, 2006; Wullimann and Mueller, 2004). These hypotheses have received functional support from studies showing that Dm in goldfish is critically involved in emotional learning and memory, for example avoidance learning and taste aversion conditioning (Broglio et al., 2005; Martín et al., 2011; Portavella et al., 2004).

Contrary to the increasing evidence concerning the striking functional similarities between Dlv and the amniote hippocampal pallium, little data are available regarding the role of Dld in spatial learning. Our results suggest that Dld is not critically

involved in spatial learning as not significant differences in the CO activity between trained and naive animals were observed in this region (Fig. 3). Indeed, the available lesion studies have reported that Dld damage in goldfish does not produce spatial learning impairments [see Fig. 7 in Rodríguez et al. (2002) and Figs. 1 and 2 in Broglio et al. (2010)]. It is important to note that the comparisons between the pallial telencephalic areas of ray-finned fish and those in other vertebrates has been hindered by the eversion or bending outward of the embryonic prosencephalic alar plate that takes place during the development of the actinopterygian forebrain (Nieuwenhuys, 1963; Northcutt and Braford, 1980; Striedter and Northcutt, 2006). Since the precise mechanisms of eversion have not been yet thoroughly understood, i.e. this process might be accompanied by a rearrangement of some of the pallial cell masses, several contending hypotheses about the homologies of some subdivisions of the teleost pallium have been proposed (see for example Braford, 1995; Butler, 2000; Nieuwenhuys, 2011; Northcutt, 2008; Mueller and Wullimann, 2009; Yamamoto et al., 2007). For example, it is currently under discussion whether the entire Dl constitutes a single pallial unit (Harvey-Girard et al., 2012; Northcutt, 2006, 2008;) or, by contrast, Dlv and Dld are two different regions (Demski, 2013; Ito and Yamamoto, 2009; Yamamoto et al., 2007). Thus, Northcutt (2006), based in the presumable uniformity of the extrinsic and intrinsic connections of Dld and Dlv, suggests that Dl should be considered as a single primary homogenous pallial division homologous to the hippocampus. In contrast, Yamamoto and colleagues (Yamamoto and Ito, 2008; Yamamoto et al., 2007), based on the topological interpretation of the pallial regions, the hodological data, and on the possibility that the preglomerular complex, i.e., the main diencephalic sensory relay station of teleosts, could be homologous to the dorsal thalamus of tetrapods, suggest that the teleostean Dlv and Dld regions are indeed two different pallial subdivisions, and could be considered homologous to the hippocampus and the dorsal cortex, respectively. Our data show that although the Dld region in naive and trained goldfish presents high levels of CO basal activity compared to Dlv and Dm, training did not increase this activity, suggesting that spatial learning does not engage this region. A high level CO basal activity in Dld compared to other pallial regions has been already described in teleosts (Saidel et al., 2001). In mammals, the highest overall levels of CO basal activity are seen in the primary sensory areas,

e.g., in the visual and somatosensorial cortices, whereas moderate to low basal CO levels are observed in associational and limbic areas (Hevner et al., 1995; Wallace, 1987). Interestingly, Saidel and colleagues (2001), using electrophysiological recording and CO histochemistry, identified a visual region in the goldfish Dld, and suggested that this area correspond to the mammalian geniculo-recipient primary visual area. Moreover, a study using voltage-sensitive dye imaging, a technique that provides high spatio-temporal resolution, showed that pallial visually related activity is restricted to a well-defined bulge of the goldfish Dld (Ocaña et al., 2009). Thus, the results in the present experiment showing a high level of CO basal activity in Dld, as well as the absence of involvement of this region in spatial learning, together with the data regarding its visual ascending inputs, its visually related activity, and its topological position -localized between the teleost hippocampal and amygdaline pallia-, argue in favor of the hypothesis that proposes that Dld is homologue to at least part of the dorsal pallium or isocortex of other vertebrates (Demski, 2003; Ito and Yamamoto, 2009; Saidel et al., 2001; Wullimann and Mueller, 2004; Yamamoto and Ito, 2008).

In summary, the present results showing that training goldfish in a spatial task increased significantly the CO activity in Dlv but did not change it in Dld nor in Dm, provides further evidence concerning the selective involvement of Dlv in spatial learning. In this regard, these data suggest that Dl is a heterogeneous pallial division, and support the hypothesis that only its ventral subdivision, Dlv, but not its dorsal subdivision, Dld, might be comparable to the hippocampus.

5. References

Bingman, V.P., Strasser, R., Baker, C. and Riters, L.V. (1998). Paired-associate learning is unaffected by combined hippocampal and parahippocampal lesions in homing pigeons. Behav Neurosci;112:533-40.

Braford, M.R.(1995). Comparative aspects of forebrain organization in the ray-finned fishes: touchstones or not? Brain Behav Evol;46: 259-74.

Broglio, C., Gómez, A., Durán, E., Ocaña, F.M., Jiménez-Moya, F., Rodríguez, F., et al. (2005). Hallmarks of a common forebrain vertebrate plan: specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Res Bull;66:277-81.

Broglio, C., Rodríguez, F., Gómez, A., Arias, J.L. and Salas C. (2010). Selective involvement of the goldfish lateral pallium in spatial memory. Behavl Brain Res;210:191-01.

Burgess, N., Jeffery, K.J. and O'Keefe, J.(1999). The hippocampal and parietal foundations of spatial cognition. London: Oxford University Press.

Butler, A.B. (2000).Topography and topology of the teleost telencephalon: a paradox resolved. Neurosci Lett;293:95-8.

Conejo, N.M., González-Pardo, H., Vallejo, G. and Arias, J.L.(2007). Changes in brain oxidative metabolism induced by water maze training. Neuroscience;145:403-12.

Conejo, N.M., González-Pardo, H., Gonzalez-Lima, F. and Arias, J.L.(2010). Spatial learning of the water maze: progression of brain circuits mapped with cytochrome oxidase histochemistry. Neurobiol Learn Mem, 2010;93:362-71.

Costa, S.S., Andrade, R., Carneiro, L.A., Goncalves, E.J., Kotrschal, K. and Oliveira ,R.F.(2011). Sex differences in the dorsolateral telencephalon correlate with home range size in blenniid fish. Brain Behav Evol;77:55-4.

Demski, L.S.(2013). The pallium and mind/behavior relationships in teleost fishes. Brain Behav Evol;82:31-4.

Demski, L.S.(2003). In a fish´s brain´s eye: Visual pallium of teleosts. In: Collin SP, Marshall NJ, editors. Sensory Processing in Aquatic Environments, New York: Springer-Verlag; 2003. p. 404-19.

Desjardins, J.K. and Fernald, R.D. (2010). What do fish make of mirror images? Biol Lett;23:744-47.

Durán, E., Ocaña, F.M., Broglio, C., Rodríguez, F. and Salas, C.(2010). Lateral but not medial telencephalic pallium ablation impairs the use of goldfish spatial allocentric strategies in a "hole-board" task. Behav Brain Res;214:480-87.

Gonzalez-Lima, F. (1992). Brain imaging of auditory learning functions in rats: studies with fluorodeoxyglucose autoradiography and cytochrome oxidase histochemistry. In: Gonzalez-Lima, F., Finkenstaedt, Th., Scheich, H., editors. Advances in Metabolic Mapping Techniques for Brain Imaging of Behavioral and Learning Functions Dordrecht: Kluwer, p.39-109.

Gonzalez-Lima, F. and Garrosa, M. (1991). Quantitative histochemistry of cytochrome oxidase in rat brain. Neurosci Lett, 1991;123: 251-53.

Gonzalez-Lima, F. and Cada, A. (1994). Cytochrome oxidase activity in the auditory system of the mouse: a qualitative and quantitative histochemical study. Neuroscience;63:559-78.

Gonzalez-Lima, F. and Jones, D. (1994). Quantitative mapping of cytochrome oxidase activity in the central auditory system of the gerbil: a study with calibrated activity standards and metal-intensified histochemistry. Brain Res;660:34-9.

Harvey-Girard, E., Giassi, A.C., Ellis, W. and Maler, L. (2012). Organization of the gymnotiform fish pallium in relation to learning and memory: IV. Expression of conserved transcription factors and implications for the evolution of dorsal telencephalon. J Comp Neurol;520:3395-413.

Hevner, R.F., Liu, S. and Wong-Riley, M.T.T. (1995). A metabolic map of cytochrome oxidase in the rat brain: histochemical, densitometric and biochemical studies. Neuroscience, 1995;65:313-42.

Ito, H. and Yamamoto, N. (2009). Non-laminar cerebral cortex in teleost fishes? Biol Lett; 5:117-21.

Kapsimali, M., Vidal, B., Gonzalez, A., Dufour, S. and Vernier, P. (2000). Distribution of the mRNA encoding the four dopamine D(1) receptor subtypes in the brain of the european eel (Anguilla anguilla): comparative approach to the function of D(1) receptors in vertebrates. J Comp Neurol;419: 20-43.

Lau, B.Y., Mathur, P., Gould, G.G. and Guo, S. (2011). Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc Natl Acad Sci;108: 2581-86.

LeDoux, J.E. (2000). Emotion circuits in the brain. Annu Rev Neurosci; 23:155-84.

López, J.C., Broglio, C., Rodríguez, F., Thinus-Blanc, C. and Salas, C. (1999). Multiple spatial learning strategies in goldfish (Carassius auratus). Anim Cogn;2:109–20.

López, J.C., Vargas, J.P., Gómez, Y. and Salas, C. (2003). Spatial and non-spatial learning in turtles: the role of medial cortex. Behav Brain Res;143:109-20.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. Ann Rev Neurosci;24: 897–31.

Martín, I., Gómez, A., Salas, C., Puerto, A. and Rodríguez, F. (2011). Dorsomedial pallium lesions impair taste aversion learning in goldfish. Neurobiol Learn Mem;96:297-05.

Melendez-Ferro, M., Rice, M.W., Roberts, R.C. and Perez-Costas, E. (2013). An accurate method for the quantification of cytochrome C oxidase in tissue sections. J Neurosci Meth;214:156-62.

Middei, S., Ammassari-Teule, M. and Marie, H.(2014). Synaptic plasticity under learning challenge. Neurobiol Learn Mem;115:108-115.

Morris, R.G. (2006). Theories of Hippocampal Function. In: The Hippocampus Book. (Editors) Andersen, R., Morris, R., Amaral, D., Bliss, T., O'Keefe, J. London: Oxford University Press;p. 581-712.

Mueller, T. and Wullimann, M.F. (2009). An evolutionary interpretation of teleostean forebrain anatomy. Brain Behav Evol;74:30-2.

Nieuwenhuys, R. (1963). The comparative anatomy of the actinopterygian forebrain. J Hirnforsch, 13:171–92.

Nieuwenhuys, R. (2011). The development and general morphology of the telencephalon of actinopterygian fishes: synopsis, documentation and commentary. Brain Struct Funct;215:141–57.

Nieuwenhuys, R. and Meek, J. (1990). The telencephalon of actinopterygian fishes. In: Jones EG, Peters A, editors. Comparative Structure and Evolution of the Cerebral Cortex. New York: Plenum Press, p. 31-73.

Northcutt, R.G. (2008). Forebrain evolution in bony fishes. Brain Res Bull;75:191-05.

Northcutt, R.G. and Braford, M.R. (1980). New observations on the organization and evolution of the telencephalon of actinopterygian fishes. In: Ebbesson SOE, editor. Comparative neurology of the telencephalon. New York: Plenum Press, p. 41-98.

Ocaña, F.M., Durán, E., Rodríguez, F. and Salas, C. (2009). Voltage-sensitive dye imaging of visually evoked responses in the goldfish telencephalic pallium. Conference Abstract: 41st European Brain and Behaviour Society Meeting: Frontiers in Behavioral Neuroscience.

O'Keefe, J. and Nadel, L. (1978). The hippocampus as a cognitive map. Oxford: Clarendon Press.

Poremba, A., Jones, D. and Gonzalez-Lima, F. (1998). Classical conditioning modifies cytochrome oxidase activity in the auditory system. Eur J Neurosci;10:3035-43.

Portavella, M., Torres, B. and Salas, C. (2004). Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. J Neurosci;24:2335-42.

Rodríguez, F., López, J.C., Vargas, J.P., Gómez, Y., Broglio, C. and Salas C. (2002). Conservation of spatial memory function in the pallial forebrain of reptiles and ray-finned fishes. J Neurosci;22:2894-903.

Ros, J., Pellerin, L., Magara, F., Dauguet, J., Schenk, F. and Magistretti, P.J. (2006). Metabolic activation pattern of distinct hippocampal subregions during spatial learning and memory retrieval. J Cereb Blood Flow Metab;26:468–77.

Saidel, W.M., Marquez-Houston, K. and Butler, A.B. (2001). Identification of visual pallial telencephalon in the goldfish, Carassius auratus: a combined cytochrome oxidase and electrophysiological study. Brain Res;919:82-3.

Salas, C., Broglio, C., Rodríguez, F., López, J.C., Portavella, M. and Torres, B. (1996). Telencephalic ablation in goldfish impairs performance in a 'spatial constancy' problem but not in a cued one. Behav Brain Res;79:193-00.

Salas, C., Broglio, C. and Rodríguez, F. (2003). Evolution of forebrain and spatial cognition in vertebrates: conservation across diversity. Brain Behav Evol;62:72–

Salas, C., Broglio, C., Durán, E., Gómez, A,. Ocaña, F.M., Jiménez-Moya, F., et al. (2006). Neuropsychology of learning and memory in teleost fish. Zebrafish,;3:157-71

Salas, C., Broglio, C., Durán, E., Gómez, A. and Rodríguez, F. (2008). Spatial Learning in Fish. In: Menzel R, editor, Learning Theory and Behavior. Vol. 1. In: Byrne, J., editor Learning and Memory: A Comprehensive Reference. Oxford: Elsevier, p. 499-28.

Sherry, D. and Duff, S. (1996). Behavioural and neural bases of orientation in food-storing birds. J Exp Biol;199:165-72.

Striedter, G.F. and Northcutt, R.G. (2006). Head size constrains forebrain development and evolution in ray-finned fishes. Evol Dev;8: 215–22.

Vargas, J.P., Rodríguez, F., López, J.C., Arias, J.L. and Salas, C. (2000). Spatial learning-induced increase in the argyrophilic nucleolar organizer region of dorsolateral telencephalic neurons in goldfish. Brain Res;865:77-4.

von Trotha, J.W., Vernier, P. and Bally-Cuif, L. (2014). Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. Eur J Neurosci;40:3302-15.

Wallace, M.N. (1987). Histochemical demonstration of sensory maps in the rat and mouse cerebral cortex. Brain Res;418:178-82.

Wong-Riley, M.T. (1989). Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. Trends Neurosci;12:94-01.

Wullimann, M.F., and Mueller, T. (2004). Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. J Comp Neurol;475:143-62.

Yamamoto, N., Ishikawa, Y., Yoshimoto, M., Xue, H.G., Bahaxar, N., Sawai, N., Yang, C.Y., Ozawa, H. and Ito, H. (2007). A new interpretation on the homology of the teleostean telencephalon based on hodology and a new eversion model. Brain Behav Evol;69:96-04.

Yamamoto, N. and Ito, H. (2008). Visual, lateral line, and auditory ascending pathways to the dorsal telencephalic area through the rostrolateral region of the lateral preglomerular nucleus in cyprinids. J Comp Neurol;508:615-47.

EXPERIMENT 2. CYTOCHROME OXIDASE HISTOCHEMISTRY REVEALS A TIME LIMITED ROLE OF THE VENTRAL REGION OF THE LATERAL DIVISION OF THE GOLDFISH TELENCEPHALIC PALLIUM IN SPATIAL LEARNING

1. Introduction

Teleost fish possess spatial abilities that are fully comparable to those of mammals and birds (for a review, see Salas et al., 2008). For example, goldfish and other teleosts use allocentric or place navigation strategies and multiple environmental features, such as landmarks and the geometries of environmental boundaries, to build map-like spatial representations for orientation (Durán et al., 2008; López et al., 1999, 2000; Reese, 1989; Rodríguez et al., 1994; Salas et al., 1996; Sovrano and Bisazza, 2008; Sovrano et al., 2003; Teyke, 1989; Vargas et al., 2004). Although it has been widely demonstrated that these map-like or relational abilities depend on the medial or hippocampal pallium and associated structures in land vertebrates (Bingman et al., 1998; Burgess et al., 1999; Cheng et al., 2006; Eichenbaum et al., 1999; O'Keefe and Nadel, 1978; Rodríguez et al., 2002, Salas et al., 2003; Sherry and Duff, 1996; Squire et al., 2004), the role of these structures in the storage and retrieval of spatial memories is currently being debated. Some authors have suggested that once a spatial task is learned, the hippocampus becomes unnecessary, and permanent storage and retrieval of consolidated spatial memories depend on broadly distributed cortical networks (Frankland and Bontempi, 2005; McClelland et al., 1995; Squire and Alvarez, 1995). Other authors claim that these memories remain dependent on the hippocampus permanently (Broadbent and Clark, 2013; Broadbent et al., 2006; Clark, 2011; Clark et al., 2005b, 2007; Martin et al., 2005; Teixeira et al., 2006).

Findings from functional studies of teleost fish have shown that the ventral subdivision of the lateral division of the area dorsalis telencephali (Dlv) shares its essential role in spatial learning and memory with the hippocampus of amniotes. Both pre- and post-training lesions of the goldfish Dlv produce severe learning and memory

impairments in locating goals based on allocentric or relational spatial strategies in a variety of experimental procedures without affecting the use of egocentric strategies (Broglio et al., 2005, 2010; Durán et al., 2010; Rodríguez et al., 2002; Salas et al., 2006). Additionally, morphofunctional studies have shown learning-related changes in the transcriptional activity of Dlv neurons after allocentric but not cued learning (Broglio et al., 2010; Vargas et al., 2000) and increases in the relative size of the Dlv that are associated with elaborated spatial behaviors in natural environments (Costa et al., 2011). In spite of these lines of evidence for the role of the Dlv in spatial processing, the comparison of the teleost pallial regions with the corresponding pallial or cortical regions of the telencephalon of land vertebrates has been hindered by the particular embryonic development that characterises the telencephalon of actinopterygian fish (eversion of the dorsal plate of the neural tube) and leads to unique morphological features, the most important of which are solid telencephalic hemispheres and the rearrangement of the main pallial divisions (Braford, 2009; Folgueira et al., 2012; Nieuwenhuys, 1963, 2011; Yamamoto et al., 2007). Although there is a general consensus that a homologue of the medial pallium homologue exists in the teleost forebrain, the identities of the regions that, together with the Dlv, comprise the teleost fish hippocampal pallium is currently a matter of discussion by comparative neurobiologists (Braford, 2009; Mueller and Wullimann, 2009; Northcutt, 2008; Nieuwenhuys, 2009; Yamamoto et al., 2007). Indeed, based on a topological criterion and the similar pattern of connections of the dorsal subdivision of the lateral division of the area dorsalis telencephali (Dld) and the Dlv, some authors have suggested that the whole teleost lateral division of the area dorsalis (Dl) should be viewed as a single primary pallial division and considered to be the homologue of the mammalian hippocampus (Harvey-Girard et al., 2012; Northcutt, 2006). In contrast, it has been suggested that the Dld is part of the dorsal pallium homologue due to its topological position, its diencephalic ascending inputs, and the visually related activity of this region (Demski, 2003; Saidel et al., 2001; Wullimann and Mueller, 2004; Yamamoto et al., 2007). Finally, the most caudal region of the teleost telencephalon, the posterior division of the area dorsalis (Dp), has also been proposed to be a specialised part of the teleost hippocampal pallium (Butler, 2000; Nieuwenhuys, 2009), although hodological studies have revealed that the Dp receives most of the secondary olfactory

projections (Levine and Dethier, 1985; Northcutt, 2006; von Bartheld et al., 1984) and recent developmental data indicate that the Dp may be homologous to the mammalian lateral or piriform cortex (Mueller et al., 2011).

In the present work, we aimed to further investigate the pallial regions of the teleost telencephalon that are functionally comparable to the mammalian hippocampus. One of the most unambiguous functions of the hippocampus that has been demonstrated in all animals that have been examined is its involvement in spatial learning. Thus, we trained goldfish in a spatial constancy task (Broglio et al., 2010; Ingle and Sahagian, 1973; López et al., 1999; Salas et al., 1996) and measured learning-related neural activity throughout the entire extent of the Dl (along its dorsoventral and rostrocaudal axes) and the Dp using quantitative cytochrome oxidase (CO) histochemistry. Additionally, we sought to study the possible time-dependent role of the putative hippocampal pallium in spatial learning. To achieve this goal, we analyzed the progressive changes in CO activity in animals that were trained for 1, 3, or 5 days and correlated regional CO levels with the behavioral scores gained each day. Given that CO is a mitochondrial enzyme that is involved in the generation of ATP and that metabolic activity is tightly coupled with neuronal activation, CO histochemistry can be used to index regional functional activity in the brain (Gonzalez-Lima and Cada, 1994; Wong-Riley, 1989). Because learning processes produce sustained energy requirements, this technique has become an accurate method that is widely used to map the local changes in neural activity that are induced by different learning situations (Agin et al., 2001; Bruchey and Gonzalez-Lima, 2008; Cada et al., 2000; Conejo et al., 2007, Deglise et al., 2003; Gonzalez-Pardo et al., 2012; Mendez-López et al., 2013; Poremba et al., 1997, 1998; Villarreal et al., 2002).

2. Methods

2.1. Subjects

Goldfish (Carassius auratus) that were 11 to 13 cm in length were obtained from a local supplier and were kept in large tanks with aerated and filtered water at 19±1 ºC on a 14/10-light/dark cycle for several weeks prior to the experiments. A week

before the experiments, 32 animals were distributed into small groups of four and kept in the same conditions. At the end of each experimental session, the fish were returned to their home aquaria and fed with Tetrapond Pondsticks (Ulrich Baensch GmbH, Germany). Fish were identified individually based on phenotypic features. All animal procedures were performed in accordance with European Directive 86/609/CEE and Spanish legislation (R.D. 53/2013).

2.2 Apparatus

The apparatus and training procedure have been described previously (Broglio et al., 2010; Salas et al., 1996). Briefly, the maze consisted of a diamond-shaped enclosure (choice compartment) and two identical, removable circular start compartments that were placed diagonally in the middle of an 85x85 cm aquarium. The aquarium was filled with aerated and filtered water at 19±1ºC to a depth of 30 cm. The experimental apparatus was made of dark grey PVC. The choice compartment, which was 324 cm2and 24 cm high, had four openings that were 6.5 cm wide and 20 cm high. One opening was placed in each corner. Two of the diagonally opposite openings lead to the start boxes, and the two remaining openings served as doors (see Fig. 1A). One of the doors remained open and provided the only exit (goal), and the other door was blocked with a transparent glass barrier. In each trial, only one start compartment was used, and the access to the opposite compartment was blocked with a sliding dark grey PVC barrier. The visual cues consisted of two removable grey PVC panels with five 2-cm-wide white vertical stripes on each that entirely covered two walls of the choice compartment. The other two remaining walls were dark grey. To ensure that no other cues were available except those provided by the experimental setting, the walls of the experimental room were homogeneously covered with dark grey curtains. Illumination was provided by an 18-W fluorescent light placed at a height of 25 cm and oriented along the axis between the two start compartments. For each trial, the observer, who was behind the start compartment, recorded the animal's behavior. Sessions were conducted with groups of four fish, but animals were trained individually. Two glass enclosures placed in the aquarium behind the start compartments served as waiting areas during inter-trial intervals. The subject

within the maze could not see the others until it completed the trial by exiting the choice compartment through the exit.

2.3. Behavioral Procedures

Fish were randomly distributed into four groups (n=8 each): naive and three groups of animals that were trained for 1, 3, or 5 sessions in the spatial constancy task (the 1-, 3-, and 5-session groups, respectively). Fish in the naive group remained in their home tanks and were not exposed to the apparatus or to the training procedure. The training procedure for the animals in the trained groups start with a habituation session where they were individually allowed to explore the experimental apparatus by swimming out of each start box five times (i.e., 10 total trials). In these trials the two start boxes were used in a pseudorandom order. Because the glass barrier was not used in these trials, the animals made spontaneous exits through either door. After the first day of habituation to the apparatus, the three groups were subsequently trained for one, three or five 25-trial sessions on consecutive days. During training, the exit door (goal) was located in a constant spatial position relative to the entire experimental setup that was formed by the choice compartment and the cues (striped panels); thus, the spatial position of the goal was independent of the entrance point. The other door was blocked with a transparent glass barrier (see Fig. 1A). To make egocentric (turn) responses irrelevant, the start boxes were selected in a pseudorandom order. In addition, to ensure that the striped panels were the only features that were relevant and spatially invariant, the choice compartment and the cues were rotated in a pseudorandom order within the aquarium between trials, but the relative position of the cues and the goal remained constant throughout the experiment. To begin a trial, the animal was carefully placed in one of the start boxes with free access to the choice compartment. A correct trial was registered when the animal passed its head through the exit without bumping against the glass barrier. An error was scored when the fish bumped against the glass barrier before reaching the exit. The fish was allowed to remain in the choice compartment until it passed through the exit (correction procedure).

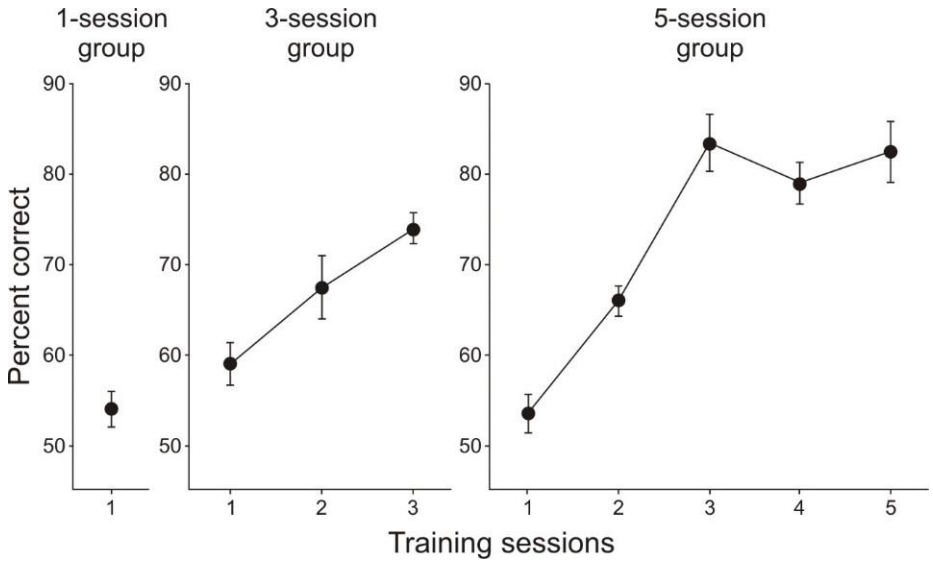

Figure 2.1. (A) Schematic representation of the spatial constancy task showing the choice compartment, the location of the cues (grey boxes), the start compartments, the exit door (goal), the glass barrier and the waiting areas. The numbers represent the percentage of trials on which each start compartment was used. The arrows mark the most appropriate trajectory to the exit during the training sessions. The glass barrier was not used during habituation. (B) The percentages of correct choices during the training sessions for the 1-, 3-, and 5-session groups. The symbols represent the mean± the SEM.

2.4. Cytochrome Oxidase Histochemistry

Immediately after the end of the behavioral procedures, animals from the three trained groups were deeply anesthetised (ethyl 3-aminobenzoate methanesulphonate, MS-222, Sigma) and perfused transcardially with cold PBS 0.1 M (pH 7.4) followed by cold fixative solution (2% paraformaldehyde and 0.5% glutaraldehyde in PBS 0.1 M, pH 7.4). Naive fish were taken from the home aquaria and processed as above. Following perfusion, the brains were removed from the skull and placed in a sucrose solution (30% in PBS 0.1 M, pH 7.4) overnight. Then, the brains were embedded in a cryoprotective gel (OCT Compound, Sakura Finetek) and stored at -70ºC.

Two series of cryostat adjacent coronal sections (30 µm, Leica CM 1850) were collected on clean slides and stored at -70 ºC. One series was processed for quantitative cytochrome oxidase (CO) histochemistry using previously described

procedures (Gonzalez-Lima and Jones, 1994), and the second series was Nissl-stained to delimitate the areas to be measured. Previously, the CO activities of goldfish brain homogenates were spectrophotometrically assessed, and sections of goldfish brain homogenate of different thicknesses (10, 30, 40, 50 and 60 µm) were used as calibration standards. Series of coronal sections from all experimental groups and a complete set of standards of known CO activity were used to perform CO histochemistry. The slides were lightly fixed for 5 min with a 1.5% glutaraldehyde solution and then rinsed three times in PBS and incubated at 37 ºC for 1 h in the dark with continuous stirring in a solution containing 0.05 g diaminobenzidine, 4 g sucrose and 0.015 g cytochrome c (type III, from horse heart, Sigma) dissolved in 100 ml PBS 0.1 M (pH 7.4). The slides were rinsed three times with PBS, dehydrated and coverslipped with DPX (Sigma).

Photographs of each telencephalic hemisphere section were taken with a digital camera mounted on a Zeiss Axioskop 2 microscope and captured using AxioVision 4 (Carl Zeiss Imaging Solutions, GmbH). Staining intensities were measured and converted to 256 grey scale values with the public domain program ImageJ 1.44p (NIH, USA). Regression curves between section thicknesses and known CO activities measured for each set of standards were calculated. Finally, relative optical density readings were converted to CO activity units (µmol of cytochrome c oxidised/min/g tissue wet weight) using the calculated regression curve for the homogenate standard.

In the present work, we were interested in mapping the CO activities of the Dp area and the rostrocaudal extent of the Dl area (including its dorsal and ventral subdivisions, Dld and Dlv, respectively; see Fig. 2). The Dp region is a restricted area of the caudal telencephalon that is located postcommissurally and discernible by a core of densely packed large neurons that define this pallial region and by the presence of the ventrally located nucleus taeniae (Northcutt, 2006; Fig. 2G-H). We defined the Dl region at three different levels (rostral, mid-telencephalic and caudal). The dorsal division of the area dorsalis (Dd), the subpallial dorsal nucleus of the area ventralis (Vd), and the anterior commissure (ac) were used as cytoarchitectonic landmarks to delimitate these levels. The rostral and mid-telencephalic levels of Dld and Dlv were precommissural, whereas the caudal level was commissural (Fig. 2A-D). The rostral Dld

and Dlv can be observed in precommissural transverse sections characterized by the presence of the Vd and the absence of the Dd areas. The mid-telencephalic level is characterized by the absence of the ac and the presence of both the Vd and Dd regions. The presence of the ac defines the transverse sections at which the caudal Dld and Dlv levels can be identified (Fig. 2E-F). Finally, the dorsal and ventral divisions of the Dl were delimitated according to Northcutt (Northcutt, 2006). Thus, in addition to the Dp, six regions in the Dl were considered: the rostral Dld and Dlv (r-Dld and r-Dlv, respectively), the mid-telencephalic Dld and Dlv (m-Dld and m-Dlv, respectively), and the caudal Dld and Dlv (c-Dld and c-Dlv, respectively). The CO activities of each of these seven regions were bilaterally measured in six adjacent sections, and these six values were averaged to a single measure for each region.

2.5. Data Analysis

The percentages of correct responses measured daily for each trained group were analyzed using a repeated-measures ANOVA. Helmert tests were used to compare the accuracy on a particular day of training to that of subsequent days. Student's t-tests were used to compare the percentages of correct responses when necessary. All group data are reported as the mean ± the SEM. A probability threshold of $p<0.05$ was used to determine statistical significance. All statistical computations were performed with the SPSS 17.00 statistical software. Group differences in CO activities measured in each brain region were evaluated by one-way ANOVAs using the experimental group as the independent variable. Tukey's HSD tests were used to assess differences between means when the ANOVAs indicated significant overall differences.

3. Results

3.1. Behavioral Results

No significant preferences for either door were observed in any group during the pretraining habituation session (paired Student's t-tests, $p>0.120$ for 1-, 3-, and 5-session groups). The performances of the animals trained in the allocentric task over 1,

3, and 5 sessions are shown in Fig. 1B. Comparison of the percentages of correct responses in the first training session showed that the three groups exhibited similar low levels of performance that were close to random (55.5% ± 1.30; $F_{2,23}$=1.967, p>0.165). A one-way repeated measures ANOVA revealed that the animals that were trained for three and five sessions improved their accuracies because the percentages of correct responses in these groups increased significantly across training ($F_{2,14}$=9.123, p<0.003; and $F_{4,28}$=31.507, p<0.0001 for the 3- and 5-session groups, respectively). Indeed, high percentages of correct responses were reached on the third training session by both groups (78.75% ± 2.12). The similarity in the percentages of correct responses between days 3 to 5 in the 5-session group suggests that acquisition reached an asymptotic level by the third session. Consequently, a Helmert contrast was performed on the data from the 5-session group to confirm this observation. Helmert contrasts are orthogonal comparisons in which each mean is compared with the combined mean of subsequent days of training; thus, the mean of the first day of training was compared to the mean of the subsequent days of training, the mean of the second day was compared to the mean of the third, fourth, and fifth day, etc. The results indicated that the animals in the 5-session group did not improve their performance between sessions 3 and 5 ($F_{1,7}$<1.607 for both, p>0.245 for both, for sessions 3 to 5; Fig. 1B). Therefore, an asymptotic level of learning was reached by the third session of training.

3.2. Cytochrome Oxidase Results

No significant differences were found between the CO activities of the left and right hemispheres (Student's t-test, p>0.06 for all). Therefore, measures from both hemispheres were averaged to obtain a single CO activity value for each of the seven regions examined here.

One-way ANOVAs with group as the independent variable were used to determine differences in CO activity in each measured region. Significant overall differences in CO activity were obtained in all Dlv levels ($F_{3,31}$=6.573, p=0.002, $F_{3,31}$=6.457, p<0.002, $F_{3,31}$=3.896, p=0.019, for r-Dlv, m-Dlv and c-Dlv, respectively; Fig.

3A). Post hoc analysis revealed significantly elevated CO activity in the r-Dlv,m-Dlv and c-Dlv in the 1-session group compared to the naive group (all ps<0.050).These results suggest that the Dlv was differentially activated by the first session of training in which the animals integrated the information necessary to solve the task.

The data for the 3- and 5-session groups revealed a clear reduction in the activity of the r-Dlv and m-Dlv with training progress. Thus, after 3 days of training, the functional activities of these precommissural levels of the Dlv continued to be elevated relative to the naive group but were below the levels of the 1-session group; however, these differences were not significant (p>0.076 for all). Examination of the CO levels of the 5-session group corroborated this decline. Indeed, post-hoc comparisons revealed that, after 5 days of training, the CO activity at both precommissural levels of the Dlv were significantly lower than those observed in the animals that were trained for one day (r-Dlv: p=0.004; m-Dlv: p=0.024), which indicates that the activity of this region declined after the animals learned the task. However, regarding the commissural level there are still significant differences between the naive group and the 3-session group (p=0.020) but not between the animals trained for 5 days and the non-exposed ones (p=0.144).

Regarding the Dld area, no significant differences between groups were found along its rostrocaudal extent ($F_{3,31}$=2.573, p=0.074; $F_{3,31}$=1.512, p=0.233; $F_{3,31}$=1.744, p=0.181, for r-Dld, m-Dld and c-Dld, respectively; Fig. 3B), which indicates that this region was not critically involved in solving this task.

Finally, an increase in CO activity was found in the Dp area of the animals in the trained groups compared to the naive group ($F_{3,31}$=6.035, p=0.003; naive vs. 1-session group: p=0.003; naive vs. 3-session group: p=0.034; naive vs. 5-session group: p=0.010). Aside from the naive group, no differences were found in the Dp area when comparing across the three trained groups (p>0.787 for all; Fig. 3C). This result suggests that the activation of this area did not increase with the number of sessions and, thus, was not related to training.

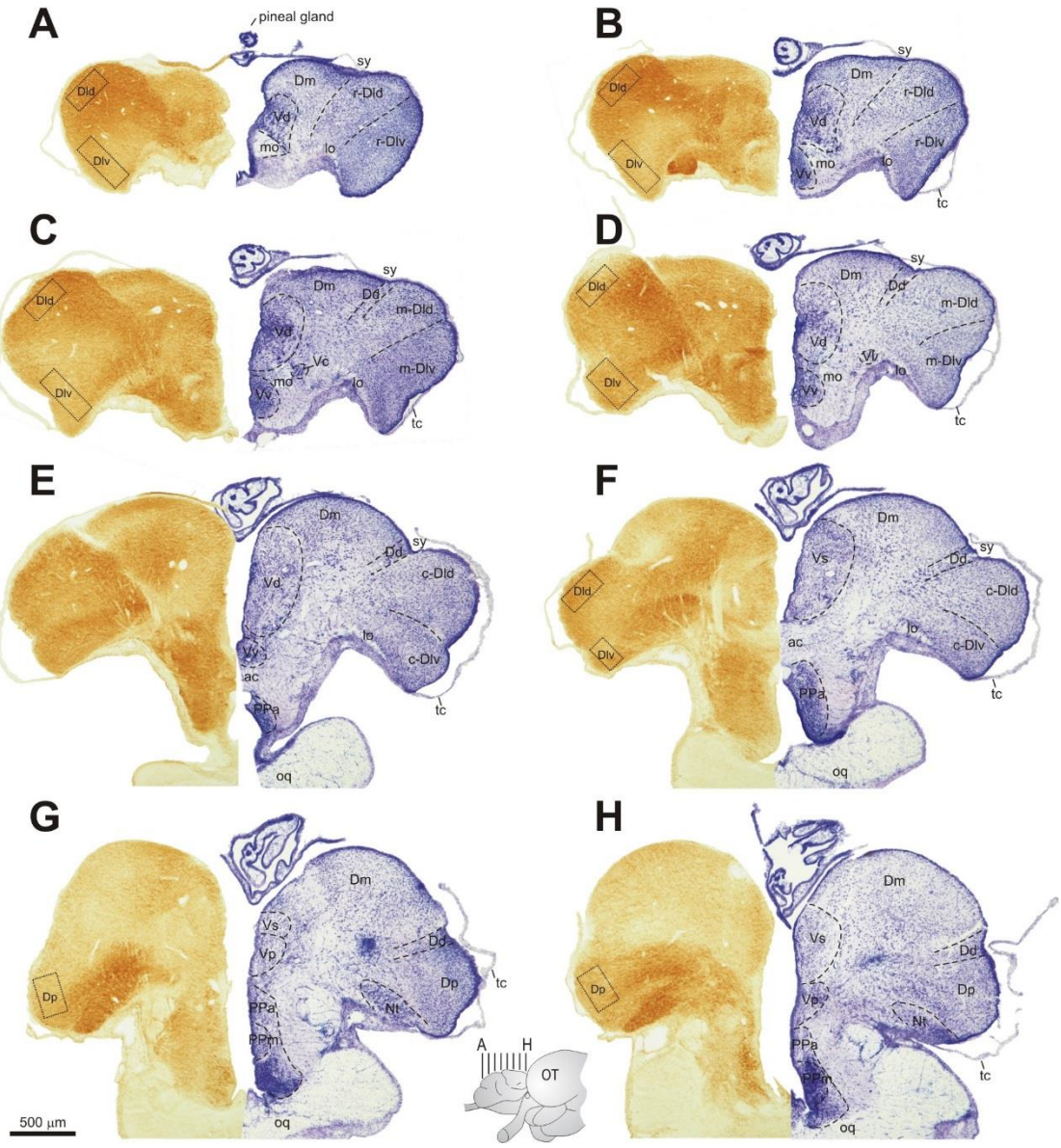

Figure 2. Transverse sections of a naive goldfish telencephalon stained for CO (left) and Nissl (right) showing the sampled areas (black boxes). The first and the last section used to measure CO activity in r-Dld, r-Dlv (A-B), m-Dld, m-Dlv (C-D), c-Dld, c-Dlv (E-F), and Dp (G-H) are shown. Each subsequent region in which CO activity was measured is approximately 450 micrometres caudal to the preceding region. The relative optical densities of the selected brain region were bilaterally measured in six adjacent sections. The drawing at the bottom depicts a lateral view of a goldfish forebrain. The relative positions of the different transverse sections are indicated by the vertical lines. Abbreviations: ac, anterior commissure; c-Dld, m-Dld, r-Dld, caudal, medial and rostral part of dorsal subdivision of lateral division of area dorsalis; c-Dlv, m-Dlv, r-Dlv, caudal, medial and rostral part of ventral subdivision of lateral division of area dorsalis; Dd, dorsal division of area dorsalis; Dm, medial subdivision of area dorsalis; Dp, posterior division of area dorsalis; lo, lateral olfactory tract; mo, medial olfactory tract; Nt, nucleus taeniae; ot, optic tract; Pin, pineal organ; PPa, nucleus preopticus parvocellularis anterioris; PMm, nucleus preopticus magnocelullaris, pars magnocellularis; sy, sulcus ypsiloniformis; tc, tela chroroidea; TEC, optic tectum; Vc, central nucleus of area ventralis; Vd, dorsal nucleus of area ventralis; Vl, lateral nucleus of area ventralis; Vp, postcommissural nucleus of area ventralis; Vs, supracommissural nucleus of area ventralis; Vv, ventral nucleus of area ventralis.

3.3. Neurobehavioral Correlations

We tested whether the metabolic changes observed across training were related to the accuracy with which the animals solved the task. The CO activity was correlated with the percentages of correct responses on the last day of training for the animals in the 1-, 3-, and 5-session groups. A significant negative correlation was observed between the final percentages of correct responses of the individual animals and the CO activities in the Dlv at the precommissural levels (r=-0.468, p=0.021; r=-0.438, p=0.032, for r-Dlv and m-Dlv, respectively; Fig. 3D), which indicates that, as the animals improved their accuracies, metabolic activity decreased. Conversely, no neurobehavioral correlations were found for Dld along its rostrocaudal extent (r>-0.290 for all, p>0.058 for all), for c-Dlv (r=-0.187, p=0.381) or for Dp (r=-0.289, p=0.171). Together, these data indicate that the CO activity changes found in the precommissural levels of the Dlv were related to the performance of the behavioral task, whereas the increase in CO activity observed in c-Dlv and Dp was not related to the efficiency acquired with training.

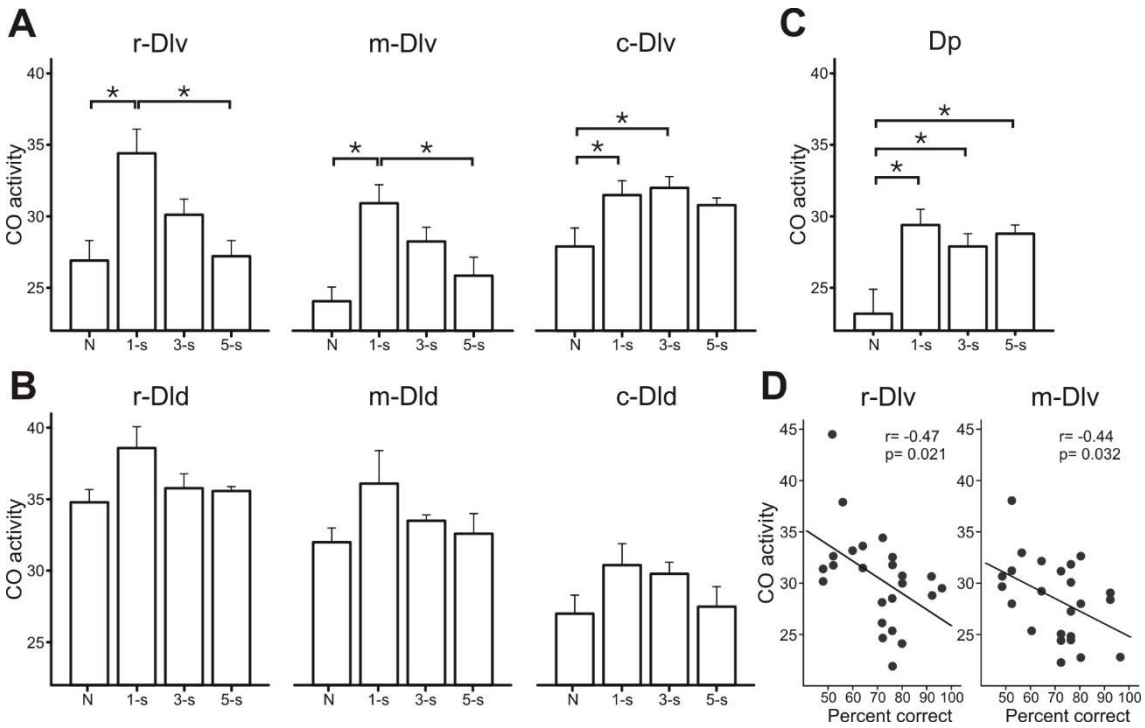

Figure 3. (A-C) Mean±SEM values for CO activity (µmol of cytochrome c oxidised/min/g tissue wet weight; n=8 fish per group) obtained from the seven regions of interest. (D) Plots showing the two significant neurobehavioral correlations between the percentages of correct responses of each trained group and the normalized CO activities measured in the r-Dlv and m-Dlv, respectively. Abbreviations: N, naive group; 1-s, 1-session group; 3-s, 3-session group; 5-s, 5-session group.

4. Discussion

In the present work, we used CO histochemistry to analyze the metabolic changes in the Dl and the Dp pallial regions that resulted from training goldfish for 1, 3, or 5 days in a spatial constancy task. This approach revealed significant changes in the CO activities of the precommissural (r-Dlv and m-Dlv), commissural Dlv (c-Dlv) and the Dp, although the patterns of activation of these regions were clearly different. No changes in CO activity were found at any level of the Dld in any of the experimental groups. Regarding Dp, its activity was enhanced from the first session to the fifth training day; however, there was no correlation between CO activity in the region and the animals' performances in the behavioural task. In contrast, CO levels in the r-Dlv and m-Dlv were correlated with the animals' behavioural performances, which suggest that this pallial region had a key role in codifying the information necessary to learn the spatial task. The present study also suggests a clear temporal-limited role of the precommissural Dlv in the acquisition of a spatial task. Henceforth, we will discuss and highlight the relevance of our functional data to the possible homologies of the cell masses in the telencephalon of actinopterygian fishes.

As has been previously reported (Broglio et al., 2010; López et al., 2000; Salas et al., 1996; Uceda et al., 2015), the results of the present work revealed that the animals required only three days of training to learn the spatial constancy task. Indeed, our data revealed that, after the third day of training, the animals reached an asymptotic level of performance (see Fig. 1B). Regarding the activities of the pallial regions analyzed here, we only observed a significant correlation between the CO levels of the precommissural region of the Dlv and the animals' performance. CO levels increased significantly in the r-Dlv and m-Dlv of animals that were trained for one day (1-session group; see Fig. 3A). Examination of the 3- and 5-session groups revealed that the CO activities of the r-Dlv and m-Dlv decreased to levels that were similar to those of the naive group; these findings indicate a clear pattern of deactivation in the precommissural Dlv that occurred after the animals reached asymptotic levels of performance. Indeed, negative linear correlations were observed between the CO

activities in the r-Dlv and m-Dlv and the percentages of correct responses (see Fig. 3D). In mammals, birds, and reptiles the hippocampus, in addition to other telencephalic structures, is critical for encoding environmental information in map-like or relational memory representations for spatial navigation (Bingman et al., 1998; Burgess et al., 1999; López et al., 2003a, b; O'Keefe and Nadel, 1978; Rodríguez et al., 2002; Sherry and Duff, 1996). Although the eversion process that takes place during the development of the forebrains of ray-finned fishes implies a reversal of the pallial medial-to-lateral topography observed in the evaginated telencephalons (for a review, see Nieuwenhuys, 2011), considerable hodological and developmental evidence suggests that the teleostean Dl is the homologue of the hippocampus or medial pallium of amniotes (Harvey-Girard et al., 2012; Northcutt, 2006, 2008; Wullimann and Mueller, 2004; Yamamoto and Ito, 2008; Yamamoto et al., 2007). Previous behavioral studies also support the homology of the teleost Dl and the medial pallium of tetrapods. Thus, specific allocentric spatial learning deficits have been consistently reported after Dlv lesions in a variety of spatial procedures (Broglio et al., 2005; Durán et al., 2010; Rodríguez et al., 2002; Salas et al., 2008); training goldfish in a spatial task identical to that employed here produces morpho-functional plasticity-related changes in the Dlv (Broglio et al., 2010), and female blenniid fish have larger Dlv regions than males because the females explore wider territories in their natural environments (Costa et al., 2011). Moreover, the present study provides further functional evidence about the regions of the teleost pallium that are involved in spatial learning. We showed that only the precommissural region of the Dlv (r-Dlv and m-Dlv) was engaged when animals were integrating the spatial information necessary to solve the task (1-session group), whereas no significant correlation between the CO activity in the c-Dlv and the percentages of correct responses were observed in any experimental group (see Fig. 3A) despite significant changes in CO activity. These findings suggest that the Dlv cannot be considered a functionally homogenous structure along its rostro-caudal axis and that the precommissural Dlv of goldfish has a time-dependent role in encoding spatial memories. At the beginning of training, when the new information was being processed, the CO activity was enhanced, meanwhile the activity of the precommissural Dlv decreased to a level similar to that of the naive group after the third session of training. These findings suggest that this structure has a minor role

after spatial memories have been acquired and, most likely, consolidated. Similarly, several studies have proposed a time-dependent role for the mammalian hippocampus in the storage and retrieval of some forms of memory (Frankland and Bontempi, 2005; Restivo et al., 2009).

Most contemporary views of system consolidation agree with the idea that the storage and retrieval of recent memory depend on the hippocampus during the initial stages, whereas at later stages, the hippocampus interacts with neocortical sites at which cortico-cortical connections progressively develop and, ultimately, self-govern the storage and retrieval of remote memories (Frankland and Bontempi, 2005; Song et al., 2011; Squire, 2004; Squire and Alvarez, 1995; Squire and Bayley, 2007; Squire et al., 2004). The marked difference that we observed in the levels of CO activity in the goldfish r-Dlv and m-Dlv before and after reaching asymptotic levels of performance may be associated with an optimization of energy utilization; i.e., increases in efficiency that occurred as performance of the task improved (Olson et al., 2006; Winocur et al., 2010). Thus, once the task has been consolidated, there is no need to maintain the sustained activity to encode the environment to which the animal has been thoroughly exposed when the requirements of the task have not changed. These results suggest a new scenario because, as system consolidation theory predicts for other species, the teleost hippocampal pallium is critical for creating new spatial memories, whereas this region is likely less important during later states. During these later stages, other structures of the teleost pallium are likely responsible for the storage and retrieval of remote memories in a manner that is similar to those of the mammalian cortical structures that are involved in these processes (e.g., the medial prefrontal cortex and the anterior cingulate cortex). Our finding of a time-dependent role of the teleost hippocampal pallium in memory along with the data demonstrating that the initial stages of memories are dependent on the antennal lobes of invertebrate species and that this dependence shifts to the mushroom bodies with time (McBride et al., 1999; Menzel, 2001), indicate that as previously suggested (Frankland and Bontempi, 2005), system consolidation might be a general organizing principle across species. However, experimental findings regarding spatial memory that appear to be in conflict with system consolidation theory have emerged in both

the human and animal literatures; some works have shown that spatial memory can remain hippocampus-dependent because spatial memories are formed and permanently stored in the hippocampus or because the expression of spatial memory requires the hippocampus (Bolhuis et al., 1994; Broadbent et al., 2006; Clark, 2011; Clark et al., 2005; Martin et al., 2005; Mumby et al., 1999; Sutherland et al., 2001). Conversely, it has been suggested that map-like or allocentric spatial representations of complex environments that are created through extensive preoperative experience can survive hippocampal damage and enable navigation (Winocur et al., 2005, 2010, 2013). A crucial element in the survival of these spatial representations appears to be the extent of experience before hippocampal lesion. Thus, in human studies, the subjects have had extensive experience with the environment before hippocampal damage (Teng and Squire, 1999, Rosenbaum et al., 2000), whereas, in most animal studies, the lesions are created immediately after reaching a learning criterion. Indeed, all studies in which the goldfish Dlv has been lesioned after training on allocentric procedures have reported retrograde amnesia (Broglio et al., 2010; Rodríguez et al., 2002). Interestingly, our data support this view because overtraining goldfish in the spatial constancy task resulted in a deactivation of the pallial region that is considered to be the homologue of the mammalian hippocampus. Future studies examining the effects of the precommissural Dlv lesions and lesions of other pallial structures after an extensive experience or training could shed light on the precise dynamics of pallial circuits in the codification, consolidation and retrieval of new and remote spatial memories in teleost fish.

Another main result of the present work is that training goldfish in a spatial constancy task did not produce any significant changes in CO activity in the r-Dld, m-Dld or c-Dld, which indicates that Dld activity is not engaged in spatial learning (Fig. 3B). Currently, there is controversy concerning whether the entire Dl constitutes a single homogenous primary pallial unit that can be considered homologous to the medial pallium or hippocampus of land vertebrates (Harvey-Girard et al., 2012; Ito and Yamamoto, 2009; Mueller, 2012; Northcutt, 2008; Yamamoto et al., 2007). Northcutt (2006) reported that the extrinsic and intrinsic connections of the Dld and Dlv are rather uniform and, consequently, it is difficult to establish a functional division

between the Dld and Dlv. In contrast, Yamamoto and colleagues (Yamamoto and Ito, 2008; Yamamoto et al., 2007), basing their conclusions on the available hodological data and the possibility that the preglomerular complex, the main diencephalic sensory relay station of teleosts, is homologous to the dorsal thalamus of tetrapods, suggested that the Dl should be split into a ventral portion (medial pallium homologue) and a dorsal portion (dorsal pallium homologue). Saidel et al. (2001) used electrophysiological and CO data to identify a visual region in the Dld and suggested that this area corresponds to the mammalian geniculo-recipient primary visual area. Similarly, voltage-sensitive dye imaging, a technique that provides high spatio-temporal resolution, showed that pallial visually related activity is exclusively restricted to a well-defined bulge of the Dld (Ocaña et al., 2009). Thus, in our opinion, the present data agree with previous functional and lesion-based behavioral studies that support the division of the Dl into two subdivisions, at least in goldfish, because the Dld and Dlv are notably functionally different. Harvey-Girard and colleagues (2012) argued that the spatial deficit observed following Dlv lesions performed in our lab could be due to the possibility that the lesions compromised the innervation of the entire Dl and disrupted the functionality of both the Dld and Dlv regions. However, our data showed that training goldfish in a relational spatial task exclusively activated the precommissural region of the Dlv and not the Dld, which precludes the argument regarding the fibres of passage. Together, these data suggest that there are clear functional differences between the Dld and Dlv and that future connectivity studies guided by the currently available functional data and developmental, imaging, electrophysiological and behavioral studies are needed to definitively describe the similarities and differences between these pallial subdivisions and the possible homology between the Dld and the dorsal pallium and between the Dlv and the medial pallium of amniotes.

Regarding the Dp, the enhanced level of CO activity in the habituation, 1-, 3-, and 5-session groups did not correlate with the behavioral scores, which indicates that the learning process per se did not modify the activity of this region. Similarly, CO increases were measured in c-Dlv but did not correlate with the animal's percentage of correct choices. This finding is in contrast to the previously described pattern of

activity of the precommissural Dlv and suggests a different functional role of the caudal Dlv and the Dp regions during spatial learning. The border between Dlv and Dp is also in debate because some authors consider Dlv and Dp an homogeneous pallial area (Butler, 2000; Nieuwenhuys, 2009). In this experiment it is shown a functional difference along the rostrocaudal extent of Dlv between the most rostral part and the commissural Dlv. Furthermore, the CO activity pattern of c-Dlv is similar to that of Dp .The Dp is the most ventrocaudal region of the teleost pallium and characterized by the fact that it receives most of the secondary olfactory projections (Levine and Dethier, 1985; Meek et al., 1998; Northcutt, 2006; von Bartheld et al., 1984). Furthermore, electrophysiological and imaging studies have provided converging evidence about the role of this pallial region in the higher-order processing of olfactory information (Blumhagen et al., 2011; Nikonov and Caprio, 2005; Nikonov et al., 2005). Despite this evidence about the olfactory role of the Dp region, there is no consensus about the origin of Dp cells and, consequently, the possible homology between this region and the lateral or piriform cortex of mammals is still a matter of discussion. Wullimann and Mueller (2004) and Yamamoto and colleagues (2007) have suggested that Dp cells and the mammalian piriform or olfactory cortex originate from the same germinative zone of the neural tube and reach a definitive position in the Dp after a caudo-lateral migration process. In contrast, it has been proposed by Nieuwenhuys (2009) that the teleost pallium develops by a simple eversion process; therefore, the Dp is situated in the immediate vicinity of its germinative zone and represents a specialized part of the hippocampal pallium of teleosts. However, a recent developmental study in zebrafish provided clear evidence about the migration of Dp cells during forebrain development that supports the homology of the Dp and the mammalian lateral or piriform cortex (Mueller et al., 2011). In this context, the sustained activation of the Dp region from the first session to the end of the training observed in the present study could be explained by the fact that chemical stimuli provide the primary information for spatial orientation over spatial scales that range from thousands of kilometers to less than a meter in both fish and birds (DeBose and Nevitt, 2008; Mitamura et al., 2005). Lesions of the piriform cortex dramatically impair homing navigation in pigeons by specifically disrupting navigational map orientation (Papi and Casini, 1990) and blocking navigational map learning (Gagliardo et al., 1997).

Furthermore, Paztke and colleagues (2010) used the expression of the neuronal activity-dependent marker ZENK to show that the olfactory system is activated during navigation over unfamiliar areas in homing pigeons. Similarly, and independently of the spatial scale, fish interpret olfactory information in fluid mediums in which odors are dispersed as filamentous patches and orient to these odorants by calibrating their odor sampling with their lateral line perception of hydrodynamic trails (DeBose and Nevitt, 2008). Mitamura and colleagues (2005) showed that black rockfish (a typical site-specific fish) experimentally displaced as little as 4 km require olfaction to return to their original familiar home range habitat, and Riedel (1998) reported that olfactory bulb ablation in blind cave fish disrupts the normal exploratory behavior observed in novel environments. Hence, similar to what has been described in birds, we suggest that the increments in CO activity observed in the Dp region could indicate an important role of the fish olfactory pallium in the specific mechanisms that link olfactory inputs to the navigational responses (Jacobs, 2012).

5. References

Agin, V., Chichery, R. andChichery, M.P. (2001). Effects of learning on cytochrome oxidase activity in cuttlefish brain. Neuroreport;12:113-6.

Bingman, V.P., Strasser, R., Baker, C. and Riters, L.V. (1998). Paired-associate learning is unaffected by combined hippocampal and parahippocampal lesions in homing pigeons. Behavioral neuroscience;112:533-40.

Blumhagen, F., Zhu, P., Shum, J., Scharer, Y.P., Yaksi, E,. Deisseroth, K., et al. (2011). Neuronal filtering of multiplexed odour representations. Nature;479:493-8.

Bolhuis, J.J., Stewart, C.A. and Forrest, E.M. (1994). Retrograde amnesia and memory reactivation in rats with ibotenate lesions to the hippocampus or subiculum. The Quarterly journal of experimental psychology B, Comparative and physiological psychology;47:129-50.

Broadbent, N.J., Squire, L.R. and Clark, R.E. (2006). Reversible hippocampal lesions disrupt water maze performance during both recent and remote memory tests. Learning & memory (Cold Spring Harbor, NY);13:187-91.

Broadbent, N.J. and Clark, R.E. (2013). Remote context fear conditioning remains hippocampus-dependent irrespective of training protocol, training-surgery interval, lesion size, and lesion method. Neurobiology of learning and memory.

Broglio, C., Gómez, A., Durán, E., Ocaña, F.M., Jiménez-Moya, F., Rodríguez, F. and Salas, C. (2005). Hallmarks of a common forebrain vertebrate plan: specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Research Bulletin, 66, 277–281.

Broglio, C., Rodríguez, F., Gómez, A., Arias, J.L. and Salas, C. (2010). Selective involvement of the goldfish lateral pallium in spatial memory. Behavioural brain research;210:191-201.

Bruchey, A.K. and Gonzalez-Lima, F. (2008). Enhanced metabolic capacity of the frontal cerebral cortex after Pavlovian conditioning. Neuroscience;152:299-307.

Burgess, N., Jeffery, K.J. and O'Keefe, J. (1999). The hippocampal and parietal foundations of spatial cognition. London: Oxford University Press.

Butler, A.B. (2000). Topography and topology of the teleost telencephalon: a paradox resolved. Neuroscience letters;293:95-8.

Cada, A., de la Torre, J.C. and Gonzalez-Lima, F. (2000). Chronic cerebrovascular ischemia in aged rats: effects on brain metabolic capacity and behavior. Neurobiology of aging;21:225-33.

Cheng, K., Spetch, M.L., Kelly, D.M. and Bingman, V.P. (2006). Small-scale spatial cognition in pigeons. Behavioural processes;72:115-27.

Clark, R.E. (2011). Eyeblink conditioning and systems consolidation: An ironic yet powerful pairing. Neurobiology of Learning and Memory;95:7.

Clark, R.E., Broadbent, N.J. and Squire, L.R. (2005). Hippocampus and remote spatial memory in rats. Hippocampus;15:260-72.

Clark, R.E., Broadbent, N.J. and Squire, L.R. (2005). Impaired remote spatial memory after hippocampal lesions despite extensive training beginning early in life. Hippocampus.;15:340-6.

Clark, R.E., Broadbent, N.J. and Squire, L.R. (2007). The hippocampus and spatial memory: findings with a novel modification of the water maze. The Journal of neuroscience : the official journal of the Society for Neuroscience;27:6647-54.

Conejo, N.M., Gonzalez-Pardo, H., Vallejo, G. and Arias, J.L. (2007). Changes in brain oxidative metabolism induced by water maze training. Neuroscience;145:403-12.

Costa, S.S., Andrade, R., Carneiro, L.A., Goncalves, E.J., Kotrschal, K. and Oliveira, R.F. (2011). Sex differences in the dorsolateral telencephalon correlate with home range size in blenniid fish. Brain, behavior and evolution;77:55-64.

DeBose, J.L. and Nevitt, G.A. (2008). The use of odors at different spatial scales: comparing birds with fish. Journal of chemical ecology;34:867-81.

Deglise, P., Dacher, M., Dion, E., Gauthier, M and Armengaud, C. (2003). Regional brain variations of cytochrome oxidase staining during olfactory learning in the honeybee (Apis mellifera). Behavioral neuroscience;117:540-7.

Demski, L.S. (2003). In a fish´s brain´s eye: Visual pallium of teleosts. In: Collin SP, Marshall NJ, editors. Sensory Processing in Aquatic Environments, New York: Springer-Verlag; p. 404-19.

Durán, E., Ocaña, F.M., Gómez, A., Jimenez-Moya, F., Broglio, C., Rodríguez, F., et al. (2008). Telencephalon ablation impairs goldfish allocentric spatial learning in a "hole-board" task. Acta neurobiologiae experimentalis.;68:519-25.

Durán, E., Ocaña, F.M., Broglio, C., Rodríguez, F. and Salas, C. (2010). Lateral but not medial telencephalic pallium ablation impairs the use of goldfish spatial allocentric strategies in a "hole-board" task. Behavioural brain research;214:480-7.

Eichenbaum, H., Dudchenko, P., Wood, E., Shapiro, M. and Tanila, H. (1999). The hippocampus, memory, and place cells: is it spatial memory or a memory space? Neuron;23:209-26.

Folgueira, M., Bayley, P., Navratilova, P., Becker, T.S., Wilson, S.W. and Clarke, J.D. (2012). Morphogenesis underlying the development of the everted teleost telencephalon. Neural development;7:32.

Frankland, P.W. and Bontempi, B. (2005). The organization of recent and remote memories. Nature reviews Neuroscience;6:119-30.

Gagliardo, A., Mazzotto, M. and Bingman, V.P. (1997). Piriform cortex ablations block navigational map learning in homing pigeons. Behavioural brain research;86:143-8.

Gonzalez-Lima, F. and Jones, D. (1994). Quantitative mapping of cytochrome oxidase activity in the central auditory system of the gerbil: a study with calibrated activity standards and metal-intensified histochemistry. Brain research;660:34-49.

Gonzalez-Lima, F. and Cada, A. (1994). Cytochrome oxidase activity in the auditory system of the mouse: a qualitative and quantitative histochemical study. Neuroscience;63:559-78.

Gonzalez-Pardo, H., Conejo, N.M., Lana, G. and Arias, J.L. (2012). Different brain networks underlying the acquisition and expression of contextual fear conditioning: a metabolic mapping study. Neuroscience;202:234-42.

Harvey-Girard, E., Giassi, A.C., Ellis, W. and Maler, L. (2012). Organization of the gymnotiform fish pallium in relation to learning and memory: IV. Expression of conserved transcription factors and implications for the evolution of dorsal telencephalon. The Journal of comparative neurology;520:3395-413.

Ingle, D.J. and Sahagian, D.E. (1973). Solution of a spatial constancy problem by goldfish. Physiol Psychol;1:83-4.

Ito, H. and Yamamoto, N. (2009). Non-laminar cerebral cortex in teleost fishes? Biology letters;5:117-21.

Jacobs, L.F. (2012). From chemotaxis to the cognitive map: The function of olfaction. Pnas;109:8.

Levine ,R.L. and Dethier, S. (1985). The connections between the olfactory bulb and the brain in the goldfish. The Journal of comparative neurology;237:427-44.

López, J.C., Broglio, C., Rodríguez, F., Thinus-Blanc, C. and Salas, C. (1999). Multiple spatial learning strategies in goldfish (Carassius auratus). Animal Cognition;2:109-20.

López, J.C., Bingman, V.P., Rodríguez, F., Gómez, Y. and Salas, C. (2000). Dissociation of place and cue learning by telencephalic ablation in goldfish. Behavioral neuroscience;114:687-99.

López, J.C., Gómez, Y., Vargas, J.P. and Salas, C. (2003). Spatial reversal learning deficit after medial cortex lesion in turtles. Neuroscience letters;341:197-200.

López, J.C., Vargas, J.P., Gómez, Y. and Salas, C. (2003). Spatial and non-spatial learning in turtles: the role of medial cortex. Behavioural brain research;143:109-20.

Martin, S.J., de Hoz, L. and Morris, R.G. (2005). Retrograde amnesia: neither partial nor complete hippocampal lesions in rats result in preferential sparing of remote spatial memory, even after reminding. Neuropsychologia;43:609-24.

Mark, R. andBraford, Jr. (2009). Stalking the Everted Telencephalon: Comparisons of Forebrain Organization in Basal Ray-Finned Fishes and Teleosts. Brain, Behavior and Evolution;74:21.

McBride, S.M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., et al. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. Neuron;24:967-77.

McClelland, J.L., McNaughton, B.L. and O'Reilly, R.C. (1995). Why there are complementary learning systems in the hippocampus and neocortex: insights from the successes and failures of connectionist models of learning and memory. Psychological review;102:419-57.

Meek, J. and Nieuwenhuys, R. (1998). Holosteans and teleosts. In: Nieuwenhuys R, ten Donkelaar HJ, Nicholson C, editors. The central nervous system of vertebrates, Berlin: Springer;. p. 759-937.

Mendez-López, M., Arias, J.L., Bontempi, B. and Wolff, M. (2013). Reduced cytochrome oxidase activity in the retrosplenial cortex after lesions to the anterior thalamic nuclei. Behavioural brain research;250:264-73.

Menzel, R. (2001). Searching for the memory trace in a mini-brain, the honeybee. Learning & memory (Cold Spring Harbor, NY);8:53-62.

Mitamura, H., Arai, N., Sakamoto, W., Mitsunaga, Y., Tanaka, H,. Mukai, Y., et al. (2005). Role of olfaction and vision in homing behaviour of black rockfish Sebastes inermis. J Exp Mar Biol Ecol;322:123-34.

Mueller, T. (2012). What is the Thalamus in Zebrafish? Frontiers in neuroscience;6:64.

Mueller, T. and Wullimann, M.F. (2009). An evolutionary interpretation of teleostean forebrain anatomy. Brain, behavior and evolution;74:30-42.

Mueller, T., Dong, Z., Berberoglu, M.A. and Guo, S. (2011). The dorsal pallium in zebrafish, Danio rerio (Cyprinidae, Teleostei). Brain research;1381:95-105.

Mumby, D.G., Astur, R.S., Weisend, M.P. and Sutherland, R.J. (1999). Retrograde amnesia and selective damage to the hippocampal formation: memory for places and object discriminations. Behavioural brain research;106:97-107.

Nieuwenhuys. R. (1963). The comparative anatomy of the actinopterygian forebrain. Journal fur Hirnforschung;7:171-92.

Nieuwenhuys, R. (2009). The forebrain of actinopterygians revisited. Brain, behavior and evolution;73:229-52.

Nieuwenhuys, R. (2011). The development and general morphology of the telencephalon of actinopterygian fishes: synopsis, documentation and commentary. Brain structure & function;215:141-57.

Nikonov, A.A., Finger, T.E. and Caprio, J. (2005). Beyond the olfactory bulb: an odotopic map in the forebrain. Proceedings of the National Academy of Sciences of the United States of America;102:18688-93.

Nikonov, A.A. and Caprio, J. (2005). Processing of odor information in the olfactory bulb and cerebral lobes. Chemical senses;30 Suppl 1:i317-8.

Northcutt, R.G. (2006). Connections of the lateral and medial divisions of the goldfish telencephalic pallium. The Journal of comparative neurology;494:903-43.

Northcutt, R.G. (2008). Forebrain evolution in bony fishes. Brain research bulletin;75:191-205.

Ocaña, F.M., Durán, E., Rodríguez, F. and Salas, C. (2009). Voltage-sensitive dye imaging of visually evoked responses in the goldfish telencephalic pallium. Conference Abstract: 41st European Brain and Behaviour Society Meeting: Frontiers in Behavioral Neuroscience.

O'Keefe, J. and Nadel, L. (1978). The hippocampus as a cognitive map. Oxford: Clarendon Press.

Olson, I.R., Rao, H., Moore, K.S., Wang, J., Detre, J.A. and Aguirre, G.K. (2006). Using perfusion fMRI to measure continuous changes in neural activity with learning. Brain and cognition;60:262-71.

Papi, F. and Casini, G. (1990). Pigeons with ablated pyriform cortex home from familiar but not from unfamiliar sites. Proceedings of the National Academy of Sciences of the United States of America;87:3783-7.

Patzke, N., Manns, M., Gunturkun, O., Ioale, P. and Gagliardo, A. (2010). Navigation-induced ZENK expression in the olfactory system of pigeons (Columba livia). The European journal of neuroscience;31:2062-72.

Poremba, A., Jones, D. and Gonzalez-Lima, F. (1998). Classical conditioning modifies cytochrome oxidase activity in the auditory system. The European journal of neuroscience;10:3035-43.

Poremba, A., Jones, D. and Gonzalez-Lima, F. (1997). Metabolic effects of blocking tone conditioning on the rat auditory system. Neurobiology of learning and memory;68:154-71.

Reese, E.S. (1989). Orientation behavior of butterflyfishes (family Chaetodontidae) on coral reefs: Spatial learning of route specific landmarks and cognitive maps. Environ Biol Fishes;25:79-86.

Restivo, L., Vetere, G., Bontempi, B. and Ammassari-Teule, M. (2009). The formation of recent and remote memory is associated with time-dependent formation of dendritic spines in the hippocampus and anterior cingulate cortex. J Neurosci.; 29,8206-14.

Riedel, G. (1998). Long-term habituation to spatial novelty in blind cave fish (Astyanax hubbsi): role of the telencephalon and its subregions. Learning & memory (Cold Spring Harbor, NY);4:451-61.

Rodríguez, F., Durán, E., Vargas, J.P., Torres, B. and Salas, C. (1994). Performance of goldfish trained in allocentric and egocentric maze procedures suggests the presence of a cognitive mapping system in fishes. Animal Learning & Behavior;22:409-20.

Rodríguez, F., López, J.C., Vargas, J.P., Broglio, C., Gómez, Y. and Salas, C. (2002). Spatial memory and hippocampal pallium through vertebrate evolution: insights from reptiles and teleost fish. Brain research bulletin;57:499-503.

Rodríguez, F., López, J.C., Vargas, J.P., Gómez, Y., Broglio, C. and Salas, C. (2002). Conservation of spatial memory function in the pallial forebrain of reptiles and ray-finned fishes. The Journal of neuroscience: the official journal of the Society for Neuroscience;22:2894-903.

Rosenbaum, R.S., Priselac, S., Kohler, S., Black, S.E., Gao, F., Nadel, L., et al. (2000). Remote spatial memory in an amnesic person with extensive bilateral hippocampal lesions. Nature neuroscience;3:1044-8.

Saidel, W.M., Marquez-Houston, K. and Butler, A.B. (2001). Identification of visual pallial telencephalon in the goldfish, Carassius auratus: a combined cytochrome oxidase and electrophysiological study. Brain research;919:82-93.

Salas, C., Broglio, C., Rodríguez, F., López, J.C., Portavella, M. and Torres, B. (1996). Telencephalic ablation in goldfish impairs performance in a 'spatial constancy' problem but not in a cued one. Behavioural brain research;79:193-200.

Salas, C., Broglio, C. and Rodríguez, F. (2003). Evolution of forebrain and spatial cognition in vertebrates: conservation across diversity. Brain, behavior and evolution;62:72-82.

Salas, C., Broglio, C., Durán, E., Gómez, A., Ocaña, F.M., Jimenez-Moya, F., et al. (2006). Neuropsychology of learning and memory in teleost fish. Zebrafish;3:157-71.

Salas, C., Broglio, C., Durán, E., Gómez, A. and Rodríguez, F. (2008). Spatial Learning in Fish. Oxford: Elsevier ed. Nueva York, Estados Unidos: Academic Press.

Sherry, D. and Duff, S. (1996). Behavioural and neural bases of orientation in food-storing birds. The Journal of experimental biology;199:165-72.

Squire, L.R. (2004). Memory systems of the brain: a brief history and current perspective. Neurobiology of learning and memory;82:171-7.

Squire, L.R. and Alvarez, P. (1995). Retrograde amnesia and memory consolidation: a neurobiological perspective. Current opinion in neurobiology;5:169-77.

Squire, L.R., Stark, C.E. and Clark, R.E. (2004). The medial temporal lobe. Annual review of neuroscience;27:279-306.

Squire, L.R and Bayley, P.J. (2007). The neuroscience of remote memory. Current opinion in neurobiology;17:185-96.

Song, Z., Jeneson, A. and Squire, L.R. (20119. Medial temporal lobe function and recognition memory: a novel approach to separating the contribution of recollection and familiarity. The Journal of neuroscience : the official journal of the Society for Neuroscience;31:16026-32.

Sovrano, V.A., Bisazza, A. and Vallortigara, G. (2003). Modularity as a fish (Xenotoca eiseni) views it: conjoining geometric and nongeometric information for spatial reorientation. Journal of experimental psychology Animal behavior processes;29:199-210.

Sovrano, V.A. and Bisazza, A. (2008). Recognition of partly occluded objects by fish. Animal cognition;11:161-6.

Sutherland, R.J., Weisend, M.P., Mumby, D., Astur, R.S., Hanlon, F.M., Koerner, A., et al. (2001). Retrograde amnesia after hippocampal damage: recent vs. remote memories in two tasks. Hippocampus;11:27-42.

Teixeira, C.M., Pomedli, S.R., Maei, H.R., Kee, N. and Frankland, P.W. (2006). Involvement of the anterior cingulate cortex in the expression of remote spatial memory. The Journal of neuroscience : the official journal of the Society for Neuroscience;26:7555-64.

Teng, E. and Squire, L.R. (1999). Memory for places learned long ago is intact after hippocampal damage. Nature;400:675-7.

Teyke, T. (1989). Learning and remembering the environment in the blind cave fish Anoptichthys jordani. J Comp Physiol A;164:655–62.

Uceda, S., Ocaña, F.M., Martín-Monzón, I., Rodríguez-Expósito, B., Durán, E. and Rodríguez, F. (2015). Spatial learning-related changes in metabolic brain activity contributes to the delimitation of the hippocampal pallium in goldfish. In press.

Vargas, J.P., Rodríguez, F., López, J.C., Arias, J.L. and Salas, C. (2000). Spatial learning-induced increase in the argyrophilic nucleolar organizer region of dorsolateral telencephalic neurons in goldfish. Brain research;865:77-84.

Vargas, J.P., López, J.C., Salas, C. and Thinus-Blanc, C. (2004). Encoding of geometric and featural spatial information by goldfish (Carassius auratus). Journal of comparative psychology;188:206-16.

Villarreal, J.S., Gonzalez-Lima, F., Berndt, J. and Barea-Rodríguez, E.J. (2002). Water maze training in aged rats: effects on brain metabolic capacity and behavior. Brain research;939:43-51.

von Bartheld, C.S., Meyer, D.L., Fiebig, E. and Ebbesson, S.O. (1984). Central connections of the olfactory bulb in the goldfish, Carassius auratus. Cell and tissue research;238:475-87.

Winocur, G., Moscovitch, M., Fogel, S., Rosenbaum, R.S. and Sekeres, M. (2005). Preserved spatial memory after hippocampal lesions: effects of extensive experience in a complex environment. Nature neuroscience;8:273-5.

Winocur, G., Moscovitch, M. and Bontempi, B. (2010). Memory formation and long-term retention in humans and animals: convergence towards a transformation account of hippocampal-neocortical interactions. Neuropsychologia;48:2339-56.

Winocur, G., Moscovitch, M., Rosenbaum, R.S. and Sekeres, M. (2010). An investigation of the effects of hippocampal lesions in rats on pre- and postoperatively acquired spatial memory in a complex environment. Hippocampus;20:1350-65.

Winocur, G., Moscovitch, M. and Sekeres, M.J. (2013). Factors affecting graded and ungraded memory loss following hippocampal lesions. Neurobiology of learning and memory.

Wong-Riley, M.T. (1989). Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. Trends in neurosciences;12:94-101.

Wullimann, M.F. and Mueller, T. (2004). Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. The Journal of comparative neurology;475:143-62.

Yamamoto, N., Ishikawa, Y., Yoshimoto, M., Xue, H.G., Bahaxar, N., Sawai, N., et al. (2007). A new interpretation on the homology of the teleostean telencephalon based on hodology and a new eversion model. Brain, behavior and evolution;69:96-104.

Yamamoto, N. and Ito, H. (2008). Visual, lateral line, and auditory ascending pathways to the dorsal telencephalic area through the rostrolateral region of the lateral preglomerular nucleus in cyprinids. The Journal of comparative neurology;508:615-47.

EXPERIMENT 3. VOLTAGE SENSITIVE DYE IMAGING OF GOLDFISH PALLIAL TELENCEPHALIC ACTIVITY DURING EMOTIONAL CLASSICAL HEART RATE CONDITIONING

1. Introduction

An ongoing goal of neuroscience is to understand the neural substrates of learning and memory, including emotional learning and memory. In this context, associative training paradigms as classical conditioning wherein a neutral stimulus called conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US), has been widely used to produce robust fear learning. After a few paired CS-US presentations, animals develop preparatory and emotional responses to the CS, i.e. freezing behavior and related changes in body physiology as heart rate deceleration (conditioned bradycardia). Considerable evidences indicate that in mammals, the pallial amygdala plays a central role in Pavlovian fear conditioning, for example when a tone and a mild electric shock are used as CS and US, respectively (Ledoux, 2000; Maren, 2005; Phelps and Ledoux, 2005). In this regard, lesions of the amygdala impair the conditioning of the heart rate (Davis et al., 1992; Gentile et al., 1986; Lee and Kim, 2004) and changes in synaptic strength at sensory inputs to the lateral nucleus of this structure occur after fear conditioning (Sigurdsson et al., 2007). On the other hand, experimental evidence show that the neocortical regions responding to the CS (i.e. the auditory cortex) are also involved in Pavlovian auditory fear conditioning particularly in the acquisition, retention, and retrieval of specific fear memories of acoustic cues (Weinberger, 2007a,b). Thus, it has been reported that immediate post-training lesions of the auditory cortex disrupt fear conditioning (Boatman and Kim, 2006), and that the retrieval of remote fear memories depends on secondary auditory cortices (Sacco and Sacchetti, 2010). In addition, fear conditioning enhances specifically the responsiveness of the neurons of the auditory cortex to the CS (Weinberger and Diamond, 1987), in particular shifting tuning toward this stimulus (Weinberger, 2004). Such plasticity extends to human auditory cortex, as revealed by fMRI and MEG neuroimaging techniques (Bröckelmann et al., 2011; Miskovic and Keil, 2012; Thiel et al., 2002).

Despite the cumulative data regarding the involvement of the mammalian amygdala and the neocortical auditory primary area in fear classical conditioning, little is known about the role of distinct telencephalic pallial regions of teleost fish in emotional learning and memory. Eversion (i.e. the outward bending of the distal walls of the prosencephalic vesicle), the unique developmental process of the teleostean telencephalon that, among other characteristics, produces the reversal of the pallial topography observed in non-actinopterygians, has historically hindered the comparison of teleostean and mammalian telencephalons (Braford, 1995; Nieuwenhuys, 1963, 2011; Northcutt, 1995, 2006; Northcutt and Braford, 1980; Striedter and Northcutt, 2006). Even though, recent studies based on the similarities in the pattern of gene expression, the neurochemical distribution, and on developmental, neuroanatomical, and functional evidence, indicate that at least part of the medial region of the area dorsalis telencephali (Dm) might be considered homologue to the mammalian amygdala (Braford, 2009; Broglio et al., 2005; Demski, 2013; Desjardins and Fernald, 2010; Lau et al., 2011; Maximino et al., 2013; Mueller and Wullimann, 2009; Northcutt, 2006, 2008; von Throtha et al., 2014; Wullimann and Mueller, 2004). Indeed, numerous studies have shown that Dm of goldfish, like the pallial amygdala of mammals, plays an important role in emotional learning and memory. For example, lesions in Dm, but not in the lateral division of the area dorsalis telencephali (Dl), i.e. the most likely teleostean region homologue of the hippocampus, impair acquisition and retention of conditioned avoidance in goldfish (Portavella et al., 2003, 2004). In addition, Dm lesions but not those of Dl cause a severe deficit in the acquisition of taste aversion in goldfish (Martín et al., 2011), which resembles that observed in mammals when the amygdala is damaged (Bermúdez-Rattoni 2004; Bernstein, 1999; Lamprecht and Dudai, 2000; Yamamoto et al., 1994). Interestingly, neuroanatomical data show that gustatory and general visceral inputs converge in the teleost fish Dm area suggesting that, like the amygdala of mammals, this region could be a site for the taste-malaise integration necessary for the formation of taste aversion memory in teleosts, and supporting the notion of its critical role in taste aversion learning (Folgueira et al., 2003, 2004; Northcutt 2006; Yoshimoto and Yamamoto, 2010). More specifically, a fear conditioning experiment conducted in our lab revealed that whereas control and sham animals exhibited a conditioned deceleration of the

heart rate after paired CS–US presentations (conditioned bradycardia), goldfish with lesions in a circumscribed region of Dm -the ventral region of Dm (Dmv)-, showed a remarkable impairment in the acquisition of this conditioned response (Broglio et., al 2005) suggesting that the goldfish Dmv region, like the pallial amygdala of mammals, is essential for emotional learning and memory.

Recent data indicates that the Dm region of goldfish could be a heterogeneous division comprising not only an area homologue to the mammalian amygdala, but also containing discrete sensory areas comparable to those present in the neocortex (Demski, 2013; Ocaña et al., 2015; Precht et al., 1998). In this line, some studies have shown that the Dm of various fishes has reciprocal connections with both the preglomerular and the thalamic nuclei, i.e. the main relay station nuclei for auditory, mechanoreceptive, electroreceptive, taste, and somatic information (Echteler and Saidel, 1981; Folgueira et al., 2004; Kanwal et al., 1988; Murakami et al., 1983, 1986a; Northcutt, 2006, 2008; Striedter, 1991; von der Emde and Prechtl, 1999; Yamamoto and Ito, 2005, 2008). Furthermore, mapping of the goldfish pallium activity by means of voltage-sensitive dye imaging has revealed that there might be a sensory pallium in goldfish and it is organized in a cortical-like manner. All together, these experiments suggest that the auditory, somatosensory and gustatory information are represented in different zones within the dorsal portion of Dm (Ocaña et al., 2015). Specifically, the primary auditory area seems to be located immediately caudal to the anterior commissure and the caudal sulcus. In addition, the auditory pallial sensory region of goldfish seems to be topologically organized since tones of different frequencies activate specific pallial domains within the auditory area.

In the present experiment we aimed to further investigate the neural basis of fear classical conditioning in goldfish. As the regions considered homologue to both the mammalian amygdala (Dmv) and the auditory cortex (Dmc) are accessible from a dorsal view of the goldfish pallium, we used in vivo voltage sensitive dye imaging during auditory fear classical conditioning. This methodological approach allows the simultaneous recording of activity in different neuron populations with high spatio-temporal resolution (Chemla and Chavane, 2010; Grinvald and Hildesheim, 2004). With this purpose, we developed an acute goldfish preparation in which we were able to

perform heart rate classical conditioning and simultaneously to record the pallial activity evoked by a 1000Hz tone (CS) before and after being paired with an unconditioned stimulus (electric shock at the base of the goldfish dorsal fin). In addition, we recorded the CS evoked activity after extinction of the acquired conditioned bradycardia.

2. Methods

2.1 Animal Preparation

Goldfish (Carassius auratus) 10-12 cm in length, measured from the mouth to the beginning of the caudal fin, were obtained from the vivarium of the University of Seville and were kept in large tanks with aerated and filtered water at 19±1 ºC, and on a 14/10 light/dark cycle for several weeks prior to experiments. The animals were anesthetized by immersion in a solution 1:20,000 of tricaine methanesulfonate (MS222, Sigma-Aldrich) and then placed in an experimental chamber. An adjustable tube connected to a pump and inserted in the mouth ensured a constant flow of aerated water through the gills. The concentration of anesthesia in the water circuit was maintained at a constant level during the surgical procedure. Animals were placed in the experimental tank and remained partially covered with water and immobilized between two curved pads placed behind its gills. Each goldfish was wrapped in sterile gauze to keep the skin moist during the entire experiment. The dorsal skin and the skull overlying the telencephalon were removed carefully under a binocular microscope (SZ61, Olympus). Subsequently, the underlying fatty tissue was aspirated, and the tela choroidea of the telencephalic ventricle was removed to expose the telencephalon. Following surgery, the anesthetic was removed by replacing the water in the chamber. Recovery of an alert state was evidenced by the reappearance of spontaneous breathing and eye and fin movements.

Di-2-ANEPEQ (JPW 1114; Molecular Probes) was used as voltage sensitive dye (VSD) because of its high water solubility, high diffusion properties, and high sensitivity to low voltage levels, good signal-to-noise ratio, and small decline of fluorescence over time (Cha et al., 2009; Onimaru and Homma, 2003; Prechtl et al., 1997). A dye stock

solution was prepared by dissolving the VSD in distilled water at 0.5 mg/ml. The stock solution was diluted in goldfish Ringer solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2 and 5 mM HEPES, pH = 7.2; all reagents were obtained from Sigma-Aldrich) to a final VSD concentration of 50 µg/ml and used as a daily staining solution. A volume of 100 µl of staining solution was applied topically to the exposed telencephalon for approximately 45 minutes (Figure 3.1). After staining, the telencephalon was washed five times with Ringer solution to eliminate the unbound dye and was kept moist with the same goldfish Ringer solution.

For the recording session, goldfish were immobilized with an intraperitoneal injection of curare (0.002 mg/g animal body weight; d-tubocurarine chloride, Sigma-Aldrich) to eliminate artifacts caused by body movements. All experiments were conducted with the approval of the ethical committee of experimentation of the University of Seville and in accordance with the European Communities Council Directive 86/609/EEC as well as Spanish legislation (R.D. 53/2013).

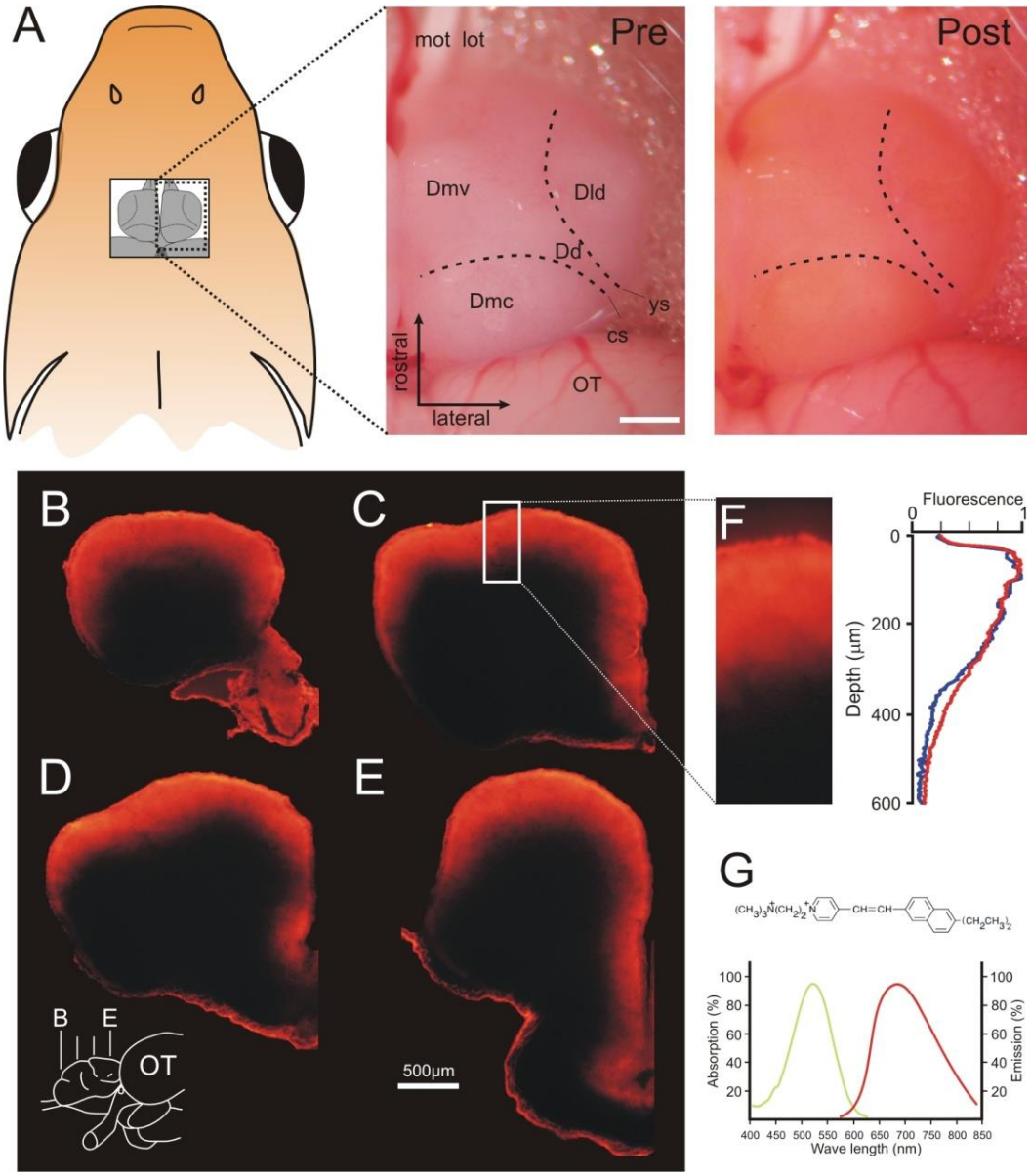

Figure 3.1. Depth penetration of Di-2-ANEPEQ. (A) Schematic representation of the dorsal view of an animal with the telencephalon exposed. The photographs on the right show the coloration effect of the VSD. Before dye application (Pre), the telencephalon surface is pale white, but after staining for 45 minutes (Post), it becomes orange. The dotted lines show the ypsiloniform (ys) and caudal (cs) sulci. (B-E) Transverse coronal sections showing the dye penetration throughout the rostro-caudal and dorso-ventral axes. (F) Depth profile of Di-2-ANEPEQ fluorescence along a radial strip of a coronal section. The photograph represents a magnification of the region signaled by the white rectangle in C. The curves represent the relative fluorescence intensity as a function of depth for the example showed on C (blue line) and averaged for three different experiments (dark yellow line). (G) Absorption (green) and emission (red) spectrums of the voltage sensitive dye Di-2-ANEPEQ. At the top is shown the chemical structure of the fluorescent. Scale bars in A and B-E represent 500 µm. Abbreviations: Dc, central region of area dorsalis; Dd, dorsal region of area dorsalis; Dld, dorsal part of the lateral region of area dorsalis; Dmv, ventral part of the medial region of area dorsalis; Dmc, caudal part of the medial region of area dorsalis; lot-mot, lateral and medial olfactory tracts; OT, optic tectum.

2.2 Optical Imaging

Optical images were acquired using a commercially available system (MiCAM01; Scimedia developed by Brain Vision; Tominaga et al., 2000; Figure 3.2). The epi-fluorescence microscope (THT; Scimedia) was mounted on a vibration-isolated table (63-540; TMC) to reduce movement noise. Epi-illumination to excite the VSD was provided by a 150 W tungsten halogen lamp (MHF-G150LR; Moritex). The light beam was passed through 530 ± 3 nm excitation filter and then through the light guide, which was equipped with a condenser and a dichroic mirror. The mirror reflects the excitation light beam through the objective lens to illuminate the telencephalon. The VSD signals emitted from the stained pallium were long-pass filtered >590 nm and collected with a CCD camera with a 2.9 x 2.1 mm imaging area consisting of 90 x 60 pixels (MiCAM01, Brain Vision). The microscope was equipped with a 0.63x objective (NA = 0.082, PLAN APO; Leica Microsystems) and a 1x projection lens, so an area of 4.6 x 3.3 mm was covered by the image sensor.

The imaging field was centered on the dorsal surface of the telencephalon with the camera axis perpendicular to this surface. The focusing plane was 200 µm below the telencephalic pallium surface. Images were acquired with MICAM software at 200 Hz (5 ms/frame). To avoid the contaminating effect caused by the shutter opening on the recorded responses, imaging acquisition was begun 300 ms after the illumination shutter was opened. During each trial, the VSD signals were collected for 3400 ms. A period of 100 ms was included before stimulation was initiated. Each trial was recorded independently and then averaged to improve the signal-to-noise ratio.

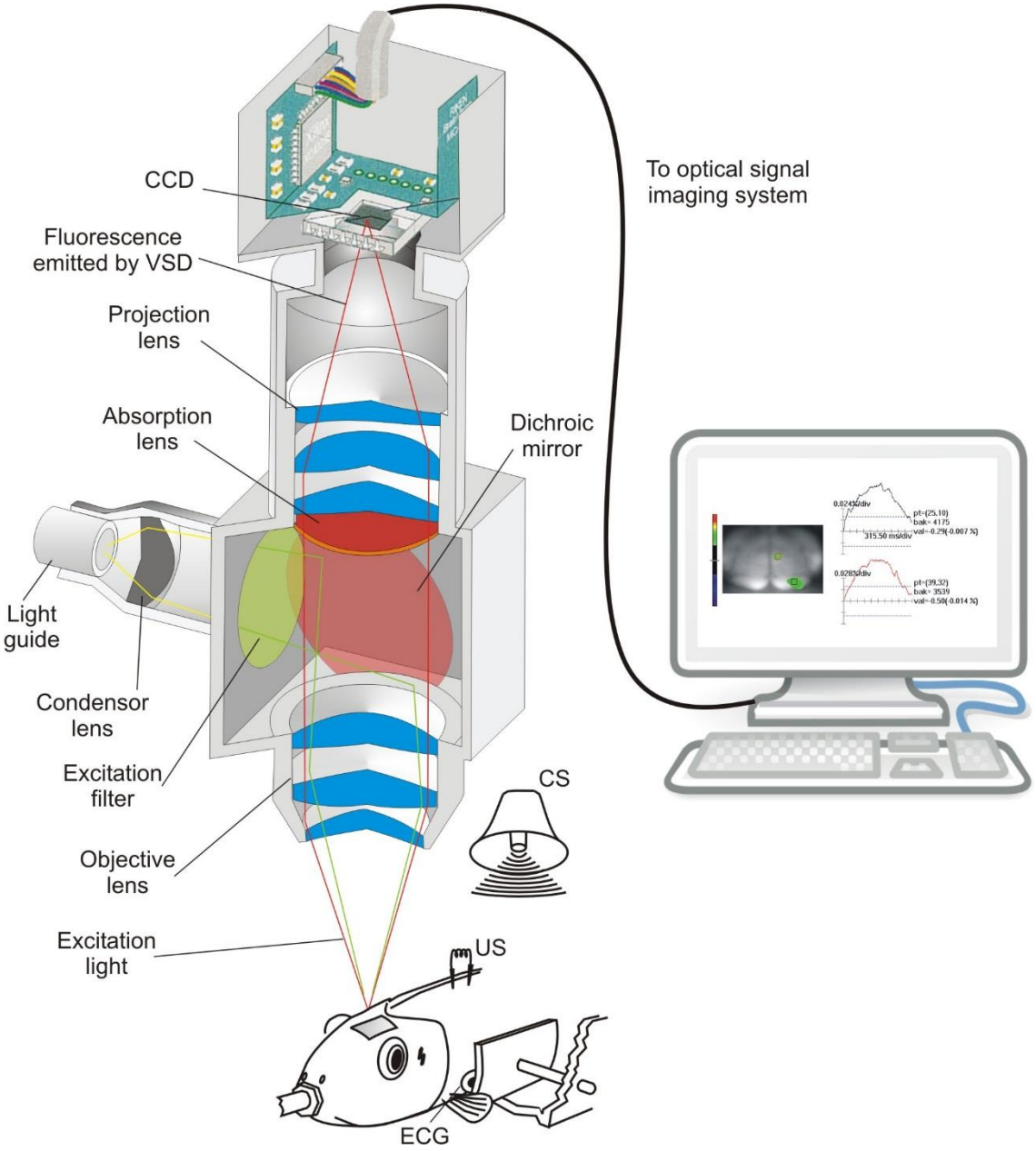

Figure 3.2. Optical imaging system and experimental preparation employed in this study. CS, conditioned stimulus; ECG, electrocardiogram electrodes; US unconditioned stimulus; VSD, voltage sensitive dye; CCD, Changed Couple Device.

2.3. Behavioral Procedures

Training was performed inside a sound attenuated chamber placed on the vibration-isolated table. A grey opaque vinyl covered the entire animal except for the exposed brain and a constant illumination during training was provided by a light placed below the animal's head (250 lux), preventing the animal from seeing the green light disposed by the optical imaging apparatus.

A 3200 ms, 70 dB, and 1 kHz pure tone (abrupt raise and fall) was used as conditioned stimulus (CS). The tone was generated by an auditory stimulator (LE-150, Letica Scientific Instruments) and presented by a loudspeaker (Ambien-20, FoneStar), placed in the air approximately 50 cm behind and above the head of the animal. The sound pressure level (SPL) was calibrated using a digital sound level meter (Velleman). The water pump and the aerator placed on the posterior side of the chamber generated a 60 dB background noise. The unconditioned stimulus (US) consisted of a mild electric shock (train duration, 100 ms; pulse rate, 50 Hz; current strength, 1-5 mA; digital stimulator DS8000, WPI) provided by a stainless steel bipolar electrode with a diameter of 100 μm that had been subdermally implanted on the left side of the rostral extreme of the dorsal fin. Presentation of stimuli was controlled by computer programs created using MED-PC behavioral programming language (MED Associates).

The animals were trained during three consecutive phases: habituation, acquisition, and extinction (Figure 3.3). Initially, the fish received 16 presentations of the pure tone to ensure their habituation to the auditory stimuli and their low responsiveness to it. After this habituation phase, the animals in the paired group were trained in a fear classical conditioning procedure with 60 trials in which the pure tone (CS) was always paired with the shock (US) except for one test trial within each 10 trials. The US was delivered 3100 ms after the onset of the CS, overlapping in time and coterminating. After these trials, 8 additional paired trials were presented during which the optical imaging was conducted. An extinction session (60 CS-alone presentations) was conducted following conditioning to test for a possible decrease in heart rate CR to repeated CS-alone presentations. At the end of extinction, animals went through a last 8 CS-alone trials presentation for imaging purposes. Regarding the Unpaired group, the behavioral procedures were conducted as above except for the unpaired phase. During this phase unpaired presentations of CS and US were used to determine whether the experience with US-alone trials might increase the number of CRs in the CS-alone trials (pseudoconditioning). For all the phases of this experiment the intertrial interval for the Paired group varied randomly between 60 and 120 s, with a mean of 90 s, to prevent conditioning to temporal stimuli. For the pseudoconditioning phase of the Unpaired group the intertrial interval varied

randomly between 30 and 60 s, with a mean of 45 s. All phases were conducted in a single day.

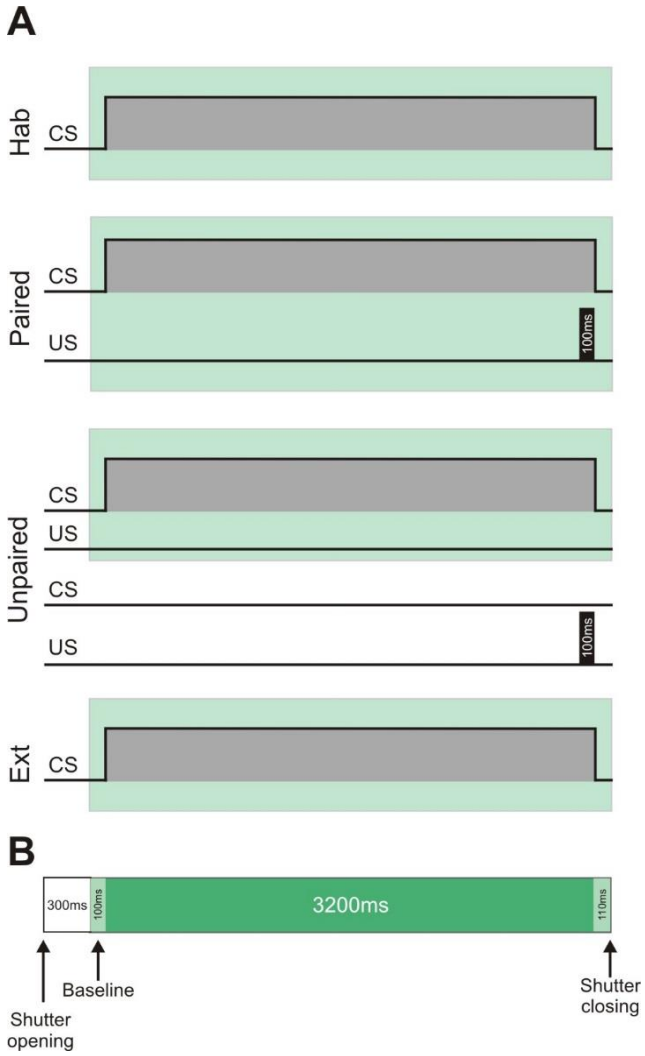

Figure 3.3. Schematic representations of the different behavioral procedures and the optical imaging timing diagram. (A) Schematic representation of habituation (CS-alone presentations), paired training (CS-US presentations), unpaired training (CS-alone and US-alone unpaired presentations), and extinction (CS-alone presentations). The light green box rectangles indicate VSD imaging period. (B) Schematic representation of the shutter opening and closing timing. Note that the excitation light was on from 400 ms before imaging onset and lasted up to the end of the imaging period.

2.4. ECG

To monitor heart rate, bipolar silver cup electrodes were placed under the pectoral fins on both sides of the ventral thoracic cavity, while the ground wire remained loose in the water of the tank. The electrocardiographic (ECG) signal was

amplified 50,000x and band-pass filtered between 0.1 Hz and 50 Hz by a differential amplifier (NL905, Digitimer), digitized at 500 Hz by an A/D converter and stored for offline analysis (Spike2, Cambridge Electronic Design).

2.5. Data Analysis

2.5.1. Heart Rate Analysis

Magnitude of the bradycardic response (heart rate deceleration) was estimated by calculating the percentage of conditioned bradycardia. This index was calculated by [(X-Y)/X)]x100, where Y was the number of beats during the 3100 ms that followed the onset of each CS, and X was the number of beats during the 3100 ms that immediately preceded the presentation of the conditioned stimulus. The percentage of conditioned bradycardia was computed for all conditioning trials. For the statistical analysis, the percentage of conditioned bradycardia was evaluated by means of a repeated measures ANOVA for each training phase (habituation, training and extinction) with the subject as the between-groups factor and the blocks of trials as the repeated measures, adjusted for multiple comparisons using a Bonferroni correction. Additionally, t-tests for unpaired samples were used to compare the percentage of CRs between Conditioned and Unconditioned groups in the optical recording phases (post-training and post-extinction). Each subject's data are reported as mean ± SEM. A probability level of $p \leq 0.05$ was used as an index of statistical significance. All of the statistical computations were performed with SPSS 17 statistical software.

2.5.2. Optical Imaging Analysis

VSD signal analysis was performed using BV-analyzer software (Brain Vision). The acquired 8 latest trials of the habituation, training and extinction phases in each experimental group per animal were averaged to improve the signal-to-noise ratio. The averaged images were detrended to compensate for dye bleaching, two-dimensionally averaged (spatial filter, 9x9 pixels) and low-pass filtered using smoothing algorithms Median filter (3x3 pixels) and Cubic filter (3x3 pixels). To evaluate the changes in optical signal intensity, we used the percentage fractional

fluorescence change (%-ΔF/F), defined as the ratio of the fluorescence intensity change (ΔF) to that of the reference image (F) obtained by averaging the activity in the initial 20 frames of the period preceding the stimulus onset. The percent fractional change was represented by pseudocolor maps in which the threshold was adjusted to 25% of the full-scale change to eliminate the background fluorescence fluctuation. In these maps, red corresponded to the largest fluorescence decrease and membrane depolarization, yellow corresponded to a medium-sized decrease, and green corresponded to the smallest decrease. Illustrative frames showing the evoked response were selected from each image sequence and saved as bitmap files. To represent the time course of fluorescence change in the region of interest, optical signals were inverted, so an upward deflection corresponds to depolarization. To characterize the evoked responses, we analyzed the maximum or peak activity and the time to peak in the Dmc and Dmv regions. The maximum or peak activity (-ΔF/Fmax) was quantified by averaging the fractional fluorescence change values of a 3x3 pixel square positioned over the more activated region of Dmc and Dmv in each animal, whereas the time to peak was defined as the time interval from the CS onset to the ΔF/Fmax. Data are represented as the mean±SEM and analyzed using Student's t-test as well as one-way and repeated measures ANOVA with SPSS version 17.0 (SPSS Inc.) when necessary. P values less than 0.05 were considered significant.

3. Results

3.1. Behavioral Results

The results of this experiment show that goldfish are able to learn to express a classically conditioned heart rate response (bradycardia, CR) in an emotional learning procedure. In the Paired group, fish showed a progressive and significant increase in the percentage of CRs to a predictive CS that is paired with a significant US. The results from paired, unpaired and extinction training converge and suggest that the increase in response following conditioning is a product of the close temporal association of the CS and US (Figure 3.4).

Figure 3.4C shows the mean percentage of conditioned bradycardia displayed by Paired and Unpaired groups throughout the training. We averaged the responses to the initial 16 CS-alone trials in two blocks of eight trials (block 1 = Orientation response; Block 2 = Habituation phase). To determine whether the Paired and Unpaired groups differed in their responses to the CS during the block 1 and block 2, a 2x2 (group x blocks of trials) repeated-measures ANOVA was conducted. The results indicated a significant main effect of block of trials ($F_{1,16}$=6.090, P=0.025) but not of group ($F_{1,16}$=16.999, P=0.899) or group x blocks of trials interaction ($F_{1,16}$=0.351, P=0.562). Thus, both Paired and Unpaired groups exhibited a significant decrease of bradycardiac response between habituation trials blocks 1 and 2 (P=0.025).

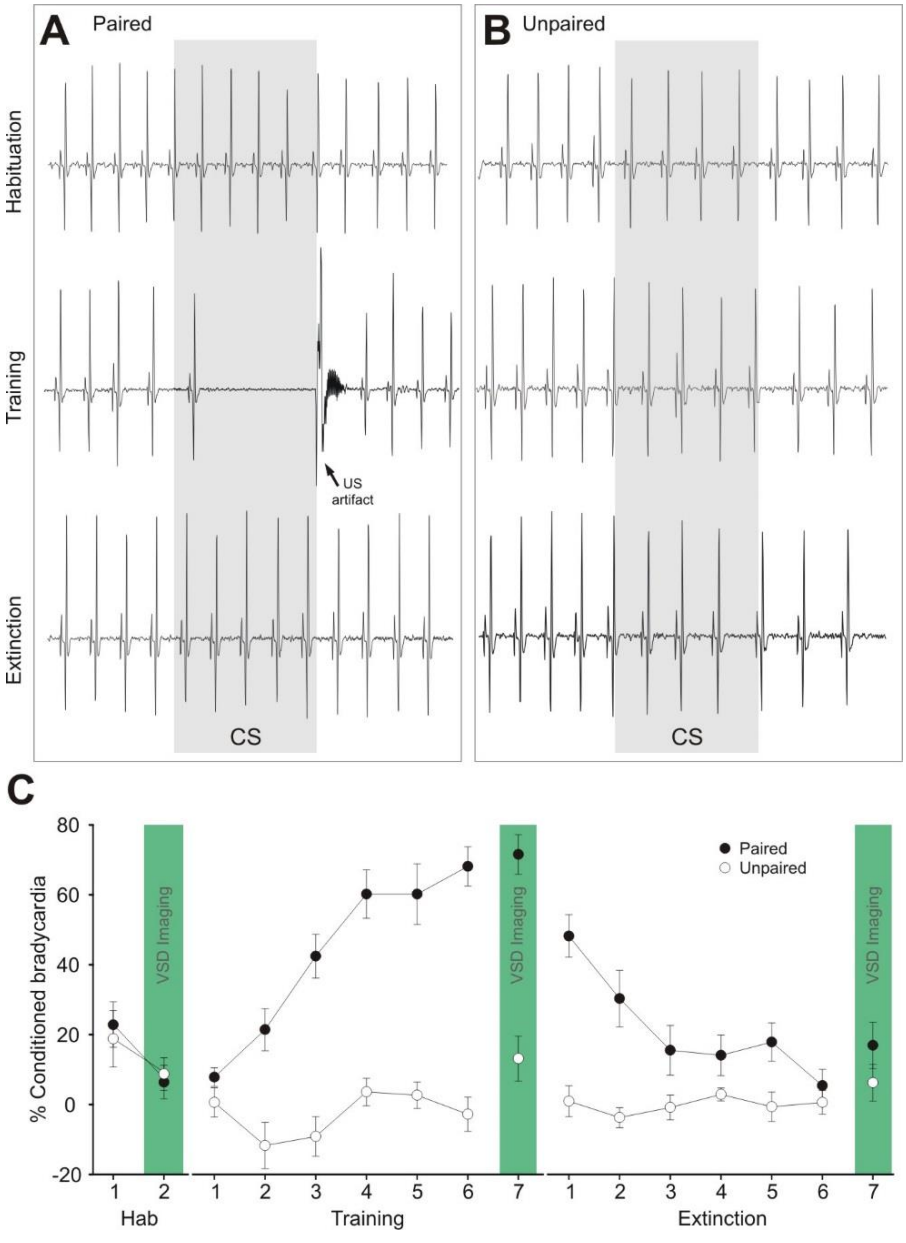

Figure 3.4. Heart rate classical conditioning. (A-B) Illustrative examples of heart rate recordings of representative goldfish trained in the Unpaired (A) and Paired (B) procedures. Note that in the paired animals the conditioned response consisted of a bradycardia (heart rate deceleration relative to pre-CS baseline). The grey boxes indicate the CS duration. (C) Percentage of conditioned bradycardia of paired and unpaired groups. The figure shows the bradycardia response during the habituation (Hab), training, and extinction. The green boxes indicate the trials in which optical imaging was conducted. Data are represented as mean ± SEM.

The percentage of conditioned bradycardia of each group during the training phase, was analyzed by means of a 2x6 (group x blocks of trials) repeated-measures ANOVA which indicated a significant main effect of group ($F_{1,16}$=75.894, P=0.000), blocks of trials ($F_{5,80}$=17.148, P=0.001) and a significant group x blocks of trials interaction ($F_{5,80}$=11.917, P=0.003). Post hoc analyses revealed that the Paired group exhibited a significant increase of bradycardiac response between acquisition trials blocks 1 and 6 (P=0.000; Figure 3.4C). The acquisition seems to reach an asymptotic level by the third block of trials as there were no significant differences in the percentage of CRs during trials blocks 3 to 6 (all Ps>0.094, Figure. 3.4C). The percentage of conditioned bradycardia of the Paired group was significantly larger compared with the values reached by the Unpaired group from the second until the last block of trials (all Ps<0.002), suggesting that the increase in response to CS observed in the Paired group cannot be attributed to a non-associative process. Moreover, the percentage of CRs in pseudoconditioned animals (Unpaired group) remained steady from the first to the last session of training (all Ps>0.264). In the last trials of the conditioning phase, where the optical recording was conducted, the percentage of conditioned bradycardia for the Paired group was significantly larger compared with the values reached by the Unpaired group (unpaired t test, t_{16}=5.316; P=0.000).

The extinction data were analyzed with a 2x6 (group x blocks of trials) repeated-measures ANOVA, which indicated a significant main effect of group ($F_{1,16}$=24.068, P=0.000) and blocks of trials ($F_{5,80}$=5.847, P=0.028) as well as a significant group x blocks of trials interaction ($F_{5,80}$=6.489, P=0.022). Further post hoc analyses indicated that there was a significant decrease in the percentage of CRs between extinction trials blocks 1 and 6 in Paired animals (P=0.001), demonstrating the

extinction of the acquired response after CS alone presentations. However, the percentage of CRs did not vary significantly between extinction trials blocks 1 and 6 in Unpaired animals (P=1.000; Figure. 3.4C). Finally, both groups exhibited the same level of bradycardia during the trials after the extinction phase in which the optical recording was conducted (unpaired t test, t_{16}=2.013; P=0.061).

3.2. Optical Imaging Results

Figures 3.5 and 3.6 show the tone-evoked activity pattern in the different phases of the experiment for a representative animal trained in the correlated procedure (paired CS-US presentations, Figure 3.5) and for a representative animal trained in the pseudoconditioning procedure (unpaired CS and US presentations, Figure 3.6). The spatiotemporal pattern of pallial activity evoked by the pure tone (CS) clearly differed among the habituation, the training, and the extinction phases. Furthermore, explicit differences existed between animals in the paired and the unpaired (pseudoconditioning) groups. In both cases, the neural activity caused by the CS was recorded at the beginning of the experimental sessions, after the habituation of the orienting response to the tone. An animal response was considered as habituated when the change in its heart rate was fewer than the 25% of its own heart rate baseline. Since the fear conditioned learning is a behavioral paradigm in which organisms learn to predict aversive events by means of pairing a neutral stimulus to an aversive stimulus and the amygdala and cortical regions (i.e. the auditory cortex) seem to mediate the conditioned response, in the present experiment, we were interested in analyzing the plastic changes induced by fear learning in the caudal region of Dm (Dmc), considered the goldfish auditory pallial region (Echteler et al., 1985; Ocaña et al., 2015; Yamamoto et al., 2007) as well as in the ventral region of Dm (Dmv), which is considered the homologue to the mammalian pallial amygdala (Broglio et al., 2005; Martín et al., 2011; Maximino et al., 2013; Wullimann and Mueller, 2004).

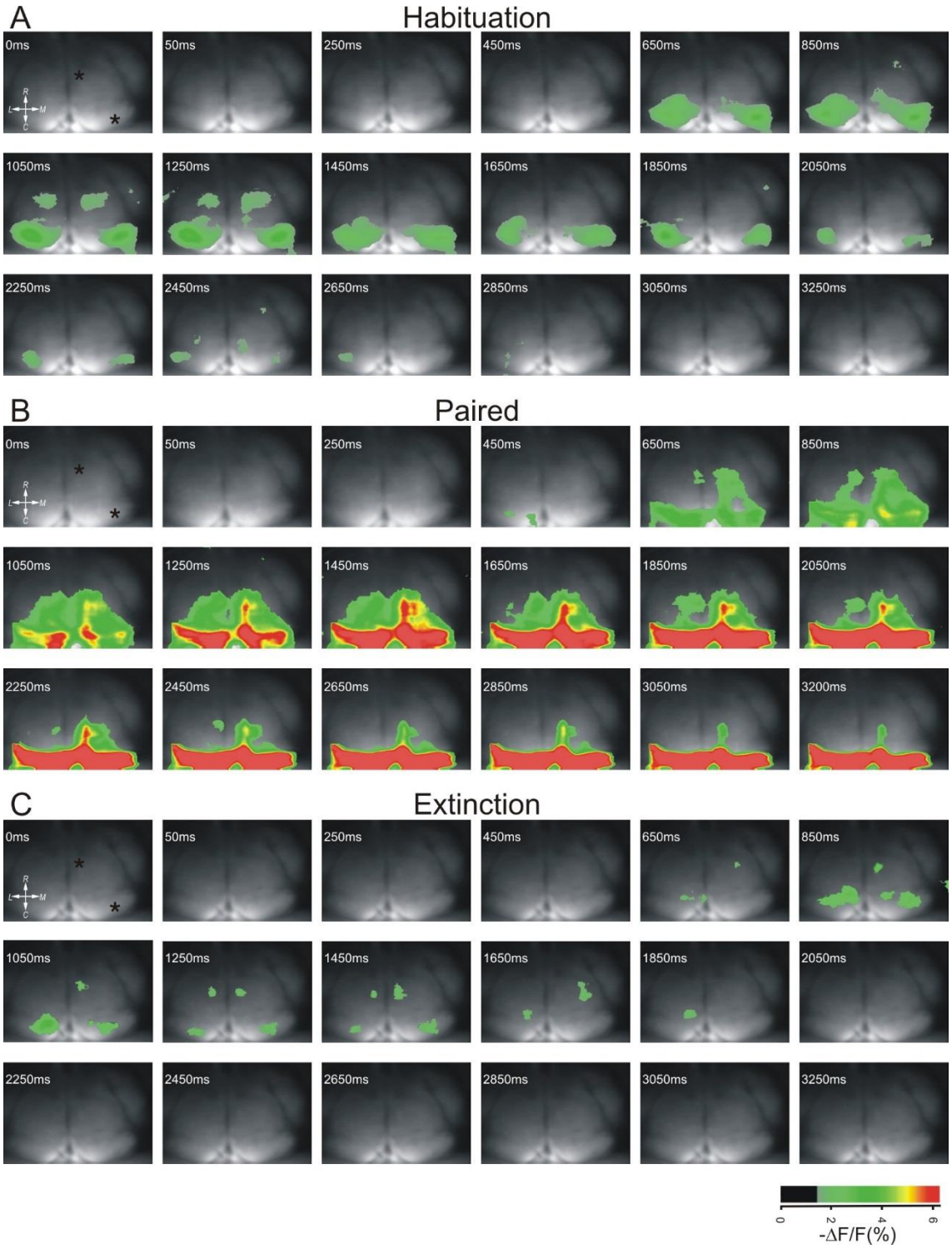

Figure 3.5. Examples of the activity pattern recorded from an animal of the Paired group along the 3200ms presentation of the tone in habituation (A), end of paired training (B), and end of extinction (C). Note that the Dmc and Dmv (asterisks) activation in the paired phase are considerably larger than in habituation and extinction. White numbers represents the time-stamp.

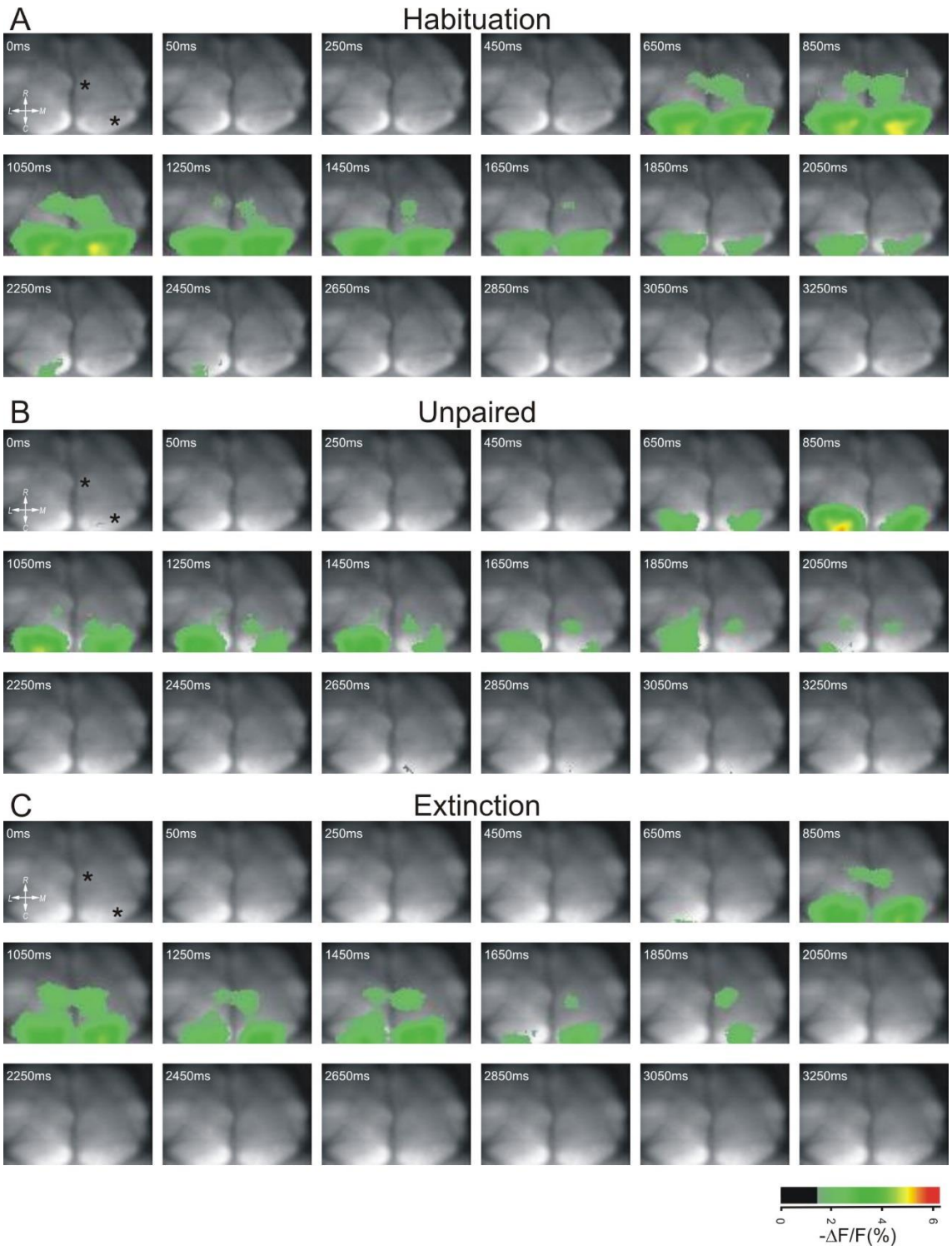

Figure 3.6. Examples of the activity pattern recorded from an animal of the Unpaired group along the 3200ms presentation of the tone in habituation (A), end of unpairing training (B), and end of extinction (C). Note that the Dmc and Dmv activation in the three phases are similar. White numbers represents the time-stamp.

During the last 8 trials of habituation, when orientation responses were not observed, the tone presentation evoked a depolarized response that appeared in the medio-lateral region of Dmc, (the auditory region, Figures 3.5 and 3.6). Over the next few milliseconds, the depolarization spread across a large part of Dmc, mainly along its latero-medial axis. Later, a second area localized rostral to Dmc, the most ventromedial part of the dorsomedial pallium (Dmv; see Figures 3.5 and 3.6), also depolarized. The analysis of the activity curves quantified by averaging the fractional fluorescence change values of a 3 x 3 pixel square positioned over the more activated region of Dmc during the latest eight trials of the habituation phase showed that the tone-evoked response was characterized by two well-defined phases, a rapid depolarization that ended with the peak or maximum activity, and a slow repolarization that finished with an optical signal similar to that recorded before stimulation (see activity curves in Figure 3.7). Furthermore, the temporal pattern of activity evoked by the tone in the Dmv region, considered the pallial region homologue to the mammalian amygdala, was similar to the pattern observed in Dmc although the peak of maximum depolarization was lower (Figure 3.7).

Following 60 CS-US paired trials, animals reached a high percentage of conditioned bradycardia and the pattern of neural activity evoked by the tone changed notably compared to the activity pattern observed in the habituation phase. Thus, after increasing its salience the CS-evoked activity enlarged in Dmc and Dmv. In fact, depolarization in both regions lasted longer, and higher depolarization levels in relation to those registered before the pairing phase were still present at the end of the CS (Figures 3.5 and 3.7). This change in the activity pattern appears to be due to an associative process related to the pairing of the CS and the US, as it was not observed in animals trained with unpaired CS-US presentations. Thus, in the Unpaired group in which the percentage of conditioned bradycardia did not change along training, we did not observe an increased depolarization associated with the repeated CS presentations in Dmc or in Dmv. In fact, the opposite effect was noted since the activity in these regions was considerable lower after training with unpaired CS-US presentations (Figures 3.6 and 3.7). Finally, a rapid shift in the spatiotemporal pattern of activity was observed after the extinction phase. In the Paired group, the extinction

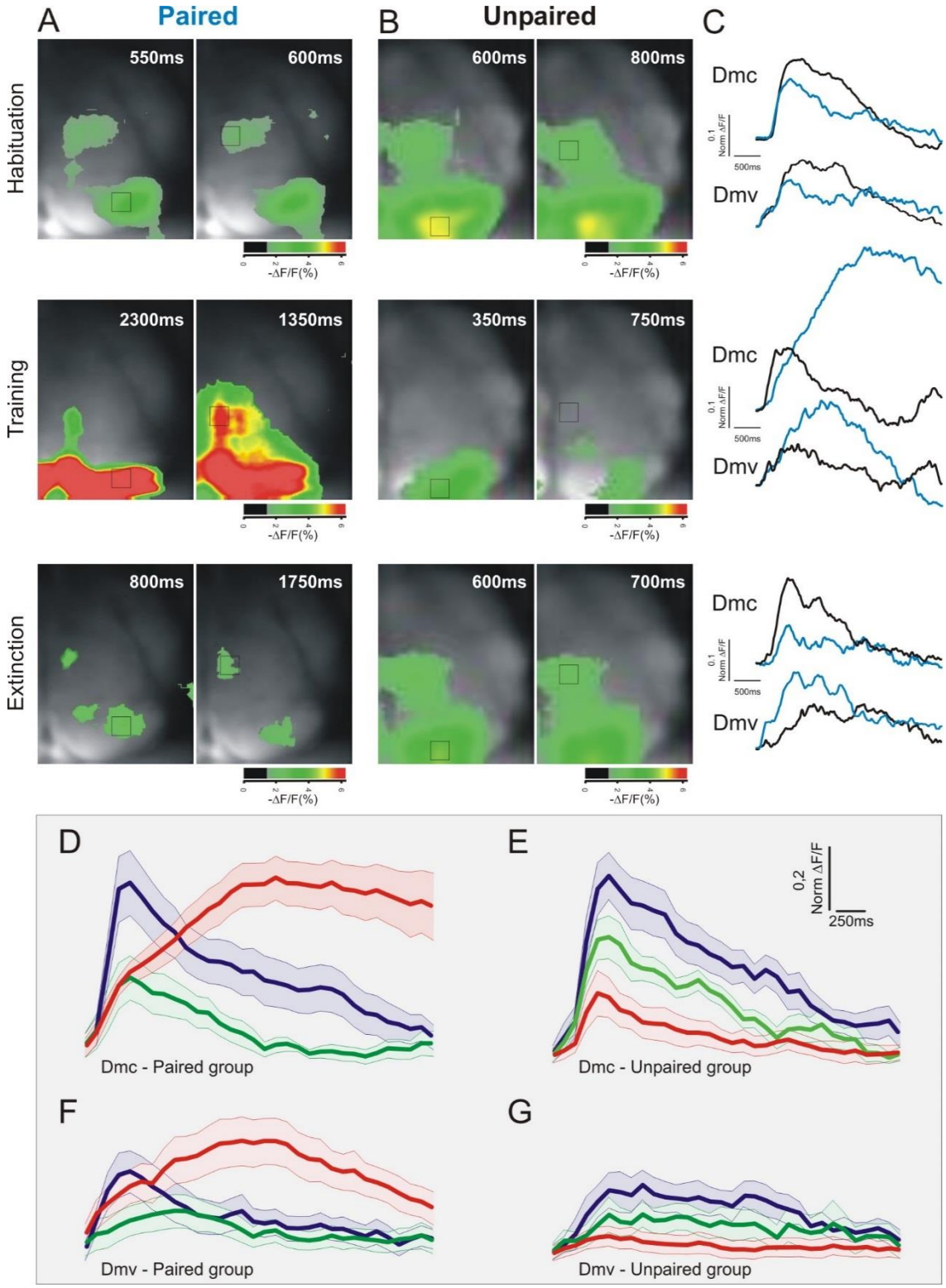

Figure 3.7. (A-B) Frames representing the peak activity of Dmc (left in each column) and Dmv (right in each column) in the Paired (A) and Unpaired (B) groups. (C) Curves of the Dmc and Dmv regions in the Paired (blue line) and Unpaired (black line) during habituation (top), end of paired (middle), and end of extinction (bottom) phases. (D-G) Averaged temporal sequence of fluorescence from each experimental group (n=9 animals for the paired and unpaired groups) and region of interest in habituation (blue line), end of training (red line), and end of extinction phase (green line).

elicited a decrease in the neural activity of Dmc and Dmv (Figures 3.5, 3.6, and 3.7), showing a pattern of activity similar to that observed during the habituation phase, where the animals did not developed a fear conditioned response to the tone.

Given that the pattern of responses observed in the animals of each experimental group was similar, we averaged fluorescence intensity data of each group to analyze the time sequence of the fluorescence signal in the Dmc and Dmv. So that, in each animal the activity peak of each region of interest was localized and the curve representing the temporal sequence of fluorescence was exported for the 3200 ms of the tone duration using MICAM01 software. These curves were normalized from 0 to 1 values to homogenate the baseline activity of each animal, and then averaged every 100ms to facilitate the statistical analysis. Figures 3.7D-G show the neural activity curves for Dmc and Dmv obtained for both groups after averaging the activity recorded in each animal in the different experimental phases. Averaged curves were compared with a 3x32 repeated measures ANOVA with experimental phase (habituation, training, and extinction) and time (32 bins of 100ms each) as within-subjects factors. Concerning the Paired group, there was significant differences in the averaged temporal sequence of fluorescence change in Dmc for both main factors [experimental phase ($F_{2,16}$=15.202, p=0.000), and time ($F_{31,248}$=7.410, p=0.000)], and the interaction between both ($F_{62,496}$=11.690, p=0.000). Subsequently, the temporal sequence of activity in this area is different in the three experimental phases (when the animal is habituated to the tone, when the animal is showing fear conditioned responses to the tone after paired training, and when the fear was extinguished after the extinction). Specifically, the paired training in comparison with the habituation and extinction phases elicited higher depolarization in Dmc. On the other hand, during extinction we observed lower activation levels than during habituation. Regarding Dmv, there are also significant differences in the averaged temporal sequence registered in animals from the Paired group for both main factors [experimental phase ($F_{2,16}$=9.392, p=0.002) and time ($F_{31,248}$=9.168, p=0.000)], and their interaction ($F_{62,496}$=7.409, p=0.000)]. Therefore, we can reject the null hypothesis and conclude that the temporal sequence of activity of that area is different in the three experimental phases. Specifically, the paired phase leads to an increment in Dmv

depolarization in comparison with the habituation and extinction phases. After extinction, a lower neural activity level than in habituation was observed.

With regard to the activity curves obtained from the animals of the Unpaired group (Figures 3.8D-E), there were statistically significant differences in the temporal sequence of activation of the Dmc region (Figure 3.8D) for the main factors [experimental phase ($F_{2,16}$=18.707, p=0.000) and time ($F_{31,248}$=31.544, p=0.000)], and the interaction ($F_{62,496}$=5.234, p=0.000). Nonetheless, the activity change observed in Dmc was different from that observed in the paired group animals. The unpaired training caused a decrement in the Dmc depolarization in comparison with the habituation and extinction, and in the extinction it was registered a lower activation than in the habituation. Concerning Dmv, there are significant differences in the maximum level of activation for experimental phase ($F_{2,16}$=4.867, p=0.022), time ($F_{31,248}$=6.069, p=0.000) and also the interaction ($F_{62,496}$=2.456, p=0.000). In particular, the neural response to the tone diminished after the repeated unpaired presentation of the CS and the US, being lower than the neural response recorded after both, habituation and extinction.

As a final point, the analysis of the peak time and the maximum depolarization confirmed the activity changes in the Dmc and Dmv regions in relation to the experimental phase and group as revealed by a 3x2 repeated measures ANOVA per region (Phase x Group; Figure 3.9). A significant main effect of phase ($F_{2,32}$=5.230, p=0.036) and interaction effect ($F_{2,32}$=7.620, p=0.014) but not main group effect ($F_{1,16}$=0.141, p=0.712) was found in Dmc. Post hoc analysis revealed that paired training produced an increment in the amount of activity in the Dmc region and that this increment rapidly disappeared after the extinction. Thus, significant higher depolarization in the Paired group in comparison with the Unpaired group after the training phase (p=0.006) but not in the habituation or extinction phases (all ps>0.067) were observed. When comparing the groups separately, there are significant differences between the habituation and training phases in relation to extinction in the paired group (all ps<0.013). However, there are no differences between habituation and training phases (p=1.000). In the Unpaired group, there are significant differences

only between habituation and training (p=0.037), with a lower depolarization level after the unpaired training.

A significant main effect of phase ($F_{2,32}$=4.904, p=0.042) and interaction effect ($F_{2,32}$=6.502, p=0.021) but not main group effect ($F_{1,16}$=1.474, p=0.242) was found in Dmv. Post hoc analysis revealed significant higher depolarization in the Paired group in comparison with the Unpaired group after the training phase (p=0.002) but not in the habituation or extinction phases (all ps>0.299). When comparing the groups separately, there are significant differences between habituation and training phases in relation to extinction in the paired group (all ps<0.004). Nonetheless, there are no differences between habituation and training phases (p=1.000). In the Unpaired group, there are no significant differences between the phases of the experiment (all ps>0.126).

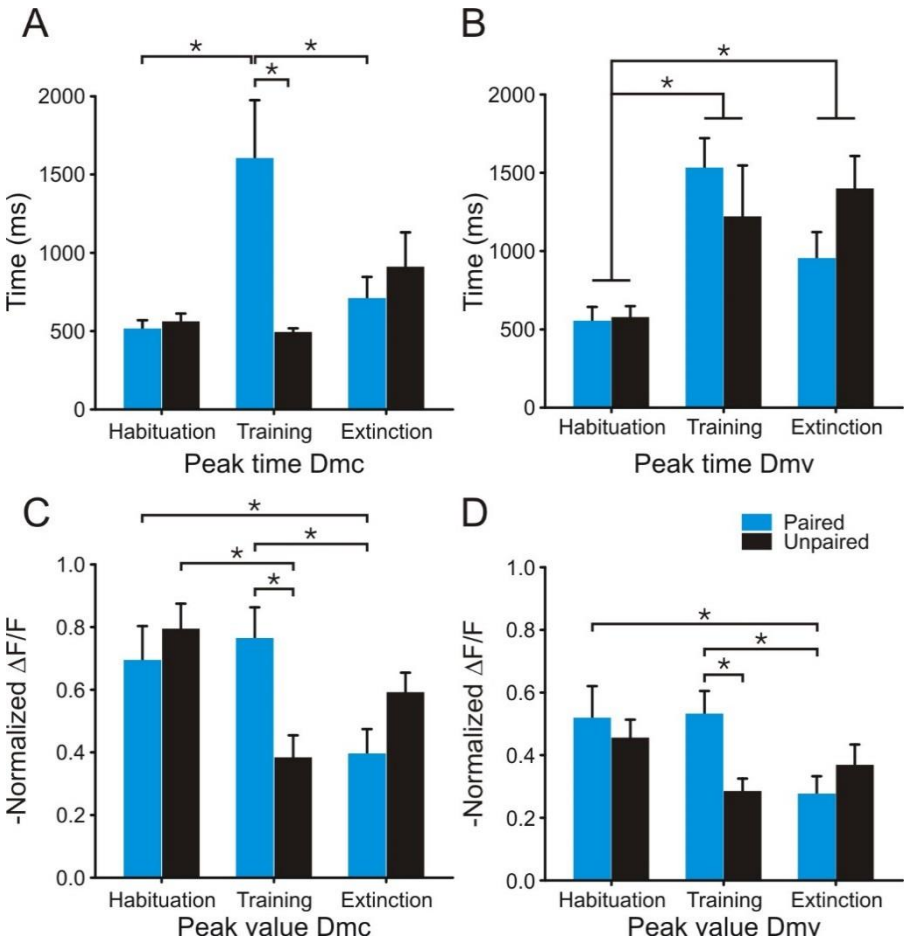

Figure 3.8. (A-B) Bars showing the average (n=9) peak latency to maximum depolarization in Dmc and Dmv in the Paired (blue) and Unpaired (black) groups. (C-D) Bars showing the average (n=9) maximum normalized activity in Dmc and Dmv in the Paired (blue) and Unpaired (black) groups. Asterisks indicate a significant comparison. Data are represented as mean ± SEM.

A 3x2 repeated measures ANOVA analysis per region of the peak time depolarization also confirmed the activity changes in the Dmc and Dmv regions (Phase x Group; Figure 3.9). A significant main effect of phase ($F_{2,32}$=4.290, p=0.022) and interaction effect ($F_{2,32}$=8.539, p=0.001) but not main group effect ($F_{1,16}$=2.819, p=0.113) was found in Dmc time to peak depolarization. Post hoc analysis revealed a change in the temporal sequence of activation. Significant higher time to peak depolarization was observed in the Paired group in comparison with the Unpaired group after the training phase (p=0.008), but not in the habituation or extinction phases (all ps>0.499). When comparing the groups separately, there are significant differences between the habituation and training phases (p=0.002), and between the training and extinction phases (p=0.015) in the Paired group. On the other hand, there are no significant differences between habituation and extinction phases (p=0.987). In the Unpaired group, there are no significant differences between all the phases of the experiment (all ps>0.281).

A no significant main effect of group ($F_{1,16}$=0.103, p=0.752) or interaction effect ($F_{2,32}$=2.117, p=0.137) but significant main effect of phase ($F_{2,32}$=10.688, p=0.000) were found in Dmv time to peak depolarization. Post hoc analysis revealed significant higher time to peak depolarization in the training and extinction phases in comparison with the habituation phase in both experimental groups (all ps<0.002).

4. Discussion

The main finding of the present experiment is that training goldfish in a fear classical conditioning procedure induce plastic changes in Dmv and Dmc pallial regions that are respectively comparable to the amygdala and auditory cortex of mammals (Braford, 2009; Desjardins and Fernald, 2010, Echteler, 1985; Lau et al., 2011; Maximino et al., 2013; Mueller and Wullimann, 2009; Northcutt, 2006, 2008; Ocaña et al., 2015; Portavella et al., 2004; von Throta et al., 2014; Wullimann and Mueller, 2004).

In this experiment we developed a preparation to classically conditioning goldfish in a procedure analogous to the auditory fear conditioning paradigm widely used in mammals. The acquisition of the conditioned bradycardia response (CR) when a predictive tone (CS) is paired with an electric shock (US) is rapid and robust. A systematic and reliable increase in the level of CRs is observed only in the CS–US paired training. The application of control procedures for conditioning such as unpaired CS–US or CS-alone presentations either failed to produce acquisition or produced the extinction of the previously acquired CRs. The sensitivity of the animal's performance to the variations in the CS–US relationships enables us to disregard that the increase in the number of CRs observed in the goldfish trained in the paired CS-US procedure could be produced by pseudoconditioning biases or other non-associative mechanisms, and indicate that in teleost fish, as in mammals, the acquisition of the CR is an associative process related to the pairing of the CS and the US (Blanchard and Blanchard, 1972, Gormezano et al., 1983, Iwata et al., 1986; Kehoe and Macrae, 2002).

Voltage sensitive dye imaging revealed learning-dependent plastic changes in the Dmc and the Dmv pallial regions evoked by the tone (CS). Indeed, the neural activity pattern in these subdivisions evoked by the tone changed significantly through training. Specifically, at the end of the CS-US paired training, when the conditioned bradycardia response has been acquired, the tone (CS) became predictive of the US and the amount of activity in the Dmv and Dmc was incremented in comparison with that activity evoked by this stimulus in these areas at the beginning of the experiment, before CS-US paired presentation (Figures 3.6, 3.7, and 3.8). Furthermore, extinction, not only produced a change in the behavioral significance of the tone (was not more a warning of the oncoming US), but also a significant decrease in the level of conditioned bradycardia responses and a rapid change in the activity pattern of Dmc and Dmv. Thus, the activity evoked by the tone after the extinction phase was weaker in these pallial regions than that recorded after the paired phase, when animals presented robust conditioned bradycardia (Figures 3.6, 3.7 and 3.8). Interestingly, the increased signal recorded in Dmc and Dmv at the end of the paired training was not observed in the animals trained in the unpaired paradigm, suggesting that this increased activity was related to a “fear memory” associated with the tone used as (CS) in this

experiment. Moreover, the present data indicate that fear memory traces can be stored in the teleost pallium and rapidly modified in relation to the significance of the stimulus.

In mammals, research on the neural circuits underlying conditioned fear responses by tone-shock pairings has identified the amygdaline nuclei as the essential center for the acquisition and storage of memories of the conditioning experience and the expression of the fear responses (Davis and Whalen, 2001; Fanselow and LeDoux, 1999; Kapp et al., 1992; LeDoux, 2000; Maren, 2001; Medina et al., 2002). The lateral nucleus (LA) is typically viewed as the sensory interface of the amygdala and as a key site for plasticity, while the central nucleus (CE) is viewed as the output region (but see Paré et al., 2004). Thus, lesions of the amygdala impair the conditioning of the heart rate (Davis et al., 1992; Gentile et al., 1986; Lee and Kim, 2004), and fear conditioning is mediated by changes in synaptic strength at sensory inputs to the lateral nucleus of the amygdala (Sigurdsson et al., 2007). Recently, on the base of connectivity patterns, genoarchitecture, chemical neuroanatomy, and functional data, it has been suggested that the most ventral portion of the dorsomedial region of the pallium of actinopterygian fish (Dmv) could be homologous to the basolateral/lateral amygdala (‘‘frontotemporal amygdaloid system’’), whereas the supracommissural and postcommissural portions of the subpallium could be homologous to the extended central amygdala (central amygdaloid nucleus and bed nucleus of the stria terminalis, see Maximino et al., 2013). Supporting this view, a recent study have shown that goldfish Dmv has an important role in the acquisition of fear classical conditioning as its inactivation by the GABAA receptor agonist muscimol abolished the acquisition of a bradycardia conditioned response (Rodríguez -Expósito, 2014). Furthermore, lesions studies have shown that Dmv lesions impair the acquisition of heart rate conditioning (Broglio et al., 2005) as well as the acquisition and retention of a conditioned active avoidance response in goldfish (Portavella et al., 2003, 2004). Finally, taste aversion learning, a behavioral paradigm in which values and motivational signals need to be codified, also depends critically of the integrity of Dmv (Martín et al., 2011, see General Introduction). As a whole, the data presented here agree with previous studies and suggest that, similarly to the mammalian amygdala, a specialized region of

goldfish dorsomedial pallium, Dmv, is critical in fear conditioning. Moreover, the present findings suggest that the increased activity in Dmv induced by fear learning could be considered as the expression of a long term potentiation (LTP) process involved in fear memory consolidation. In fact, microinjection of D-AP5, a NMDA receptor antagonist, into Dm of goldfish impairs the acquisition of active avoidance (Xu et al., 2003), an effect which is mimicked by the nitric oxide synthase inhibitor LNAME and the cyclic guanosine monophosphate inhibitor LY- 83483 (Xu et al., 2009).

Dmc pallial region is viewed by some authors as the primary auditory region of the goldfish pallium (Echteler et al., 1985; Ocaña et al., 2015). This region, like the mammalian primary auditory cortex (A1) is tonotopically organized, as a frequency map has been described along its lateromedial axis. It had long been known that primary sensory cortices develop increased responses to stimuli that gain behavioral importance due to different learning situations (e.g., classical or instrumental conditioning, reviewed in Weinberger and Diamond, 1987). Several studies in mammals have revealed that associative learning is accompanied by systematic changes in sound representation in A1. More specifically, Pavlovian CS gained increased representation, as indicated by the tuning shifts of the frequency receptive fields to the CS, the increment in the firing frequency of A1 neurons, and the increase in the area devoted to the CS frequency within the tonotopic map (Bakin and Weinberger, 1990; Diamond and Weinberger, 1986; Gonzalez-Lima and Scheich, 1986). This systematic change in the cortical representation of a stimulus parameter is called high-order (cortical) associative representational plasticity (HARP). HARP develops during associative learning in all investigated taxa, e.g., big brown bat (Gao and Suga, 1998), cat (Diamond and Weinberger, 1986), guinea pig (Bakin and Weinberger, 1990), owl monkey (Recanzone et al., 1993), rat (Hui et al., 2009; Kisley and Gerstein, 2001), and human (Molchan et al., 1994; Morris et al., 1998; Schreurs et al., 1997). The present findings suggest that HARP is also induced in the goldfish primary auditory pallial area (Dmc) by associative learning since an increment in the activity and the extension of the pallial domain activated by the tone is observed following tone-shock pairings. Furthermore, present results indicate that these learning-induced neural changes are reversed after extinction. After this phase the activity evoked in the

auditory region by tone presentations was weaker than that recorded after paired training, and in parallel the conditioned bradycardia response diminished or disappeared. Taking together, our data suggest that the role of the auditory pallial region of goldfish could be similar to that of the mammalian A1 in tone classical fear conditioning, and therefore that the teleost auditory pallium could be an important brain region for the acquisition, retention, and retrieval of specific fear memories for acoustic cues.

Understanding how neural ensembles encode behavioral programs at different time-scales and codify different types of memories is a major challenge in neuroscience. In the current experiment, we employed voltage-sensitive dye imaging of the whole goldfish pallium to identify the neural activity pattern during fear heart rate classical conditioning. This approach highlights the use of teleost fish as a model organism for studying the neural basis of fear memories as well as for analyzing the interaction between different pallial regions during emotional learning. The present results show that the responses to the conditioned auditory stimuli are increased in Dmc and Dmv due to the association of a sound with an aversive stimulus. In addition, present results add functional support to the hypothesis that teleost fish Dmv and Dmc could be homologous to the mammalian pallial amygdala and auditory cortex of mammals, respectively.

5. References

Bakin, J.S. and Weinberger, N.M. (1990). Classical conditioning induces CS-specific receptive field plasticity in the auditory cortex of the guinea pig. Brain Res;536(1-2):271-86.

Bermúdez-Rattoni, F. (2004). Molecular mechanisms of taste-recognition memory. Nature Reviews Neuroscience; 5, 209–217.

Bernstein, I.L. (1999). Taste aversion learning: A contemporary perspective. Nutrition, Immunology, Neuroscience and Behavior; 15, 229–234.

Blanchard, D.C. and Blanchard ,R.J. (1972). Innate and conditioned reactions to threat in rats with amygdaloid lesions. J Comp Physiol Psychol; 81:281–290.

Boatman, J.A. and Kim, J.J. (2006). A thalamo-cortico-amygdala pathway mediates auditory fear conditioning in the intact brain. Eur J Neurosci ;24,894 –900.

Braford, M.R. (1995). Comparative aspects of forebrain organization in the ray-finned fishes: touchstones or not? Brain, Behavior and Evolution; 46, 259-274.

Braford, M.R. Jr. (2009). Stalking the everted telencephalon: comparisons of forebrain organization in basal ray-finned fishes and teleosts. Brain Behav Evol;74(1),56-76.

Bröckelmann, A.K., Steinberg, C., Elling, L., Zwanzger, P., Pantev, C. and Junghöfer, M. (2011). Emotion-associated tones attract enhanced attention at early auditory processing: magnetoencephalographic correlates. J Neurosci; 31, 7801–7810.

Broglio, C., Gómez, A., Durán, E., Ocaña, F.M., Jiménez-Moya, F., Rodríguez, F. and Salas, C. (2005). Hallmarks of a common forebrain vertebrate plan: specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Res. Bull. 66, 277-281.

Cha, M.H., Kim, D.S., Cho, Z., Sohn, J., Chung, M., Lee, H., Nam, T.S. and Lee, B.H. (2009) Modification of cortical excitability in neuropathic rats: A voltage-sensitive dye study. Neurosci. Lett., 464, 117-121.

Chemla, S. and Chavane, F. (2010).Voltage-sensitive dye imaging: technique review and models. J. Physiol. (Paris), 104, 40-50.

Davis, M., Hitchcock, J.M. and Rosen, J.B. (1992). A neural analysis of fear conditioning, in: I. Gormezano, E. Wasserman (Eds.), Learning and Memory: The Behavioral and Biological Substrates, Lawrence Erlbaum, Hillsdale, NJ; 153–181.

Davis, M. (1992). The role of the amygdala in fear-potentiated startle: implicationsfor animal models of anxiety. Trends Pharmacol Sci. ;13(1),35-41.

Davis, M. and Whalen, P.J. (2001). The amygdala: vigilance and emotion. Mol Psychiatry;6(1),13-34.

Demski, L.S. (2013). The pallium and mind/behavior relationships in teleost fishes. Brain BehavEvol;82:31-4.

Desjardins, J.K. and Fernald, R.D. (2010). What do fish make of mirror images? Biol Lett;23,744-47.

Diamond, D.M. and Weinberger, N.M. (1986). Classical conditioning rapidly induces specific changes in frequency receptive fields of single neurons in secondary and ventral ectosylvian auditory cortical fields. Brain Res;372(2),357-60.

Echteler, S.M. and Saidel, W.M. (1981). Forebrain connections in the goldfish support telencephalic homologies with land vertebrates. Science ;212,683-5.

Echteler, S.M. (1985). Organization of central auditory pathways in a teleost fish, Cyprinus carpio. J. Comp. Neurol.; 156, 267-280.

Fanselow, M.S. and LeDoux, J.E. (1999). Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. Neuron;23(2),229-32.

Folgueira, M., Anadón, R. and Yáñez, J. (2003). Experimental study of the connections of the gustatory system in the rainbow trout, Onorhynchus mykiss. J. Comp. Neurol.; 465, 604-619.

Folgueira, M., Anadón, R. and Yáñez, J. (2004). Experimental study of the connections of the telencephalon in the rainbow trout (Onchorhynchus mykiss). II. Dorsal area and preoptic region. J. Comp. Neurol.; 480, 204-233.

Gao, E. and Suga, N. (1998). Experience-dependent corticofugal adjustment of midbrain frequency map in bat auditory system. Proc Natl Acad Sci U S A;95(21),12663-70.

Gentile, C.G., Jarrell, T.W., Teich, A., McCabe, P.M. and Schneiderman, N. (1986). The role of amygdaloid central nucleus in the retention of differential pavlovian conditioning of bradycardia in rabbits. Behav Brain Res;20(3),263-73.

Gonzalez-Lima, F. and Scheich, H. (1986). Neural substrates for tone-conditioned bradycardia demonstrated with 2-deoxyglucose. II. Auditory cortex plasticity. Behav Brain Res. ;20(3),281-93.

Gormezano, I., Kehoe, E.J. and Marshall, B.S. (1983). Twenty years of classical conditioning research with the rabbit. J.M. Sprague, A.N. Epstein (Eds.), Progress in psychobiology and physiological psychology, Vol. 10, Academic Press, New York ; 197-275.

Grinvald, A. and Hildesheim, R. (2004). VSDI, a new era in functional brain imaging of cortical dynamics. Nat. Rev. Neurosci.; 5, 873-884.

Hui, G.K., Wong, K.L., Chavez, C.M., Leon, M.I., Robin, K.M. and Weinberger, N.M. (2009). Conditioned tone control of brain reward behavior produces highly specific representational gain in the primary auditory cortex. Neurobiol Learn Mem;92(1),27-34.

Iwata, J., LeDoux, J.E., Meeley, M.P., Arneric, S. and Reis, D.J. (1986). Intrinsic neurons in the amygdaloid field projected to by the medial geniculate body mediate emotional responses conditioned to acoustic stimuli. Brain Res.;383, 195–214.

Kanwal, J.S., Finger, T.E. and Caprio, J. (1988). Forebrain connections of the gustatory system in ictalurid catfishes. J. Comp. Neurol.;278, 353-376.

Kapp, B.S., Whalen, P.J., Supple, W.F. and Pascoe, J.P. (1992). Amygdaloid contributions to conditioned arousal and sensory information processing. J.P. Aggleton (Ed.), The Amygdala, Wiley-Liss, New York ; 229–254.

Kehoe, E.J. and Macrae, M. (2002). Fundamental behavioral methods and findings in classical conditioning. J.W. Moore (Ed.), A neuroscientist's guide to classical conditioning, Springer, New York ;171–231.

Kisley, M.A. and Gerstein, G.L. (2001). Daily variation and appetitive conditioning-induced plasticity of auditory cortex receptive fields. Eur J Neurosci. ;13(10),1993-2003.

Lamprecht, R. and Dudai, Y. (2000). The amygdala in conditioned taste aversion: It's there but where? J. Aggleton (Ed.), The amygdala, Oxford University Press, New York ;331–351.

Lau, B.Y., Mathur, P., Gould, G.G. and Guo, S. (2011). Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc Natl Acad Sci,;108, 2581-86.

LeDoux, J.E. (2000). Emotion circuits in the brain, Ann. Rev. Neurosci; 23, 155-184

Lee, T.L. and Kim, J.J. (2004). Differential effects of cerebellar, amygdalar and hippocampal lesions on eye blink conditioning in rats, J. Neurosci.; 24, 3242-3250.

Martín, I., Gómez, A., Salas, C., Puerto, A.and Rodríguez, F. (2011). Dorsomedial pallium lesions impair taste aversion learning in goldfish. Neurobiol Learn Mem;96,297-05.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. Annual Review of Neuroscience, 24, 897–931.

Maren, S. (2005). Building and burying fear memories in the brain. Neuroscientist; 11,89-99.

Maximino, C., Lima, M.G., Oliveira, K.R., Batista, Ede, J. and Herculano, A.M. (2013). "Limbic associative" and "autonomic" amygdala in teleosts: a review of the evidence. J Chem Neuroanat. ; 49,1-13.

Medina, J.F., Repa, J.C., Mauk, M.D.and LeDoux, J.E. (2002). Parallels between cerebellum- and amygdala-dependent conditioning. Nat Rev Neurosci; 3,122–131.

Miskovic, V. and Keil, A. (2012). Acquired fears reflected in cortical sensory processing: a review of electrophysiological studies of human classical conditioning. Psychophysiology; 49,1230 –1241.

Molchan, S.E., Sunderland, T., McIntosh, A.R., Herscovitch, P., Schreurs, B.G. (1994). A functional anatomical study of associative learning in humans. Proc Natl Acad Sci U S A. ;91(17),8122-6.

Morris, J.S., Friston, K.J. and Dolan, R.J. (1998). Experience-dependent modulation of tonotopic neural responses in human auditory cortex. Proc Biol Sci. ;265(1397),649-57.

Mueller, T. and Wullimann, M.F. (2009). An evolutionary interpretation of teleostean forebrain anatomy. Brain Behav Evol;74,30-2.

Murakami, T., Morita, Y. and Ito, H. (1983). Extrinsic and intrinsic fiber connections of the telencephalon in a teleost, Sebastiscus marmoratus. J Comp Neurol.; 216, 115-31.

Murakami, T., Fukuoka, T. and Ito, H. (1986a). Telencephalic ascending acousticolateral system in a teleost, Sebasticus marmoratustus, with special reference to fiber connections of the nucleus preglomerulosus. The Journal of Comparative Neurology; 247, 383–397.

Nieuwenhuys, R. (1963). The comparative anatomy of the actinopterygian forebrain. Journal fur Hirnforschung;7:171-92.

Nieuwenhuys, R. (2011). The development and general morphology of the telencephalon of actinopterygian fishes: synopsis, documentation and commentary. Brain Struct Funct; 215,1415-141.

Northcutt, R.G. (2006). Connections of the lateral and medial divisions of the goldfish telencephalic pallium. J Comp Neurol;494, 903-43.

Northcutt, R.G. (2008). Forebrain evolution in bony fishes. Brain Res Bull;75:191-05.

Northcutt, R. G. and Braford, M. R. (1980). New observations on the organization and evolution of the telencephalon in actinopterygian fishes. In: Ebbesson, S. O. E. (Ed.). Comparative neurology of the telencephalon; 41-98. New York. Plenum Press.

Northcutt, R.G. (1995). The forebrain of gnathostomes: in search of a morphotype. Brain BehavEvol;46(4-5),275-318.

Ocaña, F.M., Durán, E., García, A., Broglio, C., Rodríguez, F. and Salas, C. (2015). In vivo voltage-sensitive dye imaging reveals segregated sensory areas and topographic maps in the goldfish pallium. In preparation

Onimaru, H. and Homma, I. (2003). A novel functional neuron group for respiratory rhythm generation in the ventral medulla. J. Neurosci.; 23, 1478-1486.

Paré, D., Quirk, G.J. and Ledoux, J.E. (2004). New vistas on amygdala networks in conditioned fear. J Neurophysiol;92(1),1-9.

Phelps, E.A. and LeDoux, J.E. (2005). Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron;48(2),175-87.

Portavella, M., Torres, B., Salas, C. and Papini, M.R. (2003). Lesions of the medial pallium, but not of the lateral pallium, disrupt spaced-trial avoidance learning in goldfish (Carassius auratus). Neurosci Lett. ;362(2),75-8.

Portavella, M., Torres, B. and Salas, C. (2004). Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. J Neurosci;24(9),2335-42.

Prechtl, J.C., Cohen, L.B., Pesaran, B., Mitra, P.P. and Kleinfeld, D. (1997). Visual stimuli induce waves of electrical activity in turtle cortex. Proc. Natl. Acad. Sci. USA; 94, 7621-7626.

Prechtl, J.C., von der Emde, G., Wolfart, J., Karamürsel, S., Akoev, G.N., Andrianov, Y.N. and Bullock, T.H. (1998). Sensory processing in the pallium of a mormyrid fish. J Neurosci;18(18),7381-93.

Recanzone, G.H., Schreiner, C.E. and Merzenich, M.M. (1993). Plasticity in the frequency representation of primary auditory cortex following discrimination training in adult owl monkeys. J Neurosci.;13(1),87-103.

Rodríguez-Expósito, B. (2014). Implicación diferencial del cerebelo y de las áreas dorsomedial y dorsolateral ventral del telencéfalo de los peces teleósteos en el condicionamiento clásico. Tesis doctoral. Universidad de Sevilla.

Sacco, T. and Sacchetti, B. (2010). Role of secondary sensory cortices in emotional memory storage and retrieval in rats. Science; 329,649–656.

Schreurs, B.G., McIntosh, A.R., Bahro, M., Herscovitch, P., Sunderland, T. and Molchan, S.E. (1997). Lateralization and behavioral correlation of changes in regional cerebral blood flow with classical conditioning of the human eyeblink response. J Neurophysiol;77(4),2153-63.

Sigurdsson, T., Doyère, V., Cain, C.K. and LeDoux, J.E. (2007). Long-term potentiation in the amygdala: a cellular mechanism of fear learning and memory. Neuropharmacology;52(1),215-27.

Striedter, G.F. (1991). Auditory, electrosensory and mechanosensory lateral line pathways through the forebrain of channel catfishes. J. Comp. Neurol.; 312, 311-331.

Striedter, G. F. and Northcutt, R.G. (2006). Head size constrains forebrain development and evolution in ray-finned fishes. Evolution and Development; 8, 21503-943

Thiel, C.M., Friston, K.J. and Dolan, R.J. (2002). Cholinergic modulation of experience dependent plasticity in human auditory cortex. Neuron; 35,567–574.

Tominaga, T., Tominaga, Y., Yamada, H., Matsumoto, G. and Ichikawa, M. (2000). Quantification of optical signals with electrophysiological signals in neural activities of Di-4-ANEPPS stained rat hippocampal slices. J. Neurosci. Meth.; 102, 11-23.

von der Emde, G. and Prechtl, J. C. (1999). Anatomical connections of auditory and lateral line areas of the dorsal telencephalon (Dm) in the osteoglossomorph teleost, Gnathonemus petersi. Brain Research; 818, 355–367.

von Trotha, J.W., Vernier, P. and Bally-Cuif, L. (2014). Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. Eur J Neurosci;40,3302-15.

Weinberger, N.M. (2004). Specific long-term memory traces in primary auditory cortex. Nat Rev Neurosci; 5,279 –290.

Weinberger, N.M. (2007). Auditory associative memory and representational plasticity in the primary auditory cortex. Hear Res; 229(1-2),54-68.

Weinberger, N.M. (2007). Associative representational plasticity in the auditory cortex: A synthesis of two disciplines. Learn Mem; 14(0), 1–16.

Weinberger, N.M. and Diamond, D.M. (1987). Physiological plasticity in auditory cortex: rapid induction by learning. Prog Neurobiol; 29,1–55.

Wullimann, M.F. and Mueller, T. (2004). Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. J Comp Neurol;475,143-62.

Xu, X., Bazner, J., Qi, M., Johnson, E. and Freidhoff, R. (2003). The role of telencephalic NMDA receptors in avoidance learning in goldfish (Carassius auratus). Behav Neurosci;117(3),548-54.

Xu, X., Bentley, J., Miller, T., Zmolek, K., Kovaleinen, T., Goodman, E. and Foster, T. (2009). The role of telencephalic NO and cGMP in avoidance conditioning in goldfish (Carassius auratus). Behav Neurosci;123(3),614-23.

Yamamoto, T., Shimura, T., Sako, N., Yasoshima, Y. and Sakai, N. (1994). Neural substrates for conditioned taste aversion in the rat. Behavioural Brain Research; 65, 123–137.

Yamamoto, N. and Ito, H. (2005). Fiber connections of the anterior preglomerular nucleus in cyprinids with notes on telencephalic connections of the preglomerular complex. J. Comp. Neurol.; 491, 212-233.

Yamamoto, N., Ishikawa, Y., Yoshimoto, M., Xue, H.G., Bahaxar, N., Sawai, N., Yang, C.Y., Ozawa, H. and Ito, H. (2007). A new interpretation on the homology of the teleostean telencephalon based on hodology and a new eversion model. Brain Behav Evol;69:96-04.

Yamamoto, N. and Ito, H. (2008). Visual, lateral line, and auditory ascending pathways to the dorsal telencephalic area through the rostrolateral region of the lateral preglomerular nucleus in cyprinids. J. Comp. Neurol.; 508, 615-647.

Yoshimoto, M. and Yamamoto, N. (2010). Ascending general visceral sensory pathways from the brainstem to the forebrain in a cichlid fish, Oreochromis niloticus (Tilapia). Journal of Comparative Neurology; 518, 3570–3603.

CONCLUSIONS

Previous behavioral and neurobiological evidence shows that the telencephalic pallium of teleost fish presents a complex organization not only at a neuroanatomical but also at a functional level, indicating that different pallial areas might contribute to multiple and separate memory systems (for review see Broglio et al, 2005; Northcutt, 2008). In particular, the lateral division of the area dorsalis telencephalis (Dl) of teleost fish has been proposed as homologous to the amniote medial or hippocampal pallium whereas the medial division of the area dorsalis telencephalis (Dm) is considered homologous to the amygdaline pallium. Recent experimental evidence indicates that teleost Dl is critically involved in spatial learning and memory and in temporal attribute processing (Salas et al, 2006), and that Dm is involved in emotional memory and in encoding values and motivational signals (Portavella et al., 2004; Martín et al., 2011). The present work was aimed to further analyze the implication of these pallial areas in the learning and memory abilities of goldfish. In Experiment 1 and 2 we investigated the areas involved in spatial learning by mapping the goldfish pallial activity using quantitative cytochrome oxidase (CO) histochemistry. In Experiment 3 we analyzed the areas of the telencephalic pallium involved in emotional learning and memory using optical imaging with voltage sensitive dyes.

Results from Experiment 1 and 2 showed that training goldfish in a spatial constancy task selectively increased the metabolic activity in the ventral (Dlv), but not in the dorsal (Dld) subdivision of the dorsolateral pallium or the dorsomedial pallium (Dm), suggesting that only Dlv is critically involved in spatial learning. This increased CO activity in the goldfish Dlv subdivision after long-term spatial training resembles the learning-dependent morphofunctional changes observed in the hippocampus of mammals when trained in a variety of spatial tasks, and adds further support to the hypothesis of homology between Dlv and the hippocampus of land vertebrates. Furthermore, the analysis of the metabolic activity along the rostrocaudal axis of Dld and Dlv after 1, 3, and 5 consecutive training days in the spatial constancy task, revealed that precommissural Dlv, as the mammalian hippocampus, was engaged in a time-limited manner. In addition, this timed precommissural Dlv activity correlated

with the spatial performance status exhibited by the animals,. This timed and correlated activity was selective of precommissural Dlv since although a sustained CO increase was also found in other pallial regions as the commissural part of Dlv and Dp, their activation did not changed as the behavioral performance of the animal progress.

First, these results suggest that the Dlv subdivision is a functionally heterogeneous structure along its rostrocaudal axis, and that the pre-commissural and the commissural parts of Dlv play different functional roles in the consolidation and retrieval of spatial memories. Second, a sustained spatial learning-related CO activity increase was observed in Dp, indicating that this area is also involved in spatial memory. The metabolic activity pattern observed in Dp was similar to that of the commissural part of Dlv, suggesting a functional similitude between these two pallial areas. This result is important because Dp is currently considered as the teleost homologue of the piriform cortex (Northcutt, 2006; Yamamoto et al, 2007), and argues in favor of a non-dominant alternative hypothesis proposing Dp as being part of the hippocampal pallium or the hippocampal pallium-related cortices (Nieuwenhuys, 2011; Butler, 2000). Another interesting outcome of the Experiment 1 and 2 is that Dld seemed to be not involved in spatial learning, which suggests that the dorsolateral pallium (Dl) is not a single functional division. These data are consistent with the hypotheses that consider that only Dlv is homologous to the hippocampus, and that Dld might be part of the dorsal cortex. Finally, the results showing that training goldfish in the spatial constancy task did not increase the CO activity in Dm agree with previous reports indicating that this area has not an important role in solving spatial tasks.

In Experiment 3, the paired presentations of the conditioned and unconditioned stimuli produced a robust increase in the heart rate conditioned bradycardia, which was not due to pseudoconditioning or other non-associative mechanisms as revealed by the unpaired and extinction procedures. Voltage sensitive dye imaging performed during the different phases of this emotional Pavlovian conditioning procedure revealed learning-dependent plastic changes in the auditory (Dmc) and the amygdaline (Dmv) pallial subdivisions. Thus, at the end of the CS-US paired training, when the conditioned bradycardia response has been acquired and the

tone (CS) became predictive of the US, the amount of activity in Dmc and Dmv were incremented compared to the activity evoked by this stimulus in the same areas before CS-US paired presentations. However, no increased activity in Dmc and Dmv was observed in the animals trained in the unpaired paradigm, suggesting that the increased activity observed in these regions for the animals in the paired group was selectively related to a "fear memory" associated with the tone used as CS. In addition, the activity evoked by the tone in Dmc and Dmv after the extinction phase was weaker than that recorded at the end of the paired phase, when animals presented robust conditioned bradycardia. Data from Experiment 3 suggest that the increased activity in Dmc and Dmv induced by fear learning could be considered as the expression of a long term potentiation (LTP) process involved in fear memory consolidation. Furthermore, these results significantly contribute to delimitate the areas within the Dm region involved in auditory fear Pavlovian conditioning and provide functional support to the hypotheses considering that Dmv corresponds to the basolateral amygdala, and Dmc corresponds to part of the dorsal cortex (auditory cortex, Echteler 1985; Wullimann and Mueller, 2004; Yamamoto et al., 2007).

As a whole, the present work put forwards the complex anatomical and functional organization of the telencephalic pallium of teleost fish and supports the notion of multiple and separated memory systems based on differentiated neural substrates. As in land vertebrates, the teleostean hippocampal pallium seems to be selectively involved in a memory system specialized in the processing of relational (allocentric) spatial information, whereas the amygdaline pallium is selectively involved in emotional memory. These similarities suggest the existence of a common evolutionary ancestry and that some basic functional properties of these separate memory systems evolved early in the vertebrate phylogenesis and were retained through the independent evolution of the vertebrate lineages.

References

Broglio, C., Gómez, A., Durán, E., Ocaña, F.M., Jiménez-Moya, F., Rodríguez, F. and Salas, C. (2005). Hallmarks of a common forebrain vertebrate plan: specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Research Bulletin, 66, 277–281.

Butler, A.B. (2000). Topography and topology of the teleost telencephalon: a paradox resolved, Neuroscience Letters, 293, 95–98.

Echteler, S.M. (1985) Organization of central auditory pathways in a teleost fish, Cyprinus carpio. J. Comp. Neurol., 156, 267-280.

Martín, I., Gómez, A., Salas, C., Puerto, A. and Rodríguez, F. (2011). Dorsomedial pallium lesions impair taste aversion learning in goldfish. Neurobiology of Learning and Memory, 96, 297–305.

Nieuwenhuys, R. (2011). The development and general morphology of the telencephalon of actinopterygian fishes: synopsis, documentation and commentary. Brain Structure & Function, 215, 141–157.

Northcutt, R.G. (2006). Connections of the lateral and medial divisions of the goldfish telencephalic pallium. The Journal of Comparative Neurology, 494, 903–943.

Northcutt, R.G. (2008). Forebrain evolution in bony fishes. Brain Research Bulletin, 75, 191–205.

Portavella, M., Torres, B. and Salas, C. (2004). Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. Journal of Neuroscience, 24, 2335–2342.

Salas, C., Broglio, C., Durán, E., Gómez, A., Ocaña, F.M., Jimenez-Moya, F., et al. (2006). Neuropsychology of learning and memory in teleost fish. Zebrafish;3:157-71.

Wullimann, M.F. and Mueller, T. (2004). Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. The Journal of comparative neurology;475:143-62.

Yamamoto, N., Ishikawa, Y., Yoshimoto, M., Xue, H.G., Bahaxar, N., Sawai, N., et al. (2007). A new interpretation on the homology of the teleostean telencephalon based on hodology and a new eversion model. Brain, behavior and evolution;69:96-104.